

# Impact of bacterial priming on some stress tolerance mechanisms and growth of cold stressed wheat seedlings

Mohammed E.H. Osman,<sup>1</sup>  
Wedad A. Kasim,<sup>1</sup> Mohammed N. Omar,<sup>2</sup>  
Islam A. Abd El-Daim,<sup>2,3</sup> Sarosh Bejai,<sup>3</sup>  
Johan Meijer<sup>3</sup>

<sup>1</sup>Botany Department, Faculty of Science, Tanta University, Egypt; <sup>2</sup>Department of Microbiology, Soils Water and Environment Research Institute, Agriculture Research Centre, Giza, Egypt; <sup>3</sup>Department of Plant Biology and Forest Genetics, Uppsala BioCenter, Swedish University of Agricultural Sciences and Linnean Center for Plant Biology, Uppsala, Sweden

## Abstract

The potential to enhance growth of cold stressed wheat by seed treatment (priming) with the beneficial bacteria *Bacillus amyloliquefaciens* 5113 and *Azospirillum brasilense* NO40 were tested. Results showed an improved ability of bacteria-treated seedlings to survive at  $-5^{\circ}\text{C}$  up to 12 h. Cold stress increased transcript levels of three stress marker genes and increased activity for the ascorbate-glutathione redox enzymes. However, primed and stressed seedlings generally showed smaller effects on the stress markers correlating with better growth and improved stress tolerance. Bacterial priming to improve crop plant performance at low temperature seems a useful strategy to explore further.

## Introduction

Environmental stress causes great losses in crop production where abiotic factors like soil salinity, drought and extreme temperature constitute the most fatal problems. Low temperature is the major limiting factor for crop production in regions where cold periods or freezing occurs annually. Late frost also causes major damage to seedlings in many areas. Exposure of plants to low temperatures leads to the generation of reactive oxygen species (ROS). ROS cause oxidative damage of many different compounds impairing cell function.<sup>1</sup> Since oxidants have different properties the antioxidant defense constitutes both enzymatic and non-enzymatic components distributed

over different compartments throughout plant cells to maximize protection. An important role in plant defense has been attributed to the ascorbate-glutathione pathway due to its ability to maintain high levels of antioxidants by regenerating used antioxidants.<sup>1</sup>

The synthesis of numerous biological compounds and the regulation of many metabolic processes depend on C1 metabolism.<sup>2</sup> The availability of C1 units affects the capacity to produce osmolytes and thus how plants resist abiotic stress by reducing water content, phase changes and ice formation. Methionine is a precursor for the major methyl group S-adenosyl-Met (SAM) formed by SAM synthetase (SAMS), which can be used as an indicator of the C1 status.

It is a challenge for plants to support protein folding at different temperatures to maintain protein function. Heat shock proteins (HSP) belong to the chaperone superfamily of proteins which enhance folding and stability of many proteins. HSP can also provide cryo-protective effects and maintain membrane integrity during stress conditions.<sup>3</sup> Many HSP are known to be induced by abiotic stress.<sup>3</sup>

Abiotic stress tolerance of plants is a complex process involving modifications at many levels.<sup>4</sup> Plants can be primed to enhance tolerance to stress by certain chemicals or biological agents, e.g. plant growth promoting bacteria (PGPB).<sup>5</sup> Priming has low fitness costs for the plant and of high interest to explore for durable plant protection being a cost effective biotechnological alternative to breeding. In this study we aimed to induce freezing stress tolerance in wheat using two PGPB strains. The rationale behind this is that different kinds of abiotic stress share common features such as changes in water status and osmotic balance. We have earlier found that these bacterial strains prime drought tolerance in wheat and our hypothesis was that we therefore also could prime improved cold tolerance.<sup>6</sup>

## Materials and Methods

### Plant growth and treatments

Wheat (*Triticum aestivum* L.) cv. Sids1 is a widely cultivated Egyptian spring cultivar that is relatively sensitive to abiotic stress.<sup>7</sup> Grains were surface disinfected using calcium hypochlorite.<sup>8</sup> Two bacterial strains were used for priming of wheat grains. *Azospirillum brasilense* NO40 is tolerant to osmotic stress and decreases salt stress in barley.<sup>9</sup> *Bacillus amyloliquefaciens* 5113 prime disease resistance<sup>10</sup> and abiotic stress tolerance in oilseed rape (Meijer, unpublished). Bacteria were grown in LB medium for 48 h at  $28^{\circ}\text{C}$  and the density determined by colony forming unit (CFU) analysis. Grains were soaked in solu-

Correspondence: Islam A. Abd El-Daim, Department of Forest Mycology and Pathology, Uppsala BioCenter, Swedish University of Agricultural Sciences (SLU), Box 7026, SE75007 Uppsala, Sweden.  
Tel. +46.186.72708 - Fax: +46.186.73599  
E-mail: islam.abdeldaim@slu.se

Key words: bacteria, cold stress, priming, stress tolerance, wheat.

Contributions: the authors contributed equally.

Conflict of interests: the authors declare no potential conflict of interests.

Funding: These studies were supported by The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS) and the Swedish Institute (SI). Funding for plant growth facilities were provided by KFI-VR.

Received for publication: 26 February 2013

Revision received: 4 July 2013

Accepted for publication: 5 July 2013

This work is licensed under a Creative Commons Attribution NonCommercial 3.0 License (CC BY-NC 3.0).

©Copyright M.E.H. Osman et al., 2013

Licensee PAGEPress srl, Italy

International Journal of Plant Biology 2013; 4:e8

doi:10.4081/ijpb.2013.e8

tions containing  $10^7$  bacteria  $\text{mL}^{-1}$  for 2 h at  $28^{\circ}\text{C}$  with shaking while control seeds were soaked in water. Three primed or un-primed grains were sown in  $6\text{ cm}^2$  pots containing sterile soil (50% low humidity peat and 50% average humidity peat) (Weibulls, Hammenhög, Sweden) in controlled environment with  $22/16^{\circ}\text{C}$  (day/night), 16/8 h photoperiod,  $450\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$  and 80% humidity. Plants were watered daily during 12 d and then shifted to growth cabinets (Percival Scientific, IA) with settings as above but exposed to  $-5^{\circ}\text{C}$  in order to stress the plants while another set of plants was kept under normal conditions as control. Sampling of plants was carried out at 0, 6, 12 and 24 h. Samples for laboratory analysis were frozen in liquid nitrogen and kept at  $-72^{\circ}\text{C}$ .

### Plant growth analysis

At each time point, 40 plants were randomly selected, randomly divided into two groups and kept at normal conditions stated above to allow recovery. After three days plant survival of each group was measured relative the initial number where control plants showed 100% survival. Immediately after sampling, seedlings were washed and dried between two filter papers. Fresh weight was recorded before the

samples were dried at 50°C until constant weight. The difference between fresh and dry weight was used to calculate the water content of shoots. Data based on replicates (at least six times) were subjected to analysis of variance (ANOVA) to determine the significance between the treatments using Costat (CoHort software, CA, USA).

### Enzyme and transcript assays

Ascorbate peroxidase (APX), mono-dehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) were assayed by homogenizing 200 mg frozen plant material in 1 mL extraction buffer and after centrifugation the supernatant was analyzed for enzymes activity.<sup>11</sup>

Expression of ascorbate peroxidase (*APX1*), S-adenosylmethionine synthetase (*SAMS1*) and heat shock protein (*HSP17.8*) genes was analyzed using semi-quantitative RT-PCR. Total RNA was extracted from leaves using a Plant RNA Mini Kit (Omega Bio-Tek, USA) followed by DNase I treatment. First strand cDNA was synthesized from 500 ng of RNA using SuperScript III (Invitrogen, USA). Primer3

software was used to design gene specific primers (Table 1).<sup>12</sup> The invariant *ACTIN* cytoskeleton gene was used to normalize the expression levels. As a negative control, reaction mixture without cDNA was used. The RT-PCR reaction used Dream Taq polymerase enzyme (Fermentas, Finland) and the following parameters: denaturation for 2 min at 94°C; 30 cycles of 1 min at 95°C, 50 sec at 54°C and 1 min at 72°C; and finally 10 min at 72°C. Prior experiments showed that 30 cycles provided transcript levels being in a linear range. Samples were separated on a 1% agarose gel and bands quantified using the Quantity One

software (Bio-Rad) with lane-based background subtraction and normalization of the bands to compensate for differences in loading. The band intensity was quantified using volume units (pixels/mm<sup>2</sup>) and shown as the relative fold expression at zero time.

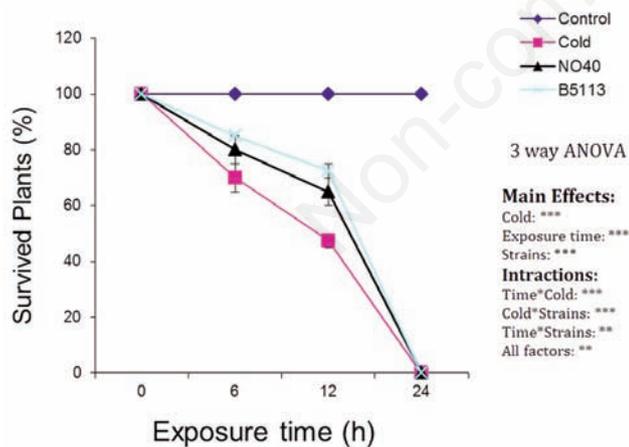
## Results

### Plant growth and survival

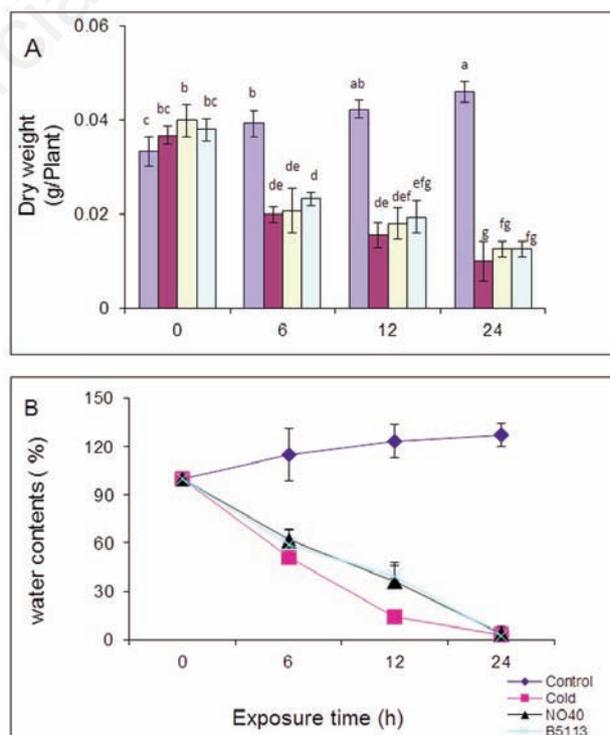
The effect of freezing on wheat growth was severe. Exposing wheat seedlings to -5°C

**Table 1. Gene specific primers and genes from *Triticum aestivum* used for RT-PCR analyses**

Gene	NCBI number	Primer sequence
<i>APX1</i>	AF387739.1	forward, 5'- GGAGGCTTCCTGATGCTG-3' reverse, 5'-CGGCCTAGTCCTTGAAGAAT-3'
<i>TmSAMS1</i>	EU399630.1	forward, 5'-GACCCAGGTGACTGTGGAGT-3' reverse, 5'-AGGCACGCCAATAGCATAAG-3'
<i>HSP17.8</i>	AF350423.1	forward, 5'-AGGATGTTCCGACTGGAGAC-3' reverse, 5'-ACGAACCTGCGCATCATCT-3'
<i>TmACTIN</i>	AB181991.1	forward, 5'-CAGTTGCCAGCAATGTATG-3 reverse, 5'-AGCAATCCAGAAACATGG-3



**Figure 1. Effect of bacterial priming on plant survival of chilling stressed wheat.** Bacterial priming with *B. amyloliquefaciens* 5113 or *A. brasilense* NO40 were tested for effects on the survival of wheat seedlings (cv. Sids1) subjected to -5°C 4 chilling stress for 0, 6, 12 and 24h. Bars indicate standard errors between tow plant groups with 20 replicates in each group. Statistical analysis based on 3 ways ANOVA was conducted on two plant groups with 20 replicates in each group. Three factors were considered for the analysis (stress, strains and stress exposure time). \*\*\*Indicates highly significant effect for the tested factor at  $P \leq 0.01$ , \*\*indicates significant effect for the tested factor at  $P \leq 0.05$ .



**Figure 2. Effect of bacterial priming on some growth parameters of chilling stressed wheat.** Bacterial priming with *B. amyloliquefaciens* 5113 or *A. brasilense* NO40 were tested for effects on growth parameters of wheat seedlings (cv. Sids 1) subjected to -5°C chilling stress for 0, 6, 12 and 24h. Bars indicate standard errors between six replicates. Treatments labelled with identical letters are not significant at  $P \leq 0.01$ . A) Shoot dry weight (g/plant); B) shoot water content (%).

resulted in a massive decrease in the survival rate where 40 % survival rate was recorded after 12 h of exposure to  $-5^{\circ}\text{C}$ , when bacterial treatment significantly improved the survival rate and growth of stressed wheat (Figure 1). The effect of both PGPB strains tested on the survival rate under freezing conditions was in the range between 60% to 80% for seedlings exposed to  $-5^{\circ}\text{C}$  for 12 h. Increasing the exposure time to 24 h caused irreversible damage with no survivors in any of the test groups.

Under cold stress conditions, wheat dry weight declined dramatically (Figure 2A). Dry weight was significantly decreased by exposing wheat to  $-5^{\circ}\text{C}$  stress but increasing the exposure time up to 24 h did not significantly affect the decline in dry weight. In most cases, both bacterial strains used for priming showed a slight increase in the accumulation of dry matter in the cold stressed wheat seedlings compared with their unprimed counterparts.

Exposing wheat to  $-5^{\circ}\text{C}$  resulted in a significant decrease in water content which correlated with the exposure time to freezing. After 24 h of cold stress severe losses in water content were recorded. Bacterial priming greatly counteracted the decrease in water content.

Priming with *B. amyloliquefaciens* 5113 improved the water content of the seedlings exposed for 12 h to cold stress more than two-fold from 15 to 39% (Figure 2B).

### Enzymes of the ascorbate-gluthathione redox cycle

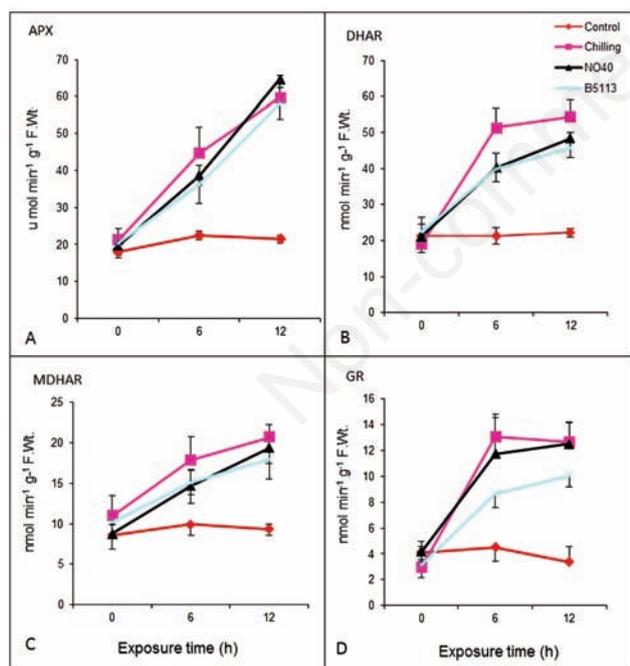
The APX activity increased significantly in the leaves of all cold stressed seedlings whatever primed or not and this was in parallel with the increase of exposure time. *B. amyloliquefaciens* 5113 primed seedlings showed lower APX activity in the leaves compared with the un-primed stressed seedlings (Figure 3A). Exposing wheat seedlings to  $-5^{\circ}\text{C}$  caused a marked increase in the activity of DHAR in the leaves at all time points (Figure 3B). It was also noticed that the effect of bacterial priming appeared as decreased activity of this enzyme in wheat leaves. Cold stress resulted in increasing MDHAR activity in the leaves of all stressed wheat seedlings (Figure 3C). Bacterial priming resulted in a slight increase in the MDHAR activity in leaf tissue compared with their un-primed stressed counterparts. Exposing wheat seedlings to freezing led to a

significant increase in the activity of GR in the leaves (Figure 3D). The highest GR activity was observed after 6 h of cold stress. GR activity was significantly decreased in the leaves of *B. amyloliquefaciens* primed seedlings compared with un-primed cold stressed seedlings.

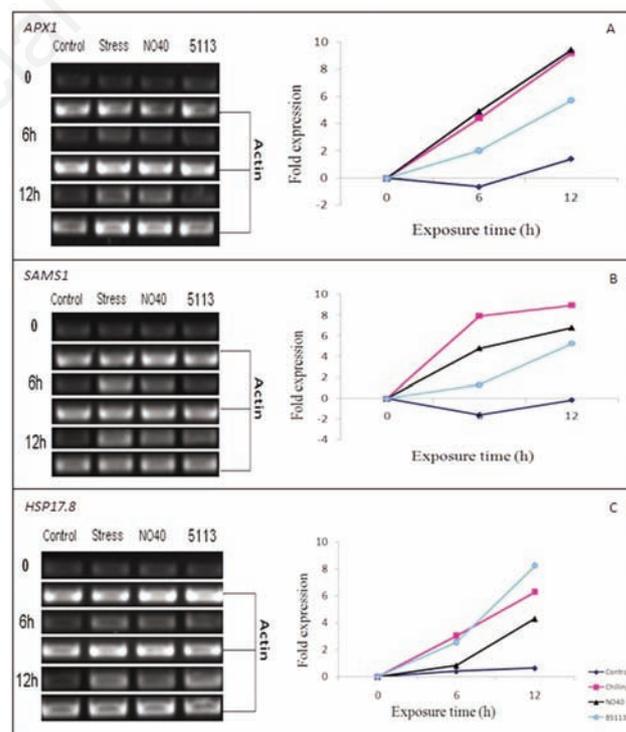
### Molecular stress markers

Transcript levels of the stress marker genes *APX1*, *SAMS1* and *HSP17.8* were measured in leaves using RT-PCR. Results indicated that exposing wheat seedlings to cold stress for 6 h led to a significant up-regulation of the *APX1* gene for all stress treatments which reached maximal levels after 12 h. The effect of bacterial treatment on *APX1* expression in stressed wheat leaves varied among the strains tested for priming. *B. amyloliquefaciens* 5113 treatment significantly decreased *APX1* levels in all stressed seedlings while for *A. brasilense* NO40 treated seedlings, *APX1* expression was not different compared with un-primed stressed plants (Figure 4A).

Exposing wheat to  $-5^{\circ}\text{C}$  significantly increased the expression of *SAMS1* in the leaves in all treatments. It was also observed



**Figure 3.** Effect of bacterial priming on the activity of redox enzymes in the leaves of cold stressed wheat. Effects of priming with *B. amyloliquefaciens* 5113 or *A. brasilense* NO40 on the activity of enzymes of the ascorbate-gluthathione redox cycle were tested in the leaves of wheat seedlings (cv. Sids1) exposed to  $-5^{\circ}\text{C}$  for 0, 6 and 12 h. A) Ascorbate Peroxidase (APX) ( $\mu\text{mol min}^{-1} \text{g}^{-1}$  fresh weight); B) dehydroascorbate reductase (DHAR); C) mono-dehydroascorbate reductase (MDHAR); and D) glutathione reductase (GR) ( $\text{nmol min}^{-1} \text{g}^{-1}$  fresh weight). Bars indicate standard errors between 3 replicates.



**Figure 4.** RT-PCR analysis of some stress marker genes in cold stressed wheat leaves. Wheat plants (cv. Sids1) pretreated with *B. amyloliquefaciens* 5113 (●) or *A. brasilense* (▲) NO40 were exposed to  $-5^{\circ}\text{C}$  for 0, 6 and 12 h. Semi-quantitative RT-PCR analysis was used to study steady state levels of *APX1* (A), *SAMS1* (B) and *HSP17.8* (C) expression. The cytoskeleton gene *ACTIN* was used to normalize expression levels. The expression is shown as the relative fold expression to the control (◆) at zero time. Cold treated plants without priming served as stress positive control (■).

that the *SAMS1* gene was responding immediately to low temperature and the expression level was always elevated with increasing stress exposure time. Generally, bacterial primed seedlings showed lower *SAMS1* levels in the leaves compared with the unprimed stressed seedlings. However, *B. amyloliquefaciens* 5113 primed seedlings showed much lower *SAMS1* levels compared to *A. brasilense* NO40 primed seedlings (Figure 4B).

Low temperature significantly induced the transcript levels of *HSP17.8* in wheat leaves. There was no difference in *HSP17.8* levels in wheat leaves when cold stressed seedlings (6 h) was compared with *B. amyloliquefaciens* 5113 primed seedlings under the same stress conditions. On the other hand, *A. brasilense* NO40 primed seedlings showed lower *HSP17.8* expression under cold stress conditions compared with its un-primed stressed seedlings (Figure 4C).

## Discussion

We chose to test low temperature tolerance without acclimation since diurnal temperature variation may vary strongly in Egypt for spring cultivars. In the present study strong stress symptoms were displayed already after 6 h at  $-5^{\circ}\text{C}$  with hanging leaves and later discolouring as well as a decline in plant growth and survival that was more pronounced after prolonged cold stress. After three days of recovery the surviving plants looked healthier and with somewhat better phenotype for the primed plants. Plant growth reduction caused by low temperature is usually a result of damage due to acute dehydration associated with freezing.<sup>4</sup> Such dehydration effects were evident from our results where dramatic losses in leaf water content were observed after exposure to freezing temperatures. On the other hand, in accordance with our hypothesis, we could show that bacterial priming significantly counteracted the deleterious effects of low temperature on plant survival and growth after 6 and 12 h of cold treatment. Although it is a well-known fact that certain bacteria promote stress tolerance of plants the mechanisms often remain unknown.<sup>5,13</sup> However, a recent study,<sup>14</sup> demonstrating priming of cold tolerance in wheat by *Pseudomonads*, attributed the effect to improved photosynthetic capacity and enhanced root growth due to bacterial IAA production. It is known that IAA can be produced by *A. brasilense* and *B. amyloliquefaciens*.<sup>15,16</sup> We observed a somewhat greater root system on the bacteria treated plants suggesting that the tested strains indeed could be working according to a similar mechanism.

Counteracting oxidative stress is critical in order to avoid cell damage. In the present study

we observed a significant increase in the activities of four antioxidant enzymes in wheat leaves experiencing freezing. In general higher antioxidant capacities correlate with elevated ROS generation. We showed that bacterial primed and stressed plants had considerably lower antioxidant enzyme activity in the leaves compared with their unprimed stressed counterparts, which suggests that priming decrease ROS generation upon stress challenge. It may be that photosynthesis, respiration and photorespiration are under better control at stress as a result of priming. Lowering of ROS levels is also important to prevent signalling for cell death.<sup>17</sup> To overcome cold stress, modifications in plant metabolism are required, e.g. synthesis of cryoprotectants.<sup>3</sup> We detected a significant up-regulation of *SAMS1* transcript levels in wheat leaves after low temperature treatment. Similar results were reported by recording elevated transcription for all C1 biosynthetic genes in the leaves of cold stressed wheat.<sup>18</sup> In the present study, it was noticed that bacterial treated and stressed seedlings had lower *SAMS1* transcript levels compared with their unprimed counterparts suggesting lower supply of methyl units and associated metabolite tuning. This could imply that the plant becomes more specific in production of protective molecules to prevent cold induced damage or those other factors are more important in primed cold tolerance. Metabolomic analysis of cold responses in plants, mostly Arabidopsis, generally showed reprogramming but for many changes the functional role remains unclear.<sup>19</sup> Many metabolites associated with cold stress are of unknown structure pointing to the role of yet unknown pathways for abiotic stress tolerance.<sup>19</sup> Most probably a combination of protective metabolites in consort with proteins are needed for appropriate temperature stress control and stress tolerant cultivars often contain higher levels of dehydrins or other stress related proteins.<sup>20</sup>

Protein folding is usually compromised by low temperatures limiting the functionality of cells.<sup>21</sup> In the present study, *HSP17.8* transcript levels were highly up-regulated in wheat leaves exposed to cold stress showing a greater need to support protein folding. However, *Azospirillum* primed and stressed seedlings showed lower levels of *HSP17.8* suggesting that a more favourable environment for protein folding and stability exists in the primed plants.

Obviously regulation of gene expression is a key issue for plants to develop stress tolerance. Various forms of abiotic stress responses share some common regulatory features in addition to unique responses.<sup>22</sup> However, changes in temperature often occur more rapidly than other abiotic stresses like drought and salinity requiring more rapid compensatory systems in the plant. Analysis of transcript

responses show that distinct sets of genes are modulated by different cold treatments but the present limitation of genome information for non-model organisms restrict in depth analysis.<sup>19</sup> However, a role for WRKY and NAC transcription factors for cold stress tolerance in wheat has been shown.<sup>23,24</sup> In Arabidopsis the NAC *NTLA* gene controls ROS generation in drought so down regulation of wheat ortholog could help to explain the primed cold stress effects on ROS.<sup>25</sup> Exposure to a low temperature for a shorter duration generally improves cold tolerance of plants (acclimation) while a longer time period of cold (vernalization) is needed for many plants to initiate reproductive growth. While adjustments to low temperatures would be expected to involve similar mechanisms, a recent investigation showed that cold acclimation and vernalization responses differ.<sup>26</sup> Another study in Arabidopsis showed that changes in transcriptome but not metabolome correlated with ability to cold acclimate while for freezing tolerance both metabolite and transcript changes was correlated illustrating the complexity of plant responses to different low temperature regimes.<sup>27</sup>

## Conclusions

In conclusion, we have demonstrated the potential of using two bacterial strains to prime abiotic stress tolerance and enhance growth of wheat experiencing low temperature, which supported our hypothesis that bacteria which counteract one type of water stress (drought and heat) also can work on other types of water related stress (cold). The data showed varying impact on the ascorbate-gluthathione redox cycle, C1 generation and the expression of small HSPs indicating that priming support stress tolerance by several changes at metabolite and protein level when plants become challenged. Future analysis need to identify factors that are needed for priming of stress tolerance to optimize this biotechnology.

## References

1. Gill SS, Tuteja N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol Biochem* 2010; 48:909-30.
2. Hanson AD, Roje S. One carbon metabolism in higher plants. *Annu Rev Plant Physiol Plant Mol Biol* 2001; 52:119-37.
3. Janská A, Maršik P, Zelenková S, Ovensá J. Cold stress and acclimation- what is important for metabolic adjustment? *Plant Biol* 2010;12:395-405.

4. Mahajan S, Tuteja N. Cold, salinity and drought stresses: an overview. *Arch Biochem Biophys* 2005;444:139-58.
5. Dimkpa C, Weinand T, Asch F. Plant-Rhizobacteria interactions alleviate abiotic stress conditions. *Plant Cell Environ* 2009;32:1682-94.
6. Kasim WA, Osman ME, Omar MN, et al. Control of drought stress in wheat using plant-growth-promoting bacteria. *J Plant Growth Regul* 2013;32:122-30.
7. Abou-Deif MH, Mohamed FI. Effect of heat stress on chromosomes and protein patterns in six hexaploid wheat varieties. *Res J Cell Mol Biol* 2007;1:42-9.
8. Miche L, Balandreau J, Effects of rice seed surface sterilization with hypochlorite on inoculated *Burkholderia vietnamiensis*. *Appl Environ Microb* 2001;67:3046-52.
9. Omar MNA, Osman MEH, Kasim WA, Abd El-Daim IA. Improvement of salt tolerance mechanisms of barley cultivated under salt stress using *Azospirillum brasilense*. In: Ashraf M, Ozturk M, Athar HR, eds. *Salinity and water stress improving crop efficiency*. Dordrecht: Springer Verlag; 2009. pp. 133-147
10. Danielsson J, Reva O, Meijer J. Protection of oilseed rape (*Brassica napus*) toward fungal pathogens by strains of plant-associated *Bacillus amyloliquefaciens*. *Microb Ecol* 2009;54:134-40.
11. Murshed R, Lopez-Lauri F, Sallanon H. Microplate quantification of enzymes of the plant ascorbate–glutathione cycle. *Anal Biochem* 2008;383:320-2.
12. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Meth Mol Biol* 2000;132: 365-86.
13. Yang J, Kloepper JW, Ryu C. Rhizosphere bacteria help plants tolerate abiotic stress. *Trends Plant Sci* 2009;14:1-4.
14. Mishra PK, Bisht SC, Ruwari P, et al. Alleviation of cold stress in inoculated wheat (*Triticum aestivum* L.) seedlings with psychrotolerant *Pseudomonads* from NW Himalayas. *Arch Microb* 2011;193:497-513.
15. Tien TM, Gaskins MH, Hubbell DH. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). *Appl Environ Microb* 1979; 37:1016-24.
16. Idris EE, Iglesias DJ, Talon M, Borriss R. Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. *Mol Plant Microbe Interact* 2007;20:619-26.
17. Jaspers P, Kangasjärvi J. Reactive oxygen species in abiotic stress signaling. *Physiol Plant* 2010;138:405-13.
18. Bhuiyan NH, Liu W, Liu G, et al. Transcriptional regulation of genes involved in the pathways of biosynthesis and supply of methyl units in response to powdery mildew attack and abiotic stresses in wheat. *Plant Mol Biol* 2007;64:305-18.
19. Hinch DK, Espinoza C, Zuther E. Transcriptomic and metabolomic approaches to the analysis of plant freezing tolerance and cold acclimation in improving crop resistance to abiotic stress. In: Tuteja N, Gill SS, Tiburcio AF, Tuteja R, eds. *Improving crop resistance to abiotic stress*. Weinheim: Wiley-VCH Verlag GmbH & Co; 2012.
20. Hanin M, Brini F, Ebel C, et al. Plant dehydrins and stress tolerance: versatile proteins for complex mechanisms. *Plant Signal Behav* 2011;6:1503-09.
21. Timperio AM, Egidio MG, Zolla L. Proteomics applied on plant abiotic stresses: Role of heat shock proteins (HSP). *J Proteom* 2008;71:391-11.
22. Qin F, Shinozaki K, Yamaguchi-Shinozaki K. Achievements and challenges in understanding plant abiotic stress responses and tolerance. *Plant Cell Physiol* 2011;52: 1569-82.
23. Niu CF, Wei W, Zhou QY, et al. Wheat WRKY genes TaWRKY2 and TaWRKY19 regulate abiotic stress tolerance in transgenic *Arabidopsis* plants. *Plant Cell Environ* 2012;35:1156-70.
24. Mao X, Zhang H, Qian X, et al. TaNAC2, a NAC-type wheat transcription factor conferring enhanced multiple abiotic stress tolerances in *Arabidopsis*. *J Exp Bot* 2012;63:2933-46.
25. Lee S, Seo PJ, Lee HJ, Park CM. A NAC transcription factor NTL4 promotes reactive oxygen species production during drought-induced leaf senescence in *Arabidopsis*. *Plant J* 2012;70:831-44.
26. Bond DM, Dennis ES, Fonnegan EJ. The low temperature response pathways for cold acclimation and vernalization are independent. *Plant Cell Environ* 2011;34: 1737-48.
27. Hannah MA, Wiese D, Freund S, et al. Natural genetic variation of freezing tolerance in *Arabidopsis*. *Plant Physiol* 2006; 142:98-111.