



Interplay of starch debranching enzyme and its inhibitor is mediated by Redox-Activated SPL transcription factor



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ABSTRACT

The germination process is of central importance across the cultivated species involving several key enzymes for mobilization of stored food reserves. Pullulanase (PUL), a starch-debranching enzyme, plays an important role in mobilizing stored endosperm food reserves during germination. Pullulanase inhibitor (PULI) hinders PUL's activity through an unknown mechanism. Barley has one *PUL* and two *PULI* genes. During the time-dependent processes of seed germination, only *PULI-1* expression shows an antagonistic relationship with that of *PUL*. Our data have indicated that the expression of *PULI-1* is modulated by SPL (Squamosa-promoter-binding Protein Like) transcription factors, known to be targeted by miR156. We show that the binding of recombinant HvSPL3 protein to the *PULI-1* promoter occurs under reducing, but not under oxidizing conditions. Replacement of Cys residues with threonine in HvSPL3 abolishes the binding, indicating an essential role of the redox state in the expression of *PULI*. Our findings may have important implications for the industrial use of starch.

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1. Introduction

Carbohydrates stored in the form of starch occupy a significant portion of the endosperm reserves to provide the energy source for seed germination and materials for building blocks for cell constituents during growth and development. Cereal starch has a widespread use in industrial applications, such as human food, animal feeds, and clean energy. By manipulating the starch properties, the modified starch leads to a remarkable potential for human use, for example, low-amylose rice [18]. Starch is comprised of two polymers, amylose and amylopectin. Amylose consists of chains of d-glucose residues linked through α -1,4 glucosidic bonds, and amylopectin is organized branched polymers through α -1,6 glucosidic linkages. Starch degradation commences with the action of aleurone layer secreted hydrolytic enzymes, α -amylase, and β -amylase, which act together in cleaving α -1,4 glucosidic linkages to mainly generate maltose molecules from a linear component as well as glucose and other oligomers. Non-reducing ends of amylose and amylopectin, leave behind other short-chain glucans

including α and β dextrins hydrolysis which require specific enzymes. Pullulanase (PUL), also called limit-dextrinase (LD), preferentially acts on α and β dextrins generated after breakage of α -1,6 linkages and converted to glucose by the action of glucosidase on these short chains [19].

In barley, pullulanase is encoded by a single gene (*HvPUL*) which is expressed during barley endosperm development and seed germination stages [6,22]. The higher expression has been observed particularly in the aleurone layer when seeds are treated with gibberellic acid, which signifies its critical role in starch debranching during seed germination and is found to be remarkably stable in broad pH range and high temperature [6,17]. In cereals, the role of pullulanase during starch synthesis overlaps with isoamylase and is yet to be fully understood [13]. Mutational analysis of pullulanase-type debranching enzyme indicates the dual function of maize PUL in starch biosynthesis and degradation [10].

Limited activity of pullulanase has been observed when it is complexed with pullulanase inhibitors (PULI), also known as limit dextrinase inhibitor (LDI) [26]. Downregulation of pullulanase inhibitor in barley revealed its impact on the starch granule size distribution, length of amylopectin chains, and amylose to amylopectin ratio [33]. The activity of pullulanase inhibitor is redox

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dependent that thioredoxin (Trx) disulfide reduction that causes structural destabilization of the PULI protein [20]. However, a 4-fold increase in the activity of pullulanase in transgenic barley overexpressing *Trxh* could not be directly correlated with the action of pullulanase inhibitor [9]. Therefore, the mechanism linking Trx, pullulanase, and its inhibitor remains elusive. Until now, it is not known how the antagonistic relationship between PUL and PULI is regulated on the transcriptional level. Given the viable activity of the PUL-PULI complex from seed dormancy to germination, we were interested in detecting if the promoters of these genes have the *cis*-regulatory element that is targeted by the specific transcriptional factors.

The SQUAMOSA-promoter binding like (SPL) transcription factors are regulated by miR156 and involved in various biological pathways and growth phase transitions in plants [35,36]. The highly conserved SBP domain of SPL, which contains two zinc-binding motifs and a nuclear localization signal (NLS) sequence, is required for binding to a *cis*-regulatory element with a GTAC core motif [21]. Among 17 barley SPL genes, only *HvSPL3* and *HvSPL23* have shown moderate expression in germinated embryos [34]. As germination represents a phase transition of an embryo from quiescent to the active stage, this prompted us to investigate the SPL transcription factors for their role in regulating the pullulanase and its inhibitor genes during seed germination.

Here, we investigated genes encoding pullulanase, its inhibitors in barely and other monocot species and analysed their phylogenetic relationships along with their spatiotemporal gene expression profiles. We further investigated the regulation of SPL on the pullulanase inhibitor during the transition from seed dormancy to germination. Our work unravels the missing link during the interplay of pullulanase and its inhibitor and demonstrates its association with redox. Overall, our findings will not only help to advance knowledge about the germination process as a key for crop production but also have value in product development and processing associated with the starch and brewing industry.

2. Material and methods

2.1. Identification of pullulanase and its inhibitors genes in barely and other monocot species

The query protein sequence of pullulanase and pullulanase inhibitor genes were obtained from the NCBI database and searched by BLASTP in the *Hordeum vulgare* Ensembl database. The Ensembl Plants database biomart function was used for retrieving gene ID's of proteins containing the respective domains of pullulanase and its inhibitor (<https://m.ensembl.org/biomart/martview/>). The selected candidate's protein domains were confirmed using the InterPRO browser. The *cis*-regulatory elements in the promoter region of pullulanase and its inhibitor genes were identified by using PLACE (Plant *cis*-acting regulatory DNA elements) database (<https://www.dna.affrc.go.jp/PLACE/>) as well as manually for the identification of potential transcription factor binding core motifs. The genomic and coding sequences of pullulanase and its inhibitor genes retrieved from Ensembl Plants release 51 (<https://plants.ensembl.org/index.html>) were aligned to generate the gene exon-intron structure diagram using the Gene Structure Display Server (<https://gsds.cbi.pku.edu.cn/>).

2.2. Phylogenetic analysis of pullulanase and its inhibitors

The putative pullulanase and its inhibitors genes were identified by BLASTP searches against the corresponding reference genomes at Phytozome (v13) (<https://phytozome.jgi.doe.gov>) and Ensembl Plants release 51 (<https://plants.ensembl.org/index.html>).

A total of 19 identified protein sequences with accession numbers were included in this study. Multiple sequence alignments of the full-length pullulanase and its inhibitors protein sequences from seven and six plant species were performed with MULTIPLE Sequence Comparison by Log-Expectation (MUSCLE), respectively. An unrooted phylogenetic tree was then constructed by the maximum likelihood (ML) method in MEGA X using Jones-Taylor-Thornton Gamma Distributed (JTT + G) model for pullulanase genes and WAG model for pullulanase inhibitors genes with a bootstrap analysis of 500 replicates [16,23].

2.3. In silico gene expression profiling analysis of pullulanase and its inhibitors genes

The RNA-seq data for pullulanase and its inhibitors genes were downloaded from Expression Atlas at (<https://www.ebi.ac.uk/gxa/home>) and Wheat Omics (<https://wheatomics.sdau.edu.cn/>). The graphs of gene expression profiling were made using GraphPad Prism (<https://www.graphpad.com/>).

2.4. cDNA synthesis and RT-qPCR analysis

Mature seeds harvested from Barley cv. Golden Promise was imbibed for 9, 24 and, 48-hour time-points, instantly frozen in liquid nitrogen, and stored at -80°C . Total RNA was extracted from finely crushed samples by using the spectrum plant total RNA kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol with minor modifications. Extracted samples were quantified on a NanoDrop ND-1000 (NanoDrop Technologies, Washington, DE, USA) and treated with DNase I (Invitrogen, USA). A total of 1 μg of extracted RNA was used in cDNA synthesis with AffinityScript QPCR cDNA Synthesis kit (Agilent technology, Canada). The qRT-PCR was carried out in the Mx3000 qPCR system (Stratagene, USA). The total volume of each reaction was 20 μl with 1 μl of 5X diluted cDNA, 5 μM gene-specific primers, and 7.5 μl of WINSENT master mix with *Hvb-actin* and *HvGAPDH* as an internal control (Table S1). Expression analyses were performed with two biological, and three technical replicates, and the relative gene expression was analysed by the $2^{-\Delta\Delta\text{CT}}$ method [25].

2.5. Electrophoretic mobility shift assays (EMSA)

The pET 32a (+) vector was used for heterologous expression of SBP-DNA binding domain sequence of *HvSPL23*, *HvSPL3*, and the six cysteine residues in the SBP domain of *HvSPL3* were mutated to threonine and the mutated protein was named as MHvSPL3 (Fig. S1). The SBP-domain of mentioned proteins was codon-optimized and cloned into *Bam*HI- and *Xho*I-digested pET 32a (+) vector by Genescript (Piscataway, USA), containing translational His tag at N- and C-terminal. The DNA probes were synthesized and labelled with biotin at the 5' end (Integrated DNA technologies, Canada) (Table S2). According to the manufacturer's instruction, all recombinant proteins were affinity-purified using a Ni-NTA fast start kit (QIAGEN). Bradford (standard procedure) method was used for recombinant protein quantification [4]. The EMSA assay was performed using the Chemiluminescent EMSA kit (Thermo Fisher, Waltham, USA) according to the manufacturer's instructions. In brief, a total of 20 μl of each binding reaction was prepared using unlabeled (specific competitors) and 5' labelled DNA probes of pullulanase and its inhibitor, and the recombinant *HvSPL3*, MHvSPL3, and *HvSPL23* protein were incubated at room temperature for 20 min. The protein-DNA complex was resolved in mini-protein 5 % TBE precast gels (Bio-rad, Canada) and then transferred to a nylon membrane (GE healthcare). Biotin signal was detected with chemi-blot function in gel

dock (Bio-rad, ChEMiDoc™ XRS +) [20]. For performing redox EMSA, the protein was treated with three oxidizing and reducing agents, 10 mM Sodium tetrathionate (Na₂S₂O₆), 20 mM Hydrogen peroxide (H₂O₂), 25 mM Oxidized glutathione (GSSG), and 5 mM Dithiothreitol (DTT), 5 mg Thioredoxin (Trx) + 1 mM Dithiothreitol (DTT), 5 mg Thioredoxin (Trx) + 7.5 mg Thioredoxin reductase (NTR) + 0.25 mM Nicotinamide Adenine dinucleotide Phosphate Hydrogen (NADPH), respectively for 30 min at room temperature then used in the preparation of binding reaction [31].

2.6. Statistical analysis

All presented qRT-PCR results were performed with the analysis of variance by Student’s *t*-test means comparison using JMP Pro V15 software.

3. Results

3.1. Identification of genes encoding pullulanase and its inhibitors in barley

The pullulanase protein consists of the pullulanase N2-domain, GH-13 N-terminal, catalytic domain, and alpha-1,6 glucosidases, C-terminal [28,1]. Using these domains as a query, ten protein sequences were retrieved from the Ensembl plant database. Analysis of ten pullulanase protein sequences revealed only one protein translated from *HvPUL* (HORVU7Hr1G027860), consisting of all the specific pullulanase domains. The 12,448 bp long genomic sequence of *HvPUL* contains 27 exons (Fig. 1). The catalytic domain of translated protein from *HvPUL* contains all the seven amino acid residues D⁴⁷³, E⁵¹⁰, W⁵¹², F⁵¹⁴, F⁵⁵³, E⁷²⁹, and D⁷³⁰, which are found to be involved in the interaction with its inhibitor (Fig. 1) [29].

Pullulanase inhibitor belongs to the chloroform/methanol soluble protein family (CM-protein family). Using bifunctional inhibitor/plant lipid transfer protein/seed storage helical inhibitor protein domain, a total of 34 protein sequences were retrieved from the Ensembl plant database. Alignment of these protein sequences displayed gene candidates, *HvPULI-1* (HORVU6Hr1G066330) and *HvPULI-2* (HORVU0Hr1G027030) and the

protein translated from them share very high similarity (98 %). Both genes possess one exon (444 bp) while the *HvPULI-1* shows the larger gene length. Identified inhibitor proteins have 5 specific amino acid residues R³⁴, R³⁸, L⁴¹, V⁴², and R⁸⁴ in the functional domains which have been involved in binding to pullulanase (Fig. 2) [29]. Translated proteins from these two genes have a differences of only 4 amino residues (Fig. 2). Our results indicated that there are two pullulanase inhibitors and only one pullulanase gene in the barley genome.

3.2. Phylogenetic relationship of pullulanase and its inhibitors in monocot species

Nine pullulanase and nine pullulanase inhibitor proteins from one dicot species and six other monocot species have been identified for phylogenetic analysis. A maximum likelihood (ML) tree was constructed with nine pullulanase and nine of its inhibitor proteins, respectively (Fig. 3). As the model plant for dicot species, *Arabidopsis thaliana* genome contains one pullulanase gene but no inhibitor gene. However, hexaploid wheat presented the largest number of pullulanase and its inhibitors, three each. Most monocot species possess only one pullulanase and one pullulanase inhibitor gene. For pullulanase, two distinct clusters are formed to separate *A. thaliana* from the monocot species. Pullulanase in wheat, barley, rice, and brachypodium clustered together in one sub-clade while another sub-clade contains ones in Mazie and Sorghum. The pullulanase inhibitors from wheat, barley, and brachypodium formed one branch whereas rice, maize, and sorghum are grouped under another branch (Fig. 3).

3.3. Pullulanase and its inhibitor express antagonistically during seed germination

Publicly available barley RNA-Seq datasets were used to analyze the expression of *PUL*, *PULI-1*, and *PULI-2* genes during 16 developmental stages such as caryopsis (15dpa and 5dpa), germinating embryo, inflorescence (5 mm), internode, root (seedling) and shoot (seedling). *HvPUL* gene exhibits expression in most of the tissues (Fig. 4). One of the pullulanase inhibitor genes, *HvPULI-1* reveals

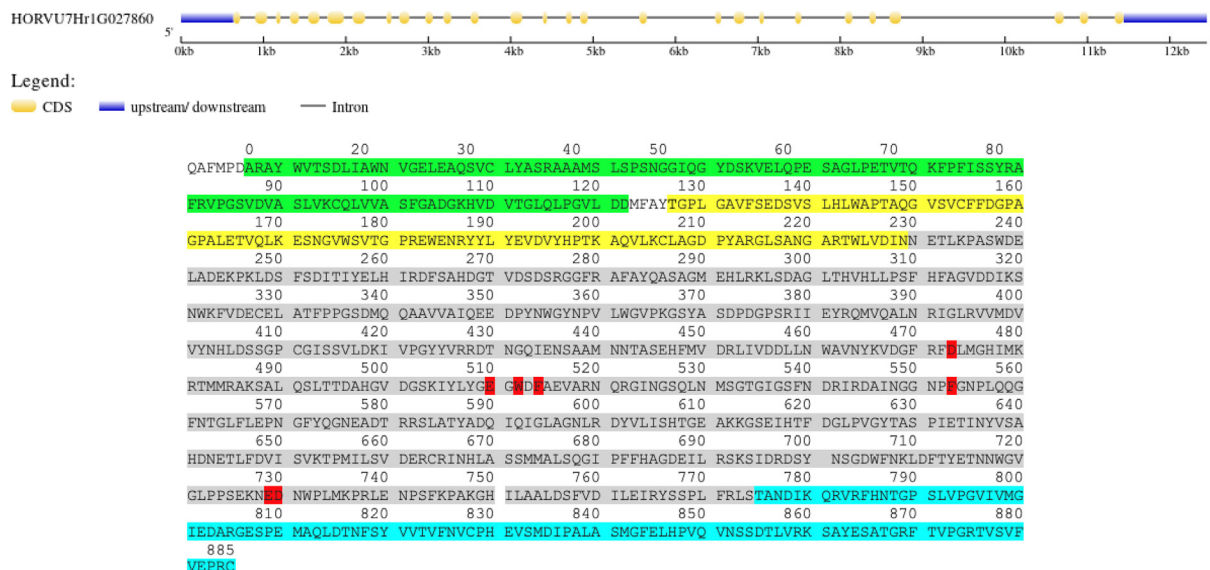


Fig. 1. The gene structure and protein sequence of barley pullulanase. Upper part showing gene structure of pullulanase gene from barley; Lower part presents functional domains of Pullulanase protein. Green, Yellow, Grey, and sky-blue represent pullulanase N2-domain, GH-13 N-terminal, catalytic domain, and alpha-1,6 glucosidases, C-terminal respectively. Amino acid residues highlighted with red denote pullulanase specific residues important for the interaction with pullulanase inhibitor. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

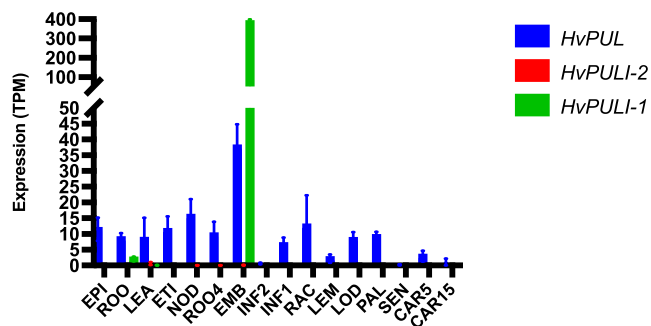


Fig. 4. Spatiotemporal expression pattern of pullulanase *HvPUL* (HORVU7Hr1G027860) and pullulanase inhibitor genes *HvPULI-1* (HORVU6Hr1G066330) and *HvPULI-2* (HORVU0Hr1G027030) in barley. Sixteen developmental stages used in the expression profiling are indicated on the bottom of X-axis, including *EPI*, epidermis (4 weeks); *ROO*, roots from seedlings (10 cm shoot stage); *LEA*, shoots from seedlings (10 cm shoot stage); *ETI*, etiolated (10 days old seedling); *NOD*, developing tillers at the six-leaf stage, 3rd internode; *ROO4*, root (4 weeks); *EMB*, 4-day-old embryos dissected from germinating grains; *INF2*, developing inflorescences (1–1.5 cm); *INF1*, young developing inflorescences (5 mm); *RAC*, rachis (5 weeks post-anthesis); *LEM*, lemma (6 weeks post-anthesis); *LOD*, lodicule (6 weeks post-anthesis); *PAL*, palea (6 weeks post-anthesis); *SEN*, senescing leaf (2 months); *CAR5*, developing grain, bracts removed (5 days post-anthesis); *CAR15*, developing grain, bracts removed (15 days post-anthesis). TPM, transcripts per million.

3.5. Interaction of HvSPLs with pullulanase and pullulanase inhibitor genes

For validating the RNA-seq data, we investigated the expression pattern of *HvSPL3*, and *HvSPL23* during the early stages of germination and found that *HvSPL3* co-expressed with *HvPULI-1* whereas the expression of *HvSPL23* was negligible (Fig. 5). Thus, co-expression of *HvSPL3* and *HvPULI-1* indicates their crosstalk which we have elucidated further via Electrophoresis Mobility Shift Assay (EMSA).

EMSA assay was performed to explore if *HvSPL3* (HORVU6Hr1G019700) and *HvSPL23* (HORVU3Hr1G094730) interact

with the promoters of pullulanase and its inhibitor genes. Gene-specific biotin labelled probes from the promoter regions were used to observe their binding with purified recombinant *HvSPL3* and *HvSPL23* proteins (Table S2). Our EMSA results indicate that probes Pi3 and Pi4 of *HvPULI-1* formed protein-DNA complexes with recombinant *HvSPL3* and retarded their mobility on the gel (Fig. 6). However, no such mobility shifts were observed with *HvPULI-2* and *HvPUL* probes (Data not shown). This binding reflects the role of *HvSPL3* in the transcriptional regulation of *HvPULI-1*.

3.6. Cysteine residues in SBP domain of HvSPL3 are critical for interaction with a cis-regulatory module of the pullulanase inhibitor

To evaluate if cysteines of the SBP domain are essential for the formation of *HvSPL3*-*HvPULI* complexes, all six cysteine residues were replaced with threonine residues. The interaction of mutated recombinant protein (*HvMSPL3*) with Pi3, and Pi4 probes from *HvPULI-1* was evaluated by EMSA. Mutated protein was unable to form complexes with the DNA probes and binding with probes was abolished, however, wild-type *HvSPL3* bound perfectly with *HvPULI-1* DNA and shifted its mobility on the gel (Fig. 6A). The results illustrated the indispensable role of cysteine residues in the SBP-DNA binding of *HvSPL3* during their interaction.

3.7. Role of redox in the interplay of HvSPL3 with HvPULI

We further investigated whether the transcription of *HvPULI* is redox-regulated through EMSA assay after redox treatment of *HvSPL3* recombinant protein using different oxidizing and reducing agents. Treatment of *HvSPL3* with oxidizing agents including Sodium tetrathionate ($\text{Na}_2\text{S}_2\text{O}_6$), Hydrogen peroxide (H_2O_2), and Oxidized glutathione (GSSG) completely abolished the binding of *HvSPL3* with the DNA of pullulanase inhibitor, however, reducing agents such Dithiothreitol (DTT), Thioredoxin (Trx) with Dithiothreitol (DTT), Thioredoxin (Trx) with NTR and NADPH favored the DNA-protein interaction of *HvSPL3* and pullulanase inhibitor. The shift in the mobility of DNA bound with *HvSPL3* has been clearly observed in the presence of reducing agents in Fig. 6B.

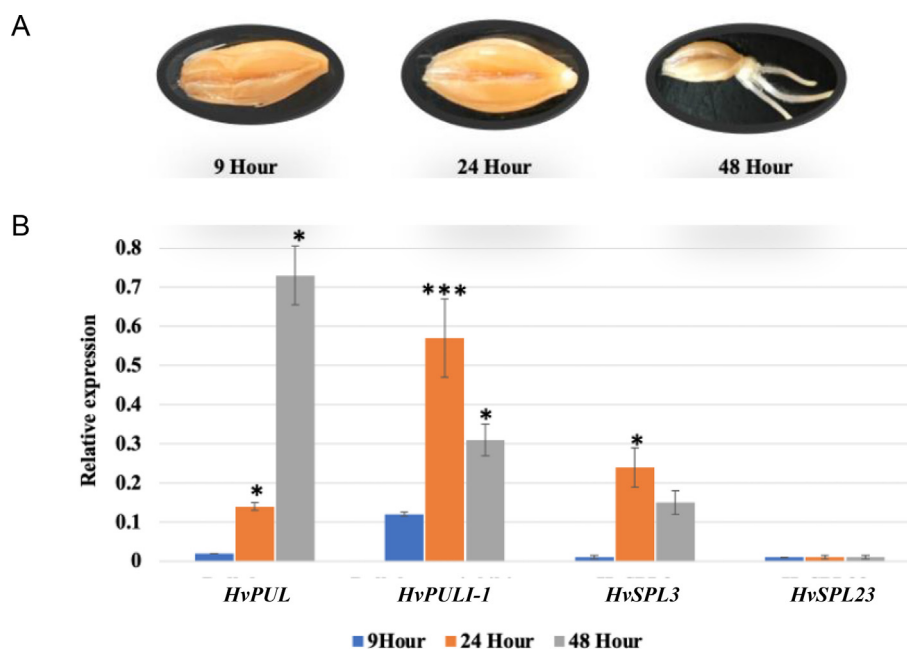


Fig. 5. Expression analysis of *HvPUL*, *HvPULI-1*, *HvSPL3* and *HvSPL23* at 9-, 24- and 48-hour of seed imbibition stage. (A) The barley seed morphology at 9-, 24- and 48-hour of imbibition; (B) The relative expression of *HvPUL*, *HvPULI-1*, *HvSPL3* and *HvSPL23* were measured by qPCR. Significance of relative expression at different timepoints is shown as student *t*-test (* $p < 0.05$, *** $p < 0.001$).

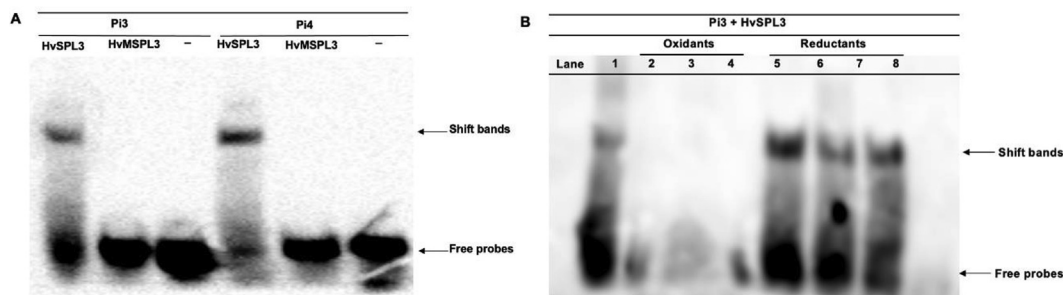


Fig. 6. The interaction of HvSPL3 with barley pullulanase inhibitor. (A) EMSA assay of pullulanase inhibitor (HORVU6Hr1G066330) probes (Pi3 and Pi4) with SBP domain of HvSPL3, and its mutated version HvMSPL3 where cysteine residues are mutated to threonine. - denotes negative control where Pi3 and Pi4 probe were electrophoresed without any protein; (B) Action of redox during interaction of Pi3 (HORVU6Hr1G066330) pullulanase inhibitor probe with recombinant HvSPL3 protein expressed in *E. coli*.; Lane 1: positive control with Pi3 probe and HvSPL3; Lane 2–7 Redox treatments; Lane 2–4 Oxidizing agents treatment 10 mM Na₂S₂O₆, 20 mM H₂O₂, 25 mM GSSG respectively; Lane 5–7 Reducing agents treatment 5 mM DTT, 5 μg Trx + 1 mM DTT, 5 μg Trx + 7.5 μg NTR + 0.25 mM NADPH respectively; Lane 8 -Negative control with Pi3 probe only.

These observations indicate the role of redox during the transcription of *HvPUL1* where reducing conditions create a favorable environment for the interaction of *cis*-regulatory elements in the promoter of *HvPUL1* with the HvSPL3 transcription factor.

4. Discussion

In this study, we show that the redox-dependent network of SPL/PUL1 modulates the transcription of pullulanase while seed transitioning towards germination. One pullulanase gene, *HvPUL* (HORVU7Hr1G027860), was identified in the barley genome and its protein contains seven amino acid residues involved in interaction with pullulanase inhibitors (Fig. 1). Previous studies reported that pullulanase is expressed as a single gene in developing endosperm and germinated grains of barley, maize, rice, and sorghum [12,2,6,14]. Our analysis also revealed two barley pullulanase inhibitor genes, *HvPUL1-1* (HORVU6Hr1G066330) and *HvPUL1-2* (HORVU0Hr1G027030), from which five amino acid residues involved in the interaction with pullulanase (Fig. 2). This is consistent with pullulanase inhibitor existed in two forms in barley, reported by Macri et al. [27].

Kristensen et al. [22] reported high pullulanase gene expression during germination and transcription level reached a maximum on day 5 of the germination. Some other studies observed maximum activity of pullulanase eight days after germination [15]. In maize, *PUL* transcript was found to be only specific to the reproductive tissues of the plant and not detected in leaf and root tissues [2]. However, further studies showed the activity of *PUL* promoter not only during seed development and germination but also in maize leaves [8]. These observations are in agreement with the spatiotemporal expression analysis in barley, in which one pullulanase, *HvPUL*, and one pullulanase inhibitor gene, *HvPUL1-1* show strong antagonistic expression patterns in various tissues and developmental stages (Fig. 5). However, the expression of *HvPUL1-2* was extremely low throughout all the stages. Although both inhibitor proteins possess high similarity in their catalytic domain, their expression pattern was completely different. We speculated that *HvPUL1-2* could be a pseudogene that needs further investigation. The qPCR results validated the antagonistic relationship between *HvPUL* and *HvPUL1-1* at 24- and 48- hours after imbibition. The literature reported the existence of pullulanase and inhibitor activity during barley germination [9,32].

To validate the role of HvSPL3 and HvSPL23 as transcription factors for *HvPUL*, *HvPUL1-1*, and *HvPUL1-2*, we performed an EMSA using biotin labelled DNA probes from the promoters *HvPUL*, *HvPUL1-1* and *HvPUL1-2* with HvSPL3 and HvSPL23 proteins expressed in *E. coli*. Although promoters of all three genes contain

the core motif 'GTAC', the interaction of HvSPL3 was only observed with *HvPUL1-1*. Previous studies also found the varying binding affinity of AtSPL1, AtSPL3, AtSPL7, and AtSPL8 with *Arabidopsis Apetala-1(AP-1)* [3]. It could be attributed to the adjoining sequences of the core motif, for example, AtSPL14 preferred 'CGTAC' instead of "GTAC" as its DNA binding motif [24]. We believe this provides specificity to the SBP-domain proteins. Further research is required to understand the effect of neighboring nucleotides around 'GTAC' on the preference of SPL transcription factors during DNA-protein interaction.

A conserved serine residue exists in the SBP domain of different SPL proteins and has been suggested as a phosphorylation site essential for their transcriptional activity [3]. Phosphorylation of OsSPL14 at Ser¹⁶³ within its DNA binding domain alters its binding specificity for activation of the expression *WRKY45* involved in enhanced disease resistance in rice [37]. To understand the role of cysteine residues within a highly conserved zinc ion binding motif, we mutated the cysteines with threonine residues in the zinc fingers of the SBP domain in HvSPL3 and verified its interaction with *HvPUL1-1*. It is clear that the mutated version of HvSPL3 did not bind with *HvPUL1-1*. This reflects that conserved cysteine residues in zinc fingers are indispensable for the transcriptional activity of HvSPL proteins. A similar observation has been made in *Arabidopsis* when mutations in zinc-coordinating cysteine and histidine residues cause the abolishment of binding. It was proposed that conserved cysteine and histidine residues are coordinating the zinc ions and affect nuclear import [3]. Therefore, it could be hypothesized that six conserved cysteine residues in the zinc fingers of the SBP domain might be involved in larger structural changes through disulfide linkages and DNA binding of HvSPL3 with *HvPUL1-1* is modulated by redox activities.

The cysteine-rich proteins with disulfide bonds are often linked to the redox status and regulated by thiol/disulphide transitions. Pullulanase and its inhibitor proteins both contain four disulphide bonds respectively and have been shown to be regulated by redox at the posttranslational level [30,20]. Redox regulation controlled by cellular redox agents such as thioredoxins, glutathione led to an alteration in the activity of proteins via thiol-disulfide exchanges [5]. Germination has often been proposed by the cellular redox status and studies have shown that redox changes in enzymes, mitochondrial components and transcription factors influence germination process [11,38,7].

To investigate the role of redox, an EMSA assay was conducted after treating recombinant HvSPL3 with different oxidizing and reducing agents. The recombinant HvSPL protein treated with oxidants was unable to form DNA-protein complexes. Our data concluded the need of reducing the environment in modulating the

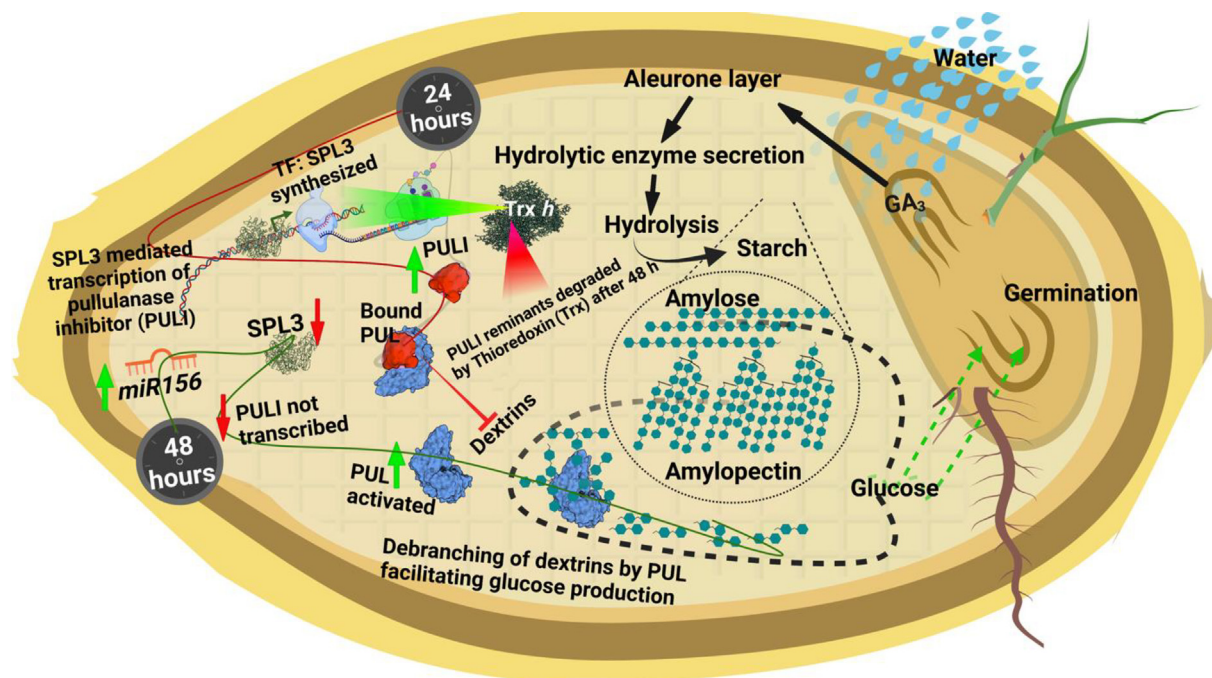


Fig. 7. A model for the regulation of PUL/PULI module for complete saccharification of starch during germination.

binding of HvSPL3 with *HvPULI-1* (Fig. 6B). It is evident from our experiments that thioredoxin reduces the recombinant HvSPL3 to facilitate the binding to the DNA probes. We speculate that Zn-ions hold the structure of the DNA-binding domain during oxidative conditions, but the DNA binding domain becomes accessible once the protein structure is disintegrated by a reducing environment. It is possible that the Zn-ions in SBP-DNA binding domain got dissociated in the oxidative environment, probably resulting in the SBP-domain deformity, and hampering the interaction. These findings provide evidence, for the first time, that the interaction of SPL transcription factors with their target DNA is redox-regulated.

5. Conclusion

Our findings, HvSPL3 binds with the promoter of *HvPULI-1*, which is important that provides new directions about the interplay of pullulanase and its inhibitor. Interestingly, cysteines in the SPL transcription factors are required during their binding with its target DNA, which we have described successfully that their interaction is redox dependent. Therefore, the cellular redox state plays a dual role in regulating the activity of pullulanase inhibitor through sulfhydryl-disulfide conversion at the post-translational level and by regulating SPL transcription factor activity through redox modulation (Fig. 7). Our study also shows that SPL transcription factors are involved in seed transition from dormant to germination phase. We are tempted to suggest that SPL transcription factors are involved globally as phase transition transcription factors.

6. Data availability

All data are available in the main text or [Supplementary Materials](#).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

JS, PGL, RD, and BBB designed research; SK and ZZ performed research; SK, ZZ, and JS contributed reagents/analytic tools; SK, ZZ, RD, and JS analyzed data; and SK, ZZ, RD, PGL, BBB, and JS wrote the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.csbj.2022.09.027>.

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