Characterization of a pollen-specific and desiccation-associated AP2/ERF type transcription factor gene from castor bean (*Ricinus communis L.*)

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Abstract

DREB transcription factors (TF) belong to the superfamily of AP2/ERF and their involvement in protein-protein interactions and DNA binding has been proposed. AP2/ERF proteins have important functions in the transcriptional regulation of a variety of biological processes related to growth and development, as well as various responses to environmental stimuli, regulating expression of plant biotic and abiotic stress-responsive genes. In this study an AP2/ERF TF gene (named *RcDREB1*) was isolated from castor bean (*Ricinus communis L.*) and its expression was analyzed in developing seeds, leaves, ovules, stems and petals of plants cultivated under field conditions. Transcripts were only observed in pollen grains, peaking during anthesis. The *RcDREB1* deduced amino acid sequence was compared to other AP2/ERF TF proteins and presented 38-78% identity. Phylogenetic analysis classified it as a member of the CBF/DREB subfamily, rooting with the subgroup A-5. The *RcDREB1* promoter was fused to the gus reporter gene and used to transform tobacco. Transgenic plants were exposed to various abiotic stress treatments (low and high temperatures, drought, salinity and exogenous ABA) and no detectable GUS expression was observed, suggesting that the *RcDREB1* promoter is not active under tested conditions. *In silico* analyses revealed the presence of three copies of the regulatory late pollen-specific element (AGAAA) in the *RcDREB1* 5´-region. Interestingly, GUS expression was only observed in pollen grains, starting when the flower opened and initiating the senescence process; at this point, desiccated mature pollen grains are released from anthers. In addition, dehydrated developing pollen grains also expressed the *gus* gene. This is the first study on a *DREB* gene presenting pollen-specific expression.

Introduction

DREB transcription factors (TF) belong to the superfamily of AP2/ERF transcription factors. Each DREB/CBF protein contains a highly conserved APETALA2/Ethylene Responsive Factor (AP2/ERF) domain, which consists of 60 to 70 amino acids. The involvement of this domain in protein-protein interactions and DNA binding has been proposed.[1] In ERF the domain specifically binds to the 11bp GCC box of the ethylene response element (ERE), a promoter element essential for ethylene responsiveness. The AP2/ERF proteins have important functions in the transcriptional regulation of a variety of biological processes related to growth and development, as well as various responses to environmental stimuli, regulating the expression of plant biotic and abiotic stress-responsive genes.[2,3] AP2/ERF genes constitute a large superfamily that has been divided into subfamilies (named AP2, RAV, CBF/DREB and ERF) based on their sequence similarities and numbers of AP2/ERF DNA binding domains.[4,5] AP2 subfamily proteins contain two repeated AP2/ERF domains and play a role in the regulation of plant developmental processes.[6-8] RAV subfamily proteins contain a single AP2/ERF domain and a B3 domain, which is a DNA-binding domain conserved in other plant-specific transcription factors. The ERF subfamily genes are mainly involved in response to biotic stresses, such as pathogenesis, by recognizing the *cis*-acting element AGCCGCC, known as the GCC box.[9]

The CBF/DREB subfamily proteins are divided into six subgroups (A-1 to A-6).[10-12] Expression of the *AtDREB1A/AtCBF3* (A-1) genes is induced by low temperature stress, but not by drought or high salt stresses, whereas *AtDREB2A* (A-2) genes are induced by drought and high salt, but not by low temperature.[13] Other *DREB* genes such as *AtTINY2* (A-4), *GhDBP1* (A-5), *GmDREB2* (A-5), and *ZmDBF1* (A-6) have also been characterized as stress-inducible proteins, since they are ABA-responsive.[14-17]

CBF/DREB subfamily transcription factors have been identified and characterized in various plant species as being associated with the response to abiotic and biotic stresses and with control of flowering.[6] In general, the CBF/DREB subfamily genes have been well studied in *Arabidopsis* and only a few members have been characterized in tropical plants, in which most of their functions remain to be determined.[11] In addition, in spite of the numerous physiological investigations into how DREB transcription factors regulate target genes, the transcriptional regulation of *DREB* genes itself has not been fully characterized.[18,19] Moreover, little is known about their spatial expression during plant organs' development under environmental stress. The objectives of this study were to isolate and characterize the expression of a CBF/DREB subfamily gene from castor bean (*Ricinus communis L.*). The promoter was fused to a report gene in order to study gene expression under different abiotic stresses and during the development of some organs. We found that the *AP2/ERF* gene (named *RcDREB1*) seems to be specifically expressed in mature pollen grains during the desiccation process.

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Contributions: TMC carried out the practical work and wrote the paper; ATM carried out the cloning of promoter and plant transformation; FJLA the research group leader, analyzed the data and finalized the manuscript.

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Materials and Methods

Cloning the *RcDREB1* promoter and coding sequence

Total genomic DNA was isolated from leaves using the DNeasy Plant Mini Kit (Qiagen Valencia, CA, USA). Degenerated primers DREB392 (5´-GCGACGTCRTGGRCACGAGCG-GC-3´) and DREB297 (5´-TGGGTGGSG-GAAATTAGAGARCC-3´) (R = G or A; S = G or C) based on conserved regions of available plant *DREB* genes were used to amplify an internal sequence from the *RcDREB* gene. Polymerase Chain Reactions (PCRs) were carried out in a thermocycler (MyCycler Thermal Cycler, BioRad) in 50 µL of solution containing 50 ng of DNA, 60 mM Tris-SO4 (pH 8.9), 18 mM

$(NH_4)2SO_4$, 2mM $MgSO_4$, 250 nM of each dNTP, 200 nM of each primer and 5 U of Platinum Taq DNA Polymerase High Fidelity (Invitrogen). The mixture was treated at 95ºC (5 min) and subjected to 35 cycles of amplification (95ºC for 1 min, 55ºC for 1 min, 68ºC for 1 min) with a final elongation cycle of 5 min at 68ºC. A 845 bp fragment was cloned into the pGEMT-Easy vector (Promega) and sequenced.

The 5´ flanking region from the *RcDREB1* gene was obtained by Thermal Asymmetric Interlaced PCR (TAIL-PCR) according to Liu with one essential modification:[20] the use of 10 mer random primers instead of degenerate 16 mer as the short primers. On the basis of the 1025 bp fragment DNA sequence of an *RcDREB1* gene, a total of two gene-specific primers in nested positions were designed (RCNESTEDR: 5´- AACAGGAGCGAATAAGAACC -3´; RCR2: 5´- ATCCTTGACCGCTTGTTC -3´). Twenty arbitrary 10 mer primers were chosen from the 10 mer primer sets obtained from Operon Biotechnologies GmbH (www.operon.com). Three rounds of TAIL-PCR were carried out on an MJ Researcher thermocycler (PTC-100) using the diluted product of the previous PCR as template for the next, and consecutively employing a common arbitrary primer and nested gene-specific primers. The primary TAIL-PCR was carried out in 25 μL of reaction mixture containing 20 ng of genomic DNA, 10 μM of the 10 mer OPE primer, 600 mM Tris-SO_4 (pH 8.9), 18 mM $(NH_4)2SO_4$, 50 mM of the $MgSO_4$, 10 mM of dNTP mixture, 5 U/μL of the Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA, USA). The final product of the primary reaction was diluted 1:50 in sterile water and secondary 1:100, which were used as templates in the secondary and tertiary reactions, respectively. In addition, each reaction provided a specific program for fragment amplification.

The secondary TAIL-PCR was carried out with the gene-specific primer RCNESTEDR in combination with the same arbitrary primer as used in the primary PCR. The reaction solution was the same as for the primary PCR, except that 1 μL of a 50-fold dilution of the primary PCR product was used as template. For the tertiary TAIL-PCR, the gene-specific primer RCR2 was used with the same arbitrary primer. The reaction solution for the tertiary PCR was the same as for the primary PCR except that 1 μL of a 50-fold dilution of the secondary PCR product was used as template, and the concentration of the arbitrary primer was 500 nM instead of 2 μM. Reactions were carried out as described by Liu,[20] except that the annealing temperature for the low-stringency cycle was set to 29°C, instead of 44°C. The PCR products were separated onto a 1% agarose gel, stained with ethidium bromide and visualized with UV light. Discrete products from the tertiary PCR were purified using the Kit Wizard SV Gel and PCR Clean-Up System (Promega) and the fragments cloned into the pGEMT-Easy vector (Promega) and sequenced. To obtain a full-length *RcDREB1* cDNA sequence, 5´- and 3´-rapid amplifications of cDNA ends (RACE)-PCR were carried out. Gene-specific primers were designed from the internal sequenced fragment and the cDNA end was amplified by using the 5´- and 3´-RACE System (Invitrogen), using the reverse specific nested primer DREBRcRACER (5´-AAGCCTTGCAGACGGGCCTC-3´) in combination with the GeneRacer 5´-Primer (Invitrogen), and the forward specific primer DREBRcRACEF (5´-CGGCCGCTCGAGCCTATGAC-3´) in combination with GeneRacer Oligo dT (Invitrogen). Nested PCRs were carried out as described above. PCR products were cloned into the pGEMT-Easy vector (Invitrogen) and sequenced. All fragments and vector were sequenced by Macrogen Inc (Korea).

Sequence and phylogenetic analyses

All similarity searches were executed locally using the BlastN, BlastX, or BlastP tools at the NCBI web site. Motif detection was performed with MEME version 4.7.0.[21] *RcDREB1* promoter was screened for *cis*-elements using the web-based PlantCARE and PLACE/Signal Scan platforms.[22,23] The relationship between the *RcDREB1* and AP2/ERF transcription factor genes was determined by aligning it with sequences available at GenBank (www.ncbi.nih.nlm.gov) and the TIGR Gene Indices. Only complete coding sequences were used for analysis. The alignment was performed using CLUSTAL W.[24] Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5.[25] Phylogenetic trees were constructed using the Neighbour-joining algorithm Bootstrap values were computed using 1000 replicates to evaluate support for the groupings.[26]

Protein sequence was *in silico* analyzed using NetStart 1.0, TargetP 1.1 and NLStradamus.[27-29]

Real time-polymerase chain reaction expression analysis

Developing seeds (5, 10, 15, and 20 d after pollination), leaves, ovules, pollen grains (from anthers of male flowers during anthesis, 1 and 2 days before anthesis and 2 days after anthesis), stems, and petals were removed from plants cultivated under field conditions and used for total RNA extraction as previously described by Abreu and Aragão.[30] Anthers were partially dehisced at the time of pollen harvest on the day of anthesis. They were completely dehisced and much reduced in size by the day after anthesis. Total RNA extraction, cDNA synthesis and RT-PCR were carried out according to Abreu and Aragão.[30] The number of amplification cycles was previously optimized in order to stop the reaction at the exponential stage, ensuring that amplification was semi-quantitative. Primers RCDREBF (5´-AGAGTCGATGCTCTTGAAACCGCT-3´) and RCDREBR (5´-TCCGAATCTTCCGGGTCGGGT-3´) were used to amplify a 227 bp fragment from the *RcDREB1*. As an internal control, primers EF1F (5´-TGTTGCTGTTAAGGATTTGAAGCG-3´) and EF1R (5´-AACAGTTTGACGCATGTCCCTAAC-3´) were utilized to amplify 358 bp within the castor bean housekeeping gene, RcEFa, elongation factor EF-1a. Fragments of the *RcDREB1* amplified from pollen were cloned into the pGEMT-Easy and sequenced. Experiments were repeated three times and 42 clones of the *RcDREB1* were sequenced.

Abiotic treatments

Transgenic and non-transgenic tobacco plants (from three lines) were subjected to various abiotic stresses according to Gutha and Reddy,[31] with modifications. Tests were performed on soil, under hydroponic and *in vitro* conditions. Tissues (leaf, root and stem) were collected and tested for GUS expression.

For experiments carried out under soil conditions, 40-day-old plants (n=12) were submitted to drought, high salinity, and low and high temperatures. For water deficit stress treatment, watering was withheld for 15 days and samples collected daily. For high-salinity treatments, plants were irrigated with 100 mL of solution containing 600 mM NaCl for seven days and samples collected daily. For low-temperature treatment, the plants were incubated at 10ºC, 75% RH, 16h-photoperiod for two days and samples collected daily. For high temperature treatment, plants were incubated at 45ºC, 15% RH, 16h-photoperiod during seven days and samples were collected at 0, 2, 6, and 24 h on the first day, followed by sample collections every 24 h for six days.

For experiments carried out under hydroponic conditions, 8-day-old plants (n=6) were cultivated at 16 h photoperiod and submitted to salinity and ABA treatments. For salinity stress treatment, plants were cultivated in contact with aqueous solution containing 250 mM NaCl for five days and samples were collected every hour for the first 6h and every 24h for five days. For hormone treatment, plants were cultivated in presence of 200 μM ABA (abscisic acid) for seven days and samples were collected every hour for the first 6 h and every 24 h for five days.

For experiments carried out under in vitro conditions, 7-day-old seedlings (n=10) were cultured in Petri dishes (9 cm diameter) containing MS medium supplemented with 100 μM ABA, or 10% PEG, or 200 mM mannitol, or 200 mM NaCl for 15 days.[32] Samples were collected every seven days.

GUS expression regulated by the *RcDREB1* promoter

The 859-bp 5´-region of the *RcDREB1* gene was cloned upstream of the *uidA* coding region (*gus*) to create a promoter-gus fusion. Primer pair RcDPNcoR (5´-TCCATGGATGGAGACAAATAATCACTC-3´) and RcDPKpn (5´-CGGTACCCCCTTAGGACTATACACCTC-3´), including the sites for *Nco*I and *Kpn*I (underlined) were used to amplify the 5´ flanking region from the *RcDREB1* by PCR. Reaction was carried out as previously described using 5 U of Platinum Taq DNA Polymerase High Fidelity (Invitrogen). The amplified fragment was cloned into the pGEMT-Easy vector (Promega) and re-sequenced. The 5´ region from the *RcDREB1* gene was excised from *pGEMT-Easy* with *Bam*HI/*Nco*I and inserted into the vector pCambia 3201 (CAMBIA, Camberra Australia), replacing the 35S CaMV promoter, to generate the plasmid pRcDREBGUS.

The plasmid vector *pRcDREBGUS* was transferred to *Agrobacterium tumefaciens* strain LBA4404, which was used to transform tobacco as previously described.[33] Transgenic plants were selected using the herbicide glufosinate ammonium and tested for presence of the phosphinothricin acetyltransferase using the Trait LL Test Kit, (Strategic Diagnostic Inc.). Regenerated transgenic plantlets were acclimatized and allowed to set seeds.

GUS histochemical assay

The gene expression in all vegetative and reproductive organs, at different developmental stages, was analyzed by a histochemical assay of the *gus* gene according to Jefferson.[34] Pollen grains were removed from anthers at eight stages (a-h) corresponding to the stages 1 (=a), 2 (=b), 6 (=c), 8 (=d), 11 (=e) and 12 (=f), as previously described.[35] Stages g and h correspond to flower senescence events.

Results

Cloning of a *DREB* gene from *R. communis*

Using PCR with degenerated primers, TAIL-PCR and 5´- and 3´-(RACE)-PCR it was possible to obtain a 1781 bp sequence from *R. communis* genome. The sequence was entered in the NCBI GenBank database with the accession number JQ361741. The cDNA contains one open reading frame of 588 bp encoding 195 amino acids with predicted molecular mass of 21.7 kDa (pI 7.08), 1,025 pb corresponding to the 5´ region and 169 bp corresponding to the 3´ region. Translation start was predicted using the NetStart 1.0.[27] The deduced amino acid sequence was used to compare the amino acid composition of the *RcDREB1* polypeptide with those of other plant AP2/ERF TFs, and presented 38-78% identity for overall amino acid sequence. *RcDREB1* was predicted to encode a single AP2/ERF domain (in the position 26-82) with 94-99% identity. No B3 domain was found. The nucleotide sequence of the intronless putative *RcDREB1* gene-coding region was compared with sequences of AP2/ERF genes from Viridiplantae available in the GenBank and TIGR databases. The amino acid residues E_{16}, W_{27}, L_{28}, and G_{29} from AP2/ERF *RcBREB1* domain were conserved when compared with all 220 AP2/ERF proteins identified in soybean and Arabidopsis thaliana. In addition, the residues R_8 and G_{29} were completely conserved when compared with all 139 AP2/ERF proteins identified in rice (*Oryza sativa*). The TargetP 1.1 algorithm predicted no chloroplast transit or mitochondrial targeting signal peptides in the N-terminal region of the *RcDREB1*.[28] The NLStradamus predicted a putative nuclear localization signal (NLS) domain.[29]

Conserved motifs outside of the AP2/ERF *RcDREB1* domain were examined using MEME. Some conserved motifs identified in the *Arabidopsis* and *Glycine max* ERF family proteins were also observed in the deduced amino acid sequences of *RcDREB1*. The ERF-associated amphiphilic repression (EAR) motif [D(L/M)NxxP] was found in the C-terminal region. However, the LxLxL type of EAR motif is not present. ERF protein signature (FKGIRMRKWGKW), the transcription initiation factor TFIID motifs (SSSNNNNNNSN and SSSSNNNNNNS) and a potential PEST motif (KVPDPEDSDVEWER) were also found.

Sequence analysis of the 5´-region of the *RcDREB1* revealed that no typical TATA boxes (TATAWAW; W=A/T) were found. However, five TATA boxes were detected that had previously been detected in the 5' upstream region of *Pisum sativum* glutamine synthetase gene and confirmed as a TATA element by *in vivo* analysis (Figure 1).[36] In addition, the erd1 (ACGTG; early response to dehydration element)[37] was found at -202 position. There were five CAAT boxes. To identify regulatory elements, the 5´ upstream sequence was analyzed by using PlantCARE database and PLACE database. Motifs AGAAA and GTGA, two *cis*-acting regulatory elements that are known to be involved in pollen/anther-specific expression, were detect-

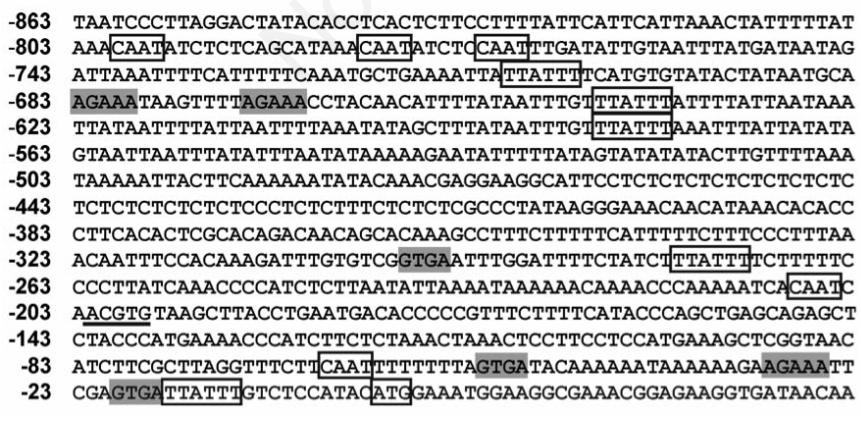

Figure 1. Nucleotide sequence of *RcDREB1* promoter. Numbering is from the first base of translation start site (+1). Putative TATA, CAAT boxes and the translation start point ATG are shown in rectangular boxes. Pollen-specific *cis*-elements (AGAAA and GTGA) (gray background) and erd1 (early response to dehydration element) (underlined) are denoted.

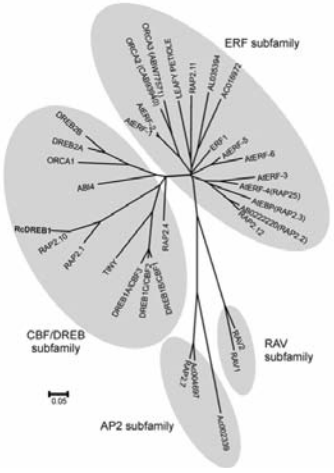

Figure 2. Phylogenetic analysis of *RcDREB1* with other AP2/ERF proteins. The *RcDREB1* grouped with the CBF/DREB subfamily. The amino acid sequences of the AP2/ERF domains were aligned by Clustal W and the phylogenetic tree was constructed using MEGA 5 with Neighbor-Joining method. The tree includes AP2 domains available in GenBank and TIGR databases. Cut-off bootstrap values >50% were considered for the consensus tree.

ed (Figure 1). There were three copies of AGAAA motifs and three copies of GTGA motifs. So many copies of AGAAA and GTGA motifs suggested that this promoter might be a specific pollen/anther promoter.

Phylogenetic analysis

To classify and to analyze the phylogenetic relationships, multiple alignment analysis of the amino acid sequences of the AP2/ERF domain in the *RcDREB1* was carried out. Based on alignment, an NJ phylogenetic tree was generated, which divided AP2/ERF proteins into clusters in agreement with the subfamily (Figure 2). This phylogenetic tree suggested that the *RcDREB1* belongs to the CBF/DREB subfamily. The second phylogenic analysis was carried out with CBF/DREB subfamily protein sequences (Figure 3). The phylogenetic tree divided the proteins into six subgroups, designated A-1 to A-6, in accordance with the classification described by Sakuma.[11] The *RcDREB1* grouped with the subgroup A-5.

Levels of *RcDREB1* transcripts in organs of R. communis

RT-PCR expression analyses were carried out to detect endogenous *RcDREB1* transcripts in different *R. communis* organs and developing seeds. The results revealed the absence of *RcDREB1* transcripts developing seeds, leaves, ovules, stems and petals of plants cultivated under field conditions (data not shown). *RcDREB1* transcripts were observed pollen grains, peaking during anthesis (Figure 4). Out of 42 clones sequenced revealed that only one sequence was present, corresponding to the *RcDREB1* transcript. However, cannot definitively conclude that only the *RcDREB1* is being expressed, because other *DREB* genes are present in the castor bean genome and these genes have a high degree of sequence similarity.

GUS expression under control of the *RcDREB1* promoter

To determine whether the *RcDREB1* promoter would be functional under stress conditions, the 3´-region of the gene was fused to the *gus* reporter gene coding sequencing. Fifteen transgenic tobacco lines were achieved, transformed with the *RcDREB1* promoter-gus fusion, and three lines were used for further experiments.

Plants cultivated in the soil, under hydroponic and in vitro conditions were exposed to various abiotic stress treatments (low and high temperatures, drought, salinity and exogenous ABA). Expression of the GUS-reporter gene was examined in different tissues of the transgenic lines. Random samples of leaves, stems and roots of the T_1 plants did not show any visible histochemical *gus* staining, suggesting that the *RcDREB1* promoter is not active under tested conditions.

Transgenic plants were allowed to develop under greenhouse conditions with normal irrigation regime and analyzed for GUS expression. No expression could be detected in leaves, stems, roots, petals, sepals and ovaries. Later, GUS expression was investigated during microspore/pollen development. Interestingly, it was possible to detect intense expression levels of the gus gene driven by the *RcDREB1* promoter (Figure 5) at some stages of pollen development. The frequency of blue-staining pollen was higher at stages f and g (99 and 97% respectively), when the flowers are open and anthers dehisced, releasing pollen grains (Figure 5f, g). At stages c, d and e it was possible to visualize a frequency of 6, 11 and 19% (respectively) of pollen grains expressing the *gus* gene (Figure 5c-e). At these stages (c to e), *gus* expression was observed only in desiccated pollen grains. It is in agreement with the expression peaking during anthesis of castor bean anthers. At stage h flowers initiated senescence and the GUS was observed in about 50% of the pollens presenting dehydration. No *gus* expression was detected in pollen of non-transgenic tobacco plants. In all cases, the positive control line transformed with the CaMV 35S promoter driving the *gus* showed intense blue staining (*data not shown*).

Discussion

In this study, the first member of the AP2/ERF transcription factor family, named *RcDREB1*, was characterized in castor bean, a perennial tropical species that presents natu-

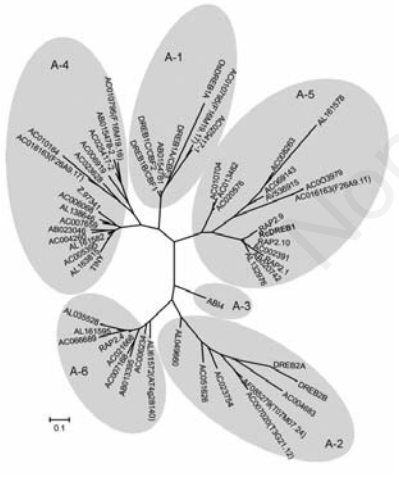

Figure 3. Phylogenetic analysis of *RcDREB1* with other DREB proteins. The *RcDREB1* grouped with the A-5 subgroup. The amino acid sequences were aligned by Clustal W and the phylogenetic tree was constructed using MEGA 5.0 and the Neighbor-Joining method. The tree includes AP2 domains available in GenBank and TIGR databases. Cut-off bootstrap values >50% were considered for the consensus tree.

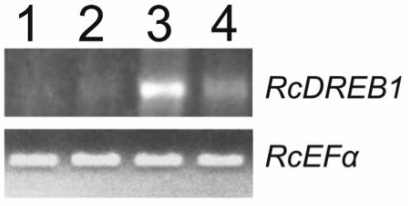

Figure 4. Expression of the *RcDREB1* gene in castor bean pollen grains from male flowers 1 and 2 days before anthesis (1, 2), during anthesis (3) and 2 days after anthesis (4). The upper bands are consistent with the expected fragment amplified from the *RcDREB1* gene and the lower band corresponds to transcripts from the RcEFα gene (elongation factor EF-1α; internal control).

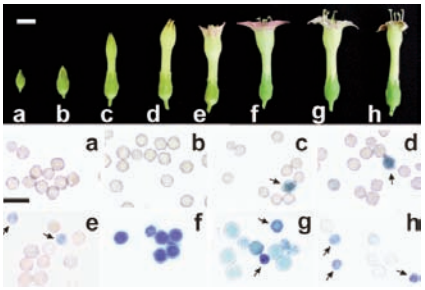

Figure 5. *RcDREB1* promoter controlling GUS expression during pollen/microspore grains development in transgenic tobacco plants. At stage a, the anthers and pistil are fully differentiated. Stage b: calyx opens slightly at top of bud; stage c: corolla tube bulges at tip of calyx; stage d: corolla is elongating and petals are green and slightly open. Stage e: corolla limb is semi open and stigma and anthers are visible. Stage f: flower open and anthers dehisced, and corolla limb is fully expanded and deep pink. Stages g and h: flower in senescent. Arrows point to dehydrated pollen grains in c to h. White bar in the upper panel corresponds to 1 cm and serves as scale for flowers. Black bar corresponds to 50 µm and serves as scale for pollen grains in a to h.

ral tolerance to drought, low fertility soils and high temperature. Plants have biochemical mechanisms to tolerate stress and respond to these conditions with an array of biochemical and physiological changes, which involve the expression of many genes to promote the ability of the plant to survive these stress conditions. A significant number of these changes was shown to be under the control of the DREB factor pathway.[38,39] These transcription factors activate/regulate the expression of a number of downstream genes that play important roles in environmental stress tolerance. Many plant species, especially those of tropical origin, have to respond and adapt to stresses at the physiological and biochemical levels, in a plastic and rapid manner. Nevertheless the isolated castor bean *DREB* gene did not presented expression in several tissues. Surprisingly, *RcDREB1* expression was only observed in pollen cells with maximum values being during anthesis.

According to Sakuma,[11] the CBF/DREB and ERF subfamilies differ by two conserved amino acid residues in the AP2/ERF domain. In AtDREB protein, a valine and glutamic acid are conserved in positions 14 and 19 respectively, while in ERF proteins alanine and aspartic acid residues are conserved at these positions. In agreement with other CBF/DREB subfamily proteins, *RcDREB1* has the V14 and E19 amino acid residues in the AP2/ERF domain, suggesting it is a member of the CBF/DREB subfamily. In addition, phylogenetic analyses revealed that the *RcDREB1* grouped in the DREB subfamily. Further phylogenetic analysis positioned *RcDREB1* in A-5 subgroup CBF/DREB of the CBF/DREB subfamily, according to the classification proposed by Sakuma.[11] Some DREB proteins from the subgroup A-5 have been characterized as stress-inducible proteins, since they are ABA-responsive.[14-17] Out of more than 140 AP2/ERF domain proteins predicted to be encoded by *Arabidopsis*, and also in *Brassica napus*, wheat, rye, and tomato, only CBFs were found to have immediately upstream (PKK/RPAGRxKFxETRHP) and downstream (DSAWR) signatures surrounding the AP2/ERF domain. The domains PK(K/R) PAGRxKFxETRHP and DSAWR were not detected in the *RcDREB1*, corroborating the suggestion that it is not a CBF protein. In addition, these signatures are not present in the *Arabidopsis* AP2/ERF protein AtDREB2a.[13,40]

The ERF-associated amphiphilic repression (EAR) motif is present in the *RcDREB1*. Zhang have shown the EAR motif sequences conserved in the C-terminal region of subgroup A-5 of the CBF/DREB subfamily proteins in soybean and *Arabidopsis*.[12]

Conserved motifs outside the AP2/ERF *RcDREB1* domain were examined using MEME. Some conserved motifs identified in the *Arabidopsis* and *Glycine max* ERF family proteins were also observed in the deduced amino acid sequences of *RcDREB1*. The ERF-associated amphiphilic repression (EAR) motif [D(L/M)NxxP] was found in the C-terminal region, but not the LxLxL type of EAR motif. ERF protein signature (FKGIRMRKWGKW), the transcription initiation factor TFIID motifs (SSSNNNNNNSN and SSSSNNNNNNS) and a potential PEST motif with 12 amino acids (KVPDPEDSDVEWER) were also found. EAR is a transcriptional regulatory motif identified in members of the ERF, C2H2, and auxin/indole-3-acetic acid families of transcriptional regulators. *Arabidopsis* EAR motif-containing proteins described in the literature are divided into two groups based on the sequence conservation pattern within the core EAR motif sites.[41] The DLNxxP motif is conserved in some members of class II ERFs, TFIIIA-type ZFPs, and ABI3/VP1 family proteins, while the LxLxL motif is conserved in AUX/IAAs and some members of the MYB and HD-Zip family proteins. ERF protein signature and the transcription initiation factor TFIID motifs were also found.

In this study, transgenic tobacco lines were generated to express the *gus* report gene under control of the *RcDREB1* promoter. Although a single ABA-responsive element (ABRE) was identified in the 5´RcDREB, the 5´*RcDREB1:gus* fusion was not functional in either in vitro or hydroponic conditions under ABA treatments. It has been shown that ABRE is necessary but not sufficient for ABA-induced gene expression. An additional element is usually required for high level ABA-induction, such as CE1 and CE3, which were not found in the 5´*RcDREB1*.[42,43] Two ABRE sequences are necessary for the expression of *Arabidopsis RD29B* in seeds and for the ABA-responsive expression of RD29B in vegetative tissue.[44]

The *RcDREB1* expression pattern is very different from the other DREB proteins from subgroup A-5. Characterization of the DREB gene from *Physcomitrella patens* (*PpDBF1*) revealed transcript accumulation under various abiotic stresses [water deficit, high salinity (NaCl), low temperature] and phytohormone treatments (ABA).[45] In addition, transgenic tobacco plants overexpressing the *PpDBF1* gained higher tolerance to salt, drought and cold stresses. Previous reports have suggested that there are different expression mechanisms among the three *AtCBF/DREB* genes. For instance, the *ICE1* gene that encodes a MYC-like bHLH protein was identified as being an expression regulator of *AtCBF3/AtDREB1A* but not of other *AtCBF/AtDREB1* genes.[46]

Flower senescence is associated with increased ethylene production in many flowers.[47] GUS expression was only observed in pollen grains, which start to appear when the flower opens and initiate the senescence process, after which desiccated mature pollen grains are dispersed from anthers onto the stigma surface covered with sticky exudates and ready for fertilization. Interestingly, the *RcDREB1* promoter analysis revealed the presence of three copies of the regulatory late pollen-specific element (POLLEN1LELAT52; AGAAA) responsible for pollen specific gene expression in tomato, tobacco and wheat.[48-52] The tomato *lat52* gene encodes a cysteine-rich protein preferentially transcribed in the vegetative cell during pollen maturation. Analyses of the organization and role of *cis*-regulatory elements in controlling the precise developmental and tissue-specific expression of *lat52* during pollen development revealed that *cis*-regulatory elements required for pollen-specific transcription were located within the upstream region 492 to 52. Three independent activator domains were identified, each sufficient to activate the minimal CaMV 35S promoter in a pollen-specific manner.[49] Similarly, a construction of gus coding sequence controlled by the *LeMAN5* promoter, which contains four copies of POLLEN1LELAT52, was used to transform *Arabidopsis*. GUS activity was not detected in roots, stems, leaves, and siliques, but intense GUS staining was observed in anthers and pollen grains of developing and open flowers of the transformants.[51]

The viability of pollen is one of the most important factors that have direct influence on the success of fertilization and seed production, being an important component of crop yield. Castor bean is indigenous to the southeastern Mediterranean Basin, Eastern Africa, and India, but is widespread throughout tropical and subtropical regions (mostly in arid zones), where temperatures remain fairly high throughout the growing season of 140 to 180 days. In general, the castor bean inflorescence is a monoecious raceme, with female flowers located at the apex and male flowers at the base. Dehiscence of anthers occurs very rapidly, pollen grains are released in 3 to 5 minutes and wind dispersed to reach other flowers of the same plant or of other plants.[53] At that moment pollen grains often have to cope with environmental stress. Consequently, the protection of the pollen grains from adverse environmental conditions is crucial. Studies on molecular mechanisms involved in pollen survival during dispersion and pollination are of ecological and agronomic relevance and castor bean could be an excellent model.

In conclusion, our results show that *RcDREB1* encodes a novel DRE-binding transcription factor that is not activated by various environmental stresses and ABA in leaf, stem and root. Interestingly, among the tests that were carried out, we observed that the *RcDREB1* promoter was only functional in dehydrated pollen grains or at the moment that desiccated mature pollen grains are ready to be dispersed from anthers, being exposed to the

external environment. To our knowledge, this is the first report on the characterization of DREB transcription factors isolated from castor bean and specifically functional in pollen grains. It has been demonstrated that moderate temperature increases could strongly influence pollen development.[54] Results of the present study could be the foundation for production of crops with highly stress-tolerant pollen, which could have an impact on productivity in a scenario of increasing temperatures, such as those projected to result from global climate change. In addition, pollen-specific promoters have the potential to target genes of biotechnological interest in pollen grains. As a potential application, engineered plants with long-life pollen grains could be produced, increasing pollination and in consequence, productivity.

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