The BdDOF24 protein from *Brachypodium distachyon* interacts with BdGAMYB in plant nuclei and regulates transcription of the *BdCathB* gene upon seed germination

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Abstract

During cereal seed germination, hydrolytic enzymes (α-amilases, proteases, etc.) synthesized in the aleurone layer in response to GA, catalyse the mobilization of storage reserves accumulated in the endosperm during seed maturation. Functional analysis of barley hydrolase gene promoters has led to the identification of a conserved tripartite GARC motif that consists on: GARE (5'-TAACAAA-3'), the pyrimidine box (5'-CCTTTT-3') and the 5'-TATCCAC-3' box, recognized by MYBR2R3, DOF and MYBR1-SAQKYF TFs, respectively. In *Brachypodium distachyon*, the *BdCathB* gene that encodes a Cathepsin B-like thiol-protease, orthologous to the wheat *Al21* and barley *HvCathB*, is highly induced in germinating seeds. The *BdCathB* promoter contains a conserved GARC, and two additional pyrimidine boxes. The expression of the *BdCathB* gene during Brachypodium seed germination is mediated, by transcription factors encoded by genes *BdDof24* and *BdGamyb*, ortologous to the barley *BPBF-HvDof24* and *HvGamyb*, respectively. Transcripts of both genes increase during germination and treatments with abscisic acid (ABA) or paclobutrazol (PAC, an inhibitor of gibberellins biosynthesis) decrease mRNA expression of *BdGamyb* but do not affect that of *BdDof24*. Besides, proteins BdDOF24 and BdGAMYB interact in plant nuclei and in transient expression assays in aleurone layers BdDOF24 is a transcriptional repressor and BdGAMYB is an activator of the *BdCathB* promoter, as occurs with barley BPBF-HvDOF24 and HvGAMYB. However, when both TFs are co-bombarded, BdDOF24 enhances the activation driven by BdGAMYB while BPBF-HvDOF24 strongly decreases the HvGAMYB-mediated activation of the *BdCathB* promoter. These different results concerning BdDOF24 and BPBF-HvDOF24 interaction with GAMYB are discussed.

Key words: *BdCathB*, *BdDof24*, *BdGamyb*, *Brachypodium distachyon*, germination, seeds, transcriptional regulation.

INTRODUCTION

During the maturation phase, seeds accumulate abundant reserves such as proteins, carbohydrates and lipids. Wheat (*Triticum aestivum*), rice (*Oryza sativa*), maize (*Zea mays*) and barley (*Hordeum vulgare*), belonging to the Poaceae family (monocotyledonous), are among the most important cereal species grown over the world for food and feed. Their main storage components, accumulated in the seed endosperm, are proteins (SSP; Seed Storage Proteins) and starch (Vicente-Carbajosa and Carbonero, 2005; Berger *et al.,* 2006). Seed storage compounds are hydrolysed upon seed germination and post-germination to provide energy, and C and N skeletons before the plant become photosynthetically active. Germination begins with the water uptake by the dry seed and ends, from a physiological point of view, when the radicle protrudes (Bewley, 1997). This complex physiological process is regulated by different external (temperature, light, etc.) and internal factors such as the hormones gibberellins (GA) and abscisic acid (ABA; Eastmond and Jones, 2005; Finch-Savage and Leubner-Metzger, 2006; Holdsworth *et al.,* 2008). In germinating barley seeds, the water imbibition process enhances GA synthesis in the embryo that diffuses to the aleurone layer where it triggers the expression of a number of hydrolase genes such as those encoding high and low pI α-amylase (*Amy6.4*, *Amy32b*), cathepsin-like enzymes (*HvCathB*, *EPB-1*), and β-glucanase isozymes (*EII*). These enzymes are secreted into the endosperm where they mobilize seed storage reserves (Sun and Gubler, 2004). In several seeds, proteases of the cathepsin-like class have been associated to SSP degradation and mobilization during seed germination and post-germination. Among them, cathepsin-like enzymes encoded by *Al21* in wheat, *EPB-1* in barley and *AtCathB3* in Arabidopsis (Cejudo *et al.,* 1992a, b; Cercós *et al.,* 1999).

Functional analysis of the promoters of hydrolase genes expressed in germinating cereal seeds has identified a tripartite *cis*-acting GA-Responsive Complex (GARC). This complex most often includes the GA-Responsive element (GARE, 5'-TAACAAA-3'), the pyrimidine box (5'-CCTTTT-3') and the 5'-TATCCAC-3' box (Sun and Gubler 2004) and several transcription factors (TFs) have been demonstrated to interact with the three motifs of the GARC. These TFs belong to

the MYBR2R3, DOF and MYBR1-SHAQKYF families (Gubler *et al.*, 1999; Mena *et al.*, 2002; Isabel-Lamoneda *et al.*, 2003; Rubio-Somoza *et al.*, 2006a, 2006b; Moreno-Risueño *et al.*, 2007). Barley HvGAMYB (MYBR2R3), acting downstream of the DELLA protein SLN1, is considered as the master regulator of hydrolase genes upon seed germination; it is highly induced by GA in aleurone cells, and through interaction with the GARE in the promoter of its target genes, is able to trans-activate the expression of several hydrolase gene such as *Amy32b*, *EII*, *Al21* (Gubler *et al.*, 1999, 2002). Its rice orthologous OsGAMYB, functions also as a positive regulator of GA-responsive genes upon germination (*RAmy1*, *REP-1*) as inferred by the study of loss-of-function mutants for *OsGAMYB* (Sutoh and Yamauchi, 2003; Kaneko *et al.*, 2004).

Several TFs of the DOF family besides being important regulators of seed reserve protein genes during the maturation phase (Haseneyer *et al*., 2010; Nogero *et al*., 2013) are part of the transcriptional complex that regulates hydrolase genes in barley germinating aleurone. These TFs that recognize the pyrimidine box of the GARC are BPBF-HvDOF24, SAD-HvDOF23, HvDOF19 and HvDOF17. Whereas SAD-HvDOF23 is a transcriptional activator of the *Al21* gene, BPBF-HvDOF24, HvDOF19 and HvDOF17 act as transcriptional repressors, and all of them interact with HvGAMYB in cell nuclei (Mena *et al.*, 2002; Isabel-Lamoneda *et al.*, 2003; Díaz *et al.*, 2005; Moreno-Risueño *et al.*, 2007). In rice, using as a bait a promoter fragment containing the pyrimidin box of the GA-regulated hydrolase gene *CPD3*, encoding a type II carboxypeptidase, five DOF TFs were identified. One of these TFs, RBPF-OsDOF10 (OsDOF3), has a synergistic effect with OsGAMYB in the trans-activation of the *RAmy1A* gene (Washio, 2001, 2003).

Brachypodium distachyon is an important model system for the grasses, since it is small in size, has a short generation time and is a self-fertile diploid. Its genome of 270 Mbp has been sequenced and a wide array of molecular resources is available (Draper *et al.*, 2001; International Brachypodium Initiative 2010). This paper reports the physiological significance and transcriptional regulation of *BdCathB*, a gene that encodes a Cathepsin B-like protease, during Brachypodium seed germination and describes that the proteins BdDOF24 and BdGAMYB are respectively transcriptional repressor and activator of the *BdCathB* gene. When

both TFs are present in transient expression assays BdDOF24 enhances the activation driven by BdGAMYB of the *BdCathB* promoter. *BdDof24* and *BdGamyb*, are highly expressed in aleurone cells upon germination, peaking at 48 hours after imbibition (hai). Whereas *BdGamyb* is repressed by abscisic acid (ABA) and by paclobutrazol (PAC, a GA biosynthesis inhibitor), an indication that is GA-inducible, *BdDof24* is not affected. Structural and functional comparison of these Brachypodium TFs with their putative orthologous in barley is discussed.

Material and methods

Plant material, growth conditions and germination assays

Brachypodium distachyon strain Bd21, a standard diploid inbred line (International Brachypodium Initiative, 2010), kindly provided by Prof. Garvin (University of Minnesota) was used in this study. Seeds were surface sterilized in 1% NaOCl for 10 minutes, washed in sterile water and placed on 9-cm Petri dishes containing two filter papers (Whatman number 3) moistened with 8 ml of sterile water and germinated in the dark at 22ºC for one week and then transferred to long-day conditions (16h /8h; light/darkness; light intensity: 155µmol photons$\cdot m^{-2} \cdot s^{-1}$) in a controlled-environmental growth chamber at 22ºC. After 4 weeks plants were transferred to pots in the greenhouse under the same conditions.

For germination assays, three replicate sets of 25 after-ripened seeds (storage at 22ºC and 30% relative humidity in the dark for 3 months) and non-stratified were surface sterilized as described above and placed on 9-cm Petri dishes containing two filter paper moistened with 8 ml of sterile water, 8 ml of ABA (10 and 25 µM) or 8ml of PAC (50, 100 and 200µM). Germination was carried out at 22ºC in the dark. Seeds were scored as germinated when the coleorhiza had emerged beyond the husk (Barrero *et al*., 2009). De-embryonated seeds containing the aleurone were collected at different time points of germination (0, 12, 24, 36, 48, 72 and 96 hours after imbibition, hai), and used for RNA and protein extraction.

Bioinformatic tools

Complete deduced protein sequences from *Cathepsin B-like*, *GAMYB* and *PBF* genes from *Brachypodium distachyon*, *Oryza sativa* and *Zea mays*, were obtained using the barley corresponding sequences (*HvCathB*, *HvGamyb* and *BPBF-HvDof24*) and the TBLASTN tool at the Phytozome v8.0 Database (Goodstein *et al*., 2012; www.phytozome.net); those of *Triticum monococum* and *Triticum aestivum* were obtained from the GenBank Database (Benson *et al*., 2005; http://www.ncbi.nlm.nih.gov).

The complete deduced amino-acid sequences of the six *Cathepsin B-like* genes, five *Gamyb* genes, and the 52 amino-acid region that spans the DOF domain of the 5 *PBF* genes from Brachypodium, barley, wheat, rice and maize, were used to construct 3 different phylogenetic dendrograms (Figs. 1A, S1B and S2) using the neighbor-joining algorithm in the Phylogeny.fr platform (Dereeper *et al*., 2008; http://www.phylogeny.fr/). The MEME program was used to identify conserved motifs and to validate the phylogenetic tree. Default parameters were used with the following exception: the maximum number of motives to find was set to 15 and the minimum width was set to 8 amino-acid residues (Bailey *et al*., 2009; http://meme.sdsc.edu/meme4_6_0/intro.htlm).

Major characteristic of proteins are listed in Table 1, Figs. S1B and S2. Signal peptide cleavage sites and subcellular location were predicted by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP) and TargetP 1.1 tools (http://www.cbs.dtu.dk/services/TargetP/), respectively (Emmanuelsson *et al*., 2007) and both pI and Mw were calculated using Compute pI/Mw tool (Gasteiger *et al*., 2005; http://www.expasy.ch/tools/pi_tool.html). Zimmerman polarity profile of PBF proteins were generated by using ProtScale tool (Gasteiger *et al*., 2005; http://web.expasy.org/protscale/). Complete sequence alignment of PBF proteins and the relative identity (%) to BPBF-HvDOF24 were calculated using CLUSTAL OMEGA (Sievers *et al*., 2011; https://www.ebi.ac.uk/Tools/msa/clustalo/).

Plant *cis* regulatory DNA elements within the *BdCathB* promoter sequence were searched through the following programs and databases: PlantCare (Lescot *et al*., 2002; http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (Higo *et al*., 1999; http://www.dna.affrc.go.jp/PLACE/).

Real time quantitative PCR analysis

Total RNA isolation from leaves, roots and spikes was performed with the classical phenol/chloroform method (Lagrimini *et al*., 1987). For the isolation of RNA from seeds at different stages of development (0, 5, 10, 15, 30 and 45 days after pollination, dap) and at different germination time points (0, 12, 24, 36, 48, 72 and 96 hai), a standard protocol described by Oñate-Sánchez and Vicente-Carbajosa (2008) was followed. RNA samples were treated with DNAse I, RNAse-free (Roche Applied Science, Mannheim, Germany) to avoid genomic DNA contamination. First-strand cDNA was synthesized with random hexameres using the High-Capacity cDNA Reverse Transcription Kit according to the manusfacture's recommendations (Applied Biosystems, Foster City, CA, USA). Samples were stored at -20ºC until used.

RT-qPCR analyses were performed in an Eco Real-Time PCR System (Illumina, San Diego, CA, USA). For each 10 µl reaction, 2 µl of the sample's cDNA was mixed with 5 µl of FastStart SYBR Green Master (Roche Applied Science) and 0.25 µl of each primer (final concentration 500 nM) plus sterile water up to final volume. Samples were subjected to thermal-cycling conditions of 95ºC for 10 min and 40 cycles of 10 sec at 95ºC and 30 sec at 60ºC for annealing and extension, respectively. The melting curve was designed to increase from 55ºC to 95ºC. The specific primers (Table S1) were designed on the 3'-non coding region using the Prime3Plus program (Untergasser *et al.,* 2007; http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The primer efficiency was calculated using a calibration dilution curve and slope calculation ($E=10^{(-1/slope)}$; Table S1). Quantification was normalized to the expression of the *BdGAPDH* gene (encoding glyceraldehyde 3-phosphate dehydrogenase; Hong *et al*., 2008; Hernando-Amado *et al.,* 2012) and calculated as the number of cycles needed for the amplification to reach a threshold fixed in the exponential phase of the PCR reaction (C_t; Pfaffl, 2001). All analyses were done in three different biological replicates and variations among samples were statistically evaluated by means of a *t*-test analysis.

Cathepsin B-like proteolytic assays

Three replicate sets of de-embrionated seeds containing the aleurone were collected at different time points during germination (0, 12, 24, 36, 48, 72 and 96 hai) and proteins were extracted with a protein extraction buffer (340 mM sodium acetate, 60 mM acetic acid, 4 mM EDTA, 8 mM DTT, pH 6). The fluorogenic substrate Z-RR-AMC (Z-Phe-Arg-7-amino-4-methylcoumarin; Calbiochem, San Diego, CA, USA) was used to determine the enzymatic activity of protein extracts following the manufacturer's instructions. Protein extracts (50 µg) and substrate (20 µM) were incubated at 36ºC for 1 hour and the emitted fluorescence was measured every 10 minutes (365 nm excitation and 465 emission wavelength) in the Genios Pro 96/384 multifunction microplate reader (TECAN®, Tecan Group Ltd., Männedorf, Switzerland).

Yeast two hybrid and LacZ assays

BdDof24 and *BdGamyb* ORFs were amplified from *B. distachyon* dry seeds cDNA by nested PCR using oligonucleotide pairs containing *attB* sites (Table S2), and cloned into the pDONR221® plasmid by the BP Gateway reaction, following manufacturer's instructions (Invitrogen, Life Technologies, Carlsbad, California, USA). Both *BdDof24* and *BdGamyb* were fused in frame both to the yeast Gal4 activation domain (AD) and to the binding domain (BD), by LR Gateway recombination reactions, into the destination vectors pDEST22® and pDEST32® (Invitrogen, Life Technologies). *BdDof24-AD/BD*, *BdGamyb-AD/BD* and *GFP-AD/AB* (as control) constructs were used to transform, in different combinations, the *Saccharomyces cerevisiae* SFY526 strain, that contains the *LacZ* gene as reporter under the control of a truncated *Gal1UAS* promoter which contains *Gal4*-responsive elements. Yeast transformation was done by the polyethylene glycol method and quantification of β-galactosidase (*LacZ*) activity in liquid culture was calculated using Miller's formula as described (Lara *et al*., 2003).

Biomolecular fluorescent complementation (BiFC)

BdDof24 and *BdGamyb* were translationally fused to the N- and C-terminal baits of the Yellow Fluorescent Protein (YFP) by LR Gateway recombination reaction between the entry vector pDONR221, harboring the *BdDof24* and *BdGamyb* ORFs, and the destination vectors pE-SPYNE-GW and pE-SPYCE-GW (Weltmeier *et al.*, 2006). Final constructs were used to co-bombard inner

epidermal layers of fresh onions (*Allium cepa*) using a biolistic Helium gun device (DuPont PDS-1000; BioRad Laboratoires, Hercules, CA, USA) as previously described (Díaz *et al*., 2005). The fluorescence emission was measured after 48 h of incubation at 22ºC in the dark, with fluorescence Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany) using the following filter parameters: excitation 450-490 nm; emission 520 nm. Images were processed with the Leica Application Suite 2.8.1 build software (Leica, Wetzlar, Germany). Each bombardment was performed in three independent plates and complementation was confirmed in two independent assays.

Transient expression assays in barley aleurone layers

BdCathB promoter sequences were amplified from *B. distachyon* genomic DNA using the primers indicated in Table S2 and cloned into the *SphI/BamHI* restriction sites of the *pUbiGUSPlus* vector (Vickers *et al*., 2003). The complete ORFs of *BdDof24* and *BdGamyb* were amplified from *B. distachyon* dry seeds cDNA (Primers in Table S2) and cloning into *BamHI* restriction site of a derived *pBlueScript* vector (Stratagene, Agilent Technologies, Santa Clara, CA, USA) containing the *CaMV35S* promoter followed by the first intron of the maize *AdhI* gene and ORFs were translationally fused to the *GFP* ORF plus the 3' NOS terminator (Moreno-Risueño *et al*., 2007). The effector constructs carrying the *HvGamyb* and *BPBF-HvDof24* ORFs were designed and constructed as described (Díaz *et al*. 2005).

Barley aleurone layers from cv Himalaya were transformed by bombardment of gold particle coated with DNA, using a biolistic Helium gun device (DuPont PDS-1000; BioRad Laboratoires) as previously described (Mena *et al*., 2002). After bombardment, aleurone layers were incubated in 20 mM Na-succinate buffer (pH 5.2) and 20 mM $CaCl_2$ with gentle shaking over-night at 22ºC in the dark. Fluorimetric GUS assays were performed according to Jefferson *et al*., 1987. Proteins were extracted from ground aleurones with GUS Extraction Buffer (GEB, 50 mM NaHPO4 buffer, 1 mM Na_2-EDTA, 0.1% Triton X-100, 100 mM β-Mercaptoethanol, pH 7). For the reaction, 50 µl of extract were added to 150 µl of GUS assay buffer containing 1mM of the fluorogenic substrate MUG (4-Methylumbelliferyl-beta-D-glucuronide trihydrate, Duchefa Biochemie, Haarlem,

The Netherlands) and incubated at 37ºC for 2 hours; every 30 minutes the reaction was stopped in 1M Na_2CO_3, and the emitted fluorescence was measured at 465 nm emission (365 nm excitation) wavelength in the Genios Pro 96/384 multifunction microplate reader (TECAN, Tecan Group Ltd.). Xylanase activity was used to normalize the data (Vickers *et al*., 2003); for this purpose, aleurone layers were transformed with *pUbiSXR* vector harboring a synthetic xylanase gene (*sXynA*) under the control of the maize ubiquitin promoter (*Ubi-I*) and with a 3' ribulose biphosphate carboxylase (RUBISCO) small subunit terminator region (*rbcS*). For the xylanase assay, 50 µl of protein extract plus 450 µl of Xylanase Assay Buffer (XAB, 50 mM sodium citrate pH 5.5) containing 10mg/ml of the substrate AZCL-xylan (Azurine-crosslinked xylan; Megazyme, Wicklow, Ireland) were incubated for 30 minutes at 40ºC and shacked at 250 rpm. Absorbance was determined at 590 nm by a Dynex Opsys MR 96 Well Microplate Reader, VS 405-690nm Spectral Range (DYNEX Technologies, Chantilly, Virginia, USA).

Results

Expression of two CathB genes in different organs of Brachypodium distachyon

Two Cathepsin B-like genes, *BdCathB* and *BdCathB** (loci *Bradi1g09730* and *Bradi1g09737*) have been annotated in the *B. disthachyon* genome and a phylogenetic tree comparing their deduced amino-acid sequences to those of other cereal *CathB* genes appear in Fig. 1A. The barley, wheat, rice, maize and Brachypodium *CathB* genes are clearly orthologous with bootstrap values higher than 80% (Fig. 1A) and all share nine consensus motives according to the MEME analysis (Table S3) with similar molecular weights and isoelectric points (Table 1). The expression profiles of these *BdCathB* genes have been analysed by RT-qPCR in different organs (Fig. 1B): 2 week-old (YL: young leaves) and 6 week-old leaves (AL: adult leaves), roots (R: 2 week-old), spikes before pollination (S) and in developing seeds (from 1 to 45 days after pollination, dap; Fig. 1C). The *BdCathB** gene is barely expressed in all the organs analysed and probably is a pseudogene.

In the aleurone of germinating seeds, the expression of the *BdCathB* gene decreases at the beginning of the imbibition period, but increases thereafter, reaching a maximum of circa 3000% (relative to the *BdGAPDH* gene) at 48 and 72 hours decreasing afterwards (Fig. 2A). As occurs in other tissues analysed, the expression of the *BdCathB** is very poor: almost three orders of magnitude less than the *BdCathB* expression (Fig. 2B). The maximum accumulation of the *BdCathB* transcripts coincides with the maximum CathB proteolytic activity (Fig. 2C) in de-embrionated germinating seeds (72 hai).

Search for TFs in Brachypodium distachyon orthologous to barley PBF (BPBF-HvDOF24) and GAMYB

Recently, the entire family of DOF transcription factors in *B. disthachyon* has been annotated, and a systematic expression profile has determined that *BdDof24* is specifically expressed in seeds, being the most highly expressed DOF TF during germination. Furthermore, in the same study the phylogenetic tree, based on the alignment of the DNA-binding domains, indicates that the BdDOF24 deduced protein is grouped with rice and barley PBFs (RPBF-OsDOF10/OsDOF3, BPBF-HvDOF24) in the same Major Cluster of Orthologous Genes, MCOG D (Hernando-Amado *et al.,* 2012), sharing only the binding domain with other members of the group. If the BdDOF24 orthologs from wheat and maize are introduced in this comparison (Fig. S1A), a new dendrogram is produced (Fig. S1B). The tree structure shows now two sub-clusters based on DOF domain similarity, with bootstrap values greater than 60%: one includes the PBF proteins from barley, wheat and maize and the other spans the Brachypodium and rice PBF (DOF24). According to the MEME analysis (Table S4) , motif 4 in rice PBF is shared also by wheat, barley and maize, and motif 7 does not appear in maize but is present in wheat and barley PBFs (Bailey *et al.,* 2008). However, it is worth mentioning that BdDOF24 is smaller in size than the rest of the DOF proteins in MCOG D (217 aa residues vs 334 in barley BPBF-HvDOF24), due to the presence of an early stop codon in its C-terminal, downstream of the DNA-binding domain. BPBF-HvDOF24, WPBF and MPBF share an asparagine-rich stretch at the C-terminus that is lacking in BdDOF24 and RPBF-OsDOF10. In summary, BdDOF24 and BPBF-HvDOF24 share similarity only in the DOF

domain (82.69%) while in the N-terminal region this % of identity is only 17.65%, and downstream of the DOF domain (C-terminal region) is only 28.70% (Fig. S1B).

The same type of analysis has been performed with HvGAMYB and its orthologous form wheat, rice, maize and Brachypodium (BdGAMYB: *Bradi2g53010*). As shown in Fig. S2 and Table S5, BdGAMYB is very closely related to barley and wheat GAMYB throughout its sequence as shown by common motives outside of the DNA-binding domain, similar molecular weight and similar isoelectric point.

Expression of BdGamyb and BdDof24 during germination is compatible with their being transcriptional regulators of the BdCathB gene.

If the *BdCathB* gene were the orthologous of the GA-induced Cathepsin B-like genes described in barley and other cereal seeds, it should present the tripartite GARC in its promoter. In order to explore this, an *in silico* search for the elements: GARE (5'-TAACAAA-3'), the pyrimidine box (5'-CCTTTT-3') and the 5'-TATCCAC-3' box, has been undertaken. As shown in Fig. 3A, the promoters of *BdCathB* and wheat *Al21* have a complete GARC conserved, besides sharing two additional pyrimidine boxes.

Gene expression analysis of *BdDof24* and *BdGamyb* has been carried out by RT-qPCR throughout germination and post-germination in de-embryonated seeds (0, 12, 24, 36, 48, 72 and 96 hai; Fig. 3B). Although with different expression levels, *BdGamyb* transcript abundance is almost one order of magnitude higher than that of *BdDof24*, the expression profiles of both show a peak at 48 hai, decreasing after that in the post-germination phase (from 48-96 hai).

Transcript quantification of *BdCathB*, *BdGamyb* and *BdDof24* in response to 10μM ABA and 200μM PAC has been explored during seed germination (24 and 48 hai; Fig. 4A). These ABA and PAC treatments (selected as described in Fig. S3) produce a decrease in the mRNA levels of *BdCathB* and *BdGamyb* in de-embrionated seeds (aleurone), while no significant alterations are detected in the expression of *BdDof24*. The higher differences ($p \leq 0.01$) are detected after 200μM PAC treatment, when the *BdCathB* and *BdGamyb* transcripts decrease by ~80% and ~60% respectively, both at 24 and 48 hai. After 10μM ABA incubation only a

reduction of ~30% in mRNA levels has been observed for both genes and the germination kinetics under the ABA and PAC treatments are significantly slower than non-treated seeds (t_{50}=38 ± 1.1 and 40.4 ± 0.5 respectively *versus* t_{50}= 30.2 ± 0.9 in the control; Fig. 4B).

BdDOF24 and BdGAMYB interact in yeast and in plant nuclei

To explore whether BdDOF24 interacts with BdGAMYB, the yeast two hybrid systems has been employed, using the *LacZ* gene as reporter. As represented schematically in Fig. 5A, the ORFs of *BdGamyb* and *BdDof24* have been translationally fused, to the yeast Gal4 binding domain (Gal4BD) and to the activation domain (Gal4AD), respectively. These constructs have been used to transform *Saccharomyces cerevisiae* yeast cells (strain SFY526) and the corresponding β-galactosidase (*LacZ*) activity, under the control of the *Gal1UAS* promoter, has been quantified in liquid medium. When yeasts are transformed (Fig. 5B) with the full length cDNA of both genes fused to the Gal4BD, β-galactosidase activity is detected, indicating that both TFs have trans-activation activity in yeast, although the activity produced by the BdDOF24 construct is 10 times weaker than the activity of BdGAMYB (20 vs 250 Miller units). When the same constructs are fused to the Gal4AD no β-galactosidase activity is detected. However, co-transformation with the cDNAs of both genes one fused to the Gal4BD and the other to the Gal4AD, a significant increase of the basal activity is produced, indicating interaction between BdDOF24 and BdGAMYB proteins in this yeast system (Fig. 5B).

To validate the BdDOF24-BdGAMYB interaction *in planta*, biomolecular fluorescent complementation experiments have been carried out. For this purpose, *BdDof24* and *BdGamyb* ORFs have been translationally fused, in the two possible combinations, to the N- and C- terminal fragments of the yellow fluorescent protein encoding gene (*YFP*; Walter *et al.*, 2004). Microscopic observations from the different TF combinations show that YFP fluorescence is reconstituted and targeted to the nucleus, indicating that BdDOF24 and BdGAMYB proteins interact in plant nuclei (Fig. 5C). As expected, no reconstruction of fluorescence is achieved in experiments with only one of the ORFs, or when the YFP fragments are tested alone (data no shown).

BdDOF24 represses transcription of the BdCathB promoter but increases the BdGAMYB activation capacity when co-bombarded in aleurone transient expression assays

The occurrence of DOF and MYB recognizing motives and their conserved position among the *BdCathB* and *Al21* promoters, together with the expression patterns of *BdGamyb* and *BdDof24* in germinating seeds, are compatible with the idea of BdGAMYB and BdDOF24 proteins acting as regulators of the expression of the *BdCathB* gene during Brachypodium seed germination, as occurs with their orthologs in barley (HvGAMYB and BPBF-HvDOF24). For this purpose, transient expression assays by particle bombardment into isolated aleurone layers of barley cv. Himalaya have been done (Fig. 6).

As reporters, two deletion fragments of the *BdCathB* promoter have been fused to the *uidA* (GUS) reporter gene: one of them contains the whole promoter (-463 bp; *P463-BdCathB::GUS*) and the other spans -292 bp upstream of the translation initiation codon (*P264-BdCathB::GUS*) and is devoid of two out of the three DOF-binding domains and the GAMYB-binding domain. As effector constructs, the *BdDof24* ORF and the *BdGamyb* ORF, under the control of the *CaMV35S* promoter, followed by the first intron of the *AdhI* gene from maize, and the 3'-non coding region of the *nos* gene, have been used (Fig. 6A). For comparison, similar effector constructs with their barley orthologous (*HvGamyb* and *BPBF-HvDof24*) have been tested (Díaz *et al.*, 2002; Mena *et al*., 2002).

As shown in Fig. 6B, when isolated aleurones are bombarded with the full promoter (*P463-BdCathB::GUS*) in combination with the effectors at a 1:1 molar ratio, the BdDOF24 acts as a transcriptional repressor since it reduces ~4-times the GUS activity of this reporter, while BdGAMYB produces a 2-fold enhancement in this activity. Surprisingly, when BdDOF24 is bombarded together with BdGAMYB, a higher transactivation activity than that obtained with BdGAMYB alone is observed (Fig. 6B). When the reporter construct used is the P264-*BdCathB::GUS*, either alone or together with the effector constructs at 1:1 molar ratio, the GUS activity lowers to almost undetectable levels.

When the reporter *P463-BdCathB::GUS* construct is co-bombarded in a 1:1 molar ratio with the barley *HvGAMYB* as effector (Fig. 6B), a dramatic increase of 7-fold in GUS activity is observed, as compared to the basal activity without

effector. Similarly, when co-bombardment is carried out using as effector the barley *BPBF-HvDOF24*, a reduction of the GUS activity is observed. However, after co-transformation with both effectors, BPBF-HvDOF24 is able to revert to the basal levels the GUS activity driven by the barley HvGAMYB, an opposite behavior as that observed when using the orthologous effectors from Brachypodium (BdDOF24 and BdGAMYB).

Discussion

Brachypodium distachyon seeds accumulate storage compounds in the endosperm cells in amyloplasts, protein bodies and cell walls (polysaccharides; Larré *et al*., 2010; Guillon *et al*., 2010, 2011). Cathepsin B-like proteins are hydrolytic enzymes that degrade and mobilize SSPs upon seed germination (Tan-Wilson and Wilson, 2012). In the *Brachypodium distachyon* genome database, two genes encoding Cathepsin B-like proteases, *Bradi1g09730* (*BdCathB*) and *Bradi1g097373* (*BdCathB**) have been found. The deduced amino-acid sequences of both genes share a high homology (~80%) between them and with those of barley HvCathB and wheat TaCathB, as shown in the phylogenetic tree and the MEME analysis. The phylogenetic tree and their close position in chromosome 1 show that the two Brachypodium genes, *BdCathB* and *BdCathB** are probably paralogous originated by a recent duplication event occurred after the diversification of the *Cathepsin B-like* cluster (Figs. 1A, S1). Transcript abundance of *BdCathB* is two orders of magnitude higher than that of *BdCathB** in all organs analysed, as well as, upon seed development and germination (Figs. 1B, 1C, 2A, 2B). All these data indicate that *BdCathB* could encode the most physiologically relevant Cathepsin B-like protein in Brachypodium.

BdCathB expression highly increases upon seed imbibition peaking between 48 and 72 hai when germination *sensu stricto* has come to the end. This expression pattern is similar to those described for its orthologs in barley (*HvCathB*) and wheat (*TaCathB-Al21*; Cejudo *et al*., 1992a; Moreno-Risueño *et al*., 2007). During Brachypodium seed germination, Cathepsin B-like protease activity in de-embryonated seeds reaches its maximum at 72 hai, supporting the role of BdCathB in the mobilization of SSPs during the post-germination phase (Fig. 2).

Recently, transcriptomic analysis of germinating barley aleurone cells has revealed the induction of several protease genes that are GA-inducible and ABA-repressed (Martínez *et al*., 2003; Moreno-Risueño *et al*., 2007; Sreenivasulu *et al*., 2008). Similarly, *BdCathB* gene expression of de-embrionated (aleurone containing) imbibed Brachypodium seeds decreases after PAC (an inhibitor of GA biosynthesis) and after ABA treatments (Fig. 4).

Promoters of several hydrolase genes expressed in cereal germinating seeds contain a tripartite *cis*-acting GA-Responsive Complex (GARC) that includes a GA-Responsive element (GARE, 5'-TAACAAA-3'), a pyrimidine box (5'-CCTTTT-3') and a 5'-TATCCAC-3' box (Sun and Gubler, 2004). A detailed *in silico* analysis of the *BdCathB* promoter has identified one GARE element (MYBR2R3 binding site), three putative pyrimidine elements (DOF binding sites) and one 5'-TATCCAC-3' box. The structure and relative position of these *cis*-elements are also present in the promoter of the *TaCathB-Al21* (Fig. 3A), suggesting that orthologous transcription factors might be involved in their regulation.

Barley HvGAMYB (MYBR2R3) is considered as the master regulator of hydrolase gene expression, through its interaction with the GARE, upon seed germination and it is highly induced by GA in aleurone cells. In de-embrionated *Brachypodium distachyon* seeds, *BdGamyb* transcripts are highly induced upon germination, reaching a maximum at 48 hai (~1500% to the *BdGAPDH* expression), just before the *BdCathB* expression peak. *BdGamyb* expression is also down-regulated by ABA and PAC as occurs with *BdCathB* transcripts and those of its putative orthologs in barley (*HvGamyb*) and rice (*OsGamyb*; Gubler *et al*., 1999, 2002; Sutoh and Yamauchi 2003; Kaneko *et al*., 2004; Washio and Morikawa, 2006).

Barley BPBF-HvDOF24 and rice RPBF-OsDOF10 (OsDOF3) proteins specifically recognize the pyrimidine box contained in the promoters of genes encoding hydrolytic enzymes implied in seed storage compound mobilization during post-germination. Their orthologous gene in Brachypodium, *BdDof24*, is the most abundant *DOF* transcript upon seed imbibition (Hernando-Amado *et al*., 2012), reaching its maximum at 48 hai and its *e*xpression is neither altered by

ABA nor by PAC (Fig. 4). However, its barley and rice othologs are induced by GA (Mena *et al*., 1998, 2002; Washio, 2003; Washio and Morikawa, 2006; Yamamoto *et al*., 2006). These data point to BdGAMYB and BdDOF24 TFs as putative regulators of the *BdCathB* gene expression upon *B. distachyon* seed germination.

In the past years, a model has been proposed for the expression control at the transcription level in the barley germinating seeds. In this model, when GA diffuses from the scutellum to the aleurone layer during germination, the expression of positive effectors, such as GAMYB, increase and the transcription of aleurone hydrolase encoding genes begins. In germinating rice seeds, the promoter expression of the α-amilase encoding gene *RAmy1A* is highly induced by GA and positively influenced by a cooperative regulatory function between OsGAMYB and RPBF-OsDOF10 (OsDOF3). Interestingly, BdGAMYB and BdDOF24 do interac in the nuclei of onion epidermal cells and in the yeast two-hybrid system as occurs with their rice and barley orthologs: OsGAMYB/RPBF-OsDOF10 (OsDOF3) and HvGAMYB/BPBF-HvDOF24, respectively. (Fig. 5; Díaz *et al*., 2002, 2005; Washio, 2003; Moreno-Risueño *et al.,* 2007). In transient expression experiments in barley aleurone layers treated with GA, while BdGAMYB positively activates the *BdCathB* gene promoter, BdDOF24 represses it. However, when BdGAMYB is co-bombarded in combination with BdDOF24, GUS activity driven by the *BdCathB* gene promoter (*P463-BdCathB::GUS*) significantly increases (compared to that when only BdGAMYB is used). Deletion of the two pyrimidine boxes and the GARE contained in the *BdCathB* promoter (*P264-BdCathB::GUS*) decreases the basal promoter activity and eliminates the effect of both BdGAMYB and BdDOF24 transcription factor over this promoter, suggesting that these *cis*- elements, and not the third DOF binding site and the 5'-TATCCAC-3' box are relevant in the transcriptional control of the *BdCathB* gene (Fig. 6). Similarly, two distal pyrimidine boxes and a GARE motif are required for the OsGAMYB and RPBF-OsDOF10 (OsDOF3) transcriptional activation of the rice *RAmy1A* gene promoter (Washio, 2003). It has been reported that the barley BPBF-HvDOF24 drastically suppresses the activation by HvGAMYB of the wheat *TaCathB-Al21* promoter and that BPBF-HvDOF24 *in vitro* binds a pyrimidine box contained in this promoter, which is located in a

position equivalent to the DOF binding site at position -392 bp of the *BdCathB* promoter (Mena *et al*., 2002). In this study, when the barley HvGAMYB and/or BPBF-HvDOF24 are co-bombarded with the *P463-BdCathB::GUS* construct in aleurone layers, HvGAMYB positively activates expression of *BdCathB* promoter and BPBF-HvDOF24 represses it, but when both TFs are added, BPBF-HvDOF24 protein negatively influences the activation provoked by HvGAMYB in this promoter. All together, the differences observed in the *BdCathB* gene regulation by DOF TFs from Brachypodium or barley might be explained by specific biochemical characteristics of their ortholog TFs more than by the promoter sequences of their target genes.

The GAMYB-like cereal proteins are highly conserved through evolution, BdGAMYB and HvGAMYB proteins have a 90% of amino-acid identity that is not restricted to the DNA binding domain, but spans also to two transcriptional activation domains (TAD; Fig. S4; Gubler *et al*., 1999). However, comparison of amino-acid deduced sequences of BdDOF24 protein with BPBF-HvDOF24 and other cereal PBFs shows that similarities among their amino-acid sequences are scarcely detectable outside of the DOF domain (Fig. S1). BdDOF24 and rice RPBF-OsDOF10 (OsDOF3) deduced protein sequences lack an asparagine-rich (N) stretch at the C-terminus that is conserved in barley BPBF-HvDOF24, wheat WPBF and maize MBPF (Fig. S5); this motif has been associated with specific transcriptional functions of PBFs proteins (Mena *et al*., 1998). Interestingly, transient expression of RPBF-OsDOF10 (OsDOF3) in de-embrionated rice seeds prolongs OsGAMYB activation function on the *RAmy1A* promoter expression (Washio, 2003). If the absence or presence on the C-terminal of the asparagine-rich motif and a polar region enriched in acidic residues (D, E) could be an explanation for the different PBF responses, is something to be elucidated.

Results presented in this work indicate that BdDOF24 acts as a repressor or an activator of the *BdCathB* gene expression during *B. distachyon* post-germination, this dual function as regulator might depend on its interaction with other TFs, such as BdGAMYB. In nature, one can found several examples of transcription factors acting as repressors or activators of the expression of a specific target gene depending on their interactions with other TFs (Ma *et al.*, 2005). In mammals, the

Myz-1 transcription factor represses or activates cell cycle progression genes such as $p21^{Cip1}$, depending on the interaction with repressors or activators (Moroy *et al*., 2011). In *Arabidopsis thaliana*, while TGA2 inhibits *PR-1* (*pathogenesis-related 1*) gene expression by forming homo-oligomers, activates its expression by heterodimerizing with NPR1 (non-expressor of pathogenesis-related gene 1; Boyle *et al*., 2009). It has also been described opposite gene regulatory functions for DOF TFs, such as the barley BPBF-HvDOF24 and HvDOF19 that are transcriptional activators of the *Hor2* gene promoter during seed maturation, but they are repressors of several genes encoding hydrolases during post-germination (Mena *et al*., 1998, 2002; Moreno-Risueño *et al*., 2007).

In summary, *BdCathB*, *BdDOF24* and *BdGAMYB* gene expression profiles, protein-protein interaction assays and trans-activation experiments suggest that BdDOF24 and BdGAMYB TFs interact to activate *BdCathB* gene expression upon Brachypodium seed germination when GA/ABA ratio is high in order to facilitate hydrolysis of SSPs needed for the growing embryo. The differences found related to gene regulatory functions for BdDOF24 and BPBF-HvDOF24 between *B. distachyon* and barley could represent a genetic basis for the developmental and morphological differences between wild and cultivated species (Hands *et al*., 2012).

Supplementary data

Supplementary Fig. S1. Sequence analysis and comparison of PBF proteins of barley (BPBF-HvDOF24), wheat (WPBF), maize (MPBF), rice (RPBF) and Brachypodium (BdDOF24).

Supplementary Fig. S2. Phylogenetic tree and schematic distribution of conserved amino-acid motives among the deduced protein sequences encoded by the GAMYB genes of *Hordeum vulgare*, *Triticum monococum*, *Brachypodium distachyon*, *Oryza sativa* and *Zea mays*.

Supplementary Fig. S3. Germination time course of *Brachypodium distachyon* seeds in the presence of different concentrations of abscisic acid (ABA) and paclobutrazol (PAC).

Supplementary Fig. S4. Comparison of HvGAMYB and BdGAMYB proteins.

Supplementary Fig. S5. Polarity profiles of PBF proteins from barley (A), wheat (B), maize (C), Brachypodium (D) and rice (E) based on Zimmerman scale (Zimmerman *et al.,* 1968; Gasteiger *et al.,* 2005).

Supplementary Table S1. Oligonucleotide sequences of primers used for RT-qPCR analyses, amplicon length and PCR efficiency.

Supplementary Table S2. List of primers used for cloning.

Supplementary Table S3. Sequences of conserved amino-acid motives (MEME; Bailey *et al.,* 2006) of the Catehsine B-like proteins from *Hordeum vulgare*, *Triticum aestivum, Brachypodium distachyon, Oryza sativa* and *Zea mays* in Fig 1A.

Supplementary Table S4. Sequences of conserved amino-acid motives (MEME; Bailey *et al.,* 2006) of the PBF proteins from *Brachypodium* distachyon, *Oryza sativa, Triticum aestivum, Hordeum vulgare* and *Zea mays* in Fig S1

Supplementary Table S5. Sequences of conserved amino-acid motives (MEME; Bailey *et al.,* 2006) of the GAMYB proteins from *Hordeum vulgare, Triticum monococum, Brachypodium distachyon, Oryza sativa* and *Zea mays* in Fig S2.

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TABLES

Table 1. Major characteristics of Cathepsine B-like proteins from Poaceae species: HvCathB (barley), TaCathB (wheat), BdCathB, BdCathB* (Brachypodium), OsCathB (rice) and ZmCathB (maize).

Protein name	Locus	Protein size		pI	Signal peptide position	SL prediction
		aa	Kda			
HvCathB	*AJ310426*	344	37.2	6.04	Cleavage site 20-21	Secretory pathway (P=0.95)
TaCathB	*X66012*	346	37.5	6.04	Cleavage site 22-23	Secretory pathway (P=0.98)
BdCathB	*Bradi1g09730*	350	38.1	5.75	Cleavage site 22-23	Secretory pathway (P=0.96)
BdCathB*	*Bradi1g09737*	351	38.6	6.84	Cleavage site 27-28	Secretory pathway (P=0.95)
OsCathB	*Os05g24550*	358	39.6	6.40	Cleavage site 22-23	Secretory pathway (P=0.64)
ZmCathB	*GRMZM2G108849*	347	38.1	5.66	Cleavage site 22-23	Secretory pathway (P=0.48)

FIGURE LEGENDS

Fig. 1. Phylogenetic analysis of Cathepsin B-like proteins from Poaceae family and global expression analysis of *Brachypodium distachyon BdCathB* genes by RT- qPCR.

(A) Phylogenetic tree and schematic distribution of conserved amino-acid motives among the deduced protein sequences of the *Cathepsin B-like* genes from *Hordeum vulgare, Triticum aestivum*, *Brachypodium distachyon*, *Oryza sativa* and *Zea mays*. Bootstraping values are indicated as percentages (when >80%) in the branches. The scale bar corresponds to 0.02 estimated amino-acids substitution per site.

(B) Transcripts analysis by RT-qPCR of *BdCathB* and *BdCathB** in different organs: young leaves (YL, from 2 week old plants), adult leaves (AL, from 6 week old plants), roots (R, from 2 week old plants) and spikes before pollination (S).

(C) Expression analyses by RT-qPCR of *BdCathB* and *BdCathB** throughout seed development at 0, 5, 10, 15, 30 and 45 days after pollination (dap).

Asterisk indicates a putative pseudogene with expression levels in the detection limit (*BdCathB**). Data are normalized to the *BdGAPDH* gene and are means ± standard error (SE) of three independent experiments.

Fig.2. Expression analysis of *Brachypodium distachyon BdCathB* genes and Cathepsin B-like proteolytic activity in de-embryonated seeds during germination: 0, 12, 24, 36, 48, 72, 96 hours after imbibition (hai).

(A) *BdCathB* gene expression analysis by RT-qPCR in de-embryonated seeds containing the aleurone during germination. Blue line indicates the germination kinetics of Brachypodium seeds with a t_{50} of 30,2±0,6h (time necessary to reach 50% of coleorhize emergence through the husk).

(B) Expression analysis of *BdCathB** gene by RT-qPCR in de-embryonated seeds during germination.

(C) Cathepsin B-like proteolytic activity in de-embryonated seeds during germination.

Data are means ± standard error SE of three independent experiments.

Fig.3. Identification of conserved *cis*- elements in *BdCathB* promoter and *BdGamyb* and *BdDof24* genes expression analysis in de-embryonated seeds during germination: 0, 12, 24, 36, 48, 72, 96 hai.

(A) Schematic representation of Cathepsin B-like gene promoters of Brachypodium and wheat (*BdCathB* and *Al2*, respectively). Positions (referred to the ATG translation start codon) and sequences of putative Pyrimidine box (grey boxes), GARE (white boxes), 5'-TATC-3' (black boxes) and TATA box (white triangles) are indicated.

(B) *BdGamyb* and *BdDof24* transcriptional abundance in de-embryonated seeds during germination: 0, 12, 24, 36, 48, 72, 96 hai. Data are normalized to the *BdGAPDH* gene and are means ± standard error (SE) of three independent experiments.

Fig. 4. *BdCathB*, *BdGamyb* and *BdDof24* expression in response to ABA and PAC treatments.

(A) Expression analysis by RT-qPCR of *BdCathB*, *BdGamyb* and *BdDof24* genes in de-embryonated seeds at 24 and 48 hours after imbibition in water (black bars), ABA 10µM (grey bars) and PAC 200µM (white bars). Data are normalized to the *BdGAPDH* gene expression and are means ± standard error (SE) of three independent experiments.

Significant differences between values are indicated as * ($p \leq 0.05$) and ** ($p \leq 0.01$).

(B) Germination time course of Brachypodium seed imbibed in water (black circles), ABA 10µM (grey circles) and PAC 200µM (white circles). Time necessary for 50% of coleorhize emergence beyond the husk, is indicated (see insets). Data are means ± SE of three independent experiments. Significant differences between values are shown as different letters ($p \leq 0.05$).

Fig.5. BdGAMYB and BdDOF24 protein interaction in yeast *Saccharomyces cerevisiae* two hybrid system (Y2HS) and in plant cell nuclei.

(A) Schematic representation of reporter and effector constructs used for the Y2HS assays: *Gal1 UAS* promoter transcriptionally fused to the *LacZ* reporter gene; Gal4BD (Gal4 DNA-binding domain); Gal4AD (Gal4-activation domain);

BdDOF24 and BdGAMYB, full-length ORFs under the control of the constitutive promoter *P35S*.

(B) Quantification of β-galactosidase activity in liquid assays to test the interaction between BdDOF24 and BdGAMYB TFs. Data are means ± SE of three independent experiments. Significant differences between values are indicated as * ($p \leq 0.05$) and ** ($p \leq 0.01$).

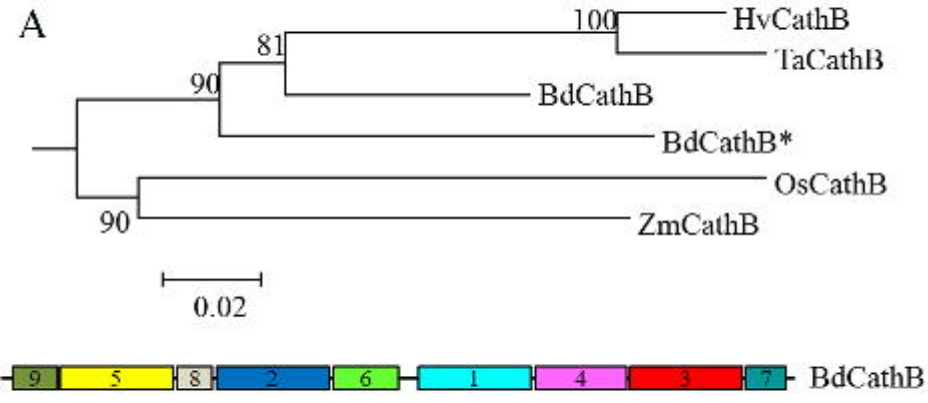
(C) Bimolecular fluorescent complementation (BiFC). YFP is reconstructed in the nuclei of onion (*Allium cepa*) epidermal cells when are transformed with combinations of P35S-NYFP-BdDOF24 and 35S-CYFP-BdGAMYB, and vice-versa. Scale bars 50µm.

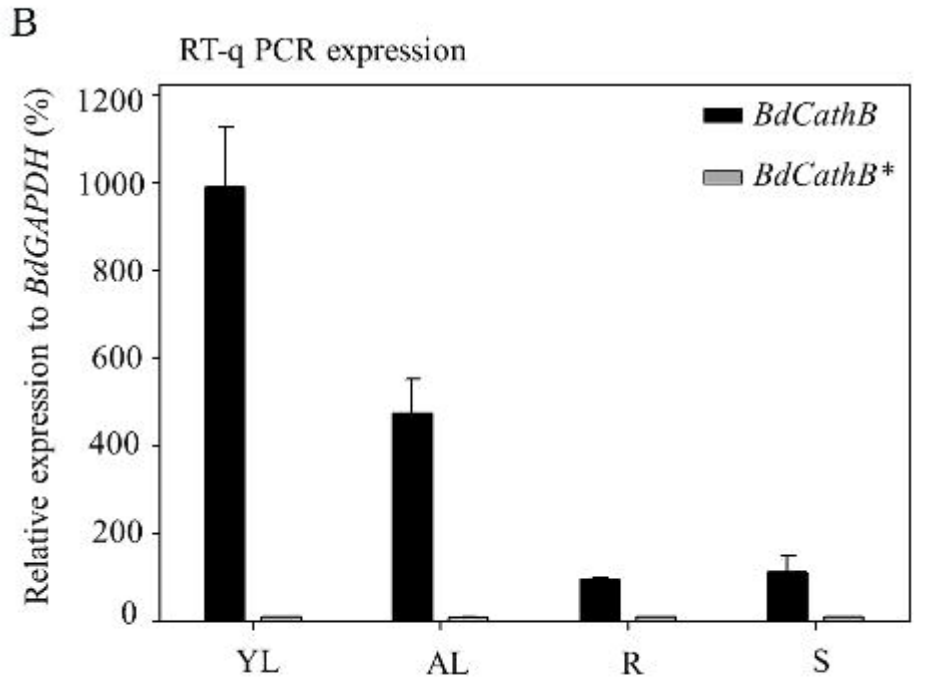
Fig.6. Transient expression assays by bombardment of barley aleurone layers.

(A) Schematic representation of reporter and effector constructs. The reporters are two deletion fragments of the *BdCathB* promoter fused to the *uidA* (GUS) reporter gene: *P463-BdCathB::GUS* (-463 bp upstream of the translation initiation codon; 1) and *P264-BdCathB::GUS* (-292 bp upstream of the translation initiation codon*;* 2). The putative *cis*-DNA binding sites of DOF (grey boxes), MYBR2R3 (white box) and MYBR1-SHAQKIF (black boxes) and the TATA box (white triangle) are indicated. As effector constructs, the *BdGamyb*, *BdDof24*, *HvGamyb* and *BPBF-HvDof24* ORFs under the control of the *CaMV35S* promoter followed by the first intron of the *AdhI* gene from maize, and the 3'-non coding region of the *nos* gene were used.

(B) Co-bombardment experiments of barley aleurone layers performed using the indicated combinations of reporter and effector plasmids at a 1:1 molar ratio in the presence of gibberellins (GA 1µM). β-gluconoridase (GUS) activity in aleurones is normalized to the xylanase activity. Values are the means ± standard error (SE) of six independent replicates and expressed as MU units/xylanase activity (Abs). Significant differences between values are indicated as * ($p \leq 0.05$).

Figure 1


A
100
81
90
HvCathB
TaCathB
BdCathB
BdCathB*
OsCathB
90
ZmCathB
0.02
9
5
8
2
6
1
4
3
7
BdCathB


B
RT-q PCR expression
Relative expression to BdGAPDH (%)
1200
1000
800
600
400
200
0
BdCathB
BdCathB*
YL
AL
R
S

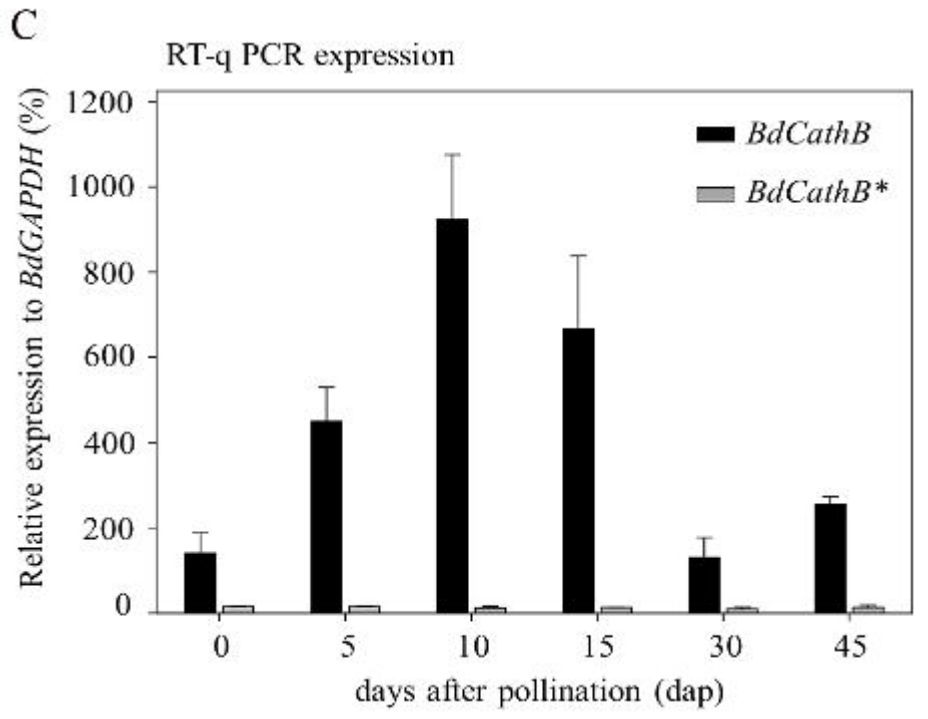

C
RT-q PCR expression
Relative expression to BdGAPDH (%)
1200
1000
800
600
400
200
0
BdCathB
BdCathB*
0
5
10
15
30
45
days after pollination (dap)

Figure 2

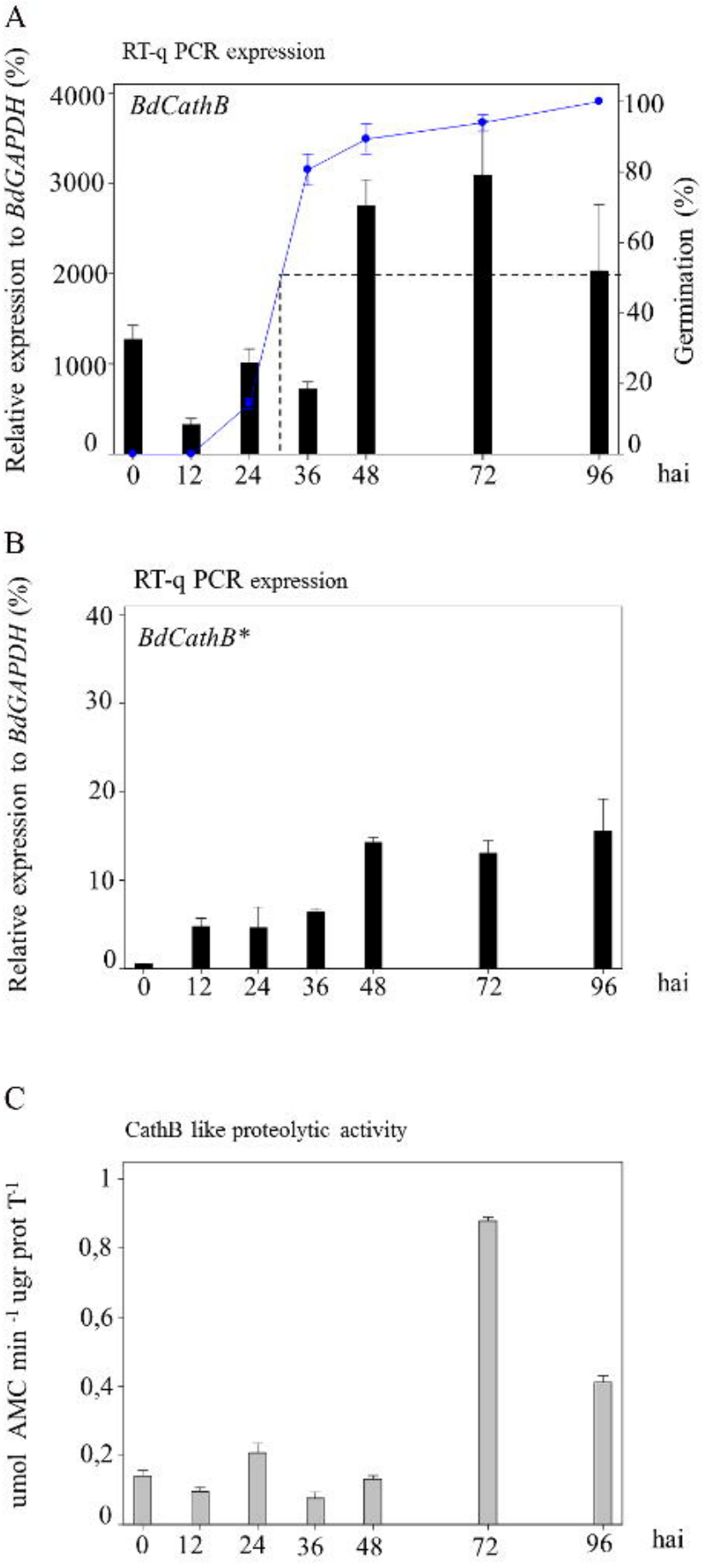
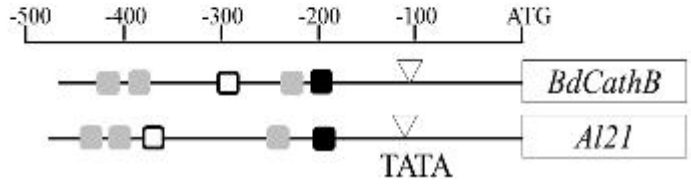

A
RT-q PCR expression
BdCathB
Relative expression to BdGAPDH (%)
Germination (%)
4000
3000
2000
1000
0
100
80
60
40
20
0
0 12 24 36 48 72 96 hai
B
RT-q PCR expression
BdCathB*
Relative expression to BdGAPDH (%)
40
30
20
10
0
0 12 24 36 48 72 96 hai
C
CathB like proteolytic activity
umol AMC min -1 ugr prot T-1
1
0,8
0,6
0,4
0,2
0
0 12 24 36 48 72 96 hai

Figure 3

A



	P-*BdCathB*	P-*Al21*
Pyrimidine box ■	-233 AAAAG (+) -393 AAAAG (+) -414 AAAAG (+)	-235 CGAAAGC (+) -401 AAAAGG (+) -434 TCTTTCA (+)
GARE □	-294 CAACGACA (+)	-367 CAACGGCAAC (+)
5'-TATCCAC-3' ■	-205 TATC (+)	-199 TATC (+)

B

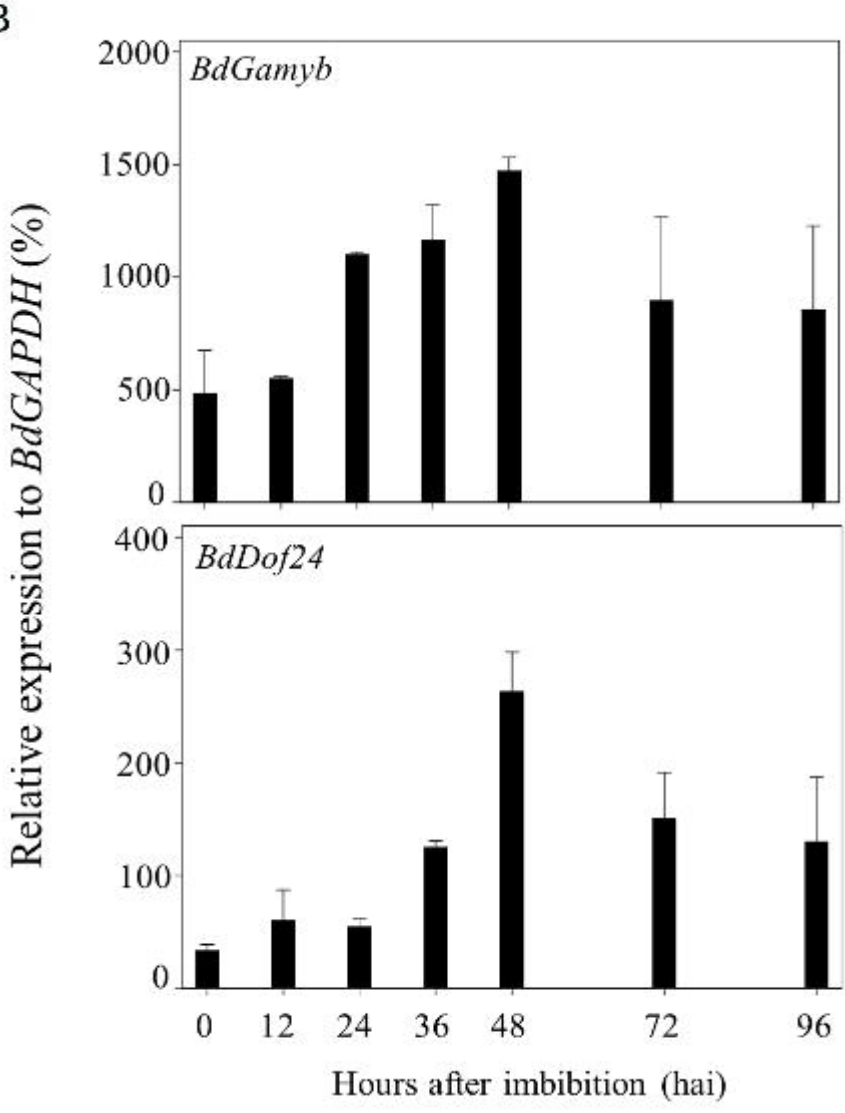
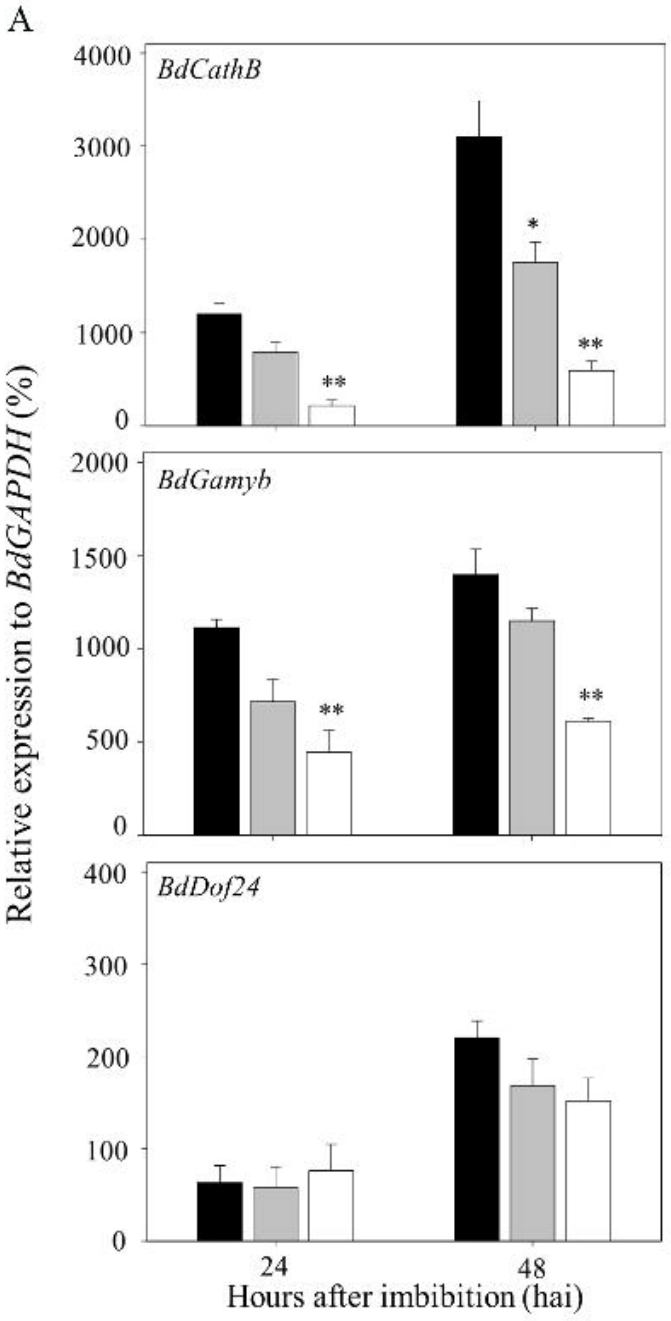


Figure 4


A
BdCathB
BdGamyb
BdDof24
Relative expression to BdGAPDH (%)
4000
3000
2000
1000
0
2000
1500
1000
500
0
400
300
200
100
0
*
**
**
**
**
24
48
Hours after imbibition (hai)

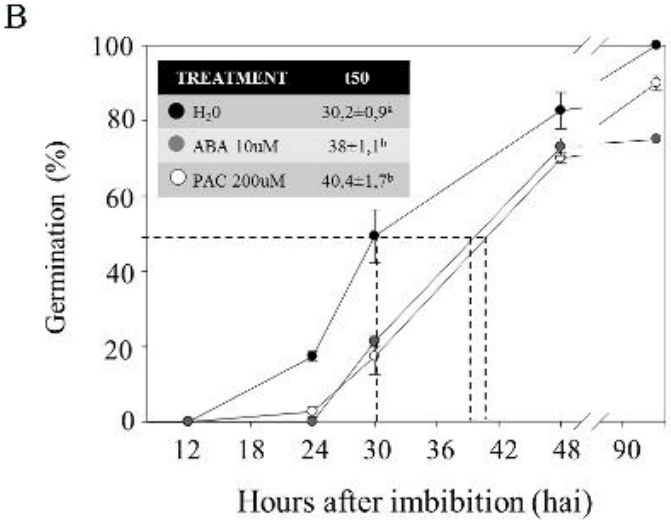

B
TREATMENT
t50
H2O
30,2±0,9a
ABA 10uM
38±1,1b
PAC 200uM
40,4±1,7b
Germination (%)
100
80
60
40
20
0
12
18
24
30
36
42
48
90
Hours after imbibition (hai)

Figure 5

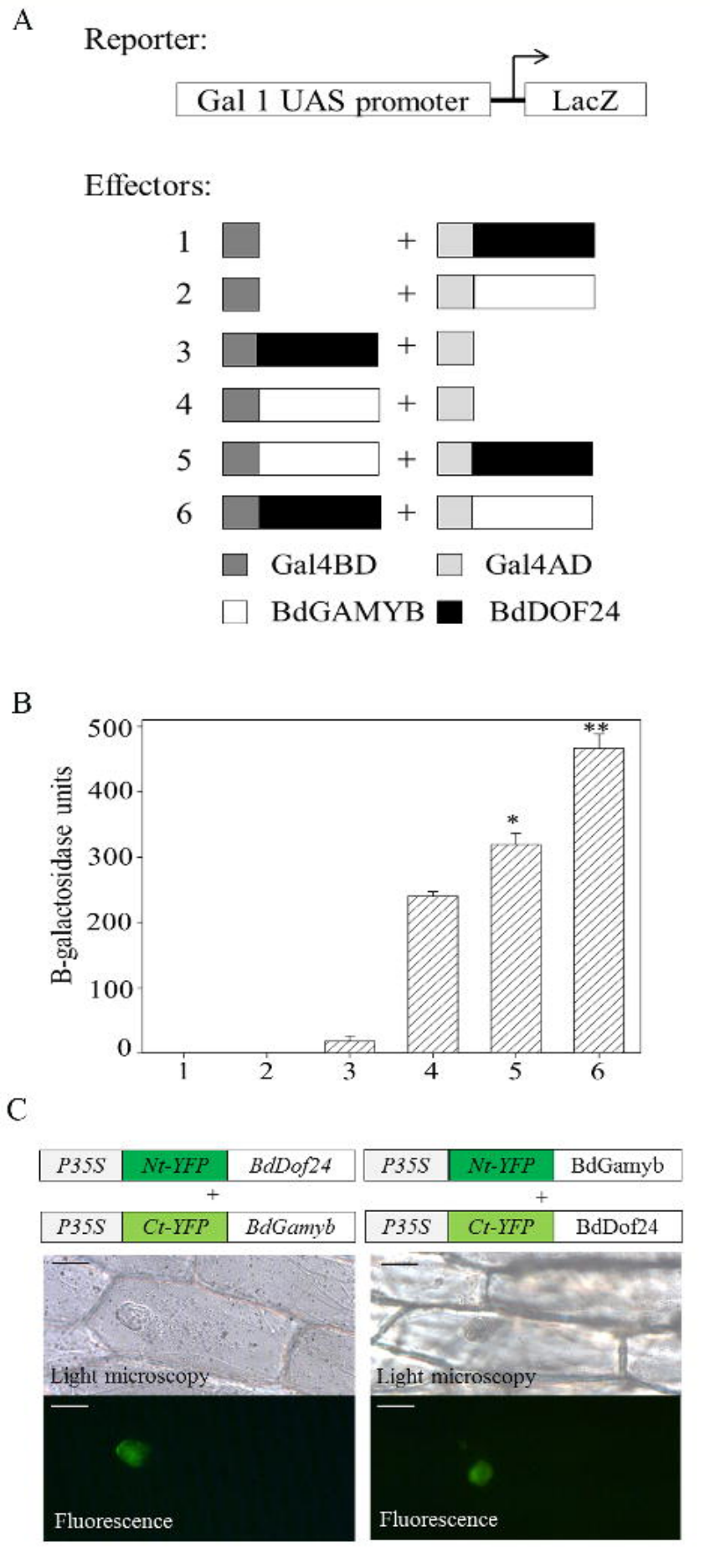
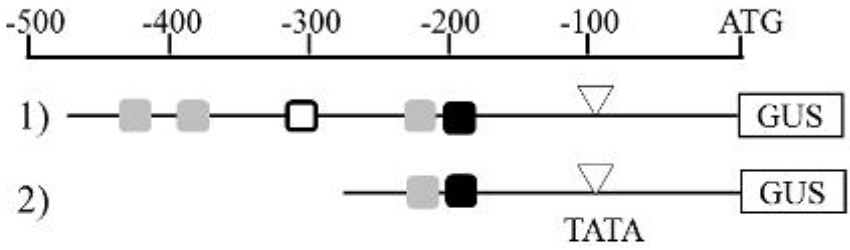

A
Reporter:
Gal 1 UAS promoter
LacZ
Effectors:
1
2
3
4
5
6
+
Gal4BD
Gal4AD
BdGAMYB
BdDOF24
B
B-galactosidase units
500
400
300
200
100
0
1
2
3
4
5
6
*
**
C
P35S
Nt-YFP
BdDof24
+
P35S
Ct-YFP
BdGamyb
P35S
Nt-YFP
BdGamyb
+
P35S
Ct-YFP
BdDof24
Light microscopy
Fluorescence
Light microscopy
Fluorescence

Figure 6

A

Reporters:



Effectors:

P35S	*I-Adhl*	*BdGamyb*	*t-nos*
P35S	*I-Adhl*	*BdDof24*	*t-nos*
P35S	*I-Adhl*	*HvGamyb*	*t-nos*
P35S	*I-Adhl*	*BPBF-HvDof24*	*t-nos*

B