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Metabolic changes in fruits of the tomato d^x mutant

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Abstract

The tomato DWARF cytochrome P450 protein catalyzes the C-6 oxidation of 6-deoxo-castasterone to castasterone. The d^x mutant does not produce a functional DWARF enzyme, and d^x shoots display severe symptoms of brassinosteroid-deficiency. However, fruits express the CYP85A3 protein which compensates for the deficiency of the DWARF protein and produce bioactive brassinosteroids. Here, we report on the metabolic characterization of d^x fruits. Fruit size, fresh weight, and pigment content were not altered. However, d^x fruits showed reduced dry mass content. Levels of starch and various sugars were reduced, amino acid levels were elevated. BR application to d^x leaves partially normalized dry mass content, sugar and amino acid levels in d^x fruits. The data demonstrate that brassinosteroid in shoots is required for fruit development in tomato.

Keywords: *Solanum lycopersicum*; Solanaceae; Tomato; Brassinosteroid; Primary metabolism; Fruit

1. Introduction

Brassinosteroids (BRs) are essential plant growth regulators (Clouse, 2002). BR-deficiency and BR-insensitivity result in extreme dwarfism in model plants such as *Arabidopsis* and crops (Bishop, 2003). The molecular mode of action of BR has been predominantly studied on the gene expression level (Müssig and Altmann, 2003; Vert et al., 2005). Genes encoding cell wall-modifying enzymes, enzymes of the BR-biosynthetic pathway, auxin response factors, signalling components, transcription factors, and further proteins are subject to BR regulation. However, causal links between physiological effects and changes of transcript levels have barely been identified. BR apparently coordinates and integrates diverse processes required for growth and development, partly via interactions with other phytohormones (Müssig, 2005). Regulation of cell division, modification of cell wall properties, organisation of microtubules and cellulose microfibrils, control of aquaporin activity, control of vacuolar H^+-ATPase activity, and further physiological processes may bring about BR-promoted growth (Sasse, 2003). Impaired growth of BR-deficient plants may also be due to reduced photosynthesis, because BR-deficiency results in a severe reduction of CO_2 assimilation and, consequently, reduced starch levels and reduced biomass production (Schlüter et al., 2002).

Two major routes of BR biosynthesis were identified, the early and late C-6 oxidation pathways, which both are connected and result in the production of the bioactive castasterone (CS) (Fujioka and Yokota, 2003). CS can be oxidised to brassinolide (BL), which is the end product of the biosynthetic pathway and shows highest biological activity. In tomato, the late C-6 oxidation pathway is likely to be the major route because only intermediates of the late C-6 oxidation pathway were detected (Bishop et al., 1999; Koka et al., 2000). The C-6 oxidation is an essential and rate-limiting step for the production of bioactive BRs. The *D* (or *DWARF*) gene in tomato encodes a cytochrome P450 enzyme termed CYP85A1 which catalyzes C-6 oxidation of 6-deoxo-castasterone (6-deoxoCS) to CS (Bishop et al., 1999; Fujioka and Yokota, 2003). The *D* gene is expressed in roots, shoots, and fruits (Montoya et al., 2005). In contrast to many other BR mutants, the

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BR-deficient d^x (*extreme dwarf*) mutant is not sterile and produces fruits. Similar to BR mutants in *Arabidopsis*, pea, rice, and other plants, the d^x mutant shows dwarfism due to reduced growth of leaves, petioles, and internodes (Bishop et al., 1999). However, tomato fruits express the *CYP85A3* gene encoding a cytochrome P450 protein which is highly similar to the DWARF protein, and the CYP85A3 protein substitutes for the DWARF protein in fruits (Nomura et al., 2005). Therefore, the CYP85A1 and CYP85A3 proteins function in parallel in shoots and fruits, and fruits of the d^x mutant plants produce bioactive CS and are capable to grow. Because the CYP85A3 protein also catalyzes the Baeyer–Villiger oxidation of CS, d^x fruits produce BL (Nomura et al., 2005). CS may represent the predominant bioactive BR during vegetative growth in tomato, whereas BL may play a specific role in fruit development (Bishop et al., 1999; Nomura et al., 2005).

In the present study, we describe metabolic changes occurring in d^x fruits. BR deficiency in shoots resulted in later flowering and delayed fruit ripening. d^x fruits exhibited reduced dry mass content, reduced sugar levels, but elevated amino acid levels. BR application to d^x leaves partly normalized the metabolic changes. Our data show that BR in shoots is required for fruit development and normal fruit metabolism.

2. Results and discussion

2.1. Ripening of d^x fruits

Similar to other BR-deficient plants, the d^x mutant flowered later in comparison to wildtype plants. On average wildtype and d^x plants developed flowers 49 and 70 days after sowing, respectively. The number of flowers and fruits of wildtype and the d^x mutant were not significantly different. Fruit development after pollination was also delayed in comparison to wildtype plants (Fig. 1). Fruit color is considered a marker for ripeness of tomato fruits. Fruit development is divided into stages: stage 0 (immature green), stage I (mature green), in which fruits have nearly their final fresh weight, stage II (breaker), in which fruits show yellowish and orange color, stage III (turning), and stage IV (red and ripe), in which fruits are edible and tasteful (Srivastava and Handa, 2005). Development of wildtype and d^x fruits was monitored according to this color standard. d^x fruits reached the stages II, III, and IV significantly later in comparison to wildtype fruits. For example, wildtype fruits reached stage IV (red and ripe) on average 47 days after anthesis, whereas d^x fruits required 61 days (Fig. 1). Wildtype and d^x fruit material of the different ripening stages was harvested at the time points given in Fig. 1. Levels of chlorophylls and carotinoids were determined by means of high performance liquid chromatography (HPLC). In agreement with the apparent color during ripening stages II, III, and IV, chlorophyll a, chlorophyll b, carotenes (lycopene, β-carotene, and phytofluene), and

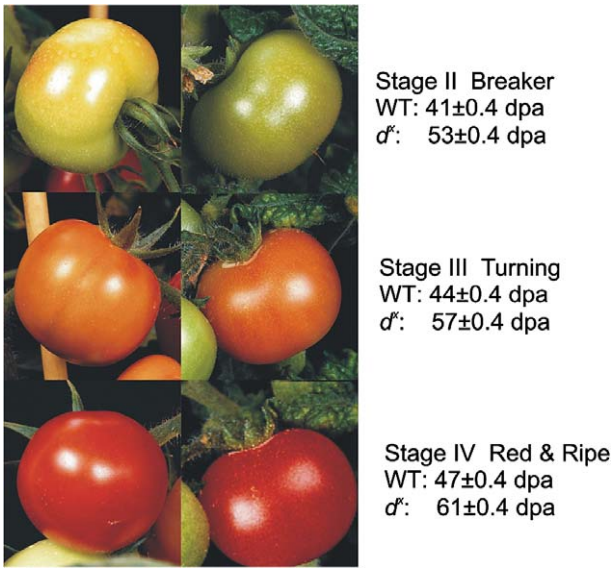

Fig. 1. Fruits of wildtype and the d^x mutant at different ripening stages. Left column shows wildtype, right column d^x mutant fruits. The d^x fruits reached the respective stages significantly later in comparison to wildtype fruits (*t*-test, $p < 0.05$, error: SE). dpa: days post-anthesis.

xanthophylls (lutein, zeaxanthin, neoxanthin, and violaxanthin) were found in similar abundance in d^x fruits in comparison to wildtype fruits (data not shown). Thus, pigment synthesis was delayed in d^x fruits, but pigment composition was not altered in other respects. Fresh weight and size of d^x fruits were determined at different ripening stages. No significant changes occurred (data not shown). This finding demonstrates that d^x fruits are capable to grow which most likely is due to formation of bioactive BRs by the CYP85A3 protein.

Climacteric ripening of tomato fruits is accompanied by a burst of ethylene (Adams-Phillips et al., 2004; Alexander and Grierson, 2002). BR-application was shown to trigger ethylene production in tomato pericarp discs (Vardhini and Rao, 2002). BR is also required for ripening of nonclimacteric fruits in which ethylene does not play a pivotal role (Symons et al., 2006). These facts indicate that BR could promote ripening of tomato fruits either directly or through altering ethylene production. However, as mentioned above, d^x fruits produce considerable amounts of bioactive BR (Nomura et al., 2005), which most likely are sufficient to trigger BR-specific processes in fruits. In fact, fruit development is also controlled by the shoot, and the lack of bioactive BR in shoots of the d^x mutant more likely caused the delay in fruit ripening.

2.2. Dry mass content and carbohydrate levels in d^x fruits

Starch accumulates early during fruit development. Starch levels were determined enzymatically in d^x and wildtype fruits and turned out to be significantly lower in d^x fruits at the ripening stage I (wildtype: 208.9 ± 16.6 μg/g fresh weight; d^x: 80.4 ± 12.7 μg/g fresh weight). Later in tomato fruit development, sugars such as glucose and

fructose accumulate. Soluble sugars are major determinants of the soluble solid content, which represents an important yield component of tomatoes. Dry mass content and relative levels of glucose, fructose, sucrose and other sugars were determined in wildtype and d^x fruits at stages II–IV. Dry mass content was significantly reduced in d^x fruits at all stages in comparison to wildtype fruits of the respective stages (Fig. 2). Gas chromatography coupled to mass spectrometry (GC-MS, Roessner et al., 2000; Schauer et al., 2005) was used to survey relative levels of various sugars. Levels of fructose, glucose, maltose, mannose, and xylose were significantly reduced in d^x fruits at stages II, III, and IV in comparison to wildtype fruits (Table 1). Sucrose levels were lower at all stages (Table 1 [difference significant at stage II] and Table S1 [differences significant at stages 0, I, II, III, and IV]). Levels of sugar alcohols were significantly lower in d^x fruits (Table 1). Galactose represented the only sugar with higher levels in d^x fruits at stages III and IV (Table 1).

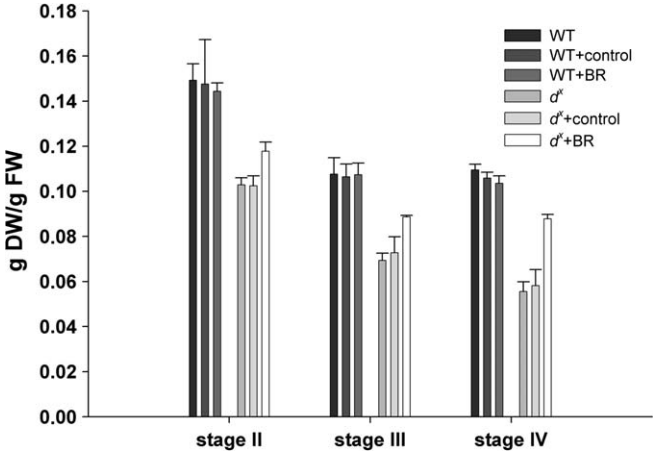

Fig. 2. Dry weight of wildtype and d^x fruits at different ripening stages. Dry mass content of d^x fruits was significantly lower at all ripening stages in comparison to wildtype fruits at the respective stages (t-test, $p < 0.05$, error: SE). BR treatment significantly increased dry mass content of d^x fruits at all ripening stages in comparison to untreated and mock-treated plants. BR-treatment did not significantly alter dry mass content of wildtype fruits.

The d^x mutant does not produce bioactive BR in the shoot (Bishop et al., 1999), whereas CS and BL were detected in fruits due to the presence of the CYP85A3 protein (Nomura et al., 2005). Metabolic changes in d^x fruits could be due to BR-deficiency of the shoot. In that case, BR-application to leaves would be expected to normalize metabolic changes. In fact, BR-application significantly increased dry mass content of d^x fruits in comparison to untreated and mock-treated plants (Fig. 2) and normalized levels of sugars such as fructose and glucose (Supplementary material, Table S1). BR-treatment of wildtype leaves did not increase dry mass content of fruits, but resulted in significantly elevated sugar levels (Table S1). These observations demonstrate that BR in leaves is required for dry mass accumulation and sugar accumulation in tomato fruits.

Sucrose is a major end product of photosynthesis and transported into sink tissues (Winter and Huber, 2000). Cleavage of sucrose into hexoses within the fruit by invertases is the driving force for carbohydrate import (Roitsch and González, 2004). Activity of cell wall acid invertases was significantly lower during stages II and III (Fig. 3). Reduced acid invertase activity likely confers reduced sink strength of d^x fruits. The importance of a fruit-specific

Table 1
Sugar and sugar alcohol composition in pericarp of wildtype and d^x mutant fruits at different ripening stages

	WT stage II		WT stage III		WT stage IV		d^x stage II		d^x stage III		d^x stage IV	
		SE		SE		SE		SE		SE		SE
Sugars												
Fructose	1.0	0.07	1.2	0.09	1.3	0.02	**0.6**	**0.02**	**0.8**	**0.04**	**0.8**	**0.03**
Fucose	1.0	0.08	1.1	0.05	n.d.		0.9	0.06	1.0	0.10	n.d.	
Galactose	1.0	0.08	1.4	0.11	4.0	0.01	0.9	0.02	**2.2**	**0.20**	**5.8**	**0.15**
Glucose	1.0	0.07	1.1	0.08	1.3	0.01	**0.6**	**0.03**	**0.8**	**0.03**	**0.7**	**0.01**
Maltose	1.0	0.11	0.7	0.05	0.3	0.02	**0.3**	**0.02**	**0.2**	**0.02**	**0.1**	**0.00**
Mannose	1.0	0.07	1.2	0.09	1.2	0.04	**0.6**	**0.03**	**0.8**	**0.04**	**0.8**	**0.03**
Rhamnose	1.0	0.06	1.2	0.07	n.d.		0.9	0.04	1.4	0.10	n.d.	
Ribose	1.0	0.07	1.2	0.07	2.6	0.05	1.1	0.06	1.2	0.08	3.1	0.07
Sucrose	1.0	0.14	0.7	0.40	0.3	0.00	**0.4**	**0.05**	0.3	0.04	0.2	0.15
Trehalose	1.0	0.11	0.8	0.60	0.5	0.00	**0.6**	**0.02**	**0.4**	**0.02**	0.4	0.00
Xylose	1.0	0.07	1.5	0.11	1.9	0.03	**0.8**	**0.02**	**0.9**	**0.08**	**1.1**	**0.13**
Sugar alcohols												
Erythritol	1.0	0.10	1.1	0.10	1.4	0.10	**0.7**	**0.00**	**0.7**	**0.00**	**1.0**	**0.10**
Mannitol	1.0	0.04	1.2	0.08	n.d.		1.1	0.09	1.2	0.11	n.d.	
Myoinositol	1.0	0.10	1.2	0.10	1.3	0.10	**0.6**	**0.00**	**0.8**	**0.00**	**1.0**	**0.00**
Ononitol	1.0	0.20	1.0	0.20	n.d.		**0.6**	**0.10**	0.6	0.00	n.d.	
Sorbitol	1.0	0.18	0.7	0.03	0.5	0.01	**0.5**	**0.06**	0.4	0.08	0.5	0.06

Values are represented as the mean and SE of six samples. Significantly altered levels of metabolites in d^x fruits in comparison to wildtype fruits of the respective stage are set in bold type (t-test, p-value < 0.05), n.d. indicates metabolites not detected.

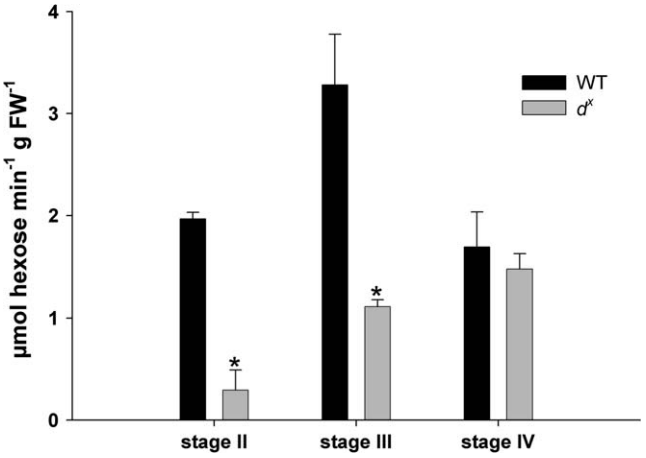

Fig. 3. Cell wall invertase activity in wildtype and d^x fruits at different ripening stages. Asterisks (*) indicate significant changes (*t*-test, $p < 0.05$, error: SE).

invertase for sugar accumulation in tomato fruits was shown by means of the analysis of tomato introgression lines that showed higher sugar yield. A single nucleotide polymorphism (SNP) was identified in the *S. pennellii Lin5* allele encoding a cell wall invertase. The SNP resulted in higher enzyme activity (Fridman et al., 2004). As a consequence, higher glucose and fructose levels were found in fruits of tomato introgression lines carrying the *S. pennellii* allele compared to those carrying the *S. lycopersicum* allele (Baxter et al., 2005; Fridman et al., 2004). However, *Lin5* transcript levels were not significantly reduced in d^x fruits (data not shown). Shoot-derived factors required for high invertase activity may be absent in d^x fruits due to BR-deficiency of shoots. Previous reports emphasized the relevance of BR for invertase activity and demonstrated that BR affects sink strength. For example, BR-application increased expression of the *Lin6* gene in the growing zone of tomato hypocotyls and promoted sugar uptake into cells (Goetz et al., 2000). Total acid invertase activity was also shown to be reduced in BR-deficient *Arabidopsis* plants (Schlüter et al., 2002).

Reduced sugar levels of d^x fruits could also be due to reduced photosynthetic capacity of d^x leaves, because carbon assimilation rates were significantly lower in comparison to tomato wildtype leaves (Müssig, C., manuscript in preparation). Furthermore, BR-deficient *Arabidopsis* plants displayed reduced CO_2 assimilation rates (Schlüter et al., 2002), and BR-application was shown to result in elevated CO_2 assimilation rates (e.g. Braun and Wild, 1984). In addition to reduced CO_2 assimilation rates (which are quoted as CO_2 uptake per time and leaf area), reduced leaf size certainly contributes to the reduced assimilatory capacity of BR-deficient plants such as the d^x mutant.

Table 2
Amino acid and amine composition in pericarp of wildtype and d^x mutant fruits at different ripening stages

	WT stage II		WT stage III		WT stage IV		d^x stage II		d^x stage III		d^x stage IV	
		SE		SE		SE		SE		SE		SE
Amino acids												
Alanine	1.0	0.04	1.3	0.08	1.8	0.00	1.2	0.09	1.2	0.11	1.9	0.00
Arginine	1.0	0.18	0.7	0.03	0.7	0.01	**2.0**	**0.06**	**1.6**	**0.08**	**0.9**	**0.06**
Asparagine	1.0	0.00	0.5	0.00	0.6	0.00	**5.3**	**0.20**	**3.8**	**0.10**	**1.8**	**0.20**
Aspartate	1.0	0.10	1.1	0.10	2.8	0.10	**1.9**	**0.10**	**3.9**	**0.30**	**7.4**	**0.30**
Cysteine	1.0	0.10	1.3	0.10	3.2	0.20	**1.6**	**0.10**	**4.3**	**0.40**	**5.2**	**0.30**
γ-Aminobutyrate	1.0	0.10	1.0	0.10	0.9	0.10	**1.9**	**0.10**	**3.3**	**0.10**	**2.9**	**0.10**
Glutamate	1.0	0.10	1.1	0.10	3.9	0.20	**2.4**	**0.10**	**8.0**	**0.60**	**13.5**	**0.50**
Glutamine	1.0	0.10	0.8	0.10	0.7	0.00	**1.2**	**0.10**	**1.2**	**0.00**	**1.0**	**0.10**
Glycine	0.9	0.00	1.0	0.00	1.3	0.00	**1.2**	**0.00**	**1.5**	**0.00**	**1.7**	**0.00**
Homoserine	1.0	0.10	1.1	0.10	1.4	0.10	**1.5**	**0.10**	**1.7**	**0.10**	**1.9**	**0.10**
Lysine	1.0	0.00	0.9	0.10	n.d.		**1.7**	**0.10**	**2.2**	**0.10**	n.d.	
Methionine	1.0	0.00	1.7	0.10	2.1	0.20	**2.0**	**0.10**	**4.0**	**0.20**	**4.0**	**0.20**
Ornithine	1.0	0.00	1.2	0.10	n.d.		**2.3**	**0.10**	**1.6**	**0.10**	n.d.	
Phenylalanine	1.0	0.10	0.8	0.10	0.9	0.10	1.0	0.00	**1.5**	**0.10**	**1.2**	**0.10**
Proline	1.0	0.00	1.0	0.00	1.9	0.00	**1.5**	**0.00**	**1.7**	**0.10**	n.d.	
Pyroglutamate	1.0	0.10	0.9	0.10	1.9	0.10	**2.5**	**0.10**	**4.6**	**0.30**	**5.9**	**0.30**
Threonine	1.0	0.10	0.9	0.00	0.9	0.00	**1.6**	**0.00**	**2.0**	**0.00**	**1.2**	**0.20**
Tryptophan	1.0	0.10	1.8	0.10	1.7	0.10	0.9	0.10	**2.6**	**0.10**	**2.2**	**0.00**
Tyrosine	1.0	0.10	1.1	0.00	1.5	0.10	0.9	0.00	1.2	0.10	1.7	0.10
Valine	1.0	0.10	0.7	0.00	0.8	0.00	**2.1**	**0.10**	**2.3**	**0.10**	**1.3**	**0.00**
Amines												
Dopamine	1.0	0.20	1.6	0.10	2.4	0.10	**2.3**	**0.00**	1.3	0.10	2.0	0.10
Putrescine	1.0	0.10	1.0	0.10	1.2	0.10	**1.9**	**0.10**	**1.8**	**0.10**	1.1	0.00
Spermidine	1.0	0.00	0.9	0.00	0.9	0.00	**1.5**	**0.00**	**1.9**	**0.00**	**1.2**	**0.00**
Tryptamine	1.0	0.10	2.5	0.20	1.9	0.10	**1.7**	**0.10**	**3.3**	**0.20**	**2.3**	**0.10**
Tyramine	1.0	0.00	1.5	0.10	n.d.		**5.1**	**0.10**	**4.0**	**0.20**	n.d.	

Values are represented as the mean and SE of six samples. Significantly altered levels of metabolites in d^x fruits in comparison to wildtype fruits of the respective stage are set in bold type (*t*-test, *p*-value < 0.05), and n.d. indicates metabolites not detected.

2.3. Amino acid and organic acid levels

GC-MS was used to monitor changes of amino acids, organic acids, and other metabolites in fruits of wildtype and d^x mutant plants. In the absence of exogenous BRs d^x fruits accumulated increased levels of amino acids in comparison to wildtype fruits. Significantly higher levels of amino acids such as arginine, aspartate, cysteine, glutamate, glutamine, methionine, threonine and valine accumulated in d^x fruits during stages II–IV in comparison to wildtype fruits of the respective stages (Table 2). None of the detected amino acids showed significantly lower levels in d^x fruits in comparison to wildtype fruits. Amines also showed higher levels in the mutant (Table 2). These observations indicate the requirement of BR for normal nitrogen metabolism in tomato fruits. In order to test whether the elevated amino acid levels in d^x fruits were caused by BR-deficiency of the shoot, BR was applied to d^x leaves. The BR-treatment resulted in a significant decrease of amino acids such as alanine, asparagine, isoleucine, leucine, methionine, and tryptophan in d^x fruits in comparison to untreated d^x plants (Supplementary material, Table S2). Only aspartate (stages II and III) and threonine (stage II) were increased. The partial normalisation of amino acid levels in fruits as a result of BR-treatment of leaves suggests that the BR deficiency of d^x shoots may cause altered nitrogen metabolism in d^x fruits.

GC-MS was used to determine relative levels of organic acids. No general trend was observed in d^x fruits, and major organic acids such as malate and citrate did not show consistent differences in independent experiments (Supplementary material, Table S3, and data not shown).

3. Conclusions

The d^x mutant produces bioactive BR in fruits but not in the shoot. It provides an option to dissect BR-dependent processes in fruits and shoots. By surveying metabolic processes in d^x fruits, reduced starch levels, reduced sugar levels, and elevated amino acid levels were found. BR-application to leaves partly normalized metabolic changes in d^x fruits suggesting that shoot-derived BR-dependent factors are required for proper fruit metabolism. Reduced cell wall invertase activity and an impaired photosynthesis in leaves of the mutant likely caused reduced sugar levels in fruits. Future work will address shoot-derived signals in tomato plants which modify primary metabolism in sink tissues, and analyze BR-effects on source efficiency on the physiological and molecular level.

4. Experimental

4.1. Plant material and growth conditions

As Craigella is the background of the d^x mutant (Accession No. LA3615), *Solanum lycopersicum* Craigella (Accession No. LA3247) was used as wildtype control. Seeds were obtained from the C.M. Rick Tomato Genetics Resource Center (TGCR, University of California at Davis). Plants were grown in potting soil in a greenhouse (16 h light [approximately 600 μmol m^{-2} s^{-1}], 26 °C, approximately 70% relative humidity/8 h night, 18 °C, approximately 70% relative humidity). All plants were grown in the same chamber at the same time in a randomized manner. d^x plants were grown on pedestals in order to adjust distance to the light source to wildtype plants. Fruit material was harvested as indicated in the text. The BR 24-epibrassinolide (EBL) (CID-tech Research Inc., Cambridge, ON) was applied as 300 nM solution to leaves. The control solution used for mock-treatments contained identical amounts of solvent (0.015% EtOH) and detergent (0.05% Tween 20). Treatments were performed twice weekly until harvest of fruit material.

4.2. Starch content

Pericarp tissue was ground to a powder under liquid nitrogen. One hundred milligrams of material was homogenized with 1 ml 90% EtOH, the samples incubated at 70 °C for 90 min, and centrifuged (15000*g*, 10 min). The pellet was washed two times with 1 ml 80% EtOH to remove soluble sugars. Starch was determined in the pellet after hydrolysis of starch with 400 μl 0.2 N KOH for 90 min at 95 °C and subsequent neutralization with 70 μl 1 N HCl using a commercially available kit (R-Biopharm, Darmstadt, Germany) based on the photometric determination of glucose.

4.3. Invertase activity

Proteins were prepared from frozen pericarp powder. The extraction buffer contained 50 mM Hepes-KOH (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 2 mM benzamidine, 2 mM 6-aminocaproic acid, 0.5 mM PMSF, 10% glycerol, and 0.1% triton-X-100. Five millilitres of extraction buffer were added to 5 g pericarp powder from three tomato fruits. Samples were allowed to thaw, transferred to 15 ml Falcon tubes, and centrifuged at 4 °C, 12000*g* for 10 min. Bound invertases were extracted from the pellet by adding 450 μl 1 M NaCl and 50 μl glycerol (Doehlert and Felker, 1987). After centrifugation, the supernatant was aliquoted and assayed for activity of acid invertases. The invertase activity assay was as follows: 70 μl 100 mM sucrose in 20 mM NaOAc (pH 4.7) was added to 30 μl buffer. Samples were incubated at 30 °C for 60 min. Ten microlitres of 1 M Na_2PO_4 (pH 7.2) was added to stop the reaction, and all samples were incubated for 6 min at 95 °C. 588.5 μl containing 100 mM immidazole, pH 6.9, 5 mM $MgCl_2$, 2 mM $NADP^+$, 1 mM ATP, and 2 U/ml glucose-6-phosphate dehydrogenase were added to 10 μl of the reaction mixture. 1.5 μl hexokinase (1500 U/ml) were added to each reaction and absorption increase was fol-

lowed at 340 nM. Subsequently, 2 µl phosphoglucoisomerase (700 U/ml) were added and absorption increase was followed at 340 mM.

4.4. Metabolite sample extraction and preparation

Relative levels of metabolites were determined by a method modified from that described by Roessner et al. (2000). Samples were extracted in 1500 µl of methanol. Fifty microlitres of internal standard (2 mg of ribitol in 1 ml water) were added for quantitative standardisation for the polar stage. The mixture was extracted for 15 min at 70 °C, mixed vigorously with 1 volume of water, centrifuged at 2200*g*, and subsequently reduced to dryness *in vacuo*. The residue was redissolved and derivatized for 5 h at 45 °C (in 80 µl of 20 mg ml^{-1} methoxyamine hydrochloride in pyridine) followed by a 30 min treatment at 37 °C (with 80 µl of *N*-methyl-*N*-[trimethylsilyl]trifluoroacetamide). 40 µl of a retention time standard mixture (0.5 mg heptanoic acid, 0.5 mg nonaoic acid, 0.5 mg undecanoic acid, 0.5 mg tridecanoic acid, 0.5 mg pentadecanoic acid, 1 mg nonadecanoic acid, 1 mg tricosanoic acid, 1.5 mg heptacosanoic acid, and 3 mg triacontanonoic acid dissolved in tetrahydrofuran) were added before trimethylsilylation. Sample volumes of 1 µl were injected with a split ratio of 25:1, using a hot needle technique.

The GC-MS system was composed of an AS 2000 autosampler, a GC 8000 gas chromatograph, and a Voyager quadrupole mass spectrometer (ThermoQuest, Manchester, UK). The mass spectrometer was tuned according to the manufacturer's recommendations using tris-(perfluorobutyl)-amine (CF43). GC was performed on a 30 m SPB-50 column with 0.25 µm film thickness (Supelco, Bellfonte, CA). The injection temperature was set at 230 °C, the interface at 250 °C, and the ion source adjusted to 200 °C. Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. The analysis was performed under the following temperature program: 5 min of isothermal heating at 70 °C, followed by a 5 °C min^{-1} oven temperature ramp to 310 °C, and a final 1 min of heating at 310 °C. The system was then temperature equilibrated for 6 min at 70 °C before injection of the next sample. Mass spectra were recorded at 2 scan s^{-1} with a scanning range of 50–600 m/z. Both chromatograms and mass spectra were evaluated using the Masslab program (ThermoQuest). Six samples (each with 3 fruits) per stage and genotype were analyzed.

4.5. Statistical analysis

Data were analyzed using the Microsoft Excel software. If two observations are described in the text as different their difference was determined to be statistically significant ($p < 0.05$) by performance of Student's *t*-tests. Errors in the text, figures, and tables are given as standard error (SE).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2006.07.008.

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