Postharvest Biology and Technology 143 (2018) 13–27

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Genome-wide identification and expression analyses of the calmodulin and calmodulin-like proteins reveal their involvement in stress response and fruit ripening in papaya

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ARTICLE INFO

Keywords:
Calmodulin
Calmodulin-like protein
Papaya
Gene expression
Fruit ripening
Stress response

ABSTRACT

Calcium (Ca^{2+}) is an important second messenger involved in diverse developmental and adaptive processes in plants. Calmodulin (CaM) and calmodulin-like (CML) proteins are primary Ca^{2+} sensors that control diverse cellular functions. In this study, 41 genes encoding CaM and CML proteins were identified in the papaya genome, three of which were *CaM* and others were *CML*. Sequence alignment, gene structural and phylogenetic analyses revealed that all CaM/CMLs contained the EF-hand, but not other functional domains, and CaM proteins were quite conservative while CML proteins exhibited sequence diversity and structural multiformity. Promoter analysis identified different types of cis-elements related to plant growth and development, hormone response, light response, stress response and transcriptional enhancement in promoter regions of *CpCaM/CML* genes. Gene expression analysis showed distinctive expression profiles of the *CaM/CMLs* in different tissue types, different fruit developmental stages and different fruit storage conditions. Several groups of *CaM/CML* genes were positively or negatively regulated by high and low temperature stresses, such as *CML16, CML17.1, CML24* and *CML36*, indicating that they may play a role in temperature stress adaption. Notably, some *CaM/CML* genes were rigorously regulated by ethylene (ethephon and 1-MCP treatment), either in a positive or a negative manner, such as *CaM7, CML15, CML16, CML17.1, CML37* and *CML46*. All these results indicated that *CaM* and *CML* gene families might play important roles in fruit development, in response to temperature stresses and in fruit ripening process.

1. Introduction

Plant growth and development are adversely affected by various environmental stresses, including biotic stresses such as pathogen and insect attacks, and abiotic stresses such as water shortage, unfavorable temperatures, salinity stress, nutrient imbalance, chemical and heavy metal toxicity, etc. (Vert and Chory, 2011). Unfavorable growth and storage condition also affect fruit production and shelf-life (Martínez-Romero et al., 2007). Fruits are subjected to various environmental and artificial stresses during their postharvest handling and storage period, which usually cause considerable loss in their economic values.

Calcium (Ca^{2+}) is a crucial second messenger in plants, which couples diverse stimuli to trigger various plant physiological responses (Ranty et al., 2016). Ca^{2+} plays a very important role in the signal transduction pathways, and Ca^{2+} signal needs to be transduced and decoded by Ca^{2+} sensors, including calmodulin (CaM) and calmodulin-like (CML) proteins (Reddy et al., 2011; Kamthan et al., 2015), calcineurin B-like proteins (CBL) and calcium-dependent protein kinases (CDPK) (Mccormack et al., 2005). CaM proteins are the most extensively studied Ca^{2+} sensors and have been known to play important roles in the regulation of plant growth, development and resistance to abiotic stresses (Zeng et al., 2015). CaM proteins are highly conserved and share 95–100 % identity with each other. There are only a few differences in the amino acid sequence among CaMs, which contribute to the specificity of their interactions with different target proteins (McCormack and Braam, 2003; Bhattacharya et al., 2004). CaM is composed of two pairs of EF-hand motif. The EF-hands are joined by a linker domain and each EF-hand binds a single Ca^{2+} ion (McCormack and Braam, 2003). Most plant CaMs contain 149 amino acid residues and four EF-hand domains (Zhu et al., 2015). *CaM* genes have been found in all plant species such as *Arabidopsis* where seven distinct genomic loci of *CaM* have been identified in the genome, which encode

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https://doi.org/10.1016/j.postharvbio.2018.04.010
Received 8 December 2017; Received in revised form 7 April 2018; Accepted 13 April 2018

four protein isoforms (McCormack and Braam, 2003; Mccormack et al., 2005). Five *CaM* genes have been identified in rice and they encode three CaM isoforms, and *OsCaM2* and *OsCaM3* proteins, which differ only in two amino acid residues (Boonburapong and Buaboocha, 2007). In potato, the full-lengths of four *StCaMs* genes are identified as *StCaM 1–4* that encode three different *StCaM* isoforms (Zhao et al., 2013). Thirteen *CaM* genes have been isolated in tobacco and they encode three groups of *CaM* isoforms responding to various stresses such as wounding and tobacco mosaic virus-induced hypersensitive cell death (Yamakawa et al., 2001). *CaM* gene family in *Solanaceous* species shows a high nucleotide sequence homology with each other, ranging from 79% to 98% (Giammaria et al., 2011; Zhao et al., 2013).

In addition to conserved CaMs, plants also possess a unique expanded family referred to calmodulin-like proteins (CMLs). Similar to CaMs, CMLs share typical EF-hand domain for Ca^{2+} binding but no other identifiable functional domains (Zhu et al., 2015). In *Arabidopsis*, 50 *CMLs* have been identified in the genome and they share 16%–75% amino acid sequence identity with typical CaMs (McCormack and Braam, 2003; Mccormack et al., 2005). These CMLs usually contain 80–318 amino acid residues with 1–6 EF-hand domains, implicating the specificity of calcium affinity among them. In tomato, fifty-two *CML* genes have been identified (Munir et al., 2016). Thirty-two and nineteen *CML* genes have been identified in the rice (Boonburapong and Buaboocha, 2007) and *Lotus japonicas* (Liao et al., 2017) genomes, respectively. For other species, different numbers of *CMLs* have been identified for further analysis (Zhu et al., 2015).

CaMs are involved in various plant development (Zhao et al., 2014) and abiotic and biotic stress responses (Park et al., 2007; Reddy et al., 2011; Wan et al., 2012; Peng et al., 2014). *CaM2* has been reported to be involved in pollen germination and the loss-of-function mutant results in a significant reduction in pollen germination (Landoni et al., 2010). *CaM3* genes are induced by heat shock, indicating that they may also participate in heat stress response (Zhang et al., 2009). It has been reported that the Ca^{2+}-CaM signaling pathways play a role in regulating the intracellular ROS level and the viability of *C. guilliermondii* under oxidative stress via acting on the DRM proteins (Bang et al., 2014).

Compared to *CaMs*, less information about *CMLs* is available. However, increasing lines of evidence have shown that they are also involved in distinct activities within different stages of plant development and stresses adaption (Virdi et al., 2015; Ranty et al., 2016; Zhu et al., 2017b). It has been reported that *AtCML23* and *AtCML24* participate in plant flowering (Delk, 2006). Other closely related paralogs, AtCML4 and AtCML5, are membrane proteins and play a role in vesicle transport within the endomembrane system in plants (Ruge et al., 2016). *CML25* plays an important role in pollen grain germination, pollen tube growth and seed setting (Wang et al., 2015). *AtCML9* plays dual roles in *Arabidopsis* by acting as a negative regulator in drought stress response (Magnan et al., 2008) and as a positive regulator in plant innate immunity upon bacterial infection (Leba et al., 2012). Our previous study also showed that *AtCML8* played a positive role in plant immunity against *P. syringae* as *AtCML9*, but through different pathways (Zhu et al., 2017a; Zhu et al., 2017b). Recent reviews show that CaMs and CMLs are the key hubs in plant response to biotic and abiotic stresses (Zeng et al., 2015; Ranty et al., 2016). The roles of different CaMs and CMLs involved in different stress response have been summarized in the work of Zeng et al. (2015). It is well known that calcium signal plays an important role in the regulation of fruit ripening (Ferguson, 1984; Aghdam et al., 2012), but the underlying mechanism is still not clear. Previous studies showed that *SlCaM2* in tomato participates in ethylene coordinated fruit ripening (Yang et al., 2014) and that *MaCDPK7* is involved in banana fruit ripening and temperature stress response (Wang et al., 2017).

Papaya (*Carica papaya* L.) is a nutritionally-rich fruit and is one of the most popular tropical fruit. However, due to its climacteric characteristics, it ripens rapidly and is very susceptible to pathogens (Paull et al., 1997). Improper growing and storage environment accelerate fruit aging or result in ripening disorders (Qiu et al., 1995; Jgd and Vitoria, 2011). Papaya fruit are highly sensitive to improper temperature that can cause chilling injury (Yuan et al., 2014), heat injury as well as biotic stresses during postharvest storage (Li et al., 2013b). Little information about CaM/CML and their functions in papaya is known to date. This work aimed to identify *CaM/CML* genes in papaya, analyze their characteristics and expression profiles under different experimental conditions and to reveal their specific roles in fruit development, ripening and stress response. Our results showed that *CaM/CML* gene families might play a role in fruit development and ripening and might also respond to temperature stress stimuli and ethylene triggering. The results presented herein will provide an important foundation for further understanding of the *CaM/CML* gene family function in papaya fruit.

2. Materials and methods

2.1. Database searches for CaMs and CMLs in papaya

The *C. papaya* genome and proteome were downloaded from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Cpapaya) for analysis. To identify potential CaM and CML proteins in *C. papaya*, one typical CML protein sequence from *Arabidopsis thaliana* (CML23(At1g66400)) was used as a query in BLASTP search for the candidate CaM and CML proteins in papaya proteome with default parameters in Phytozome. All the potential sequences were downloaded for further analysis. For CML identification in papaya, CML9 was used as a query in the same BLASTP search and similar results were obtained.

We obtained abundant sequences as the original data and subsequently used them as queries in BLASTP searches in the AtCML pool (all CaMs and CMLs in *Arabidopsis*). We selected candidate CaM/CMLs with the highest sequence similarity, and all the other candidates with EF-hand domains were removed (CBLs and CDPKs). Then all the sequences were manually checked to ensure that comprehensive sequences of high quality were obtained. The corrected protein sequences obtained were pooled and used as queries in BLASTP search in the papaya protein database for an exhaustive identification of the members of divergent gene families. The sequences already in the sequence pool were eliminated and the new candidate CaM and CML sequences were selected. To look for other paralogs or pseudogenes, the typical AtCaM2 or AtCML23 were used as queries to perform tBLASTn in the papaya genome database for seeking any possible non-predicted genes.

2.2. Prediction of EF-hands and analysis of gene structures

PROSITE (http://prosite.expasy.org/) was used for the prediction of EF-hand domains. Normally CaMs possess four EF-hand domains and CML have multiple EF-hands ranging from 1 to 6. CaM and CML only have the EF-hand domain but no other functional domains. Thus, the sequences containing other domains were eliminated. Gene exon-intron structures were extracted from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Cpapaya) in *Carica papaya* species and visualized in Fancy Gene software (http://bio.ieo.eu/fancygene/).

2.3. Putative cis-acting element analysis

The 2-kb upstream genomic sequences of all *CpCaM/CML* genes were retrieved from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Cpapaya) and used for putative cis-acting element analysis in the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).