An R2R3-MYB gene regulates pungency in chili pepper

Corresponding author:
Neftalí Ochoa-Alejo
Mailing address: Departamento de Ingeniería Genética de Plantas, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (Cinvestav-Unidad Irapuato), Km. 9.6 Libramiento Norte Carretera Irapuato-León; Apartado Postal 629; 36821-Irapuato, Gto., México.
Phone: (+52) 462 623 9654
Fax: (+52) 462 624 5849
E-mail: nochoa@ira.cinvestav.mx

An R2R3-MYB Transcription Factor in Capsaicinoid Biosynthesis
Magda L. Arce-Rodríguez¹ and Neftalí Ochoa-Alejo¹, ²

Departamento de Ingeniería Genética de Plantas¹, and Departamento de Biotecnología y Bioquímica², Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Unidad Irapuato, Km 9.6 Libramiento Norte Carretera Irapuato-León, 36821, Irapuato, Guanajuato, México.

One sentence summary:
CaMYB31, an R2R3-MYB transcription factor, regulates the capsaicinoid biosynthetic pathway in chili pepper fruits, and is regulated by plant hormones, wounding, temperature and light.

Financial source:
The National Council of Science and Technology (Conacyt, Mexico) financially supported this research, through the project 177063, and an MLA-R scholarship.

Corresponding author e-mail: nochoa@ira.cinvestav.mx

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Neftalí Ochoa-Alejo (nochoa@ira.cinvestav.mx).

Author's contributions:
MLA-R. designed and carried out the experimental work, analyzed the data and wrote the manuscript. N.O-A. designed, directed and supervised the experiments, and complemented and corrected the article.
Capsaicinoids are responsible for the hot taste of chili peppers. They are restricted to the genus *Capsicum* and are synthesized by the acylation of the aromatic compound vanillylamine (derived from the phenylpropanoid pathway) with a branched-chain fatty acid by the catalysis of the putative enzyme capsaicinoid synthase. R2R3-MYB transcription factors have been reported in different species of plants as regulators of structural genes of the phenylpropanoid pathway; therefore, we hypothesized that MYB genes might be involved in the regulation of the biosynthesis of pungent compounds. In this study, an *R2R3-MYB* transcription factor gene, designated *CaMYB31*, was isolated and characterized in *Capsicum annuum* L. cv. Tampiqueño 74. Bioinformatic analysis suggested that *CaMYB31* could be involved in secondary metabolism, stress and plant hormone responses, and development. *CaMYB31* expression analysis from placental tissue of pungent and non-pungent chili pepper fruits showed a correlation positive with the structural genes *Ca4H*, *Comt*, *Kas*, *pAmt* and *AT3* expression and also with the content of capsaicin and dihydrocapsacacin during fruit development. However, *CaMYB31* was also expressed in vegetative tissues (leaves, roots and stems). Moreover, *CaMYB31* silencing significantly reduced the expression of capsaicinoid biosynthetic genes and the capsaicinoid content. Additionally, *CaMYB31* expression was affected by the plant hormones indoleacetic acid, jasmonic acid, salicylic acid and gibberellic acid or by wounding, temperature and light, factors known to affect the production of capsaicinoids. These findings indicate that *CaMYB31* is indeed involved in the regulation of structural genes of the capsaicinoid biosynthetic pathway.
INTRODUCTION

Chili peppers are members of the *Capsicum* genus, which belongs to the Solanaceae family. *Capsicum annuum* is the most cultivated species in the world. The importance of this crop is based on a great variety of industrial applications, such as food, pharmaceutical, cosmetic and agronomic uses (Ochoa-Alejo and Ramírez-Malagón, 2001). One of the most valued compounds of chili pepper fruits are the capsaicinoids, which are synthesized and accumulated in the placental tissue through the convergence of phenylpropanoid and the branched-chain fatty acid pathways (Aza-González et al., 2011) (Fig. 1). Capsaicin and dihydrocapsaicin are responsible for 90% of the pungency in the fruit, and their accumulation depends on the genotype, stage of fruit development and environmental conditions (Lindsey and Bosland, 1996; Kim et al., 2009; Arce-Rodríguez and Ochoa-Alejo, 2015).

Research on the capsaicinoid biosynthesis pathway has uncovered key aspects of its biochemistry and molecular biology (Aza-González et al., 2011). The generation of chili pepper cDNA libraries and comparative gene expression analysis of pungent and non-pungent fruits has allowed the identification of several structural genes of the capsaicinoid biosynthesis pathway, such as phenylalanine ammonia-lyase (*Pal*), cinnamic acid 4-hydroxylase (*Ca4H*), 4-coumarate-CoA ligase (*4CL*), hydroxycinnamoyl transferase (*HCT*), caffeic acid O-methyltransferase (*Comt*), hydroxycinnamoyl-CoA hydratase/ligase (*HCHL*) and a putative acyltransferase (*AT3*) proposed to be the possible capsaicinoid synthase (*CS*) (Curry et al., 1999; Kim et al., 2001; Stewart et al., 2005; Mazourek et al., 2009; Liu et al, 2013).

The role of transcription factors in the capsaicinoid biosynthesis regulation is underexplored. Stewart et al. (2005) reported a differential expression analysis of two bZIP (Basic Leucine Zipper) transcription factors in pungent and non-pungent fruits at different developmental stages, but their expression pattern did not correlate positively with the expression of the structural genes *Pal*, *Ca4H*, *Comt*, *pAMT*, *BCAT*, *Kas*, *Acl*, *FatA* and *AT3*. Recently, Keyhaninejad et al. (2014) reported two ERF (Ethylene Response Factor) transcription factors whose
expression correlated positively with the pungency level in nine chili pepper cultivars and they were proposed as possible regulators of capsaicinoid biosynthesis.

Previous studies have shown that the phenylpropanoid pathway is regulated by R2R3-MYB transcription factors in different plant species such as *Arabidopsis thaliana*, *Antirrhinum majus*, and *Pinus taeda* (Tamagnone et al., 1998; Patzlaff et al., 2003; Zhou et al., 2009). R2R3-MYB transcription factors represent one of the largest family in plants and regulate different biological processes such as primary and secondary metabolism, responses to biotic and abiotic stresses, developmental processes, and hormonal responses (Dubos et al., 2010; Ambawat et al., 2013; Liu et al., 2015). Since the phenylpropanoid pathway is involved in the biosynthesis of precursors of capsaicinoid biosynthetic pathway, we hypothesized that the R2R3-MYB family transcription factors participate in the biosynthesis of capsaicinoids. Here, we report on the isolation of a CaMYB31 cDNA, which encodes a putative R2R3-MYB protein, and its role in the regulation of capsaicinoid biosynthetic genes and in the accumulation of capsaicinoids in chili pepper fruits. Furthermore, we characterized CaMYB31 regarding its gene organization, phylogeny, function and its responses to hormones, light, temperature and wounding.

**RESULTS**

**Identification of CaMYB31 by Differential Gene Expression in Placental Tissue from Pungent and Non-Pungent Fruits**

The accumulation of capsaicin and dihydrocapsaicin in placental tissue of chili pepper fruits of *C. annuum* cv. Tampiqueño 74 (pungent) and cv. California Wonder (non-pungent) during fruit development is presented in Table I. Capsaicin content was twice as high as dihydrocapsaicin in ‘Tampiqueño 74’ fruits, but these two capsaicinoids were not detected in California Wonder. In ‘Tampiqueño 74’,
capsaicinoids were not recorded at 10 days post-anthesis (DPA), began accumulating at 20 DPA, peaked at 30-40 DPA, and decreased at 50-60 DPA.

The expression of Ca4H, Comt, pAmt, AT3 and Kas genes was positively correlated with capsaicinoid accumulation in ‘Tampiqueño 74’ placental tissue. The expression of pAmt, Kas, AT3 and Comt was very low or even undetectable at 10 DPA, increased to a maximum between 30 and 40 DPA, decreased at 50 DPA, and was very low or undetectable at 60 DPA. Ca4H was expressed moderately at 10 DPA, increased to a maximum between 20 and 40 DPA, and then diminished slightly between 50 and 60 DPA. In California Wonder, the expression of AT3 was undetectable at all stages of fruit development, while that of Kas and pAmt was very low at 30-50 DPA. However, Comt and Ca4H were expressed at all stages of the non-pungent fruits (Fig. 2).

Partial MYB sequences were identified from a cDNA library of C. annuum cv. Tampiqueño 74. Only sequences that showed >40% homology and that expressed in pungent placental tissue were selected for qRT-PCR analysis (see Supplemental Figure 1). CaMYB31 showed a clear expression pattern that correlated positively with both the expression levels of the capsaicinoid biosynthetic structural genes and with capsaicinoid content during fruit development (Fig. 2).

**CaMYB31 Structure and Phylogenetic Sequence Analyses**

CaMYB31 contains a 747 bp reading frame encoding a putative R2R3-MYB protein of 249 amino acids that is preceded by a 5’ UTR of 148 bp and followed by a 3’ UTR of 189 bp. CaMYB31 cDNA sequence was compared to the chili pepper genome database (Qin et al., 2014) and three exons of 133, 130 and 484 bp, and two introns were identified. The first intron of 724 bp was localized between aa 44 and 45 of the R2 domain, and the second one of 425 bp was between aa 88 and 89 of the R3 domain (Fig. 3A).

A phylogenetic tree of 167 plant MYB proteins was constructed using the neighbor joining method, based on alignment of the MYB domain, with a bootstrap
test (n = 1000) (Fig. 3B). The full-length amino acid sequence of CaMYB31 was
used to look for MYB proteins from RefSeq and Swiss-Prot NCBI protein database,
and the MYB Arabidopsis proteins were also included (See Supplemental Table II).
The phylogenetic analysis was congruent with the subgroups defined for
Arabidopsis MYB proteins (Kranz 1998; Stracke et al., 2001).

According to our phylogenetic tree, CaMYB31 belongs to a cluster of MYB
Solanaceae proteins with no assigned biological functions at this time
(XP_016580728.1 with 98% identity to XP_015081732.1 with 67% identity) (Fig.
3B). The nearest neighbor clade to CaMYB31 was the subgroup 14 of Arabidopsis
thaliana proteins (AtMYB36 with 63% identity to AtMYB68 with 46% identity), which
are mainly related to development, while some of them are implicated in hormone
response, stress response and in the regulation of the lignin biosynthesis (Fig. 3B).
We also constructed the phylogenetic tree using the IT3F software online (Bailey et
al., 2008) (see Supplemental Fig. 2) and the results were consistent with our
phylogenetic analysis.

CaMYB31 alignment with other MYB plant regulatory proteins showed a
highly conserved R2R3 MYB domain in the N-terminal region, and a highly variable
C-terminal region (see Supplemental Fig. 3A). As a complement to the
phylogenetic analysis, we analyzed the C-terminal region of CaMYB31 by
comparison with the nearest neighbor clade to search for conserved motifs using
MEME motif-detection software, thinking that such motif might be important for the
function of CaMYB31. The C-terminal region of CaMYB31 share one motif with
these proteins related to development (see Supplemental Fig. 3B). Moreover,
PlantPAN 2.0 (Chow et al., 2016) was used as a database for signal scanning of
MYB binding sites in the putative promoter sequences of the structural
capsaicinoid genes, and we found MYB cis-elements on all predictive promoters of
the capsaicinoid structural genes (see Supplemental Fig. 4).

Differential CaMYB31 Expression Analyses in Different Organ Tissues of
Capsicum spp.
The expression of *CaMYB31* and that of the structural genes *Kas*, *pAmt*, *Comt* and *Ca4H* were analyzed by qRT-PCR in different tissues of the fruit (seeds, pericarp and placenta) and plant (roots, stems, flowers and leaves) from pungent and non-pungent cultivars. *Kas* and *pAmt* genes expression was detected exclusively in placental tissue (Fig. 4), although transcript levels were much higher in placental tissue from the immature fruits of Habanero than from ‘Tampiqueño 74’, which correlated with the pungency level, whereas *Comt* and *Ca4H* were also expressed in seeds, pericarp and vegetative tissues (Fig 4).

*CaMYB31* showed the highest expression in placental tissue of Habanero immature fruits, which are considered highly hot (Fig. 4). Moreover, the characteristic expression pattern of *CaMYB31* during the development of ‘Tampiqueño 74’ fruits was confirmed. The *CaMYB31* expression observed in placental tissue of California Wonder fruits was much lower. *CaMYB31* presented very low expression in the seeds and pericarp.

In the case of vegetative tissues, *CaMYB31* expressed at very low levels in flower tissues of ‘Tampiqueño 74’ and was undetectable in flowers of California Wonder (Fig. 4). However, *CaMYB31* was expressed moderately in roots and highly in leaf and stem tissues when compared to the placenta of ‘Tampiqueño 74’ chili pepper fruits.

**Effect of *CaMYB31* Silencing on the Expression of Capsaicinoid Biosynthetic Genes and on Capsaicinoid Content**

*CaMYB31* silencing experiments were carried out using a *Tobacco rattle virus* (TRV)-derived vector to generate the construct pTRV2-*CaMYB31*, which contained a 234 bp region from 90 aa of the R3 domain towards the C-terminal region (Fig. 3A). Fruits of 40 DPA were collected to investigate the effect of *CaMYB31* silencing.

Fruits from plants infected with the pTRV2-*CaMYB31* construct exhibited a significant diminution of the *CaMYB31* gene expression (82%) compared to uninfected plants (Fig. 5A). *CaMYB31* silencing caused a significant reduction in
the expression of the capsaicinoid biosynthetic genes \( Ca4H \) (81.8%), \( 4CL \) (80.6%), \( C3H \) (76.4%), \( HCT \) (49%), \( Comt \) (42.7%), \( pAmt \) (71%), \( BCAT \) (88.6%), \( BCKDH \) (48.5%), \( Kas \) (70%) and \( Acl \) (30.3%), compared to fruits from uninfected plants (Fig. 5A). It has been previously shown that agroinfection with the empty pTRV2 vector did not cause statistically significant changes in the expression patterns of tested structural genes in chili pepper fruits (Arce-Rodríguez and Ochoa-Alejo, 2015).

Capsaicin and dihydrocapsaicin contents in fruits of plants agroinfected with pTRV2-\( CaMYB31 \) showed a significant reduction of 74.2 and 73%, respectively, compared with fruits from uninfected plants (Fig. 5B). Fruits from plants agroinfected with the empty pTRV2 vector exhibited capsaicin content statistically similar to that of uninfected plants; however, dihydrocapsaicin content was significantly higher. Therefore, fruits from plants agroinfected with pTRV2-\( CaMYB31 \) had an 82.4% decrease in capsaicin and dihydrocapsaicin compared to fruits from plants infected with the empty pTRV2 vector (Fig. 5B).

Effect of Plant Hormones, Light, Temperature and Wounding on the Expression of \( CaMYB31 \) and Capsaicinoid Biosynthesis Structural Marker Genes

Environmental stimuli and some plant hormones affect capsaicinoid accumulation in chili pepper fruits (Kim et al., 2009; Gutiérrez-Carbajal et al., 2010). In order to test whether these factors regulate the \( CaMYB31 \) expression, several physical stimuli and plant hormones were applied to 30 DPA fruits of Serrano ‘Tampiqueño 74’. The expression of \( CaMYB31 \) and the capsaicinoid biosynthetic gene markers \( Kas \) and \( pAmt \) were analyzed in placental tissue by qRT-PCR.

Fruits were treated with light or dark conditions with varying exposure time, and the transcript levels were examined. Dark exposure caused a significant diminution on \( CaMYB31 \) expression similar to the expression pattern for \( Kas \) and \( pAmt \) (Fig. 6). Temperature was another tested stimulus; we monitored gene
expression at high temperature (37°C), low temperature (4°C) and standard growth temperature (25°C) at different exposure times. \textit{CaMYB31}, \textit{Kas} and \textit{pAmt} expression at 37°C was significantly lower, while that in fruits at 4°C was higher compared to standard growth conditions (Fig. 7). The expression of \textit{CaMYB31}, \textit{Kas} and \textit{pAmt} in wounded fruits had a significant decrease when compared to non-wounded fruits (Fig. 8).

In addition, the transcript levels of \textit{CaMYB31} in fruits treated with 100 µM of jasmonic acid (JA), salicylic acid (SA), gibberellic acid (GA3) or indoleacetic acid (IAA) compared to control fruits (treated with MS) were determined. \textit{CaMYB31}, \textit{Kas} and \textit{pAmt} expression increased significantly by IAA, SA and GA3 treatment, whereas a significant diminution by JA treatment was detected, except at 3 h of exposure (Fig. 9). The effect of these treatments was dependent on the time of exposure. In general, \textit{CaMYB31} expression correlated with the expression of \textit{Kas} and \textit{pAmt} under exposure to different environmental stimulus.

\section*{DISCUSSION}

The generation of chili pepper cDNA libraries and comparative gene expression analysis of pungent (Serrano cv. Tampiqueño 74) and non-pungent fruits (Bell pepper cv. California Wonder) allowed us to identify \textit{CaMYB31}, an R2R3-MYB transcription factor gene, as a strong candidate to regulate the capsaicinoid pathway.

In previous studies, the positive correlation between the expression pattern of structural genes (\textit{Pal}, \textit{Ca4H}, \textit{Comt}, \textit{Acl}, \textit{Fat}, \textit{Kas} and \textit{AT3}) of the capsaicinoid biosynthetic pathway and the level of pungency of chili pepper fruits has been reported (Curry et al., 1999; Aluru et al., 2003, Arce-Rodríguez and Ochoa-Alejo, 2015). It has been shown that capsaicinoid accumulation is dependent on the developmental stage of chili pepper fruits, and this synthesis and accumulation is highly likely to be regulated and coordinated at the transcriptional level.
Capsaicinoid Content and Expression of Genes Involved in the Capsaicinoid Pathway in Chili Pepper Fruits

A positive correlation was observed between the expression pattern of the structural genes Kas, pAmt and AT3 and the pungency level during the development of Serrano and Bell pepper fruits, indicating that these are specific genes of the capsaicinoid pathway. On the other hand, Comt and Ca4H genes were also expressed in non-pungent fruit and in vegetative tissues, suggesting that they participate in other biological process, such as lignin biosynthesis (Humphreys and Chapple, 2002). CaMYB31 showed an expression pattern similar to that of the Kas, pAmt and AT3 genes in the placental tissue of fruits that correlated with pungency, which strongly suggested it as a candidate to regulate the structural genes of the capsaicinoid pathway. However, CaMYB31 was also expressed in vegetative tissues such as roots, leaves and stems, indicating a possible role in more than one biological process, such as lignin biosynthesis or other metabolites derived from the phenylpropanoid pathway.

Bioinformatic Analysis of CaMYB31

Bioinformatic analysis of R2R3-MYB proteins showed that the putative CaMYB31 protein was closer to unknown function proteins of chili pepper, tomato, potato and tobacco. Moreover, the subgroup 14 of MYB Arabidopsis proteins was the nearest clade to CaMYB31, which shared one motif in the C-terminal region, and therefore might have some similar functions (lignin biosynthesis, stress and plant hormone response or development). The lignin and capsaicinoid biosynthetic pathways have common steps at the early stages of the phenylpropanoid biosynthesis, suggesting that CaMYB31 is probably implicated in the early regulation of the capsaicinoid pathway. Additionally, we found putative MYB binding sites on all the predicted promoters of the structural genes of the capsaicinoid pathway, suggesting that they are potential target genes of CaMYB31.
Effect of *CaMYB31* Silencing on the Expression of Capsaicinoid Biosynthesis-Related Genes and Capsaicinoid Content

Virus induced gene silencing (VIGS) was used to investigate the function of *CaMYB31* in the capsaicinoid pathway. *CaMYB31* silencing caused a significant decrease in capsaicinoid accumulation and on the expression of all structural genes of the phenylpropanoid pathway, except that of *Pal*, and also on the early genes of the branched-chain fatty acids pathway (*BCAT, BCKDH, Kas* and *Acl*) of capsaicinoid biosynthesis, showing strong evidence of its participation in the regulation of this metabolic process. Based on these results, we propose that the structural genes that showed a significant decrease in expression when *CaMYB31* was silenced are potential target genes of *CaMYB31*. Future studies of *CaMYB31* interaction with promoters of the structural genes whose expression was affected should provide more evidence on the direct transcriptional effect.

Effect of Hormones, Light, Temperature and Wounding on *CaMYB31* Expression

The biosynthesis and accumulation of capsaicinoids in chili pepper fruits is sensitive to environmental factors such as temperature and light, by stress conditions (wounding, drought), and also by plant hormones (Lindsey and Bosland, 1996; Suresh et al., 2005; Kim et al., 2009; Gutiérrez-Carbajal et al., 2010). For this reason, we analyzed the effect of plant hormones and stress on *CaMYB31* expression and two of the possible target genes: *Kas* and *pAmt*.

In previous studies, it was reported that SA induced the production of capsaicinoids and intermediary compounds (Gutierrez-Carbajal et al., 2010; Altuzar-Molina et al., 2011; Rodas-Junco et al., 2013), while JA induced or repressed the accumulation of capsaicinoids and intermediary metabolites depending on the concentration and exposure time (Suresh et al., 2005; Gutierrez-Carbajal et al., 2010; Altuzar-Molina et al., 2011). *CaMYB31*, *Kas* and *pAmt*
expression was significantly increased with SA treatment, while JA treatment caused a significant decrease in its expression, except at 3 h of exposure. In general, a typical antagonistic effect between SA and JA treatment was revealed, similar to that observed in genes related to defense against pathogens.

Wounding stress has been reported to promote the formation of JA while repressing SA production (Lee et al., 2004). A significant decrease in CaMYB31, Kas and pAmt expression in wounded chili pepper fruits was consistently observed with JA treatment, except at 3 h.

Other hormones, such as gibberellic acid and auxin, interact with the SA and JA pathways, increasing the complexity of the signaling pathway in the mechanism of defense in plants (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). CaMYB31, Kas and pAmt expression was significantly incremented under IAA and GA3 treatment. It has been reported that gibberellins suppress JA signaling and induce SA signaling (Pieterse et al., 2012), consistent with our results. Conversely, it has been reported that the auxin response was antagonistic to the SA response (Pieterse et al., 2012); however, we did not observe an antagonistic effect on the expression of CaMYB31, Kas and pAmt. The concentration of the plant hormone and exposure time highly influence gene expression.

Temperature is another factor that affects positively or negatively the level of pungency in chili pepper fruits and the expression of capsaicinoid structural genes (Rahman and Inden, 2012; Kim et al., 2009; González-Zamora et al., 2013). The expression of CaMYB31, Kas and pAmt was significantly increased at 4°C and was reduced at 37°C compared to 25°C; thus, Serrano ‘Tampiqueño 74’ perhaps is a variety in which the accumulation of capsaicinoids is negatively affected by high temperature.

Light intensity has been found to exert a significant effect on capsaicinoid content in chili pepper fruits (Iwai et al., 1979; Gangadhar et al., 2012). Kim et al. (2009) reported that the enzymatic activity of AT3 was induced by exposure to light and repressed in the dark. Similarly, the expression of CaMYB31, Kas and pAmt was significantly higher under light compared to dark conditions in the present investigation.
CONCLUSION

Our work showed strong evidence of the involvement of a MYB transcription factor in the regulation of capsaicinoid biosynthesis and its stress response in chili pepper fruits. Future studies are necessary to investigate whether CaMYB31 acts directly on the promoter of structural genes of the capsaicinoid pathway or through the formation of a protein complex with other transcription factors.

MATERIALS AND METHODS

Plant Growth Conditions

Chili pepper (Capsicum annuum L.) cv. Tampiqueño 74 (Serrano type) and cv. California Wonder plants were grown in greenhouse conditions and fertilized every two weeks with FerviaFol (Agroquímicos Rivas) solution (N:P:K 30:20:10). Chili pepper fruits were harvested at 10, 20, 30, 40, 50 and 60 days post-anthesis (DPA) from at least six plants. Only placental tissue was collected and was immediately frozen in liquid nitrogen, stored at -80°C and used for qRT-PCR expression analysis to select MYB gene candidates.

Capsicum annuum L. cv. Tampiqueño 74, cv. California Wonder and Capsicum chinense Habanero BG-3821 plants were grown for differential expression analysis in different organs. Chili pepper fruits were harvested at 10, 20, 40 and 60 DPA from Capsicum annuum and immature and mature stages from Capsicum chinense were dissected to separate the seeds, placenta and pericarp, which were stored separately. Roots, leaves, stem and flowers of adult plants from the different genotypes of C. annuum were collected. All tissues were frozen and stored at -80°C.

Seeds of cv. Tampiqueño 74 were germinated in a growth chamber at a temperature of 28°C under a 16 h photoperiod, a photon flux of 70 µmol m⁻² s⁻¹ (T8W/starcoat GE fluorescent lamps) and a relative humidity of 66% for silencing experiments. Three-week-old chili pepper seedlings were agroinfected and after 6
weeks were transferred to greenhouse conditions until 40 DPA chili pepper fruits were collected for each treatment.

Seeds of *Capsicum annuum* L. cv. Tampiqueño 74 were germinated and grown in a greenhouse for the studies of environment stimuli in entire fruits. Placental tissue was collected from 30 DPA chili pepper fruits for each treatment.

**Identification and Isolation of CaMYB31 Gene**

We selected 24 MYB partial sequences from a cDNA library of *Capsicum annuum* L. cv. Tampiqueño 74 with more than 40% identity to MYB proteins (see Supplemental Table I). The expression of these MYB genes was analyzed by qRT-PCR, comparatively with the expression of the capsaicinoid biosynthetic genes AT3, Kas, pAmt, Ca4H and Comt in placental tissue of fruits at different developmental stages (10, 20, 30, 40, 50 and 60 DPA) of *C. annuum* L. cv. Tampiqueño 74 (pungent) and cv. California Wonder (non-pungent). Moreover, the levels of capsaicin and dihydrocapsaicin were quantified at the same stages of development in pungent and non-pungent fruits. CaMYB31 gene was selected as candidate based on the positive correlation of its expression pattern with capsaicinoid content and the expression levels of the structural genes.

The full-length CaMYB31 gene was predicted using GeneScan web server from the pepper genome (Quin et al., 2014) and primers were designed (forward: 5’-TACACGTGATGGTGAGAACAACCTTGCT-3’ and reverse: 5’-CGCACGTGTTAATAATTTAAAATGATCAA-3’) for amplification by RT-PCR from placental tissue of 40 DPA chili pepper fruits of cv. Tampiqueño 74. The PCR conditions for amplification were 94°C for 3 min, followed by 30 cycles (94°C for 30 s; 57.5°C for 30 s; 72°C for 1 min), and 72°C for 7 min. The PCR product was diluted (1 µL of PCR product with 19 µL of sterile water) and used as template for a second PCR under the same conditions. PCR product was cloned into the pCR 8 vector (Invitrogen) according to the manufacturer’s instructions and sequenced.

**Phylogenetic and Sequence Analysis**
BLASTp was used to search MYB protein sequences from RefSeq_protein and Swiss-Prot NCBI database using the full-length amino acid sequence of CaMYB31. The characterized Solanaceae proteins (SIMYB12, SBLIND, CaBLIND, CaMYB, CaMYB1, CaA and CaMYB3) and all the Arabidopsis thaliana MYB proteins reported in Stracke et al., (2001) and in the IT3F Website (Bailey et al., 2008) were also included (See Supplemental Table II). The sequences were aligned with CLUSTAL W using default parameters, and it was manually adjusted. Based on the MYB domain alignment, a phylogenetic tree was constructed with the neighbor joining method, model JTT+G, and a bootstrap test (1000 replicates) using MEGA 6 software. In addition, we constructed a phylogenetic tree using the IT3F Website with CaMYB31 and the MYB proteins of Arabidopsis thaliana. The clades were grouped as previously reported (Kranz et al., 1998; Stracke et al., 2001). MEME (Multiple Em for Motif Elicitation) version 4.11.3 (http://meme-suite.org/tools/meme) was used to discover putative motifs in the C-terminal region of CaMYB31. PlantPAN 2.0 (Chow et al., 2016) was used to search MYB binding sites in the predictive promoters of the structural genes of the capsaicinoid biosynthesis pathway.

Virus-induced gene silencing of CaMYB31 gene

A 234 bp fragment of CaMYB31 was amplified (bases 269 to 503) using the primers forward 5´-AGTCTAGATCCTTTGGTGACCATTCTGA-3’ and reverse 5´-AGTCTAGAGCGATTGCTGCTCACTT-3’. Amplification conditions were 94°C for 5 min, followed by 30 cycles (94°C for 30 s; 55°C for 30 s; 72°C for 1 min) and extension at 72°C for 7 min. The CaMYB31-viral silencing plasmid construction and agroinfiltration was carried out as reported for tomato (Liu et al., 2002), with some modifications (Arce-Rodríguez and Ochoa Alejo, 2015).

Quantification of Capsaicinoids
Capsaicin and dihydrocapsaicin were quantified in placental tissue from *C. annuum* cv. Tampiqueño 74 (pungent) and cv. Bell pepper California Wonder (non-pungent) chili pepper fruits in three biological samples composed of 10 placentas each in the case of 10 DPA fruits, and 3 placentas for the 20, 30, 40, 50 and 60 DPA fruits. The extraction and quantification of capsaicin and dihydrocapsaicin was carried out as reported by Arce-Rodríguez and Ochoa-Alejo (2015).

**Plant Hormones, Light, Temperature and Wounding Chili Pepper Fruit Treatments**

Chili pepper fruits of *C. annuum* cv. Tampiqueño 74 at 30 DPA were collected to evaluate the effect of different factors on the expression of *CaMYB31* and the capsaicinoid biosynthetic genes *Kas* and *pAmt* for 3, 6, 12 and 16 h. For light/dark response assays, complete fruits were first wrapped with plastic film and foil for 24 h and then were uncovered and exposed to fluorescent light conditions (50 µM m\(^{-2}\) s\(^{-1}\)). For temperature treatments, entire fruits were wrapped with plastic film and foil and incubated at 4°C, 25°C and 37°C. For the wounding responses experiment, a scalpel blade was introduced approximately 6 mm deep several times into whole fruits, which were then covered with plastic film and incubated at 25°C under standard light conditions. To apply plant hormone treatments, placental tissue was separated from 30 DPA fruits and submerged into MS (Murashige and Skoog 1962) liquid medium containing 0 or either 100 µM jasmonic acid, salicylic acid, gibberellic acid or indoleacetic acid (SIGMA) and incubated at 25°C under standard light conditions. After incubation, the placental tissues from all treatments were immediately frozen in liquid nitrogen and stored at -80°C for gene expression analysis.

**Total RNA Extraction and cDNA Synthesis**

Total RNA was extracted from placenta, pericarp, seeds, flowers, stems, leaves and roots with Trizol (Invitrogen) according to the protocol provided by the
manufacturer. The extraction was performed in triplicate for each sample. RNA was purified with the PureLink™ Micro-To-Midi kit (Invitrogen) according to the instructions. cDNA was synthesized from purified RNA with the Super Script II or Super Script III reverse transcriptase (Invitrogen) following the protocol provided by the manufacturer.

**Real-Time Quantitative PCR Assays**

Total RNA was extracted, purified and treated with DNase I (Invitrogen) following the manufacturer’s instructions. cDNA was synthesized with Super Script III reverse transcriptase (Invitrogen) and adjusted to 100 ng mL⁻¹. qRT-PCR was performed as reported by Arce-Rodríguez and Ochoa-Alejo (2015) using the primer pairs described in Supplemental Table III.

**Statistical Analysis**

The data generated in silencing experiments and the effect of different factors on the expression of *CaMYB31* were subjected to Analysis of Variance (ANOVA) with the Tukey test (P ≤ 0.05) to find statistically significant differences between the mean values.

The coding sequence of *CaMYB31* can be found in GenBank under accession number XXX.

**Supplemental Data**

**Supplemental Table I.** Sequences from the cDNA library of *Capsicum annuum* L. cv. Tampiqueño 74 (Serrano type) for qRT-PCR analysis.

**Supplemental Table II.** Accession numbers of MYB proteins listed in Figure 3.

**Supplemental Table III.** Primers used for qRT-PCR analysis.

**Supplemental Figure 1.** qRT-PCR expression assays of the putative MYB transcription factor genes in placental tissue from chili pepper fruits of *Capsicum annuum* cv.
**Supplemental Figure 1.** qRT-PCR expression assays of the putative MYB transcription factor genes in placental tissue from chili pepper fruits of *Capsicum annuum* cv. Tampiqueño 74 and cv. California Wonder at different developmental stages. The data points represent the means of three biological replicates ± SD.

**Supplemental Figure 2.** Phylogenetic relationship of CaMYB31 with MYB transcription factors of *Arabidopsis thaliana*. Phylogenetic tree was constructed online using the IT3F website. The clades were grouped as previously reported for *Arabidopsis thaliana* (Kranz et al., 1998; Stracke et al., 2001) (highlighted with colored diamonds for each subgroup). The nearest neighbor clade to CaMYB31 (highlighted with blue letters) was the subgroup 14. Accession numbers for all protein sequences are listed in Supplemental Table II.

**Supplemental Figure 3.** Alignment of amino acid sequences of CaMYB31 with others plant R2R3-MYB proteins. A, Comparison of R2R3-MYB proteins. CaMYB31 (XXX), AtMYB36 (AT5G57620), AtMYB37 (AT5G23000), AtMYB35 (AT3G28470), AtMYB80 (AT5G56110) OsMYB4 (Q7XBH4), CaBLIND (F5C7S0), and SiMYB12 (B4YAJ8). Dark gray indicates identical amino acids and gray shows similar amino acids. Lines above the sequence highlight the R2 and R3 domains. B, Schematic diagram of the comparison of CaMYB31 with proteins of the nearest

**Supplemental Figure 4.** Schematic diagram of the putative MYB binding sites localization in the predicted promoter of capsaicinoid structural genes.

**Supplemental Table I.** Sequences from the cDNA library of *Capsicum annuum* L. cv. Tampiqueño 74 (Serrano type) for qRT-PCR analysis.

**Supplemental Table II.** Accession numbers of MYB proteins listed in Figure 3.

**Supplemental Table III.** Primers used for qRT-PCR analysis.
clade in the phylogenetic tree showing the putative motifs. Each box indicates a putative motif, and specifically the red box indicates the motif that CaMYB31 shared with these proteins.

**Supplemental Figure 4.** Schematic diagram of the putative MYB binding sites localization in the predicted promoter of capsaicinoid structural genes. The horizontal black line indicates the length of promoter sequence from ATG codon to -1500 pb. The vertical red lines indicate the putative TATA box, and the vertical green and black lines indicate the putative MYB binding sites TAACAAA and [AG]GATT, respectively; and the orientation towards up or down indicates the position in the positive or negative strand, respectively.

**ACKNOWLEDGMENTS**

We thank Yolanda Rodríguez for technical assistance in the HPLC analysis; Dr. Cesar Aza-González, Dr. María del Rocío Gomez-García, and José Luis Pablo-Rodríguez for technical assistance in the agroinfiltration experiments; and Dr. Luis Delaye, Dr. Octavio Martínez de la Vega and Magdalena Rivera for their important insights in bioinformatic analysis.

**Figure legends:**

**Figure 1.** Capsaicinoid biosynthetic pathway. PAL, phenylalanine ammonia lyase; Ca4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; HCT, hydroxycinnamoyl transferase; C3H, coumaroyl shikimate/quinate 3-hydroxylase; COMT, caffeic acid O-methyltransferase; pAMT, aminotransferase; BCAT, branched-chain amino acid transferase; KAS, ketoacyl-ACP synthase; ACL, acyl-CoA synthetase; FAT, acyl-ACP thiosterase; CS, capsaicinoid synthase; AT3, acyltransferase. The potential target genes of CaMYB31 transcription factor are marked in red.
Figure 2. qRT-PCR expression assays of the capsaicinoid biosynthetic genes Kas, pAmt, AT3, Comt and Ca4H and CaMYB31 in placental tissue from chili pepper fruits of Capsicum annuum cv. Tampiqueño 74 and cv. California Wonder at different developmental stages. The data points represent the means of three biological replicates ± SD.

Figure 3. Structural organization of the CaMYB31 gene and phylogenetic relationship of CaMYB31 with other plant MYB transcription factors. A, Schematic diagram of CaMYB31 showing the coding regions (black boxes), 5' and 3' UTR regions (gray boxes), introns (solid lines), R2R3-MYB domains (dotted lines), and the fragment sequence of CaMYB31 used for VIGS. B, Phylogenetic tree was constructed using the neighbor joining method based on the MYB domain alignment using MEGA 6 software. The bootstrap values are shown as percentages (1000 replicates) when greater than 50%. The clades were grouped as previously reported for Arabidopsis thaliana (Stracke et al., 2001) (highlighted with colored diamonds for each subgroup), while CaMYB31 (highlighted with blue letters) grouped with solanaceous sequences (marked with red circles). Accession numbers for all protein sequences are listed in Supplemental Table II.

Figure 4. Differential expression assays of CaMYB31 and of the capsaicinoid biosynthetic genes pAmt, Kas, Comt and Ca4H in different tissues of Capsicum annuum cv. Tampiqueño 74 and cv. California Wonder, and in fruits of Capsicum chinense Habanero BG-3821. M, mature; I, immature; Pe, pericarp; Se, seed; Pl, placenta; Fl, flower; Le, leaf; St, stem; Ro, root. The data points are the means of three biological replicates ± SD.

Figure 5. Effect of CaMYB31 gene silencing on biosynthetic structural gene expression and on capsaicinoid content in fruits of chili pepper. A, qRT-PCR analysis of capsaicinoid biosynthetic genes and CaMYB31 in fruits of chili pepper plants infected with the viral construct pTRV2-CaMYB31 compared to the uninfected control plants. B, HPLC analysis of capsaicin and dihydrocapsaicin in
fruits of uninfected plants and pTRV2 and pTRV2-CaMYB31 infected plants. The data points are the means of three biological replicates ± SD. Asterisks indicate significant differences between the control (uninfected) and infected plants ($P \leq 0.05$; Tukey test).

**Figure 6.** Effect of light on CaMYB31, Kas and pAmt expression in placental tissue from chili pepper fruits at 30 DPA. Gene expression was measured at different incubation times (3, 6, 12 and 16 h). The data points are the means of three biological replicates ± SD. Asterisks show significant differences between fruits incubated under dark and light conditions ($P \leq 0.05$; Tukey test).

**Figure 7.** Effect of temperature on CaMYB31, Kas and pAmt expression in placental tissue from chili pepper fruits at 30 DPA. Gene expression was quantified at different incubation times (3, 6, 12 and 16 h) and different temperatures (4, 25 and 37°C). The data points are the means of three biological replicates ± SD. Asterisks show significant differences between fruits incubated at standard temperature (25°C), and high temperature (37°C) or low temperature (4°C) ($P \leq 0.05$; Tukey test).

**Figure 8.** Effect of wounding on CaMYB31, Kas and pAmt expression in placental tissue from chili pepper fruits at 30 DPA. Gene expression was quantified at different incubation times (3, 6, 12 and 16 h). The data points are the means of three biological replicates ± SD. Asterisks show significant differences between control (no injuries) and wounded fruits ($P \leq 0.05$; Tukey test).

**Figure 9.** Effect of plant hormones on CaMYB31, Kas and pAmt expression in placental tissue from chili pepper fruits at 30 DPA. Gene expression was quantified at different incubation times (3, 6, 12 and 16 h) in fruits treated with 100 µM IAA (A), GA3 (B), SA (C) or JA (D). The data points are the means of three biological replicates ± SD. Asterisks show significant differences between control (MS medium) and treated fruits ($P \leq 0.05$; Tukey test).
Table I. Accumulation of capsaicin and dihydrocapsaicin in chili pepper fruits of *C. annuum* cv. Tampiqueño 74 and cv. California Wonder at different developmental stages

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Stages of fruit development (DPA, Days post-anthesis)</th>
<th>Capsaicin (mg g⁻¹Lpt)</th>
<th>Dihydrocapsaicin (mg g⁻¹Lpt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Tampiqueño 74’</td>
<td>10</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>6.73 ± 0.55</td>
<td>3.43 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>9.91 ± 1.42</td>
<td>5.44 ± 1.59</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>11.21 ± 1.19</td>
<td>5.85 ± 0.91</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>7.94 ± 0.61</td>
<td>4.98 ± 0.87</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>5.44 ± 0.46</td>
<td>3.93 ± 0.49</td>
</tr>
<tr>
<td>‘California Wonder’</td>
<td>10</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>
Separation and determination of capsaicin and dihydrocapsaicin was performed by HPLC. Lpt, lyophilized placental tissue. Each value is the mean of six biological replicates ± SD. N.D., Not detected.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>30</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>40</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>50</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>60</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

**LITERATURE CITED**


Figure 1. Capsaicinoid biosynthetic pathway. PAL, phenylalanine ammonia lyase; Ca4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; HCT, hydroxycinnamoyl transferase; C3H, coumaroyl shikimate/quinate 3-hydroxylase; COMT, caffeic acid O-methyltransferase; pAMT, aminotransferase; BCAT, branched-chain amino acid transferase; KAS, ketoacyl-ACP synthase; ACL, acyl-CoA synthetase; FAT, acyl-ACP thiosterase; CS, capsaicinoid synthase; AT3, acyltransferase. The potential target genes of CaMYB31 transcription factor are marked in red.
Figure 2. qRT-PCR expression assays of the capsaicinoid biosynthetic genes Kas, pAmt, AT3, Comt, Ca4H, and CaMYB31 in placental tissue from chili pepper fruits of Capsicum annuum cv. Tampiqueño 74 and cv. California Wonder at different developmental stages. The data points represent the means of three biological replicates ± SD.
Figure 3. Structural organization of the CaMYB31 gene and phylogenetic relationship of CaMYB31 with other plant MYB transcription factors. A, Schematic diagram of CaMYB31 showing the coding regions (black boxes), 5’ and 3’ UTR regions (gray boxes), introns (solid lines), R2R3-MYB domains (dotted lines), and the fragment sequence of CaMYB31 used for VIGS. B, Phylogenetic tree was constructed using the neighbor joining method based on the MYB domain alignment using MEGA 6 software. The bootstrap values are shown as percentages (1000 replicates) when greater than 50%. The clades were grouped as previously reported for Arabidopsis thaliana (Stracke et al., 2001) (highlighted with colored diamonds for each subgroup), while CaMYB31 (highlighted with blue letters) grouped with solanaceous sequences (marked with red circles). Accession numbers for all protein sequences are listed in Supplemental Table II.
Figure 4. Differential expression assays of *CaMYB31* and of the capsaicinoids biosynthetic genes *pAmt*, *Kas*, *Comt* and *Ca4H* in different tissues of *Capsicum annuum* cv. Tampiqueno 74 and cv. California Wonder, and in fruits of *Capsicum chinense* Habanero BG-3821. M, mature; I, immature; Pe, pericarp; Se, seed; Pl, placenta; Fl, flower; Le, leaf; St, stem; Ro, root. The data points are the means of three biological replicates ± SD.
Figure 5. Effect of CaMYB31 gene silencing on biosynthetic structural gene expression and on capsaicinoid content in fruits of chili pepper. A, qRT-PCR analysis of capsaicinoid biosynthetic genes and CaMYB31 in fruits of chili pepper plants infected with the viral construct pTRV2-CaMYB31 compared with the uninfected control plants. B, HPLC analysis of capsaicin and dihydrocapsaicin in fruits of uninfected plants and pTRV2 and pTRV2-CaMYB31 infected plants. The data points are the means of three biological replicates ± SD. Asterisks indicate significant differences between the control (uninfected) and infected plants (P ≤ 0.05; Tukey test).
Figure 6. Effect of light on CaMYB31, Kas and pAmt expression in placental tissue from chili pepper fruits at 30 DPA. Gene expression was measured at different incubation times (3, 6, 12 and 16 h). The data points are the means of three biological replicates ± SD. Asterisks show significant differences between fruits incubated under dark and light conditions ($P \leq 0.05$; Tukey test).
Figure 7. Effect of temperature on CaMYB31, Kas and pAmt expression in placental tissue from chili pepper fruits at 30 DPA. Gene expression was quantified at different incubation times (3, 6, 12 and 16 h) and different temperatures (4, 25 and 37°C). The data points are the means of three biological replicates ± SD. Asterisks show significant differences between fruits incubated at standard temperature (25°C), and high temperature (37°C) or low temperature (4°C) ($P \leq 0.05$; Tukey test).
Figure 8. Effect of wounding on CaMYB31, Kas and pAmt expression in placental tissue from chili pepper fruits at 30 DPA. Gene expression was quantified at different incubation times (3, 6, 12 and 16 h). The data points are the means of three biological replicates ± SD. Asterisks show significant differences between control (no injuries) and wounded fruits ($P \leq 0.05$; Tukey test).
Figure 9. Effect of plant hormones on CaMYB31, Kas and pAmt expression in placental tissue from chili pepper fruits at 30 DPA. Gene expression was quantified at different incubation times (3, 6, 12 and 16 h) in fruits treated with 100 μM IAA (A), GA3 (B), SA (C) or JA (D). The data points are the means of three biological replicates ± SD. Asterisks show significant differences between control (MS medium) and treated fruits (P ≤ 0.05; Tukey test).


Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title


Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title


Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title


Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title


Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title


Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title


Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title


Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title


Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title


Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title


Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title


Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title


Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title


Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title


