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Identification of candidate genes involved in the response to biotic and abiotic stress in almond

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Abstract. Almond is commonly considered as tolerant to diverse biotic or abiotic factors, but little is known concerning the molecular mechanisms involved in the response to stress. In this study we have focused on the search for candidate disease resistance genes (*R*), and on the isolation of almond homologues to the CBF/DREB1 family of transcription factors, known to play an important role in abiotic stress response. A set of resistance-gene candidate sequences (RGCs) were isolated from one almond cultivar and two wild almond ecotypes (*P. webbii*). Phylogenetic analysis revealed that RGCs from both cultivar and wild ecotypes, clustered together in 5 different groups. This observation suggested a great similarity of the genetic backgrounds from both species, regarding their biotic resistances. The isolated RGCs are being mapped in the Texas x Earlygold *Prunus* genetic map. A PCR-based approach was used to isolate almond CBF/DREB1 homologous sequences. Preliminary RT-PCR analysis showed that the isolated almond CBF/DREBs are expressed in cold- and drought-induced tissues. Complete characterization of functional CBFs will provide valuable information regarding the molecular mechanisms involved in drought and cold stress responses in almond.

Keywords. Almond – *Prunus webbii* – *R* genes – DREB1/CBF – Cold – Drought.

Identification de gènes candidats intervenant dans la réponse au stress biotique ou abiotique chez l'amandier

Résumé. *L'amandier est généralement considéré comme tolérant à divers facteurs biotiques ou abiotiques, mais on ne sait que peu de chose sur les mécanismes moléculaires impliqués dans la réponse au stress. Dans cette étude nous nous sommes concentrés sur la recherche de gènes candidats pour la résistance aux maladies (R), et sur l'isolement d'homologues d'amandier pour la famille CBF/DREB1 de facteurs de transcription, connue pour son rôle important dans la réponse au stress abiotique. Un ensemble de séquences de gènes candidats pour la résistance (RGC) ont été isolées chez un cultivar d'amandier et deux écotypes sauvages d'amandier (*P. webbii*). L'analyse phylogénétique a indiqué que les RGC du cultivar et des écotypes sauvages pouvaient être répartis en 5 groupes différents. Cette observation a suggéré une grande similitude des fonds génétiques des deux espèces, concernant leurs résistances biotiques. Les RGC d'isolement sont tracés dans la carte génétique du* Prunus *Texas X Earlygold. Une approche basée sur PCR a été employée pour isoler les séquences homologues d'amandier CBF/DREB1. L'analyse préliminaire RT-PCR a prouvé que les CBF/DREB isolés d'amandier sont exprimées en tissus induits par le froid et la sécheresse. La caractérisation complète de CBF fonctionnels fournira des informations très intéressantes concernant les mécanismes moléculaires impliqués dans la réponse aux conditions adverses de sécheresse et de froid sur l'amandier.*

Mots-clés. *Amandier* – Prunus webbii – *Gènes R – DREB1/CBF – Froid – Sécheresse*

I – Introduction

Biotic and abiotic stresses can directly or indirectly affect plant growth and crop production. To cope with these constrains, plants have developed several response mechanisms at the molecular and cellular levels.

Plant disease *R* genes code for proteins that mediate the recognition of pathogen virulence proteins. This recognition activates signal transduction cascades that lead to the activation of plant defence mechanisms. *R* genes can fall into five main classes and the most frequent are the NBS-LRR class, characterized by the presence of a Nucleotide Binding Site - Leucine-Rich Repeat (NBS-LRR) domain structure (Bent *et al.,* 1994; Whitham *et al.,* 1994). The central NBS domain consists of several conserved motifs, namely a P-loop/kinase-1a [GGV(I/M)GKTT], a kinase-2 [LVLDDVW(D)], a kinase-3a (GSRIIITTRD) and a hydrophobic domain [GL(F)PL(F)AL] (Traut, 1994).

The *R* genes with NBS-LRR domain are thought to function as both elicitor recognition and activation of downstream signal pathways leading to disease resistance responses (McHale *et al.,* 2006). NBS-LRR R proteins are divided in two sub-families, according to sequence motifs present in their N-terminal domain. In the first group, named TIR-NBS-LRR (TNL), this domain shows homology to the Drosophila Toll and mammalian Interleukin-1 Receptor (TIR) proteins (Meyers *et al.,* 1999). The second sub-family, the non-TIR-NBS-LRR (CNL) is characterized by the presence of a coiled-coil (CC) motif at its N-terminal region, instead of the TIR (Pan *et al.,* 2000). Due to the short conserved regions present in the NBS domain, PCR-targeting of NBS sequences became a useful technique for the identification of *R* gene candidate sequences (RGCs). Mapping analysis have shown that RGCs are widely distributed in plant genome, often organized in clusters and sometimes strongly linked to known resistance loci (Kanazin *et al.,* 1996; Meyers *et al.,* 1999).

Plant response to environmental stress involves either up- or down-regulation of specific subsets of genes. The C-repeat Binding Factor / Dehydration Responsive Element-Binding protein (CBF/DREB1) is an example of a family of TFs that can be induced by different abiotic stresses, playing a key role in stress-responsive mechanisms (Gilmour *et al.,* 1998). These TFs interact with the specific motifs present in the promoter region of many stress regulated genes, controlling their expression (Yamaguchi-Shinozaki and Shinozaki, 1994). These late-response genes usually encode enzymes or structural components that will participate in the direct protection of cells. The importance of *CBF/DREB1* genes in stress tolerance, initially addressed by several studies in *Arabidopsis*, has lead to the search of similar genes in many other plants. Further characterization showed some structural and regulatory differences in DREB/CBF cold response pathway among plant species. An interesting new insight is the effect of light on DREB1/CBF regulation by cold stress in *Eucalyptus*, which can be an adaptive trait associated with overwintering on perennial woody plants (El Kayal *et al.,* 2006).

Almond (*Prunus dulcis* [Mill.] D.A. Webb) is an important fruit crop, originated from the domestication of one or more wild species that evolved in the deserts of Central Asia. Given its high genetic variability, almond has been considered to be tolerant to diverse biotic and abiotic factors, but little is known concerning the molecular mechanisms involved in the response to stress. In this study we have focused on the search for candidate resistance genes (*R*) and on the isolation of almond homologues to the CBF/DREB transcription factors.

II – Materials and methods

1. Plant material and nucleic acid isolation

The almond cultivar Primorskyi and two *P. webbii* ecotypes, C4 and A4, were used for the identification of resistance gene candidates. The search for almond CBF sequences was performed using cultivars Masbovera and Verdeal. Leaf samples from Primorskyi and Masbovera were provided from Centre de Mas Bové (IRTA, Spain); the cultivar Verdeal, obtained from Direcção Regional de Agricultura de Trás-os-Montes (Portugal), was previously established *in vitro* in the host lab; the *P. webbii* ecotypes were obtained from the University of Bari (Italy). Genomic DNAs were extracted according to a modified method described by Martins *et al.* 2003).

Stress treatments were performed using two week-old plantlets (after replication) from cv. Verdeal. Drought treatment was performed exposing the plants to dehydration conditions through partial drying, inside empty closed tubes, during 12h in the 22ºC growth chamber. For cold treatment, plantlets were transferred from 22ºC to 5º C by the end of the light period and maintained in dark conditions for 10h. After this period, plants were transferred to 10ºC under light conditions. Plantlets were collected at defined intervals and frozen in liquid N_2. RNA samples were extracted using the RNeasy® Plant Mini Kit (Qiagen, Germany) with minor modifications. RNA samples were quantified and stored at -80ºC.

Bin mapping analysis in the *Prunus* reference map (almond x peach) was performed using the two parental cultivars, Texas and Earlygold plus the F1 hybrid and a set of 6 individuals from the F2 (namely 5, 12, 23, 30 34 and 83). The corresponding genomic DNAs were kindly provided by Dr. Pere Arús, from IRTA-CSIC (Cabrils, Spain).

2. Isolation of almond RGC sequences

A. PCR amplification and cloning

PCR amplification of almond RGC sequences was performed using combinations of degenerate primers (Kanazin *et al.* 1996; Yu *et al.* 1996; Aarts *et al.* 1998). PCR amplification was performed with 25 ng of template DNA, 1x PCR Buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.75 μM of each primer and 2 U *Taq* DNA polymerase (Invitrogen, USA). PCR products were electrophoresed on agarose gel, stained with ethidium bromide and visualized under UV light. The expected fragment was excised from the gel, purified and cloned into the pCR®2.1 vector (Invitrogen). Clones were transformed in competent *E. coli* INVαF' and sequenced by Beijing Sunbiotech Co., Ltd (Beijing, China).

B. Sequence alignment and phylogenetic analysis

The obtained nucleotide sequences were compared with the GenBank database using the BLASTX algorithm (Altschul *et al.* 1997). Clones that shared homology with published RGCs were translated and sequences containing open-reading frames were selected for further analysis. A multiple sequence alignment with CLUSTAL W (Thompson *et al.,* 1994) was performed along with the corresponding NBS domain of known R genes from other species. Phylogenetic analysis was conducted using PHYLIP software package, version 3.5 (Feldstein, 1993). A bootstrap analysis with 500 replications was performed to evaluate the reliability of tree branches (Felsenstein, 1985).

C. Mapping analysis using Single-Strand Conformation Polymorphism

Clones with more than 95% nucleotide similarity were grouped into 18 sub-groups and specific markers were designed for each one. Until now, only 6 markers were screened in the Texas x Earlygold mapping population. PCR amplifications were performed with about 12.5 ng of genomic DNA, 1x GoTaq® Flexi Buffer (Promega, USA), 2.0 mM MgCl2, 0.2 mM dNTPs, 0.5 μM of each forward and reverse primers and 1 U GoTaq® DNA polymerase (Promega). For SSCP analysis, about 10% of total PCR product was mixed with an equal volume of 2x SSCP gel loading buffer. Samples were denatured and analyzed in polyacrylamide gels. Electrophoresis was carried out at 15ºC in a Double Wide Mini-Vertical Gel unit (CBS Scientific, USA).

3. Isolation of almond CBF/DREB1 candidate sequences

A. Amplification and sequence of almond CBF/DREB1 homologues

The primers PaCBF-Fw1, PaCBF-Fw2 and PaCBF-Rv were designed based on the nucleotide sequence of the *P. avium DREB1* cDNA sequence (Kitashiba *et al.*, 2004). PCR amplification was performed using 20 ng of genomic DNA (cv. Masbovera), 1x GoTaq® Flexi Buffer (Promega), 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of each primer combinations (PdCBF-Fw1/Rv and PdCBF-Fw2/Rv) and 1 U GoTaq® (Promega). Preliminary expression studies were also carried out using stress induced almond plantlets. About 2μg of total RNA samples, extracted from cold- and drought-induced tissues were used for reverse transcription. The cDNA samples were used as template for PCR amplification with PaCBF-Fw1/Rv. Amplification with primers for *Actin* gene was used as internal control. The fragments of interest obtained from both genomic and cDNA templates were purified from the gel, cloned into pCR 2.1 and transformed in competent *E. coli* DH5α. Clones of interest were sequenced by STABVida (Portugal). The obtained sequences were analysed as mentioned above.

III. Results and discussion

1. Isolation a mapping of almond RGC

Almond RGCs were isolated from Primorskyi, which is commonly described as resistant to several diseases, and two wild almond ecotypes (*P. webbii*). A major band of around 500 bp was amplified in all the three accessions used (Primorskyi, C4 and A4, data not shown). The band of interest was excised from the gel and cloned. A total of 86 clones (30 from 'Primorskyi', 27 from A4 e 32 from C4) showed significant homologies with known RGCs after searching in GenBank database. After translation, clones containing STOP codons or frame-shift mutations were excluded from further analysis. Additionally, several sequences showed to be identical at the amino acid level and only one was considered for the phylogenetic analysis.

Multiple alignment was performed using 56 sequences together with NBS domains of previously reported *R* genes. The phylogenetic tree (Fig. 1) obtained from the multiple alignment indicated that almond RGCs grouped in two main clusters, along with the NBS sequences used as reference. One of the clusters consists of the CNL sub-class (non-TIR NBS-LRR) and includes 8 RGCs. The second cluster consists of the TNL sub-class of resistance genes and groups the majority of the almond RGCs. A similar distribution was obtained for *P. persica* RGCs (Lalli *et al.,* 2005) and for *P. armeniaca* (Soriano *et al.,* 2005). These results emphasize the hypothesis of a greater abundance of TNL sub-class of RGCs in the *Prunus* genome. However, this distribution pattern can be also related to bias in the amplification of RGCs, due to limitations of primer design.

Additionally, phylogenetic analysis also showed that RGC sequences from the *P. dulcis* cultivar and its *P. webbii* relatives clustered together within the five groups (RGCI to V) (Fig. 1). Interestingly, phylogenetic analysis of RGC sequences from several members of Rosaceae showed that *Prunus* members (namely, *P. persica*, *P. davidiana*, *P. domestica* and *P. armeniaca*) didn't group closely with each other, as it happened with *Malus* and *Rosa* RGCs (Xu *et al.,* 2007). In almond, amino acid identities between RGCs from Primorkyi and *P. webbii* ecotypes reached, in some cases, 99% to 100% (data not shown). These results suggest a high similarity of the genetic backgrounds from both species, regarding their resistances. Similar results were already obtained by Sanchez *et al.* (submitted) when analyzing *P. dulcis* and *P. webbii* S-RNase gene sequences. These observations reinforce the hypotheses of occurrence of several hybridization events between *P. webbii* and *P. dulcis* along its evolution. These

phenomena could have lead to the introgression of self-compatibility in some almond cultivars (Socias i Company, 2002) as well as of some resistant traits.

For mapping analysis, RGC sequences with more than 95% nucleotide similarities were grouped. A total of 18 sub-groups were obtained and, to date, only 6 were mapped. Specific markers were designed and individual polymorphisms were assessed by PCR-SSCP. The markers were used to screen the Texas x Earlygold F2 mapping population (Joobeur *et al.,* 1998) according to the selective (bin) mapping strategy described by Howad *et al.* (2005). Further analysis showed that three almond RGC sub-groups were co-localizing with resistance loci or QTLs already mapped in the *Prunus* reference map (data not shown). The mapping of the remaining 9 RGC sub-classes in underway and the more interesting markers will be further studied in more detail, using the whole T x E population.

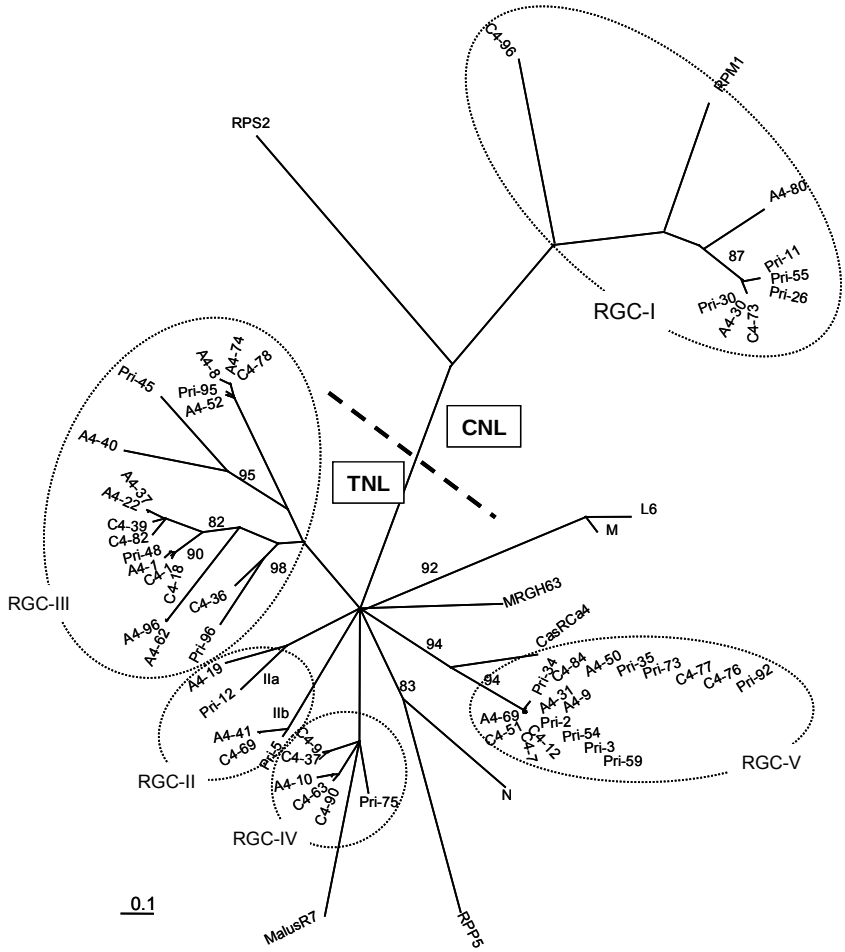

Fig. 1. Phylogenetic tree (unrooted) obtained from the alignment of 56 almond deduced amino acid RGCs and analogous domains of 6 *NBS-LRR* gene sequences (*N*, *RPS2*, *L6, RPP5, M, RPM1, MalusR7, RCa4, MRGH63*). Bootstrap values based on 500 replications are indicated. Clusters of almond RGCs are numbered from I to V. Pri - Primorskyi. The scale bar displays a distance of 0.1 amino acid substitutions per site.

2. Identification of almond CBF/DREB1 sequences

PCR amplification of almond genomic DNA with primer combinations PaCBF-F1/Rv and PaCBF-F2/Rv originated fragments of approximately 600 and 500 bp, respectively. These fragments were cloned and sequenced.

The putative expression of *CBF/DREB1* genes in almond was addressed by preliminary RT-PCR experiments. The results obtained showed a differential expression of putative almond *CBF*s during both stress treatments (Fig. 2). During cold treatment, it was possible to detect *CBF/DREB1* expression from 1h until the end of the treatment. In this case, expression was shown to increase from 1h to 4h and decrease after 10h exposure, which can be correlated to the change of temperature and light conditions (from 5ºC to 10ºC). During drought treatment, *CBF/DREB1* expression was faintly detected from 20min to 1h of treatment. These results suggest that putative CBF/DREB1 transcripts are quickly accumulated during drought stress, being involved in the early stages of response. However, in response to cold, CBF/DREB1 transcripts are expressed at a higher fold, along the 12h. This expression pattern is somewhat similar to most DREB1/CBF genes from other plants (Jaglo *et al.,* 2001; El Kayal *et al.,* 2006). RT-PCR fragments obtained for each treatment were isolated and sequenced.

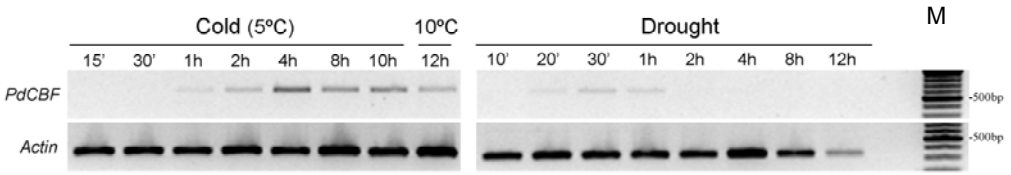

Fig. 2. Expression analysis of putative almond *CBF/DREB1* (PdCBF) and *Actin* (control) genes, during cold and drought stress. Levels of *PdCBF* transcripts were determined by RT-PCR. M- 100bp DNA Ladder (Fermentas).

Sequences obtained from both cDNA amplifications and genomic DNA (cv. Masbovera) were compared with sequences from GenBank database and several showed significant homology with published CBF/DREB1 sequences. A total of 4 different clones, showing high homology with *DREB*-like genes from *P. avium* and *P. persica*, could be translated to peptide sequences without STOP codons. The clones PdCBF10 and PdCBF11 were obtained from cold induced cDNAs. Another clone, PdCBF12, was obtained from drought induced cDNA amplification and showed to be identical to PdCBF10. PdCBF3 and PdCBF6 were isolated from genomic DNA amplification with primer combinations PaCBF-F1/Rv and PdCBF-F2/Rv, respectively.

At least two different clones seem to be involved in the response to cold and drought (PdCBF10 and PdCBF11), since they were obtained directly from stress-induced tissues. However, the involvement of PdCBF3 and PdCBF6 in stress response needs further investigation. Isolation of the full length cDNA sequences of the each PdCBF clones is underway using 3' and 5' RACE-PCR (rapid amplification of cDNA ends).

IV – Conclusions

Almond is commonly known as having an improved performance under several environmental constrains and diseases. These interesting aspects can be related to the inheritance of particular traits from its wild ancestors. Regarding disease resistance genes, the comparative analysis of RGCs from both Primorskyi and *P. webbii* ecotypes suggested a high similarity between both. Three of the mapped RGCs seemed to be co-localizing with known loci involved in disease resistance. Furthermore, mapping of the remaining almond RGCs and further

characterization studies could provide valuable information for development of new markers to be used in breeding programs towards disease resistance.

Additionally, we have isolated 4 different almond homologs to the DREB1/CBF TFs, and two of them seemed to be activated during cold and drought treatments. More detailed studies are underway in order to clarify the importance of DREB1/CBF and to identify novel players involved in abiotic stress signalling in almond. This will provide valuable information regarding the complex interaction between this woody tree and its habitat.

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