Research Report

Changes in Cell Ca^{2+} Distribution in Loquat Leaves and Its Effects on Cold Tolerance

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Abstract: Calcium has been associated with improved cold tolerance in many crops. The aim of this study was to investigate the changes in leaf cell Ca^{2+} distribution and cell organelle ultrastructure of loquat (*Eriobotrya japonica* Lindl.) plants in response to cold stress at -3°C , using transmission electron microscopy (TEM). Two loquat accessions, Zaozhong 6 (a commercial cultivar) and oakleaf loquat (a wild relative) were used. Cold tolerance, as measured by leaf browning rate, was higher in oakleaf plants, and calcium treatment improved cold tolerance in both species. Cold stress first induced inward transport of Ca^{2+} from the intracellular space. Then, the imported Ca^{2+} was aggregated around the chloroplast membrane, finally entering the chloroplast. This pattern of Ca^{2+} distribution in leaf cells occurred earlier in Zaozhong 6 than in the wild loquat. With increasing time of cold exposure, the chloroplast membranes of Zaozhong 6 leaves were damaged, blurred and even disappeared, while those of wild oakleaf loquat leaves maintained their structure longer. In Zaozhong 6, cold stress induced a clear cavity between poorly structured granal thylakoids and vesicles appearing inside the chloroplast, while in oakleaf leaves cold stress had little effect on the ultrastructure of chloroplasts (although chloroplast membranes looked blurred). Loquat leaves accumulated free calcium ions around chloroplasts in response to cold stress, with earlier calcium accumulation occurring in the cold-sensitive cultivar Zaozhong 6 than in wild oakleaf loquat. These results demonstrate that these two loquat species have differences in both cold tolerance and calcium accumulation dynamics.

Additional key words: cold stress, injury, low temperature treatment, subcellular localization, ultrastructure

Introduction

Loquat (*Eriobotrya japonica* Lindl.) is a subtropical evergreen fruit crop originated from southeastern China (Hueso and Cuevas, 2008). It has been introduced to many other countries, such as Japan, India, Australia, New Zealand, Madagascar, South Africa and Mediterranean countries in Europe (He et al., 2011). The fresh fruits of loquat can be consumed directly or processed into jam and jelly. Its leaves have been documented to possess medicinal values and have been used to treat skin diseases, diabetes, chronic bronchitis, coughs, phlegm and ulcers (Ferrerres et al., 2009).

China is the world's largest producer of loquat with a cultivation area of 120,000 ha, producing more than 400,000 tons of fresh fruits per annum (Hueso and Cuevas, 2008). Putian city of Fujian province is one of the largest loquat producing regions in China with 80,000 tons of annual production (Jiang et al., 2010). Loquat plants bloom in autumn, and fruits develop during winter and mature in early spring (Reig et al., 2011). Hence fruit development is at high risk to the cold damage during winter. Generally, cold damage to loquat occurs when temperature drops to less than -6°C at flowering stage and to less than -3°C at the early fruiting stage (Yang et al., 2003). Jiang et al.

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(2010) reported that more than 80% loquat trees in Putian regions were cold damaged in 2004, causing 13,300 tons of yield reduction and 65 million Chinese Yuan of economic loss. Cold damage is therefore a major limiting factor for loquat production and area expansion (Wang et al., 2008).

Calcium plays an important regulatory role in plant's response to multiple environmental stresses (Kader and Lindberg 2010; Shao et al., 2008; Yoon et al., 2010). It plays an important role in the signal reception, transduction and responses of plant to the external biotic and abiotic stresses (Guo et al., 2004; Snedden and Fromm, 2001). The role of calcium has been implicated in plant responses to cold tolerance (Braam and Davis, 1990), mechanical stimulation (Botella and Arteca, 1994; Braam and Davis, 1990), drought stress (Shao et al., 2008) and oxidative stress (Harding et al., 1997). Calcium also assists in maintaining stabilization and integrity of cell membrane and its function (Msadek et al., 1998), reducing lipid peroxidation (Zeng et al., 1987), and inducing gene expression and synthesis of specific proteins (Gao and Chen, 2002; Kim et al., 2009). The alleviatory effect of Ca^{2+} on the damage induced by different abiotic stresses has been reported in many fruit trees such as apple (*Malus pumila* Mill), pear (*Pyrus* spp.) and mango (*Mangifera indica* L.) (Chen and Liu, 2001). Pan et al. (2000) found that Ca^{+} regulated the activities of superoxide dismutase (SOD) by activating cellular calmodulin (CaM), thereby reducing the membrane injury of mango leaves by water stress. It has been reported that CaCl_2 sprays or application of $\text{Ca}(\text{NO}_3)_2$ fertilizer increased Ca^{+} concentrations in leaves and consequently increased cold tolerance in pear trees, reducing the incidence of fruit disorders (Raese, 1996).

Cytosolic Ca^{2+} increases rapidly and its distribution in cell changes accordingly in response to environmental stresses, which is a critical step in the transduction of stress signal and the response of plant to stress (Guo et al., 2004; Liu et al., 2010). Biyaseheva et al. (1993) showed that heat shock resulted in increased intracellular Ca^{2+} in pea mesophyll protoplasts. However, little is reported about the changes of Ca^{2+} distribution in loquat during low temperature stress. The cytochemical method of transmission electron microscopy (TEM) has been widely used for subcellular calcium localization (Jian et al., 1999; Meng et al., 2000). The present study was to determine cold tolerance, changes in cell Ca^{2+} distribution and cell superstructure in the leaves of two distinct loquat accessions under cold stress. The possible role of Ca^{2+} in the alleviation of cold damage was also discussed.

Materials and Methods

Plant Materials

A commercial loquat cultivar Zaozhong 6 (*Eriobotrya japonica* Lindl.) and a wild accession oakleaf loquat (*Eriobotrya prinoidea* Rehd) were obtained from the National Germplasm Nursery, Fujian Academy of Agriculture Sciences, China. The scions were grafted onto the rootstock of Jiefangzhong (*E. japonica*) and grown in pots (one plant per pot) in an orchard in Fujian Agricultural and Forestry University, Fujian, China. The grafted trees were placed randomly, irrigated and fertilized according to common district practices. Three years after the grafting, trees with uniform and vigorous growth were selected and used as experimental materials.

Calcium Pre-treatment

Calcium pre-treatment was conducted at 9:00 am on 15 September, 2009. CaCl_2 solution at 10 mM was sprayed onto each loquat plant until the solution began to run off from the leaves. The spraying treatments were made once every three days and repeated four times. This study used a randomised complete block design with two loquat varieties, two levels of CaCl_2 (water and 10 mM) and three replicates. Each pot was properly labelled to identify the treatments and irrigated when necessary during the experimental period.

Low Temperature Treatment

The cold stress experiment was carried out in the Horticultural Laboratory of Fujian Agriculture and Forestry University from March to April, 2009. The cold treatment procedure was adopted from the methods of Xie and Li (2006) and Wang et al. (2008). The pre-treated loquat plants (whole plants in pots) with or without CaCl_2 were placed in a temperature controllable freezer (ETC-100, Shanghai Jingchuang Electronics Manufacturing Co., Ltd. China). The temperature was initially set to 10°C and gradually decreased to 4°C at a rate of 10°C/h and then remained constant at 4°C for 10 h. The temperature was further decreased to -3°C at a rate of 2°C/h. The fourth and fifth leaves from the top were sampled after 0, 1.5, 3, 4.5, 6 and 7.5 h of the cold treatment at -3°C and used as the materials for ultrastructure observation. The treated plants were thawed under room temperature. Leaf browning was observed after 12 h. The leaf with more than 50% browning in total leaf area was considered as cold-damaged browning leaf.

TEM Observation

TEM observation was performed as previously described (Borgers et al., 1983) with minor modifications. Briefly, leaf tissue from one third of the top section of the harvested leaves was cut into pieces (1 mm × 2 mm) and prefixed in 3% (v/v) glutaraldehyde in 2% (w/v) potassium antimonite solution containing 0.1 M phosphate-buffered saline (PBS, pH 7.6) at 4°C for 24 h. Then the prefixed leaf samples were washed four times, 30 min each, with 2% (w/v) potassium antimonite (0.1 M PBS, pH 7.6) and postfixed in 1% (w/v) osmium tetroxide containing 2% (w/v) antimonite and 0.1 M PBS (pH 7.6) at 4°C for 4 h. The postfixed samples were washed four times, 30 min each, with double distilled water (pH 6.0) and then two times, 30 min each, with double distilled water (pH 10.0). After this, the samples were dehydrated in a gradient series of ethanol and acetone, and finally embedded in Epon 618 resin. The sections (50 nm in thickness) were obtained by using an LKB-5 ultramicrotome (LKB, Bromma, Sweden), stained with uranyl acetate, and observed in a JEM 1010 TEM (JEOL, Tokyo, Japan) at 80 kV.

In order to confirm that the presence of Ca^{2+} deposits, chelation of calcium ions with ethylenediamine tetraacetic acid (EDTA) was performed. The mounted tissue sections with the presence of potassium antimonite previously confirmed by TEM were immersed in 100 mM EDTA (pH 8.0) and incubated at 37°C for 1.5 h. After incubation, the grids were rinsed briefly with distilled water and stained with uranyl acetate. EDTA chelates with Ca^{2+} resulted in the removal of Ca^{2+} . The disappearance of deposited Ca^{2+} was examined under TEM.

Results

Loquat Leaves Damaged by Cold Treatment

The wild accession oakleaf loquat was more tolerant than the commercial loquat cultivar Zaozhong 6 when exposed to the cold treatment at -3°C (Fig. 1). The leaves of the wild loquat accession remained intact after 1.5 h of the cold treatment, while those of the Zaozhong 6 were damaged by 12-18%, depending on the calcium treatment. Similarly, the nil-calcium control Zaozhong 6 plants had a frost injury index of 95.23% at 4.5 h after the cold treatment, as compared to 65.87% for oakleaf loquat.

The CaCl_2 pre-treatment improved cold tolerance in both Zaozhong 6 and the wild oakleaf loquat. After 3 h, the CaCl_2 treatment reduced the leaf browning rate by 9% in Zaozhong 6 and by 4% in the wild oakleaf loquat when

compared with the untreated control. These results indicated that the CaCl_2 treatment increased the cold tolerance of both accessions.

The treatment duration also had significant impact on the browning rate. The leaf browning rate increased as the treatment time progressed. For example, the leaf browning rate of Zaozhong 6 was 18.23%, 75.67%, 95.25% and 100% at 1.5, 3, 4.5 and 6 h after cold treatment. All leaves of Zaozhong 6 were damaged after 6 h of cold treatment, irrespective to the CaCl_2 pre-treatment.

Subcellular Calcium Localization

The free Ca^{2+} in plant cells reacted with antimonite and resulted in the formation of a calcium antimonate precipitate. The precipitate was present in black granules under TEM, indicating the presence of free Ca^{2+} , which was clearly seen in the leaf cells of oakleaf loquat (Fig. 2A). After the EDTA treatment, the black granules disappeared, leaving a transient part on the same area (Fig. 2B). This was due to the chelating reaction of EDTA with Ca^{2+} , resulting in the removal of Ca^{2+} . This procedure verified the presence of free Ca^{2+} in loquat cells.

Changes in Cellular Ca^{2+} Level by CaCl_2 Treatment

The calcium levels in the leaf cells of loquat were increased by foliar CaCl_2 application, as clearly shown in the leaf cells of Zaozhong 6 loquat plants (Fig. 3B) compared to the nil-calcium control (Fig. 3A). As shown in the Fig. 3A, only a few black granules were observed in the intracellular

Fig. 1. Browning rate (%) of the loquat leaves cold-stressed at -3°C, as determined 12 h after incubation at room temperature. Bars represent standard error of the mean.

Fig. 2. Cell ultrastructures of the leaves of oakleaf loquat plants pretreated with 10 mM CaCl_2 , observed by a transmission electron microscopy. A, mesophyll cell, without cold stress (bar = 1 μm); B, mesophyll cell after decalcification with EDTA, without cold stress (bar = 1 μm); C, chloroplast, without cold stress (bar = 0.2 μm); D, chloroplast, 1.5 h exposure at -3°C (bar = 0.2 μm); E, chloroplast, 3 h exposure at -3°C (bar = 0.1 μm); F, chloroplast, 3 h exposure at -3°C (bar = 1.5 μm); G, mesophyll cell, 4.5 h exposure at -3°C (bar = 1 μm); H, chloroplast, 6h exposure at -3°C (bar = 0.2 μm); and I, chloroplast, 7.5 h exposure at -3°C (bar = 0.2 μm); Cw, cell wall; Cp, cytoplasm; Ch, chloroplast; Is, intracellular space.

Fig. 3. Cell ultrastructures of the leaves of Zaozhong 6 plants pretreated with 10 mM CaCl_2 , observed by a transmission electron microscopy. A, mesophyll cell, without cold stress and CaCl_2 (bar = 1 μm); B, mesophyll cell, without cold stress (bar = 1 μm); C, chloroplast, without cold stress (bar = 0.5 μm); D, chloroplast, 1.5 h exposure at -3°C (bar = 0.2 μm); E, chloroplast, 3 h exposure at -3°C (bar = 0.2 μm); F, chloroplast, 4.5 h exposure at -3°C (bar = 0.2 μm); G, chloroplast, 6 h exposure at -3°C (bar = 0.2 μm); and H, chloroplast, 7.5 h exposure at -3°C (bar = 0.2 μm); Cw, cell wall; Cp, cytoplasm; Ch, chloroplast; V, vacuole; Is, intracellular space.

space and the vacuole of the untreated control, while more black granules were observed in the CaCl_2 -treated cells, especially in the intracellular space (Fig. 3B).

Changes in the Chloroplast Ultrastructure and Ca^{2+} Distribution by Cold Treatment

In the absence of cold stress, the stroma lamella and granal thylakoids of the chloroplast in Zaozhong 6 were

clearly visible. The grana lamella was short while the starch granules were relatively large (Figs. 3B and 3C). The cells in oakleaf loquat were relatively larger with a compacted arrangement. They were rich in cell inclusion. The additional calcium precipitated in vacuoles and intracellular spaces (Fig. 3B). The cold treatment (-3°C) changed the chloroplast ultrastructure and Ca^{2+} distribution in Zaozhong 6, depending on the treatment durations. After 1.5 h of the cold treatment, the chloroplast ultrastructure of Zaozhong 6 was not affected and there was little change in calcium precipitates in the chloroplast (Fig. 3D). However, calcium precipitates in the cytoplasm around the chloroplast membrane increased, with about 10 black spots counted after 1.5 h cold treatment (Fig. 3D) as compared to virtually no calcium precipitates prior to the cold exposure (Fig. 3C). After 3 h of exposure, the chloroplast membrane was seriously damaged with abundant precipitates inside the chloroplast. There were about 40 black spots counted (Fig. 3E) as compared to only about 7 black spots after 1.5 h exposure (Fig. 3D), indicating the movement of calcium from the cytoplasm into the chloroplast. After 4.5 h of exposure, chloroplast membrane almost disappeared, causing leakage of stroma thylakoid and the aggregation of calcium precipitates (Fig. 3F). A clear void appeared between poorly structured grana lamella, and the calcium precipitates aggregated when the exposure time progressed to 6 h, which was indicated by the presence of hollow circles (Fig. 3G). Vesicles started to appear inside the chloroplast and calcium accumulation continued when the exposure time reached to 7.5 h (Fig. 3H).

As for the oakleaf loquat, there were only a few calcium precipitates which were evenly distributed in the chloroplast, with almost no precipitates found in the cytoplasm (Fig. 2C) prior to the cold treatment. No obvious changes occurred in chloroplast ultrastructure although there was slight increase in calcium precipitates at the 1.5 h exposure time (Fig. 2D). Meanwhile, calcium was found to have accumulated around cell wall (16 black spots) and plasma membrane (39 smaller black spots). At the 3 h exposure time, the chloroplast ultrastructure remained intact, while a large area with black granules and some calcium precipitates around the edge of the chloroplast and plasma membrane were observed, indicating the accumulation of calcium (Figs. 2E and 2F). After 4.5 h treatment, no obvious effect on chloroplast was observed and only limited calcium precipitates (about 3 small light-coloured black spots) was found inside the chloroplast, but the calcium precipitates aggregated in hollow circles around the chloroplast (Fig. 2G). After 6 h treatment, the thylakoid structure still

remained intact and clear but chloroplast membrane became blurred. Large calcium precipitates (4 black spots) appeared (Fig. 2H). After 7.5 h exposure, the chloroplast membrane was hardly distinguished but the internal structure of chloroplast was still clearly defined. At this stage, the enlarged calcium precipitate granules (black spots) and starch grains (white spots) were observed (Fig. 2I).

Discussion

The leaf injury on loquat leaves increased with increasing exposure time under cold stress at -3°C . CaCl_2 treatment reduced the leaf injury on both accessions. Similar results were observed in other plants (Chen and Liu, 2001; Raese, 1996; You et al., 2001). Raese (1996) reported that calcium foliar application increased calcium concentrations in leaves and fruits of apple (*Malus domestica*) and pear (*Pyrus communis* L.), resulting in improved cold hardiness and yield of the fruit trees.

The cold damage also varied with loquat accessions. Oakleaf loquat demonstrated higher cold-tolerance than Zaozhong 6. Qu and Li (2006) also found that different pear varieties differed in their tolerance to cold stress. The differences in cold injury between cultivars and species of fruit trees have been frequently reported (Rodrigo, 2000). Calcium application could alleviate the cold damage, which could be due to the improved osmotic stress through the signal transduction process (Liu et al., 2010). Chloroplasts are believed to be the most sensitive cell organelles to low temperatures (Kimball and Salisbury, 1973). The present study also showed that calcium application could reduce chloroplast damage during the initial phase of cold treatment. However, further increase in cold treatment duration would eventually result in the collapse of the chloroplasts even though loquat plants responded with the accumulation of calcium around chloroplasts.

TEM observations on two loquat accessions treated with CaCl_2 indicated that free Ca^{2+} level in loquat leaves were very low under normal temperatures. However, under cold stress, Ca^{2+} level in cytoplasm was significantly increased, followed by the accumulation of Ca^{2+} around the chloroplast membrane. Similar changes in subcellular Ca^{2+} localization have also been observed in maize mesophyll cells under drought stress (Ma et al., 2011). The changes in cell Ca^{2+} distribution could be related to the signal transduction associated with Ca^{2+} . It has been well documented that Ca^{2+} plays an important role in signal transduction in response to environmental changes (Chen and Liu, 2001; Kim et al., 2009; Shao et al., 2008). Liu et al. (2010) documented

that Ca^{2+} participated in the signal transduction process of osmotic stress in maize. Generally, plant maintains a low level of Ca^{2+} in cytoplasm by regulating membrane Ca^{2+} transport and the intracellular Ca^{2+} pools (Song and Fu, 1997). Under abiotic stresses, Ca^{2+} was released into cytoplasm from cell walls and vacuoles through Ca^{2+} channel, the activation of Ca^{2+} pools, the reduction of Ca^{2+} pump functions and the Na^+ - Ca^{2+} exchange system (Liu et al., 2010; Saunders and Hepler, 1981; Williamson and Ashley, 1982). Our observation also indicated that Ca^{2+} level rose accordingly with increasing exposure time to the cold stress, resulting in the accumulation of Ca^{2+} in cytoplasm. Further increase in exposure time led to the collapse of chloroplast despite of the responsive accumulation of Ca^{2+} around the chloroplast membrane. The calcium ions were then transported into chloroplast through inward Ca^{2+} channels and Ca^{2+} transport protein (Wang and Zhang, 2002).

Low temperatures have significant impacts on the ultra-structures of chloroplasts (Kimball and Salisbury, 1973). As observed in our experiments, the cold damages on the chloroplast structure such as membrane, stroma lamella and granal thylakoids were more severe in Zaozhong 6 than in wild oakleaf loquat at each respective cold treatment duration. It was also found the Ca^{2+} level was lower in oakleaf loquat than in Zaozhong 6 and the calcium accumulation after cold treatment occurred much earlier in Zaozhong 6 than in wild oakleaf loquat. These results suggest that the cold sensitive cultivar Zaozhong 6 had a quicker response to the cold stress by the rapid accumulation of Ca^{2+} , as compared to the cold-tolerant wild oakleaf loquat.

In conclusion, calcium foliar application improved cold tolerance in both loquat accessions. Loquat plants accumulated calcium around chloroplasts in response to cold stress and such accumulation of calcium occurred earlier in the cold sensitive cultivar Zaozhong 6 than in the cold-tolerant wild oakleaf loquat. In addition, cold damage symptoms to chloroplasts also appeared earlier in Zaozhong 6 than in oakleaf loquat. These results suggest that the two loquat species differed in cold tolerance and calcium accumulation dynamics.

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