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The rice *Osmyb4* gene enhances tolerance to frost and improves germination under unfavourable conditions in transgenic barley plants

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Abstract The *Osmyb4* rice gene, coding for a transcription factor, proved to be efficient against different abiotic stresses as a trans(cis)gene in several plant species, although the effectiveness was dependent on the host genomic background. Eight barley transgenic lines carrying the rice *Osmyb4* gene under the control of the Arabidopsis cold inducible promoter *cor15a* were produced to test the efficiency of this gene in barley. After a preliminary test, the best performing lines were subjected to freezing at −11°C and −12°C. Frost tolerance was assessed measured the F_v/F_m parameter widely used to indicate the maximum quantum yield of photosystem II photochemistry in the dark adapted state. Three transgenic lines showed significantly increased tolerance. These selected lines were further studied under a complex stress applying cold and hypoxia at germinating stage. In these conditions the three selected transgenic lines outperformed the wild type barley in terms of germination vigour. The transgenic plants also showed a significant modification of their metabolism under cold/hypoxia conditions as demonstrated through the assessment of the activity of key enzymes involved in anoxic stress response. None of the transgenic lines showed dwarfism, just a slight retarded growth. These results provide evidence that the cold dependent expression of *Osmyb4* can efficiently improved frost tolerance and germination vigour at low temperature without deleterious effect on plant growth.

Keywords Barley · Frost · Germination · *Osmyb4* · Transcription factor · Transformation

Abbreviations

GP	Golden Promise
RWC	Relative water content
CSVT	Complex stressing vigour test
AMY	Alpha-amylase
ASAT	Aspartate aminotransferase
LDH	Lactate dehydrogenase

Introduction

Abiotic stresses are often the most limiting factors reducing yield and quality of agricultural crops. Beside drought, cold and frost stresses are the most crucial factors that the cereal plants must cope with. Low temperature limits the growth, the productivity and the distribution of many crops, including important cereals such as maize, wheat, rice or barley.

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Cereals, during field germination, can undergo low temperatures associated with hypoxia due to the heavy raining conditions of sowing season in many cereal growing regions, a stress combination that frequently limits the establishment of the young plants. An important early indication of crop establishment is seed vigour that can be defined as 'the inherent ability of seeds to establish normal (or usual) seedlings under adverse growing environments' (Geneve 2005). A vigourous seed germinates and emerges evenly and quickly, even under a variety of field conditions. Variable environmental conditions, such as temperature or moisture can have major effects on germination quality which is a prerequisite of good productivity. Biochemical and physiological changes during germination are well characterized (Bewley and Black 1994; Bradford and Nonogaki 2007); these phenomena have also been studied in cereals, such as in barley (*Hordeum vulgare*) (Fincher and Stone 1993). Novel techniques make it possible to identify these alterations at the gene expression level. A recent microarray analysis determined the temporal differences in gene expression patterns in germinating barley embryos. It was found that almost 95% of the genes exhibited similar expression levels after 4 and 24 hours of imbibitions. However, considerable fluctuations in gene expression occurred between 24 and 96 h of imbibitions. After 48 h the ratio of the down- and upregulated genes was high (45% vs. 8%). This ratio gradually diminished: after 72 h 19% of the genes were down- and 12% were upregulated, while after 96 h the same percentage (7.8% vs. 7%, respectively) was recorded (Watson and Henry 2005). An analysis of germinating barley expressed sequence tags (ESTs) led to the assumption that the transcription factors belonging to the AP2 and Myb family are presumably the major regulators of germination (Zhang et al. 2004).

Plant adaptation to cold stress has been studied at physiological and molecular levels as well. Effectors, regulatory genes and gene networks, involved in the low-temperature response have been described pointing out the key role of a small number of regulatory genes, mainly transcription factors (Chinnusamy et al. 2007; Galiba et al. 2009; Nakashima et al. 2009; Winfield et al. 2010; Zhou et al. 2010). Since the transcription factors act as master switches for the main regulatory networks in response to stresses, they are considered the best and safest candidate genes for engineering these complex traits (Nakashima et al. 2009; Zhang 2003; Saibo et al. 2009).

The effect of the ectopic expression of the rice *Osmyb4* gene (*Oryza sativa Myb4* gene, accession number Y11414), coding for a transcription factor, has been studied in homologous and heterologous transgenic systems. In transgenic rice, the supra-optimal expression of the *Osmyb4* up-regulated 4193 and down-regulated 5362 genes. About 4% of the total *Osmyb4*-regulated genes codes for transcription factors making *Osmyb4* a major central point of a large transcriptional network with multiple subregulons (Park et al. 2010). When *Osmyb4* overexpressing Arabidopsis (*Arabidopsis thaliana*) lines were exposed to different stresses (cold, frost, drought, salt, UV, ozone, viruses, bacteria and fungi) the plants showed improved tolerance/resistance to all conditions, as well as the activation of signal transduction process involved in many different stress responses (Vannini et al. 2004, 2006; Mattana et al. 2005). Transgenic plants also accumulated high amounts of several compatible solutes (e.g. glucose, fructose, sucrose, proline, glycine) (Mattana et al. 2005). The ectopic expression of *Osmyb4* gene improved the physiological and biochemical adaptation to cold and drought in apple (*Malus pumila*) (Pasquali et al. 2008), or cold in *Osteospermum ecklonis* (Laura et al. 2010). *Osmyb4* transgenic tomato (*Solanum lycopersicum*) plants acquired a higher tolerance to drought stress, but not cold (Vannini et al. 2007). Overall, the data obtained in different species indicate that the specificity and the degree of *Osmyb4* activity might also depend on the host genomic background.

These results prompted us to study the effectiveness of the *Osmyb4* gene in barley. Several reports described detrimental effects (e.g. low transformation efficiency, dwarfism and sterility) when different plant species were transformed with *Osmyb4* gene, driven by constitutive promoters. Such phenomena were also recorded when the *Osmyb4* gene was driven by a constitutive promoter in transgenic tomato and were overcame (Vannini et al. 2007) with the replacement of the cold, drought and ABA inducible promoter, isolated from Arabidopsis *Cor15a* (AT2G42540) gene (Baker et al. 1994). These considerations, moreover the fact that this promoter had proved to be effective in cold-related stress studies (Vannini et al. 2007) led us to ensure the expression of *Osmyb4* transgene by the *cor15a* promoter in the present work. Transgenic plants were generated and subjected to frost and to a complex abiotic stress (cold + hypoxia) during germination to test the effectiveness of *Osmy4* transgene in barley.

Materials and methods

Vector construction and plant transformation

Barley (*Hordeum vulgare* L.) plants, cv. Golden Promise, were grown in a growth chamber under controlled environment at 18°C with a 16 h light/8 h dark period (light intensity: 300 μmol $m^{-2}s^{-1}$, 70% relative humidity). Immature embryos were transformed by *Agrobacterium tumefaciens* strain AGL1 following the removal of the embryogenic axis. The expression cassette *cor15a-Osmyb4* used for the transformation was based on the

Osmyb4 full length cDNA (accession number Y11414) driven by the Arabidopsis *cor15a* stress-inducible promoter (Vannini et al. 2007). The *cor15a-Osmyb4-NOS* (promoter-gene-terminator) fragment was cloned into the pENTR™/SD/D-TOPO® cloning vector (Invitrogen) and then recombined with the Gateway-compatible pMDC99 *Agrobacterium sp*. binary vector (Curtis and Grossniklaus 2003). The hygromicin resistance gene under the control of the constitutive *CaMV35S* promoter was employed for the selection of the transformed plants. The barley transformation protocol was carried out as described by Tingay et al. (1997) with the modifications introduced by Matthews et al. (2001).

Molecular characterization of the transgenic barley plants

Eight T_0 (i.e. the regenerated plants from tissue culture) putative transgenic hygromycin resistant plants (L1 – L9, L6: proved to be un-transformant) were verified for the presence of the *cor15a* promoter and *Osmyb4* transgene by PCR analysis. Plant genomic DNA was extracted from the leaves using the CTAB method (Doyle and Doyle 1990). Transgene copy number was evaluated by Southern hybridization of the genomic DNA digested with *Eco*R V enzyme. This enzyme was selected since it does not cut in the probe sequence stemmed from vector construct (*in silico* analysis) and gave polymorphic results. Digested DNA samples were fractioned electrophoretically on a 0.8% (w/v) agarose gel and blotted onto a Hybond membrane (Amersham GE Healthcare). The membrane was prehybridized in hybridization solution at 65°C for 6h and hybridized overnight at 65°C in the hybridization solution (Sambrook and Russel 2001) containing α-^{32}P-CTP-labelled *cor15a-Osmyb4* probe, PCR amplified from the *cor15a-Osmyb4* construct (primers are listed in Table 1). The membrane was washed once with 2X SSC solution containing 0.1% SDS for 20 min, then with 1X SSC (0.1% SDS) and twice with 0.5X SSC (0.1% SDS) for 30 min at 65°C. The membrane was exposed to x-ray film (Kodak) at −80°C.

Cold treatment and freezing test

Five T_2 plants of each transgenic line and two Golden Promise plants were grown in control conditions at 20°C/15°C under a light intensity of 200 μmol $m^{-2}s^{-1}$ (10 h light/14 h dark) and 70% relative humidity. After ten days the plants were moved into a cold chamber (4°C/2°C, 8/16 h light/dark). Leaf samples were taken before cold treatment (control sample) and after one-day of cold treatment (cold-treated sample) and used for RNA isolation.

All T_2 transgenic plants together with Golden Promise (spring barley used for plant transformation) and Nure (winter barley with high level of frost resistance, used as internal control) were first phenotyped for frost tolerance. Then, selected T_3 transgenic lines (L1, L5, L8, L9) were subjected to two further freezing tests to confirm the phenotype of the transgenic lines. The plants were grown at 20°C/15°C, with a 10 h light/14 h dark period, under a light intensity of 200 μmol $m^{-2}s^{-1}$ and 70% relative humidity for one week, then plants at the first-leaf stage were cold hardened for 3 weeks at 4°C, 8 h light/16 h dark period, afterwards subjected to −11°C or −12°C freezing treatments according to Crosatti et al. (2008). In brief: the temperature was gradually (2°C/h) reduced to −3°C, held for 16 h, and then reduced (2°C/h) to the freezing temperatures, where plants were kept for 16 h, in the dark. The frost-induced damage was measured in leaves as a decrease in the maximum quantum efficiency of photosystem II (PSII) photochemistry, using the chlorophyll fluorescence parameter Fv/Fm, which is the ratio of variable (Fv) to maximal (Fm) fluorescence in dark adapted state (Butler and Kitajima 1975). F_v/F_m was determined using a pulse amplitude-modulated fluorometer (PAM 2000, Walz, Effeltrich, Germany), before exposure to hardening (control plants), at the end of the hardening period, immediately after freezing and 24 hours later, after exposing plants to the same conditions employed for growth (Rizza et al. 2001).

The freezing tests were arranged as randomised block with 15 and ten replications in the first and second experiments, respectively.

Table 1 List of primers used for PCR based works. These primer pairs were used for Southern probe preparation, *Osmyb4* expression analysis and for gene expression analysis after CSVT

	GB	GI	Left	Right	PS
Southern probe	–	–	ACATTTAGGCTTGCAACCTTGTCGG	TGGAGAAGTTGCCCCGCTTGAT	839
Osmyb4 expression	–	–	TTACCGTTTCTTCGGTTAATTGATTTG	CATCGCATCGCATGATTCGC	134
actin	AY145451	24496451	TCGCATGTTCCTGGGTTTTT	TCCCCCCACGCTAGCA	55
AMY1	FN179389	229610880	ACAAGGTCATGCAGGGCTAC	CTGGTCCTTAAACCCCCAGT	95
AMY2	FN179390	229610882	CTCTGGGCAAGTCCTGTTTC	CTTGCCCATCAGGAAGTTGT	82
AMY3	FN179391	229610884	GGTGACCTTTGTGGACAACC	ATATCCCTGCATGACCCTGT	82
AMY4	FN179392	229610886	CCCAATATCATGGGACGAAC	TGGGAACCCCATCAAAGTTA	89

GB: GenBank accession number, GI: GenInfo identifier, PS: Predicted size of the amplicon. *AMY*: alpha-amylase

Complex stressing vigour tests

Complex stressing vigour tests (CSVT) were carried out as described by Barla-Szabó and Dolinka (1988). 200 seeds of three T_3 transgenic lines (L1, L5 and L8) and of Golden Promise were soaked in 200 ml distilled water for 48 h at 20°C, and for additional 48 h at 2°C. After the soaking, seeds were placed onto a moist filter paper with the radicle pointing downwards and rolled tightly (25 seeds/roll). To ensure germination, the rolls were placed into plastic bags and put into 20°C for 96 h, vertically. After the germination period, the length of the 200 seedlings was measured and the seedlings were classified into the following categories:

I. *Normal seedlings* (N): seedlings, which developed both shoot and roots.
   High vigour (Hv): seedlings longer than a quarter of the mean of the five longest ones.
   Medium vigour (Mv): seedlings shorter than a quarter of the mean of the five longest ones.
II. *Abnormal germs* (Abn): (Low vigour. Abnormal germ has only shoot or only root.)
III. *Not-germinated* or rotten seeds (Ng)

Samples for RNA isolation and enzymatic assays were taken during the experiment. After 48 h soaking at 20°C as well as after 48 h soaking at 2°C, the embryos were excised and collected from 25 seeds/line and used for RNA isolation, while whole seed samples were collected for enzymatic assays. Shoot and root samples were collected separately from the normal seedlings at the end of the experiment for both analysis. The CSVT experiment was repeated three times, and the plants were grown under the same conditions for every repetition to ensure the same grain development and filling.

RNA expression analyses

Total RNAs were extracted with TRIzol® Reagent (Invitrogen) from leaves, shoots, roots and embryos and 3 μg were reverse-transcribed using Superscript™ II RT reagent kit (Invitrogen). The concentration of the cDNA samples were evaluated using Qubit fluorometer with Quant-iT™ dsDNA HS Assay Kit (Invitrogen, Life Technologies), and subsequently diluted to 0.3 ng/μl concentration. The expression of the transgene was checked by RT-PCR using *Osmyb4*-specific primer pair (Table 1). The samples were subjected to 30 amplification cycles with an annealing temperature of 57°C.

The expression of several genes involved in the anoxic response was also analysed. The genes and the primers used for the analysis are listed in Table 1 and in the supplemental material (online resource 1). Equal amount (1.5 ng) of cDNA was applied in each RT-PCR reaction. The amplification products were separated by electrophoresis on 1.2 % agarose gels. The actin housekeeping gene was used as reference in all reactions (Table 1).

Assessment of enzymatic activities

The activities of the following enzymes were tested:

Alpha-amylase (AMY; EC 3.2.1.1): 1 g fresh plant sample or four seeds were homogenized in 4 ml 0.01 M phosphate buffer (pH 6.7, 1 mM $CaCl_2$, 1 mM PMSF) at 4°C using power-driven potter. The extracts were centrifuged at 3500 g for 20 min. Aliquots of these clear supernatants were used for determination of AMY activity measured with Phadebas® (Magle Life Sciences, International) α-amylase test.

Aspartate aminotransferase (ASAT; EC 2.6.1.1): 1 g fresh plant sample or four seeds were homogenized in 4 ml 0.01 M phosphate buffer (pH 7.8, 120 mM KCl, 0.2 mM pyridoxal phosphate, 1 mM PMSF) at 4°C using power-driven potter. The extracts were centrifuged at 3500 g for 20 min. Aliquots of these clear supernatants were used for determination of ASAT activity in a reaction mixture containing 12.5 mM l-aspartate, 1 mM α-ketoglutarate, 0.2 mM NADH and 9.6 Units of malate dehydrogenase in 100 mM phosphate buffer pH 7.8 (Cazzulo et al. 1977; Sauvage et al. 1991).

Lactate dehydrogenase (LDH; EC 4.1.1.27): 1 g fresh plant sample or four seeds were homogenized in 4 ml 0.01 M phosphate buffer (pH=9.2; 1mM PMSF) at 4°C using power-driven potter. The extracts were centrifuged at 3500 g for 20 min. Aliquots of these clear supernatants were taken for enzyme assay based on the method described by Hoffman et al. (1986). The reaction mixture contained 0.5 mM Na-lactate, 0.4 mM hydrasine, 0.5 mM glycine and 0.5 mM NAD^+. LDH was assayed spectrophotometrically in the lactate → pyruvate direction by monitoring NAD^+ reduction at 340 nm.

Statistical analysis

One-way and two-way analysis of variance (ANOVA) were performed using the SPSS 16.0® (for Windows) software. From the data of the ANOVA table, the least significant difference (LSD) was calculated with t-test. Statistically significant differences between the lines (compared to the wild type Golden Promise) are signed by asterisks; *; **; *** meaning that the difference is significant at the P value≤ 0.05, 0.01, 0.001 respectively.

Results

Development and molecular characterization of the transgenic lines

Golden Promise barley plants were transformed with a binary vector carrying the coding sequence of *Osmyb4* under the control of the stress inducible c*or15a* promoter. Eight T_0 *cor15a-Osmyb4*-transformant barley lines were regenerated. Beside a slightly retarded growth, the transgene did not influence significantly the development of transgenic plants, and all of them were fertile and set seeds. The successful transformation was confirmed by PCR amplification of an 839 bp region from *cor15a-Osmyb4* sequence and by Southern blot (Fig. 1). Six transgenic lines showed a single copy of the transgene while in L5 and L9 two copies of *cor15a-Osmyb4* were detected (Fig. 1.)

Since the stress inducible (cold, drought and abscisic acid inducible) *cor15a* promoter (Baker et al. 1994) was employed to drive the expression of *Osmyb4* gene, the cold dependent expression of *Osmyb4* was tested in the transgenic barley lines. RNA was isolated from leaf samples collected before and after one day of cold treatment from five plants (P1-P5) from each transgenic line and used for RT-PCR expression analysis. The results showed that the *Osmyb4* mRNA was up-regulated in most plants after cold treatment, although a basal expression level was detected in several transgenic lines (e.g. in L5, Fig. 2). A few plants did not show *Osmyb4* mRNA expression (e.g. P4 plant in L1 and P3 plant in L2, Fig. 2) suggesting a possible segregation of the traits in T_2 progenies. No *Osmyb4* amplification was detected in RNA samples isolated from Golden Promise plants (Fig. 2).

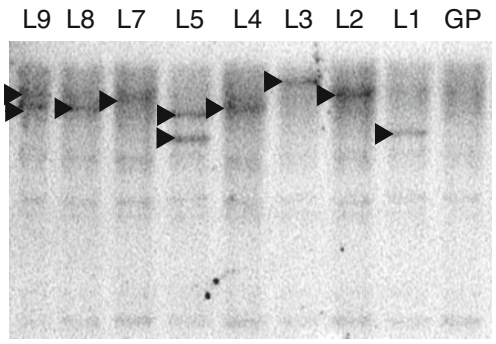

Fig. 1 Transgene copy number estimation by Southern blot hybridization. Genomic DNA was digested with *Eco*R V enzyme, separated, blotted and probed with radioactively labelled DNA fragment of the construct used for transformation. L1-L9: transgenic barley lines, GP: Golden Promise (wild type barley). Triangles indicate the presence of the transgene

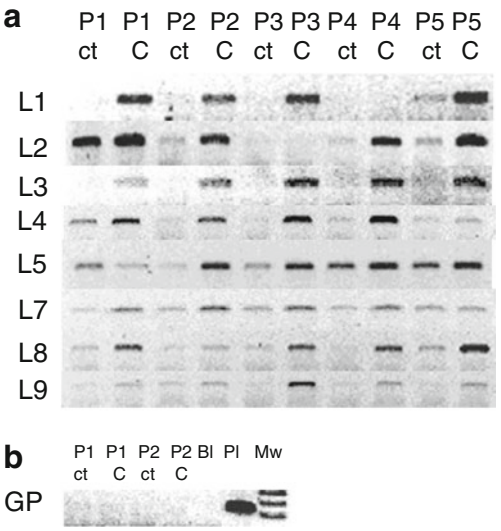

Fig. 2 The expression of the *Osmyb4* transgene in the transgenic barley lines grown under control or cold conditions. Five plants (P1-P5) from each transgenic line (box A) and two from the wild type (box B) were subjected to RT-PCR. 'ct': control samples, 'C': cold treated plants, 'Bl' is the sign of blank (no template), the negative control, while 'Pl' stands for the positive control (plasmid, containing the construct used for transformation)

Freezing test

Preliminary freezing experiments were carried out to identify the discriminating temperatures. In the first one the applied stress temperatures −10°C and −11°C proved to be lethal (data not shown) for the wild type Golden Promise. To test whether *Osmyb4* gene increases freezing tolerance, T_2 transgenic plants were exposed to −12°C after three weeks of cold hardening. All transgenic lines were evaluated in comparison with the spring barley cultivar Golden Promise and with the winter barley cultivar Nure. The degree of damage was assessed through the analysis of the chlorophyll florescence parameter F_v/F_m (Rizza et al. 2001). Before freezing, F_v/F_m values around 0.73–0.74 were recorded for both the transgenic and non transgenic plants, showing that no damage had occurred in the functionality of PSII due to genetic transformation. The F_v/F_m ratio, recorded immediately after the freezing stress, was not affected, as previously reported by Rizza et al. (2001). Significant (statistical analysis: online resource 2) differences were instead detected 24 h after the end of the frost treatment at −12°C. As expected, the spring cultivar Golden Promise was significantly damaged by frost (F_v/F_m=0.414) while the winter cultivar Nure recorded an F_v/F_m value not significantly different from un-frozen plants. The transgenic lines showed a behaviour, intermediate between Golden Promise and Nure, and some transgenic lines (e.g. L1, L5, L8 and L9) were significantly more tolerant than the wild type Golden Promise, although none of them reached the same level of frost tolerance of the winter cultivar Nure (Table 2).

Table 2 Preliminary frost test results carried out at −12°C on T_2 transgenic lines. The F_v/F_m ratio was measured on all transgenic lines and evaluated in comparison with the spring barley cultivar Golden Promise (GP). The winter cultivar Nure was also involved as an internal standard

Lines	F_v/F_m		
	cold hardened	after stress	24h after stress
L1	0.734	0.721	0.570**
L2	0.736	0.733	0.478
L3	0.747	0.739	0.511
L4	0.735	0.736	0.635***
L5	0.743	0.748*	0.701***
L7	0.738	0.733	0.538*
L8	0.724**	0.73	0.694***
L9	0.733	0.733	0.630***
GP	*0.746*	*0.731*	*0.414*
Nure	0.745	0.757**	0.751***

*, **, ***: significantly different (P≤0.05, P≤0.01 and P≤0.001, respectively) compared to Golden Promise

Based on the T_2 preliminary results, selected T_3 lines, including three of the best performing ones (L5, L8 and L9), and one with a medium performance (L1) were subjected to further freezing tests carried out at two temperatures. These transgenic lines, together with Golden Promise and Nure were cold hardened and frozen at −12°C, and, since this temperature proved to be severe, at a milder one, at −11°C. All the four transgenic lines showed significantly (statistical analysis: online resource 3) higher F_v/F_m values than the wild type, when assessed 24 hours after frost treatment, confirming that these transgenic lines are more frost tolerant than the wild type (Table 3).

CSVT

Cereals frequently subjected to low temperature are associated with hypoxia during field germination, a stress combination that limits the establishment of the young plants. To assess the effect of an improved cold tolerance on the response to a combined cold + hypoxia stress, the complex stressing vigour test (CSVT) was applied, an assay for the analysis of plant ability to germinate after exposure to hypoxia and low temperature, to study the performance of the transgene on seed vigour (i.e. the ability of a seed to establish a seedling under adverse growing conditions).

CSVT was applied on three selected transgenic lines (L1, L5, and L8). Seeds were soaked in distilled water (hypoxia) at 20°C for two days then for an additional 48 hours at 2°C (hypoxia and cold stress). Then the seeds were germinated between wet filter papers for 4 days at 20°C (Fig. 3). The length of the shoots and roots were measured and the vigour for each line was calculated as described under the materials and methods section. The data of the measurements are summarized in Table 4 and the statistical analysis is presented as online resource 4. All transgenic lines showed a much longer shoot length compared to wild type seedlings, suggesting a superior vigour of the plant over-expressing the *cor15a-Osmyb4* sequence. Even though L8 did not perform well in control condition (80% germination), its vigour did not decrease under complex stress condition, and the length of the shoot was the same as that of the other two transgenic lines. Some non-

Table 3 Frost test results on selected T_3 transgenic lines. Selected T_3 transgenic lines were cold hardened and frozen at −11°C and −12°C. F_v/F_m ratio was evaluated by comparing the transgenic plants with the spring barley cultivar Golden Promise (GP). The winter cultivar Nure was also involved as an internal standard

Lines	F_v/F_m								
	cold hardened			after stress			24 h after stress		
	−11°C	−12°C	Av.	−11°C	−12°C	Av.	−11°C	−12°C	Av.
L1	0.776	0.788	0.782	0.738	0.723	0.731	0.546***	0.365	0.456**
L5	0.774	0.788	0.781	0.762***	0.752***	0.757***	0.617***	0.474**	0.546***
L8	0.778	0.785	0.782	0.762***	0.736*	0.749***	0.613***	0.403	0.508***
L9	0.784*	0.792	0.788*	0.753**	0.732	0.743*	0.590***	0.417*	0.504***
GP	*0.775*	*0.788*	0.782	*0.732*	*0.717*	0.725	*0.317*	*0.294*	0.306
Nure	0.789***	0.795*	0.792***	0.772***	0.74**	0.756***	0.724***	0.686***	0.705***
Sig(g)	0.024			0.010			0.010		
Sig(e)	0.001			0.002			0.010		
Sig(g x e)	0.544			0.341			0.022		

*, **, ***: significantly different (P≤0.05, P≤0.01 and P≤0.001, respectively) compared to Golden Promise, Av: averages, Sig: significance level, g: genotype, e: environment.

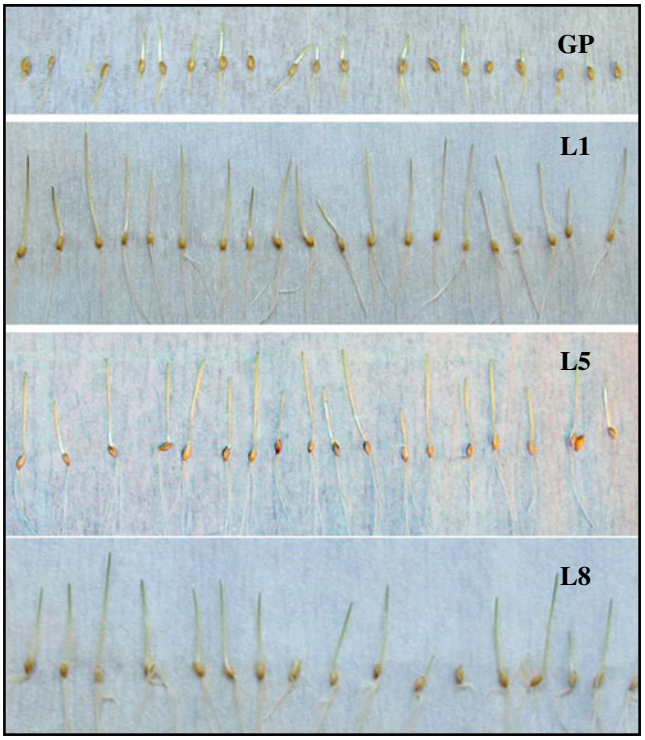

Fig. 3 Seedlings of the transgenic (L1, L5, L8) and wild type (GP) plants after the CSVT. Seeds (200 seeds for Golden Promise and 199, 199 and 197 for the transgenic lines, respectively) were soaked in distilled water for 48 h at 20°C, and for additional 48 h at 2°C. After the soaking, seeds were placed onto a moist filter paper and kept at 20°C for 96 h

germinated and abnormal seeds (which developed root or shoot, but not both) were observed after complex stress-treatment. The ratio of the abnormal seedlings was lower in all the transgenic lines (between 2.0% and 4.5% for the transgenics, while it was 9.0 for GP), and the percentage of non-germinating seeds was lower (1% and 4%) in two *Osmyb4* transgenic lines than in GP (10%). All three transgenic lines showed better performance than Golden Promise for all the parameters tested. The differences in the vigour and in the length of the seedlings between the transgenic lines and wild type were significant. The CSVT data were confirmed in two additional experiments.

Analysis of enzyme activity involved in tolerance to hypoxic stress

Since the CSVT suggested a role of *Osmyb4* in the tolerance to hypoxic (or hypoxic combined with cold) conditions, the activities of several enzymes known to play crucial role in the tolerance to hypoxic/anoxic stress were assayed. Alpha-amylase (AMY) activity was measured in the seeds, subjected to hypoxia (H), hypoxia and cold (H + C), or in the shoot (S) and root tissues of the young seedlings. No differences were found between the root samples (data not shown) and no significant differences were recorded when the seeds were subjected to H + C. Indeed, the AMY activity was significantly higher in all three transgenic lines subjected to H, and also significant increase was detected for L1 and L8 in the shoot tissues (Fig. 4a).

In seeds, subjected to H, the activity of lactate dehydrogenase (LDH) enzyme was significantly ($P \leq 0.05$) higher in one, and it was also higher in two transgenic lines when subjected to H + C. A higher LDH activity was also recorded in the shoot tissue of two transgenic lines (Fig. 4b).

The aspartate aminotrasferase (ASAT) activity was significantly higher ($P \leq 0.05$) in the seeds subjected to hypoxia (H) and hypoxia + cold (H + C) in the transgenic lines studied. Also, higher activities were detected in the shoot samples; however, these differences were significant only for L8 (Fig. 4c).

The statistical analyses for enzymatic assays are available as supplemental material (online resource 5).

The differences found in the enzyme activities were studied at gene expression level by RT-PCR. It was found that the expression level of alpha-amylase 2 and alpha-amylase 3 genes is higher in the shoot samples in two (L1 and L8) transgenic lines, compared to the wild type Golden Promise (Fig. 5). However, no differences were recorded for amylase 1 and amylase 4 mRNAs (data not shown). Also, no differences were found for lactate dehydrogenase, nor in the expression of other genes (such as alcohol dehydrogenase, aldehyde dehydrogenase, pyruvate dehydrogenase etc.) that are known to be involved in the anaerobic stress response (data not shown).

Table 4 Summarization of CSVT data showing the vigour-related and germination-related parameters

Genotype	Nr of seeds for CSVT	N			Abn [%]	Ng [%]	control
		Hv [%]	SL [mm]	Mv [%]			germ
L1	199	91.44***	32.94***	3.00	4.5	1*	100%
L5	199	89.48***	39.87***	3.00	3.5*	4	100%
L8	197	77.80	34.02***	7.57	2.0**	12.5	80%
GP	*200*	*76.00*	*19.46*	*5.00*	*9.0*	*10*	*95%*

Hv, Mv: percentage of seedlings with high or medium vigour (respectively), SL: seedling length, Abn: percentage of seedlings with abnormal seeds, Ng: percentage of none germinated seeds. *, **, ***: significantly different ($P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively) compared to Golden Promise.

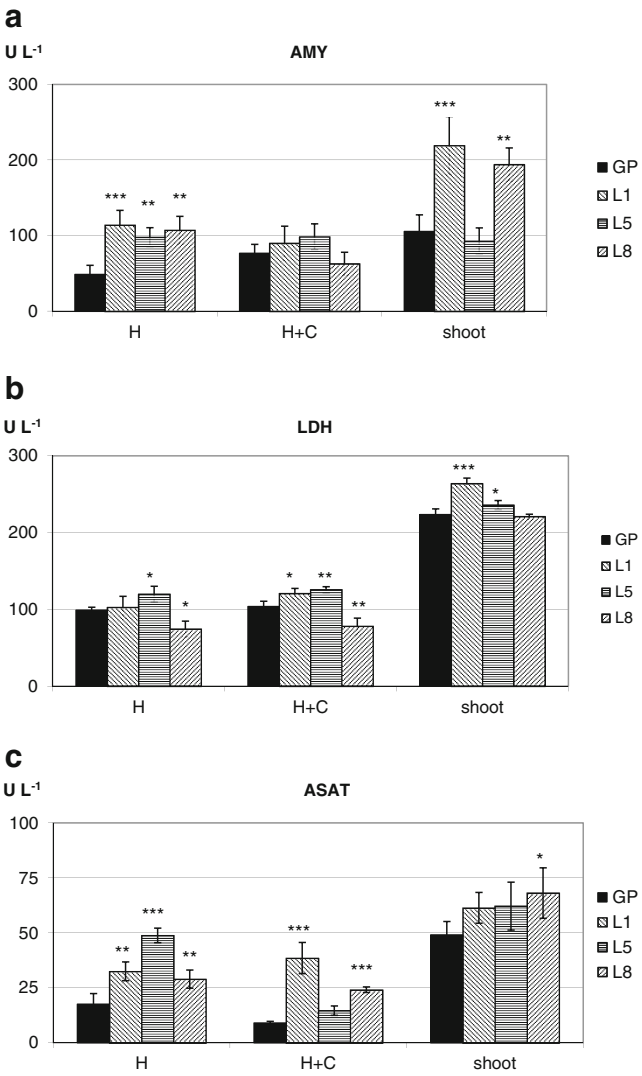

Fig. 4 Enzyme activity assay on alpha-amylase, lactate dehydrogenase and aspartate aminotransferase enzymes during CSVT. 4a: Alpha-amylase (AMY), 4b: lactate dehydrogenase (LDH), 4c: aspartate aminotransferase (ASAT) activity in three transgenic (L1, L5, L8) and in the wild type plants under hypoxia (H), hypoxia and cold (H + C) or in the shoot tissue.*, **, ***: significantly different (P≤0.05, P≤0.01 and P≤0.001, respectively)

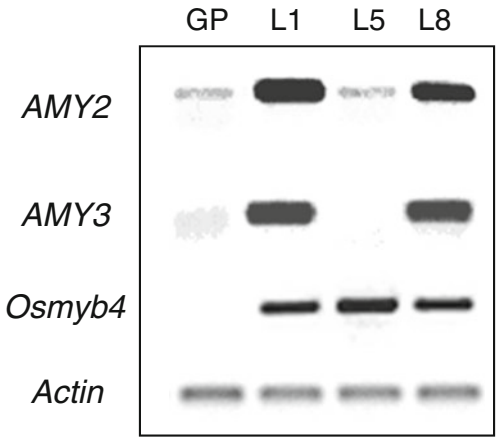

Fig. 5 RT-PCR of alpha-amylase gene expression in the shoot tissues in three transgenic and in the wild type plants. Expression of two alpha-amylase genes (*AMY2* and *AMY3*) and the *Osmyb4* transgene in the shoot tissues. *Actin* was used as reference gene

Discussion

Several plant species, namely Arabidopsis (Vannini et al. 2004), tomato (Vannini et al. 2007), *Osteospermum* (Laura et al. 2010) and apple (Pasquali et al. 2008) have already been successfully transformed with the rice *Myb4* transcription factor, controlled by a constitutive promoter. In these species the expression of the transgene enhanced the tolerance to abiotic and biotic stresses, although the activity of *Osmyb4* gene was, to some extent, dependent on the host's genomic background. Nevertheless, the transformation efficiency was quite low in these earlier experiments and the transgenic plants showed more or less severe dwarf phenotype depending on the level of the transgene expression, and some of the plants did not produce seeds. The utilization of the stress-inducible Arabidopsis promoter *cor15a* led to the production of transgenic *Osmyb4* tomato plants with minimized negative effects and increased stress tolerance (Vannini et al. 2007).

Therefore, based on the results obtained in tomato, *Osmyb4* was introduced in barley under the control of *cor15a* promoter. As expected, the transgene expression was induced by cold-treatment (Fig. 1), although a certain level of mRNA was found even under control conditions, and in some cases the expression level of *Osmyb4* did not appreciably differ between control and cold-treated samples from the same transgenic plant. This background expression level was most likely due to the effect of the *CaMV35S* promoter, which ensured the highly constitutive expression of the hygromycin selection marker gene. It has already been described that the 35S enhancer sequence, which ensures a strong expression of the selectable marker gene, may trans-effect and alter the expression pattern of the downstream transgene (Yoo et al. 2005).

The frost tolerance of the barley spring cultivar Golden Promise transformed with *cor15a-Osmyb4* was significantly improved (P≤0.05) for some of the transgenic lines (although it did not reach the frost tolerance level of the winter standard Nure).

These results confirm the conserved role of *myb4* gene in different plant species and suggest that the effect *Osmyb4* cannot overcome the role of the winter growth habit gene on cold acclimation. Improved frost tolerance was also reported for *Osmyb4*-transgenic Arabidopsis (Vannini et al. 2004) and *Osteospermum* (Laura et al. 2010), while the *cor15a-Osmyb4* transgene did not improve frost tolerance in tomato (Vannini et al. 2007). The effectiveness of the *Osmyb4*

transgene on frost tolerance depends on the transformed species, most likely because not all plants carry the downstream genes required to activate the cold acclimation response controlled by *Osmyb4* transcription factor.

Numerous transformation studies have already been engaged in *Osmyb4* functional analysis, and the transgenic Arabidopsis plants effectively demonstrated increased tolerance to different abiotic and resistance to biotic stresses, namely chilling, frost, drought, salt, UV, ozone, virus, bacteria and fungi. However, result on the effect of *Osmyb4* transgene in the face of anoxic or hypoxic stress has not been published yet. Nevertheless, the role of genes belonging to *Myb* family in response to flooding is not preposterous because, for example, in *Arabidopsis thaliana*, *AtMYB2* was found to be involved in the response to low oxygen by the regulation of *AtADH1* (alcohol dehydrogenase1) gene (Hoeren et al. 1998).

CSVT, accepted by International Seed Testing Association (ISTA) Vigour Test Committee (Hampton and TeKrony 1995) is based on complex stress condition described by Barla-Szabó and Dolinka (1988). It imposes temperature and oxygen deficiency stress by soaking seeds at moderate temperature for 2 days followed by another 2 days soak at low temperature (2°C). When *Osmyb4* transgenic barley seeds were subjected to this complex stress treatment they showed significantly better performance compared to the wild type. Transgenic seeds developed longer shoots and they had enhanced vigour as well. Moreover, in two transgenic barley lines an improved alpha-amylase activity was demonstrated at the level of gene expression and also at the enzymatic level. In spite of its increased vigour, surprisingly, no increased level of alpha-amylase activity was detected at enzymatic or at gene expression level in the L5 transgenic line. One explanation could be that in this line the insertion (or less likely the expression) of the transgene led to a repressed (compared to L1 and L8) alpha-amylase activity; meanwhile other (unidentified) mechanisms ensured its increased vigour. The other explanation might be that the enlarged alpha-amylase activity is not (directly) related to the increased vigour, it is just a coincidence. However, the involvement of alpha-amylase activity in hypoxic-related stresses is supported by the facts that carbohydrates are required to deliver energy to shoot elongation, and the required carbohydrates can be reached by the degradation of starch via enhanced alpha-amylase activity (Bailey-Serres and Voesenek 2008).

In our experiment it was shown that the introduction of a rice gene (*Osmyb4*, coding for a transcription factor) into barley plants resulted in an increased alpha-amylase activity during germination in all three transgenic lines tested under hypoxic conditions. It has been proved that cereal seeds (such as barley) fail to produce alpha-amylase and do not germinate under hypoxic conditions; however, rice is able to synthesize this enzyme under anoxia (Perata et al. 1993). Gubler et al. (1995) isolated a gibberellin-regulated Myb (GAMyb) transcription factor in barley, which binds to the GA response complex (GARC) in the promoter of the high-pI alpha-amylase gene, and trans-activates it in the aleurone cells, suggesting that GAMyb is a part of the GA-response pathway, leading to alpha-amylase gene expression. Rice and barley differ distinctly in their ability to respond to GA-treatment under anaerobic conditions, because GA-treatment does not result in induction of alpha-amylase transcript in barley half-grains under anoxia, while rice is able to produce it (Loreti et al. 2002).

Lactate dehydrogenase converts pyruvate, the final product of glycolysis to lactate when oxygen is absent or in short supply. Increase in LDH activity under hypoxia and anoxia was found in crop seedlings (Mustroph and Albrecht 2003) and the anaerobic induction of LDH in barley aleurone layers was demonstrated by Hanson and Jacobsen (1984). In our experiments one transgenic line showed an improved level of LDH activity in seeds under hypoxia, two lines under hypoxia combined with cold stress, and those same two lines showed an increased LDH activity in their shoot tissues as well. However, when the expression of LDH genes was studied by RT-PCR, no differences were observed. Considering the increasing amount of evidence proves that beside the transcriptional, the post-transcriptional and post-translational mechanisms are also important factors in the abiotic stress response (reviewed in Mazzucotelli et al. 2008; Floris et al. 2009), this finding can be explained by post-transcriptional modifications.

ASAT facilitates the conversion of aspartate and alpha-ketoglutarate to oxaloacetate and glutamate, and vice-versa. Glutamate is the common precursor of transamination, and it is believed to play a central role in anaerobic amino acid metabolism. There are several controversial data on changing ASAT activity under hypoxia. Good and Muench (1992) did not find increase in aspartate aminotransferase activity in barley root, in contrast to data of de Sousa and Sodek (2003) in soybean (*Glycine max*) roots. In *Osmyb4* transgenic barley lines we found significantly increased levels of ASAT enzyme activity under hypoxia and under hypoxic stress combined with cold, or (significant for one line only) in the young seedlings, suggesting that, indeed, ASAT has an important role in the developing tolerance to hypoxia.

Our freezing data proved that *Osmyb4* transgenic barley lines are more tolerant to low temperature stresses, and, on the other hand, we also demonstrated that these lines have higher vigour to hypoxia combined with cold stress during germination. These data suggest a possible role of *Osmyb4* in the development of flooding tolerance and in the facilitation of germination under unfavourable conditions. Such unfavourable conditions, i.e. flood with simultaneous cold, are often found at the time of sowing in spring or autumn, when young seedlings are just emerging. Our results show that by enhancing seedling vigour, *Osmyb4* gene is a promising tool to increasing plant tolerance against these abiotic stresses.

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References

Bailey-Serres J, Voesenek LACJ (2008) Flooding stress: acclimations and genetic diversity. Annu Rev Plant Physiol 59:313–339

Baker SS, Wilhelm KS, Thomashow MF (1994) The 5'-region of *Arabidopsis thaliana* cor15a has cis-acting elements that confer cold-, drought-, and ABA-regulated gene expression. Plant Mol Biol 24:701–713

Barla-Szabó G, Dolinka B (1988) Complex Stressing Vigour Test: a new method for wheat and maize seeds. Seed Sci Technol 16:63–73

Bewley JD, Black M (1994) Seeds: Physiology of Development and Germination. 2nd ed. Springer, Berlin

Bradford K, Nonogaki H (2007) Seed Development, Dormancy and Germination. Annual Plant Reviews, Volume 27, Wiley, New York

Butler WL, Kitajima M (1975) Fluorescence quenching in photosystem II of chloroplast. Biochimica et Biophysica Acta 376:116–125

Cazzulo JJ, Juan SM, Segura EL (1977) Glutamate dehydrogenase and aspartate aminotransferase in *Trypanosoma cruzi*. Comp Biochem Physiol 56:301–303

Chinnusamy V, Zhu J, Zhu JK (2007) Cold stress regulation of gene expression in plants. Trends Plant Sci 12:444–451

Crosatti C, Pagani D, Cattivelli L, Stanca AM, Rizza F (2008) Effect of growth stage and hardening conditions on the association between frost resistance and the expression of the cold-induced protein COR14B in barley. Environ Exp Bot 62:93–100

Curtis MD, Grossniklaus U (2003) A Gateway cloning vector set for high-throughput functional analysis of genes *in planta*. Plant Physiol 133:462–469

de Sousa CAF, Sodek L (2003) Alanine metabolism and alanine aminotransferase activity in soybean (*Glycine max*) during hypoxia of the root system and subsequent return to normoxia. Environ Exp Bot 50:1–8

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Fincher GB, Stone BA (1993) Physiology and biochemistry of germination in barley. In: MacGregor AW, Bhatty RS (eds) Barley: chemistry and technology. American Association of Cereal Chemists, MN, pp 247–295

Floris M, Mahgoub H, Lanet H, Robaglia C, Menand B (2009) Post-transcriptional regulation of gene expression in plants during abiotic stress. J Mol Sci 10:3168–3185

Galiba G, Vágújfalvi A, Li CX, Soltész A, Dubcovsky J (2009) Regulatory genes involved in the determination of frost tolerance in temperate cereals. Plant Sci 176:12–19

Geneve RL (2005) Vigour testing in flower seeds. In: McDonald MB, Kwong FY (eds) Flower seeds: biology and technology, CAB, Wallingford, UK, pp. 311–332

Good AG, Muench DG (1992) Purification and characterization of an aerobically induced alanine aminotransferase from barley roots. Plant Physiol 99:1520–1525

Gubler F, Kalla R, Roberts JK, Jacobsen JV (1995) Gibberellin-regulated expression of a *myb* gene in barley aleurone cells: evidence for Myb transactivation of a high-pI α-amylase gene promoter. The Plant Cell 7:1879–1891

Hampton JG, TeKrony DM (1995) ISTA - Handbook of Vigour Test Methods, 3rd edn. International Seed Testing Association, Zurich, Switzerland

Hanson AD, Jacobsen JV (1984) Control of lactate dehydrogenase, lactate glycolysis, and alpha-amylase by O_2 deficit in barley aleurone layers. Plant Physiol 75:566–572

Hoeren FU, Dolferus R, Wu Y, Peacock WJ, Dennies ES (1998) Evidence for a role for AtMYB2 in the induction of the *Arabidopsis thaliana* alcohol dehydrogenase gene *(ADH1)* by low oxygen. Genetics 149:479–490

Hoffman NE, Bent AF, Hanson AD (1986) Induction of Lactate Dehydrogenase isozymes by oxigen deficit in barley root tissue. Plant Physiol 82:658–663

Laura M, Consonni R, Locatelli F, Fumagalli E, Allavena A, Coraggio I, Mattana M (2010) Metabolic response to cold and freezing of *Osteospermum ecklonis* overexpressing O*smyb4*. Plant Physiol Biochem 48:764–771

Loreti E, Vernieri P, Alpi A, Perata P (2002) Repression of α-amylase activity by anoxia in grains of barley is independent of ethanol toxicity or action of abscisic acid. Plant Biol 4:266–272

Mattana M, Biazzi E, Consonni R, Locatelli F, Vannini C, Provera S, Coraggio I (2005) Overexpression of *Osmyb4* enhances compatible solute accumulation and increases stress tolerance of *Arabidopsis thaliana*. Physiol Plantarum 125:212–223

Matthews PR, Wang MB, Waterhouse PM, Thornton S, Fieg SJ, Gubler F, Jacobsen JV (2001) Marker gene elimination from transgenic barley, using co-transformation with adjacent 'twin T-DNAs' on a standard *Agrobacterium* transformation vector. Mol Breed 7:195–202

Mazzucotelli E, Mastrangelo AM, Crosatti C, Guerra D, Stanca AM, Cattivelli L (2008) Abiotic stress response in plants: When post-transcriptional and post-translational regulations control transcription. Plant Sci 174:420–431

Mustroph A, Albrecht G (2003) Tolerance of crop plants to oxygen deficiency stress: fermentative activity and photosynthetic capacity of entire seedlings under hypoxia. Physiol Plantarum 117:508–520

Nakashima K, Ito Y, Yamaguchi-Shinozaki K (2009) Transcriptional regulatory networks in response to abiotic stresses in Arabidopsis and grasses. Plant Physiol 149:88–95

Park MR, Yun KY, Mohanty B, Herath V, Xu F, Wijaya E, Bajic VB, Yun SJ, De los Reyes BG (2010) Supra-optimal expression of the cold-regulated *Osmyb4* transcription factor in transgenic rice changes the complexity of transcriptional network with major effect on stress tolerance and panicle development. Plant Cell Environ 33:2209–2230

Pasquali G, Biricolti S, Locatelli F, Baldoni E, Mattana M (2008) *Osmyb*4 expression improves adaptive responses to drought and cold stress in transgenic apples. Plant Cell Rep 27:1677–1686

Perata P, Geshi N, Yamaguchy J, Akazawa T (1993) Effect of anoxia on the induction of alpha-amylase in cereal seeds. Planta 191:402–408

Rizza F, Pagani D, Stanca AM, Cattivelli L (2001) Use of chlorophyll fluorescence to evaluate the cold acclimation and freezing tolerance of winter and spring oats. Plant Breed 120:389–396

Saibo NJ, Lourenço T, Oliveira MM (2009) Transcription factors and regulation of photosynthetic and related metabolism under environmental stresses. Ann Bot 103:609–623

Sambrook J, Russel DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York

Sauvage FX, Romieu CG, Flanzy C, Robin JP (1991) Purification and characterization of an aerobically induced alanine aminotransferase

from barley roots. Isolation and characterization of aspartate aminotransferase Am J Enol Vitic 42:209–218

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S, Brettel R (1997) *Agrobacterium tumefaciens*-mediated barley transformation. The Plant J 11:1369–1376

Vannini C, Locatelli F, Bracale M, Magnani E, Marsoni M, Osnato M, Mattana M, Baldoni E, Corragio I (2004) Overexpression of the rice *Osmyb4* gene increases chilling and freezing tolerance of *Arabidopsis thaliana* plants. The Plant J 37:115–127

Vannini C, Iriti M, Bracale M, Locatelli F, Faoro F, Croce P, Pirona R, Di Maro A, Corragio I, Genga A (2006) The ectopic expression of the rice *Osmyb4* gene in Arabidopsis increases tolerance to abiotic, environmental and biotic stresses. Physiol Mol Plant Pathol 69:26–42

Vannini C, Campa M, Iriti M, Genga A, Faoro F, Carravieri S, Rotino GL, Rossoni M, Spinardi A, Bracale M (2007) Evaluation of transgenic tomato plants ectopically expressing the rice *Osmyb4* gene. Plant Sci 173:231–239

Watson L, Henry RJ (2005) Microarray analysis of gene expression in germinating barley embryos (*Hordeum vulgare* L.). Funct Integr Genomics 5:155–162

Winfield MO, Lu C, Wilson ID, Coghill CA, Edwards KJ (2010) Plant responses to cold: transcriptome analysis of wheat. Plant Biotech J 8:749–771

Yoo SY, Bomblies K, Yoo SK, Yang JW, Choi MS, Lee JS, Weigel D, Ahn JH (2005) The 35S promoter used in a selectable marker gene of a plant transformation vector affects the expression of the transgene. Planta 221:523–530

Zhang JZ (2003) Overexpression analysis of plant transcription factors. Curr Opin Plant Biol 6:430–440

Zhang H, Sreenivasulu N, Weschke W, Stein N, Rudd S, Radchuk V, Potokina E, Scholz U, Schweizer P, Zierold U, Langridge P, Varshney RK, Wobus U, Graner A (2004) Large-scale analysis of the barley transcriptome based on expressed sequence tags. Plant J 40:276–290

Zhou MQ, Shen C, Wu LH, Tang KY, Lin J (2010) *CBF*-dependent signaling pathway: A key responder to low temperature stress in plants. Crit Rev Biotechnol 31:186–192