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ORIGINAL PAPER

Manipulation of cold stratification and endophytic effects on expression patterns of RSG and KAO genes in coleorhiza of wheat seeds

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Abstract Stratification is the exposure of seeds to conditions which break dormancy and enhance germination. Currently stratification is limited to abiotic conditions such as cold, acidity, light, salinity and osmotic priming. Here, the role of biological stratification on mycovitality was uncovered by assessing the effect of fungal endosymbiont on its host plant during dormancy release and germination commencement in seeds. The impact of the endophytic fungal SMCD 2206 strain on germination energy and vigor as compared to untreated seeds was evaluated. The expression patterns of plant growth promoting genes, repression of shoot growth (RSG) protein and *ent*-kaurenoic acid oxidase (KAO) of gibberellins (GAs), during the early breakage of seed dormancy and germination were assessed and indicated the involvement of a GA-mediated negative feedback regulation in treated seeds. The GA levels in all treated seeds reached optimum before 24 h of germination with direct interactions between seeds and endophyte generating maximum GA levels during the early germination stage. Our findings bring a significant contribution to plant prenatal care of cereal crops; they demonstrate endophyte-mediated vitality, a reprogramming of pre- and post-germination events in wheat seeds, which enhances dormancy breakage and germination.

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Abbreviation

GAs	Gibberellins
ABA	Abscisic acid
KAO	*Ent*-kaurenoic acid oxidase
RSG	Repression of shoot growth
SMCD	Saskatchewan microbial collection and database
PDA	Potato dextrose agar
GE	Germination energy
GV	Germination vigor

Introduction

Stratification is a pre-treatment of seeds to break seed dormancy and stimulate germination. Cold stratification is one of the most common seed germination enhancement practices that exposes moisten and pre-chilled seeds to fluctuating temperature between 0 and 10 °C (Offord and Meagher 2001; Hartmann et al. 1990). Cold stratification on wheat seeds significantly improves germination rates as well as salinity tolerance (Chauhan et al. 2006; Wang et al. 2011). In addition to the cold stratification, many other abiotic factors such as acidity, light quality, salinity and osmotic priming have an effect on seed germination rate (Lynch 1980; Ghersa et al. 1994; Almansouri et al. 2001; Akbarimoghaddam et al. 2011). Furthermore, biotic stratification using symbiotic fungal endophytes (Hubbard et al. 2011) on seed germination was recently demonstrated by Banerjee et al. (2014). This myco-mediated improvement of seed germination parameters, also known as

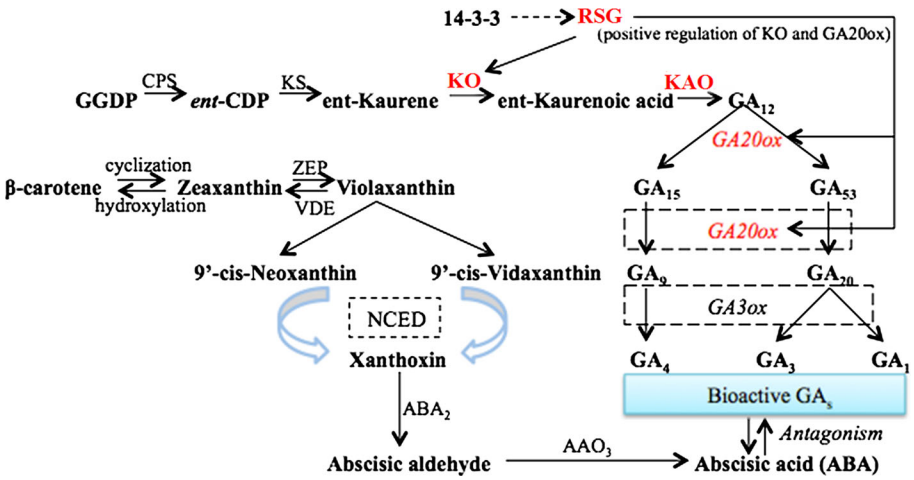

Fig. 1 The major GA biosynthetic pathway in wheat. RSG regulates the expression of ent-kaurene oxidase and GA 20-oxidase, directly or indirectly. CPS, ent-copalyl diphosphate; KS, ent-kaurene synthase; KO, ent-kaurene oxidase; KAO, ent-kaurenoic acid oxidase; GA20ox, GA 20-oxidase; GA3ox, GA 3-oxidase; GGDP; geranylgeranyl diphosphate, CDP; ent-copalyl diphosphate. (Modified from Spielmeyer et al. 2004 and Yamaguchi 2008)

mycovitalism (Vujanovic and Vujanovic 2007; Hubbard et al. 2012), alters expression of genes involved in phytohormonal metabolic pathways such as gibberellic acids or gibberellins (GAs) and abcisic acid (ABA) in germinating seeds (Banerjee et al. 2014).

Seed dormancy enforces a block to the germination and prevents seed pre-harvest sprouting in cereals. Dormancy can be categorized as coat-enhanced dormancy and embryo dormancy, which release may be associated with various environmental and chemical stimuli (Bewley 1997). During early germination, seeds are imbibed under condition of particular atmospheric water content to activate metabolic activities in the embryo (Bryant 1985). After 12 h imbibition, the elongation of coleorhiza is first observed followed by the elongation of coleoptile after 24 h (Barrero et al. 2009). Coleorhiza was initially recognized as a protective root sheath that shields radicula (Sargent and Osborne 1980). Furthermore, the coleorhiza absorbs water and nutrients to assist embryo emergence. Alternatively, during dehydration of seed, coleorhiza blocks radicula emergence and enhances seed dormancy. Barrero et al. (2009) demonstrated that the expression level of genes responsible for abscisic acid (ABA) biogenesis, which inhibits germination and enhances seed dormancy in coleorhiza (Noda et al. 1994) is reduced, and the catabolism of ABA is promoted during the early stages of imbibitions (Arc et al. 2013).

In addition to ABA catabolism, the bioactive gibberellic acids (GA_s), such as GA_1 and GA-$_4$, promote a series of distinct growth stages including seed germination, dormancy breaking, stem elongation, leaf expansion and seed maturity (Yamaguchi 2008). The GA biosynthesis pathway was defined in *Arabidopsis*, maize, wheat and barley, and consists of seven key enzymes: *ent*-copalyl diphosphate synthase (CPS), *ent*-kaurene synthase (KS), *ent*-kaurene oxidase (KO), *ent*-kaurenoic acid oxidase (KAO), GA 20-oxidase, GA 3-oxidase, and GA 2-oxidase (Yamaguchi and Shinozaki 2006; Yamaguchi 2008; Song et al. 2011; Huang et al. 2012; Spielmeyer et al. 2004). A simplified biosynthesis pathway of GA_s is presented in Fig. 1, as modified version from Spielmeyer et al. (2004) and Yamaguchi (2008). *Ent*-kaurenoic acid oxidase (KAO) is a cytochrome P450 monooxygenase which plays critical role in bioactive GA synthesis by converting *ent*-kaurenoic acid to GA_{12}, an intermediate involved in biosynthesis of GA_1, GA_3, and GA_4. Although only a few studies focused on KAO gene expression patterns in wheat previously, measuring KAO gene expression levels is critical to the understanding GA biosynthesis. Three homologues KAO genes have been found with different exonic sequences and they were located at the chromosomal arms 7AS, 4AL and 7DS respectively in wheat (*Triticum aestivum* L.) (Khlestkina et al. 2010).

The regulation of GA biosynthesis is controlled, at least in part, by the repression of shoot growth (RSG) protein which functions as leucine zipper (bZIP) transcriptional activator to enhance cell elongation (Fukazawa et al. 2000). The expression of KO and KAO is regulated coordinately by RSG, since the expression patterns of the KO1 and the two KAO mRNAs in *Arabidopsis* are similar (Helliwell et al. 2001). In addition, RSG plays a role in the homeostasis of GAs through direct binding to the GA20ox1 promoter triggered by a decrease in GA levels in the cell (Fukazawa et al. 2011).

Cold stratification is proven to be an effective technique to assist seed germination in weeds (Milberg and

Andersson 1998), grass (Baskin et al. 1992; Schutz and Rave 1999), pine (Carpita et al. 1983), himalayan balsam (Mumford 1988), tobacco (Wu et al. 2008), rice (Mukhopadhyay et al. 2004), and wheat (Wang et al. 2011). In addition, endophytic fungal symbiosis was shown to enhance wheat germination (Hubbard et al. 2012). However, the molecular mechanisms that are driving improvement in germination are currently undefined. Thus, our objective was to study KAO and RSG gene expression patterns associated with GA biosynthesis pathways in wheat coleorhiza tissues when exposed to conditions of cold stratification and endophytic fungal association. Since expression patterns of a gene involved in the late stage of GA biosynthesis (GA3-ox2) in wheat coleorhiza under cold and biological stratification was previously described (Banerjee et al. 2014), we focused on gene expression patterns of the two upstream genes involved in the GA biosynthesis pathway, KAO and RSG. Since fungal endophytes form a unique interaction with plant or seeds, and due to the particular characteristics of coleorhiza tissues in early germination of wheat seeds, we hypothesized that (1) endophytic treatment will benefit the host (wheat) by enhancing seed vitality and vigor that corresponds to mycovitality and increase the efficacy of wheat seed germination; (2) Fungal endophytes will break seed dormancy by enhancement of GA biosynthesis which would be reflected by up-regulation of the KAO gene in coleorhiza of wheat seeds; and (3) The timeline of expression and expression levels of KAO and RSG genes in biologically stratified seeds are different from those exposed to cold stratification.

Materials and methods

Description of wheat seeds

Seeds of the durum wheat cultivar AC Avonlea were produced under greenhouse conditions and certified to be free of microbes and low resistance to environmental stresses (Hubbard et al. 2012). Seeds were kept in sterile ziplock bags and stored in the dark in dry 4 °C conditions. Good quality seeds were selected uniformly and pre-treated with chlorine gas as an efficient sterilization process (Desfeux et al. 2000), which minimized the failure of germination as opposed to other sterilization methods (Zhang et al. 2007, 2008; Abdul Baki 1974; Abdellatif et al. 2009).

Germination of wheat seeds under cold and biological stratification

Cold stratification: Surface sterilized seeds were assembled and placed under moist conditions initially. A bulk of seeds ($\sim$200) were cold stratified at 4 °C for 48 h. After cold stratified treatment, ten seeds were randomly transferred to 90 mm petri dish with potato dextrose agar (PDA) and stored in room temperature for 3 days. Biological stratification: Two treatments were designed according to different fungal-seed interactions, and termed as endophyte-seed indirect (volatile/diffusible) interaction and endophyte-seed direct interaction (Banerjee et al. 2014). Endophyte-seed indirect interaction is based on an indirect-contact between seeds and endophyte through volatile organic and/or diffusible compounds (Zamioudis et al. 2013) produced by endophyte. In this experiment, an endophytic *Penicillium* sp. SMCD 2206 was cultured on PDA (Banerjee et al. 2014). PDA petri dishes with SMCD 2206 mycelia plug were allowed to grow in darkness under room temperature for at least 3 days. A mycelia agar plug (5 mm^2) of SMCD 2206 was dissected from the margins of a parent colony and placed on the center of a new PDA petri dish. Ten moistened and sterilized seeds were placed with equally spaces between each other and encircled the center mycelia plug with equivalent distance of approximately 4 cm (Abdellatif et al. 2009). Direct endophyte-seed interaction is the physical contact between fungal mycelia and seeds that may induce physiological transformations of seeds. Unlike indirect endophyte-seed interaction, moistened and sterilized seeds were distributed on PDA plate with (3 mm^2) agar plug between two adjacent seeds and 5 mm^2 plug in the centre. A set of non-treated seeds was used as a control in this experiment. For stratification, all experiments were done in triplicate. Following inoculation, all plates were carefully sealed with five layers of Parafilm® (Pechiny Plastic Packaging, Menasha, WI) to avoid any biological contamination and diffusion of volatile/diffusible compounds. All the plates were incubated in a bench-top incubator (Precision Thermo Scientific, model 3522, Nepean, Ont., Canada) at 20 °C in darkness and the germination conditions were recorded at 24, 48, and 72-h.

Germination

Seeds germination was determined visually when coleorhiza broke through the seed coat or by emergence of radicles. Germination rates were calculated as the number of germinated seeds out of 100 seeds that were planted.

Germination energy is the percent, by number, of seeds which germinate up to the time of peak germination, generally taken as the highest number of germinations in a 24 h period. It is calculated as:

$$\text{Germination energy} = \text{cumulative germination percentage} \div \text{days since sowing date} \times 100\ \%$$

In our experiments, 50 % germination energy was considered to be high germination energy.

The efficacy of germination is indicated as germination vigor, which is described as the potential of a seed to emergence and develop rapidly and uniformly (Rajjou et al. 2012). Germination vigor is calculated as:

$$\text{Germination vigor} = \%\,\text{germination in a treatment} - \%\,\text{germination in control}$$

All replicates were recorded with the same method and the mean values were taken as representative data used in analysis.

Isolation of coleorhiza under sterile condition

Wheat seeds were observed for germination rate before isolating coleorhiza at the growing points over 3 days (24, 48 and 72 h) following the method used by Banerjee et al. (2014). Wheat seeds were carefully dissected under the microscope and layers of coleorhiza were cleaved off using sterilized needle and scalpel. Isolated coleorhiza tissues were placed in an RNase-free sterilized microcentrifuge tube and weighted approximately 20 mg for each treatment. All tubes with coleorhiza tissues were centrifuged at 8000 rpm for 30 s to ensure all the tissues were deposited at the bottom of the tube and enhanced handling in later RNA extraction steps. Tubes with coleorhiza tissues were stored in liquid nitrogen as soon as coleorhiza tissues were isolated to preserve the cells and prevent denaturation of RNA.

Extraction of RNA and cDNA synthesis

Total RNA was extracted from 20 mg plant tissues on ice using the Aurum™ Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, CA). Extracted total RNA was directly loaded with premixed cDNA synthesis agents obtained from the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Reverse transcription was carried out at 42 °C for 30 min with a final denaturation at 85 °C for 5 min. cDNA concentration was measured by Nanodrop spectroscopy (Thermo Scientific, Wilmington, DE) and diluted or concentrated to 100 ng/µl.

Quantitative RT-PCR and Statistical Analysis

The quantitative real-time PCR (qRT-PCR) was performed on a MiniOpticon™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) with iQ™ SYBR® Green supermix kit (Bio-Rad Laboratories, Hercules, CA). In order to normalize qRT-PCR data, the *Actin* gene (131 bp length fragment) was selected as a reference gene and served as internal control to avoid fluctuation bias of gene expression under low cDNA concentration (Zhang et al. 2007; Nicot et al. 2005). Primers used in amplification were TAs (TA for *Triticum aestivum*): TA-KAO 5′-ACAACTGCCTGGCCAAGATCACCAG-3′ and 5′-GTGACAACTTTGACTCATCCGCGACAACAA-3 for KAO (184 bp) gene and TA-RSG 5′-TGGGCTACCG-GACTACGCCAAG-3′ and 5′-CCTTGGAACTTGACCT GCCGCTT-3′ for RSG (367 bp) gene according to Zhang et al. (2007). 100 ng/µl cDNA samples were further diluted to 10 ng/µl and 2 µl cDNA were used for each 25 µl reaction. In addition, 12.5 µl of iQ™ SYBR® Green supermix, 8.5 µl sterile milli-Q water, 1 µl of each forward and reverse primer (10 pmol) were made up to 25 µl reaction mix. 95 °C for 10 min and 40 cycles of 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 1 min were used for cycling conditions. All reactions were performed in triplicate with the appropriate no-template control reactions. The gene expression levels referred to quantitative curves were carried out by CFX Manager™ Software (Bio-Rad Laboratories, Hercules, CA). Cycle quantification (Cq) values from the recorded fluorescence measurements were adjusted manually for baseline. Relative quantification is the statistical method chosen in this study (Ginzinger 2002). Gene of interest relative to the endogenous control gene was used to compare with different treatments using the ΔΔCT method described previously (Bustin et al. 2009; Jurado et al. 2010).

KAO and RSG genes

Amplified KAO and RSG gene fragments were purified by using BioBasic PCR Purification Kit (Bio Basic Inc., Ontario, Canada) and sent for sequencing (NRC-PBI, Saskatoon, Canada). Gene sequences were identified by Basic Local Alignment Search Tool (BLAST) analyses (http://blast.ncbi.nlm.nih.gov). High identity or similar genes corresponding to different homologous organisms were assembled and aligned by software MEGA5 (Molecular Evolutionary Genetics Analysis). A phylogeny tree was generated using the statistical method of Neighbor-joining (Saitou and Nei 1987) based on the aligned genes.

Results and discussion

Germination rate and efficacy

To determine if our combined cold and endophyte stratification treatments were superior compared to standard treatments, we measured the germination rate and efficacy and compared them to control groups. Germination rates for cold stratified (85 %), endophyte indirect (87 %) and endophyte direct (89 %) stratification were all above that

of the control group (70 %) by the end of the day 3 (Fig. 2a). During the entire 3-day experiment, seeds in direct contact with endophyte showed the highest germination rate. Cold stratified and seeds affected by endophyte's volatile/diffusible compounds exhibited the lowest germination rate on day 1 and slight increase within next 2 days. Although the germination rates for the cold stratified and seeds affected by endophyte's volatile/diffusible compounds on day 2 and 3 were lower than the control, the final germination rates on day 3 reached and even exceeded the control values. Germination energy is used to measure the speed of germination and the capacity of the seeds to break dormancy (Hossain et al. 2005). On the first day, seeds in direct contact with endophyte showed a 50 % germination energy that was significantly ($P < 0.05$) higher than other treatments including the control (Fig. 2b). The germination energy of seeds directly interacting with endophytes remained higher on day 2 and 3 (Fig. 2b). Control and endophyte direct interaction were noticed for maximum increase in germination energy on the first day to the minimum on the last day of the treatment (Fig. 2b). However, cold stratification and endophyte indirect treatments gradually showed increase in germination energy from day 1 to day 3. Therefore, endophyte-seed direct interaction showed the most rapid germination among all treatments on any of these days. In contrast, cold stratification did not significantly impact speed of germination or dormancy breaking during the first 2 days. However, the germination speed was increased on the third day. To the best of our knowledge, previous studies of cold stratification on seeds did not measure the germination energy during the early stage of germination events. Instead, the general observation was that cold stratification improves germination rate. Subsequent research demonstrated that cold stratification could potentially enhance the production of the growth promotion hormone GA and inhibit the biosynthesis of dormancy-induced hormone abscisic acids (ABA) in general, as germination begins when GA levels become higher relative to ABA level (Pipinis et al. 2009). Nevertheless, the germination activities after cold stratification during early stage of germination events were never studied or followed traced by any of researches. Our results showed that cold stratified seeds need to recover from cold treatment in order to release germination energy to increase germination rate. Increase in germination rate will further cause the positive germination vigor on the third day of germination (Fig. 2c). According to the germination energy of endophyte-seed direct and endophyte-seed indirect interactions (Fig. 2b), endophyte-seed direct interaction can be considered an

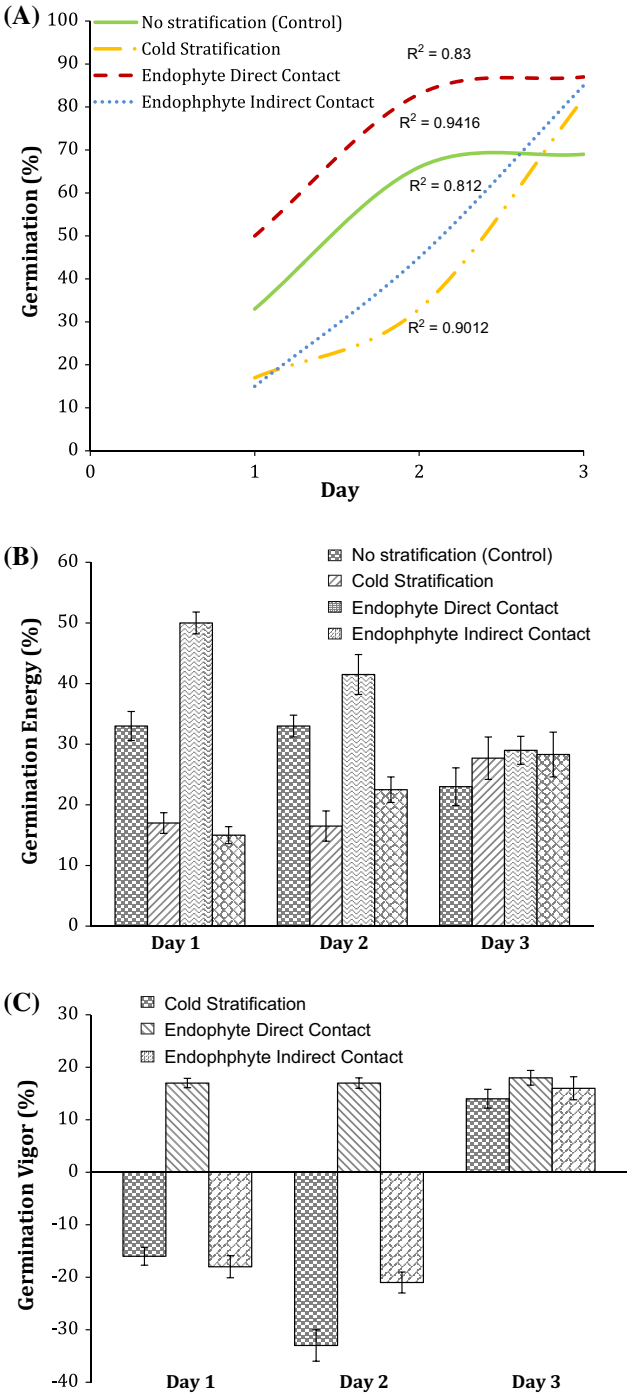

Fig. 2 Germination of wheat seeds in vitro after 3 days on potato dextrose agar at 20 °C in darkness. Cold stratification was applied by keeping seeds at 4 °C for 48 h. For endophyte indirect and endophyte direct treatments, seeds were germinated at approximately 4 cm distance and in direct contact with a mycelia agar plug (5 mm^2) of SMCD2206, respectively. **a** Germination rate, **b** Germination energy, and **c** Germination vigor. Germination vigor was calculated by subtracting the germination rate of control from treated seeds on day 3. In all experiments each value is the average of three replicates with ten seeds per replicate

efficient method to affect the seeds physically and physiologically via hyphae penetration through seed pores within a short period of time (Hill and West 1982). In contrast, endophyte-seed indirect interaction is based on effect of endophitic volatile/diffusible compounds on physiological status of the treated seeds (Strobel et al. 2004). Thus, endophyte-seed indirect interaction required a longer time to affect seeds, but by the end of the treatment improvement of a germination rate relatively to the control is observed. Although germination vigor (Fig. 2c) was improved through indirect endophyte-seed interaction, our results demonstrate that endophyte-seed direct interaction is the most efficient treatment to enhance germination rate. These results corroborate with our previous work demonstrating that SMCD2206 improved wheat seed germination under abiotic stresses such as heat and drought by improving seed vitality and vigor via "mycovitality" (Hubbard et al. 2012). Seed germination, however is still a complex process involving many molecular and biochemical changes whose underlying mechanisms are still being investigated (Nonogaki et al. 2010).

Gene phylogeny and expression patterns of RSG and KAO in coleorhiza

GA is the most common phytohormone associated with dormancy breaking, seed germination and germinant growth events. For those events to take place properly, seed developmental genes need to be silenced whereas vegetative growth genes are activated (Molitor et al. 2014). Thus, expression levels of GA biosynthesis related KAO and RSG genes were monitored.

The biosynthesis of GA in higher plants, such as wheat, is divided into three stages (Zhang et al. 2007). Repression of shoot growth (RSG), ent-kaurene oxidase (KO) and ent-kaurene acid oxidase (KAO) are involved in the first and second phases (Fig. 1), whereas GA20ox, GA3ox and GA2ox are involved in the third phase of GA biosynthesis. Genbank database with the KAO and RSG sequences has identified close similarity to particular sequences; KAO showed 100 % similarity to KAO-A1 (GU980891) and RSG showed 98 % similarity to bZIP type transcription factor (GQ266689). Phylogenetic analyses based on KAO sequence (Fig. 3a) unambiguously showed a close

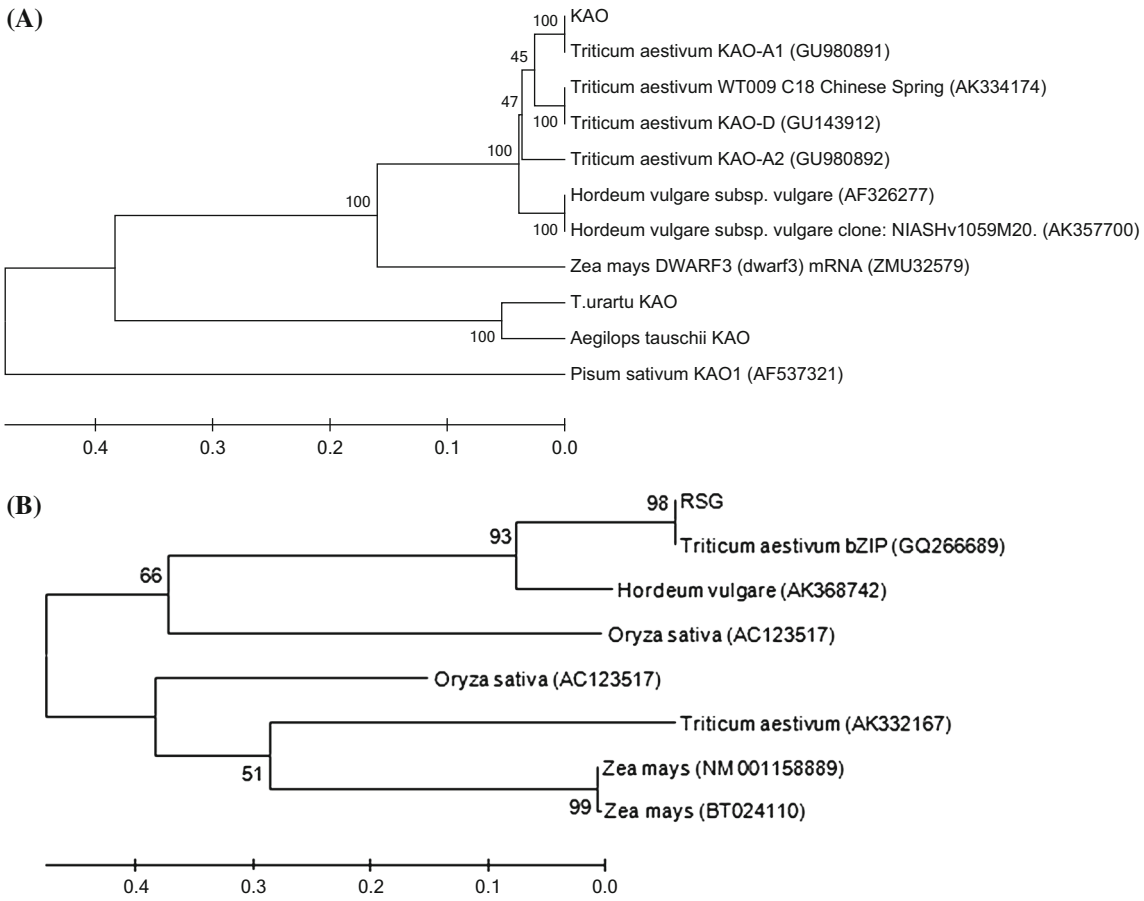

Fig. 3 Homologous **a** KAO and **b** RSG phylogeny trees constructed using DNA sequences by Neighbor-joining method

evolutionary relation between *Triticum* and *Hordeum*, whereas RSG (Fig. 2b) showed a relationship between *Hordeum* and *Oryzae* sequences. Khlestkina and colleagues (Khlestkina et al. 2010), identified KAO-A1 must be situated on wheat chromosome 7A, whereas no data were reported for RSG sequences. However, our basic BLAST search against Wheat-URGI database (http://wheat-urgi.versailles.inra.fr/Seq-Repository/BLAST) indicate that RSG sequences are mainly located on chromosomal arms 4 (A, B and D), 1B, 5B and 6D. Although this research did not exactly locate the RSG sequence position, the highest probability scores generated by Wheat-URGI search algorithm suggested the sequence location on chromosome 4D in wheat (*Triticum aestivum* L.).

RSG is recognised as a transcriptional activator, which positively regulates the expression of KO and KAO as well as GA20ox genes in biosynthesis of endogenous GAs in tobacco (Fukazawa et al. 2000, 2011). The relative gene expression of RSG was determined in seeds treated directly and indirectly with endophyte and in the seeds exposed to cold during the 3 days course. RSG expression was down-regulated after day 1 and day 2 for all treatments except in endophyte indirect treatment where RSG was up regulated on day 2 (Fig. 4A). On day 3, the expression level of RSG was up-regulated for all treatments. Moreover, the relative gene expression of RSG were significantly ($P < 0.05$) down-regulated only in endophyte direct treatment. RSG regulates transcription of KO and GA20ox genes and promotes the biosynthesis of GAs (Fig. 1). The expression of RSG gene and the function of RSG transcriptional factor are in direct correlation with bioactive GA levels. When GA levels reach optimum, the GA negative feedback regulation will become dominant and represses the transcription of GA20ox gene in order to block the GA biosynthesis pathways. In response to high GA level RSG gene will be suppressed. Otherwise, RSG will be up-regulated to promote transcriptions of KO and GA20ox genes and enhance GA biosynthesis (Fukazawa et al. 2010). The down-regulation of RSG gene during the day 1 and day 2 (Fig. 4a) may comes with two possibilities. Cold stratification and endophytic treatments of seeds may repress the expression of RSG as a result of excessive endogenous amount of GAs or GAs level reaches optimum even before 24 h of germination in all treatments. Germination data (Fig. 2a) does not support hypothesis that the expression level of RSG was repressed due to the high germination rate in endophyte direct treatment on day 1. More likely, the down-regulation of RSG on day 1 and day 2 is due to GA negative feedback regulation and GA levels might reach optimum within 24 h of germination. The most significant increase in GA level within 24 h of germination is observed in endophyte direct treatment. The cold stratification and endophyte indirect treatments did not show

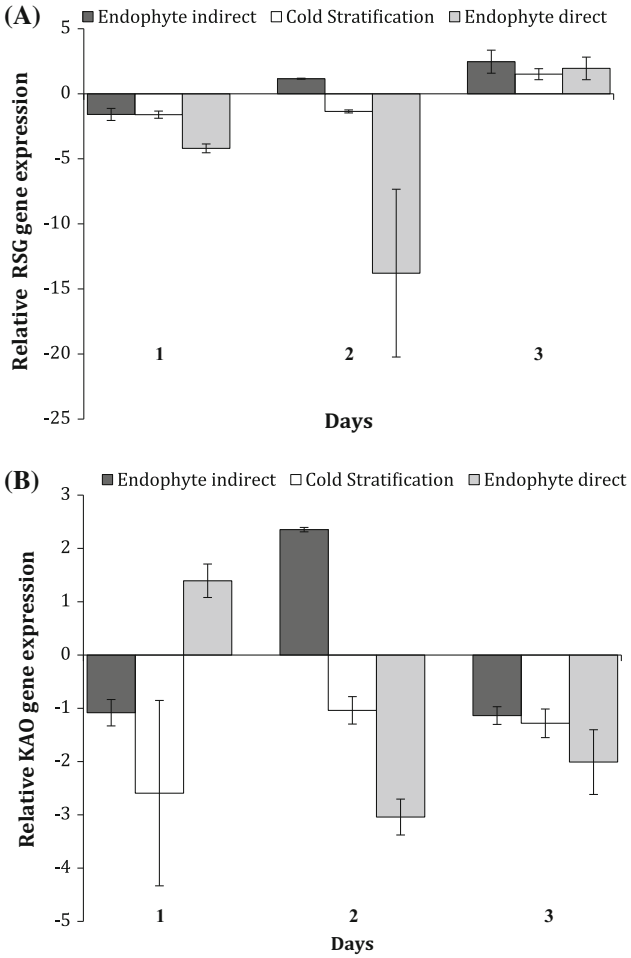

Fig. 4 The expression pattern of RSG and KAO genes in coleorhiza of germinating wheat seeds for 3 days under cold and biological stratification. **a** Relative RSG gene expression and **b** relative KAO gene expression. Gene expression was calculated as $2^{\Delta\Delta CT}$ using cycle threshold (Ct) value (Zhang et al. 2007). Wheat actin gene was used as a reference gene to normalize gene expression results. Gene of interest relative to the reference gene was used to compare within different treatments. The quantification (ΔCT) was done relative to the subtraction from Cq value of the gene of interest to Cq value of the control gene. ΔCT was further subtracted by calibrator value and generated corresponding ΔΔCT values which were transformed to log2 (doubling function of PCR) to synthesize relative gene expression levels

significant relative RSG expression for days 1 and 2. That can potentially indicate the GAs levels are relatively low as opposed to endophyte direct treatment. It also explains why the germination rate of endophyte direct treatment is significantly higher compare to other treatments. The relative expressions of RSG for all treatments were turned to up-regulation on day 3, which indicated that the endogenous GA levels were reduced due to the seeds' metabolism and RSG was required to involve in enhancement of GA biosynthesis. Although RSG and KAO are not directly associated with each other, biosynthesis of *ent*-kaurenoic acid has to be guaranteed with the assistance of RSG

according to Fig. 1. Also related to seed mycovitalism, Benerjee et al. (2014) reported the low expression of 14-3-3 gene, as negative regulator of GA biosynthesis, in wheat coleorhiza. These results suggest that 14-3-3 signaling protein partner has not been produced to negatively modulate the bZIP transcriptional activator RSG, which is involved in the regulation of endogenous amounts of GA (Igarashi et al. 2001; Ishida et al. 2004).

Therefore, the KAO expression is somewhat indirectly associated with RSG. The relative KAO expression on day 1 (Fig. 4b) was down-regulated for all treatments except endophyte direct, which indicate that the penetration of endophyte mycelia could potentially maximize the biosynthesis of GA against GA negative feedback regulation or drive up the seed metabolic rate in response to the reduction of GA levels. Nevertheless, the relative expression of RSG on day 1 was shown as down-regulated and double confirmed the homeostasis of GA was remained the same and endophyte direct association can enhance the biosynthesis of GA by elevating KAO expression levels. On day 2 the relative expression of KAO in cold stratification and endophyte direct treatment was down-regulated while endophyte indirect treatment showed up-regulation on the same day. This indicated that GA level reached maximum on day 1 in endophyte direct treatment of seeds and the expression of KAO is slowing down regarded with the shortage of *ent*-kaurenoic acid which was synthesized with the assist of RSG. The up-regulation of KAO expression in endophyte indirect treatment of seeds can be considered for the same reason as up-regulation of KAO in endophyte direct treatment on day 1; however, unlike direct contact, volatile/diffusable compounds from endophyte took longer time to cause effects on seeds in terms of physiological changes. Thus, the GA level was maximized after 48 h of endophytic indirect treatment. Besides, the cold stratification did not take actions to enhance the GA biosynthesis at all. The germination rate of endophyte direct and indirect treatment were above the other treatments on both days, 1 and 2 (Fig. 2a), which is supported by the relative expression data of KAO as explained above. The relative expressions of KAO on day 3 were down-regulated for all treatments and GA level will be reduced due to the metabolic reactions of seeds, which also include the up-regulated expression of RSG to recover the GA biosynthesis pathways.

Fungal endophytes have been studied over many years and proved to break dormancy and enhance germination rate in plant seeds such as wheat and grasses (Hubbard et al. 2012; Gundel et al. 2006). Endophyte-seed direct interaction was shown as a most efficient method as compared to other treatments in this study. The endophytic hyphae of SMCD 2206 strain were penetrated into seed coat after approximately 12 h, compared to the direct penetration of coleorhiza tissues which occurred within 24 h of co-culture on PDA plate. The physical contact followed by physiological changes drives up biosynthesis of GA to stimulate dormancy release and trigger germination. Thus, this study on endophytic fungi-seed interaction demonstrates an important reprogramming effect on pre- and post-germination events of wheat seed towards an enhanced dormancy breakage and germination, effectively contributing to the prenatal care of cereal crops.

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