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ORIGINAL ARTICLE

Receptor-like kinase HAESA-like 1 positively regulates seed longevity in *Arabidopsis*

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Abstract

Main conclusion **Based on the phenotypic, physiological and transcriptomic analysis, receptor-like kinase HAESA-like 1 was demonstrated to positively affect seed longevity in Arabidopsis.**

Abstract Seed longevity is very important for both genetic resource conservation and crop production. Receptor-like kinases (RLKs) are widely involved in plant growth, development and stress responses. However, the role of most RLKs, especially in seed longevity, is largely unknown. In this study, we report that *Arabidopsis* HAESA-like 1 (AtHSL1) positively regulated seed longevity. Disruption of HSL1 significantly decreased the germination rate to 50% at 7 days after cold stratification (DAC), compared with that of the wild type (93.5% at 7 DAC), after accelerated aging treatment. Expression of the *HSL1* gene in *hsl1* basically restored the defective phenotype (86.3%), while *HSL1*-overexpressing lines (98.3%) displayed slower accelerated aging than WT (93.5%). GUS staining revealed *HSL1* was highly expressed universally, especially in young seedlings, mature seeds and embryos of imbibed seeds, and its expression could be induced by accelerated aging. No difference in the dyeing color and area of mucilage were identified between WT and *hsl1*. The soluble pectin content also was not different, while the adherent pectin content was significantly increased in *hsl1*. Global transcriptomics revealed that disruption of HSL1 mainly downregulated genes involved in trehalose synthesis, nucleotide sugar metabolism and protection and repair mechanisms. Therefore, an increase in adherent pectin content and downregulation of genes involved in trehalose synthesis may be the main reasons for decreasing seed longevity owing to disruption of HSL1 in *Arabidopsis*. Our work provides valuable information for understanding the function and mechanism of a receptor-like kinase, AtHSL1, in seed longevity.

Keywords Heasa-like 1 (HSL1) · Leucine-rich repeat receptor-like kinase (LRR-RLK) · Nucleotide-sugar metabolism · Pectin · Seed longevity · Trehalose synthesis

Abbreviations

DAC	Days after cold stratification
DEGs	Differentially expressed genes
GGLT	Golgi nucleotide sugar transporter 3
HAE	HAESA
HSL1	HAESA-like 1
LRR	Leucine-rich repeat
RHM	Rhamnose synthase
RLKs	Receptor like kinases
SSPs	Seed storage proteins

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Introduction

Seeds are the key reproductive organ of most flowering plants. Dry seeds greatly reduce metabolic activity and are maintained at a very low (almost static) level, which allows seeds to maintain germinability for a long time (Buitink and Leprince 2008). Seed longevity is defined as the period during which seeds maintain vigor and germination ability (Probert et al. 2007). However, seed aging or seed deterioration is an inevitable and irreversible process during storage (Zhou et al. 2020). Understanding the mechanism underlying seed longevity is of great significance to plant germplasm conservation and crop production.

Although the mechanisms underlying seed longevity are still unclear, the accumulation of reactive oxygen species (ROS) and membrane lipid peroxidation are proposed to be the most important factors affecting seed deterioration

(Bailly et al. 2008). To prolong seed longevity, plants have evolved complex protection and repair mechanisms to scavenge excessive ROS. Vitamin E (tocopherols and tocotrienols) can prevent nonenzymatic lipid oxidation, and disruption of the key enzyme genes involved in the synthesis leads to a decrease in seed longevity in *Arabidopsis* and rice (Sattler et al. 2004; Chen et al. 2016). Protective proteins, such as seed storage proteins (SSPs), late embryogenesis abundant proteins and small heat shock proteins, have significant roles in protecting macromolecules and cell membranes against ROS attack (Prieto-Dapena et al. 2006; Wu et al. 2017). The seed coat serves as a protective layer of seeds against external damage, and the structure and components influence seed longevity (Renard et al. 2020). *TRANSPARENT TESTA* regulates the polysaccharide composition in the seed coat, and its disruption affects seed longevity (Debeaujon et al. 2000). Overexpression of *ARABIDOPSIS THALIANA HOMEOBOX 25* (*ATHB25*) positively regulates GA synthesis and increases mucilage formation and then prolongs seed longevity in *Arabidopsis* (Bueso et al. 2014), where mucilage is a pectin complex secreted by specialized epidermal cells of the seed coat (Arsovski et al. 2010). Furthermore, phytohormones such as ABA and IAA are also involved in regulating seed longevity. For example, *ABSCISIC ACID INSENSITIVE 5*, the key component of the ABA response signaling pathway, significantly reduces seed longevity (Zinsmeister et al. 2016). In single deletion mutants with tryptophan-dependent auxin biosynthesis enzymes, seed longevity was affected (Pellizzaro et al. 2020).

Receptor-like kinase proteins (RLKs) usually localize to the plasma membrane, perceive signals from the environment, and lead to a series of signal transductions (Shiu and Bleecker 2001). According to their extracellular domains, RLKs can be classified into several subclasses, in which leucine-rich repeat (LRR) RLKs constitute the largest family. In *Arabidopsis*, approximately 225 LRR-RLK members belonging to 23 subfamilies have been identified. Sixty of them have been reported for their biological functions (Huang et al. 2016). For instance, *BRASSINOSTEROID-INSENSITIVE 1* is the main receptor of brassinolide (BR), and its loss-of-function mutant is insensitive to BRs and shows the phenotype of dwarfing, delayed flowering and serious growth defects (Li et al. 2002). The only RLK involved in seed longevity is RLK7, and the loss-of-function mutant *rlk7* showed a disruption of HSL1 rate than the WT after natural aging (Pitorre et al. 2010). However, the underlying molecular mechanism remains unclear.

Our previous work revealed that *Os02g0227600* was significantly upregulated in rice tocopherol and tocotrienol synthesis mutant seeds after aging treatment (unpublished data), and its *Arabidopsis* homologous gene is *HAESA-like 1* (*HSL1*). HSL1, with its homologous members HSL2 and HAESA (HAE), constitutes the *Arabidopsis* LRR-RLK XI subfamily (Patharkar and Walker 2016). The function and signaling pathways of *AtHAE* and *AtHSL2* have been extensively reported (Jinn et al. 2000; Shiu and Bleecker 2001; Patharkar and Walker 2016). HAE and HSL2 function in the regulation of floral organ abscission, cell separation, lateral root development and biotic stresses (Meng et al. 2016; Patharkar and Walker 2016; Wang et al. 2017; Zhu et al. 2019). *INFLORESCENCE-DEFICIENT IN ABSCISSION* (*IDA*) encodes a small secreted peptide that acts as the upstream ligand and triggers HAE and HSL2 activity (Cho et al. 2008). AGAMOUS-like 15, a MADS-domain transcription factor that is positioned downstream of HAE, is phosphorylated by MAPK KINASE4 (MKK4)/MKK5 and MAPK3/MAPK6, resulting in a transcriptional increase in HAE and therefore creating a positive feedback loop (Patharkar and Walker 2015). Recently, HSL3, another member of the group, was reported to negatively regulate plant tolerance to drought stress and control H_2O_2-mediated stomatal closure in response to ABA signaling (Liu et al. 2020). However, the function of HSL1 is rarely reported. Only recently, HSL1 has been reported to recognize the CLE9/10 peptide, recruit SERKs as coreceptors, and phosphorylate the transcription factor SPEECHLESS, eventually decreasing meristemoid mother cells and affecting stomatal development (Qian et al. 2018). Here, we report that HSL1 positively regulates seed longevity by affecting adherent pectin content and the expression of trehalose synthesis genes in *Arabidopsis*.

Materials and methods

Plant materials and growth conditions

Arabidopsis T-DNA insertion mutant *hsl1* (SALK_108127) (Col-0 background) was obtained from the Arabidopsis Biological Resource Center. Functional reversion (RE, *hsl1 proHSL1::HSL1-GFP*), overexpression (OE, *proHSL1::HSL1-GFP*) and promoter-GUS (*proHSL1::GUS*) transgenic lines were generated using the methods described below. Seeds were germinated as described below in an incubator (22 °C, 16 h: 8 h, light: dark, 70% humidity). After 7days, seedlings were transferred to the soil and grown under the same conditions as described above.

Mutant isolation

Genomic DNA was isolated from leaf tissues using a modified cetyl trimethylammonium bromide (CTAB) method. Specific primers for targeting genomic fragments and T-DNA insertion fragment were utilized for genotyping. Total RNA was isolated from the leaves of homozygous plants using an Eastep® Super Total RNA Extraction Kit

(Promega, Shanghai, China). cDNAs were synthesized using Reverse Transcriptase M-MLV (RNase H, TaKaRa, Dalian, China) and SMART 3'-end primers. The expression of the target genes was analyzed by RT-PCR. A 515-bp fragment of *Arabidopsis* actin 2 (ACT2) mRNA (NM_112764) was amplified as the quantitative control using the primer pair AtActin-qF3/AtActin-R4. All the primers used for genotyping and expression analyses are provided in Table S1.

Accelerated aging and germination assay

Seed longevity was evaluated by accelerated aging. Seeds that had been collected and stored dry in paper bags at room temperature for at least 1 month to avoid the effect of dormancy on germination were used for the experiment, as *Arabidopsis* wild-type seeds are not dormant anymore after 20 days after ripening (data not shown). Seeds put in tubes were placed in the top layer of an aging boxes while its lower layer containing 0.01% NaClO solution. After equilibrated for 2 days at room temperature, the accelerated aging treatment was performed in a seed aging tank (Zhejiang Top Yunnong Technology Co., Ltd., Hangzhou, China) at 42 °C with 70% RH for 4 days. Seeds were then dried at room temperature for 2 days. Seeds were sterilized with 10% bleach, stratification at 4 °C in dark for 3 days, and then sown on 1/2 Murashige and Skoog medium as the aged *Arabidopsis* seeds would be infected if germination occurred under non-controlled condition (data not shown). The plates were then placed in an incubator under the condition as described above. Germination was recorded daily until the 10th day. Seeds were considered germinated when showing > 2 mm radicle.

Vector construction and plant transformation

A 1902 bp promoter fragment of *HSL1* was amplified from Col-0 genomic DNA by using primers 28440-PstI-F/28440-NcoI-R and then subcloned into pCAMBIA3301 for promoter-GUS fusion. As the coding sequence of *HSL1* (2991 bp) is too long, segmented cloning was used. The ORF1 and ORF2 of *HSL1* were amplified with primers 28440-ORF1-F/28440-ORF1-R and 28440-ORF2-F/28440-ORF2-R, respectively. The *ORF1-GFP* recombinant fragment was first inserted into pCAMBIA3301-HSL1::GUS using the *Nco*I/*Pml*I site. ORF2 was then inserted into pCAMBIA3301-HSL1::ORF1-GFP using the *Nco*I site to generate the pHSL1::HSL1-GFP construct. All the primers used for vector construction are provided in Table S1. The constructs were subsequently transformed into *Agrobacterium tumefaciens* LBA4404 by electroporation. The promoter-GUS, RE and OE lines were generated by the standard floral dip method to transform the wild-type and *hsl1* plants. The transgenic plants were selected by spraying basta to T_1 seedlings and purified by multiple generations of self-crossing.

Histochemical GUS assay

The HSL1::GUS T2 lines with the strongest expression were grown, roots, stems, leaves, flowers, fruit pods, and seeds at different developmental stages were collected. Samples were first incubated in cold acetone for 10 min and then incubated in GUS staining solution (0.5 mg/mL X-Gluc, 1% Trition X-100, 0.5 mM $K_4Fe(CN)_6{\cdot}3H_2O$, 0.5 mM $K_3Fe(CN)_6$, 0.05 M sodium phosphate buffer) at 37 °C overnight. The stained tissues were decolorized using a solution of ethanol and glacial acetic acid (3:1, v/v) and photographed using a Leica S9i stereomicroscope (Leica Microsystems, Weztlar, Germany).

Ruthenium red staining and pectin content determination

For ruthenium red staining, seeds were hydrated in water at room temperature for 2 h, and then incubated in 0.01% (w/v) ruthenium red for 0.5 h (Mendu et al. 2011). The staining was observed and photographed using a Leica S9i stereomicroscope (Leica Microsystems), and the area was measured by ImageJ v.1.8.0 software (National Institutes of Health, Bethesda, MD, USA).

For pectin content determination, approximately 50 mg of seeds were hydrated in water, and extracted by shaking at 150 rpm for 0.5 h twice, and centrifuged to obtain the supernatant as water-soluble pectin. The remained seed residues were then extracted with votex at 1500 rpm for 3 h, extracted with water for 0.5 h twice, and then again centrifuged to obtain the supernatant as adhesive pectin. The content of uronic acids was determined using the m-hydroxybiphenyl colorimetric uronic acid assay (Blumenkrantz and Asboe-Hansen 1973).

qRT-PCR analysis

Total RNA isolation and first-strand cDNA synthesis were carried out according to the methods described above. qRT-PCR was performed on a CFX Connect TM real-time PCR system (Bio-Rad, Hercules, CA, USA) using a SYBR Premix *Ex* Taq™ II Kit (TaKaRa) in a total volume of 20 µL containing 10 µL SYBR Premix Ex Taq II, 1 µg cDNA, and 10 µM primers. A 113 bp of ACT2 (at3g18780) was used as an internal control for leaf samples, while a 111 bp of UBC (at5g25760) was used as an internal control for seed samples as it is believed to be more stable in seeds (Dekker et al.

2012). The primers used in qRT-PCR analysis are listed in Table S1.

RNA-seq analysis

Freshly harvested seeds (200 mg) of WT and *hsl1* were dried for at least 1 month at room temperature, and then treated by artificial aging. Total RNA was extracted using the RNA prep Pure Plant Kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. mRNAs were purified using polyT oligo-attached magnetic beads. First-strand cDNA was synthesized using random hexamer primers and M-MLV Reverse Transcriptase (TaKaRa). The second strand of cDNA was synthesized using DNA polymerase I and RNase H. The cDNA library was constructed by PCR enrichment and purified with the AMPure XP system (Beckman Coulter, Beverly, USA). Library quality was assessed on the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) and sequenced using the Illumina platform. The clean reads were submitted to the NCBI Read Archive database under the accession number PRJNA793755. The differentially expressed genes (DEGs) were identified by DESeq2 with $P < 0.05$ and fold change > 1.3 for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.

Results

Disruption of *HSL1* showed faster-accelerated aging phenotype in *Arabidopsis*

Our previous global transcriptomic analysis revealed that *Os02g0227600* was significantly upregulated in accelerated aged rice seed embryos (unpublished data). To investigate the function of *HSL1* in seed longevity, its *Arabidopsis* homologue, we isolated a *hsl1* mutant with a T-DNA insertion in the first exon of *HSL1* (Fig. 1a). Molecular analysis revealed that the insertion caused no detectable *HSL1* expression in the leaves (Fig. 1b). The wild type and the mutant showed no phenotypic difference during the growth and development stages (Fig. S1).

To explore the role of *HSL1* in seed longevity, we compared the germination rate and seedling growth of *hsl1* seeds with those of WT, before and after aging treatment. Before aging, both genotypes began to germinate at 1 DAC and nearly reached 100% germination at 2 DAC (Fig. 1c,

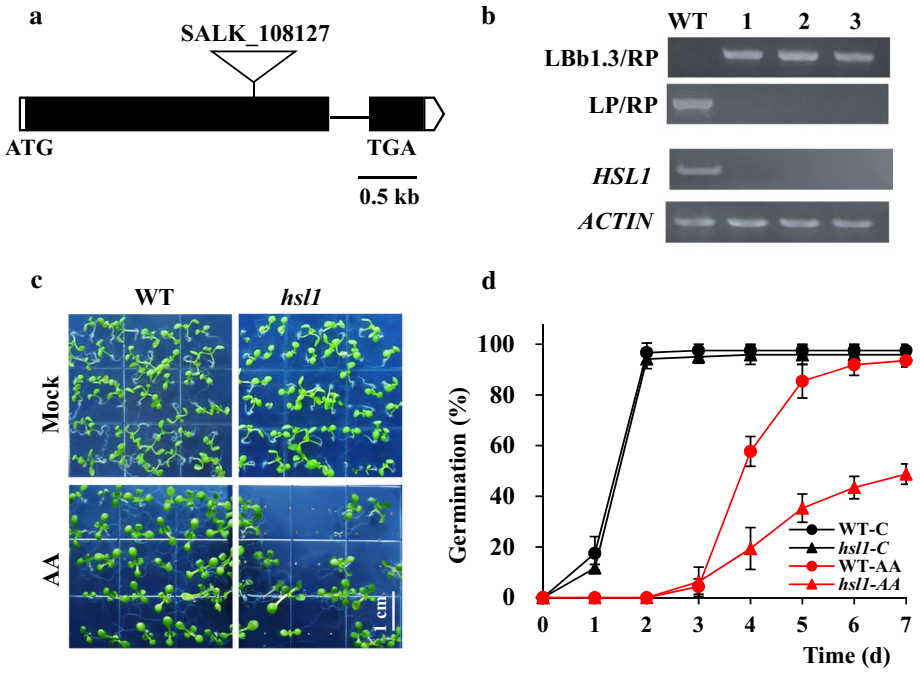

Fig. 1 Loss of function of *hsl1* results in faster-accelerated aging phenotype in *Arabidopsis*. **a** Schematic representation of the T-DNA insertion in the *hsl1* mutant. The black box represents exons, the horizontal line represents introns, the white box represents UTRs and the inverted triangle represents insertion sites. **b** Analysis of *hsl1* mutant. *Upper panel*: PCR analysis of the T-DNA insertion in the gene. Lanes 1, 2, 3, homozygous mutants; LP, left genomic primer; RP, right genomic primer; LBb1.3, left border primer of the T-DNA insertion. *Lower panel*: RT-PCR analysis of *HSL1* transcript levels in leaves of wild-type and *hsl1* plants. **c** Germination status at 7 DAC. Bars = 1 cm. **d** Germination percentage of WT and *hsl1* seeds after aging treatment. Mock, before aging. AA, accelerated aging. At least 100 seeds were used for each treatment. Values are shown as mean ± SD ($n = 3$)

d). After aging treatment, the germination rate of WT was 57.7% at 4 DAC and 93.5% at 7 DAC, while *hsl1* was reduced to 19.4% at 4 DAC and 50% at 7 DAC (Fig. 1c, d). These results indicate that *HSL1* may positively affect seed longevity in *Arabidopsis*.

Expressing *HSL1* basically recovered the accelerated aging of *hsl1*, while overexpressing *HSL1* improved accelerated aging phenotype

To confirm the role of *HSL1* in seed longevity, we generated RE and OE lines by introducing a *HSL1::HSL1* construct (Fig. 2a). qPCR analysis was performed to screen RE lines (RE-1, RE-7 and RE-8) with *HSL1* expression close to WT (Fig. 2b), and the OE lines (OE-1, OE-3 and OE-8) with

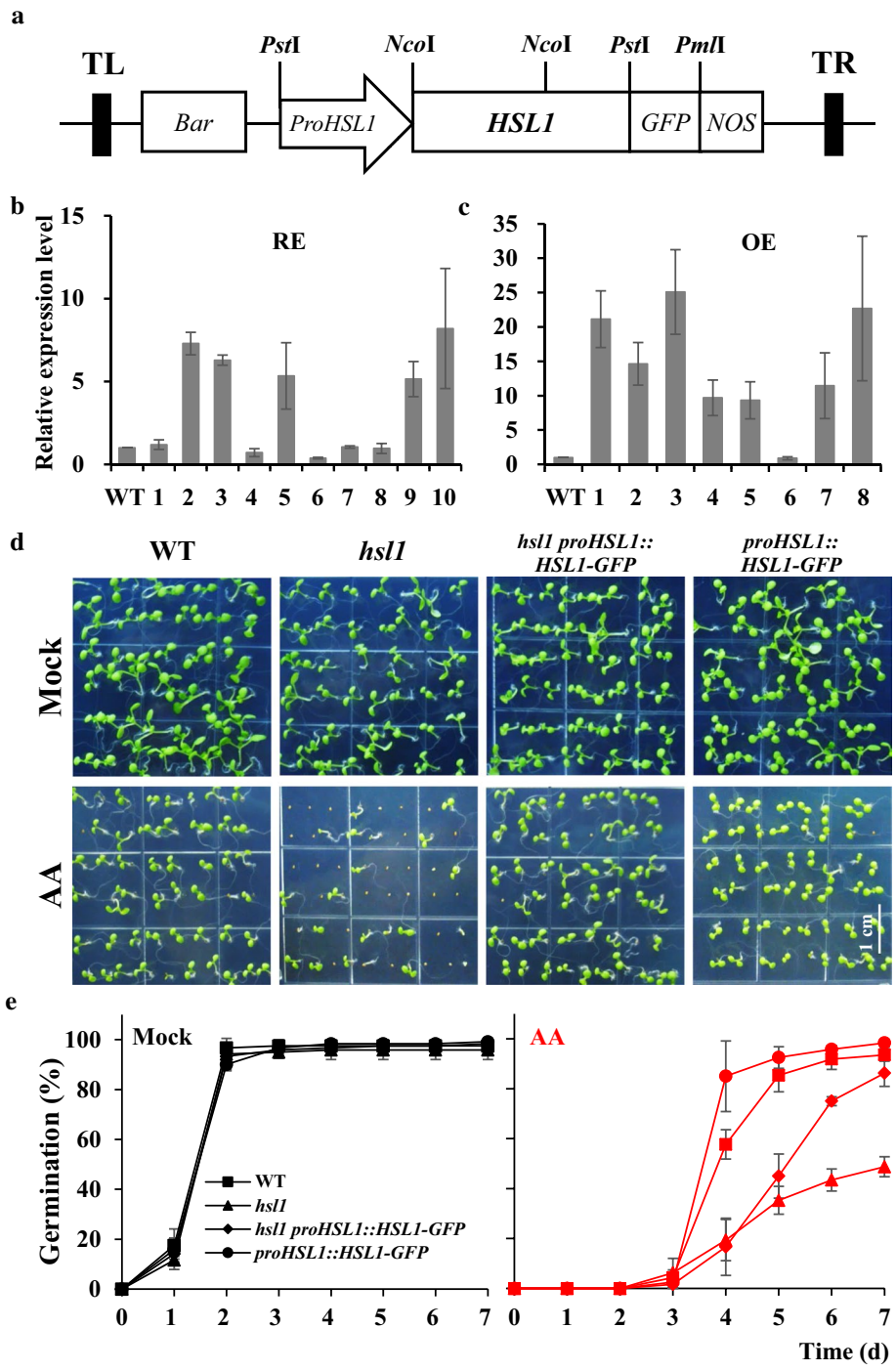

Fig. 2 Expression of *HSL1* in *hsl1* basically rescued the accelerated aging phenotype, while overexpression of *HSL1* improved seed longevity. **a** Schematic representation of the *proHSL1::HSL1-GFP* vector used for the reversion (RE) and overexpression (OE) experiments. In this vector, the *HSL1-GFP* fusion gene is driven by the HSL1 promoter, and Bar is used as the selection marker. **b, c** qRT-PCR analysis of RE **b** and OE **c** transgenic lines. **d** Germination status at 7 DAC. Bars = 1 cm. **e** Germination percentage of WT, *hsl1* mutant, RE and OE lines after aging treatment. For simplicity, only 1 RE and OE line were shown here. Mock, before aging. AA, accelerated aging. At least 100 seeds were used for each treatment. Values are shown as mean ± SD ($n = 3$)

the highest *HSL1* expression (Fig. 2c) for further research. Subsequently, we compared their germination rates before and after aging treatment.

Before aging, WT, *hsl1*, RE and OE lines began to germinate at 1 DAC, almost all germinated at 2 DAC, and their germination rates showed no difference at any time point (Fig. 2d, e). After aging, at 4 DAC, the germination rates of *hsl1* and RE were significantly lower (16.7%-19.4%) than those of the WT and OE (57.7%-85.0%). At 7 DAC, the germination rates of WT and OE reached 93.5%-98.3%, while that of RE was increased to 86.3%, and *hsl1* had the lowest germination (48.8%) (Fig. 2d, e).

Taken together, expressing *HSL1* in *hsl1* basically recovered the accelerated aging of *hsl1*, while overexpressing *HSL1* improved the accelerated aging phenotype of WT, which further confirmed that *HSL1* positively regulated seed longevity.

HSL1 is highly expressed in seed embryos and can be induced by seed aging

To further investigate the role of *HSL1* in seed longevity, we developed a large number of pHSL1::GUS transgenic lines by introducing a *HSL1::GUS* construct (Fig. S2a). Based on the high expression in seeds, we performed GUS staining on the seeds of each transgenic line at 24 h after imbibition (HAI) and screened the lines (G-1, G-2) with a darker color for subsequent expression analysis (Fig. S2b).

GUS staining revealed that *HSL1* was highly expressed in whole plants (especially leaves and roots) in 10-day-old

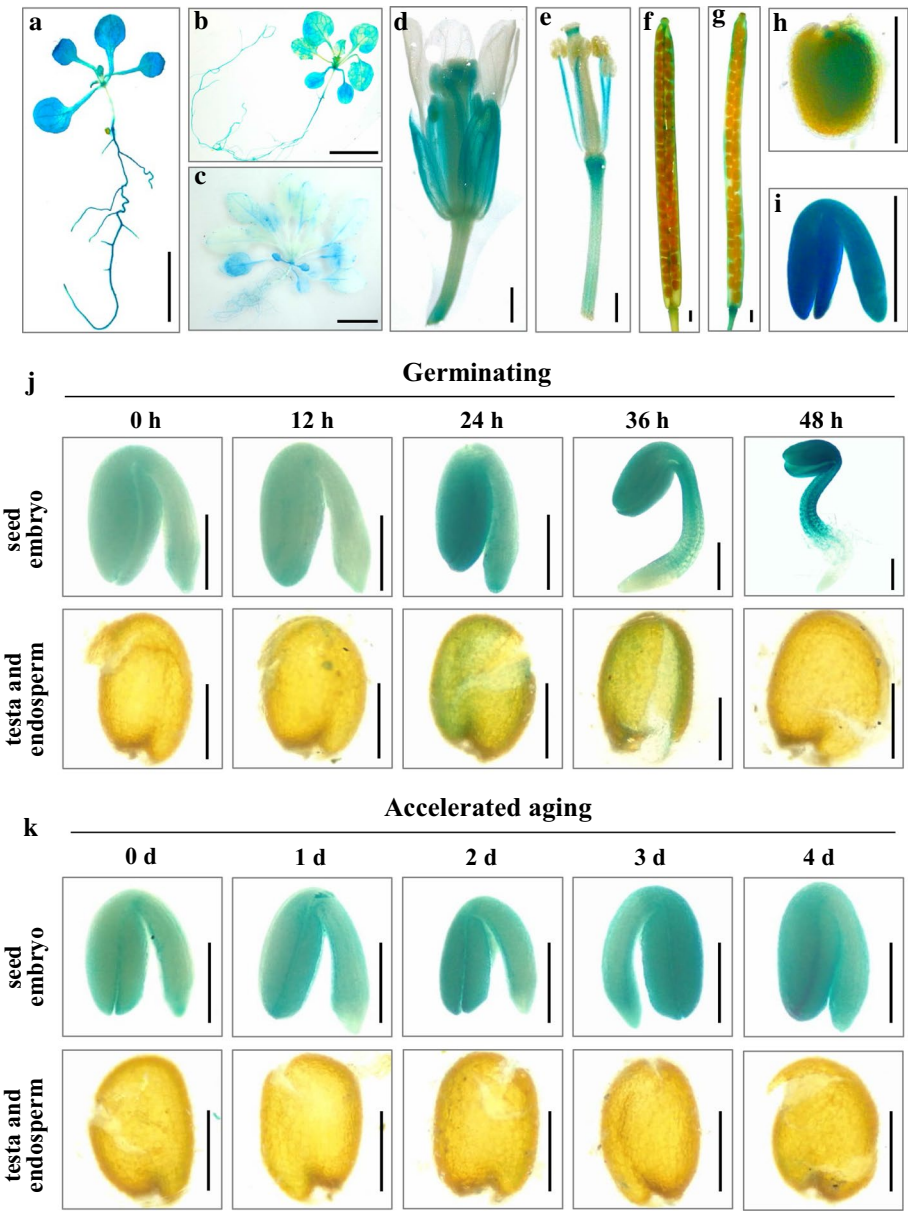

Fig. 3 Tissue-specific and induction pattern of HSL1 using GUS staining. **a-c** The expression of *HSL1* on 10- (**a**), 25- (**b**) and 45-day-old (**c**) plants. **d**–**g** Flowers at 1 day after pollination (DAP) (**d**) and at 3 DAP (**e**); silique at 9 DAP (**f**) and at 15 DAP (**g**). **h, i** Seeds in silique at 15 DAP (**h**), and mature seeds at 24 h after imbibition (**i**). **j, k** Expression of HSL1 in germinating seeds (**j**) and under seed aging treatment (**k**). Experiments were repeated three times, and representative images are displayed. Bars = 5 mm (**a**–**c**), 0.5 mm (**d**–**i**), 0.25 mm (**j**–**k**)

seedlings (Fig. 3a), and significantly reduced in 25-day-old plants (Fig. 3b), but only expressed slightly in 25-day-old plants (Fig. 3c). *HSL1* was highly expressed in the calyx, stamen and stigma at 1–3 days after pollination (DAF) (Fig. 3d, e), but not in the silique at 9 and 15 DAF (Fig. 3f, g). *HSL 1* was highly expressed in the seeds at 15 DAF and seed embryos at 24 HAI (Fig. 3h, i).

We also determined *the HSL1* induction expression pattern. During germination, *HSL1* was expressed slightly in embryos at 0–12 HAI, but gradually increased in embryos at 24–48 HAI as germination progressed. *HSL1* was expressed slightly in endosperm at 24–36 HAI, and no expression was found in the seed coat (Fig. 3j). In response to aging induction, *HSL1* expression was increased on the 1st day of aging and continued to increase and peaked on the 3th day of aging (Fig. 3k). GUS activity was also primarily expressed in the embryo but not in the seed coat or endosperm (Fig. 3k).

Taken together, expressing *HSL1* in *hsl1* basically recovered the accelerated aging of *hsl1*, while overexpressing *HSL1* improved the seed longevity of WT, which further confirmed that HSL1 positively regulated seed longevity.

Disruption of HSL1 increased the adherent pectin content of mucilage

Previous reports showed that *Arabidopsis* IDL6-HAE/HSL2 positively affects pectin degradation of the leaf cell wall and decreases resistance to *Pseudomonas syringae* (Tomato DC3000 (Wang et al. 2017). When *Arabidopsis* dry seeds are hydrated, mucilage secretory cells (MSCs) quickly secrete mucilage around the seeds. To investigate whether HSL1 is involved in the mucilage content, the seeds of the WT and *hsl1* mutant were hydrated and stained with ruthenium red. No difference in the dyeing color and area was identified between WT and *hsl1* (Fig. 4a, b). Subsequently, we measured the soluble and adherent pectin content, which is the main component of mucilage. No difference in the soluble pectin content was shown between WT and *hsl1*, while the adherent pectin content was significantly increased in *hsl1* compared to WT (Fig. 4c). In summary, our data demonstrated that disruption of *HSL1* increased the adherent content in *Arabidopsis*.

Transcriptome analysis of WT and *hsl1* seeds before and after accelerated aging

To further understand the function of HSL1 in seed longevity, global transcriptomics were compared using unaged and aged seeds of WT and *hsl1*. Twenty-five DEGs (10 upregulated, 15 downregulated) were identified between the unaged seeds (Fig. 5a, Table S2). Two hundred and five DEGs (110 downregulated, 95 upregulated) were identified between the

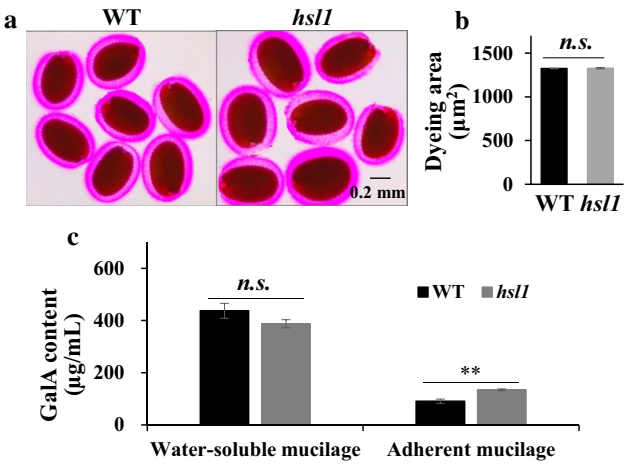

Fig. 4 Disruption of HSL1 decreases the adherent pectin content in seed mucilage. **a**, **b** Pictures (**a**) and the mucilage area (**b**) revealed by ruthenium red staining of WT and *hsl1* seeds after hydration. Bars = 500 μm. **c** Soluble and adherent pectin content in seeds of WT and *hsl1* mutant. Values are shown as mean values ± SD ($n = 3$), and analyzed using Student's t test. Asterisks indicate significant differences (**$P < 0.01$; *n.s.* not significant)

treated seeds (Fig. 5a, Table S3). The expression patterns of these DEGs were confirmed by qRT-PCR analysis (Fig. S3).

When Gene Ontology (GO) term and KEGG pathway enrichment analyses were performed on the 25 DEGs between unaged seeds, six DEGs were enriched in five biological processes (BPs), and two KEGG pathways ($P < 0.05$). The enriched BPs were positive regulation of kinase activity, DNA metabolic process (DNA integration, DNA recombination), trehalose biosynthetic process and photosynthesis (light harvesting), and other types of *O*-glycan biosynthesis (Fig. 5b, Table S4). The enriched KEGGs were "other types of *O*-glycan biosynthesis" and "photosynthesis-antenna proteins" (Fig. 5b, Table S4). Interestingly, four downregulated DEGs related to SSPs (seed storage albumin 1, AT4G27140; seed storage albumin 2, AT4G27150; seed storage albumin 3, AT4G27160; legume lectin family protein, AT3G01345) were not enriched in the analysis (Table S2).

When GO enrichment analysis was performed on the 205 DEGs between the treated *hsl1* and WT seeds, 54 DEGs were enriched in 29 BPs and 5 KEGG pathways ($P < 0.05$). The 14 most important BPs ($P < 0.02$) were nucleotide-sugar metabolic process, DNA recombination, ATP synthesis coupled proton transport, phospholipid scrambling, interphase microtubule nucleation by interphase microtubule organizing center, N-terminal protein amino acid methylation, cellular response to molecule of fungal origin, cellular response to hypoxia, protein folding, aerobic electron transport chain, SNARE complex assembly, microtubule severing, ER to chloroplast lipid transport, detoxification, and oxidation–reduction process (Fig. 5c, Table S5). The

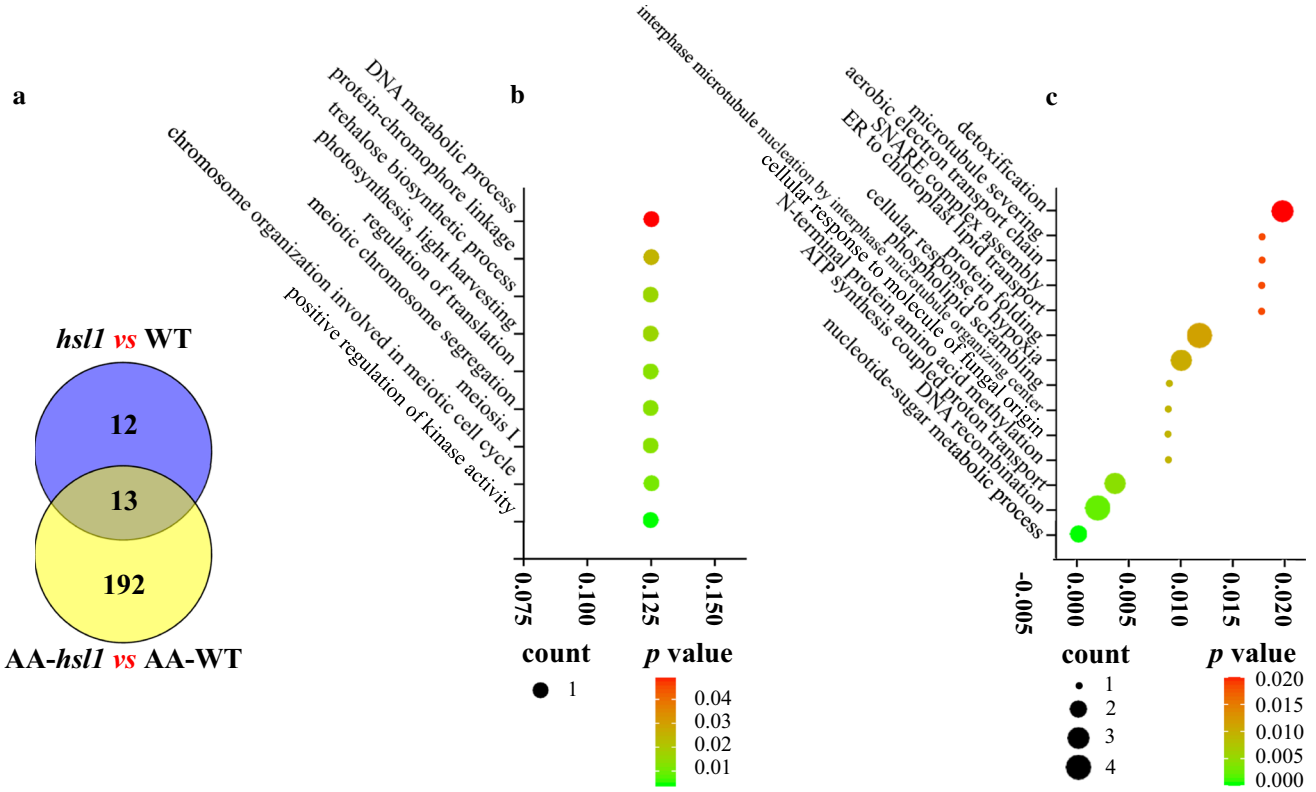

Fig. 5 Transcriptome analysis of the unaged and aged *hsl1* seeds relative to the wild type seeds. **a** Venn diagram of differentially expressed genes (fold change > 1.3 and $P < 0.05$) in the unaged and aged *hsl1* seeds. The detailed information on differential genes is shown in Table S2 and Table S3. **b, c** Gene ontology (GO) enrichment analysis of biological processes of differentially expressed genes (DEGs) in the unaged ($P < 0.05$) (**b**) and aged ($P < 0.02$) (**c**) *hsl1* seeds. The detailed information of biological pathways is shown in Table S4 and Table S5

enriched KEGG pathways were protein processing in the endoplasmic reticulum, other types of *O*-glycan biosynthesis, biotin metabolism, protein export and fructose and mannose metabolism (Table S5).

To reveal the mechanisms related to seed longevity owing to the disruption of HSL1, we further investigated the DEGs enriched in the above-mentioned BPs and KEGG pathways. Of them, 87% of DEGs (47/54) could be divided into four categories. The nucleotide-sugar metabolism group contained eight DEGs involved in three BPs and two KEGGs (Fig. 6). Interestingly, only two DEGs (ILITYHIA and glycosyltransferase family 61 protein) involved in the positive regulation of kinase activity and other types of *O*-glycan biosynthesis were upregulated, indicating that compensation of other kinase pathways was induced in *hsl1*. Among the DEGs that were downregulated, trehalose-6-phosphate phosphatase J (TPPJ), which catalyzes trehalose 6-phosphate into trehalose (Lunn et al. 2014), was downregulated in both unaged and aged *hsl1* seeds. The downregulation of TPPJ would result in a decrease in trehalose and an increase in trehalose 6-phosphate. Moreover, three DEGs involved in nucleotide-sugar metabolism and transport were downregulated. Rhamnose synthase (RHM) is a trifunctional enzyme that catalyzes UDP-Glc into UDP-Rha (Usadel et al. 2004; Oka et al. 2007). UDP-Rha could then be delivered by Golgi nucleotide sugar transporter 3 (GGLT1) into the Golgi lumen, and serve as a precursor to synthesize rhamnose-containing polysaccharides (Rautengarten et al. 2014). Rhamnose residues are usually incorporated as part of the rhamnogalacturonan I (RG-I) backbone, or provide sidechain decorations of rhamnogalacturonan II (RG-II) (Mohnen 2008). The downregulation of these genes (RHM2, RHM3, GGLT1) would result in a decrease in rhamnose-containing polysaccharides in the seed cell wall of *hsl1*. In addition, the downregulation of phosphofructokinase may decrease glycosis and result in changes in cellular metabolism.

The protection and repair group contained ten DEGs involved in four BPs (Fig. 6). Of them, three antioxidant enzyme genes (dehydroascorbate reductase 2, *DHAR2*; glutathione *S*-transferase TAU 1, *GSTU1*; chloroplast aldehyde reductase, *CHLADR*), one regulatory gene (NmrA-like negative transcriptional regulator family protein) and one gene involved in the cellular response to a molecule of fungal origin (*BPA1-LIKE 1*) were downregulated. Furthermore,

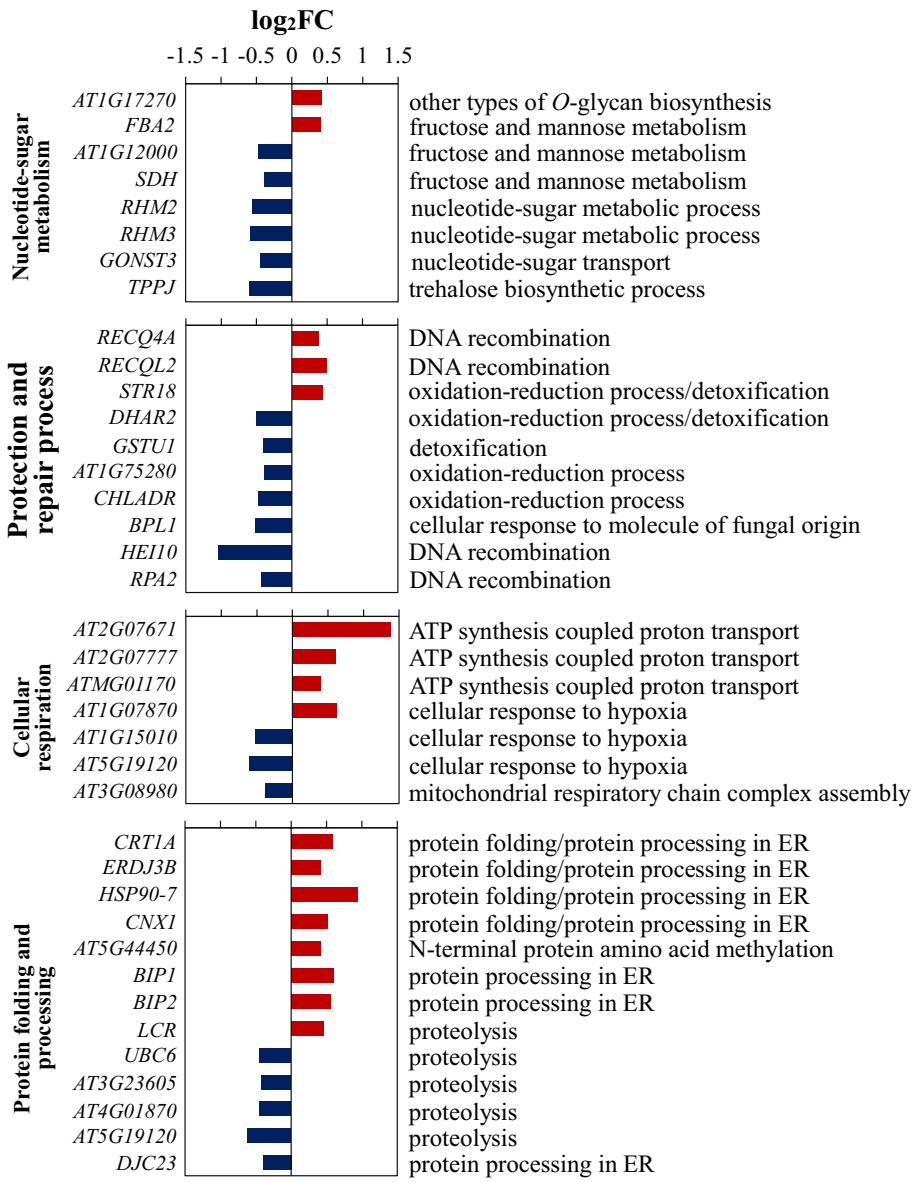

Fig. 6 Disruption of HSL1 modulates the expression of genes involved in nucleotide-sugar metabolism, protection and repair, cellular respiration and protein folding and processing. *BIP1* luminal binding protein 1, *BIP2* luminal binding protein 2, *BPL1* BPA1-LIKE 1, *CHLADR* chloroplast aldehyde reductase, *CNX1* calnexin 1, *CRT1A* calreticulin 1A, *DHAR2* dehydroascorbate reductase 2, *DJC23* DNA J protein C23, *ECQL2* rrecq helicasel 2, *ER* endoplasmic reticulum, *ERDJ3B* J domain protein localized in ER lumen DNAJ heat shock family protein, *FBA2* fructose-bisphosphate aldolase 2, *GONST3* Golgi nucleotide sugar transporter 3, *GSTU1* glutathione *s*-transferase tau 1, *HEI10* homolog of human HEL10 (enhancer of cell invasion No. 10), *HSP90-7* heat shock protein 90-7, *LCR* leaf curling responsiveness, *RECQ4A* DNA helicase (RECQl4A), *RHM2* rhamnose biosynthesis 2, *RHM3* rhamnose biosynthesis 3; *RPA2* replicon protein a2, *SDH* sorbitol dehydrogenase, *STR18* sulfurtransferase 18, *TPPJ* trehalose-6-phosphate phosphatase j, *UBC6* ubiquitin-conjugating enzyme 6

one regulatory gene (RING/U-box superfamily protein) and replicon protein A2 genes involved in DNA recombination were downregulated. The downregulation of these genes in *hsl1* may decrease the capability of seeds to encounter oxidative stress and repair DNA damage, which may be another reason for decreasing seed longevity.

The cellular respiration group contained seven DEGs involved in three BPs (Fig. 6). Of them, three ATP synthases involved in oxidative phosphorylation and ATP synthesis were upregulated in *hsl1* which would enhance aerobic respiration. Mediators of RNA polymerase II transcription subunit 1 protein and aspartyl proteases involved in the cellular response to hypoxia were downregulated. Similarly, the protein folding and processing group contained 12 DEGs involved in three BPs and two KEGG pathways. Of them, seven DEGs (hypothetical protein AXX17, calreticulin 1A, DNAJ heat shock family protein, heat shock protein 90-7, calnexin 1, luminal binding protein 1 (BIP1), BIP2) were involved in protein folding and protein processing in the endoplasmic reticulum, indicating that endoplasmic reticulum stress occurred in *hsl1*. Moreover, most proteolysis genes (4/5) (ubiquitin-conjugating enzyme 6, ubiquitin-like superfamily protein, tolB protein-like protein, eukaryotic aspartyl protease family protein) were downregulated, which would result in the accumulation of abnormal, damaged, and short-lived proteins.

Taken together, our transcriptional profiling revealed that disruption of HSL1 downregulated genes involved in trehalose synthesis, nucleotide-sugar metabolism, protection and repair mechanisms and responses to hypoxia and

proteolysis, while upregulated genes involved in cellular respiration, protein folding and protein processing in the endoplasmic reticulum.

Discussion

The *Arabidopsis* LRR-RLK XI subfamily contains three members, HAE, HSL1 and HSL2 (Patharkar and Walker 2016). The function and signaling pathways have been extensively reported (Meng et al. 2016; Qian et al. 2018; Zhu et al. 2019), but their functions in seed longevity remain unknown. In this study, using the *hsl1* mutant, we revealed that disruption of HSL1 resulted in a faster-accelerated aging phenotype than that of WT (Fig. 1), while overexpressing *HSL1* in *hsl1* basically recovered the accelerated aging phenotype of *hsl1* (Fig. 2). GUS staining revealed that *HSL1* is highly expressed in the embryos of mature seeds and could be induced by accelerated aging (Fig. 3). These data clearly demonstrated that HSL1 positively regulates seed longevity in *Arabidopsis*.

Arabidopsis mature seeds consist of an embryo surrounded by endosperm cells containing storage reserves, and associated with a brown seed coat. Mucilage is the outermost layer of the seed coat of cruciferous plants. Pectin is the main component of mucilage. Mucilage plays an important role in seed dispersal and settlement, seed germination, and seedling survival and growth (Shi et al. 2017). It was reported that overexpression of *ATHB25* upregulates the gibberellin synthase gibberellic acid 3-oxidase 2 gene, increases GA1 and GA4 content in the seeds, and then influences the mucilage content and increased seed longevity in *Arabidopsis* (Bueso et al. 2014). In this study, we revealed that disruption of HSL1 did not affect the mucilage content (dyeing color and area) but increased the adhesive pectin content of the mucilage compared with the wild type (Fig. 4). Our data is consistent with the previous observation that IDL6-HAE/HSL2 controls the degradation of leaf cell wall pectin, thereby affecting the development of lateral roots (Kumpfa et al. 2013) and its resistance to *Pseudomonas syringae* (Tomato DC3000) in *Arabidopsis* (Wang et al. 2017). Therefore, an increase in adherent pectin content may be one of the mechanisms that decreased seed longevity owing to disruption of HSL1 in *Arabidopsis*.

To further reveal the mechanism by which HSL1 regulates seed longevity, we performed global transcriptomics of the unaged and aged seeds of WT and *hsl1*. Interestingly, only 25 DEGs were identified between the unaged *hsl1* and WT seeds, indicating that limited gene expressions are affected in *hsl1* when seeds are quiescent. However, a large number of DEGs were identified between the aged *hsl1* and WT, indicating that *HSL1* targets a number of genes during the accelerated aging process.

Generally, analysis of the downregulated DEGs could reveal the mechanisms related to seed longevity owing to the disruption of HSL1. Our transcription analysis showed that trehalose phosphatases (TPPJs) were downregulated in unaged *hsl1* seeds. The downregulation would consequently result in a decrease in trehalose levels and an increase in trehalose 6-phosphate (Tre6P) levels. Trehalose is a quantitatively important compatible solute and stress protectant, as well as carbon storage and transport (Benaroudj et al. 2001). Some desiccation-tolerant resurrection plants accumulate massive amounts of trehalose in response to drought, and may persist in metabolic stasis for several years until rewatering (Iturriaga et al. 2006). On the other hand, Tre6P is a signal of sucrose availability, and the elevated levels would decrease the sucrose level, an important carbon source for the synthesis of SSPs, which was also downregulated in our transcription analysis. The function of SSPs in seed longevity was intensively reported (Prieto-Dapena et al. 2006; Wu et al. 2017). Therefore, downregulation of TTPJs may decrease seed longevity by decreasing the synthesis of SSPs owing to the disruption of HSL1 in *Arabidopsis*.

Our transcription analysis also revealed that two RHMs and the nucleotide sugar transporter GGLT1 were downregulated in treated *hsl1* seeds. Rhamnose, a deoxy monosaccharide, is required as a building block for synthesizing pectic polymers. The downregulation of these genes (RHM2, RHM3, GGLT1) would result in a decrease in rhamnose-containing polysaccharides in the seed cell wall of *hsl1*. Disruption of AtRHM2 was also reported to result in defect in mucilage formation and ~ 50% decrease in Rha content in *Arabidopsis* mucilage (Usadel et al. 2004; Western et al. 2004), which is quite different from our mucilage staining result.

The above down-regulated genes in *hsl1* were enriched in the trehalose biosynthetic process and nucleotide-sugar metabolism, which relates to carbohydrate metabolism. Carbohydrate metabolism-related genes (though different) were also downregulated in the transcriptomic analysis of *haehsl2* floral receptacles (Niederhuth et al. 2013), where HAE and HSL2 are homologue genes of HSL1. As a majority of the hydrolases, cell wall degradation and remodeling, defense genes against pathogens were downregulated in *haehsl2*, the regulation of HAE and HSL2 on the downstream genes involved in cell separation and root emergence has been substantially studied (Patharkar and Walker 2016; Wang et al. 2017; Zhu et al. 2019), while no reports on carbohydrate metabolism genes afterwards. However, as plant cell wall is composed of various carbohydrate polymers, the regulation of carbohydrate metabolism genes by these receptor-like kinases may

be a common feature of LRR-RLK XI subfamily. Further research is required to clarify whether and how the downregulation of the carbohydrate metabolism-related genes decreases seed longevity owing to the disruption of HSL1 in *Arabidopsis*.

In addition, genes involved in protection and repair mechanisms and responses to hypoxia and proteolysis were also downregulated. However, it is unclear whether the downregulation of these genes is simply a response to accelerated aging, as it is difficult to imagine the direct relationship owing to the disruption of HSL1.

Our transcriptomic analysis also revealed that genes involved in oxidative phosphorylation and ATP synthesis, protein folding and protein processing in the endoplasmic reticulum were upregulated. Generally, enhanced aerobic respiration consumes seed storage compounds, which is not good for maintaining seed vigor. Similarly, the upregulation of protein folding-related genes indicated that ER stress occurred in *hsl1*. ER stress then induces the unfolded protein response and further induces the expression of molecular chaperones and stress-related genes to cope with cell difficulties (Schröder and Kaufman 2005). As *hsl1* has lower seed longevity than the wild type, we proposed that the upregulated DEGs involved in cellular respiration and protein folding and processing are the early response to accelerated aging.

In conclusion, our study demonstrated that HSL1 played a positive role in seed longevity. Further analysis indicates that an increase in adherent pectin content and downregulation of trehalose synthesis genes may be the main reasons decreasing seed longevity in *Arabidopsis* owing to disruption of HSL1. As accelerated aging is just a mimicked method of natural aging, the question of whether the mechanisms found by the transcriptome analysis are artefacts of accelerated aging, requires further research. Moreover, alternative methods, such as mass spectrometry, are also required to screen the interacting proteins of HSL1 and clarify the possible downstream regulatory pathways.

Author contribution statement DC and XC designed and advised the study. HG, SC, QY and PW performed the experiments. DC, HG, SC and XC analyzed the data. SC and XC wrote the paper. All authors read and approved the manuscript.

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Data availability The RNA-seq raw data were stored on the NCBI Read Archive database under the accession number PRJNA793755, and will be made publicly accessible after the publication of the manuscript. Other datasets and plant materials generated during the study are available from the corresponding author upon request.

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