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Crosstalk between calcium and melatonin affects postharvest physiological deterioration and quality loss in cassava

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ABSTRACT

Rapid postharvest physiological deterioration largely reduces the quality and marketability of cassava. The molecular mechanism underlying cassava postharvest physiological deterioration and quality loss is largely unknown. The present study aimed to investigate the role of calcium and its relationship with melatonin in cassava postharvest physiological deterioration. Transcriptomic analyses indicate that most of the calcium ion (Ca^{2+}) sensor genes are upregulated in cassava tuberous roots at different postharvest stages. Exogenous $CaCl_2$ reduces postharvest physiological deterioration, increases the endogenous levels of Ca^{2+} and melatonin, reduces the degradation of ascorbic acid and starch, and induces the expression of genes related to melatonin biosynthesis after harvest. These effects are reversed by the exogenous application of a Ca^{2+} chelator (EGTA). Exogenous melatonin also increases endogenous melatonin levels and reduces ascorbic acid and starch degradation during postharvest physiological deterioration but do not affect endogenous Ca^{2+} content. Together, these findings demonstrate that calcium-induced activation of melatonin biosynthesis plays a role in reducing postharvest physiological deterioration and quality loss in cassava. Additionally, pretreatment with EGTA arrests the melatonin-induced reduction of postharvest physiological deterioration, suggesting the possible crosstalk between melatonin and calcium during postharvest physiological deterioration.

1. Introduction

Cassava is the sixth most important crop in terms of global production following wheat, maize, and rice and is mainly grown for its edible tuberous roots (Zhang et al., 2010; Zidenga et al., 2012). Based on its high starch production, cassava is also considered as a potential biofuel crop (Zidenga et al., 2012). However, the rapid postharvest physiological deterioration of cassava tuberous root that occurs within 72 h postharvest largely reduces its quality and marketability (Zidenga et al., 2012; Vanderschuren et al., 2014). During harvest and storage, physiological deterioration is induced by mechanical injury and progresses from the proximal site of damage to the distal end, resulting in unpalatable roots (Zidenga et al., 2012; Vanderschuren et al., 2014). Extensive studies have been conducted on elucidating the physiological and biochemical mechanisms underlying postharvest physiological deterioration of cassava (Reilly et al., 2001, 2004; Iyer et al., 2010; Zidenga et al., 2012; Xu et al., 2013; Vanderschuren et al., 2014). During postharvest physiological deterioration of cassava, reactive oxygen species (ROS) levels increased, followed by the regulation of genes and activities related to the antioxidant system (Reilly et al., 2004). Moreover, genetic modification of various genes encoding antioxidant enzymes, including AtAOX1A, MeCu/ZnSOD, MeCAT1 and MeGPX, delays postharvest physiological deterioration and extends shelf life of cassava tuberous roots (Zidenga et al., 2012; Xu et al., 2013; Vanderschuren et al., 2014). Collectively, these evidences highlight that ROS-induced oxidation leads to symptoms of postharvest physiological deterioration, and the reduction in ROS accumulation could reduce postharvest physiological deterioration.

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Melatonin (N-acetyl-5-methoxytryptamine) was first isolated from the pineal gland of cow in the late 1950s (Lerner et al., 1958, 1959). In 1995, melatonin was subsequently identified in plants (Dubbels et al., 1995; Hattori et al., 1995), and melatonin has been isolated from various plant species, including *Arabidopsis*, rice, apple, beestrawberry, cucumber, tobacco, and cassava (Ma et al., 2016; Shi et al., 2016). Further studies have shown that melatonin is involved in multiple plant biological processes such as root architecture, photoprotection, flower development, seed germination, leaf senescence, fruit ripening, vegetative growth, and responses to stress (Hu et al., 2016b; Shi et al., 2016). Melatonin also acts as an antioxidant by activating the antioxidant system, directly scavenging ROS, and augmenting the efficiency of other antioxidants (Hu et al., 2016b). A recent study has shown that the exogenous application of melatonin delays cassava postharvest physiological deterioration as well as reduces H_2O_2 accumulation by activating antioxidative enzymes (Ma et al., 2016), which is in agreement with the findings of physiological and transcriptomic analyses (Hu et al., 2016b). Calcium plays an important role in regulating various biological processes. Cellular responses to abiotic stress include a significant increase in Ca^{2+} levels, which in turn activates Ca^{2+} receptors and the Ca^{2+} signaling network (Gilroy et al., 2014). Exogenous application of Ca^{2+} could alleviate stress-induced injury, as it has a protective role in plant responses to abiotic stress. One important event underlying this particular strategy for protection is activation of the antioxidative system and repression of ROS accumulation. Physiological and biochemical studies suggest that the exogenous application of Ca^{2+} increases plants tolerance to cadmium, chilling, acid rain, and hypoxic stresses by activating antioxidant enzymes and inhibiting ROS accumulation (He et al., 2012; Shi and Chan, 2014; Srivastava et al., 2015; Hu et al., 2016a). Further genetic investigations also support the positive role of calcium in inducing the antioxidant system in stress conditions by overexpressing or repressing genes that encode Ca^{2+} receptors (Verslues et al., 2007; Deng et al., 2013a, 2013b; Zou et al., 2015). Calcium is involved in reducing cellular ROS levels and improving plant tolerance to abiotic stress; but, the role and mechanism of calcium in postharvest physiological deterioration of cassava remains unclear. The relationship between calcium and melatonin during cassava postharvest physiological deterioration has yet to be determined.

The present study aimed to elucidate the role of calcium in regulating postharvest physiological deterioration and tuberous root quality of cassava, as well as establish the relationship between calcium and melatonin during cassava postharvest physiological deterioration using transcriptome and physiological analyses.

2. Material and methods

2.1. Plant materials and treatments

Ten-month-old cassava tuberous roots (*Manihot esculenta* Crantz cv. SC8) were harvested to cut into 0.005-m-thick slices that were subsequently transferred into Petri dishes lined with wet filter paper (Vanderschuren et al., 2014). To investigate the role of calcium in postharvest physiological deterioration, root slices were incubated in water (control), 0.01 M $CaCl_2$, or 0.01 M ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). All the slices of each treatment were placed in a 2 L volume of the solution for 2 h. Then, the root slices were removed from the solution and placed in an incubator in the dark (28 °C and 60% relative humidity). After incubation for 0 h, 2 h, 6 h, 12 h, 24 h, 48 h, and 72 h, the slices were frozen in liquid nitrogen until total RNA extraction or physiological analysis. Each sample (each treatment at each time point) had three replicates (each replicate contained one slice from one tuberous root), which constituted one biological experiment. Totally, three biological experiments were performed for each sample. To study the role of melatonin in postharvest physiological deterioration, root slices were incubated in water (control) or different concentrations (1×10^{-4} M, 3×10^{-4} M, and 5×10^{-4} M) of melatonin for 2 h. To test the combined effect of melatonin and EGTA on postharvest physiological deterioration, root slices were pretreated with 0.01 M EGTA for 2 h, then exposed to 1×10^{-4} M melatonin for 2 h. The material composition and the subsequent incubation were the same as earlier described. To test the effect of calcium on whole tuberous roots, roots were incubated in water (control), 0.01 M $CaCl_2$, or 0.01 M EGTA for 2 h. Then, the roots were placed in an incubator in the dark (28 °C and 60% relative humidity). After incubation for 0 d, 14 d, and 21 d, the roots were cut into 0.005-m-thick slices and then imaged.

2.2. Transcriptomic analysis

Total RNA was isolated using a plant RNA extraction kit (TIANGEN, China) and used for cDNA library construction. Sequencing was performed with an Illumina GAII system following the manufacturer's instructions. Adapter sequences were removed using a FASTX-toolkit. Clean reads were generated by removing low-quality sequences using FastQC. Tophat v.2.0.10 was used to map the clean reads to the cassava genome (Trapnell et al., 2009). Transcriptome data was assembled using cufflinks (Trapnell et al., 2012). Fragments Per Kilobase of transcript per Million mapped reads (FPKM) was employed to calculate gene expression levels. Differentially expressed genes were identified with DEGseq (Wang et al., 2010). Each sample had three biological replicates (each replicate contained one slice from one tuberous root).

2.3. Quantitative real-time PCR analysis (qRT-PCR)

Changes in the expression of *MeTDC1* (Manes.12G079500), *MeTDC2* (Manes.12G038600), *MeSNAT* (Manes.08G168900), *MeT5H* (Manes.06G111700), *MeASMT1* (Manes.13G140900), *MeASMT2* (Manes.17G050500), *MeASMT3* (Manes.13G140500) were measured by qRT-PCR on a Stratagene Mx3000P real-time PCR system using SYBR® Premix Ex Taq™ (TaKaRa, Japan). The primer pairs were examined based on the melting curve, agarose gel electrophoresis, and sequencing PCR products (Table S1). The qRT-PCR was conducted as follows: 95 °C for 10 min; followed by 40 cycles at 95 °C for 10 s, 55 °C for 15 s, and 72 °C for 20 s. The amplification efficiency was within the range of 0.92–1.05. The expression of the target genes was normalized with the *TUB* and *EF1* genes. The $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative expression of the target genes. Each sample had three biological replicates (each replicate contained one slice from one tuberous root).

2.4. Evaluation of postharvest physiological deterioration of cassava tuberous roots

Postharvest physiological deterioration evaluation was determined according to the method of Zidenga et al. (2012). Tuberous roots were carefully harvested, and then cut into slices approximately 0.005-m-thick, followed by various treatments. After treatment, the slices were stored at a growth chamber with 28 °C and 60% relative humidity in the dark. After incubation up to 72 h, the slices were collected to evaluate the deterioration rate using ImageJ image processing and analysis software (http://rsb.info.nih.gov/ij/, NIH, MD, USA). The root slices were photographed under standard illumination settings. Color images were converted into gray images. Gray values that represent the area of postharvest physiological deterioration in each root slice were calculated. The entire area of the root slices was also calculated. The ratio between gray values and entire area in each root slice was used to evaluate deterioration rate, which ranged from 0 to 1.

2.5. Quantification of endogenous melatonin, Ca^{2+}, ascorbic acid, and starch

Samples from treated or control root slices were frozen in liquid

nitrogen and then ground into powder. About 1×10^{-4} kg of tuberous roots powder was used to extract melatonin according to the acetone-methanol method (Pape and Luning, 2006). Melatonin was measured using a plant melatonin enzyme-linked immunosorbent assay (ELISA) kit (Jianglai Biotechnology, Shanghai, China) according to the manufacturer's protocols. Endogenous Ca^{2+} and ascorbic acid levels were determined using kits (C004-2 for Ca^{2+} and A009 for ascorbic acid, Jianchen, Nanjing, China). Starch concentrations were determined as described by Gu et al. (2013).

2.6. Statistical analyses

SPSS (SPSS Inc., Chicago, IL, USA) was employed to perform ANOVA analyses. Subsequently, Duncan's multiple range test was used to analyze physiological data, in which means denoted by the same letter do not significantly differ at $P < 0.05$ (n = 3). Transcriptomic data were analyzed by the student's *t*-test at a significance level of $P < 0.05$ (n = 3).

3. Results

3.1. The expression patterns of cassava CAMs, CMLs, CBLs, and CPKs

Calcium signals are sensed and decoded by Ca^{2+} sensors, including CAMs, CMLs, CBLs, and CPKs (Hu et al., 2015, 2016a). To elucidate the role of calcium sensors in physiological deterioration of the roots, expression profiling of genes encoding Ca^{2+} sensors were performed using previous transcriptomic data (Hu et al., 2016b). Compared with 0 h after harvest, 19, 31, and 12 calcium sensor genes showed upregulation (log_2-based fold-change > 1; *P*-value < 0.05), whereas 1, 3, and 3 genes showed downregulation (log_2-based fold-change < −1; *P*-value < 0.05) at 6 h, 12 h, and 48 h after harvest, respectively. In contrast to different stages of control samples, no calcium sensor genes showed significant changes at transcriptional levels after melatonin treatment (Fig. 1; Table S2). These results suggest that most of the calcium sensor genes were upregulated in roots at different postharvest stages.

3.2. The role of calcium in regulating postharvest physiological deterioration and the quality of cassava tuberous roots

To study the effect of calcium on regulating postharvest physiological deterioration and the quality of roots, slices were treated with water (control), $CaCl_2$, or EGTA for 2 h, then postharvest physiological deterioration symptoms and several quality indices were measured up to 72 h (Figs. 2 and 3). Under normal conditions, the physiological deterioration symptoms were observed as slight 'vascular streaking' at 6 h, then 'vascular discoloration' symptoms with brown color spread from the vascular regions to the entire surface of the cassava tuber slices with 12 h–72 h incubation. Further physiological measurements showed that the Ca^{2+}, melatonin, and ascorbic acid levels had two peaks at 2 h and 24 h of incubation, and starch concentration decreased during the incubation period. Compared with the controls, the application of exogenous $CaCl_2$ resulted in reduced physiological deterioration during the 6 h–72 h incubation period, increased concentration of cellular Ca^{2+} during 0 h–48 h and melatonin during 0 h–48 h, and slowed down the decrease of ascorbic acid during 0 h–72 h and of starch during 12 h–72 h. Conversely, the exogenous application of EGTA, a Ca^{2+} chelator, aggravated postharvest physiological deterioration development after 6 h–72 h of treatment and decreased the concentration of cellular Ca^{2+} after 0 h–48 h treatment, melatonin 0 h–48 h, ascorbic acid 0 h–24 h, and starch after 12 h–72 h. These results suggest that calcium can reduce physiological deterioration and quality loss in cassava tuberous roots after harvest. Moreover, the effects of Ca^{2+} and EGTA on physiological deterioration were validated in whole tuberous roots after 0 d–21 d storage (Fig. S1).

To provide molecular evidence for calcium-induced increase of melatonin, the expression levels of genes related to melatonin synthesis (*MeTDC1*, *MeTDC2*, *MeT5H*, *MeASMT1*, *MeASMT2*, *MeASMT3*, and *MeSNAT*) were examined under untreated, $CaCl_2$ or EGTA treatments. The expression levels of all the tested genes were significantly affected by $CaCl_2$ and EGTA. Notably, compared to the control samples, these genes, except for *MeASMT1*, were upregulated after $CaCl_2$ treatment, whereas downregulated after EGTA treatments at various time points (Fig. 4). These results suggest that the application of exogenous calcium induces changes in the expression of melatonin synthesis-related genes.

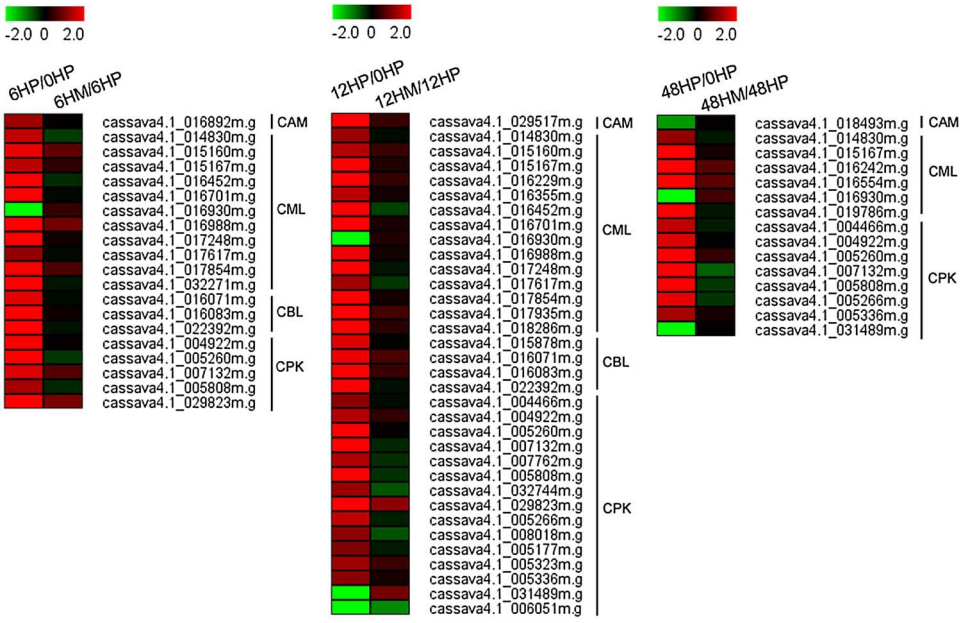

Fig. 1. Expression profiles of *CAMs*, *CMLs*, *CBLs* and *CPKs* in cassava tuberous roots and in response to melatonin treatment at different postharvest stages. Whole tuberous roots were incubated in water (control) or 1×10^{-4} M melatonin for 2 h. Subsequently, roots were cut into slices and placed in an incubator in the dark (28 °C and 60% relative humidity). After incubation for 0 h, 6 h, 12 h and 48 h, slices were sampled to perform transcriptomic analysis. *CAMs*, *CMLs*, *CBLs* and *CPKs* genes that showed significant expression changes (log_2-based fold-change > 1 or < − 1; *P*-value < 0.05) at 6 h, 12 h, and 48 h after harvest in comparison to 0 h after harvest (6HP/0HP, 12HP/0HP, and 48HP/0HP) were extracted from our transcriptomic data. Then, the expression of these genes in response to melatonin treatment was also identified from the transcriptomic data. The heat map was constructed according to the Log2 based FPKM values of cassava *CAMs*, *CMLs*, *CBLs* and *CPKs* from three biological replicates. The scale represents the relative signal intensity of Los2 based FPKM values. HP, hour postharvest; HM, hours after melatonin treatment.

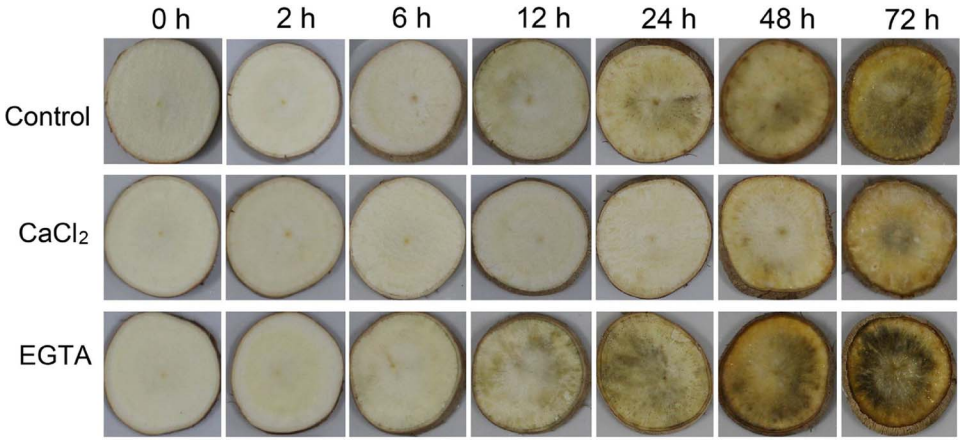

Fig. 2. Effects of $CaCl_2$ and EGTA on postharvest physiological deterioration of tuberous roots. Cassava tuberous roots were cut into slices approximately 0.005 m thick, and incubated in water (control), 0.01 M $CaCl_2$, or 0.01 M EGTA for 2 h. Subsequently, the root slices were incubated at 28 °C and 60% relative humidity in the dark for 0 h, 2 h, 6 h, 12 h, 24 h, 48 h and 72 h.

3.3. The role of melatonin in regulating postharvest physiological deterioration and the quality of cassava tuberous roots

To study the effect of melatonin on regulating physiological deterioration, root slices were treated with water (control) or different concentrations of melatonin for 2 h. Compared with the control samples, melatonin treatment reduced deterioration of tuberous roots, and the eff ;ect was dose-dependent (Fig. 5). Furthermore, several quality indices were measured at 0 h, 2 h, 6 h, 12 h, 24 h, 48 h, and 72 h after treatment (Fig. 6). Compared with the control samples, the application of exogenous melatonin increased melatonin accumulation after 0–48 h of treatment and slowed down the reduction in ascorbic acid concentration at 0 h–24 h and starch at 12 h–72 h after harvest. However, treatment with melatonin did not result in any changes in cellular Ca^{2+} concentration. These results suggest that melatonin can not only reduce physiological deterioration, but also slow down loss of endogenous ascorbic acid and starch concentration.

3.4. The combined effect of melatonin and EGTA on regulating postharvest physiological deterioration of cassava tuberous roots

Exogenous melatonin treatment reduced postharvest physiological deterioration after 12 h–72 h. However, EGTA treatment aggravated deterioration after 6 h–72 h. The combined application of EGTA and melatonin resulted in a reduction in melatonin-induced deterioration, as well as alleviated EGTA-triggered deterioration (Fig. 7). These results indicate the possible crosstalk between melatonin and calcium in deterioration.

4. Discussion

Quality and marketability of cassava is largely reduced by rapid postharvest physiological deterioration. Increasing evidence shows that oxidative burst is one of the earliest events during postharvest physiological deterioration (Iyer et al., 2010; Reilly et al., 2001, 2004; Zidenga et al., 2012; Xu et al., 2013; Vanderschuren et al., 2014;). Genetic modification is an effective strategy to delay postharvest physiological deterioration and extend the shelf life of cassava tuberous roots (Zidenga et al., 2012; Xu et al., 2013; Vanderschuren et al., 2014), as is chemical application (Bajwa et al., 2015; Ma et al., 2016). However, our understanding of the control of postharvest physiological deterioration by chemical application is limited.

Calcium, as an important second messenger, plays crucial roles in various aspects of plant biological processes. During plant growth and

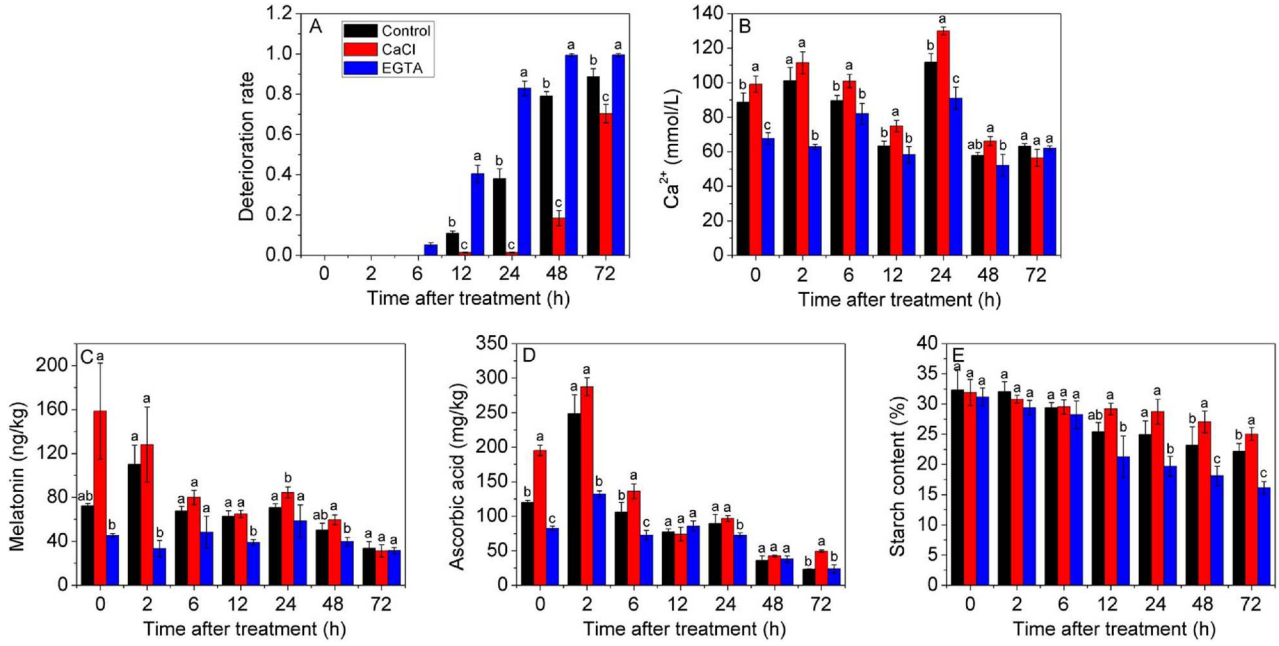

Fig. 3. Deterioration rate (A) and concentrations of Ca^{2+} (B), melatonin (C), ascorbic acid (D) and starch (E) of cassava tuberous roots under untreated, $CaCl_2$ or EGTA treatments. Data are means ± SD calculated from three biological experiments. Means denoted by the same letter do not significantly differ at $P < 0.05$ as determined by Duncan's multiple range test.

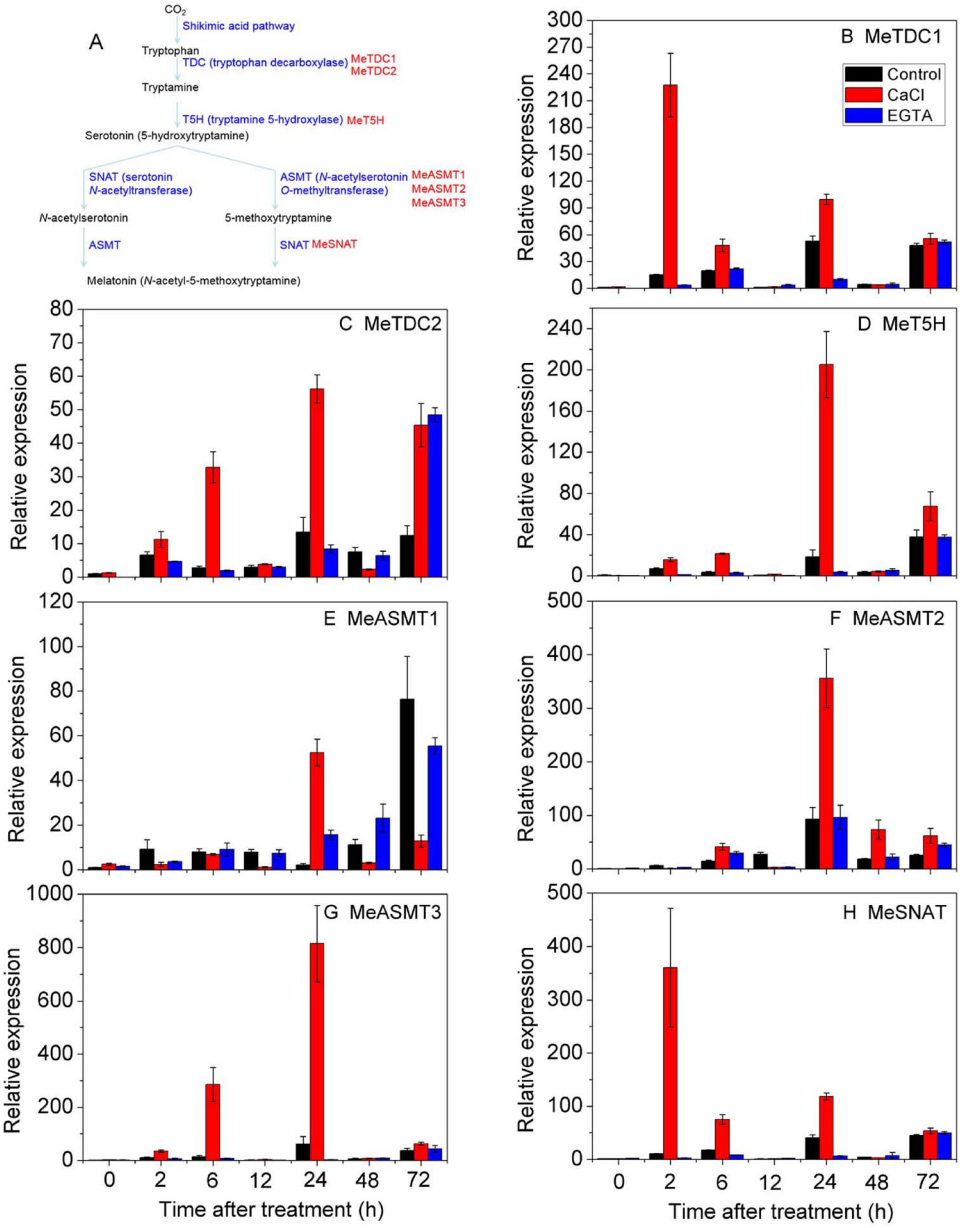

Fig. 4. Expression profiles of the genes responsible for melatonin synthesis under untreated, $CaCl_2$ or EGTA treatments. (A) the genes involved in melatonin synthesis pathway. (B–H) the expression patterns of those genes under untreated, $CaCl_2$ or EGTA treatments. The mRNA fold difference was relative to that of control samples at 0 h. Data are means ± SD of n = 3 biological replicates.

development and in response to light, pathogens, abiotic stresses and hormones, cellular calcium levels change (Hu et al., 2015, 2016a). Calcium signals are sensed and decoded by Ca^{2+} sensors, including CAMs, CMLs, CBLs, and CPKs, and transduced by various types of transcription factors (Mittler et al., 2004; Hu et al., 2015, 2016a). Transcriptome analysis indicates that most of the Ca^{2+} sensor genes are upregulated in cassava tuberous roots at different stages after harvest (Fig. 1), which is in agreement with the findings of a recent study that CaM transcript and protein expression levels are upregulated during this time (Qin et al., 2017).

The exogenous application of $CaCl_2$ resulted in a reduction of deterioration, whereas it was aggravated by EGTA, indicating the important role of calcium in reducing deterioration (Fig. 2). A series of physiological and biochemical changes occur in the cassava tuberous roots after harvest, including decrease in starch, ascorbic acid, dry matter, and protein content and an increase in soluble sugar and organic acid content (Uarrota et al., 2016). Based on the importance of starch and ascorbic acid in cassava, these two indices were examined after harvest. The results indicate that the application of $CaCl_2$ reduces the degradation of ascorbic acid and starch, whereas EGTA elicited the opposite effect (Fig. 3). Overall, the evidence suggests that calcium is involved in reducing physiological deterioration and quality loss in cassava tuberous roots after harvest.

Melatonin reduces postharvest physiological deterioration in cassava tuberous roots (Ma et al., 2016). Transcriptomic analysis has also shown that melatonin activates genes that are involved in ROS-scavenging and ROS signal transduction pathways in cassava after harvest (Hu et al., 2016b). In present study, melatonin application reduced degradation of ascorbic acid and starch (Figs. 5 and 6). These findings indicate that melatonin is also a regulator of cassava quality after harvest, which is coincides with the results of our previous transcriptomic analysis that show that the expression of starch degradation-related genes is downregulated at the middle and late stages of melatonin-treated cassava tuberous roots (Hu et al., 2016b).

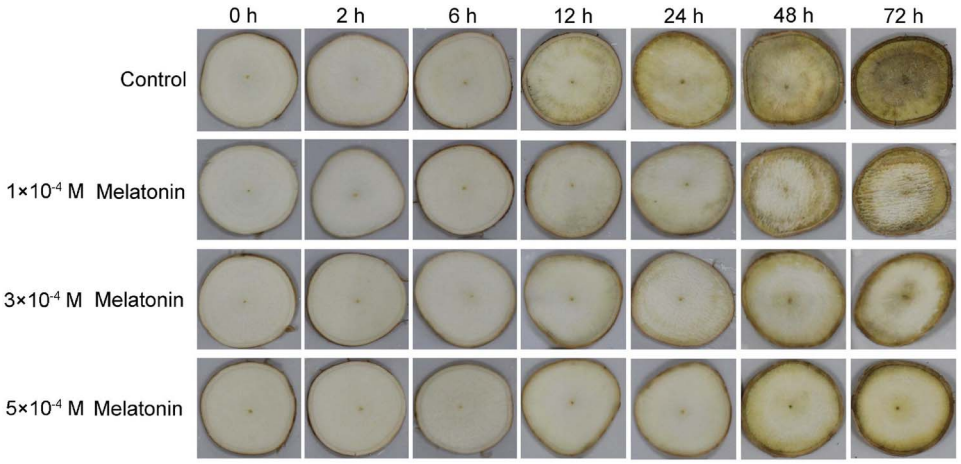

Fig. 5. Effect of melatonin on postharvest physiological deterioration of tuberous roots. Cassava tuberous roots were cut into slices approximately 0.005 m thick. Then, the root slices were incubated in water (control) 1×10^{-4} M, 3×10^{-4} M, and 5×10^{-4} M melatonin for 2 h. Subsequently, the root slices were incubated at 28 °C and 60% relative humidity in the dark.

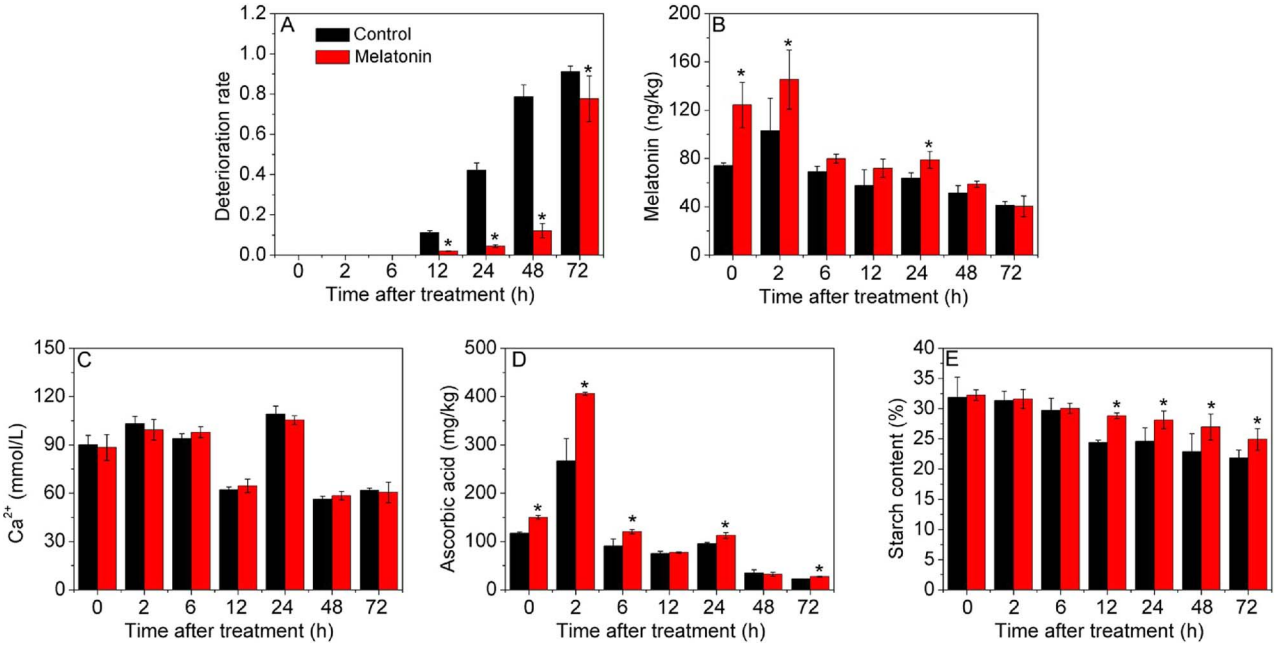

Fig. 6. Quantification of deterioration rate (A), melatonin (B), Ca^{2+} (C), ascorbic acid (D) and starch (E) of cassava tuberous roots under untreated or melatonin treatment (1×10^{-4} M) during postharvest physiological deterioration. Data are means ± SD calculated from three biological experiments. Asterisks indicate significant difference ($P < 0.05$) compared with the control treatment.

$CaCl_2$ and melatonin treatments had similar effects on the regulation of physiological deterioration and tuberous root quality. The exogenous application of $CaCl_2$ and EGTA increased and decreased cellular melatonin content and the expression of genes that are related to melatonin synthesis, respectively (Figs. 3 and 4). However, the exogenous application of melatonin did not affect the cellular Ca^{2+} concentration (Fig. 6). Calcium and melatonin are crucial regulators of cellular ROS levels by activating antioxidant enzymes that are involved in various biological processes (Tan et al., 1993, 2003; Kolar and Machackova, 2005; He et al., 2012; Shi and Chan, 2014; Zhang and Zhang, 2014; Srivastava et al., 2015; Hu et al., 2016b; Ma et al., 2016). It is possible that calcium induces melatonin biosynthesis, which activates antioxidant enzymes and represses ROS accumulation, thereby leading to a reduction in physiological deterioration and quality loss. Additionally, pretreatment with EGTA arrested the melatonin-induced reduction in deterioration, suggesting the possible crosstalk between melatonin and calcium (Fig. 7). Although $CaCl_2$ and melatonin have positive effects on deterioration in cassava, there are some potential challenges that hinder its commercial use. Firstly, production of melatonin needs high cost. Secondly, exogenous application of $CaCl_2$ and melatonin can only reduce physiological deterioration to a certain extent. Further efforts should be made to decrease the production cost of $CaCl_2$ and melatonin as well as to enhance its effect by the combined application of multiple chemicals.

In summary, this is the first report that shows the role of calcium in regulating postharvest physiological deterioration and tuberous root quality of cassava, as well as a relationship between calcium and melatonin. Calcium-induced activation of melatonin biosynthesis may play a crucial role in reducing physiological deterioration of cassava roots. Further studies to elucidate the mechanism underlying the effect of calcium and melatonin on postharvest physiological deterioration and quality are warranted.

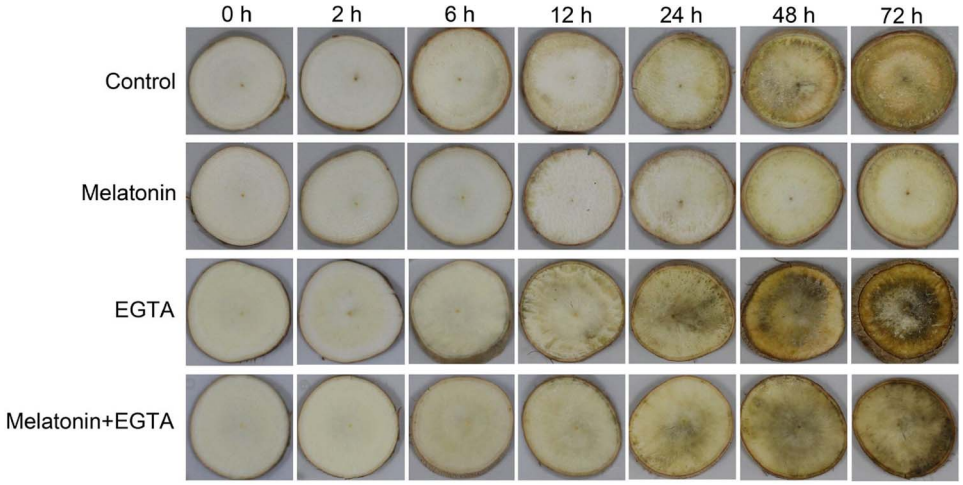

Fig. 7. The combined effect of melatonin and EGTA on postharvest physiological deterioration of cassava tuberous roots. Cassava tuberous roots were cut into slices approximately 0.005 m thick. The root slices were pretreated with 0.01 M EGTA for 2 h, then exposed to 1×10^{-4} M melatonin for 2 h. Subsequently, the root slices were incubated at 28 °C and 60% relative humidity in the dark.

5. Competing interests

The authors declare that they have no competing interests.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2018.02.007.

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