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Gene transcript accumulation and enzyme activity of β-amylases suggest involvement in the starch depletion during the ripening of cherry tomatoes

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ABSTRACT

The flavor of tomato fruits is mostly influenced by the accumulation of sugars and organic acids. During fruit ripening a conversion of starch to sugars occurs, which modulates significantly the taste and consequently the quality of the ripe tomato fruits. β-Amylases, a group of major starch hydrolytic enzymes involved in starch degradation were examined in developing cherry tomatoes. Our results suggest that the enzyme activity and the gene transcript accumulation of plastidial β-amylase isoenzymes were elevated during the late stages of fruit development indicating a participation of the enzyme in starch depletion and in the increase of total soluble sugar levels in ripe tomatoes.

1. Introduction

The flavor of tomato fruits, one of the most important quality traits, is mostly influenced by the accumulation of sugars and organic acids (Klee and Giovannoni, 2011). During the early stages of fruit development, tomato fruits accumulate high levels of starch coinciding with rapid fruit growth, cell division and enlargement (Wang et al., 1993; Moore et al., 2002). In contrast, during fruit ripening the conversion of starch to sugars occurs along with other metabolic changes and contributes to the texture, flavor and nutrient composition of the fruits (Moore et al., 2002). It is reported that fruits mostly accumulate starch up to 20 to 25 days after flowering, while starch degradation occurs 35 to 40 days after flowering (Luengwilai and Beckles, 2009).

Beta-amylases (EC 3.2.1.2-bAmy) are a class of hydrolases that remove β-maltose units from the non-reducing end of polyglucans and are involved in starch degradation in all plant tissues (Sparla et al., 2006; Peroni et al., 2008). bAmy is mostly associated with the later stages of fruit development and has been extensively studied in fruits containing high levels of starch (15–20% of fresh weight), such as mango and banana (do Nascimento et al., 2006; Peroni et al., 2008). In tomato fruits, starch phosphorylase was considered to be the predominant enzyme of starch breakdown during fruit maturation (Robinson et al., 1988). However, later research found that bAmy played an important role in the hydrolytic starch breakdown pathway (Purgatto et al., 2001; Fulton et al., 2008). Furthermore, Bassinello et al. (2002) emphasized the importance of bAmy in the hydrolytic breakdown pathway in mangoes, pears and also in corn.

In contrast to fruits such as mango and banana where the role of bAmy during ripening has been adequately investigated, in most fruits with lower starch levels, such as the tomato, only scarce information exists about the actual role of bAmy during fruit maturation (do Nascimento et al., 2006; Peroni et al., 2008). However, according to Schaffer and Petreikov (1997), the process of starch breakdown could also considerably affect the sugar levels, and consequently the quality, of the low-starch fruits.

The present study attempts to investigate the role of bAmy, probably one of the most important enzymes of the hydrolytic starch breakdown pathway during fruit development and maturation of cherry tomatoes. For this purpose, the starch content, the bAmy enzyme activity and the transcript accumulation of bAmy genes were examined in developing cherry tomatoes.

Abbreviations: bAmy, β-amylase; UBQ, ubiquitin; ImG, immature green; MG, mature green; Br, breaker; RR, red ripe.

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2. Materials and methods

2.1. Plant material and growth conditions

Plants of cherry tomato (*Solanum lycopersicum* L. var. *cerasiforme* cv. 'Conchita F1' de Ruiter seeds, Melbourne Australia), a productive hybrid with a long shelf life, were cultivated in a glasshouse of the Agricultural University of Athens, Greece between December and May. Mean minimum and maximum temperatures in the greenhouse were 15.7 ± 2.0 and 26.6 ± 4.3 °C, respectively, (Spring, {March–May}) and 12.9 ± 1.9 and 23.9 ± 4.4 °C (Winter, {Oct.–Feb.}). Average solar radiation was 318.75 μmol/m^2 s. Fruits were harvested systematically at the following stages: immature green (ImG — diameter 15 mm) (25 DAF — days after flowering), mature green (MG — 38 DAF), breaker (Br — 44 DAF) (<10% red color than red ripe-measured with a Konica — Minolta CR-400 Chroma Meter), and red ripe (RR — 52 DAF). Each harvest was carried out at 11 am and replicated three times. For the experiments at least 20 whole fruits of each lot were ground to a fine powder with liquid nitrogen except for the assessment of the starch content.

2.2. Assessment of starch content

Glucose, fructose, and sucrose contents were measured by HPLC in ethanolic extracts of tomato pulp, following the method of Piccaglia and Galleti (1988), with slight modifications. Sugars were extracted from 0.5 g of homogenized flesh in 2 ml 80% (*v*/*v*) ethanol at 65 °C for 25 min. The suspension was centrifuged at 5500 *g* for 15 min and the supernatant was retained for the analysis of sugars. The procedure was then repeated on the resulting pellet and the supernatants from both extractions were combined and evaporated to dryness at 65 °C with the aid of continuous ventilation (N2). The pellet was then dissolved in 3 ml water (HPLC-grade) and the aqueous solution was filtered (Macherey–Nagel Chromafil PET 20/15 MS; pore size 0.20 μm) and injected into an HPLC equipped with a refractive index detector (ERC-7511; Erma, Tokyo, Japan) and a Supelco Supelcosil LC-NH2 column (5 μm; 25 cm 4.6 mm; Sigma-Aldrich, St. Louis, MO, USA) maintained at 30 °C. The eluent was 80% (*v*/*v*) acetonitrile + 20% (*v*/*v*) H_2O (HPLC-grade; Fisher Scientific, Hampton, NH, USA), at a flow rate of 1 ml min^{-1}. The ethanol-insoluble residue was used to measure starch content, according to the method of Dekker and Richards (1971) using amyloglycosidase (EC 3.2.1.3) from *Aspergillus niger* (Sigma-Aldrich) to release glucose. The glucose content was subsequently determined colorimetrically following the method of Barham and Trinder (1972), using a glucose oxidase/peroxidase (GOD-POD) kit (Biosis Ltd., Athens, Greece), and a spectrophotometer set at 510 nm (Lambda 1A; Perkin-Elmer, Waltham, MA, USA). A reference curve was created using standard starch concentrations (0, 25, 50, 100, 150, 250, 500, 750, 1000 mg/l).

2.3. Enzyme extraction and bAmy activity

For the enzyme activity assessment, 1 g of powdered fruits from all stages of fruit development was powdered using liquid nitrogen, and mixed at a 1:1 (*w*/*v*) with an extraction medium as proposed by Stenzel et al. (2003) with some modifications. The extraction buffer contained 50 mM Tris HCl pH 7.5, 15% glycerol, 0.02% bovine serum albumin, 2 mM reduced glutathione, 4% PVP-40 (MP Biochemicals, Eschwege Germany), 0.1% Triton X and 20 mM Na_2SO_3. The extracts were centrifuged at 15,000 × *g* for 15 min at 4 °C. The protein content of supernatants was determined using the Bradford method, before the enzyme activity tests. The protocol Betamyl-3 (Megazyme Wicklow, Ireland) was used for the determination of beta amylase activity according to the manufacturer's instructions. The produced p-nitrophenol was assessed photometrically at 400 nm in a Shimadzu UV 160A spectrophotometer.

2.4. qPCR analysis

By performing BLAST searches (Altschul et al., 1997) among the databases of the National Center for Biotechnology Information (NCBI) using the appropriate keywords, the registered nucleotide sequences for tomato *bAmy* and for the reference genes were identified and recorded (Supplementary data). With the use of the online alignment tool of NCBI the highly similar sequences were grouped (Supplementary data) and gene specific primers were designed for the identified sequences coding for isoenzymes of tomato bAmy using Beacon designer v 7.01 software (Premier Biosoft, Palo Alto, USA) (Table 1). For the topology prediction of the bAmy isoenzymes the online tool TargetP 1.1 was used (Emanuelsson et al., 2000). For each identified tomato *bAmy* sequence the name of similar *bAmy* sequence from *Arabidopsis thaliana* is provided. No significantly similar *bAmy* sequence *Arabidopsis thaliana* was found for tomato *bAmy3* (Table 1, Supplementary data). Total RNA was isolated from each sample using RNeasy extraction Kit following the manufacturer's instructions (Qiagen, Hilden Germany). The quantity and quality of total RNA were assessed by spectrophotometric and electrophoretic analysis, measuring the absorbance at 260 nm and the absorbance ratio of 260/280 nm in a Nanodrop spectrophotometer (Thermo, Wilmington USA) and by 1.5% *w*/*v* agarose-gel electrophoresis. To eliminate total DNA, samples were treated with RNAse free DNase I (Takara, Otsu Shiga Japan) according to the manufacturer's instructions.

Table 1
Primer sequence of genes used for qPCR.

Gene	Encoded enzyme/protein	Primer sequence	Accession no.
bAmy1	Putative plastidial beta amylase 1	Sense: GTTCCACTTGCTGGGGAGAA Antisense TGTTCGCGGCATTTGTTAGC	NM_001247627.1 Similar to AAY89374 from tobacco (Ren et al., 2007) and NM_113297.2 from Arabidopsis.
bAmy2	Putative plastidial beta amylase 2	Sense: GTG CAA ACT GCT CAC CAG AA Antisense: CTT CCG ACA TGC TCT TCA CA	XM_004245796 similar to AJ250341 ct-bAmy from Arabidopsis (Lao et al., 1999)
bAmy3	Putative plastidial beta amylase 3	Sense: CAA ACA GTA TGC CGA GAG CA Antisense: AGG AAG TTT GCC ACA AAT GG	NM_001247123.1 (Aoki et al., 2010) similar to NM_121872.2 from Arabidopsis.
bAmy4	Putative cytosolic beta amylase1	Sense: AGGGAGCTGAAGAACCAAGC Antisense: ATAGCTCTTCGCTGTCGCTC	XM_004229839.2 Similar to XM007051752 bamy 7 from *Theobtoma cacao* and NM_130151.6 from Arabidopsis (Lin et al., 1999)
bAmy5	Putative plastidial beta amylase 4	Sense: GAGCAATCCCCATCACCACA Antisense: TGACGAAGTCCAGCAAGCAT	XM_004244394.1 Similar to XM_007040835 bamy 2 from Theobtoma cacao. Translation similar to NM_123898.2 from Arabidopsis (Motamayor et al., 2013)
UBQ	Ubiquitin	Sense: gcagactataacatccagaaagag Antisence: aacaacaaagcacacacagccatc	X73156.1
18s	18s ribosomal	Sense: ccgtcgcttggcattttcat Antisence: ttggttcccattcccagacg	AF179442