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Arabidopsis galactinol synthases 1 (AtGOLS1) negatively regulates seed germination

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ABSTRACT

Seed germination begins the growth phases of plants and its rate is affected not only by plant hormones, including abscisic acid (ABA), gibberellin (GA) and brassinosteroids (BRs), but also by environmental factors. In this study, we searched for additional chemical reagents that affect seed germination, using the *det2-1* and *ga1-3* mutants that showed reduced seed germination due to defective BR- or GA- biosynthesis, respectively. We found that the reducing reagent dithiothreitol (DTT) specifically enhanced seed germination of *det2-1* compared with that of *ga1-3*. To further investigate the underlying molecular mechanism for this phenomenon, we identified *AtGOLS1* as a differentially expressed gene in germinating seeds treated with DTT by GeneFishing analysis. *AtGOLS1* encodes a galactinol synthase, critical for the first step in raffinose family oligosaccharides synthesis during seed maturation. We observed that expression of *AtGOLS1* decreased when conditions were favorable for seed germination. We also determined that the seed germination rate was faster in T-DNA knockout *atgols1* mutant and transgenic plants transformed with an RNA interference construct targeting *AtGOLS1* compared with wild type plants. The double mutant of *det2-1* and *atgols1* also suppressed the reduced seed germination of the *det2-1*. Taken together, our results suggest that AtGOLS1 acts as a negative regulator in seed germination.

1. Introduction

A mature Arabidopsis seed is surrounded by two layers: a thin inner endosperm layer and an outer testa layer called the seed coat [1,2]. Overall, seed germination begins with water uptake by the mature dried seed and is completed when the radicle has emerged through the ruptures of the endosperm and testa layers [3,4]. The determination of seeds to be dormant or to germinate is critical to plant survival. To control the germination, many intrinsic signals are integrated into the seeds. In particular, the balance of two plant hormones, gibberellin (GA) and abscisic acid (ABA,) is critical to regulating seed germination [5]. ABA keeps the seeds dormant during embryo maturation and GA breaks the ABA-induced dormancy, resulting in a germinating seed [6–8]. In addition to this overall antagonistic regulation by ABA and GA, other plant hormones also affect the seed germination. Brassinosteroids (BRs), plant-specific steroid hormones, play critical roles in a broad range of plant development programs including cell growth, xylem differentiation, pollen tube growth, seed germination, and flower development [9,10]. Several BR-biosynthetic or BR-signaling mutants, such as *det2-1*, *bri1-1*, and *bri1-301*, show reduced seed germination, although their most distinguished phenotypic changes appear in their shoot morphology, with a dwarfed stature and compact and dark-green epinastic rosette leaves [11,12]. BR mutants show hypersensitivity to ABA, resulting in lower germination rate than that for wild type plants when treated with ABA [11]. Inhibitory effects of ABA on seed germination are exerted through delayed radicle expansion and weakening of the endosperm [13]. Actually, BRs, GA, and ethylene induce seed germination through facilitating testa and endosperm rupture [14,15]. Recently, a BR mutant was reported to be impaired in the synthesis of bioactive GA due to down-regulation of the genes encoding GA20ox and GA3ox, key enzymes for GA biosynthesis [12].

During water uptake, reactive oxygen species (ROS) bursts occur in seeds through the action of apoplastic peroxidases and NADPH oxidases. ROS function to decrease cell wall crosslinking, leading to increased cell wall elasticity and radicle extension [16,17]. Notably, nitric oxide ($^{\bullet}$NO) was reported to attenuate ABA-induced inhibition of germination through degradation of ABI5 via *S*-nitrosylation of a cysteine residue [18]. Redox changes in storage proteins in seeds also affect germination velocity. Generally, reduced sulfhydryl-containing proteins showed better solubility than oxidized proteins [19]. In barley, the reduced form of the seed storage protein hordein was increased in a thioredoxin *h*-overexpressing transgenic plant, resulting in acceleration of germination [20]. Wong et al. [20] reported that treatment of barley seeds with dithiolthreitol (DTT) enhanced seed germination, similar to

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the thioredoxin *h* overexpression. Therefore, these results imply that small proteins like thioredoxin or reducing molecules, like DTT, modulate seed germination through changing the redox status of the proteins that are involved in metabolic processes upon germination [20].

DTT is a reducing agent for thiolated DNA or various proteins [21,22]. When it is oxidized, DTT forms a stable six-membered ring with an internal disulfide bond with proteins. Because of its redox properties, DTT is often used for the full denaturation of proteins, along with sodium dodecylsulfate (SDS), in protein separation techniques. In addition, DTT is used as a potent antioxidant. Inhibition of seed germination by the heavy metal mercury (Hg) was caused by oxidation of an aquaporin, important proteins for water uptake in germinating seeds. The oxidative stress exerted on this specific protein was suppressed by the addition of DTT. When DTT was added, ROS-scavenging enzyme activities were increased [23]. However, in another aspect, DTT causes oxidative stress and induces expression of defense genes [24]. DTT is sometimes used as an endoplasmic reticulum (ER) stress-inducing agent through reduction of Cys–Cys bonds in the ER. DTT treatment changed sets of the unfolded protein response (UPR) genes in Arabidopsis [25] and in *C. elegans* [26].

In this study, we report that treatment with DTT enhanced seed germination of *det2-1*, a BR-biosynthetic mutant that showed reduced seed germination compared with the wild type. We further examined differentially expressed genes (DEGs) by DTT treatment using GeneFishing analyses and identified the *AtGOLS1* gene (*At2g47180*) as one of the genes whose expression was down-regulated by DTT. We determined for the first time that the expression of the *AtGOLS1*, encoding a galactinol synthase, was inversely correlated with germination capacity of seeds and showed that AtGOLS1 plays a negative role in seed germination, by analyzing the T-DNA knockout mutant and transgenic knock-down plants. Further, we showed seed germination of the *det2-1* was greatly enhanced by the lack of the *AtGOLS1* expression.

2. Materials and methods

2.1. Plant materials and growth conditions

We used *Arabidopsis thaliana* Columbia-0 (Col-0) as the wild type in all experiments. The BR-biosynthetic mutant *det2-1* and the GA-biosynthetic mutant *ga1-3* used in this study were in the Col-0 background. The *atgols1* T-DNA insertional knock-out mutant seeds (Salk_128044) were obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA). To generate transgenic plants, plants were transformed with an *AtGOLS1* RNA interference construct. Wild type cDNA was amplified with two primer sets (AtGOLS-RNAiF1/AtGOLS1-RNAiR1 and AtGOLS-RNAiF2/AtGOLS1-RNAiR2) separately to generate two 250-bp fragments covering bases 20–270 of the *AtGOLS1* gene. These two fragments were cloned into the vector *pHannibal* [27], and then re-cloned into the binary vector *pPZP212*. To generate transgenic plants overexpressing *AtGOLS1* driven by the native promoter, genomic DNA of wild type plants was amplified with a primer set (AtGOLS1 pro F/AtGOLS1 R) to produce a PCR fragment containing an approximately 2-Kb promoter region. The resulting PCR fragment was cloned into the *pPZP212*. All the completed plasmid constructs were transformed into wild type Col-0 using the *Agrobacterium*-mediated floral dipping method.

For the growth of plants on plates, seeds were sterilized with 75% ethanol containing 0.05% Tween-20 for 15 min and washed twice with 95% ethanol [28]. The sterilized seeds were germinated on 1/2 Murashige and Skoog (MS) medium (Duchefa, Haarlem, Netherlands) containing 0.8% phytoagar. For growth of plants in soil, seeds were sown directly onto Sunshine #5 soil. All plants were grown at 22 °C under long-day conditions (16 h light/8 h dark)

2.2. Assessment of seed germination

Twenty sterilized seeds were placed on 1/2 Murashige and Skoog (MS) medium, and then the plate was sequentially exposed to far-red light (3.2 μmol/m^2/s) and red light (20 μmol/m^2/s) as described (Oh et al., 2006). When needed, each of 1 μM of brassinolide (BL) (Sigma-Aldrich, St Louis, MO, USA), 1 μM of brassinazole (BRZ) (Santa Cruz Biotechnology, Dallas, TX, USA), 20 μM of gibberellin A3 (GA3) (Sigma-Aldrich), 1 mM dithiothreitol (DTT) (Sigma-Aldrich), 100 μM of glutathione (GSH) (Sigma-Aldrich), or 500 μM of ascorbic acid (AsA) (Sigma-Aldrich) were prepared in 1/2 MS medium. All plates were placed in thegrowth room set to the long day condition. Each day, germinated seeds with protruding radicles were counted [29].

2.3. Measurement of raffinose family of oligosaccharides (RFOs)

RFOs contents were determined using the Raffinose/D-galactose assay kit (Megazyme, Ireland). 500 mg of seeds were homogenized in 5 ml 95% (v/v) ethanol followed with the incubation under 85 °C for 5 min to inactivate endogenous enzymes. RFOs were extracted by adding 45 ml of 50 mM sodium acetate buffer (pH 4.5) and incubated at room temperature over 15 min 5 ml of the extraction solution/slurry was thoroughly mixed with 2 ml chloroform to remove the lipids. After centrifugation (1000*g*) for 10 min, absorbance of the upper aqueous solution was determined at a wavelength of 510 nm using a spectrophotometer (DU730; Beckman Coulter, Inc., Fullerton, CA, USA) and the RFOs contents were calculated by the manufacturer's procedure.

2.4. RNA expression analysis

RNA was isolated from imbibed seeds of indicated lines. To investigate differentially expressed genes (DEGs) by DTT treatment in *det2-1* compared with wild type seeds, a GeneFishing experiment was performed using an annealing control primer (ACP)-based PCR reaction following manufacturer's recommendations (Seegene, Korea). Reverse transcription-PCR (RT-PCR) analysis was performed according to the procedure described by Kang et al.[28]. First-strand cDNAs were synthesized using RNA treated with RNase-free RQ1 DNases (Promega, Madison, WI, USA) and adding SuperScript^{III}-MMLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and the Oligo(dT)-15 primer. An aliquot of the first-strand cDNA synthesis was used as a template in the second PCR. Quantitative RT-PCR was performed and analyzed with the Step-One Plus Real Time PCR system (Applied Biosystems, Foster City, CA, USA) using the same cDNA and a SYBR Green PCR Master Mix. The expression of the *Ubiquitin* gene (*At3g62250*) was used to normalize the data.

2.5. Primer sequences

Primer sequences for the genes used in this study are listed in Supplemental Table 1.

3. Results and discussions

3.1. The reducing agent DTT specifically promotes the seed germination of BR-biosynthetic mutant det2-1

BRs exert their physiological effect by alone and in combination