Microbiological Research 169 (2014) 616–622

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Gene expression patterns in wheat coleorhiza under cold- and biological stratification

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ARTICLE INFO

Article history:
Received 11 July 2013
Received in revised form
28 September 2013
Accepted 30 September 2013
Available online 8 October 2013

Keywords:
Mycovitality
Biological stratification
Fungal endophyte
Coleorhiza
Gene expression

ABSTRACT

This study assessed germination of wheat seeds under cold and biological stratification and determined the expression level of gibberellins (GA) and abscisic acid (ABA) genes in coleorhiza. Both cold and biological stratification significantly ($P < 0.05$) enhanced the rate and efficacy of germination. The spatial distance between the fungal endophyte and the seed can be a determining factor of biological stratification as seeds in direct contact with fungal endophyte showed the highest rate and efficacy of germination. Consistently high expression of GA3ox2 gene was found in wheat coleorhiza throughout the tested period of germination. The expression of ABA biosynthesis gene, TaNCED, was substantially higher in cold stratification seeds, reflecting the role of abscisic acid in stress-adaptation. Overall, this study provides molecular evidence of the importance of coleorhiza in germinating wheat seeds, in addition to reporting that the spatial distance between symbiotic partners may be a critical factor driving mycovitality.

1. Introduction

During last three decades, world wheat production declined 5.5% due to climate changes, threatening food security (Lobell et al. 2011). Wheat is one of the most widely used cereal crops in the world. Although Canada produces nearly 5% of world's total wheat, it exports more than 75% of its total production (Canadian Wheat Board, 2010). Climate is a key driver of plant ecophysiology, phenophases, and reproduction efficacy. Hence, consequences of a global warming on each stage along the plant developmental continuum seem likely (Cleland et al. 2007). In a mature seed, the non-vascularized multicellular embryonic tissue that shields the quiescent meristematic tissue of radicle and elongates during imbibition and early germination before radicle emergence in monocot seeds is called coleorhiza (Sargent and Osborne, 1980). Although coleorhiza was initially perceived as a mere protective layer, its diverse morphology, anatomy, functioning, and key role in seed dormancy or germination have recently been elucidated (Barrero et al. 2009). Seed germination is one of the vital phases in plant life cycle; it regulates plant emergence and early adaptation to natural or agricultural ecosystems and as such it is the foundation of crop production (Weitbrecht et al. 2011). The impact of fungal endophytes on seed germination is well-established (Warcup, 1985; Vujanovic et al. 2000). Fungal endophytes help seed break their morphophysiological dormancy and maintain vitality, leading to successful germination; this critical phenomenon is known as 'mycovitality' (Vujanovic and Vujanovic, 2007). The use of hydrothermal modeling demonstrated that rate and energy of germination in wheat can be significantly improved by employing fungal endophytes (Hubbard et al. 2012). Furthermore, mycobionts can enhance heat and drought tolerances of wheat, which in turn aid seeds attain 50% germination in ~2 days (Hubbard et al. 2013). This is particularly important as seeds are the key generative organs in regeneration and dispersal of flowering plants (Baskin and Baskin 2004). The beneficial impact of fungal endophytes on crop seed germination has considerable implications for agronomy and plant biotechnology. Seeds are a fundamental constituent of the world's diet and cereal grains add up to half of the global per capita energy intake (Bewley, 1997). Hence, assessment of conditions that improve symbiotic seed germination is of strategic importance.

Stratification is a long-known process of "activation" that facilitates the release of dormancy and onset of germination (Koller et al. 1962). In wild conditions, seeds endure natural stratification that

Abbreviations: ABA, abscisic acid; GA, gibberellins; PDA, potato dextrose agar; qPCR, quantitative real-time PCR.

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0944-5013/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.micres.2013.09.016

is seed-testa softens up on frozen ground in winter and triggers embryo to rupture the testa. Thus, stratification was traditionally perceived as a cold related signaling mechanism of breaking seed dormancy, without considering biological or fungal symbiotic activators. Cold stratification in which seeds are kept at dark and cold (~4 °C) conditions is a well-acknowledged method of seed germination enhancement. It is often reported as the most effective way of promoting seed germination (Schutz and Rave, 1999). Cold stratification has been studied in many species including weeds (Milberg and Andersson, 1998), grass (Schutz and Rave, 1999), pine (Carpita et al. 1983), tobacco (Wu et al. 2008), and rice (Mukhopadhyay et al. 2004). Recent transcriptomic studies discovered importance of coleorhiza in regulating dormancy in cereals (Barrero et al. 2009). Because of the obvious importance of cold stratification it is imperative to understand the molecular mechanisms and drivers of this process. However, very few studies have examined the effect of cold stratification on seed germination and expression patterns of functional genes, especially in wheat. Since many of the functional genes associated with seed germination in wheat are well-established, wheat can serve as a model plant for disentangling the impact of stratification. For example, the genes GA3-oxidase 2 and *14-3-3* are known as GA biosynthetic gene and negative regulator of GA biosynthesis pathway, respectively (Ji et al. 2011; Zhang et al. 2007). The NCED gene is well known for its role in ABA biosynthesis pathway whereas ABA 8′-hydroxylase gene is involved in ABA catabolic pathway (Ji et al. 2011). These key functional genes can be employed to assess the effect of stratification on seed germination in wheat.

Microorganisms that colonize and cause asymptomatic infections in healthy plant-tissues are called endophytes (Strobel et al. 2004). Considerable number of studies have shown that fungal endophytes are capable of producing volatile compounds that can affect various phenophases in plants (Mitchell et al. 2010; Strobel et al. 2001, 2004). Although early physiological studies confirmed that spore germination and growth of microorganisms have been enhanced by volatile compounds released from germinating seeds (Schenck and Stotzky, 1975), it is still poorly known if volatiles discharged by microbial endophytes or microbe-seed physical contact have the same effect on seed response and germination (Vujanovic et al. 2000). The role of fungal endophytes in enhancing seed germination was recently recognized in *Gramineae* including wheat, as a natural phenomenon with potentially important biotechnological implications (Clay, 1987; Hubbard et al. 2012; Vujanovic, 2007). Although both cold and symbiotic stratification are individually well-acknowledged for last few decades, comparison of these mechanisms with regard to seed germination and functional gene expression patterns remains elusive. This study aims to disentangle the GA-ABA expression patterns in wheat seed during cold and biological stratification to link germination rate and gene expression patterns in this critical phenophase. The overarching goal of this study is to understand the mechanisms by which endophytes affect seed germination and to assess if the volatile compounds produced by endophytes influence plant gene expression that in turn may affect seed dormancy and germination. Specifically, the following research questions were asked: (1) are the rate and efficacy of symbiotic seed germination stimulated by physical contact and/or spatial distance between two symbiotic (photobiont and mycobiont) partners; and (2) is the percentage of seed germination linked to different expression patterns of GA and ABA biosynthesis genes when exposing the seed to biological *vs.* cold stratification?

This study provides evidence for the hypothesis that fungal endophytes improve wheat seed germination by modulating the expression of germination-related genes.

2. Materials and methods

2.1. Wheat seeds and sterilization protocols

Seeds of the durum wheat cultivar AC Avonlea produced by Agriculture and Agri-Food Canada Seed Increase Unit Research Farm (Indian Head, Saskatchewan) were used in this study. The seeds used were produced under greenhouse conditions, and were certified to be free of microbes (Hubbard et al. 2012). To identify the best suitable protocol that efficiently sterilizes seed-surface without affecting seed quality and vitality, we compared four widely acknowledged seed-sterilization methods: sterilization by 95% ethanol (Zhang et al. 2007, 2008), sterilization by 5% sodium hypochlorite (Abdul-Baki, 1974), combined 95% ethanol and 5% sodium hypochlorite (Abdellatif et al. 2009), and sterilization by chlorine gas (Desfeux et al. 2000). Chlorine gas sterilization protocol was the most effective method showing 80% germination and completely free of contaminants and thus, it was selected to sterilize the seeds.

2.2. Cold and biological stratification

For cold stratification, surface sterilized seeds were kept on moist filter paper at 4 °C cold-room for 48 h (Mukhopadhyay et al. 2004; Wu et al. 2008). For biological stratification, sterilized seeds were incubated in presence of SMCD 2206, an endophytic Ascomycota mitosporic fungal isolate deposited in the Saskatchewan Microbial Collection Database (SMCD). SMCD 2206 strain was selected by Dr. Vujanovic from a much larger collection of hundreds of fungi because of it positive impacts on wheat agronomic traits including yield subjected to biotic stress (Hubbard et al. 2013). This fungus differs from other wheat endophytes in terms of macroscopic and microscopic appearance, growth rate as free-living organisms and tolerance for heat or drought stress (Hubbard et al. 2012). Both *in vitro* and *in planta* studies demonstrated the endophytic nature of SMCD 2006 being colonizer of asymptomatic wheat (Hubbard et al. 2012, 2013). Because of its positive impact on seed germination and stress tolerance in wheat, SMCD 2206 was selected in this study. Fungal endophyte was grown on potato dextrose agar (PDA) at room temperature in darkness for at least three days before use. To assess indirect fungal stratification, an agar plug (5 mm^2) of the endophyte dissected from the margins of a parent colony was placed in the center of a 90 cm petri dish with PDA, ensuring an indirect contact *via* exchange of volatiles. Then 10 surface sterilized seeds were place at the periphery of the petri dish encircling the fungal agar plug at approximately 4 cm distance. All petri dishes were sealed with 5 layers of Parafilm® (Pechiny Plastic Packaging, Menasha, WI) to avoid diffusion of volatile/gaseous compounds. The impact of direct-contact of the fungal endophyte was elucidated by placing a 3 mm^2 agar plug between two adjacent surface sterilized wheat seeds and 5 mm^2 plug in the center of the PDA plates (Abdellatif et al. 2009). All treatments were carried out with three replicates of PDA plates with 10 surface sterilized wheat seeds on each plate. Petri dishes were incubated at ~20 °C in darkness.

2.3. Germination percentage

Wheat seed germination was recorded during 3 days after sowing on agar plates. At that time, seeds in direct-contact were colonized by fungus hyphae while seeds in indirect-contact assays were non-colonized meaning that fungus-seed communication was only possible *via* diffusible or volatile chemical signals transferred from the sender to the receiver cells (Bruns and Read, 2000). The germination assessment was applied to distinguish developmental and gene expression changes during the early seed

germination events. Percentage of germination was calculated by estimating the number of seeds germinated out of 10 wheat seeds on each PDA plate. The 50% germination rate was assumed as the energy of germination (Hubbard et al. 2012). The efficacy of germination in different treatments was calculated as: efficacy = % germination in a treatment – % germination in control. In this study, we assessed the early germination events during the first 3days of germination as appropriate phenophase to depict a shift in genes expressions related with mycovitalism. Since some of initial germination percentages showed zero, we calculated and presented germination efficacy over three days of incubation using specific formula for 'seed vigor'. Seed vigor depends on multiple biochemical and molecular variables; it is a precondition of seed germination performance and healthy seedling emergence (Ulmanova et al. 2013). Differences in germination rate or seedling emergence between treated seeds and seeds that germinate under standard or control conditions referred to as differences in seed vigor (Hampton and TeKrony, 1995).

2.4. Isolation of coleorhiza and RNA extraction

Wheat seeds were carefully dissected under compound microscope and layers of coleorhiza were cleaved off using sterilized needle and scalpel. Isolated coleorhizas were stored in an RNase-free sterilized microcentrifuge tube and RNA extraction was performed immediately. Total RNA was extracted from 20 mg of coleorhiza samples using Aurum™ Total RNA Mini Kit according to manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). RNA concentration was spectrophotometrically measured by Nanodrop (Thermo Scientific, Wilmington, DE). Immediately after RNA extraction, cDNA synthesis was performed using iScript cDNA Synthesis Kit following manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

2.5. Quantitative real-time PCR and sequencing

Expression of gibberellin and abscisic acid functional genes was estimated by relative quantification using quantitative real-time PCR (qPCR). Various catabolic and biosynthetic genes were selected to assess their respective roles in cold and biological stratification (Table 1). Wheat actin gene of 131 bp length fragment was used as the internal control (Nakamura et al. 2010). qPCR was performed using a MJ-Mini Gradient Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) following manufacturer's instructions. The PCR condition was 1 cycle of 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. Each 25 μl reaction contained 12.5 μl of iQ™ SYBR® Green supermix, 6.5 μl sterile milli-Q water, 1.5 μl bovine serum albumin, 2.5 μl (25 ng) of template cDNA, 1 μl of each forward and reverse primer. Relative quantification was performed according to Zhang et al. (2007). To ensure the specificity and consistency of amplicons, melting curve analysis and agarose gel electrophoresis were performed after each qPCR run. Amplicons were purified using BioBasic PCR Purification Kit (Bio Basic Inc., Ontario, Canada) and were sent for sequencing at Plant Biotechnology Institute (NRC-PBI, Saskatoon, Canada). Sequences were identified by Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov).

3. Results and discussion

3.1. Percentage and efficacy of germination

Both cold stratification and biological stratification treatments significantly enhanced the rate of germination with all three treatments exhibiting higher germination percentage than control (Fig. 1A). Energy of germination is a critical parameter of seed vitality determining the capacity of seeds to break dormancy and start germination. Endophyte-direct showed highest germination percentage reaching the energy of germination (50%) at day 2. Throughout the assessed germination period of 3 days, it demonstrated significantly ($P < 0.05$) higher germinability than other three treatments. Moreover, some replicates of direct and indirect endophyte treatments attained 50% germination at ~20 and ~32 h of incubation respectively. In terms of efficacy of germination, endophyte-direct showed highest performance followed by endophyte-indirect and cold stratification (Fig. 1B). Although the overall efficacy was not significantly different among the treatments, individual trends are apparent throughout the growing period. Moreover, on Day 2 which is a key stage in seed germination, the efficacy was significantly ($P < 0.05$) different among the treatments.

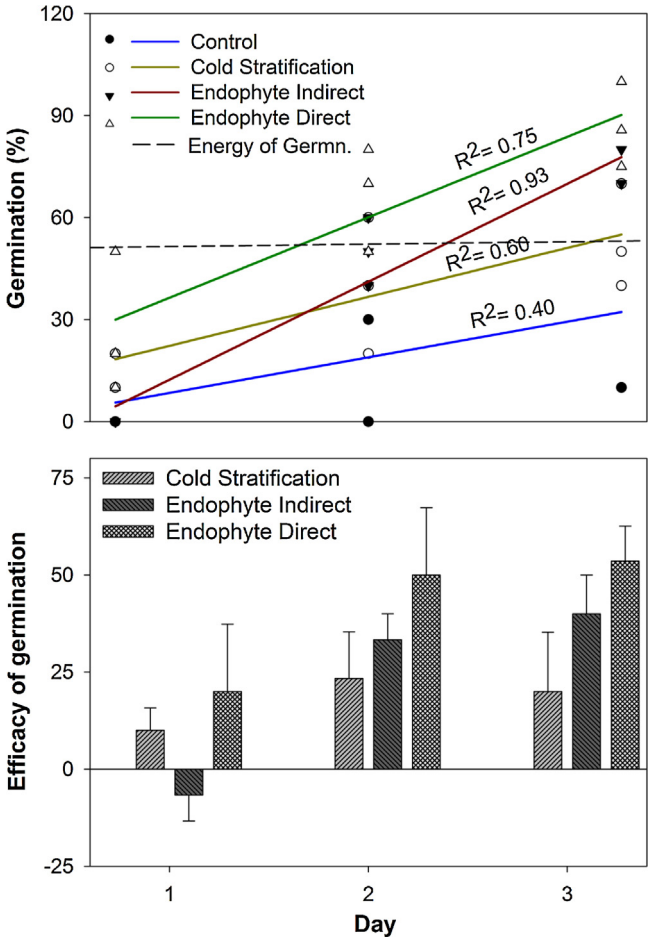

Fig. 1. Germination of wheat seeds *in vitro* after three days on potato dextrose agar at 20° in darkness. Cold stratification was imposed by keeping seeds at 4 °C cold-room for 48 h. For endophyte-indirect and endophyte-direct treatments, seeds were germinated at approximately 4 cm distance and in direct contact respectively. (A) Percentage of germination in comparison with energy of germination (≥50% germination). Each value is the average of 3 replicates with 10 seeds per replicate. (B) Efficacy of germination of wheat seeds subjected to cold and biological stratification. Efficacy was calculated by subtracting the germination percentage of control from treated seeds at the end of experiment, day 3.

Alleviation of seed dormancy and improvement of germination through cold stratification have been achieved in many species. This study found that the effect of cold stratification requires an initially longer period of dormancy, making seed germination not significantly different from control on Day 1. However, it demonstrated considerable impact on germination from Day 2 and percentage of germination increased as much as 20% higher than the control (Fig. 1A). The time period of cold stratification in this study was

Table 1
List of genes, their respective primers, similarity of sequenced amplicons with known genes.

Gene	Function	Primer name	Primer sequence (5′ → 3′)	Reference	Length of amplicon (bp)	Similarities with homologue genes
Actin	Internal control	*Actin F* *Actin R*	GACCCAGACAACTCGCAACT CTCGCATATGTGGCTCTTGA	Nakamura et al. (2010)	131	*Triticum aestivum* actin gene, partial cds Identities = 99/100 (99%)
ABA 8′-hydroxylase gene	ABA catabolic pathway	*TaABA8′OH1F* *TaABA8′OH1R*	ACAGATGGTCCACCTCCAAG CCTCTATCGTGCCGTTGATT	Ji et al. (2011)	122	*Triticum aestivum* ABA 8′-hydroxylase (CYP707A1) gene Identities = 108/108 (100%)
NCED gene	ABA biosynthesis pathway	*TaNCED2F* *TaNCED2R*	ACGGCTACGTGCTGTCCTT GATTAGTGCTGGGCTTCGAG	Ji et al. (2011)	130	*Triticum aestivum* cDNA clone: WT005_L05 Identities = 122/140 (87%)
GA3-oxidase 2 gene	Gibberellin biosynthesis pathway	*TaGA3ox2-3F* *TaGA3ox2-3R*	GTACATGGGCGTGCGCAAGAAG GCTAATCTAACAGCCCGCCACCAT	Zhang et al. (2007)	260	*Triticum aestivum* gibberellin 3-oxidase 2–3 (GA3ox2-3) Identities = 211/212 (99%)
14-3-3 gene	Negative regulator of gibberellin biosynthesis pathway	*14-3-3 F* *14-3-3 R*	CACTATGTCTGGGGTCG ATTTAGGACTTGCTGGCA	Zhang et al. (2007)	340	*Triticum aestivum* cDNA, mRNA: DR740841 Identities = 387/480 (81%)

selected from previous reports (Wu et al. 2008; Mukhopadhyay et al. 2004). Earlier studies showed that the impact of cold stratification is proportional to its time-length (Cavieres and Arroyo, 2000). The findings from present study supports this and further extends the notion to report that slightly longer stratification period (~4 days) may be required for wheat to attain maximum germinability.

Several reports have shown the enhancement of seed germination through the application of fungal endophytes (Vujanovic et al. 2000; Hubbard et al. 2012). The present study also supports the concept of "mycovitalism" (Vujanovic and Vujanovic, 2007), which is the increase of vitality through fungal colonization. This study shows that fungal contact is not necessary for seed germination, which also suggests that some type of diffusible or volatile compound is involved (Bruns and Read, 2000), Thus, endophytes may be capable of affecting seed germination even when they are not in direct contact with seeds, and this attribute is particularly useful in field conditions (Song et al. 2010). Nonetheless, seeds in direct contact with fungal endophyte are undoubtedly more benefitted than

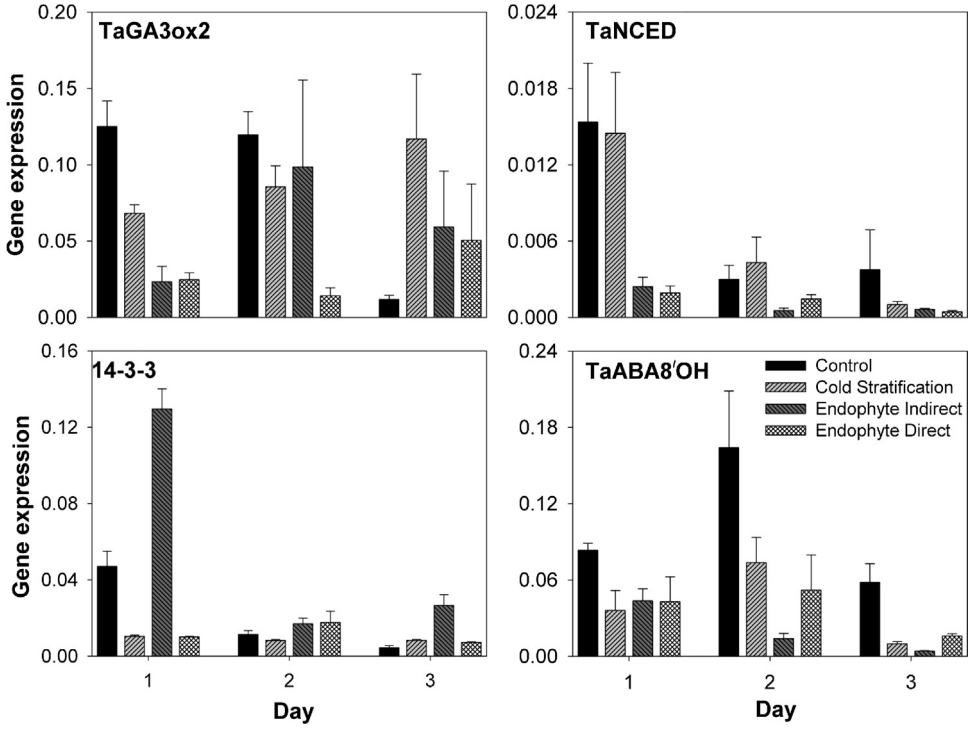

Fig. 2. Differential expression patterns of gibberellin (*TaGA3ox2* and *14-3-3*) and ABA (*TaNCED* and *TaABA8′OH*) genes in coleorhiza of germinating wheat seeds for three days under cold and biological stratification. Gene expression was calculated as $2^{-\Delta CT}$ using cycle threshold (Ct) value (Zhang et al. 2007). Wheat β actin gene was used as a reference gene to normalize gene expression results. The difference between the Ct values of target gene and β actin gene (Ct^{target}–Ct^{actin}) was estimated as ΔCt and then the expression level was calculated as $2^{-\Delta Ct}$. Mean of three replicates of $2^{-\Delta Ct}$ were used to assess difference in expression between control and stratification treatments.

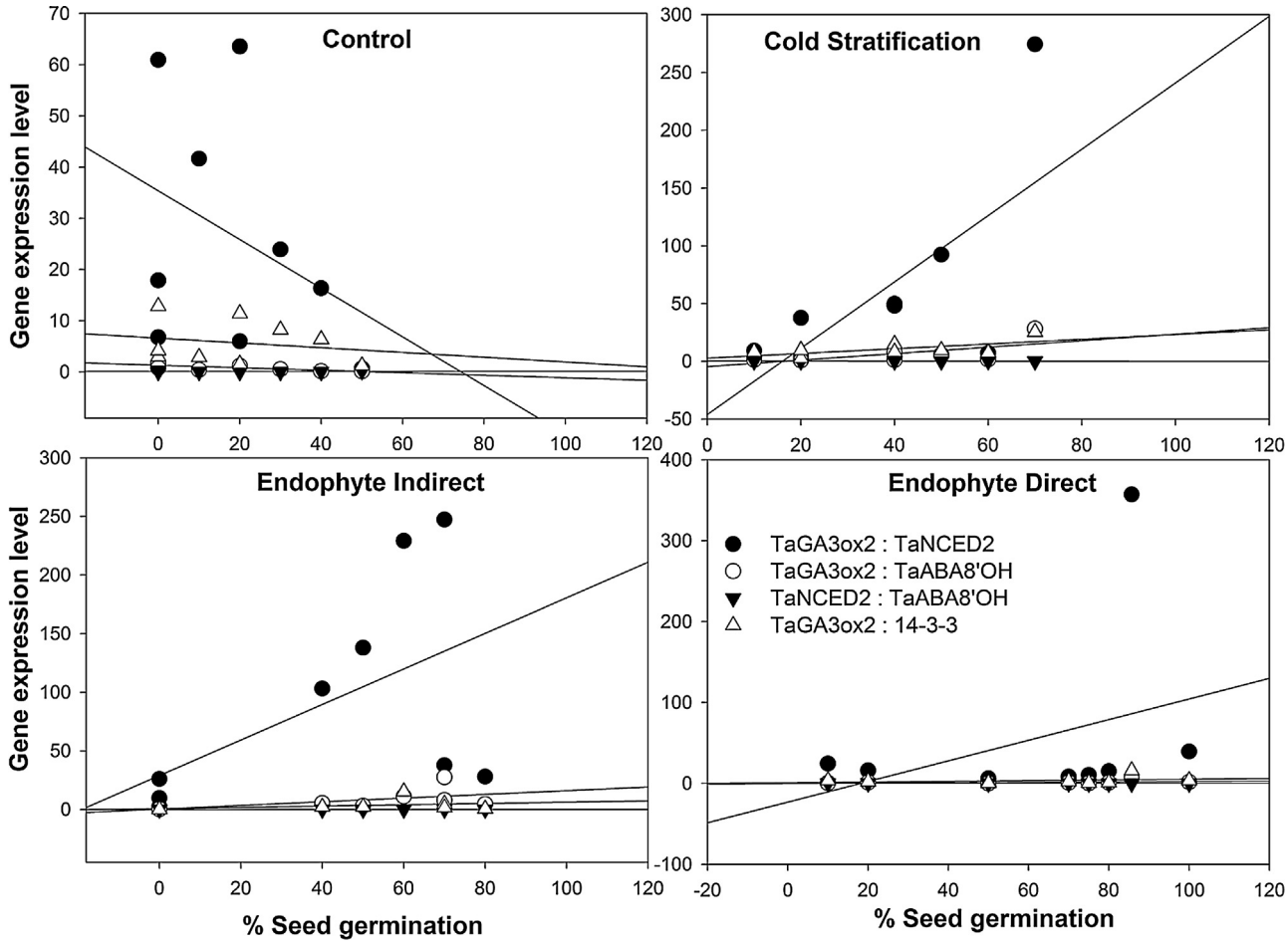

Fig. 3. Regression plots of seed germination and the ratio of expression levels ($2^{-\Delta CT}$) of gibberellin (*TaGA3ox2* and *14-3-3*) and ABA (*TaNCED* and *TaABA8′OH*) genes in coleorhiza of germinating wheat seeds for three days under cold and biological stratification.

their counterparts. However, it is important to note that it is possible that volatile compounds produced by fungal endophytes may take slightly longer period to accumulate and reach the optimum level where they can diffuse to seeds properly and demonstrate its maximum effect on germination. Therefore, similar to cold stratification, endophyte indirect may also exert its highest impact on seed germination if the study is longer than 3 days. Future studies will explore these aspects using multiple fungal endophytes and various spatial distances.

3.2. Expression level of gibberellin and abscisic acid genes in coleorhiza

We found that the expression of different functional genes varied significantly ($P < 0.05$) among the treatments (Fig. 2). The *GA3ox* (*GA3ox2* or *GA4H*) is exclusively expressed during seed germination indicating involvements of colorhiza tissue expansion associated with hormonal activities in breaking seed dormancy (Phillips et al. 1995; Hedden and Phillips, 2000). Similar to previous reports, this study also demonstrates high expression of *GA3ox2* gene in wheat coleorhiza throughout the germination period. Potentially, this implies consistent production of the bioactive GA molecule GA3 in wheat coleorhiza during germination. On the other hand, the low expression of *14-3-3* gene, a negative regulator of GA biosynthesis, was also detected in coleorhiza. With gradual seedling growth and increase in *GA3ox2* expression, the transcript level of *14-3-3* also declined. On Day 1, endophyte indirect treatment had the highest the expression of *14-3-3* gene and lowest efficacy of germination, suggesting a strict congruence between seed germination and expression of functional genes. These results were in accordance with previous report by Zhang et al. (2007), who showed GA biosynthesis and catabolic genes closely linked to GA content and shoot growth. However, further studies are needed to include more GAs or GA's regulator genes to validate these findings.

Expression patterns of the ABA pathway genes have been studied in a wide range of cereals and pulses including rice (Oliver et al. 2007), wheat (Ji et al. 2011; Nakamura et al. 2010), and bean (Qin and Zeevaart, 1999). Our results show that except control and cold stratification on Day 1, expression of TaNCED gene did not vary among treatments (Fig. 2). Abscisic acid plays a pivotal role in plant stress-adaptation pathways (Nakamura et al. 2010). Since the cold stratification seeds were kept at 4 °C for 48 h prior to their incubation at room temperature, the abscisic acid content may have been higher. On the other hand, high TaNCED expression in control may have resulted in higher ABA synthesis and thereby in slower germination rate. It should be noted that the expression level of TaGAox2 and TaABA8′OH genes in controlled seeds on Day 1 may seem inconsistent with percentage of germination, however, it may also indicate that GA production and seed germination is governed by an array of functional genes and the impact of their individual expression level may be overridden by combined or relative expression. For example, on Day 1, expression of both TaGAox2 and TaNCED genes in controlled seeds were high and this may have attributed to low seed germination. Moreover, controlled seeds also had high 14-3-3 expression. Similarly, endophyte direct showed highest germination rate and efficacy despite low TaGAox2 but also

lowest *14-3-3* expression levels. Thus, actual seed germination may be governed by expression of several genes and not a particular gene. Moreover, the effect of functional gene expression level may not be immediate and may take several hours before showing any apparent changes. For example, expression of ABA catabolic gene, TaABA8′OH, was moderately high during Day 1 and 2 which might have slowly facilitated the germination rate to reach maximum on Day 3. This data highlights the importance of early days in seed germination. However, further research is required to corroborate these patterns in detail and to identify the underlying drivers.

While ratio of other genes was moderately linked to percentage of seed germination, the ratio of GA and ABA biosynthesis genes demonstrated significant ($P < 0.01$) relationships (Fig. 3). Although, TaGA3ox2:TaNCED did not vary remarkably on Day 1, it was highest in endophyte-indirect on Day 2 owing to its significant increase. On the other hand, all three stratification treatments showed considerable up-regulation of TaGA3ox2:TaNCED on Day 3, which has also reflected in their germination. Similarly, on Day 3, cold stratification and endophyte indirect had the highest TaGAox2: TaNCED ratio, indicating the role of ABA in seed dormancy. In case of endophyte-direct, only TaGA3ox2:TaNCED was consistently correlated with seed germination. However, the fact that relationships were slightly weaker than cold stratification and endophyte indirect suggests that direct contact of fungal endophyte may up-regulate some other potential functional genes that were not assessed in this study.

The underlying mechanisms of biological stratification are still relatively unknown and could reveal how plant–fungus interactions take place in the early stages of germination. This study demonstrates that fungal endophyte can stimulate seed germination significantly, and this mycovitality is proportional to the physical distance between the seed and fungal endophyte. Moreover, the effect of biological stratification mediated by fungal endophyte is considerably higher than cold pre-treatment. Previous studies have shown that initiation of germination is proportional to the time of cold stratification (Cavieres and Arroyo, 2000); considering this, future study may extend cold stratification period (>48 h) to increase seed germinability in wheat.

We report that seed germinability can be substantially enhanced through the application of fungal endophytes and the spatial distance between seed and endophyte is a critical factor that governs this mycovitality. The fact that fungal contact is not necessary to improve seed germination suggests that diffusible or volatile compound of fungal origin was also involved in mycovitality, which allowed the expansion of coleorhizae and rapid seed germination. Results also indicate that to break dormancy and increase germination *via* mycovitality might positively influence dynamics of seedlings recruitment. To the best of our knowledge, no study has compared germination and gene expression patterns in wheat coleorhiza under cold and biological stratification. Coleorhiza has been shown as a highly active component of germinating seed (Barrero et al. 2009). Similarly, here we also found high expression of various functional genes in coleorhiza of germinating wheat seeds. However, we specifically assessed the expression of four genes in coleorhiza. Future studies may corroborate these relationships by incorporating more functional genes and other plant organs affected by developmental, hormonal, and environmental factors that influence seed germination process (Bassel et al. 2011). Overall, this study would serve as a baseline for future studies wishing to disentangle the effects of stratification and plant-fungi interactions during seed germination.

Acknowledgements

This research was financially supported by Natural Sciences and Engineering Research Council of Canada – Discovery Grant and Saskatchewan Ministry of Agriculture – ADF Fund to Dr. Vladimir Vujanovic and Dr. Jim Germida. We thank Dr. M. Hubbard and Dr. P. Daida for their assistance in laboratory. We also thank the editor and two anonymous reviewers for their perceptive comments.

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