The grape berry-specific basic helix–loop–helix transcription factor VvCEB1 affects cell size

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Abstract

The development of fleshy fruits involves complex physiological and biochemical changes. After fertilization, fruit growth usually begins with cell division, continues with both cell division and expansion, allowing fruit set to occur, and ends with cell expansion only. In spite of the economical importance of grapevine, the molecular mechanisms controlling berry growth are not fully understood. The present work identified and characterized Vitis vinifera cell elongation bHLH protein (VvCEB1), a basic helix–loop–helix (bHLH) transcription factor controlling cell expansion in grape. VvCEB1 was expressed specifically in berry-expanding tissues with a maximum around veraison. The study of VvCEB1 promoter activity in tomato confirmed its specific fruit expression during the expansion phase. Overexpression of VvCEB1 in grape embryos showed that this protein stimulates cell expansion and affects the expression of genes involved in cell expansion, including genes of auxin metabolism and signalling. Taken together, these data show that VvCEB1 is a fruit-specific bHLH transcription factor involved in grape berry development.

Key words: auxin, bHLH, cell expansion, fruit, grape, transcription factor.

Introduction

Determination of the final fruit size depends on both cell growth and proliferation. Cell division activity determines the final cell number, but cell expansion is critical, as it allows the increase in volume determining the final size of the fruit. Cell divisions occur immediately after fertilization and during the early phase of fruit development. This is followed by cell expansion involving cell-wall loosening and uptake of solutes and water (Coombe, 1992). Cell expansion requires primary cell-wall loosening and incorporation of newly synthesized cell-wall material. Cell-wall loosening results from the disruption of chemical bonds between the structural components of the cell wall through either acidification or the action of hydrolysing enzymes. All these modifications require a finely tuned and coordinated transcriptional regulation of genes involved in cell-wall biosynthesis and modification.

Fruit development is a complex process involving numerous physiological and biochemical changes that are initiated by hormonal signals generated after pollination (Coombe and McCarthy, 2000; Conde et al., 2007). Their abundance at specific stages of fruit development and ripening indicates their possible role during these developmental stages (Conde et al., 2007). In the berry of grapevine (Vitis vinifera L.), the first steps of development, from fertilization to nouaison (fruit set) are under the control of developmental hormones (auxins, cytokinins, and gibberellins) promoting cell division and expansion. Although part of these hormones can...
be imported into the berry, they are mostly produced by the seeds, or by the maternal tissues (unfertilized ovules) in case of seedless cultivars. The concentration of these hormones decreases from their maximum at flowering to a low level at veraison and throughout ripening (Coombe 1992; Blouin and Guimberteau, 2000; Böttcher et al., 2011).

Basic helix–loop–helix (bHLH) proteins are found throughout eukaryotic organisms (Pires and Dolan, 2010). Due to their propensity to form homodimers or heterodimers, bHLH proteins can participate in an extensive set of combinatorial interactions leading to the regulation of multiple transcriptional programmes. Some bHLH proteins only form homodimers or restrict their heterodimerization to closely related members (Toledo-Ortiz et al., 2003), while others can form heterodimers with one or several different partners (Littlewood and Evan, 1998). These properties allow bHLH proteins to participate in the regulation of a myriad of essential developmental and physiological processes. In plants, bHLH proteins function as transcriptional regulators modulating secondary metabolism pathways, fruit dehiscence, carpel and epidermal development, phytocrome signalling, and stress responses (Ramsay and Glover 2005; Castillon et al., 2007; Pires and Dolan, 2010; Feller et al., 2011). Recent studies have described the involvement of bHLH proteins in the determination of plant organ size. The SPATULA protein was shown to control cotyledon, leaf, and petal expansion by affecting cell proliferation in Arabidopsis thaliana (Ichihashi et al., 2010). The Capsicum annuum protein Upa20 (upregulated by Capsicum annuum) from A. thaliana limits petal growth by reducing cell size (Kay et al., 2007), whereas BIGPETALp (BPEp) from A. thaliana (Szécsi et al., 2007; Matus et al., 2010). The present study identified and characterized Vitis vinifera ‘Chasselas’x Vitis berlandieri according to Lecourieux et al. (2010). An embryogenic cell suspension culture was initiated as described previously (Coutos-Thévenot et al., 1992a). This cell suspension was subcultured weekly in 25 ml of glycerol/maltose culture medium (Coutos-Thévenot et al., 1992b) supplemented with naphthoxyacetic acid at 1 mg l⁻¹ in the dark. Embryogenic cells were transformed using an Agrobacterium tumefaciens co-cultivation method (Mauro et al., 1995), and, after selection, the transgenic cells were subcultured in the same conditions in a medium supplemented with paromomycin at 2 mg ml⁻¹ and cefotaxime at 200 mg ml⁻¹ (Duchefa). Embryos were initiated from these 41B cells by removing auxin from the culture medium.

Transgenic tomato plants (Solanum lycopersicum L. cv. Wta106) were generated by A. tumefaciens-mediated transformation of tomato cotyledons as described by Gonzalez et al. (2007). Tomato plants were grown in a culture chamber with a 14 h/10 h day/night cycle. The temperature was 25 °C during the day and 20 °C during the night. Individual flowers were tagged on the day of anthesis (flower opening). Fruits were harvested at different developmental stages (expressed in days post-anthesis, d p.a.).

Materials and methods

Isolation of VvCEB1 cDNA and construct production

VvCEB1 full-length clone was generated from a cDNA library of grape Cabernet Sauvignon berries (veraison stage) by PCR using synthetic oligonucleotide primers designed to

begin and end at the start and stop codons of the open reading frame (forward primer, 5'-TAGAATTCCCCGATGGGACGCTTTTCTCATGCTCACCAC-3'; reverse primer, 5'-ATGGATCCCCGGTCAGCGCAGCAGAAGATGATCGTTGCTGAAACCATA-3'). This VvCEB1 complete open reading frame was amplified and cloned into the pGEM-T Easy vector (Promega) for DNA sequencing, prior to subcloning into a stable expression binary vector downstream of the 35S promoter of cauliflower mosaic virus (CaMV).

Plant transformation and culture

Grapevine transformations were made with the 41B rootstock (V. vinifera ‘Chasselas’xVitis berlandieri) according to Lecourieux et al. (2010). An embryogenic cell suspension culture was initiated as described previously (Coutos-Thévenot et al., 1992a). This cell suspension was subcultured weekly in 25 ml of glycerol/maltose culture medium (Coutos-Thévenot et al., 1992b) supplemented with naphthoxyacetic acid at 1 mg l⁻¹ in the dark. Embryogenic cells were transformed using an Agrobacterium tumefaciens co-cultivation method (Mauro et al., 1995), and, after selection, the transgenic cells were subcultured in the same conditions in a medium supplemented with paromomycin at 2 mg ml⁻¹ and cefotaxime at 200 mg ml⁻¹ (Duchefa). Embryos were initiated from these 41B cells by removing auxin from the culture medium.

RNA and cDNA production

Roots, shoots, leaves, and inflorescences were collected from Cabernet Sauvignon fruit cuttings grown in a greenhouse. Berries from different varieties (Tite de crabe, Dodrelabi, Dabouki, Cardinal, Candicans 10089, Riparia 10202, Riparia 10525, Sylvestris 38, Rubra 10924, Cinerea 10137, and Cabernet Sauvignon) were harvested in Domaine du Grand Parc (INRA, Lastresne, France) or in Domaine de la Grande Ferrade (INRA, Villenave d’Ornon, France). In order to compare berries at the same level of maturity, Cabernet Sauvignon berries were sorted by weight before veraison, and on a NaCl density gradient after veraison. All samples collected were quickly frozen in liquid nitrogen, ground to a fine powder, and stored at -80 °C until use. Total RNA from grape organs and berries was extracted according to the method of Lecourieux et al. (2010). Total RNA from grape embryos was extracted using a Spectrum™ Plant Total RNA kit (Sigma) following the manufacturer’s protocol. RNA isolation was followed by DNase I treatment. Reverse transcription was performed from 2 μg of purified RNA using Moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer’s instructions. The cDNA obtained was diluted 1:20 in distilled water.

Gene expression analysis

Quantitative real-time RT-PCR (qRT-PCR) expression analysis was carried out using a CFX96 Real-Time PCR Detection system (Bio-Rad). Reaction mixes (10 μl) were prepared, which included 5 μl of IQ™ SYBR Green Supermix (Bio-Rad), 0.2 μM of each primer, and 2 μl of diluted cDNA. In this study, three V. vinifera reference genes were evaluated, elongation factor EF1 γ-chain (EF1γ; GenBank accession no. AF176496), glyceraldehyde-3-phosphate dehydrogenase
In silico promoter region identification and analysis

Identification of the potential promoter regions and Myc-like bHLH binding factor (MYCL) binding sites was conducted using the Genomatix suite of programs (http://www.genomatix.de, Genomatix Software GmbH, Munich, Germany) (Quandt et al., 1995). The Gene2promotor program from the Genomatix software package was used to define 1000 bp of the promoter regions (1000 bp upstream of the transcription start site) for each gene. The 1000 bp sequences obtained from the Gene2promotor program were then used as the target sequences for putative bHLH transcription factor recognition site identification using the MatInspector version 8.06 program (Cartharius et al., 2005). The parameters used were the Matrix Family Library version 8.4 (June 2011), the standard (0.75) core similarity, and the optimized matrix similarity.

Light microscopy and stereomicroscopy analyses

Grape embryos were placed in a quick-clearing solution of chloral hydrate:H2O:glycerol (8:2:1, w:v:v) on a microscope slide for 4–24 h. Grape embryos were placed in a quick-clearing solution of chloral hydrate:H2O:glycerol (8:2:1, w:v:v) on a microscope slide for 4–24 h. The samples were then examined under differential interference contrast optics (Nomarski) using a light microscope (Axiophot, Zeiss). Photographs were taken using a Spot RTKE camera (Diagnostic Instruments).

β-Glucuronidase (GUS) histochemical staining

Tissues from stably transformed tomato plants were fixed in cold acetone, washed twice in sodium phosphate buffer (0.1 M NaH2PO4, pH 7.2), soaked, vacuum infiltrated, and incubated overnight at 37 °C in GUS staining solution [1 mM X-Gluc, 0.1 M NaH2PO4 (pH 7), 1.5 mM K3Fe(CN)6, 1.5 mM K4Fe(CN)6, 0.05% Triton X-100]. GUS-stained tissues were cleared for 2 d in 96% ethanol and stored in 70% ethanol. For this experiment, five independent transgenic tomato lines were used, but only one representative staining pattern of each tissue or developing stage is shown.

Sequence analysis

Amino acid sequence alignments were performed using ClustalW (Thompson et al., 1994; http://www.ebi.ac.uk/Tools/msa/clustalw2/). The phylogenetic tree was constructed using MEGA version 4 (Tamura et al., 2007) with full-length protein sequences. The optimal tree with the sum of branch length (32.66E+899) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). There were a total of 1167 positions in the final dataset.

Results

Identification of a novel bHLH-like protein from grape

A transcriptomic analysis of developing grape berries was performed in order to identify new transcription factors that could affect berry development (D. Glissant and S. Delrot, unpublished data). Different genes encoding transcription factors whose expression was upregulated during berry development were identified. Among these, a bHLH-like gene was isolated and analysed further. The full-length cDNA was amplified by PCR using a grape berry cDNA library and was named VvCEB1 (GenBank accession no. JQ823168).

To identify the cluster to which VvCEB1 belongs, a rooted phylogenetic tree was constructed from the alignment of 67 full-length bHLH protein sequences of different plant species (Fig. 1). The grapevine sequences were identified by performing a BLASTP similarity search on the NCBI database using different Arabidopsis bHLH proteins as queries (Pires and Dolan, 2010). This phylogenetic analysis revealed that, among the 26 distinct plant bHLH protein subfamilies described by Pires and Dolan (2010), VvCEB1 belonged to subfamily XII and particularly to a cluster containing proteins involved in growth regulation such as UPA20 (Kay et al., 2007), BPEp (Szécsi et al., 2006) and bHLH137 (Zentalla et al., 2007).

The full-length sequence alignment of VvCEB1 with its closest homologues from other plant species revealed that VvCEB1 exhibited 64% amino acid sequence similarity with SlbHLH1 (Sly014317, S. lycopersicum), 59% with UPA20 (C. annuum), and 46% with BPEp (A. thaliana). The VvCEB1 transcript was 810 bp and encoded a protein of 270 aa. VvCEB1 contained a nuclear localization site between aa 68 and 96 and a typical bHLH domain, which usually serves as a DNA binding and dimerization domain (Toledo-Ortiz et al., 2003), located in the region between aa 115 and 164 (Fig. 2).

Transient expression experiments using tobacco protoplasts expressing green fluorescent protein (GFP) fused in frame to the C terminus of VvCEB1 showed that, unlike the GFP control that was expressed throughout the cell, VvCEB1–GFP was only detected in the nucleus (Supplementary Fig. S1 at JXB online), in agreement with a putative role in the control of transcription.

Expression analysis of VvCEB1 in grapevine

The expression profile of VvCEB1 was determined in different grapevine organs by qRT-PCR with RNA extracted from Cabernet Sauvignon roots, stems, leaves, flowers, and mature berries (80 d p.a.). VvCEB1 was expressed almost exclusively in berries (Fig. 3A). Indeed, in mature berries, VvCEB1 was...
Fig. 1. Phylogenetic analysis of VvCEB1. VvCEB1 (black circle) and its homologues (bold) belong to a distinct bHLH gene subfamily. The different bHLH gene subfamily numbers and Arabidopsis thaliana (At), Thellungiella halophila (Th), Petunia hybrida (Ph), Oryza sativa (Os),
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highly expressed, whereas it was barely detectable in leaves, stems, roots, and inflorescences. VvCEB1 transcript accumulation was also assessed during berry development (Fig. 3B). VvCEB1 expression showed a strong and gradual increase starting from nouaison (20 d.p.a., berry set) and reached a maximum after veraison (60 d.p.a., fruit ripening). Transcript levels were maintained maximal until 80 d.p.a. and then slowly decreased until the mature stage (100 d.p.a.).

VvCEB1 transcript amounts were also monitored in seeds and in different berry tissues (mesocarp and exocarp) after veraison. VvCEB1 transcripts were most abundant in the mesocarp, moderately abundant in the exocarp, and weakly detected in seeds (Fig. 3C).

Finally, a time-course study of VvCEB1 expression in response to 20 µM treatment with different hormones revealed that, at this concentration, only auxin significantly affected VvCEB1 expression and decreased the abundance of its transcripts (Supplementary Fig. S3 at JXB online).

Relationship between VvCEB1 expression and grape berry size

To investigate further the relationship between VvCEB1 expression and grape fruit size, VvCEB1 transcript abundance was determined in grapevine varieties exhibiting differences in berry size. Five varieties producing small berries (Vitis rubra cv. 10924, Vitis cinerea cv. 10137, Vitis riparia

Fig. 2. Sequence analysis of VvCEB1. Full-length sequence comparison of VvCEB1 and its closest homologues SibHLH1, CaUPA20, AthHLH137, AtBIGPETAL and AtBEE1 using the ClustalW program with default parameters. Conserved residues are shaded in black; dark grey shading indicates conserved residues in at least four out of six of the sequences, and light grey shading indicates conserved residues in three out of six of the sequences. Basic residues that putatively function as a nuclear localization site for VvCEB1 are indicated by arrows above the alignment. Putative protein–protein binding domains and a bHLH domain for VvCEB1 are labelled above the alignment in black circles and open squares, respectively. Another conserved region with predicted secondary structure was identified for VvCEB1: an acidic α-helical domain, indicated by a black line.

and Hordeum vulgare (Hv) GenBank accession numbers reported in this figure are as described by Pires and Dolan (2010). The phylogenetic tree represents a non-exhaustive list of bHLH transcription factors; each bHLH subfamily is generally illustrated by a known A. thaliana bHLH and its closest homologues in V. vinifera (Vv), Solanum lycopersicum (Sl), and Populus trichocarpa (Pt). Sequence data were obtained from: http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/ (V. vinifera), http://planttfdb.cbi.edu.cn/family.php?sp=Sly&fam=bHLH (S. lycopersicum), and http://www.ncbi.nlm.nih.gov/genbank/ (P. trichocarpa). The phylogenetic tree was constructed with MEGA version 4 (Tamura et al., 2007) using the neighbour-joining method with 2000 bootstrap replicates.
cv. 10525, V. riparia cv. 10202, and V. vinifera cv. Sylvestris 38) and five varieties producing large berries (Vitis candicans cv. 10089, V. vinifera cv. Tite de crabe, V. vinifera cv. Cardinal, V. vinifera cv. Dodrelabi, and V. vinifera cv. Dabouki) were analysed. This study was performed with berries collected 3 weeks after veraison had occurred, when VvCEB1 expression peaks. The results are summarized in Fig. 4. In small berries, VvCEB1 expression varied from a relative expression level of 1 to 1.2, whereas it was almost tripled in large berries (relative expression level from 2.8 to 3.2). These data suggested a relationship between VvCEB1 expression level and berry size.

VvCEB1 overexpression affects grapevine embryo development

To investigate the function of VvCEB1 in grape, transgenic cells overexpressing VvCEB1 were produced using 35S promoter-driven VvCEB1 constructs. After stabilization of the cell suspension, expression of VvCEB1 was tested by qRT-PCR using VvCEB1-specific primers. In cells expressing 35S::VvCEB1, VvCEB1 transcripts accumulated 75-fold more than in cells expressing the empty vector (Fig. 5A).

To study the effect of VvCEB1 on grape development, regeneration was initiated from VvCEB1 overexpressing cells. The development of control somatic embryos and of embryos expressing 35S::VvCEB1 was observed under a light microscope (Supplementary Fig. S2 at JXB online). 35S::VvCEB1 lines displayed strong phenotypical defects when compared with control embryos. Up to 7 d, embryos of similar appearance were formed in both control and 35S::VvCEB1 embryos. Between 7 and 14 d, control embryos switched from the heart to the torpedo stage (d 14) and to mature embryos, with fully formed cotyledons; the hypocotyls-root axis was visible at d 19. During the same period, the transgenic embryos rapidly
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 elongated and still failed to develop cotyledons and to acquire bilateral symmetry (Supplementary Fig. S2). Moreover, secondary embryos started to develop from the primary transgenic ones and led to abnormal somatic embryos that could never develop into grape plantlets (Fig. 5B). Detailed measurements revealed that 35S::VvCEB1 embryos grew faster than the control and could reach, during the growth period, a size exceeding 11-fold the size of the control embryos (i.e. 17 d after initiation of embryogenesis) (Fig. 5C). In addition, bright-field microscopy observations after chloral hydrate treatment showed that the increased size of the transgenic embryos was associated with the presence of larger cells than in the control embryos (Fig. 6A). The difference in cell size increased during embryo development (Fig. 6B).

These phenotypic changes suggested that VvCEB1 might be involved in cell and organ growth, and also that its ectopic overexpression might affect organogenesis.

Specific activity of the VvCEB1 promoter in tomato fruits

VvCEB1 transcripts accumulated predominantly in the berry, indicating a fruit-specific expression of this gene (Fig. 3A). To investigate further the expression of VvCEB1 in planta and to confirm this observation, the activity the VvCEB1 promoter fused to the GUS reporter gene was examined in transgenic tomatoes. Strong GUS activity was observed in all 35Spro:GUS organs, while no staining was detected in wild-type controls (Fig. 7A). The VvCEB1 promoter only resulted in fruit-specific expression, with no GUS staining in vegetative

Fig. 4. Correlation between VvCEB1 expression and grape berry size. qRT-PCR analysis showing VvCEB1 transcript accumulation (black squares) in different grape varieties exhibiting different berry weight (grey bars). Berries were harvested 3 weeks after veraison and seeds were removed for this analysis. Gene expression was normalized against VvEF1γ. All data are means ±SD of four replicates from two independent experiments (summer 2009 and 2010).

Fig. 5. Overexpression of VvCEB1 strongly affects grape embryo development. (A) Relative expression level of VvCEB1 transcript accumulation in transgenic grape 41B embryos. The VvCEB1 transcript level was quantified by qRT-PCR in control (pFB8 empty vector) and VvCEB1-overexpressing (35S::VvCEB1) lines. These embryos were collected 7 d after initiation of embryogenesis. Gene expression was normalized against VvEF1γ. Data are means ±SD of three independent experiments. (B) Control (pFB8 empty vector) and VvCEB1-overexpressing (35S::VvCEB1) embryos lines were observed with a stereomicroscope 32 d after the initiation of embryogenesis. Bars, 1 mm. (C) Embryos sizes were measured at different stages of 41B embryo development, from 7 to 32 d after initiation of embryogenesis (DAE), both for control (pFB8 empty vector, black circles) and VvCEB1-overexpressing (35S::VvCEB1, open circles) lines. Thirty embryos were measured for each
organs (Fig. 7), confirming that, even when expressed in tomato, VvCEB1 keeps its fruit specificity. Analysis of VvCEB1pro:GUS during fruit development showed GUS staining from early stages until the mature green stage (30 d.p.a.), after which the activity of the VvCEB1 promoter decreased (Fig. 7B). These data were consistent with a specific expression of VvCEB1 in the fruit during the expansion phase spanning 10–40 d.p.a. (Lemaire-Chamley et al., 2005).

Modulation of cell expansion- and auxin-related gene expression in grape embryos overexpressing VvCEB1

To understand how VvCEB1 can affect cell expansion and/or auxin responses, the expression of several genes involved in these processes was tested in transgenic embryos overexpressing VvCEB1. These included some members of early auxin-responsive genes [auxin/indole-3-acetic acid (AUX/IAA), small auxin upregulated (SAUR) and Gretchen Hagen3 (GH3)], cell-wall metabolism (XET, PECL, AMY, AGP, and EXP) and aquaporin (AQP).

This expression analysis, assessed by qRT-PCR, showed that all the auxin signalling components tested were affected (Table 1). Indeed, VvIAA9 and VvIAA17 genes were downregulated in the transgenic embryos overexpressing VvCEB1, whereas VvIAA14, VvIAA16, VvIAA19, VvSAUR1, and
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VvSAUR5 were strongly upregulated compared with the control lines. The genes VvGH3-2 and VvGH3-6, encoding auxin-conjugating enzymes, were also dramatically upregulated in the transgenic line.

qRT-PCR experiments confirmed that most of the cell expansion genes tested were strongly upregulated in 35S::VvCEB1 transgenic embryos compared with the control lines (Table 2). These include the cell-wall modification genes encoding expansins (VvEXPI, VvEXP8, VvEXP11, VvEXP12 and VvEXP17), α-amylase (VvAMY1), xyloglucan endotransglycosylase (VvXET2), pectate lyase (VvPECL1 and VvPECL8), arabinogalactan protein (VvAGP20) and three out of four aquaporin genes tested (VvAQP1, VvAQP3, and VvAQP4).

Some of the genes that were significantly affected in the grape transgenic lines were also analysed throughout berry development. In this context, expression of the auxin-responsive transcription factor genes VvIAA49 and VvIAA17 that were negatively regulated by VvCEB1 overexpression was significant at the green stage and dropped during berry development. By contrast, the VvIAA19, VvIAA16, VvEXP11, VvAQP4, VvXET2, VvAMY1, VvPECL8, and VvSAUR genes that strongly accumulated in 35S::VvCEB1 lines were shown to be upregulated during the time course of VvCEB1 expression in the developing berry (Supplementary Fig. S4 at JXB online).

A region of 1000 bp of upstream sequence for all of auxin and expansion-related genes was analysed for putative bHLH regulatory elements (MYCL) using the MatInspector version 8.06 program (Cartharius *et al.*, 2005). The results are presented in Tables 1 and 2. The promoter regions of three genes analysed (VvGH3-6, VvEXP11, and VvAMY1) did not show any MYCL binding sites, whereas the 23 others exhibited one or more (up to nine) MYCL binding sites, suggesting that they might be direct targets for VvCEB1.

Taken together, these data suggested a coordinated regulation of several genes encoding proteins affecting cell expansion and VvCEB1 expression, and reinforce the conclusion that the berry-specific transcription factor VvCEB1 may affect cell expansion processes during grape berry development.

**Discussion**

This work identified and characterized VvCEB1, a new bHLH transcription factor from grape that is preferentially expressed in the berry and particularly in the expanding tissues. Its expression starts at fruit set, is maximal at veraison, and is maintained until harvest. VvCEB1 overexpression strongly affected cell size in grape embryos. Together, these results suggest a key role for VvCEB1 in cell expansion during fruit development.
VvCEB1 is a nuclear bHLH transcription factor clustering with genes controlling cell growth and organ size

Phylogenetic analysis revealed that VvCEB1 belongs to subfamily XII of the bHLH transcription factors (Pires and Dolan, 2010), which contains proteins previously described as being involved in growth regulation. This cluster includes, among others, UPA20 and BPEp, both known to regulate cell growth and organ size. BPEp affects cell expansion and petal growth in an auxin-dependent manner (Szécsi et al., 2006; Varaud et al., 2011), whereas UPA20 is involved in cell elongation in C. annuum (Kay et al., 2007). The A. thaliana bHLHI137 was reported as a DELLA-responsive gene that may repress gibberellic acid signalling (Zentalla et al., 2007). The other known proteins of this subfamily are involved in brassinosteroid (BR) signalling (BEE) (Friedrichsen et al., 2002) and in cryptochrome interaction (CIB) (Liu et al., 2008). Like auxins, BRs promote plant growth and participate in a wide array of plant developmental processes (Yang et al., 2011). Recently, Chung et al. (2011) made a direct link between auxin and BRs. They showed that auxin regulates BR biosynthesis in Arabidopsis, and suggested that some of the growth-promoting effects of auxin are mediated through BR biosynthesis. Taken together, these data suggest similarities in the mode of action of proteins belonging to clade XII on the regulation of cell elongation. This hypothesis is further supported by data from the literature indicating that members of the same bHLH subfamily are frequently involved in the same biological process and present partially or totally redundant functions (Pires and Dolan, 2010).

The predominant expression of VvCEB1 in the fruit suggests a key role in berry development

VvCEB1 was expressed almost exclusively in berries and more particularly in the expanding tissues (mesocarp and exocarp) (Fig. 3). Its expression increased gradually throughout berry development, without following the bimodal expression pattern usually attributed to the two phases of berry enlargement (stages I and III). This maintained accumulation of VvCEB1 transcripts during the lag phase (stage II) may suggest that its role in cell expansion is linked to its interaction with other proteins and/or that VvCEB1 is involved in additional aspects of berry development when combined with other proteins.

The heterologous analysis of the activity of the VvCEB1 promoter in tomato also showed that VvCEB1 is expressed specifically in the fruit. Interestingly, this fruit-specific
expression was maintained during the fruit expansion period of phase III, again underlining a link between VvCEB1 expression and cell expansion. Finally, VvCEB1 expression studies in grape varieties exhibiting differences in berry size revealed a relationship between VvCEB1 transcript accumulation and fruit size.

Together, these data strengthen evidence for the role of VvCEB1 in regulating berry size during development.

**Overexpression of VvCEB1 affects embryo development**

The production of grape embryos overexpressing VvCEB1 also showed that its overexpression affects cell growth. Indeed, the transgenic lines ectopically overexpressing VvCEB1 exhibited drastic phenotypic differences. The 35S::VvCEB1 embryos rapidly elongated and failed to develop cotyledons and to acquire bilateral symmetry, thus leading to abnormal somatic embryos that could never develop into grape plantlets. Compared with the controls, these embryos grew faster and reached a more important size because of the presence of larger cells. These differences may result from a stimulation of cell-expansion processes and from an alteration of hormone fluxes, especially of auxin fluxes that are necessary for the establishment of bilateral symmetry, growth, and organogenesis (Liu et al., 1993; Jenik and Barton, 2005; Moller and Weijers, 2009; Vanneste and Friml, 2009; de Smet et al., 2010).

VvCEB1 strongly affects the expression of genes involved in early auxin response and cell expansion

In order to understand better how VvCEB1 might affect cell size and interact with auxin, expression analyses were performed on 35S::VvCEB1 embryos. The data showed that cell-expansion gene expression was affected, and indicated that auxin could be involved through some signalling components related to fruit growth and development. Additionally, a time-course study of VvCEB1 expression in response to different hormones revealed that, at the concentration used, only auxin significantly affected VvCEB1 expression and decreased the abundance of its transcripts (Supplementary Fig. S3).

In this context, the early auxin-responsive genes AUX/IAA, SAUR, and GH3 were tested. These genes encode very low-abundance nuclear proteins with short half-lives that control secondary downstream genes (Hagen and Guilfoyle, 2002; Knauss et al., 2003). In the VvCEB1 overexpressing line, the VvIAA4 genes tested were either induced or repressed, whereas the VvSAUR and VvGH3 genes tested were only upregulated. These disparities in the transcript levels of auxin-regulated genes may reflect their complex regulation, which involves various combinations of transcriptional regulators, some of which are modulated by cell specificity, developmental stage, or abiotic signals (Paponov et al., 2008). Among the AUX/IAA genes tested in this study, VvIAA19 caught our attention because, in addition to its high expression in the 35S::VvCEB1 line, this gene was strongly expressed during berry development. Furthermore, a recent study (Kohno et al., 2012) showed that, although no morphological change was observed, transgenic Arabidopsis plants overexpressing VvIAA19 grew faster and flowered earlier than control plants, which indicates that the constitutive expression of VvIAA19 promotes growth. SAUR genes that are upregulated in the 35S::VvCEB1 line are abundant in the elongation zone of soybean hypocotyls and are expressed most strongly in epidermal and cortical cells (Gee et al., 1991). Very recently, Spartz et al. (2012) showed that the SAUR19 subfamily function as positive effectors of cell expansion. Together, these data suggest that VvCEB1 might contribute to the stimulation of cell expansion by regulating the expression of these genes.

To coordinate auxin-mediated processes, plants need to maintain the endogenous pool of auxins at an appropriate level. This can be achieved by regulating auxin biosynthesis and distribution among different organs and by conjugation (Berleth et al., 2004; Woodward and Bartel, 2005). Although the physiological importance of conjugates in auxin homeostasis is not yet fully understood, it is generally accepted that conjugate formation plays a critical role in auxin action. In grape berries, Böttcher et al. (2011) recently identified a GH3-1 gene that displays a developmental expression pattern correlated with the third phase of berry development and suggested its involvement in ripening processes. In Solanum, another non-climacteric fruit, the role of GH3 genes was also investigated (Kuang et al., 2011). The authors suggested that DIGH3.1 and DIGH3.2 are involved in fruit growth and particularly in pericarp growth and fruit ripening. Our data showed a high transcript accumulation of two VvGH3 genes in the 35S::VvCEB1 lines, suggesting the presence of a high amount of conjugated auxin and possible control of GH3 expression by VvCEB1. Together, this transcriptional analysis suggested that VvCEB1 could participate in berry growth, and reinforces the hypothesis that auxin is involved in the mode of action of VvCEB1.

Cell expansion involves changes in cell-wall composition and the accumulation of different compounds maintaining both osmotic pressure and water flow in the expanding cells (Carrari and Fernie, 2006). This process implies, among other aspects, cell-wall modification proteins (e.g. expansin, arabinogalactan proteins, xyloglucan endotransglycosylases), starch degradation enzymes (α-amylase and pectate lyase), and water channels (AQPs). In agreement with these data, the present study showed that VvCEB1 overexpression strongly upregulated expansion-related genes (Table 2) and therefore stimulated cell-expansion processes. These results also agree with the observations showing that overexpression of VvCEB1 in grape embryos enhanced cell size (Fig. 5). In addition, the effect of VvCEB1 on auxin-associated genes also fits with its putative role in cell expansion. Indeed, the classical effect of auxin is described as a very rapid stimulation of cell expansion by modifying the cell-wall network, followed by sustained growth over a longer time period, although this hormone is also important in other responses, such as cell division and differentiation (Schenck et al., 2010).

In conclusion, this work showed that VvCEB1 is a bHLH transcription factor expressed specifically in the expanding tissues of the fruit and is probably involved in the regulation of cell size. The present work also suggests that genes involved in
cell expansion and auxin responses are potential direct and/or indirect targets of VvCEB1. These data linking a fruit-specific bHLH transcription factor to cell expansion and auxin responses open new perspectives for the understanding of fleshy fruit development. However, additional work is needed to strengthen the hypothesis of VvCEB1 function in planta.

Supplementary data
Supplementary data are available at JXB online.

Fig. S1. Nuclear localization of GFP-VvCEB1 fusion protein in tobacco protoplasts.

Fig. S2. Kinetics of development of control (pFB8 empty vector) and VvCEB1-overexpressing (35S::VvCEB1) 41B grape embryos.

Fig. S3. Study of the hormonal regulation of VvCEB1 expression in grape cell suspension.

Fig. S4. Time-course study of the expression of VvCEB1 putative target genes during grape berry development.

Fig. S5. List of GenBank accession numbers used for qRT-PCR analysis.

Table S1. PCR primers used to amplify gene-specific regions for expression analyses.

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