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PROOF OF THE FUNCTION OF THE OSMYB4, TACBF14 AND TACBF15 TRANSCRIPTION FACTORS REGULATING ABIOTIC STRESS ADAPTATION IN TRANSGENIC BARLEY

MAIN POINTS OF THE PHD THESIS

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1. BACKGROUND AND AIMS

One fundamental problem facing agriculture is that yields vary from year to year depending on what environmental stress factors are experienced. These include winter frosts, drought, high salt content on saline areas, low or high temperatures, etc. Overcoming the damaging effects of abiotic stress may also be complicated by the simultaneous occurrence of various stress factors or by changes in the intensity of the stress over space and time, even within a single vegetation period. Abiotic stress tolerance is a quantitative trait, determined by a number of genes. As complex phenomena can be expected to occur, often simultaneously, it is extremely important to obtain information on the metabolic processes involved in the development of tolerance of a given stress factor and on the genes that control them. In the case of plants there are a number of general stress responses, induced by many different kinds of stress. This means that an understanding of the mechanism of one stress process often helps to clarify how another stress response works.

Studies on the physiological and biochemical processes leading to the development of abiotic stress tolerance, and on their genetic background, have been underway for several decades. The molecular analysis of genetic regulation is a relatively new field, while investigations on the role of transcription factors are now in the focus of research. Some genes are expressed constitutively in plant cells, while other genes are only induced or repressed by a specific signal. By binding to certain DNA regions, transcription factors are able to switch on, amplify or inhibit the transcription of RNA. A single transcription factor may switch a number of genes on or off, and the individual factors may be activated by various different signals.

The *OsMYB4* gene isolated from rice codes for a transcription factor with a role in the response to cold stress; it regulates several thousand genes either directly or via intermediate transcription factors (Park et al. 2010). In the case of low temperature stress the role of the CBF/DREB1 transcription factors has been studied in the greatest detail. The genes coding these factors are expressed transiently, in an early phase of cold acclimation, in response to low temperature. It has been proved in both homologous and heterologous experimental systems that plants transformed with the *CBF/DREB1* gene exhibit greater cold or frost tolerance and an increase in drought tolerance. Two *CBF* genes with an outstanding role in the frost tolerance of cereals (*CBF14* and *CBF15*) have been detected in Martonvásár (Vágújfalvi et al. 2005, Knox et al. 2008).

One way of analysing gene functions is to cause the candidate gene to overproduce in transgenic plants. Transformation with transcription factors may also influence the functioning of numerous other genes present in the host plant, leading to a measurable effect on stress adaptation. In the course of the present work the function of genes coding for the transcription factors *OsMYB4*, originating from rice, and *TaCBF14* and *TaCBF15*, isolated from wheat, was investigated in

transgenic barley plants. The candidate genes were overproduced in spring barley and the effect of the increased gene expression on the abiotic stress adaptation of the transgenic plants was analysed in response to hypoxia, low temperature, frost and osmotic stress.

The aims of the PhD thesis can be summarised as follows:

1. To incorporate the *OsMYB4* gene isolated from rice into a binary vector containing the *cor15a* stress-inducible promoter originating from *Arabidopsis thaliana*, and the *TaCBF14* and *TaCBF15* genes isolated from wheat into a binary vector containing a promoter driving constitutive expression.

2. To adapt the transformation technique using *Agrobacterium tumefaciens* for spring barley and to use the above-mentioned constructs to develop stable barley lines transformed with *OsMYB4*, *TaCBF14* and *TaCBF15*.

3. To prove the incorporation and expression of the given transgene and to determine the copy number using molecular methods.

4. To study the role of the OsMYB4 transcription factor by testing the frost tolerance of stable barley lines transformed with *cor15a-OsMYB4* and by analysing their tolerance of cold and hypoxia.

5. To prove the role of the TaCBF14 and TaCBF15 transcription factors in the development of frost tolerance by means of frost tests on transgenic barley lines, and to investigate the role of the genes in protection against osmotic stress.

2. MATERIALS AND METHODS

2.1. Plant material

The transformation of barley (*Hordeum vulgare* L.) is greatly dependent on the genotype. The variety that can be most successfully transformed is the spring barley Golden Promise, immature embryos of which were transformed with the mediation of *Agrobacterium tumefaciens* in the present work. Using the pMDC99-*cor15a-OsMYB4-NOS* construct, the barley transformation was carried out by an Italian partner. Using the constructs pBract202 (a control vector containing only the Hygromycin selection marker gene), pBract214-*TaCBF14* and pBract214-*TaCBF15*, the transformation of immature embryos of spring barley and tissue culture were performed using the protocol of Bartlett et al. (2008) and Harwood et al. (2008).

2.2. Preparation of the transformation constructs

The rice *OsMYB4* gene (gene bank accession number: Y11414), driven by the *cor15a* stress/ABA-inducible promoter isolated from *Arabidopsis thaliana*, was obtained from Dr Immacolata Coraggio in an expression cassette (pUC-*cor15-MYB4*). This initial construct was used to prepare a new construct suitable for the *Agrobacterium tumefaciens*-mediated transformation of barley in the following steps:

- Amplification of the *cor15a-OsMYB4-NOS* sequence (Accuprime™ *Pfx* DNA polymerase, Invitrogen) and the validation of the PCR product by gel electrophoresis.
- TOPO cloning reaction (pENTR™ Directional TOPO Cloning Kit, Invitrogen) and validation of the pENTR/SD/D-TOPO-*cor15a-OsMYB4-NOS* cloning construct using PCR and digestion with the restriction enzyme *Not*I (Fermentas).
- LR recombination reaction between the cloning (donor) vector and the pMDC99 recipient vector (Institute of Plant Biology and Zurich-Basel Plant Science Centre, University of Zurich, Zurich, Switzerland), using Gateway LR clonase (Invitrogen).
- Validation of the PMDC99-*cor15a-OsMYB4-NOS* construct using PCR, followed by sequencing.
- Transformation of the validated new construct into the AGL1 *Agrobacterium tumefaciens* strain using the pSoup helper plasmid.

The main steps in the incorporation of the *TaCBF14* (accession number: EU076382) and *TaCBF15* (accession number: EU076383) genes from wheat into the binary vector were as follows:

- Amplification of the *TaCBF14* and *TaCBF15* genes (Accuprime™ *Pfx* DNA polymerase, Invitrogen) from cDNA, which was transcribed by means of reverse transcription (M-MLV RT, Promega) from RNA (TRIzol® Reagent, Invitrogen) treated with DNase I enzyme (Promega), and isolated from the cold-treated Cheyenne winter wheat genotype.
- TOPO cloning reaction; validation of the pENTR/D-TOPO-*TaCBF14* and pENTR/D-TOPO-*TaCBF15* cloning constructs using PCR, followed by sequencing.
- LR recombination reaction between the cloning (donor) vectors and the pBract214 binary recipient vector (http://www.bract.org/constructsavailable/pBractVectors/Constructs/constructs.html).
- Validation of the pBract214-*TaCBF14* and pBract214-*TaCBF15* constructs by means of PCR, digestion with restriction enzymes (*Bam*HI and *Sac*I, Fermentas) and sequencing.
- Transformation of the two validated plasmids into the AGL1 *Agrobacterium tumefaciens* strain using the pSoup helper plasmid.

The sequence alignment and maps for the cloning and expression vectors were prepared using the Vector NTI® (Invitrogen) software.

2.3. Molecular proof of barley transformation

In the case of the *OsMYB4* barley transformants, genomic DNA was isolated from the leaves of candidate plants using the CTAB method (Doyle and Doyle 1990). The copy number was determined by Southern blot hybridisation (Sambrook et al. 1989).

Genomic DNA was isolated from the leaves of candidate hygromycin-resistant *TaCBF* barley transformants using a DNeasy Plant Mini Kit (Qiagen). The presence of the transgene was proved with PCR, using a combination of primer pairs that amplified fragments of various lengths from the promoter-transgene and transgene-terminator sequence segments. The determination of the copy number in *TaCBF* barley transformants in the T_0 generation and of whether the transgene was inherited in the homozygous or heterozygous state in the progeny plants (T_1 generation) were carried out at IDNA Genetics Limited, UK, using quantitative Real-Time PCR with primers specific for the hygromycin gene.

2.4. Frost tests

Frost tests on the *OsMYB4* transformant barley lines involved a week of preliminary growth (20/15°C day/night temperature, 10/14 h light/dark photoperiod, 200 µmol m^{-2} s^{-1} light intensity, 70% relative humidity) followed by three weeks of cold hardening at 4°C (8/16 h light/dark photoperiod). The hardened plants were then frozen at –10°C, –11°C and –12°C, as described by Crosatti et al. (2008).

Frost tests on the *TaCBF* barley transformants were performed according to Vágújfalvi et al. (2003). After three weeks of preliminary growth under control conditions (17/13°C day/night temperature, 16 h illumination, 240 µmol m^{-2} s^{-1} light intensity, 75% relative humidity), the plants were cold hardened for three weeks at 4°C (under the same illumination conditions). In the case of frost testing without cold hardening, plants grown under control conditions were subjected to the frost test. Hardened plants were frozen at –11°C and –13°C and non-hardened plants at –6°C.

The degree of frost tolerance was determined indirectly by measuring chlorophyll fluorescence induction parameters (F_v/F_m ratio and F_0 value) and by recording conductance. Frost damage to the plants was scored directly on the basis of regeneration ability. The survival rate was evaluated one and two weeks after freezing on a scale ranging from 0 (perished by cold) to 5 (no frost damage). The survival percentage for each line was calculated as the ratio of surviving plants to the number of plants subjected to freezing.

2.5. Low temperature stress treatment

Five plants from each of the *OsMYB4* barley transformant lines (T_1 generation) were grown under control conditions (20/15°C day/night temperature, 10 h illumination, 200 µmol m^{-2} s^{-1} light intensity, 70% relative humidity). After ten days the temperature was reduced to 4/2°C (8/16 h light/dark period). Samples were taken from the leaves both under control conditions and after the one-day cold treatment for expression studies.

In the case of the frost tests on *TaCBF* barley transformants, samples for expression studies were taken under control conditions (17/13°C day/night temperature, 16 h illumination, 240 µmol m^{-2} s^{-1} light intensity, 75% relative humidity) and during cold hardening.

2.6. Complex stressing vigour test (CSVT)

The complex stressing vigour test (CSVT) was carried out as described by Barla-Szabó and Dolinka (1988). In the course of the experiment samples were taken for expression studies and for the measurement of enzyme activity [alpha-amylase (AMY), aspartate aminotransferase (ASAT), lactate dehydrogenase (LDH)]. At the end of the 48 h hypoxia treatment and after a further 48 h of complex stress treatment (hypoxia and cold), the embryos were excised from the seeds for RNA isolation, while whole seeds were collected for enzyme activity measurements. After 96 h germination, samples were taken from the shoots.

2.7. Osmotic stress experiment

The experiment was carried out under controlled conditions (17/13°C day/night temperature, 16/8 h day/night illumination, 220 µmol m^{-2} s^{-1} light intensity) in a growth chamber (Conviron, Canada). The germinated seeds were placed on half-strength Hoagland solution. Osmotic stress was induced by a stepwise increase in the concentration of polyethylene glycol (PEG 6000, Sigma) in the medium, as reported by Molnár et al. (2004), with the following modification: 18% PEG for 3 days, 21% PEG for 4 days, 24% PEG for 3 days and finally 27% PEG for 4 days. Each time the PEG solution was changed, the relative water content (RWC) of the leaves was determined according to Schonfeld et al. (1988).

2.8. Gene expression studies

Total RNA was isolated from the plants using TRIzol® Reagent (Invitrogen) according to the instructions of the manufacturer, and the samples were treated with DNase I enzyme (Promega).

For the *OsMYB4* samples, cDNA was transcribed by reverse transcription from 3 µg RNA using a Superscript™ II RT reagent kit (Invitrogen), while cDNA was synthesised from the RNA samples of *TaCBF* transformants using M-MLV Reverse Transcriptase (Promega). The concentration of each cDNA was determined with a Qubit fluorometer using a Quant-iT™ dsDNA

HS Assay Kit (Invitrogen). Equal quantities (1.5 ng) of cDNA were used for all the semi-quantitative RT-PCR reactions.

For the *TaCBF* barley transformants, the expression level of a number of target genes (*HvCOR14b, HvA22, HvCBF9, HvDHN5, HvDHN8*) was determined in the *TaCBF14* and *TaCBF15* transgenic lines exhibiting the greatest frost tolerance (lines 24/1/1 and 9A/1/1) by means of Real-Time PCR, after normalisation for the barley cyclophylin gene with the $\Delta\Delta C_t$ method, using the primer pairs reported by Morran et al. (2011). The reactions were run on an ABI 7500 Fast Real-Time PCR instrument (Applied Biosystems) in normal operation mode. The relative Fold Change (FC) and Log_2FC values were calculated as described by Bookout and Mangelsdorf (2003).

2.9. Statistical analysis

The statistical evaluation of the data was performed with the help of the SPSS statistical program package version 16.0. Anomalous values (outriders) were eliminated (α=0.05). The normality of the data sets was checked with the single-factor Kolmogorov-Smirnov test and the equality of variances using Levene's test.

Pair-wise comparison (transgenic lines compared with the wild type or the transgenic control) was performed using the Analyze/ Compare means/ One-Way ANOVA/ Post Hoc Multiple Comparisons menu of SPSS, applying the least significant difference (LSD) method when the variances were homogeneous, while the Tamhane test was used to find significant differences between the lines if the variances were unequal. The t-test was used to compare two measuring points, and the data were evaluated using the Analyze/ Compare means/ Independent–Samples T-Test menu (α=0.05).

3. RESULTS

3.1. Proof of the role of the OsMYB4 transcription factor by transformation

The presence of the transgene was demonstrated in eight of the nine candidate *cor15a-OsMYB4* transformant plants regenerated (T_0 generation). The lines originating from these plants were labelled L1–L9; L6 (line 6) proved to be non-transformant. The determination of copy number showed that the transgene was present in a single copy in six lines, while two copies were detected in lines L5 and L9. In these *OsMYB4* barley transformants the transgene is driven by *cor15a*, a cold- and ABA-inducible promoter isolated from *Arabidopsis thaliana*. In order to determine whether the incorporated transgene was functional, i.e. the gene was expressed, the plants had to be placed in an environment capable of switching on the promoter. The results indicated that the mRNA of *OsMYB4* was overproduced after cold treatment in the majority of the transformant plants, although in some of the plants a certain level of expression was detected even under control

conditions, while in a number of plants there was no great difference in the transgene expression level between the control and the cold-treated samples. In certain plants the expression of the transgene could not be detected, suggesting that these plants were not transformants due to segregation in the progeny generation (T_1).

3.1.1. Testing of the frost tolerance of the *OsMYB4* transgenic lines

It was established in a preliminary experiment that wild-type Golden Promise spring barley (GP) perished by cold at –10°C after a week of preliminary growth and three weeks of cold hardening. The frost tolerance of the transformant lines was tested at freezing temperatures of –11°C and –12°C in the T_2 and T_3 generations and was compared with that of GP. The damaging effect of frost was illustrated by measuring chlorophyll fluorescence induction parameters (F_v/F_m ratio). There was a significant decrease in the F_v/F_m values recorded in GP leaves 24 hours after the freezing treatment, and similar damage was detected for a few transformant lines (e.g. L2, L3). By contrast some of the lines (L1, L5, L8 and L9) had significantly higher values of F_v/F_m than GP, confirming that these lines were more tolerant of frost than the wild-type Golden Promise.

3.1.2. Complex stressing vigour test on the *OsMYB4* transgenic lines

The possible effect of the OsMYB4 transcription factor on the vigour of germinating seeds from the transformant lines was investigated using the complex stressing vigour test, which involves hypoxia, followed by combined hypoxia and cold stress. The test was carried out on three transgenic lines (T_3 generation, lines L1, L5 and L8) shown by the frost test to have enhanced low temperature stress tolerance due to the presence of the transgene. The mean shoot length of all three transgenic lines was significantly greater than that of GP, and seedlings with great vigour were found in a higher proportion in these lines than in the wild type. The ratio of abnormal seedlings was considerably smaller in the transgenic lines than in the wild type. The results were confirmed when the experiment was replicated twice.

An analysis of the functioning of enzymes involved in the anaerobic metabolism in transgenic barley demonstrated higher activity for AMY, LDH and ASAT. The functioning of the genes coding for these enzymes was investigated at the gene expression level, using semi-quantitative RT-PCR. In the case of the *AMY1* gene (GB: FN179389) there was no difference in expression between GP and the transformants, but for *AMY2* (GB: FN179390) and *AMY3* (GB: FN179391) a high level of expression was detected in samples isolated from the shoots of lines L1 and L8, while the two genes were not expressed in GP. No difference was found between the *OsMYB4* transformant lines and the wild type in the expression of the *LDH* genes, or in that of numerous other genes (e.g. alcohol dehydrogenase, aldehyde dehydrogenase, etc.).

3.2. Proof of the role of the transcription factors TaCBF14 and TaCBF15 by transformation

In the course of the work, immature barley embryos were transformed using the pBract202 vector and the constructs pBract214-*TaCBF14* and -*TaCBF15*. Two independent transgenic controls and 10 independent *TaCBF14* and 18 independent *TaCBF15* stable transformant lines were developed (the transformation efficiency being 3.33%, 7.29% and 12.32%, respectively). The integration of the transgene was proved with PCR in all the candidate transformants.

In the *TaCBF14* and *TaCBF15* transformants the transgene is regulated by the ubiquitin promoter, which ensures strong constitutive expression. Samples were thus taken under control conditions in the T_1 generation to prove the functioning or expression of the transgene. An analysis was also made of whether the transgene was present in the given plant in the homo- or heterozygous form. Based on the results, homozygous plants were selected for multiplication for use in later phenotyping and expression studies.

3.2.1. Frost tests on *TaCBF* transformant lines

In order to select the most tolerant *TaCBF* transgenic barley lines, all 28 independent transformants were tested for frost tolerance and compared with wild-type Golden Promise barley. Freezing temperatures of –11°C and –13°C were used in frost tests on the T_1 generation. Some of the transformants survived the lower freezing temperature. To confirm the results the frost test at –13°C was repeated. The most resistant lines were identified on the basis of regeneration (scoring), survival percentage, chlorophyll fluorescence measurements and conductance tests. The lines selected for further tests were 4A/1/1, 6A/1/2, 21B/1/1, 23A/1/2, 23A/2/1 and 24/1/1 for *TaCBF14* and 9A/1/1, 12B/2/1, 12-18/2/1, 12-18/4/1 and 17/3/1 for *TaCBF15*.

In the homozygous T_2 generation the selected lines were tested at –11°C and –13°C. Judging by the conductance measurements, the leaves of the *TaCBF14* and *TaCBF15* transformants were less severely damaged by freezing than those of the wild type. The F_v/F_m values were significantly higher in the leaves of transgenic plants than in the wild type, indicating less damage to PSII. The six selected *TaCBF14* transformants and line 9A/1/1 from among the *TaCBF15* transformants also proved to be more frost-resistant than wild-type Golden Promise on the basis of regeneration (scoring) and survival percentage. The other *TaCBF15* transformants tested exhibited a slight increase in frost tolerance. The results obtained in the homozygous T_2 generation were better than those recorded in frost tests on the T_1 generation, and the differences compared with the wild type were more pronounced.

As the *TaCBF14* and *TaCBF15* transgenes are constitutively expressed, frost tests were also performed on the selected lines without a preliminary hardening period (inductive conditions). Treatment at –6°C was lethal for the wild type, while the transgenic lines exhibited a low

percentage of regeneration after freezing. In this experimental system lines 24/1/1 (*TaCBF14* transformant) and 9A/1/1 (*TaCBF15* transformant) proved to have the best frost tolerance.

3.2.2. Osmotic stress tolerance of *TaCBF* transgenic plants

The experiments were carried out on two *TaCBF14* lines (6A/1/2 and 24/1/1) and two *TaCBF15* lines (9A/1/1 and 12-18/2/1) that performed well in the frost tests, on the transgenic control line (2A/2/4) and on the wild-type GP. Under control conditions the relative water content of the leaves of all the lines was around 97%, and this declined slightly to 95% after treatment with 18% PEG. When the PEG concentration was increased to 21% the RWC values of the transgenic control and the 6A/1/2 *TaCBF14* line declined significantly compared to the GP value. After treatment at 24% PEG the RWC of 9A/1/1, 12-18/2/1 (*TaCBF15* transformants) and 24/1/1 (*TaCBF14* transformant) was significantly higher than that of GP. During the regeneration period following osmotic stress treatment, the leaves of the various lines regained their original (control) water content; no significant differences could be observed between the RWC values of the individual lines.

3.2.3. Gene expression studies on the *TaCBF* transformants

One of the largest groups of genes induced by drought, cold and salt stress consists of the late embryogenesis abundant (LEA) proteins, involved in late embryo development (Ingram and Bartels 1996, Thomashow 1999). The functioning of four different genes belonging to the LEA protein group (*HvCOR14b, HvA22, HvDHN5, HvDHN8*) and one barley CBF gene (*HvCBF9*) was tested on control and cold-treated samples of wild-type GP and the transgenic lines 24/1/1 (*TaCBF14*) and 9A/1/1 (*TaCBF15*), which proved to be most resistant in previous tests. The constitutive expression of the *TaCBF* transgene induced the expression of the cold-inducible *HvCOR14b* gene in samples of the transgenic lines raised under control conditions (without cold treatment); the level of expression approached that caused by cold treatment in the wild type. The *HvCOR14b* gene was induced in the wild type only by cold treatment, while in the transgenic lines its expression was enhanced on by cold stress. The *HvCBF9* gene was expressed to the same extent under control conditions in all the lines tested, but was induced four times as strongly by cold treatment in GP than in the transgenic lines. In the case of the *HvA22* gene the expression level recorded in the 24/1/1 line under control conditions was 13 times as great, and the cold induction of *HvA22* was also the most pronounced in this line, being three times as great as that detected in the wild type and in 9A/1/1. The expression of the *HvDHN5* gene was four and nine times greater, respectively, in lines 9A/1/1 and 24/1/1 than in the wild type. In response to cold treatment the expression of the *HvDHN5* gene increased a thousand times in GP, but was induced to a lesser extent in the transgenic lines. In line 24/1/1 eight times greater *HvDHN8* expression was recorded under control

conditions than in GP, but this gene was expressed most intensively in the wild type after cold treatment.

3.2.4. Development of *TaCBF* transgenic barley lines

Compared to the wild type, the majority of selected barley lines over-producing the *TaCBF14* and *TaCBF15* transcription factors exhibited retarded development, slower growth and late flowering. The transgenic control and a small number of transformant lines entered in the heading phase at the same time as the wild type, and no difference in plant height was observed in the course of development.

3.3. New scientific results

1. The constructs pMDC99-*cor15a-OsMYB4-NOS*, pBract214-*TaCBF14* and pBract214-*TaCBF15* were prepared and were used for the *Abrobacterium tumefaciens*-mediated transformation of spring barley (*Hordeum vulgare* L. cv. Golden Promise). The transformant barley lines developed included eight independent *OsMYB4* lines, 10 independent *TaCBF14* lines and 18 independent *TaCBF15* lines, together with two independent transgenic control barley lines.

2. In the course of frost tests on the pMDC99-*cor15a-OsMYB4-NOS* transgenic barley lines, the analysis of photosystem II proved that the OsMYB4 transcription factor increased the frost resistance of spring barley.

3. It was proved that the OsMYB4 transcription factor increases the vigour of transgenic barley plants exposed to complex stress involving both hypoxia and cold. Gene expression studies and enzymatic analyses proved that the activity of the enzymes alpha amylase, lactate dehydrogenase and aspartate aminotransferase, which play an important role in plants exposed to low oxygen levels, increased in the transgenic lines in comparison with the wild type.

4. The TaCBF14 and TaCBF15 transcription factors were proved to be involved in the development of frost tolerance. Conductance studies demonstrated that the leaves of transgenics suffered less severe damage from frost than those of the wild type. Significantly higher F_v/F_m values were recorded in the leaves of the transgenic lines than in the wild type, suggesting that PSII was less damaged in the transgenic lines. Based on the extent of regeneration (scoring) and the survival percentage, the selected *TaCBF14* transformants and the 9A/1/1 line (*TaCBF15*) proved to be more frost tolerant than wild-type Golden Promise. The most resistant *TaCBF* transgenic barley lines survived temperatures a few degrees Celsius lower than the freezing temperature that proved lethal to the wild type.

5. Changes were detected in the expression of certain genes belonging to the CBF regulon in the *TaCBF* transgenic barley lines, and the gene *HvCOR14b*, which is induced by low temperature stress, exhibited enhanced expression even under control conditions, compared with the wild type.

6. Preliminary experiments indicated that the TaCBF14 and TaCBF15 transcription factors play a role in the protection against osmotic stress.

7. It was shown that the TaCBF14 and TaCBF15 transcription factors had a pleiotropic effect, causing retarded development, slower growth and late flowering in the transgenic barley lines.

8. New germplasm containing the transgenes *TaCBF14* and *TaCBF15* was selected after repeated frost tests. This could be used in future to continue these studies and as a basic material for experiments aimed at clarifying the functioning of the CBF regulon.

4. CONCLUSIONS AND RECOMMENDATIONS

4.1. The OsMYB4 transcription factor enhances the frost resistance of transgenic barley lines

The role of the OsMYB4 transcription factor in low temperature stress tolerance was examined by means of frost tests on the transgenic barley lines. The significant difference found between the chlorophyll induction parameters of wild-type Golden Promise and the various transgenic lines gave a clear indication that the *OsMYB4* transgene enhanced the stability of PSII. It was found that the frost tolerance of Golden Promise was slightly increased by the *OsMYB4* transgene.

4.2. The OsMYB4 transcription factor increases the vigour of transgenic barley plants

A number of transformation studies have dealt with the functional analysis of the *OsMYB4* gene, but no reports were found on the effect of the transgene on germination. The present experiments proved that *OsMYB4* transgenic barley plants exposed to the complex stressing vigour test developed significantly better than the wild type. The transgenic lines had greater vigour and the tested seeds produced longer shoots. When the enzymes involved in the anaerobic metabolism were examined in transgenic barley, increased activity was recorded for alpha amylase, lactate dehydrogenase and aspartate aminotransferase, which could be attributed to the functioning of the OsMYB4 transcription factor originating from rice.

4.3. The TaCBF14 and TaCBF15 transcription factors improve the frost tolerance of the spring barley Golden Promise

The experiments revealed that the *TaCBF* transgenic barley plants had better frost tolerance than the wild type, thus proving the role of the *TaCBF14* and *TaCBF15* transcription factors in the development of frost resistance. The results of frost tests on the selected, homozygous T_2 generation showed that the transgene increased the frost tolerance to such an extent that the transgenic plants were capable of surviving temperatures several degrees Celsius lower than the freezing temperature that proved lethal for Golden Promise.

4.4. The TaCBF14 and TaCBF15 transcription factors may have a role in the development of osmotic stress tolerance

The promoter of certain osmotic stress-inducible genes contains a sequence designated by the abbreviation CRT/DRE (D-repeat/drought-responsive element), to which CBF/DREB type transcription factors bound specifically, thus regulating their functioning (Thomashow 1999, Shinozaki and Yamaguchi-Shinozaki 2000). As it has been proved that frost leads to a state of relative water deficiency in plant cells, the osmotic stress tolerance of the two most frost-resistant lines produced with each of the transgenes was investigated. The results suggested that the TaCBF14 and TaCBF15 transcription factors could play a role in the development of drought tolerance and in enhancing the level of osmotic stress tolerance. Further experiments will be required to prove this hypothesis.

4.5. The TaCBF14 and TaCBF15 transcription factors influence the expression of genes belonging to the CBF regulon in transgenic barley

The data proved that the constitutively expressed transgene influenced the functioning of genes demonstrated to play a role in the cold hardening of cereals and in the development of frost resistance. The greatest effect was experienced for the *HvCOR14b* gene. The *TaCBF14* and *TaCBF15* transgenes induced the expression of the gene even under control conditions, resulting in a similar level of gene expression to that observed in the wild type in response to low temperature treatment. Morran et al. (2011) observed similarly intense *HvCOR14b* expression in *TaDREB3* transformants of Golden Promise barley. As in the experiments of these authors, the frost tests in the present work also revealed increased frost tolerance in the transgenic plants, even without a cold hardening period. This suggests that the *CBF/DREB* genes investigated influence the development of frost resistance by regulating the *HvCOR14b* gene. Many genes other than the *COR* genes also play an important role in cold hardening and in the development of frost tolerance (for a review of *Arabidopsis*, see Thomashow 1999, for wheat, see Winfield et al. 2010). The *HvCBF9* gene was

repressed in *TaDREB2* and *TaDREB3* transgenic barley lines (Morran et al. 2011), while in the *TaCBF14* and *TaCBF15* lines examined in the present work the gene was induced to a lesser extent in response to cold treatment than in the wild type. This raises the question of whether the *CBF/DREB* genes are able to regulate each other, and what effect the over-production or silencing of one *CBF/DREB* gene may have on the expression of the other *CBF/DREB* genes coded in the genome. The present expression studies can be regarded as a preliminary experiment; the experiments will be repeated in the future to include all the *CBF* genes, using a larger number of biological samples in order to reduce the deviation in the results and to obtain a clearer picture of changes in the expression patterns of the genes belonging to the *CBF* regulon. The transcriptome changes caused by the *TaCBF14* and *TaCBF15* transgenes will be clarified by cDNA-microarray analysis, after which the changes in the expression of the most interesting target genes will be validated using the Real-Time PCR method.

Biolistic transformation was also performed on the spring wheat variety Cadenza using the pBract214-*TaCBF14* and -*TaCBF15* constructs designed in the present work and other constructs based on RNA interference (RNAi). These experiments are still in the early stages, so the results were not included in the thesis. As rice is much more sensitive to cold than barley or wheat, and is relatively easy to transform compared with barley and especially wheat, it is planned to develop rice lines over-producing the *TaCBF14* and *TaCBF15* transgenes. This will allow the role of the two *TaCBF* genes in the development of abiotic stress tolerance to be examined both directly and indirectly in a complex transformation system.

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