Phenotypic and transcriptomic characterization of an ABA-sensitive mutant generated by microspore embryogenesis in barley

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ABSTRACT

Barley (Hordeum vulgare L.) has been recognized as an ideal model plant to study the mechanism of environmental adaptation for crop improvement owing to its wide adaptability to abiotic stresses. Abscisic acid is an essential hormone involved in many abiotic stresses. Here, an ABA-sensitive barley mutant (abas1) was generated by microspore embryogenesis. On exogenous ABA treatment, the seedlings of abas1 presented less plant height, root length, shoot and root dry matter than the wild type, and more genes showed higher expression intensity and up-regulation models by RNA-Seq profiling. The gene ontology analysis revealed that the regulation of DEGs may lead to the activation of the ROS pathway and consumption of more energy in abas1 specifically. Furthermore, the up-regulation of two candidate genes (P450 and NsLTP) in abas1 may contribute to the variation of ABA sensitivity. Our findings provide more valuable material and information toward the understanding of the mechanism of ABA response in barley.

Introduction

Plants experience many different kinds of abiotic stresses, including salinity, cold, heat, and drought over their life cycles (Tuteja 2007). These stresses show significant adverse impacts on the yield of crops. The actual yield of most crops is about half of their potential due to environmental stimuli (Tuteja et al. 2012). Breakthroughs in the molecular mechanisms of environmental adaptation have opened new avenues for crop breeding with good tolerance to abiotic stresses. Among the major cereal crops, barley (Hordeum vulgare L.) exhibits a high adaptive capacity. Given its natural tolerance to drought and salinity, barley is also a model plant for cereal crops in stress biology research (Gürel et al. 2016). Thus, a comprehensive understanding of the molecular mechanisms of barley tolerance to abiotic stress will benefit the breeding of other important cereal crops such as wheat and rice. To characterize the molecular basis, any mutant sensitive to stress is ideal for the study to establish a link between phenotype and the corresponding genes.

Plants have evolved sophisticated innate systems to protect themselves from abiotic stresses. Membrane receptors (usually receptor-like kinase) first perceived the stress signals under extracellular stress, such as abscisic acid (ABA), which activates the complex intracellular signaling cascade, and subsequently, results in the generation of secondary signal molecules such as inositol phosphates, Ca2+, reactive oxygen species (ROS). Then, the stress signals transduce to the nucleus and induce stress-responsive genes, ultimately regulating the biologic process conferred plant tolerance to stresses (Tuteja 2007). Under abiotic stress, hormone networks are efficiently regulated in plants. Among various plant hormones, ABA is defined as an important stress-related hormone and plays a central role in many abiotic stresses to maintain cellular homeostasis (Zhang et al. 2006). The accumulation of ABA in plant organisms could regulate the stomatal aperture through the stress-responsive genes, as well as the plant response to seed maturation and water shortage (Yoshida et al. 2019). Although ABA is well characterized in model plants such as Arabidopsis, the understanding of the mechanisms in crops is still needed, especially for the crops with good environmental adaptation such as barley.

Microspores retain the potential to form haploid embryos (Microspore embryogenesis) that eventually develop into whole plants under specific conditions. Microspore embryogenesis has emerged as an efficient tool to produce double haploid (DH) plants and is widely exploited for plant breeding (Touraev et al. 1997; Soriano et al. 2013). Microspore embryogenesis was induced under abiotic stress, and the haploid cells are far more prone to mutation during in vitro culture. During cultures, the variation further increases in the plant cells, because the cell cultures are heterogeneous with regard to the metabolic productivity of plant cells (Bhojwani and Dantu 2013). Moreover, the somaclonal variation was easily fixed in a complete homozygous state via chromosomal doubling (Szarejko and Forster 2007; Lu et al. 2008). In addition, chemical mutagenesis has been
successfully applied in several cruciferous plants through microspore embryogenesis (Barro et al. 2001; Beath et al. 2005; Ferrie et al. 2008; Liu et al. 2010; Gao et al. 2018). In our previous work, an efficient protocol of isolated microspore culture has been established for barley variety Hua 30 (Lu et al. 2008). In the present study, one ABA-sensitive mutant (under 75 μM ABA) was identified (named as abas1) from the DH lines via microspore embryogenesis for barley cv. Hua 30.

In this study, we found a mutant type that is more sensitive to ABA when received 75 μM ABA treatment. RNA-seq was employed to compare the gene expression patterns between the mutant and its wildtype Hua 30. Our data showed the distinct phenotypic changes of the mutant compared to its wild type upon ABA treatment, and identified a few candidate genes that might contribute to the sensitive ABA signals, which provides deep insight into the understanding of the molecular mechanism of ABA tolerance in barley.

Materials and methods

Plant materials and microspore culture

Barley (Hordeum vulgare L.) cv. Hua-30, a popular cultivar cultivated in the area of Yangtze River Delta of China, was grown at the farm of Shanghai Academy of Agricultural Sciences, Shanghai, China. Microspore culture was performed as previously described by Lu et al. (2008). Briefly, the collected spikes were subjected to cold pretreatment at 4°C for 2 weeks, and the microspores were collected by crushing the anthers from the sterilized spikes. The collected microspores were isolated by filtration and centrifugation, and finally, cultured on the induction medium (N6 basal medium supplemented with 2.0 μM/L 2.4-D, 2.3 μM/L KT, and 0.25 M/L maltose) for embryogenic callus induction. After 21 days of culturing, the formed embryogenic calli in each dish were transferred to the differentiation medium (MS basal medium supplemented with 2.2 μM/L 6-BA, 7.0 μM/L KT, 0.25 μM/L NAA, and 83.3 mM/L maltose) for plant regeneration.

ABA treatments and phenotype analysis

The seeds of barley Hua 30 and abas1 were germinated on wet filter paper, and the seedlings were grown hydroponically in a modified Hoagland’s solution described by Tavakkoli et al. (2012) at 20–22°C with a photoperiod of 12 h. To find an appropriate concentration for screening DH lines, the seedlings of Hua 30 at the two-leaf stage were treated with ABA at concentrations of 0, 50, 75, 100, and 150 μM/L. After 7 days treatment, the plant height and root length were measured. For ABA treatments to screen the DH plants, two-leaf stage old DH plants generated from Hua 30 by microspore embryogenesis and the original Hua 30 were subjected to 75 μM/L ABA, the plant height was measured after 7 days treatment. For ABA treatments on abas1, two-leaf stage old hydroponically grown Hua 30 and abas1 seedlings were subjected to 75 μM/L ABA for 7 days. The plant height, root length, shoot and root dry weight were measured after 7 days treatment. The leaves of plants with and without ABA treatment were sampled at 7 days post treatment for RNA isolation. All experiments except ABA treatments for DH plants have three biological repetitions.

RNA extraction and preparation of cDNA library

The total RNA of Hua 30 and abas1 plant samples was extracted using TRIzol regent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. RNA integrity was confirmed using the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA libraries can be constructed when 28S/18S of each sample ≥ 0.7 and RIN of each sample ≥ 7. The RNA libraries for transcriptome sequencing were constructed according to the Illumina RNA Seq library kit (Illumina, Inc.). The total RNA was digested by DNase I. Then, poly-A containing mRNA was enriched by Oligo (dT) attached magnetic beads, followed by random fragmentation of mRNA into small segments. The first and the second strand cDNA were synthesized using the fragments as templates, then followed by end repairing. The ends of DNA fragments were modified and ligated with adapters, and the cleaned ligation products (300–350 bp) were enriched by the PCR (15 cycles) with random primers (random hexamers), followed by gel purification. Amplified libraries were checked by the Agilent 2100 Bioanalyzer (Agilent, Inc.).

RNA sequencing and data analysis

RNA sequencing was performed using Illumina HiSeqTM 4000 platform (Illumina, Inc.) for 150 bp paired-ends sequencing in Shanghai OE Biotech Co., Ltd. Quality control (QC) for the raw data were trimmed by removing all empty and low-quality reads (Q<30 and length <50 bp), as well as all adaptor sequences to obtain clean reads. Putative transcripts annotations were identified by searching the listed annotations of high confidence (HC) genes (2016) (http://webblast.ipk-gatersleben.de/barley_ibsc/downloads/Hv_IBSC_PGSB_r1_HighConf.gtf.gz). The levels of gene expression were calculated by fragments per kilo bases per million reads (FPKM) using the reads mapped to the reference sequence. DEGs were identified using the DESeq functions estimate size factors and the nbinom Test to the reference sequence. The resulting P-values were adjusted using Benjamini and Hochberg’s approach for controlling the false discovery rate. Genes with an adjusted P value <0.05 were set as the threshold for significantly differential expression. The GO enrichment analysis was performed using the DEGs. The GO enrichment analysis was performed to assign possible functional categorization using the AgriGO tool (http://systemsbiology.cau.edu.cn/agriGOv2/).

Quantitative RT–PCR analysis

To validate the expressions of DEGs, 11 identified candidate genes were randomly selected for the qRT-PCR analysis. The sequences of these candidate DEGs were obtained from the website (http://plants.ensembl.org/Hordeum_vulgare/Info/Index), and the primer pairs were designed using Primer3 (http://www.premierbiosoft.com/) according to the reference sequences. The selected gene name and primer
information is listed in Supplementary Table S1. First-strand cDNA was synthesized from about 1 µg of total RNA using Super Script™ reverse transcriptase (Takara, Dalian, China). The amplification reactions were performed in the ABI 7500 fast instrument (Applied Biosystems, USA) and the SYBR Premix Ex Taq™ Kit (Life, USA) was used following the manufacturers’ instructions. The house-keeping gene of beta-actin in barley was used as an internal control (Goddall et al. 2013). The comparative CT method (ΔΔCT method) of quantification was used to quantify the relative expression of specific genes (Livak and Schmittgen 2001).

Results

Morphological screening for barley ABA-sensitive mutant

To determine an appropriate concentration in examining the sensitivity of barley seedlings to ABA stress, the original barley cultivar Hua 30 was first subjected to hydroponic culture with different concentrations of ABA. As shown in Figure 1, when treated with more than 75 µM/L ABA, a distinct phenotypic change was observed in the two-leaf-old seedlings of Hua 30 (Figure 1(A)). The plant height and root length were significantly decreased when received treatment with 75 µM/L ABA or more (Figure 1(B and C)). Therefore, the concentration of 75 µM/L was sufficient to detect the phenotypic changes in the screening of ABA sensitivity mutants within the regenerated double haploid (DH) plants.

Microspores were isolated from barley cv. Hua30 anthers and subjected to in vitro culture. Finally, 30 DH lines were produced via microspore embryogenesis. All the DH lines were treated with 75 µM/L ABA for a screening. It was found that one DH line (DH12) possessed the lowest of plant height among all DH lines as well as Hua 30 (Figure 2), and it showed more sensitivity to the ABA treatment (Figure 3).

Multigenerational analysis on the inheritance of barley ABA-sensitive mutant

To check whether the mutant’s alteration on the sensitivity to ABA treatment was heritable, the progenies of M1 and M2 generations from DH line 12 were further treated with or without ABA. The DH line presents distinct phenotypic changes with smaller plant height after 7 days of ABA treatment (Figure 3). In M1 and M2 generations, the plant height, root length, and the dry weight of shoots and roots of seedlings, all decreased in both DH line 12 and Hua 30, after being treated with ABA, but the performance of the DH line was significantly lower than that in Hua 30 (Figure 4). The results showed that the growth of DH line 12 was significantly inhibited by ABA treatment, and revealed more sensitivity to ABA than the wildtype Hua 30 with a stable inheritance across multigeneration. Consequently, we named the ABA-sensitive mutant of DH line 12 as abas1.

Data analysis on the RNA sequencing of barley ABA-sensitive mutant

To compare the gene expressional patterns between abas1 and the wildtype Hua 30 under ABA stress from the whole genomic level, the cDNA libraries were constructed from
12 samples separately and sequenced on the Illumina sequencing platform. On average, 49.39 million raw reads were generated from each library. After removing the low-quality reads, the total number of remained reads per library ranged from 46.87 to 47.62 million. The "Phred value" > 30 (Q30) of each library ranged from 92.64% to 93.27%. The number of reads that ranged from 95.33% to 95.79%, were mapped to the barley reference genome. Furthermore, the GC percentage of each library ranged from 57% to 58.08% (Table 1). Therefore, the transcriptome data of all samples presented a high quality and were reliable for further bioinformatics analysis.

### Comparative analysis of abas1 and Hua 30 transcripts

To characterize the specificity of gene expresional patterns in the abas1 mutant, the whole transcripts of abas1 and Hua 30 with or without ABA treatment were compared. The average expresional intensity of whole genomic genes (FPKM > 1) was different in both abas1 and Hua 30 under ABA stress. Interestingly, the expresional intensity became lower in Hua 30 after ABA treatment; however, it became higher in abas1 (Figure 5(A)).

**Table 1.** An overview of sequencing and assembly of Hua 30 and abas1 samples.

| Samples     | Raw reads | Clean reads | Total mapped | Q30    | GC    |
|-------------|-----------|-------------|--------------|--------|-------|
| H30-1       | 49.54M    | 47.39M      | 45223337(95.44%) | 92.92% | 57.52%|
| H30-2       | 49.08M    | 47.02M      | 44971974(95.65%) | 93.04% | 57.11%|
| H30-3       | 49.67M    | 47.46M      | 45328530(95.52%) | 92.65% | 57.03%|
| H30-ABA-1   | 49.65M    | 47.61M      | 45117222(95.60%) | 93.14% | 57.16%|
| H30-ABA-2   | 48.99M    | 46.96M      | 449346(95.69%)   | 93.01% | 57.50%|
| H30-ABA-3   | 48.97M    | 46.87M      | 4493770(95.79%)  | 93.04% | 57.87%|
| abas1-1     | 49.59M    | 47.31M      | 45179611(95.49%) | 92.76% | 57.00%|
| abas1-2     | 49.21M    | 47.34M      | 45423190(95.57%) | 93.27% | 57.33%|
| abas1-3     | 49.06M    | 47.02M      | 4493715(95.36%)  | 93.05% | 57.43%|
| abas1-ABA-1 | 49.70M    | 47.62M      | 4516404(95.38%)  | 92.92% | 58.08%|
| abas1-ABA-2 | 49.82M    | 47.55M      | 45324029(95.33%) | 92.64% | 58.06%|
| abas1-ABA-3 | 49.43M    | 47.33M      | 45180290(95.45%) | 92.98% | 57.77%|

**Figure 3.** Phenotype of Hua 30 (H30) and abas1 with and without ABA treatment. Bar = 10 cm.

**Figure 4.** Plant height, root length, shoot dry weight, and dry weight of roots in Hua 30 and DH plant (abas1) with or without ABA treatment. Mock indicates a plant without ABA treatment. M1 and M2 indicate DH plant generation. Different letters indicate statistically significant differences (P < 0.05, one-way ANOVA).
The number of DEGs was counted for abas1 and Hua 30 separately under ABA treatment against their mocks. The expression patterns of DEGs displayed a large difference in the up-regulated group. There were 577 down-regulated genes and 236 up-regulated genes in Hua 30, while there were 495 down-regulated genes and 1670 up-regulated genes for the abas1 mutant (Figure 5(B)). It was found that the number of up-regulated genes increased about 7 folds in the abas1 mutant over that in Hua 30.

Venn diagrams were further constructed between abas1 and Hua 30 using the DEGs (Figure 5(C)). In summary, 534 DEGs were specifically detected in Hua 30, 1886 DEGs in abas1, and 244 DEGs (68 up- and 176 down-regulated) were detected in both groups. These results indicated that gene expression changed dramatically in abas1 under ABA treatment.

Gene ontology (GO) analysis on DEGs between abas1 and Hua 30

To detect the significant biological processes in abas1 and Hua 30 under ABA stress, GO enrichment was performed using DEGs based on the Venn diagram analysis. The DEGs were separated into three parts: part A consisted of 534 DEGs specifically detected in the group of Hua30-ABA vs Hua30-mock, part B consisted of 1886 DEGs specifically detected in the group of abas1-ABA vs abas1-mock, and part C consisted of 244 DEGs shared within two groups.

For part A (specifically in Hua 30 group), 6 functional groups were enriched in two GO categories including cellular component and molecular function (Figure 6(A)). Heme binding, monooxygenase activity, and metal ion binding were three functional groups under the category of molecular function. An extracellular region, integral component of the membrane, and intracellular membrane-bounded organelle were other functional groups under the category of cellular components.

For part B (specifically in the abas1 group), 23 functional groups were enriched in all three GO categories, including biological process, cellular component, and molecular function (Figure 6(B)). Xylan catabolic process, guard cell differentiation, reactive oxygen species metabolic process, cell wall organization, peptide biosynthetic process, response to oxidative stress, carboxylic acid biosynthetic process, and ubiquitin-dependent protein catabolic process were eight functional groups under the category of biological process. Plastid, plasmodesma, cytosol, ribosome, apoplast, plasma membrane, cell wall, and endomembrane system were eight functional groups under the cellular component category. Xylanase activity, catalytic activity, rRNA binding, peroxidase activity, heme binding, metal ion binding, and ubiquitin protein ligase activity were seven functional groups under the category of molecular function.

For part C (shared within Hua 30 and abas1 groups), 12 functional groups were enriched in all three GO categories, including biological process, cellular component, and molecular function (Figure 6(C)). Response to stress, cellular response to starvation, response to external stimulus, RNA catabolic process, and lipid metabolic process were five functional groups under the category of biological process. An extracellular region, membrane-bounded organelle, and cytoplasm were three functional groups under the cellular component category. Catalytic activity, nuclease activity, hydrolase activity, and phosphatase activity were four functional groups under the category of molecular function. These results showed the common and the difference of interest genes between abas1 and Hua 30 in response to ABA stress. Especially, the enriched interests under the category of biological processes showed an obvious difference between abas1 and Hua 30 under ABA stress.

Comparison of gene expression patterns between abas1 and Hua 30

The genes of interest with significant change (log2(fold change) ≥ 1 or ≤ −1) in the groups (H30-ABA vs H30-mock, abas1-ABA vs abas1-mock) were selected for a further analysis of gene expression patterns (Figure 7, Supplemental data 1). The fold-change values were used to generate heat maps. When fold change was less than 1, the result was negative inverse. The expression patterns were classified into three types, including up-regulation (fold change) ≥ 2), down-regulation (fold change ≤ −2), and unchanged (fold change between −2 and 2). As to the genes of interest unchanged in Hua 30 under ABA stress, most of them were up-regulated in abas1, and a small number of them were down-regulated (Figure 7(A)). As to the genes of
interest up-regulated in Hua 30 under ABA stress, about one-third of them kept similar patterns, and more than half of them were unchanged in abas1 (Figure 7(B)). As to the genes of interest down-regulated in Hua 30 under ABA stress, about half of them kept similar patterns, one-third of them unchanged, and a small number of genes were up-regulated in abas1 (Figure 7(C)). These genes with different expression patterns in abas1 may play important roles in the sensitivity to ABA stress.

Identification of candidate genes related to abas1 sensitivity to ABA

We further identified the genes that related to the ABA sensitivity of abas1. Based on the above results, DEGs which were down-regulated in Hua 30 but up-regulated in abas1 under ABA treatment (Figure 7(C)) were selected for further analysis. Using the threshold of FPKM in abas1-ABA ≥1, log2 (fold change) ≤−1 in Hua 30 and ≥1 in abas1, two up-regulated genes of interest were identified following the strategy. These genes encoded cytochrome P450 and non-specific lipid transfer protein (NsLTP). Heat maps of the two genes were constructed using fold-change values in abas1 and Hua 30 (Figure 8(A)). They display a clear trend that the expression levels of two genes were significantly decreased in Hua30, while significantly increased in abas1 under ABA treatment.

Previous studies have identified many genes that were involved in plant response to the ABA pathway (Todaka et al. 2019). In our data, we examined their expression patterns in abas1 and Hua 30 under ABA stress (Figure 8(B–E)). Heat maps of SNRK (SNF1-related kinases), PYR (Pyrabactin resistance 1-like protein), ABI (Abscisic Acid insensitive), and PP2C gene families were constructed using the fold-change values in abas1 and Hua 30. The results showed that most of these genes have similar expression patterns in two materials under ABA treatment. One PP2C gene (HORVU7Hr1G079310) was significantly up-regulated in abas1.

Figure 6. Functional classification of DEGs in Hua 30 and abas1. (A) GO enrichment using DEGs from Hua 30-ABA vs Hua 30-mock specifically (part A). (B) GO enrichment using DEGs from abas1-ABA vs abas1-mock specifically (part B). (C) GO enrichment using DEGs overlapping in Hua 30-ABA vs Hua 30-mock and abas1-ABA vs abas1-mock (part C). The y axis represents a rich ratio. The x axis represents enriched function categories.
after ABA treatment, which also has remarkable accumulation in Hua 30 under ABA treatment.

Validation of RNA-seq results by quantitative RT-PCR

To validate the results of the gene expression from RNA-seq data, 11 DEGs were selected for the qRT-PCR analysis. These DEGs include two candidate genes cytochrome P450 (HORVU2Hr1G072400), NsLTP (HORVU5Hr1G104570), and 9 randomly selected genes. There was a good concordance ($R^2 = 0.84$) between the RNA-seq data and qRT-PCR analysis (Figure 9(A, B)), indicating that the gene expression levels by the DGE analysis were reliable.

Discussion

In this study, the growth of $abas1$ and Hua 30 was inhibited under ABA treatment, but $abas1$ exhibited more sensitivity than Hua 30. The root is the first organ to sense exogenous ABA. A low concentration of ABA promotes root development, while a high concentration of ABA acts as an inhibitor of plant development (Zhang et al. 2010). Root growth was obviously suppressed in both materials under ABA stress, but the root length and root dry weight of $abas1$ were less than that of Hua 30. Similar tendencies appeared in plant height and shoot dry weight of both materials. Microspores are single haploid cells, so plants from microspore embryogenesis are in complete homozygous status. The ABA-sensitivity of $abas1$ was heritable across M1 and M2 generations.

Based on these data, the DH line 12 was identified as an ABA-sensitive mutant ($abas1$) of Hua 30.

Plants do not have a nervous system, and signaling requires long-distance transduction in whole plants in response to abiotic stress. The expression of thousands of genes involved in the signaling transduction was regulated during the response. Thus, it is essential to elucidate the molecular mechanisms of plant response to abiotic stress at late phases. In this study, DEGs were carefully examined in $abas1$ and Hua 30 compared with each mock. The number of total DEGs was decreased in Hua 30, but increased in $abas1$ under ABA stress. More down-regulated genes were detected in Hua 30 than up-regulated genes under long-time ABA treatment (7 days). Urano et al. (2017) showed that more genes were down-regulated under a long time (3 days) of drought stress in Arabidopsis seedlings with high ABA accumulation. The gene expression patterns were similar in rice under 3 days of drought stress accompanied by a significant accumulation of ABA (Maruyama et al. 2014). Interestingly, more up-regulated genes were detected than down-regulated genes under a long time ABA stress in $abas1$. Moreover, $abas1$ has a different response to ABA stress compared to wildtype Hua 30, and the mechanism of ABA stress in the two materials may be entirely different.

GO analyses were enriched to distinguish the different mechanisms between $abas1$ and Hua 30 under long-time ABA stress. There is no doubt that the common function categories in both materials were response to stress, cellular response to starvation, response to external stimulus, RNA catabolic process, and lipid metabolic process. But in $abas1$, more genes were related to the xylan catabolic process, cell wall organization, oxidation–reduction process, and carboxylic acid biosynthetic process. It was suggested that more genes participated in stress response and plant growth regulation in $abas1$ under long-time exogenous ABA stress.
Oxidation-reduction process is a critical process in plant response to environmental stress (Shao et al. 2008). Exogenous ABA leads to the accumulation of ROS (reactive oxygen species), including superoxide anion (O2-) and hydrogen peroxide (H2O2) (Jiang and Zhang 2001). ROS usually acts as a second messenger in many biological processes, but a large amount of ROS will cause oxidative stress (Borland et al. 2006). In plant cells, oxidative stress generally increased the production of carbonylated proteins (Møller and Kristensen 2004). Protein synthesis overload in cells caused endoplasmic reticulum (ER) stress which is initiated by different types of stimulators, such as biotic or abiotic stresses. ER stress also leads to the accumulation of ROS (Janssens et al. 2014). Protein synthesis also needs more energy in plant cells. Carbohydrates produced by photosynthesis in plants are major energy resources. Previous research showed that the application of exogenous ABA increased the accumulation of carbohydrates in wheat and up-regulated the genes encoding key enzymes involved in the carbohydrate metabolic process (Liu et al. 2013). In summary, these results suggested that long-time ABA stress caused severe oxidative stress in abas1, and more energy was used for anti-oxidative stress, eventually leading to the inhibition of plant growth.

Gene expression patterns in abas1 and Hua 30 after ABA treatment exhibited a clear difference between the two materials. The expression of most DEGs was up-regulated in abas1 but unchanged in Hua 30. This may contribute to ABA sensitivity in the mutant of abas1. Most genes from SNRK, PYR, ABI, and PP2C families showed similar patterns between the two materials after a long time of ABA treatment. It was suggested that these genes function as normal in the ABA-responsive pathway of abas1 and Hua 30. A few genes were down-regulated in Hua 30 but up-regulated in abas1 under long time ABA stress. Two candidate genes involved in the ABA-sensitivity of abas1 were further validated by the qRT-PCR assays, which showed different expression patterns between abas1 and Hua 30. One of the candidate genes encoded cytochrome P450 protein (CYPs). In plant cells, ABA is mainly inactivated by 8'-hydroxylation, which is catalyzed by cytochrome P450 (Kushiro et al. 2004). Moreover, NAD(P)H-dependent electron transport involving CYPs produces ROS in endoplasmic reticulum (Mittler 2002). Overexpression of SlCYP707A1 in tomatoes caused smaller leaf surface area and slower growth than that in wildtype (Nitsch et al. 2009). It is possible to explain that the up-regulation of P450 in abas1 to cope with ABA stress might suppress the growth more greatly than that in Hua30 under ABA treatment. Another candidate gene encodes a non-specific lipid transfer protein (nsLTP), which was reported as a small, basic protein associated with the functions of the cell wall in stress tolerance (Yubero-Serrano et al. 2003). Xing et al. (2020) showed that cell wall organization is one of the main processes in response to exogenous ABA in sugar beetroots. Similarly, in our study, the functional group of the cell wall was enriched from the DEGs, specifically in the abas1 group by the GO analysis. The growth of roots was suppressed more heavily and the expression of nsLTP was significantly up-regulated in the mutant of abas1, which is consistent with the sensitivity of abas1 in response to ABA.

In conclusion, we found one barley ABA-sensitive mutant (abas1) generated from microspore mutagenesis, which showed smaller plant size after long-term ABA stress. A putative network underlying the mechanism of abas1 sensitive to ABA was proposed. Upon long-term ABA stress, more genes involved in the biological processes, such as
cell wall organization, oxidation–reduction biological process, and carbohydrate metabolic process were up-regulated in abas1, which may lead to the activation of ROS pathway and consumption of more energy. In addition, the up-regulation of P450 and nsLTP may involve in the adaption of ABA stress and the adjustment of root development. All these transcriptomic changes uncover the sensitive response to ABA in the mutant of abas1. The mutation site or gene(s) in abas1 awaits further investigation.

Data availability statement
The datasets generated and analyzed during the current study are available in the National Center for Biotechnology Information. The raw data for the RNA-seq can be downloaded at https://www.ncbi.nlm.nih.gov/sra/PRJNA771492.

Disclosure statement
No potential conflict of interest was reported by the author(s).

Funding
This work was supported by the National Key Research and Development Program of China [grant number 2018YFD0100702-5], Shanghai Agriculture Applied Technology Development Program, China [grant number G2016060102], the National Natural Science Foundation of China (31801353), and the Earmarked fund for China Agriculture Research System [grant number CARS-05-01A-02].

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