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The changes of intracellular calcium concentration and distribution in the hard end pear (*Pyrus pyrifolia* cv. 'Whangkeumbae') fruit☆

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ABSTRACT

Hard end is a physiological disorder of pear fruit that is frequently observed in the 'Whangkeumbae' (*Pyrus pyrifolia*) variety, however, the mechanisms that are involved in its development are poorly understood. In this study, we explored the causes of hard end disorder in pear fruit in relation to calcium deficiency. During fruit development, the ratio of Ca/N, Ca/K, Ca/Mg and the content of B were significantly lower in the hard end fruit as compared to normal fruit. However, no calcium deficiency was detected in the soil and leaves of the orchard where the hard end fruit were located. Additionally, the Ca^{2+} influx in the calyx of hard end fruit was lower than that of normal fruit at 90 d after anthesis. The free Ca^{2+} and storage Ca^{2+} in the flesh cells of hard end fruit were less than that of normal fruit during fruit development, while an opposite tendency was observed at 120 d after anthesis (harvest day). In hard end fruit, the Ca^{2+} transport-related gene, *PpCNGC1* (Cyclic nucleotide-gated ion channel 1), was up-regulated; whereas the Ca^{2+} sensor-related genes of *PpCIPKs*, *PpCDPK28* and *PpCML41* were all down-regulated. Spraying with a 2% calcium chloride ($CaCl_2$) solution inhibited the incidence rate of hard end disorder and decreased fruit firmness and lignin content during storage. Additionally, the ratio of Ca/N, Ca/K, Ca/Mg and the content of B all increased on harvest day. Our study suggests that low Ca^{2+} influx leads to less Ca^{2+} into the pear fruit, which results in an intracellular imbalance of Ca^{2+} and consequently triggers the development of hard end disorder.

1. Introduction

Hard end is a pear fruit disorder in which the tissue of the calyx end of the ripe fruit is hard and dry [1]. The disorder has been reported primarily in most of the European cultivars, such as Bartlett, Anjou and Winter Nelis [2]. In recent years, it has been frequently observed within the 'Whangkeumbae' (*Pyrus pyrifolia*) variety from several pear orchards in the Laiyang district of the Shandong Province of China. In hard end pear fruit, the sclereid and lignin content was increased, which resulted in higher firmness of pear fruit and decreased the fresh market value furtherly [3]. The hard end disorder of pear fruit was postulated to be caused by certain metabolites that are produced in Asian pear rootstocks and translocated to the fruit, additional possible causes for the disorder are water stress, lignin accumulation and calcium deficiency [3–6].

Calcium (Ca) has been considered to be one of the pre-harvest factors that significantly influence the development of disorders [7]. In fruit, many physiological disorders are closely related to Ca deficiency [8–10]. For example, in tomato fruit, Ca deficiency was shown to induce blossom end rot [11]. In apple fruit, a low level of Ca increased the sensitivity to bitter pit [12]. Additionally, the 'Anjou' pear fruit with several disorders, such as stony pit, green stain, alfalfa greening, cork spot and black end; were lower in Ca levels as compared to normal fruit [13]. Nevertheless, the involvement of Ca in hard end development is still not well understood. Although many studies have revealed that several pear disorders were related to a lower Ca content [8,9,14], there are no comprehensive analyses on Ca content, Ca^{2+} localization and its flux in hard end 'Whangkeumbae' pear fruit. Besides Ca, the levels of other mineral nutrients could also influence fruit susceptibility to calcium deficiency disorders [7]. Previous studies suggested a high level of N reduced the movement of Ca towards the fruit, which was possibly one of the causes for the development of the hard end disorder [15–20]. Additionally, the K^+ and Mg^{2+} could compete with Ca^{2+} for binding sites at the plasma membrane, which limited the Ca content in the fruit [21,22]. Therefore, the nutrient concentration ratios such as N/Ca, K/Ca, and Mg/Ca are usually more precise to predict Ca deficiency

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disorders than total fruit Ca concentration alone [12,23,24]. Studies also have shown that B deficiency is capable inducing the accumulation of lignin in plants [25–27], which is an obvious symptom of hard end disorder.

In plants, Ca deficiency disorders have puzzled researchers for many years, and at the present time, little is known pertaining to the mechanisms that are involved with these disorders [15,28,29]. In addition, there are no comprehensive and thorough studies on the hard end disorder which occurs in 'Whangkeumbae' pear fruit. In this study, we performed the first in-depth analyses which aimed to reveal the causes of this disorder from the point of view of Ca deficiency.

2. Materials and methods

2.1. Plant material

Orchards containing 10-year-old 'Whangkeumbae' pear trees were located in Laiyang City, Shandong Province, People's Republic of China. Standard irrigation and fertilization conditions were applied and were identical throughout the orchards. One orchard (Orchard 1) of 'Whangkeumbae' pear without hard end pear fruit and another orchard (Orchard 2) with many hard end pear fruit were used in the study to collect samples, respectively. Fruit from Orchard 1 and 2 were designated as control and hard end fruit, respectively.

After a period of 75, 90, 105 and 120 days (d) after anthesis, fruit were sampled from the two orchards; with 10 fruit examined in each of the three replicates. Flesh tissue samples were taken from the calyx-end of the fruit (which did not contain kernels or peels), were sliced and immediately frozen in liquid nitrogen and maintained at −70 °C.

In a separate experiment, $CaCl_2$ treatment was performed on Orchard 2 by spraying 2% $CaCl_2$ on the fruit at 30, 45 and 75 d after anthesis, respectively. Fruits of the two orchards were harvested at 120 d after anthesis and were subsequently transported to the laboratory within a period of 2 h. The fruits of uniform size and without pests or diseases were stored at 2 °C. During the storage period, 10 fruits from each of the three replicates were sampled every 30 d. The flesh tissue without kernels or peels were sampled as described above at 0, 60 and 120 d after harvest.

2.2. Determinations of the total contents of Ca, Mg, K, B and N

Samples were dried in an oven at 70 °C until a constant weight was achieved. In this study, 0.5 g of flesh and leaf tissues (taken from near the fruit) were mixed with 2 mL of perchloric and 10 mL nitric acids, and 0.5 g of soil sample (taken from near the fruit tree, 10 cm depth from the ground) were mixed with 2 mL perchloric, 10 mL nitric and 5 mL of hydrofluoric acids. After digestion and dissolution, the total contents of Ca, Mg, K and B were analyzed by using anatomic absorption spectrophotometer (AAnalyst100, PerkinElmer Inc., USA).

For the determination of N content, 1 g of dried flesh samples were mixed with 0.3 g $CuSO_4$, 3 g anhydrous Na_2SO_4 and 10–12 mL concentrated H_2SO_4. After digestion and dissolution, the N content was assayed using a Kjeldahl nitrogen apparatus (FOSS-2100, FOSS, Switzerland).

2.3. Observations of Ca^{2+} localization

The localization of free Ca^{2+} was observed by fluorescence imaging as previously described by Qu et al. [30] with some modifications. Thin slices of flesh were collected from the same calyx region under the peel tissue of normal and hard end fruit by using a razor blade. The flesh tissues were initially washed twice with HEPES buffer solution, which were loaded with fluo-3/AM at 4 °C for 2 h and then subsequently washed three times with HEPES buffer solution. After maintaining the tissue in the dark at 25 °C for 2 h, we visualized fluo-3 fluorescence (488 nm excitation laser light and 525–530 nm long-pass emission filter) using a laser scanning confocal microscope (TCSSP5 II, Leica, Germany). The fluorescence results were analyzed using Image-Pro Plus software and Microsoft Excel 2010 [31].

The localization of storage Ca^{2+} was observed with cytochemical methods as described by Suzuki et al. [32] with slight modifications. The flesh samples were obtained from the calyx of control and hard end pear fruits. Each sample was obtained from the same region under the peel tissue and was cut into 1 mm^3 sections using a razorblade. The samples were fixed in 2.5% (v/v) glutaraldehyde in 60 mM potassium phosphate buffer (pH 7.8) containing 2% (w/v) potassium antimonite and 2% (w/v) paraformaldehyde at 4 °C for 4 h. After washing in the same buffer with 2% (w/v) potassium antimonite every 30 min for three times, the samples were post-fixed in 1% (v/v) osmic acid in 60 mM potassium phosphate buffer (pH 7.8) containing 2% (w/v) potassium antimonate at 4 °C for 8 h, and then dehydrated in a graded series of acetone (30%, 50%, 70%, 80%, 90%, 100%). Finally, the samples were embedded in epoxy resin (EPON812, SPI supplies division of structure probe Inc., USA). To examine the Ca^{2+} localization, 80-nm-thick sections were prepared using a diamond knife on an ultramicrotome (UC7, Leica, Germany). Next, the sections were examined using a transmission electron microscope (HT7700, Hitachi, Japan) at an operating voltage of 80 kV. To confirm the specificity of the potassium-pyroantimonate precipitation reaction, a control was obtained using sections that were treated with 0.1 M methylene glycol tetraacetic acid (EGTA) at 60 °C for 1 h to remove the calcium pyroantimonate precipitation.

2.4. Measurements of Ca^{2+} flux

The Ca^{2+} fluxes were measured in the Younger USA Xuyue (Beijing) BioFunction Institute by using Non-invasive Micro-test Technology (NMT100 Series, Younger USA LLC, USA; Xuyue Sci. & Tech. Co., Ltd., China) and imFluxes V2.0 software (Younger USA, LLC, Amherst, USA). A Ca^{2+}-microsensor was prepared as previously described [33]. Prior to the flux measurement, the microsensor was calibrated with cultural media having different concentrations of Ca^{2+}, 0.5 mM and 0.05 mM, respectively. Only microsensors with a Nernstian slope > 22 mV/decade were used in this study. The probes measured the voltage at two additional points: close to the tip and 30 μm farther away. The probe oscillation was 0.3 Hz. The Ca^{2+} fluxes were calculated by using the JCal V3.3 (a free MS Excel spreadsheet, youngerusa.com or xbi.org) from Fick's law of diffusion: $J = -D_0 \cdot (dc/dx)$, where J is the ion flux (positive represents outflow, negative represents inflow), D_0 is the ion diffusion constant in a particular medium, and dc/dx is the ion concentration gradient.

2.5. Determination of fruit firmness and lignin content

During the storage period, fruit firmness was determined for ten fruits in each of the three replicates on the fruit calyx. A texture analyzer (CT3; Brookfield, USA) was used with a 2 mm diameter probe, 10 mm penetration depth, and 0.5 mm/s penetration rate. Measurements were made on four sides of each fruit after the removal of a small piece of peel, and the data were expressed in kg/cm^2.

The lignin content was measured as previously described by Dyckmans and others [34].

2.6. RNA-Seq protocol

At 120 d after harvest, the flesh tissue of normal and hard end fruit without kernels or peels were sampled and sequenced using an Illumina HiSeq™ 2500 system at the Biomarker Technologies Corporation (Beijing, China). The clean reads were subsequently mapped to the pear reference genome (https://www.rosaceae.org/species/pyrus/pyrus_communis/genome_v1.0). In this study, the RNA-Seq project for 'Whangkeumbae' pear was initiated (http://www.ncbi.nlm.nih.gov/