Polymorphism of HvDRO1 and HvqSOR1 associated with root growth angle in barley accessions

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Abstract: The root growth angle (RGA) is an important breeding target that confers high crop adaptability to deleterious environments. In barley, natural variations in RGA among accessions have been observed, but many of the genetic factors that cause this variation remains unclear. In this study, we explored the orthologs of OsDRO1 (DEEPER ROOTING 1) and OsqSOR1 (quantitative trait locus for SOIL SURFACE ROOTING 1), which play a critical role in RGA regulation in rice, from barley genome and analyzed the polymorphisms of these genes among barley accessions. BLASTP search detected putative orthologs of OsDRO1 and OsqSOR1 in barley (HvDRO1 and HvqSOR1) with more than 60% amino acid similarity. Sequence analysis identified SNPs causing mis-splicing and nonsynonymous amino acid substitution in HvDRO1 and HvqSOR1, respectively. These SNPs were associated with RGA variation among the 47 barley accessions. Phylogenetic analysis using the 105 barley accessions revealed that the alleles of HvDRO1 and HvqSOR1 are related to the genetic background of the accessions. Furthermore, the mutant allele of HvDRO1 is mainly shared in the Hokuriku/Nagano subpopulation, suggesting that the mutant allele is involved in local adaptation of barley cultivars to the soil environment of the region. Our findings suggest that the polymorphisms of HvDRO1 and HvqSOR1 are possible determinants of RGA variation in barley, at least in Japanese accessions, and provide information on allelic variants of the genes for marker-assisted selection to genetic improvement of RGA of barley.

Keywords: barley (Hordeum vulgare L.), local adaptation, natural variation, root growth angle

Abbreviations: ORF, open reading frame; QTL, quantitative trait locus; RGA, root growth angle; RSA, root system architecture

Introduction

Root system architecture (RSA) contributes to the adaptation of plants to various soil environments. The root growth angle (RGA; a measure of downward growth) is a component of RSA that determines the distribution of the root system in the vertical dimension, which is closely related to efficient nutrient acquisition and avoidance of abiotic stresses (Uga et al. 2015). For example, previous studies have demonstrated that crops with deeper root systems have an advantage for drought avoidance due to water uptake from the lower soil layers (Uga et al. 2013), whereas shallow root systems enhance the acquisition of phosphorus distributed in the topsoil where available phosphorus is abundant in agricultural soil (Lynch and Brown 2001). Thus, RGA is an important breeding target to develop a crop with great adaptability to various environments for sustainable crop production.
Natural variations in RGA have been observed in various plants (Kitomi et al. 2015, Ito et al. 2016, Burrage et al. 2017, Uga et al. 2018, Jia et al. 2019). The genetic architecture of the variation has been dissected by genetic and molecular level studies. *OsDRO1 (DEEPER ROOTING 1)* was identified as a key gene regulating the RGA of rice by quantitative trait loci (QTL) mapping using recombinant inbred lines derived from the lowland and upland cultivars that showed shallow and deep root systems, respectively (Uga et al. 2011, Uga et al. 2013). It was demonstrated that introducing *OsDRO1* of deep rooting cultivar into the shallow rooting cultivar resulted in increased deep rooting and enhanced drought tolerance (Uga et al. 2013). Additionally, the *OsDRO1* homolog *OsqSOR1* (*quantitative trait locus for SOIL SURFACE ROOTING 1*) was identified as a gene regulating soil surface rooting, which play a role in avoidance of the reducing stress in subsoil caused by waterlogging and salinity (Mano and Omori 2007, Kitomi et al. 2020). Both genes independently regulate the gravitropic responses of roots and act additively to determine RSA (Kitomi et al. 2020). *DRO1* homologs, including *qSOR1*, are conserved in a wide range of plant species and influence the RGA in monocots and dicots (Uga et al. 2013, Guseman et al. 2017, Ashraf et al. 2019, Kitomi et al. 2020). Therefore, the allelic variation of *DRO1* homologs would be a powerful resource for genetic improvement of RSA in various crops.

Barley is the fourth major cereal crop worldwide. Barley has a typical fibrous root system like rice. We previously evaluated RGA variation among Japanese barley accessions using hydrogel polymer medium, and revealed that the RGA are related to the geographic distribution of the accessions (Konishi et al. 2021). QTL mapping and GWAS have identified several loci associated with RGA variation in barley (Robinson et al. 2016, Sayed et al. 2017, Jia et al. 2019). However, many of the genetic factors underlying RGA variation remain unclear in barley.

Although barley has a large genome size (~5,000 Mbp), accurate whole-genome sequencing has been released owing to advances in next-generation sequencing technology (Monat et al. 2019, Jayakodi et al. 2020). This information enables us to rapidly isolate the orthologs of key genes controlling RGA that have been previously characterized in other plant species. In this study, we explored the orthologs of *OsDRO1* and *OsqSOR1* from barley genome and analyzed the association between polymorphisms of the genes and RGA among the 47 barley accessions. Furthermore, we investigated the relationships between the alleles of genes and the genetic background of barley accessions. These analyses suggest that the polymorphisms of *DRO1* homologs contribute to RGA variation among barley accessions.

**Materials and Methods**

**Plant material**

The barley accessions used in this study were obtained from the National Agriculture and Food Research Organization (NARO) Central Region Agricultural Research Center, NARO Institute of Crop Science, NARO Kyushu-Okinawa Agricultural Research Center, and NARO Genebank.

**BLASTP search and phylogenetic analyze of *OsDRO1* and *OsqSOR1* orthologs in barley**

BLASTP search were conducted using the IPK Barley BLAST Server (https://webblast.ipk-gatersleben.de/barley_ibsc/) with the “Barley AA (HC and LC) Morex v2.0 2019” database. The amino acid sequences of *OsDRO1* (Os09t0439800-01) and *OsqSOR1* (Os07t0614400-01) obtained from the Rice Annotation Project Database (RAP-DB; https://rapdb.dna.affrc.go.jp/index.html; Sakai et al. 2013) were used as query sequences.

Phylogenetic tree was constructed using the maximum likelihood method with MEGA X (Kumar et al. 2018). Amino acid sequences of *DRO1* family proteins were obtained from the UniProtKB (https://www.uniprot.org/; The UniProt Consortium 2021) and the TAIR database (https://www.arabidopsis.org/index.jsp).

**Sequence analysis of *HvDRO1* and *HvqSOR1***

Open reading frames (ORFs) of *HvDRO1* and *HvqSOR1* in barley accessions were sequenced by direct sequencing of PCR amplicons. The ORF regions of both genes were amplified using TaKaRa Ex Taq Hot Start Version (Takara Bio, Kusatsu, Japan) according to the manufacturer’s instructions. The amplicons were sequenced using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, United States) by Takara Bio Inc. (Kusatsu, Japan) and Fasmac Co. Ltd. (Atsugi, Japan). The primers used are listed in Table S1. Potential phosphorylation sites were predicted using NetPhos3.1 (http://www.cbs.dtu.dk/services/NetPhos3.1; Blom et al. 1999).

**Root growth angle**

The information of the RGA of 47 barley accessions...
was obtained from Konishi et al. (2021). The RGA was evaluated using the angle between the soil surface and the root tip of the longest root. \( P \)-values for the associations between RGA and polymorphisms of \( HvDRO1 \) and \( HvqSOR1 \) were calculated by Watson Williams test using the “circular” package (Agostinelli and Lund 2013) in R version 4.0.3 (R Core Team 2020).

**RNA extraction and sequence analysis of cDNA for \( HvDRO1 \)**

Total RNA was extracted from the roots of the barley accessions. Seedlings of each accession were grown on 1% (w/v) water agar plates kept at a vertical angle under dark conditions at 20°C for five days. Subsequently, roots from three individuals were collected and immediately frozen in liquid nitrogen. Total RNA extraction and reverse transcription were performed using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) and ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan) according to the manufacturers’ instructions. The \( HvDRO1 \) cDNA was amplified using TaKaRa Ex Taq Hot Start Version (Takara Bio) and sequenced by direct sequencing, as described above. The primers used are listed in Table S1.

**Phylogenetic analysis of barley accessions**

We mapped RNA-seq data (DRA012162) reported by Tanaka et al. (2019) to the second version of the reference genome sequence assembly of barley cv. Morex (V2) downloaded from IPK (https://doi.ipk-gatersleben.de/DOI/83e8e186-dc4b-47f7-a820-28ad37cb176b/d1067eba-1d08-42e2-85ec-66bd5112cd8/2) by hisat2-2.0.5, with the options “--min-intronlen 20 --max-intronlen 10000 --downstream-transcriptome-assembly --rna-strandness RF” (Kim et al. 2015). Mapping results were converted from SAM-formatted files to BAM-formatted files using samtools-1.4 (Li 2011) and indexed by Sambamba 0.6.8 (Tarasov et al. 2015). Genotyping of each sample was performed using HaplotypeCaller in GenomeAnalysisTK (GATK) v3.7-0-gcfed67 with the options “-ERC GVCF -variant_index_type LINEAR -variant_index_parameter 128000 -filter_reads_with_N_cigar”, and all results were integrated using CombineGVCFs and GenotypeGVCFs in GATK (McKenna et al. 2010). All identified SNPs were filtered using vcftools (Danecek et al. 2011) with the option “-max-missing 1 --maf 0.1 --not-chr chr\(n\) --max-alleles 2 --remove-indels”. After eliminating three accessions (Rokkaku Chevallier, Mona, and Shiratamahadaka) that contained many heterozygous alleles (> 1,000), all SNP sites with heterozygous alleles were removed. Eventually, 16,988 SNPs were included in the analysis.

Admixture analysis of the 105 barley accessions was performed using the ADMIXTURE software (Alexander et al. 2009). The proportional membership of each accession was inferred for \( K = 2–15 \), and the \( K \) with the lowest cross-validation error was selected as the optimum \( K \) value (\( K = 8 \)). The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987) with the PHYLP software. Evolutionary distances were calculated using the Kimura 2-parameter model (Kimura 1980). The phylogenetic tree was drawn using R version 4.0.3 (R Core Team 2020) with the “ggtree” package (Yu et al. 2017).

**CAPS and dCAPS marker**

Cleaved-amplified polymorphic sequence (CAPS) and derived CAPS (dCAPS) markers were designed using dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/; Neff et al. 2002). The SNPs causing mis-splicing and nonsynonymous amino acid substitutions in \( HvDRO1 \) (G1438T) and \( HvqSOR1 \) (A1074C) were used to develop the markers. In the CAPS marker for \( HvDRO1 \), the primer pair amplified a 340-bp fragment, and only the functional allele (G) could be digested by EcoO109I (New England Biolabs, Ipswich, MA, United States). In the dCAPS marker for \( HvqSOR1 \), the primer pair amplified a 342-bp fragment, and only the mutant allele (C) could be digested by SalI (New England Biolabs). Hot Start Taq DNA Polymerase (New England Biolabs) was used for PCR amplification of CAPS and dCAPS markers. The primers used are listed in Table S1.

**Results**

**BLASTP search of OsDRO1 and OsqSOR1 orthologs in barley**

Orthologs of OsDRO1 and OsqSOR1 in barley were explored using BLASTP search at the IPK barley BLAST Server (https://webblast.ipk-gatersleben.de/barley_ibsc/) based on the “Barley AA (HC and LC) Morex v2.0 2019” database. The BLASTP searches detected 12 and 7 proteins for OsDRO1 and OsqSOR1 in barley, respectively (E-value < 10, Table S2). Two genes (HORVU.MOREX.r2.5HG0398200 and HORVU.MOREX.r2.2HG0103490) were commonly detected in both BLASTP searches for OsDRO1 and OsqSOR1. Among the detected proteins, five proteins contain a
Fig. 1. Phylogenetic tree of DRO1 family proteins from monocots and Arabidopsis. Bold font represents barley proteins containing CCL domain detected by BLASTP search. The scale bar indicates the relatedness of proteins.

Fig. 2. Sequence analysis of HvDRO1 and HvqSOR1. (a) Polymorphism of HvDRO1 and HvqSOR1 among the 47 barley accessions. Black rectangles represent the exon region. Only variants that caused mis-splicing and nonsynonymous amino acid substitutions are shown. (b) Alignment of HvDRO1 cDNA sequence of the accessions with functional (G) and mutant (T) allele.
Fig. 3. Phosphorylation potential of HvqSOR1 predicted by NetPhos 3.1. The yellow box indicates the site of amino acid substitution by SNP in the accessions with mutant allele (S249R).

CCL (the conserved C terminus in the LAZY1 family of proteins) domain (Table S2), which is highly conserved among DRO1 homologs in terrestrial plants (Ashraf et al. 2019, Kitomi et al. 2020). To reveal the relatedness between the barley proteins containing CCL domain and DRO1 family proteins from other plants, we performed phylogenetic analysis using amino acid sequences of DRO1 family from monocots (rice, sorghum, wheat and Brachypodium) and Arabidopsis (Fig. 1). The DRO1 family proteins were grouped into DRO1 subgroup, qSOR1/DRL1 subgroup and DRL2 subgroup, consistent with previous report (Kitomi et al. 2020). This analyze showed that HORVU.MOREX.r2.5HG0398200 and HORVU.MOREX.r2.2HG0103490 are grouped in the DRO1 subgroup and qSOR1/DRL1 subgroup, respectively. HORVU.MOREX.r2.5HG0398200 consists of 251 amino acids, similar to OsDRO1, and had a 75% amino acid sequence identity with OsDRO1, while HORVU.MOREX.r2.2HG0103490 consists of 276 amino acids and had a 61% sequence identity with OsqSOR1 (Fig. S1 and Table S2). These results suggest that the proteins are the most likely orthologs of OsDRO1 and OsqSOR1 in barley (referred to as HvDRO1 and HvqSOR1).

Polymorphism analysis of HvDRO1 and HvqSOR1

According to the released barley genome (Monat et al. 2019), the ORFs of HvDRO1 and HvqSOR1 are 2,308 bp and 1,314 bp long, respectively. We sequenced the ORF regions of the 47 barley accessions that showed different root growth angles (Table S3) to identify polymorphisms in the proteins. In HvDRO1, sequence analysis identified 16 and two variants in the intron and exon regions with minor allele frequency > 10% (i.e., N > 5), respectively (Table S4). Among the variants, an SNP in the exon region caused nonsynonymous amino acid substitutions (M165I, Fig. 2a). In addition, an SNP in the intron region was located on the 3’ splice site of the second intron that probably causes mis-splicing. Subsequent sequence analysis of HvDRO1 cDNA confirmed that the SNP caused mis-splicing, leading to a 7-bp deletion resulted in a premature stop codon produced by a frameshift (Fig. 2b). This suggests that the HvDRO1 of the accessions with the mutant allele is truncated. In addition, sequence analysis of HvqSOR1 detected two SNPs in the exon and intron regions with minor allele frequency > 10%. The SNP located in the exon region caused a nonsynonymous amino acid substitution from Ser to Arg (Fig. 2a and Table S5). The Ser residue is a potential phosphorylation site inferred by NetPhos 3.1, and the potential was predicted to be lower by substitution with Arg (Fig. 3). These results indicate that there are several
polymorphisms that may affect the function of HvDRO1 and HvqSOR1 in barley accessions.

**Association analysis between RGA and polymorphisms of HvDRO1 and HvqSOR1**

We examined the associations between the RGA of barley accessions and the polymorphisms of HvDRO1 and HvqSOR1. The RGA of the 47 barley accessions used in this study ranged from 0 to 57.5° (Table S3). In HvDRO1, the SNP causing mis-splicing was significantly associated with the RGA (Fig. 4), and the mean RGA of the accessions with mutant allele (T) was 15.6° shallower than that with the reference allele (functional allele; G). This result suggested that the loss-of-function mutation in HvDRO1 resulted in decreased RGA. No significant association was detected between RGA and the SNP causing nonsynonymous amino acid substitution in HvDRO1 (M165I, Fig. 4). On the other hand, the SNP causing nonsynonymous amino acid substitution in HvqSOR1 was slightly but significantly associated with RGA (Fig. 4). The mean RGA of the accessions with the mutant allele (C) was 10.8° deeper than that with the reference allele (A). These results suggest that the polymorphisms of HvDRO1 and HvqSOR1 are associated with variations in RGA among barley accessions.

**Relationships of the genetic background of barley accessions and the alleles of HvDRO1 and HvqSOR1**

We then investigated the relationships between the genetic background of barley accessions and the alleles of HvDRO1 and HvqSOR1 to gain insight into the contribution of the alleles to local adaptation. We performed admixture analysis using 105 barley accessions with 16,988 SNPs detected from RNA-seq data (Tanaka et al. 2019). The admixture analysis inferred eight ancestral subpopulations (K = 8, Fig. 5 and 6), which roughly corresponded to the geographic distribution and pedigree of the accessions (Fig. 6). This grouping was supported by the phylogenetic tree constructed by the neighbor-joining method using the same SNP set (Fig. 6).

We defined the alleles of HvDRO1 and HvqSOR1 of the 105 barley accessions using CAPS and dCAPS markers. This analysis revealed that 18 out of the 105 accessions possessed the mutant allele of HvDRO1 (Fig. 6). All the accessions with the mutant allele were six-rowed hulled barley that developed in the Hokuriku/Nagano and Kanto regions. There were no accessions carrying the mutant allele in the other regions. This result suggests that the mutant allele of HvDRO1 is shared in the local subpopulation among Japanese accessions. In contrast, eight out of the 105 accessions carried the mutant allele of HvqSOR1. Although the accessions with the mutant allele were developed in various regions, most of the accessions were non-Japanese accessions or Japanese cultivars developed using non-Japanese accessions as ancestors. There were no accessions carrying the mutant alleles of both genes.

**Discussion**

**Orthologs of OsDRO1 and OsqSOR1 in barley**

Improving RSA is an effective approach to confer high adaptability to various deleterious environmental conditions in crops; however, direct observation of root traits is difficult because it is an underground phenotype. To improve such traits during the breeding process, marker-assisted selection is used to select individuals without direct observation of the root phenotype. In this study, we found polymorphisms in HvDRO1 and HvqSOR1 associated with RGA among barley accessions and developed DNA markers to distinguish the alleles. Furthermore, phylogenetic analysis revealed that the allele of the genes was related to the developed region of barley accessions. This suggests that the polymorphism of DRO1 orthologs may contribute to local adaptation in Japanese barley accessions.

DRO1 homologs play an important role in regulating RSA among various plant species (Uga et al. 2013, Guseman et al. 2017, Ashraf et al. 2019, Kitomi et al. 2020). We found orthologs of OsDRO1 and OsqSOR1 in barley (HvDRO1 and HvqSOR1) by BLASTP searches (Fig. S1 and Table S2).
Fig. 6. Phylogenetic tree of the 105 barley accessions constructed by the neighbor-joining method using 16,988 SNPs. Color circles represent the allele of HvDRO1 (left circle) and HvqSOR1 (right circle) of each accession. A horizontal bar represents the proportion of membership of each accession. Each color corresponds to the inferred membership in K = 8 ancestry subpopulations estimated by ADMIXTURE (K = 8).
HvDRO1 and HvqSOR1 are composed of five exons and four introns like other orthologs, and the protein contain the CCL domain (Fig. 2a and Table S2). Furthermore, phylogenetic analysis revealed that HvDRO1 and HvqSOR1 are grouped with DRO1 and qSOR1 orthologs of other monocots, respectively (Fig. 1). These results suggest that HvDRO1 and HvqSOR1 may be involved in the regulation of RGA in barley. In addition to HvDRO1 and HvqSOR1, BLASTP search detected other three proteins containing the CCL domain (Table S2). Among the proteins, HORVU.MOREX.r2.4HG0337310 and HORVU.MOREX.r2.6HG0525530 were grouped with DRL2 and LAZY1 orthologs, respectively (Fig. 1). It was demonstrated that DRL2 play a role in RGA regulation and LAZY1 is involved in controlling tiller angle in rice (Li et al. 2007, Kitomi et al. 2020), suggesting that the orthologs of DRL2 and LAZY1 might be also involved in gravitropic responses of roots and shoots in barley.

Polymorphisms of HvDRO1 and HvqSOR1 associated with RGA

Sequence analysis revealed several polymorphisms of HvDRO1 and HvqSOR1 that possibly affect gene function. An SNP located in the 3’ splice site of the second intron of HvDRO1 was associated with RGA among barley accessions (Fig. 2 and 4). The SNP caused extension of the intron up to the next splice site (i.e., AG) located 7-bp downstream that produced a premature stop codon caused by a frameshift and resulted in a truncated protein lacking the CCL domain (Fig. 2b). A rice accession IR64 carrying a mutant allele of OsDRO1, which also lacks the CCL domain, showed a shallower RGA than the rice accession with the functional allele (Uga et al. 2013). In addition, it was reported that a wild barley ISR42-8 showed a weaker HvDRO1 expression level and shallower RGA compared to the cultivar Scarlet (Ashraf et al. 2019). These results suggest that the loss-of-function allele of HvDRO1 is a determinant of RGA variation among barley accessions. In addition, an SNP causing nonsynonymous amino acid substitution in HvqSOR1 was associated with RGA, and the mean RGA of the accessions with the mutant allele was slightly deeper than that with the reference allele (Fig. 2a and 4). OsqSOR1 regulates the development of soil-surface roots, and the loss-of-function allele of OsqSOR1 results in increased soil surface roots (Kitomi et al. 2020). The SNP in HvqSOR1 caused Ser to Arg substitution, which potentially led to changes in protein phosphorylation (Fig. 3). Phosphorylation is an important mechanism for regulating protein function (Ranjeva and Boudet 1987, Sakuma et al. 2017), suggesting that changes in the phosphorylation status of HvqSOR1 might result in increased RGA. Further analysis is needed to evaluate the effect of the HvqSOR1 polymorphism on protein function. The loci of HvDRO1 and HvqSOR1 were detected as QTL region by GWAS for several root traits, such as root spreading angle and root system depth (Jia et al. 2019). However, it is unclear whether the polymorphisms of HvDRO1 and HvqSOR1 identified in this study are shared among barley accessions worldwide.

Allele distribution of HvDRO1 and HvqSOR1 in Japanese barley population

Phylogenetic analysis revealed that the genetic background of the 105 mostly Japanese barley accessions was roughly related to their geographic distribution (Fig. 6). The climate of Japan is diverse due to its latitudinal variation so, the required crop variety adaptability is different depending on the local environment. We found that the mutant allele of HvDRO1 (shallow root type) is mainly shared among the accessions that were developed and cultivated in the Hokuriku/Nagano region (Fig. 6), where upland paddy fields composed of heavy clay soil are mainly used for barley cultivation in a double-cropping system with rice, especially in Hokuriku region (Obara and Mitsuchi 1990, Ikenaga et al. 2012). In such fields, root hypoxia caused by inadequate drainage is a major environmental stress factor that disturbs barley growth. It has been suggested that the shallow root system is beneficial for waterlogging tolerance because the shallow root system allows more oxygen to be obtained from the topsoil and avoid toxins that accumulate in subsoil under reducing condition (e.g., Fe$^{2+}$ and H$_2$S; Mano and Omori 2007, Kitomi et al. 2020). In fact, Oyanagi et al. (2004) demonstrated that a wheat strain with a shallow root system tends to show higher grain yield in wet paddy fields than that with a deep root system. Furthermore, it has also been suggested that the shallow root system contributes to adaptation to hypoxic conditions caused by snow coverage and temporal flooding due to snowmelt (Waidmann et al. 2019). These results suggest that the shallow root system caused by the mutant allele of HvDRO1 may contribute to the adaptation of clayey soils in the Hokuriku/Nagano region. In addition, several accessions belonging to the Kanto group carried the mutant allele of HvDRO1 (Fig. 6). These accessions were recently developed using the Silksnow cultivar, which carries the mutant allele and was developed in Nagano. Therefore, the mutant allele of HvDRO1 may have been selected together...
with other characteristics of Silksyosnow. On the other hand, the mutant allele of HvqSOR1 (deep root type) was shared among non-Japanese accessions (Fig. 6). In addition to the non-Japanese accessions, several Japanese accessions also harbored the mutant allele (Fig. 6 and Table S3). These accessions were developed using a non-Japanese cultivar Mona, which carried the mutant allele (data not shown), as a common ancestor; therefore, it might be the origin of the allele in Japanese accessions. Deep root system are known to contribute to drought avoidance by enhancing water uptake from deep soil layers (Uga et al. 2013, Uga et al. 2015). Drought is a major environmental stressor for crop growth worldwide (Cattivelli et al. 2008). Therefore, conferring higher drought tolerance is an important target for barley breeding, especially in regions where upland fields with high drainage capacity are mainly used for barley cultivation. These results suggest that the deep root type allele of HvqSOR1 may contribute to adaptation to drought environments. However, there are only a few reports on the relationship between RSA and environmental stress tolerance in barley. Further investigation is required to uncover the role of RSA in the adaptation to deleterious environments.

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Fig. S1. Orthologs of OsDRO1 and OsqSOR1 in barley. (a) Amino acid alignment of OsDRO1 and HvDRO1 (HORVU.MOREX.r2.5HG0398200). (b) Amino acid alignment of OsqSOR1 and HvqSOR1 (HORVU.MOREX.r2.2HG0103490). Orthologs were identified by BLASTP search using the IPK Barley BLAST Server (https://webblast.ipk-gatersleben.de/barley_ibsc/) using the “Barley AA (HC and LC) Morex v2.0 2019” database.
### Table S1. List of primers

| Object                                      | Name                      | Sequence                           |
|---------------------------------------------|---------------------------|------------------------------------|
| **Amplification for**                       | HvDRO1_ATG-25bp-F         | forward 5' CCTCCTCCATTCCTAAGCAAG 3' |
| **HvqSOR1 ORF**                             | HvDRO1_TAA+19bp-R         | reverse 5' ACACCTGGCCTCAATGGAAC 3' |
|                                             | HvDRO1-13935-F            | forward 5' CTTCTGCTACATCTCTCTTCT 3' |
|                                             | HvDRO1-13332-R            | reverse 5' GGAACCAGAAAAATCCACTTC 3' |
|                                             | HvDRO1-12568-F            | forward 5' TCAGGATGGAAGGACATGG 3'  |
|                                             | HvDRO1-11406-R            | reverse 5' TCCTGATAGTCGAGTTGAA 3'  |
|                                             | HvDRO1_CDSATG+468bp-R     | reverse 5' GTTAGTGTAGGAGCAAAGCAAC 3' |
| **Sequence analysis for**                   | HvDRO1-13935-F            | forward 5' CTTCTGCTACATCTCTCTTCT 3' |
| **HvqSOR1 ORF**                             | HvDRO1-12568-F            | forward 5' TCAGGATGGAAGGACATGG 3'  |
|                                             | HvDRO1_ATG+315bp-F        | forward 5' CGGTGATCCTTTGATCTCTGC 3' |
|                                             | HvDRO1_ATG+1527bp-R       | reverse 5' GCAGCTGGCTACCTCTCTCT 3' |
|                                             | HvDRO1_ATG-25bp-F         | forward 5' CCTCCTCCATTCCTAAGCAAG 3' |
|                                             | HvDRO1_ATG+908bp-R        | reverse 5' TGATCCATGCGCCCTAGCG 3'  |
|                                             | HvDRO1_TAA-424bp-F        | forward 5' AGGAGCTTTCTCCCTAATC 3'  |
| **Amplification for**                       | HvDRO1_ATG-25bp-F         | forward 5' CCTCCTCCATTCCTAAGCAAG 3' |
| **HvDRO1 cDNA**                             | HvDRO1_TAA+19bp-R         | reverse 5' ACACCTGGCCTCAATGGAAC 3' |
| **Sequence analysis for**                   | HvDRO1_CDSATG+24bp-F      | forward 5' AAAAGATCAGGGGAACATG 3'  |
| **HvDRO1 cDNA**                             | HvDRO1_CDSATG+445bp-R     | reverse 5' CTTCTTCCACGGAATCTC 3'   |
|                                             | HvDRO1_CDSATG+424bp-F     | forward 5' AGGAGCTTTCTCCCTAATC 3'  |
| **Amplification for**                       | HvqSOR1_ATG-33bp-F        | forward 5' CCAGCAGCTGTACTTGAAGG 3' |
| **HvqSOR1 ORF**                             | HvqSOR1_TAA+24bp-R        | reverse 5' TAAACCTTTGTTGCTCTCTCG 3' |
| **Sequence analysis for**                   | HvqSOR1_ATG+411bp-F       | forward 5' GACTTCACACCATCGGGAG 3'  |
| **HvqSOR1 ORF**                             | HvqSOR1_ATG+557bp-R       | reverse 5' CAGTTGGAACCTGTGAG 3'   |
| **HvDRO1 CAPS marker**                      | HvDRO1_CAPS-F             | forward 5' GTGGTCACTGCTCGGACCTT 3' |
|                                             | HvDRO1_CAPS-R             | reverse 5' GCGGGTACATGGCCTGTGGT 3' |
| **HvqSOR1 dCAPS marker**                    | HvqSOR1_ATG+777bp-F       | forward 5' AGCCTCAAAGATGCGATTG 3'  |
|                                             | HvqSOR1_dCAPS-Sall-R      | reverse 5' ACTTGAAAGATACACATGTAATCTCTGTGTCAGT 3' |
Table S2. Result of BLASTP search of OsDRO1 and OsqSOR1 orthologs in barley

| Query               | Subject                  | E-value     | Identity | CCL domain |
|---------------------|--------------------------|-------------|----------|------------|
| OsDRO1 (Os09t0439800-01) | HORVU.MOREX.r2.5HG0398200 | 1.69E-139   | 74.6     | contain    |
| OsDRO1 (Os09t0439800-01) | HORVU.MOREX.r2.2HG0103490 | 2.18E-14    | 31.0     | contain    |
| OsDRO1 (Os09t0439800-01) | HORVU.MOREX.r2.4HG0337310 | 0.014       | 24.3     | contain    |
| OsDRO1 (Os09t0439800-01) | HORVU.MOREX.r2.5HG0414410 | 0.14        | 25.3     | contain    |
| OsDRO1 (Os09t0439800-01) | HORVU.MOREX.r2.1HG0031030 | 0.66        | 34.0     |            |
| OsDRO1 (Os09t0439800-01) | HORVU.MOREX.r2.6HG0525530 | 1.7         | 26.1     | contain    |
| OsDRO1 (Os09t0439800-01) | HORVU.MOREX.r2.6HG0451960 | 2           | 44.8     | contain    |
| OsDRO1 (Os09t0439800-01) | HORVU.MOREX.r2.5HG0359290 | 2.7         | 28.6     |            |
| OsDRO1 (Os09t0439800-01) | HORVU.MOREX.r2.2HG0116110 | 3.4         | 34.2     |            |
| OsDRO1 (Os09t0439800-01) | HORVU.MOREX.r2.1HG0049030 | 5.1         | 30.4     |            |
| OsDRO1 (Os09t0439800-01) | HORVU.MOREX.r2.2HG0131660 | 6.9         | 35.5     |            |
| OsDRO1 (Os09t0439800-01) | HORVU.MOREX.r2.4HG0307680 | 9.9         | 28.8     |            |
| OsqSOR1 (Os07t0614400-01) | HORVU.MOREX.r2.2HG0103490 | 1.73E-104   | 60.9     | contain    |
| OsqSOR1 (Os07t0614400-01) | HORVU.MOREX.r2.5HG0398200 | 5.49E-16    | 30.4     | contain    |
| OsqSOR1 (Os07t0614400-01) | HORVU.MOREX.r2.2HG0098000 | 0.65        | 26.7     |            |
| OsqSOR1 (Os07t0614400-01) | HORVU.MOREX.r2.1HG0062480 | 1.5         | 30.0     |            |
| OsqSOR1 (Os07t0614400-01) | HORVU.MOREX.r2.3HG0195650 | 2           | 29.2     |            |
| OsqSOR1 (Os07t0614400-01) | HORVU.MOREX.r2.6HG0463880 | 2.5         | 35.4     |            |
| OsqSOR1 (Os07t0614400-01) | HORVU.MOREX.r2.3HG0275420 | 8.2         | 41.7     |            |
Table S3. List of accessions for sequence analysis

| Accession                  | RGA (°) | Distance from ATG (Putative effect of the SNP) | HvDRO1 | HvqSOR1 |
|---------------------------|---------|-----------------------------------------------|--------|---------|
|                           |         | 1438 (mis-splicing) 1848 (M165I) 1074 (S249R) |        |         |
| Hokurikukawa48            | 0.0     | T G A                                         |        |         |
| Shunrai                   | 0.0     | T G A                                         |        |         |
| Yumesakiboshi             | 1.1     | G G A                                         |        |         |
| Fiber-Snow                | 2.7     | T G A                                         |        |         |
| CDC Battlefield           | 3.5     | T G A                                         |        |         |
| Aizuhadaka3               | 5.0     | T G A                                         |        |         |
| Kumogatashirazu           | 5.5     | G A A                                         |        |         |
| Hokurikukawa39            | 6.2     | G G A                                         |        |         |
| Alexis                    | 11.2    | G A A                                         |        |         |
| Ichibanboshi              | 11.7    | G G A                                         |        |         |
| AC Metcalfa               | 11.7    | G A A                                         |        |         |
| Kirarimochi               | 12.9    | G G C                                         |        |         |
| Carlsberg II              | 13.2    | G A C                                         |        |         |
| Kaiki76                   | 14.0    | T G A                                         |        |         |
| Nishinohoshi              | 14.3    | G G A                                         |        |         |
| Hokurikukawa35            | 14.4    | T G A                                         |        |         |
| Stein                     | 14.7    | G G C                                         |        |         |
| Koharuniyo                | 15.1    | G G A                                         |        |         |
| Golden Melon              | 16.1    | G G A                                         |        |         |
| Mikamo Golden             | 17.6    | G G A                                         |        |         |
| Miharu Gold               | 18.3    | G G A                                         |        |         |
| CDC Fiber                 | 19.6    | G G A                                         |        |         |
| Silksnow                  | 19.8    | T G A                                         |        |         |
| Hungarian                 | 20.0    | G G A                                         |        |         |
| Hokurikukawa50            | 21.4    | T G A                                         |        |         |
| Sukai Golden              | 21.7    | G G A                                         |        |         |
| Mokusekikou3              | 22.9    | G G A                                         |        |         |
| Shikokuhadaka110          | 25.5    | G G A                                         |        |         |
| Toyonokaze                | 26.7    | G G A                                         |        |         |
| Miyukiomugi               | 27.6    | G G A                                         |        |         |
| Daishimochi               | 28.9    | G G A                                         |        |         |
| Chariot                   | 29.3    | G G C                                         |        |         |
| Benkeimugi                | 29.6    | G G A                                         |        |         |
| Schooner                  | 29.8    | G A C                                         |        |         |
| New Golden                | 30.3    | G A A                                         |        |         |
| Chikurinibaraki1          | 30.4    | G G A                                         |        |         |
| Ryofu                     | 30.6    | G G A                                         |        |         |
| Dorirumugi                | 30.9    | G G A                                         |        |         |
| Aizu1                     | 31.0    | G G A                                         |        |         |
| Minorimugi                | 32.3    | G G A                                         |        |         |
| Hokurikukawa38            | 35.8    | G G A                                         |        |         |
| Mannenboshi               | 36.0    | G G A                                         |        |         |
| Kashimamugi               | 36.5    | G G A                                         |        |         |
| Taishomugi                | 38.7    | G G A                                         |        |         |
| Karl                      | 40.8    | G A C                                         |        |         |
| Chevallier                | 49.7    | G A C                                         |        |         |
| Beau Fiber                | 57.5    | G A C                                         |        |         |

The information of RGA of barley accessions was obtained from Konishi et al. (2021)
| Accession       | Distance from ATG | Intron | Intron | Intron | Intron | Intron | Intron | Intron | Intron | Intron | Exon | Intron | Intron | Intron | Intron |
|-----------------|-------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|------|--------|--------|--------|--------|
| Chevallier      | T A               | CCT    | T A    | A T    | A A    | A G    | A C    | A T    | G T    | T A   |      |        |        |        |        |
| Scooner         | T A               | CT     | G T    | A G    | A G    | A C    | A T    | G T    | T A   |      |      |        |        |        |        |
| New Golden      | T A               | CCT    | T A    | A T    | A A    | A G    | A C    | A T    | G T    | T A   |      |      |        |        |        |        |
| AC Metcalfe     | T A               | CCT    | T A    | A T    | A A    | A G    | A C    | A T    | G T    | T A   |      |      |        |        |        |        |
| Kumogatashirazu | T A               | CCT    | T A    | A T    | A G    | A G    | A C    | A T    | G T    | T A   |      |      |        |        |        |        |
| Karl            | T A               | CCT    | T A    | A T    | A A    | A G    | A C    | A T    | G T    | T A   |      |      |        |        |        |        |
| CarlsbergII     | T A               | CCT    | T A    | A T    | A A    | A G    | A C    | A T    | G T    | T A   |      |      |        |        |        |        |
| Alexis          | T A               | CCT    | T A    | A T    | A A    | A G    | A C    | A T    | G T    | T A   |      |      |        |        |        |        |
| Yumesakiboshi   | C A               | GTACGT | T T    | A A    | A G    | A G    | C C    | G T    | T C    |      |      |      |        |        |        |        |
| Toynokaze       | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | G C    | C C    | T G    |      |      |      |        |        |        |        |
| Sukai Golden    | C T               | GTACGT | TT    | C A    | G T    | A G    | A G    | G T    | T C    | T T    |      |      |      |        |        |        |        |
| Stein           | T A               | GTACGT | TT    | C G    | G T    | A G    | A G    | G C    | C T    | T T    |      |      |      |        |        |        |        |
| Shikokuhadaka110| C A               | GTACGT | TT    | C G    | A G    | G G    | G G    | G C    | C G    | T C    |      |      |      |        |        |        |        |
| Ryofu           | C T               | GTACGT | TT    | C G    | G T    | A G    | A G    | G T    | T C    | T T    |      |      |      |        |        |        |        |
| Nishinohoshi    | C T               | GTACGT | TT    | C G    | G T    | A G    | A G    | G G    | T C    | T T    |      |      |      |        |        |        |        |
| Mokuseikoku3    | C A               | GTACGT | TT    | C G    | A C    | G G    | G G    | G C    | C C    | G T    |      |      |      |        |        |        |        |
| Minorimugi      | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | G C    | C G    | T C    |      |      |      |        |        |        |        |
| Mikamo Golden   | C T               | GTACGT | TT    | C G    | A G    | T A    | G G    | T C    | T T    | T T    |      |      |      |        |        |        |        |
| Mihara Gold     | C T               | GTACGT | TT    | C G    | G T    | A G    | A G    | G T    | T C    | T T    |      |      |      |        |        |        |        |
| Mammenboshi     | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | G C    | C G    | T C    |      |      |      |        |        |        |        |
| Koharanjiyo     | C T               | GTACGT | TT    | C G    | A G    | T A    | G A    | G G    | T C    | T T    |      |      |      |        |        |        |        |
| Kirarimochi     | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | G C    | C C    | G T    |      |      |      |        |        |        |        |
| Ichibamushii    | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | G C    | C C    | G T    |      |      |      |        |        |        |        |
| Hungarian       | C T               | GTACGT | TT    | C G    | A A    | T A    | G A    | G G    | T C    | T T    |      |      |      |        |        |        |        |
| Hokurikakawa59  | C A               | GTACGT | TT    | C G    | A C    | G G    | G G    | G C    | C C    | T C    |      |      |      |        |        |        |        |
| Hokurikakawa38  | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | G C    | C C    | T C    |      |      |      |        |        |        |        |
| Golden Melon    | C T               | GTACGT | TT    | C G    | G G    | T A    | G A    | G G    | T C    | T T    |      |      |      |        |        |        |        |
| Daishimochi     | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | G C    | C C    | T C    |      |      |      |        |        |        |        |
| Chariot         | C T               | GTACGT | TT    | C G    | G G    | T A    | G A    | G G    | T C    | T T    |      |      |      |        |        |        |        |
| CDCFiber        | C T               | GTACGT | TT    | C G    | G G    | T A    | G A    | G G    | T C    | T T    |      |      |      |        |        |        |        |
| Beau Fiber      | C A               | GTACGT | TT    | C G    | A T    | G G    | G G    | G G    | C C    | T C    |      |      |      |        |        |        |        |
| Benkeimugi      | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | G G    | C C    | G T    |      |      |      |        |        |        |        |
| Aizu1           | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | G G    | C C    | G T    |      |      |      |        |        |        |        |
| SilkySnow       | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | T G    | C C    | G T    |      |      |      |        |        |        |        |
| Shunrei         | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | T G    | C C    | G T    |      |      |      |        |        |        |        |
| Kaisei76        | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | T G    | C C    | G T    |      |      |      |        |        |        |        |
| Hokurikakawa50  | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | T G    | C C    | G T    |      |      |      |        |        |        |        |
| Hokurikakawa48  | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | T G    | C C    | G T    |      |      |      |        |        |        |        |
| Hokurikakawa35  | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | T G    | C C    | G T    |      |      |      |        |        |        |        |
| Fiber Snow      | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | T G    | C C    | G T    |      |      |      |        |        |        |        |
| CDC Battlefold  | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | T G    | C C    | G T    |      |      |      |        |        |        |        |
| Aizuhadaka3     | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | T G    | C C    | G T    |      |      |      |        |        |        |        |
| Taishomugi      | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | G G    | C C    | G T    |      |      |      |        |        |        |        |
| Miyukimugi      | C A               | GTACGT | T C    | G G    | T A    | G G    | G G    | G G    | C C    | G T    |      |      |      |        |        |        |        |
| Kashimamugi     | C A               | GTACGT | T C    | G G    | C G    | G G    | G G    | G G    | C C    | G T    |      |      |      |        |        |        |        |
| Dorirumugi      | C A               | GTACGT | T C    | G A    | C G    | G G    | G G    | C C    | G T    | C T    |      |      |      |        |        |        |        |
| Chikurinibarakil| C A               | GTACGT | T T    | A A    | T A    | A G    | G G    | C C    | G T    | C T    |      |      |      |        |        |        |        |
Table S5. Polymorphisms of *HvqSOR1* among 47 barley accessions

| Accession               | Distance from ATG |
|-------------------------|-------------------|
|                         | Exon  | Intron |
| 1074                    |       |        |
| 1275                    |       |        |
| Yumesakiboshi           | A      | A      |
| Toyonokaze              | A      | A      |
| Taishomugi              | A      | A      |
| Shunrai                 | A      | A      |
| Stein                   | C      | C      |
| Sukai Golden            | A      | A      |
| Chevallier              | C      | A      |
| Silksnow                | A      | A      |
| Shikokuhadaka110        | A      | A      |
| Schooner                | C      | C      |
| Ryofu                   | A      | A      |
| Nishinohoshi            | A      | A      |
| New Golden              | A      | A      |
| Mokusekikou3            | A      | A      |
| Miyukiomugi             | A      | A      |
| Minorimugi              | A      | A      |
| Mikamo Golden           | A      | A      |
| Miharu Gold             | A      | A      |
| Mannenboshi             | A      | A      |
| Kumogatashirazu         | A      | A      |
| Koharunijyo             | A      | A      |
| Kirarimochi             | C      | C      |
| Kashimamugi             | A      | A      |
| Karl                    | C      | A      |
| Ichibanboshi            | A      | A      |
| Hungarian               | A      | A      |
| Hokurikukawa50          | A      | A      |
| Hokurikukawa48          | A      | A      |
| Hokurikukawa39          | A      | A      |
| Hokurikukawa38          | A      | A      |
| Hokurikukawa35          | A      | A      |
| Golden Melon            | A      | A      |
| Fiber-Snow              | A      | A      |
| Doririmugi              | A      | A      |
| Daishimochi             | A      | A      |
| Chikurinibaraki1        | A      | A      |
| Chariot                 | C      | C      |
| CDC Fiber               | A      | A      |
| CDC Battlefield         | A      | A      |
| Carlsberg II            | C      | C      |
| Beau Fiber              | C      | C      |
| Benkeimugi              | A      | A      |
| Alexis                  | A      | A      |
| Aizuhadaka3             | A      | A      |
| Aizu1                   | A      | A      |
| Kaiseki76               | A      | A      |
| AC Metcalfa             | A      | A      |