Generation of drought-resistant transgenic cereals using transcription factors isolated from wheat grain

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INTRODUCTION

Transcription factors have been shown to control the activity of multiple stress response genes in a coordinated manner and therefore represent attractive targets for application in molecular plant breeding. Several families of transcription factors, such as DREB/CBF, ERF, MYK, MYB, AREB/ABF, NAC and HDZip class I and II, were found to be involved in the regulation of drought response in plants[1] These factors bind specific *cis*-elements on the promoters of drought regulated genes. DREB factors were among the first discovered families of transcription factors responsible for gene regulation under conditions of water deficiency. The members of the family contain a single AP2 DNA-binding domain, which strongly and specifically interacts with the dehydration-responsive element (DRE)[2]. The DRE (TACCGACAT) was used by several groups as a bait for isolation of DREB proteins from vegetative tissues of different plant species subjected to drought stress[3,4,5]. DREB proteins were stably overexpressed under constitutive[6,4,7,8] and/or drought inducible[6,9,10] promoters in some dicotyledonous and monocotyledonous species. The resulting transgenic plants frequently demonstrated increased resistance to drought and/or cold stress at the seedling stage in laboratory tests[9,10,11,12]. Only one paper thus far has described overexpression of a DREB factor from Arabidopsis in the heterologous species, wheat[10]; no DREB factors have been overexpressed in barley.

Previously, we isolated two new DREB factors from a cDNA library prepared from immature grain of unstressed wheat using Arabidopsis DRE as a bait[13]. In the current paper we describe the generation of transgenic barley and wheat plants overexpressing *TaDREB2* and *TaDREB3* under constitutive (35Sx2) and drought inducible (ZmRab17) promoters. Transgene integration and expression was confirmed by Southern and northern blots, respectively, and selected transgenic plants with strong overexpression of *DREB* genes were phenotyped for: developmental abnormalities in the absence of stress; drought tolerance; water use efficiency (WUE); and freezing tolerance at the seedling stage. Transgenic plants with elevated levels of *TaDREB2* or *TaDREB3* demonstrated both a remarkable resistance to drought and enhanced tolerance to freezing. Undesirable developmental phenotypes were mitigated by the application of the ZmRab71 drought inducible promoter. The results of this work will be discussed.

MATERIALS AND METHODS

Coding regions of *TaDREB2* and *TaDREB3* cDNAs were cloned into: (i) the pMDC32 vector[14], downstream of the vector's duplicated 35S promoter; and (ii) a pMDC32 vector in which the 35Sx2 promoter was excised using *Hind*III – *Kpn*II restriction sites and replaced with a 634bp fragment of the ZmRab17 promoter[15]. The construct was transformed into barley (*Hordeum vulgare* L. cv. Golden Promise) using *Agrobacterium*-mediated transformation [16 17]. Wheat (*Triticum aestivum* L. cv. Bobwhite) was transformed using biolistic bombardment [18]. Transgene presence and expression in T_0 transgenic plants and/or T_1 generation plants were analysed by Southern and northern blot hybridization [20]. For phenotypic analysis plants were grown in glasshouse conditions with an average day/night temperature of 25°C/16°C and 15 h day length. T_1 and T_2 generation plants were monitored for phenotypic changes, such as growth rate, plant height, heading time, number of tillers, spike phenotype, grain phenotype and yield. Seedlings for drought tolerant testing were grown in growth rooms, with a 16 h day length at day/night temperatures of 24°C/16°C. T_1 and T_2 generation plants were grown in 4 inch pots for four weeks and the volumetric water content (VWC) of the pots was monitored. At 4 weeks water was withheld. Seven days later the pots reached 1-2% VWC and clear wilting was observed; at this point the plants were re-watered. Plants were assessed for recovery after one and two weeks of re-watering and stress tolerant plants were transferred to the glasshouse for generation of seeds. Water use efficiency (WUE) of transgenic plants was determined using a seedling assay where seeds of similar size were sown in 450 ml pots (15cm height x 7cm diameter). Each pot contained 400g of soil. The pots were covered with a plastic sheet to prevent surface evaporation. The plants were grown until there was no more extractable water left. Total plant water use was calculated by subtracting the final pot weight from starting weight and factoring in water loss through evaporation. WUE was then calculated in grams of dry shoot biomass per ml of water used. For the cold tolerance assay T_2 generation plants were grown for three weeks at which point they were placed at 4°C for 24 h. After this treatment plants were cold stressed over 17h with a slow decrease in temperature to approximately -6°C. Plants were then transferred to a glasshouse and the number of recovered plants was recorded two weeks after the cold stress.

RESULTS AND DISCUSSION

Using the yeast one-hybrid system and the DRE as bait, we have identified and cloned cDNAs of two novel DREB transcription factors from unstressed developing wheat grain, designated *TaDREB2* and *TaDREB3*[13]. Deduced protein sequences of these transcription factors have the highest level of identity with a product of the *TINY* gene from Arabidopsis and *HvCBF5*, respectively. Involvement of *TINY* in drought and cold stress response regulation was recently demonstrated[21]. There are no data reported so far about the role of *HvCBF5* in stress response.

Both *TaDREB2* and *TaDREB3* were over-expressed in wheat and barley transgenic plants under constitutive (35Sx2) and drought inducible (ZmRab17) promoters. Barley plants (cv. Golden Promise) were transformed using *Agrobacterium*-mediated transformation while biolistic transformation was used to produce transgenic wheat (cv. Bobwhite). The presence and expression of the transgenes in T_0 plants has been confirmed by PCR, Southern and northern blot hybridization.

The developmental phenotypes of plants transformed with either gene were analysed using T_1 and T_2 progeny of at least four independent transgenic lines with the highest levels of transgene expression. Plants with constitutive over-expression of *TaDREB2* showed a slower growth rate and delayed germination and flowering relative to control plants; however, the plants reached normal size, and were darker than control plants. Plants with ectopically up-regulated *TaDREB3* showed a slightly different phenotype. They had retardation in growth and were approximately 1/3 smaller than wild type plants at their maximum size. Plants with the strongest phenotype produced twice as many tillers as control plants and had slightly shorter spikes. Constitutive overexpression of *TaDREB2* or *TaDREB3* did not result in any changes in grain size, shape, colour and yield, nor in germination rate.

Transgenic plants were tested for drought tolerance and WUE. T_1 progeny showed a clear enhancement of drought tolerance at the seedling stage. The levels of transgene expression were analysed in all plants used in this experiment. The drought tolerant phenotype correlated with the strength of transgene expression; and null segregants exhibited the same tolerance as control plants. The water use efficiency of transgenic plants overexpressing *TaDREB3* was significantly increased compared to control plants. Transgenic plants overexpressing *TaDREB2* did not show an increase in water use efficiency. This suggests different mechanisms of drought tolerance conferred by these transcription factors. This suggestion is supported by the fact that some drought and cold inducible genes like *HvDhn8* were up-regulated in plants with constitutive up-regulation of *TaDREB3*, but the level of expression of this gene remained the same in transgenic plants with constitutive up-regulation of *TaDREB2*.

The most drought tolerant transgenic barley lines with ectopic overexpression of *TaDREB2* and *TaDREB3* were also analysed for enhancement of freezing and salt tolerance. Transgenic plants with up-regulated *TaDREB2* showed a clear increase in the freezing tolerance: 80-90% plants survived the treatment vs. 0-10% of control plants. The percentage of surviving barley plants with up-regulated *TaDREB3* was lower: 50-60% vs. 0-10% of control plants. Expression of the transgene in all surviving transgenic lines was confirmed by northern blot hybridization.

Supported hydroponics was used for the analysis of the salt tolerance of transgenic barley lines with constitutive overexpression of *TaDREB2* and *TaDREB3*. Plants were grown on media with and without 100 mM NaCl. After 6 weeks the increase in biomass of control and transgenic plants was measured and compared. It was found that no increase in salt stress tolerance was conferred by either transgene. The growth of both control and transgenic plants was strongly but similarly suppressed by high salt concentration in the media. However, we also have not found any negative effect of transgene overexpression on the salt stress tolerance.

To prevent undesirable developmental phenotypes in transgenic wheat plants, we prepared constructs in which the coding regions of *DREBs* were downstream of the drought inducible promoter of the maize *Rab17* gene. Transformants containing the *ZmRab17* promoter::*TaDREB3* construct exhibited no differences, relative to control plants, with respect to germination, speed of development and plant size in six week old plants. T_1 progeny of seven independent transgenic wheat lines with *TaDREB3* under the *ZmRab17* promoter demonstrated slightly lower levels of wilting under drought stress, which was detected in at least three transgenic lines one week after relative water content in soil reached 1%. However, one week after re-watering transgenic plants from all seven lines quickly recovered and started to grow vigorously. In contrast, only one plant from 17 control plants was able to recover. The presence and activity of the transgene in all plants was confirmed using Southern and northern blot hybridization. A very good correlation between the level of *TaDREB3* expression and increased tolerance to the prolonged absence of water was observed.

Detailed analysis of drought, cold and salt tolerance in barley and wheat transgenic plants are currently in progress. This work includes assessment of grain yield under mild stress at the reproductive stage of plant development and thorough analysis of downstream genes and mechanisms of increased drought tolerance.

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