# Coordinated regulation of enzymes involved in secondary cell wall biosynthesis by a homeodomain transcription factor

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## INTRODUCTION

Homeodomain-leucine zipper transcription factors (HDZip TFs) are known to be modulators of morphogenesis in response to environmental stimuli and plant development programs[1]. To understand the role of HDZip TFs in grain development several HDZip class II (HDZipII) TFs were isolated from a cDNA library prepared from developing wheat embryos[2] and liquid endosperm of wheat at 3-6 days after pollination (DAP)[3], using the yeast one-hybrid system and the synthetic *cis*-element -CAAT(G/C)ATTG-[4] as bait. One of the isolated wheat TFs, *TaHDZipII-1*, had very low levels of the expression in all tested tissues[3]. The closest homologues of this factor are the transcription repressor from rice *Oshox1*[5] and the Arabidopsis protein *HAT4/AtHB-2*[6,7]. The *Oshox1* was originally isolated by screening cDNA library of 7-day old etiolated Indica rice seedlings using *HAT4* as a probe[5]; it was later isolated from the rice. *Oshox1* is expressed at different developmental stages, with the highest level of expression in embryos and leaves[5]. It can homodimerise and act as a transcriptional repressor of the reporter genes in transient assays[5]. *Oshox1* gene expression regulates critical, early stages of provascular ontogenesis[8]. Overexpression of *Oshox1* in transgenic Arabidopsis leads to severe retardation in growth, smaller and thinner leaves, difficulties in rooting and seed set. However, seeds were not obtained and primary transformants were used for the analysis[5]. Ectopic overexpression of *Oshox1* in rice leads to no obvious changes in phenotype, but enhances polar auxin transport (PAT) and the sensitivity of PAT toward auxin[9]. The promoter of *Oshox1* directs vascular, auxin- and sucrose-responsive gene expression in Arabidopsis and rice[10]. It was also shown that embryonic vascular expression of this gene is achieved mainly through the suppression of expression in non-vascular tissues[6]. In contrast to *Oshox1*, the *HAT4/AtHB-2* transcription factor from Arabidopsis demonstrate clear developmental phenotypes if ectopically up- or down-regulated[6,11]. Among the characteristic features of the phenotype are enhanced longitudinal cell expansion in the hypocotyls, inhibited secondary growth of the vascular system and lateral root formation, smaller and fewer leaves, a darker green colour, and inability of more than 50% seeds to germinate[6,11]. Another feature of this gene that makes it different from *Oshox1* is its strong inducibility by far red light and involvement in the regulation of the shade avoidance response[11,12].

In this research we overexpressed *TaHDZipII-1* ectopically in barley plants. The transgenic plants showed strong developmental phenotypes similar to those described above for transgenic Arabidopsis plants with elevated levels of *HAT4/AtHB-2* and *Oshox1*. Further investigations of the phenotype revealed features typical for the phenotypes of some *irx* mutants in Arabidopsis[13]. Detection in over-expressing transgenic plants of strong down-regulation of genes encoding secondary cell wall biosynthesis enzymes and lower deposition of lignin in several plant tissues confirmed the common origin of phenotypes of *TaHDZipII-1* plants and *irx* mutants. The possible function of *TaHDZipII-1* as a negative regulator of secondary cell wall biosynthesis will be discussed.

## RESULTS AND DISCUSSION

The transgenic analysis of the wheat *TaHDZipII-1* transcription factors has been undertaken in barley, phylogenetically very close to wheat. Wheat transformation protocols were not developed in our laboratory at the time when we started this work. Constitutive over-expression of *TaHDZipII-1* in transgenic barley leads to plants with greatly reduced growth rates, lower numbers of shoots and smaller spikes compared to control plants. The transgenic plants are shorter, darker, and have smaller thicker and more erect leaves than control plants (Fig. 1). To understand better the dark colour of transgenic plants, chlorophyll content was measured. The data obtained with a spadmeter revealed nearly twice as much chlorophyll per gm fresh weight in transgenic plants compared to controls, although this might be partly attributable to the thicker leaves. Transgenic plants had fewer tillers than control plants; flowering of transgenic plants was delayed up to five weeks. The spikes of transgenic plants were three fold shorter than spikes of control plants and had an oval shape. Plants with a strong phenotype usually were not able to release spikes from the flag leaf. Transgenic plants produced much less seed than control plants, often no more than one to two seeds per spike. The reason of the unsuccessful fertilisation most can be explained by aberrant development of anthers, which were shorter, had irregular shapes and often were not able to open and release pollen. Seeds were gray in colour and about thirty percent failed to germinate. During germination on filter paper, seeds were quickly killed by strong fungal infestation, although the control seeds obtained from plants grown in the same glasshouse at the same time were clearly much less susceptible to fungal infection. Overall, the phenotype of barley plants with up-regulated *TaHDZipII-1* had a lot of similar features with the phenotype of Arabidopsis plants with constitutively up-regulated *HAT4/AtHB2*[6,7]. Up-regulation of *HAT4/AtHB2* led to elongation of cells in coleoptiles of transgenic plants. Using scanning electron microscopy we demonstrated that the epidermal

cells in the stem of transgenic barley plants with up-regulated *TaHDZipII-1* were about 2-3 fold longer than epidermal cells of control barley plants. The phenotype of transgenic plants with ectopic up-regulation of *TaHDZipII-1* have a lot in common with phenotypes of transgenic plants overexpressing *HAT4/AtHB2* and *Oshox1*. This suggests a similar function of the three genes. It was shown that elevated *AtHB2* enhances longitudinal cell expansion in the hypocotyls[11]. However, no mechanism of such enhancement was proposed and downstream genes regulated by this transcription factor remain unknown.

Microscopic examination of stem sections of transgenic plants with elevated levels of *TaHDZipII-1* revealed collapse of cell walls in vascular bundle sheath extension cells, similar to collapsed xylem vessels in irregular xylem (*irx)* mutants previously described for genes encoding enzymes involved in secondary cell wall and particularly lignin biosynthesis[13]. Further tests revealed differences in cell wall formation in control and transgenic barley plants. It was found that transgenic barley plants had reduced stem strength compared to control plants, although stems of transgenic plants were thicker. Staining with phloroglucinol failed to reveal lignin in stems, and only low levels were found in vascular tissue in grain and bracts (lemma and palea) in the transgenic plants showing the strongest phenotype. The reduced levels of lignin correlated with the strength of the altered phenotype, and with the level of transgene expression. It was previously shown that changes in lignin biosynthesis pathways make plants more susceptible to pathogens[14]. Low levels or changed structures of lignin in bracts might be an explanation of why grain from transgenic plants is more susceptible to fungal infection.

Several genes, which are already known to be involved in secondary and primary cell wall biosynthesis were tested as possible candidate genes acting downstream of *TaHDZipII-1*. Q-PCR comparisons of gene expression in control and transgenic plants revealed strong and coordinated down-regulation of several genes encoding enzymes specific to secondary cell wall biosynthesis. These included coumarate ligase and laccase, enzymes involved in the lignin biosynthesis pathways, as well as several cellulose synthases: HvCesA4, HvCesA8, and HvCesA7. These cellulose synthases were previously shown to be coordinately transcribed in different barley tissues and are most similar to the cellulose synthases of Arabidopsis that have been implicated in secondary cell wall synthesis[15]. The expression of the Myb transcription factor and COBRA gene were also down-regulated in transgenic plants. Some members of these gene families were shown to act as positive regulators of secondary cell wall biosynthesis. In contrast, levels of expression of genes involved in the primary cell wall biosynthesis either remained the same or were slightly elevated. All data suggest that *TaHDZipII-1* may function as a negative regulator of secondary cell wall biosynthesis. We suggest that through up-regulation in non-transgenic plants by yet unknown environmental or developmental stimuli *TaHDZipII-1* might inhibit formation of secondary cell walls, and thereby allowing cells to grow more rapidly and for longer periods. In the absence of stimuli, *TaHDZipII-1* most probably is expressed in tissues or groups of cells where formation of secondary cell walls should be prevented. For example, *in situ* hybridization showed that *TaHDZipII-1* was expressed in endosperm and maternal vascular tissue at early stages of grain development, but was not detected in the lignin containing layers of maternal tissue and bracts.

## MATERIALS AND METHODS

The full-length coding region (CDS) of the *TaHDZipII-1* cDNA (Acc No DQ353857)[3] was cloned into the pMDCUbi (pUbi) vector. pUbi is a derivative of pMDC32 vector[16] in which 2X35S promoter was cut out using *Hind*III and *Kpn*I restriction sites and replaced with maize polyubiquitin promoter[17]. Resulting construct was designated pUbi-TaHDZipII-1 and was transformed into the *Agrobacterium tumefaciens* strain AGL1 by electroporation. The presence of plasmid in selected bacterial clones was confirmed by PCR using specific primers derived from CDS of plant gene. Construct, pUbi-TaHDZipII-1 was transformed into barley (*Hordeum vulgare* L. cv. Golden Promise) using *Agrobacterium tumefaciens*-mediated transformation developed by[18] and modified by[19]. Transgenic plants were grown in PC2 glasshouse with 10-hr light photoperiod. Plant phenotype was studied in $T_0$, $T_1$ and $T_2$ generations of several transgenic lines. Transgene integration in barley plants was confirmed by Southern blot hybridization. Genomic DNA from selected barley lines was digested with *Xho*1 and probed with the coding sequence of hygromycin phosphotransferase. Total RNA was isolated from wheat and barley samples using TRI REAGENT (Molecular Research Centre, Inc., Cincinnati, OH) and used in Northern blot hybridization as it described elsewhere. Pools of single strand cDNAs for Q-PCR were prepared using SuperScript III reverse transcriptase (Invitrogen). The forward primer for TaHDZipII-1 was designed for the wheat variety Chinese Spring using 3'UTR sequence of cDNA. To provide specific recognition of transgene cDNA the reverse primer sequence was selected from Nos terminator of vector plasmid. The primer pairs for cell wall enzymes were designed for the barley variety Golden Promise using 3'UTR sequences. The Q-PCR amplification was performed in the RG 2000 Rotor-Gene Real Time Thermal Cycler (Corbett Research, NSW, Australia) using QuantiTect SYBR Green PCR reagent (Qiagen, VIC, Australia), as described in[15]. The Rotor-Gene V4.6 software (Corbett Research) was used to determine the optimal cycle threshold (CT) from dilution series and the mean expression level and standard deviations for each set of four replicates for each cDNA were calculated.

For light microscopy stems and leaves of control and transgenic plants were fixed in TEM fixative (0.25% glutaraldehyde, 4% paraformaldehyde, 4% sucrose in 1xPhosphate Buffered Saline). After fixation, plant

material was rinsed (2-3 changes in 8 hours to remove fixative) in 1 M sodium phosphate. Tissues were rinsed and dehydrated in a successive ethanol series (70, 90, 95, 100%), and infiltrated step-wise with xylene (25, 50, 75, 100% in ethanol). 7 µm thick sections were stained with 0.01% (w/v) toluidine blue in 0.1% aqueous sodium tetraborate for 1-5 mins. To visualise lignin, hand-cut sections from fresh barley stems or leaves were stained with phloroglucinol - HCl. Each section was covered with 2-3 drops of 1% w/v solution of phloroglucin in 95% ethanol, followed by the addition of 1 drop of 25 % (w/v) HCl. The results were observed using a Laser Dissection microscope (Leica AS LMD). To compare size and shape of epidermal cells in leaf and stem of transgenic and wild type plants, we used a low voltage SEM procedure under low temperature[20]. Three T2 plants of three independent transgenic lines and three control plants were examined in this experiment. Middle regions of leaves (adjacent to midrib) and pieces excised 1 cm over second stem node (direction down from spike) were examined. Digital images were acquired from the same or similar regions on the plant leaves of transgenic and wild type barley plants.

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