Antisense suppression of an acid invertase gene (MAI1) in muskmelon alters plant growth and fruit development

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Received 30 January 2008; Revised 3 May 2008; Accepted 6 May 2008

Abstract
To unravel the roles of soluble acid invertase in muskmelon (Cucumis melo L.), its activity in transgenic muskmelon plants was reduced by an antisense approach. For this purpose, a 1038 bp cDNA fragment of muskmelon soluble acid invertase was expressed in antisense orientation behind the 35S promoter of the cauliflower mosaic virus. The phenotype of the antisense plants clearly differed from that of control plants. The transgenic plant leaves were markedly smaller, and the stems were obviously thinner. Transmission electron microscopy revealed that degradation of the chloroplast membrane occurred in transgenic leaves and the number of grana in the chloroplast was significantly reduced, suggesting that the slow growth and weaker phenotype of the transgenic plants may be due to damage to the chloroplast ultrastructure, which in turn resulted in a decrease in net photosynthetic rate. The sucrose concentration increased and levels of acid invertase decreased in transgenic fruit, and the fruit size was 60% smaller than that of the control. In addition, transgenic fruit reached full-slip at 25 d after pollination (DAP), approximately 5 d before the control fruit (full-slip at 30 DAP), and this accelerated maturity correlated with a dramatic elevation of ethylene production at the later stages of fruit development. Together, these results suggest that soluble acid invertase not only plays an important role during muskmelon plant and fruit development but also controls the sucrose content in muskmelon fruit.

Key words: Acid invertase, Cucumis melo, chloroplast ultrastructure, ethylene production, sucrose.

Introduction
Sugars are the most important biochemical components for fruit quality. The type and amount of the sugars directly influence fruit flavour components, such as sweetness. As the first step towards genetic improvement of the quality of muskmelon fruit, it is necessary to determine the sugar components accumulated in fruit, to elucidate the enzymes involved in sugar metabolism, and to clarify the relationship between sugar accumulation and the activity of some related enzymes (McCollum et al., 1988; Moriguchi et al., 1992; Roitsch and González, 2004; Sakalo and Kurchii, 2004).

In the middle stage of fruit development, muskmelon fruit undergoes a metabolic transition marked by both physical and compositional changes such as netting of the exocarp, softening of the mesocarp, and the onset of sucrose accumulation (Lester and Dunlap, 1985). Attempts to elucidate the changes in metabolism that lead to the accumulation of sucrose have focused on sucrose metabolizing enzymes during fruit growth and development (Lingle and Dunlap, 1987; Schaffer et al., 1987; Winter and Huber, 2000; Geromel et al., 2006). It has been reported that both acid invertase (EC 3.2.1.26) and sucrose phosphate synthase (EC 2.4.1.14) are determinants of sucrose accumulation in muskmelon fruit. The activity of acid invertase in muskmelon fluctuates throughout the whole period of fruit development; it reaches a high level in immature fruit and then declines.
to a low level in the further developing fruit (Schaffer et al., 1987; McCollum et al., 1988; Hubbard et al., 1989). Synchronously, the levels of sucrose are low in immature fruit and high in mature fruit, indicating that acid invertase activity in muskmelon fruit shows a positive correlation with fruit growth and a negative correlation with levels of sucrose.

The optimum pH of acid invertase and neutral invertase activities is pH 3–5 and pH 7, respectively. Acid invertases are either ionically bound to the cell wall (cell wall invertase) or accumulate as soluble proteins in the vacuole (vacuolar invertase), with basic (Lauriere et al., 1988) and acidic (Unger et al., 1992) pIs, respectively. Genes encoding acid invertase have been cloned from many plant species (Klann et al., 1992; Elliott et al., 1993; Davies and Robinson 1996; Haouazine-Takvorian et al., 1997; Kim et al., 2000; Yu et al., 2003; Ji et al., 2005), all sharing homology to the active site of yeast invertase (Unger et al., 1994). It is known that two larger families encode the acid invertases of the cell wall and vacuole (Sherson et al., 2003; Cho et al., 2005) and the neutral/alkaline invertases of the cytosol (Vargas et al., 2003; Murayama and Handa, 2007).

In the present study, the contributions of MAI1 soluble acid invertase during plant growth and fruit development were investigated by expressing a chimeric antisense MAI1 gene in transgenic muskmelon plants. The T2 generation of transformants was analysed throughout plant growth and fruit development, addressing not only the effects on acid invertase activity and sucrose content in fruit, but also the effects on plant growth and fruit development.

**Materials and methods**

**Muskmelon transformation**

A 1038 bp fragment (nucleotides 1–1038) from the 5' end of the MAI1 cDNA (GenBank accession number EU260044) was inserted in the antisense orientation into the binary vector pBI121 (Clontech, Palo Alto, CA) behind the 35S promoter of cauliflower mosaic virus (CaMV). The construct was introduced into Agrobacterium tumefaciens LBA4404 (Hoekma et al., 1983) via direct DNA transformation (Höfgen and Willmitzer, 1988). Cotyledons of muskmelon inbred line M01-3 were transformed as described by Fang and Grumet (1990). Three independent transformants were obtained and analysed, and the T-4 transgenic line, with higher sucrose levels in the fruit, was studied in more detail. The phenotypes of the T1 transgenic plants from the T0 transgenic lines after self-pollination were identical. To obtain homozygous plants, T1 plants were also self-pollinated and T2 transgenic plants from three T1 independent lines were analysed in this study.

Untransformed M01-3 muskmelon plants were used as controls. Transgenic and control muskmelon plants were grown in a greenhouse on an experimental farm at Shandong Agricultural University in Tai’an, China from February to the end of May 2007, with a spacing of 50 cm between the plants and 120 cm between the rows. Average day/night temperatures were about 30/20 °C. The average daylight was about 12 h. Fertilizer was applied at two stages: a preplant broadcast application of 900 kg ha⁻¹ of 14:6:29.9 N:P:K, followed by a sidedress application of 150 kg ha⁻¹ N at the flowering stage. Irrigation by furrows was applied as needed. Freshly opened female flowers were tagged on the day of hand-pollination to identify fruit of a known age. The vines (main shoots) were trained vertically and topped at the 23rd node. The lateral shoots were all removed except the lateral shoots setting fruit. Fruit set was recorded 10 d after pollination and the fruit were thinned to one per plant.

**Plant growth and net photosynthetic rate (Pn) measurement**

Plant height, stem diameter, and leaf area were determined during the development of transgenic and control plants. Twenty plants were sampled for each time point. The net photosynthetic rate (Pn) was measured with a portable photosynthesis system (Li-Cor 6400; Li-Cor Inc., Lincoln, NE, USA). Measurement was made on the uppermost youngest, fully expanded mature leaves of the main stem of transgenic and control plants, and was repeated at least six times on each.

**Microscope observation**

The leaf cellular structure and subcellular structure were analysed by photomicroscopy and electron microscopy, respectively, on sections from the middle portions of fully expanded leaves of the main stem. Three leaves from three plants of the 20 plants used to measure plant growth and Pn of transgenic and wild-type muskmelons were sampled, and two pieces (2×2 mm) of each leaf were taken and used for analysis. These pieces were first fixed with 4% glutaraldehyde in phosphate buffer (pH 7.2) at room temperature overnight, post-fixed using 1% osmic acid, dehydrated through an ethanol series, and embedded into Spurr’s low viscosity resin (Kutik et al., 1999). Transverse ultrathin and thin sections were then prepared from the embedded objects that were first contrasted with a saturated uranyl acetate solution followed by lead citrate solution (Reynolds, 1963). The thin section microphotographs were taken using a photomicroscope and the ultrathin section microphotographs were taken using a Jeol 1200X electron microscope (Jeol System Co., Akishima Tokoyo, Japan). At least 20 cells/ultrathin section were photographed and analysed. The characteristics of the chloroplasts were analysed from the photographs by digitizing the areas of starch granules and chloroplasts, and calculating the number of the starch granules and chloroplasts.

The data were analysed using a factorial analysis of variance. Least significant differences (LSDs) at P = 0.05 and P ≤ 0.01 were used to distinguish significantly different means.

**Fruit sampling**

Female flowers of T2 generation plants and control plants were hand pollinated and tagged. The control fruit were harvested at 5, 10, 15, and 25 DAP and at full-slip (approximately 30 DAP), but the transgenic fruit were harvested at 5, 10, 15, and 20 DAP and at full-slip (approximately 25 DAP). Five fruit samples of the appropriate age from 20 plants were pooled to analyse each data point. The same fruit samples were analysed for sugars, weight, enzymatic activity, and northern blot analysis. Sampling continued until the five fruit samples selected were all mature.

**Northern blot analysis**

For RNA gel-blot analysis, 20 µg of total RNA were separated on 1.2% agarose gels containing formaldehyde. RNA gel blot analysis was performed according to the DIG Northern Starter Kit Manual (Roche Corp., Basel, Switzerland). The 3’ UTR fragment of MAI1 cDNA (about 300 bp) was labelled with digoxigenin (DIG) using a DIG nucleic acid labelling kit and used as hybridizing probe. RNA transfer and fixation were performed by capillary transfer with
20× SSC overnight and baking the membrane at 80 °C for 2 h. Prehybridization and hybridization were done at 68 °C in DIG Easy Hyb Buffer with gentle agitation for 30 min and 12 h, respectively. The blot was washed twice with 2× SSC and 0.1% SDS at 25 °C under constant agitation for 5 min. Then the blot was washed twice in 0.1× SSC and 0.1% SDS at 68 °C under constant agitation for 15 min. The hybridized probes were immunodetected with antidiogoxigenin-AP and Fab fragments and then visualized with the chemiluminescence substrate CDP-Star. Enzymatic dephosphorylation of CDP-Star by alkaline phosphatase led to a light emission at a maximum wavelength of 465 nm, which was recorded on X-ray films for 30 min.

**Acid invertase assay**

Acid invertase extraction and assay were performed in a manner similar to that previously described by Lowell et al. (1989) and Hubbard et al. (1989) with some minor modifications. Acid invertase was extracted from about 1 g of minced frozen muskmelon tissue by homogenizing for 1 min on ice in 200 mM HEPES/NaOH (pH 7.5), 5 mM MgCl2, 1 mM EDTA, 0.5 mg ml⁻¹ BSA, 0.05% Triton X-100 (Sigma-Aldrich Corp., St Louis, MO, USA), 1 M NaCl, 1% insoluble PVP, and 5 mM DTT. Homogenates were quickly desalted at 4 °C. Desalted buffer contained 20 mM HEPES/NaOH (pH 7.5), 0.25 mM MgCl2, 0.01% 2-mercaptoethanol, 1 mM EDTA, 0.05% BSA, and 0.2% glycerine. The desalted extract was assayed immediately.

Acid invertase was assayed in 50 mM Na-citrate buffer (pH 5.0), using 50 mM sucrose as a substrate. The control was boiled for 10 min immediately after the addition of the desalted enzyme extract. The reactions were incubated for 30 min at 37 °C and then stopped by transfer to a boiling water bath. Absorbance was measured with a microplate reader (3550-UV Bio-Rad Laboratory Inc., Hercules, CA, USA) at 570 nm and compared to glucose standards.

**Sugar measurements**

 Sugars were extracted by grinding flesh tissues (10 g fresh weight) in 80% ethanol, adjusted to pH 7.0 with 0.1 N NaOH, and heated for 5 min at 80 °C. They were then analysed as described by Lingle and Dunlap (1987).

**Ethylene measurement**

Five fresh fruit samples of the appropriate age were placed in an 8.0 l jar and sealed with a rubber serum cap. The headspace gas was allowed to accumulate for 2–3 h at 25 °C. Headspace samples (1 ml) were withdrawn and analysed using a GC-9A gas chromatograph (Shimadzu Corp, Kyoto, Japan), which was equipped with an FID detector (Dunlap, 1988).

**Fig. 1.** Plant height (A), stem diameter (B), leaf area (C), and net photosynthetic rate (D) during transgenic and control plant development. The antisense MAI1 transgenic line T-4 plants and control plants were grown in a greenhouse on an experimental farm of Shandong Agricultural University in Tai'an, China from February to the end of May 2007. Twenty plants were used to measure plant height, stem diameter, and leaf area for each time point. The net photosynthetic rate was measured with a portable photosynthesis system (Li-Cor 6400; Li-Cor Inc., Lincoln, NE, USA). Measurements were made on the uppermost youngest, fully expanded mature leaves of the main stem of transgenic and control plants, and was repeated at least six times on each. Bars indicate SE.
Results

Transgenic muskmelon plants are distinctly weaker than control plants

The antisense MAI1 transgenic plants and control plants were grown in a greenhouse with the culture conditions described in the Materials and methods. 20 transgenic plants and control plants, respectively, were chosen to measure the plant height, stem diameter, and leaf area. The results showed that the plant height of the transgenic plants was slightly shorter than that of control plants at the early stages of plant development, but were similar at later stages (Fig. 1A). By contrast, the stem diameter of the transgenic plants was much thinner (Fig. 1B); the leaf area was much smaller throughout plant development and the fully expanded mature leaf area was approximately 30% smaller than that of the control plants (Fig. 1C). Moreover, the net photosynthetic rate of transgenic plants was slightly slower than that of control plants throughout plant development (Fig. 1D). The transgenic muskmelon plants were distinctly weaker than the control plants (Fig. 2A).

In order to understand the influence of reduced soluble acid invertase activity on transgenic plant growth and development, the leaf cellular and subcellular structure of the transgenic and control plants were analysed. Microscope observation showed that the spongy cells were in a cluster and had very large cellular interspaces (Fig. 3B; arrows) in the transgenic leaves, but the epidermal cells and palisade cells were similar to those of the control plants (Fig. 3A, B). The electron microscope analysis revealed good chloroplast membrane integrity (Fig. 3E; arrow) and the structure of the grana lamella was clear and in good order in the leaves of control plants (Fig. 3C, E), whereas the membranes of some chloroplasts were completely disaggregated (Fig. 3F; arrow) and the grana became thinner and the grana lamella was not clear in the transgenic plant leaves (Fig. 3F). Surprisingly, it was found that many chloroplasts had altered shapes (Fig. 3G, D; up arrow); some were consumed by the vacuoles (Fig. 3D), and some had been completely disaggregated (Fig. 3H, D; down arrow). The number of grana, chloroplast width, and granule width of the transgenic plant leaves were significantly less or smaller than that of the control plants. However, no significant difference was observed in the number of chloroplasts between the transgenic and control plant leaves (Table 1).

Expression of antisense MAI1 in transgenic muskmelon markedly reduces acid invertase activity and fruit size and increases sucrose concentration

To verify the efficiency of the antisense MAI1 strategy, the soluble acid invertase mRNA expression level and enzyme activity were determined using different mesocarp tissues from 5 DAP to full-slip. The northern blot results showed that the expression pattern of the soluble acid invertase gene was the same in both the transgenic fruit and the control fruit. However, the mRNA levels of acid invertase genes in antisense plants decreased obviously compared with the controls during fruit development (Fig. 4A). In both transgenic fruit and control fruit, the acid invertase activity became lower and lower as the fruit grew and matured, which is in agreement with the previous reports (Schaffer et al., 1987; McCollum et al., 1988). Just as in the case of the mRNA level, the activities of acid invertase in transgenic fruit also decreased very quickly during fruit development (Fig. 4B). The neutral invertase activities during fruit development were also analysed. The result showed that the neutral invertase activities were not affected during transgenic fruit development compared with that of the wild-type fruit (data not shown).

To determine the impact of the antisense MAI1 strategy on fruit quality in muskmelon, the fruit size, growth rate, and sugar composition were analysed. The results showed that the fruit sizes of the three transgenic lines in the T2 generations at harvest were reduced by approximately...
60% relative to the controls (Figs 2B, 5). It was observed that the control fruit increased in size at a steadily higher rate throughout fruit development, whereas the transgenic fruit maintained a slow growth rate. In both T2 transgenic and control fruit, the contents of fructose and glucose were almost the same during fruit development. However, the sucrose levels in mature transgenic fruit were significantly higher (approximately from 19% to 40%) than that in control fruit (Fig. 6).

Muskmelon with reduced acid invertase activity and shorter fruit development time has elevated fruit ethylene production

In our study, it was observed that transgenic fruit reached full-slip at 25 DAP, which is 5 d earlier than the control (fruit reached full-slip at 30 DAP). To elucidate the effects of the antisense MAI1 strategy on fruit development in muskmelon, ethylene production in transgenic and control fruit was determined. The results indicated that inbred M01-3 muskmelon fruit undergoes a respiratory climacteric during ripening that is triggered by ethylene, and the MAI1 antisense gene altered the respiratory behaviour during fruit ripening. The transgenic fruit differed dramatically from the control fruit during the respiratory climacteric. Ethylene production was approximately 260% lower in the transgenic unripe fruit than in the control at the same stage, whereas it was

Table 1. Length, width, and number of chloroplasts and number of grana, and length, width, and number of starch granules measured per chloroplast (n=20)

The data were analysed using factorial analysis of variance. Least significant differences (LSDs) at $P<0.05$ and $P<0.01$ were used to distinguish significantly different means (the letters ab and AB denoting the differences at $P<0.05$ and $P<0.01$, respectively; only those parts marked with different letters significantly differ).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild type</th>
<th>Antisense (T-4)</th>
</tr>
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<tr>
<td>Number of chloroplast/cell</td>
<td>17.1 aA</td>
<td>16.7 aA</td>
</tr>
<tr>
<td>Number of starch granules/chloroplast</td>
<td>19.8 aA</td>
<td>19.4 aA</td>
</tr>
<tr>
<td>Length of chloroplast (μm)</td>
<td>16.9 aA</td>
<td>16.6 aA</td>
</tr>
<tr>
<td>Width of chloroplast (μm)</td>
<td>5.6 aA</td>
<td>5 bA</td>
</tr>
<tr>
<td>Length of starch granules (μm)</td>
<td>3 aA</td>
<td>2.6 aA</td>
</tr>
<tr>
<td>Width of starch granules (μm)</td>
<td>1.3 aA</td>
<td>0.9 bB</td>
</tr>
</tbody>
</table>

Fig. 3. Leaf cellular and subcellular structure of wild-type and transgenic line T-4 plants. Three expanded leaves of the main stem from different plants of each type were collected, and two pieces (2×2 mm) of each were taken and used for analysis. (A) Photomicrograph of wild-type plants; bar = 10 μm. (B) Photomicrograph of antisense plants. The spongy cells have very large cellular interspaces (arrows); bar = 10 μm. (C) Electron micrograph of wild-type plants ×2500; bar = 2 μm. (D) Electron micrograph of antisense plants ×2500; bar = 2 μm. Many chloroplasts appear to have altered shape (up arrow) and some of them had become disaggregated (down arrow). (E) Electron micrograph of wild-type plants. The chloroplast membrane appears integrated (arrow) ×25 000; bar = 200 nm. (F) Electron micrograph of antisense plants ×10 000; bar = 500 nm. (H) Electron micrograph of antisense plants ×10 000; bar = 500 nm.
approximately 150% higher in the transgenic ripe fruit than in the control (Fig. 7). The evidence suggests that a rapid rise in ethylene production at the later stage of fruit development results in a shorter period of fruit development.

Discussion

Plant tissues frequently exhibit a positive correlation between soluble acid invertase activity and rates of cell expansion (Morris and Arthur, 1984; Pfeiffer and Kutschera, 1995; Chopra et al., 2003, 2005; Koch, 2004). In kidney bean the specific activity of soluble acid invertase increases during the early stages of leaf expansion and peaks at the time of the most rapid cell expansion (Morris and Arthur, 1985). The rapid phase of lamina expansion is characterized by high concentrations of hexose sugar and low concentrations of sucrose. In this study, a reduction of soluble acid invertase activity in MAI antisense plants makes muskmelon growth weaker during the whole plant growth period, which leads to thinner stems and a smaller leaf area (Fig. 1B, C). It was reported that a reduction of acid invertase expression in carrot (Daucus carota L.) decreases plant growth, but the antisense plants become malformed and have more leaves than the control plants (Tang et al., 1999). By contrast, expression of antisense mRNA for acid invertase in tomato (Lycopersicon esculentum L.) (Ohyama et al., 1995; Klann et al., 1996) and potato (Solanum tuberosum L.) (Zrenner et al., 1996) did not lead to phenotypic and plant growth changes in transgenic plants. A possible explanation for this difference is that the genes down-regulated by antisense mRNA expression in tomato and potato are expressed only at a late stage of plant development, such as the periods during fruit ripening in tomato and during cold sweetening of stored tubers in potato. In muskmelon, the acid invertase gene may be expressed at a very early stage of plant development like in carrot (Sturm et al., 1995). These findings suggest distinct functions of acid invertase in different plant species.
To our knowledge, this is the first report that the spongy cells were in a cluster and many chloroplasts were consumed and damaged in transgenic plant leaves according to microscope and electron microscope observations (Fig. 3B, D, F). This cellular evidence suggests that the weaker phenotype of MAI1 antisense plants may be due to the damage to chloroplast ultrastructure resulting in the decrease of net photosynthetic rate. Certainly, the molecular mechanism of acid invertase affecting muskmelon leaf cellular and subcellular structure in MAI1 antisense muskmelon needs to be elucidated.

In general, the acid invertase activity in muskmelon fruit is correlated with sucrose accumulation. Stepansky et al. (1999) reported that total sugar levels in the broad spectrum of C. melo genotypes were correlated only with sucrose levels and within C. melo all high sugar genotypes are mainly based on the accumulation of sucrose but not hexose. In the present study, the introduction of a MAI1 antisense gene in muskmelon altered the soluble sugar composition, resulting in a dramatic increase of sucrose concentration. These results agree with those from the previous studies in tomato fruit; however, in their studies hexose concentrations were reduced in the transgenic tomato fruit (Ohyama et al., 1995; Klann et al., 1996).

The developmental losses of sucrose hydrolysing activity (acid invertase) in the muskmelon fruit has been proposed as the genetically controlled metabolic determinant of sucrose accumulation (Schaffer et al., 1987; Hubbard et al., 1989). Our results indicated that the unripe fruit of control and transgenic muskmelon had a high activity of acid invertase but a low sucrose concentration, which was contrary to ripe fruit (Figs 4, 6). Also, the levels of acid invertase activity in transgenic plants were much lower than in the control plants during the whole period of fruit development, but the concentration of sucrose in the transgenic mature fruit was increased by approximately 19–40% (Fig. 6), thus suggesting that acid invertase plays a crucial role in determining sucrose accumulation in muskmelon fruit.

Ethylene production plays an important role in fruit ripening, including colour changes, sweetness
accumulation, the formation of volatile flavour, and firmness (Theologis, 1992; Flores et al., 2002; Defilippi et al., 2005). It is shown for the first time that transgenic muskmelon fruit reached full-slip at 25 DAP, which was 5 d earlier than control fruit (full-slip at 30 DAP), probably due to a rapid elevation in ethylene production (Fig. 7). This is not consistent with a previous report that ethylene production in tomato was 100% higher in antisense fruit than in control fruit due to a rapid elevation in ethylene production (Fig. 7).

In conclusion, the results presented here demonstrate that the expression of a MAI antisense gene results in higher sucrose concentrations and a shorter time for fruit development in transgenic muskmelon fruit, suggesting that muskmelon quality improvement could be realized by modifying the expression of a single gene encoding soluble acid invertase.

References


