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Original Scientific Paper

A biochemical and proteomic approach to the analysis of tomato mutant fruit growth

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ABSTRACT:
To assess the effects of ABA deficiency on tomato fruit growth, the ABA mutant *flacca* was grown in an optimal soil water regime and various analyzes were performed, including morphological (fruit number, diameter and fruit biomass), physiological (duration of growth and fruit growth rate), biochemical (ABA accumulation, enzyme cell wall peroxidase activity) as well as proteomics. The fruit growth analysis showed that the slower fruit growth rate and development resulted in smaller *flacca* fruits in comparison to the wild-type fruits. The comparison of the temporal dynamics of cell wall peroxidase activity and ABA content in our experiment indicated an opposite relationship during fruit development. Proteomic analysis and the down-regulation of most proteins from carbon and amino acid metabolism, the translation and processing of proteins, energy metabolism and cell wall-related metabolism in the *flacca* fruits compared to the wild type, indicated reduced metabolic flux which reflected a slower fruit growth and development and reduced fruit size in the ABA mutant. These findings also indicated that ABA limited carbon sources, which could be responsible for the reduced fruit growth and size of ABA-deficient tomato fruits. The up-regulation of sulfur and oxygen-evolving enhancer proteins in the *flacca* fruits implicated the maintenance of photosynthesis in the late expansion phase, which slows down transition to the ripening stage. The majority of antioxidative and stress defence proteins were down-regulated in the *flacca* fruits, which could be related to the role of ABA in the activity of different antioxidative enzymes as well as in regulating cell wall expansion and the cessation of fruit growth.

Keywords:
ABA, cell wall peroxidase, *flacca* mutant

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INTRODUCTION

The tomato (*Solanum lycopersicum* L.) is one of the most widely grown vegetables in the world and its fruits are of special economic importance because they are used both as fresh vegetables and as a component in the food processing industry (Faostat 2019). Moreover, it is well known that the consumption of the tomato fruit in the human diet, as a source of important bioactive compounds, is associated with significant positive effects on human health.

In addition to its economic and nutritional importance, the tomato has also become a model for the study of fleshy fruit development and growth (Quinet *et al.* 2019). Tomato fruit growth is a complex process depending on the interaction between different factors including physiological, biochemical and metabolic processes which are under the influence of internal (genotypic)

and external (environmental) control. According to the study carried out by Azzi *et al*. (2015), final fruit size depends on developmental phases of cell division and cell expansion, which are under the control of complex interactions between hormone signaling and carbon partitioning, which establish the determinants of the quality of ripe fruit. Also, fruit development and fruit weight are intimately connected to its composition of primary and secondary metabolites (Tohge *et al*. 2014).

Considerable attention has been directed toward elucidating the hormonal regulation during fruit development and ripening. A large amount of data in the literature has demonstrated the significant role of other hormones such as ABA and ethylene (in fruit set processes and the antagonistic effect on ripening) as well as polyamines and other plant growth regulators (Kumar *et al*. 2014; Mou *et al*. 2016).

Hormone-deficient mutants serve as a useful model for studying the role of a specific hormone and its interactions with other hormones. In our presented research we investigate the ABA-deficient *flacca* mutant. The biochemical mutation of *flacca* and low ABA content are a result of the deletion of 6 base pairs in the molybdenum cofactor sulfurase, thus reducing the oxidising capacity of aldehyde oxidases (AOs) (Sagi *et al*. 2002).

Although the mutants have been characterized at the molecular level, there is still not enough information about the effects of these ABA mutations on fruit growth. Nitsch *et al*. (2012) reported the phenotypic characterization of *not/flc* double mutant lines. The fruits of these double mutants have considerably reduced ABA levels, and displayed smaller fruit size and cell size, especially within the pericarp. The consequence of increasing ethylene levels while lowering ABA suggested that ABA stimulates fruit growth by restricting ethylene levels and limiting its growth inhibitory action.

Tomato fruit growth, similar to the growth of other plant organs, is regulated by the properties of the cell walls and the associated activities of the cell wall enzymes. These enzymes include particularly important cell wall peroxidases whose role has been implicated in "locking" together cellulose microfibriles through the formation of phenolic cross-linkages between the cell wall components, thus decreasing the ability of the cell wall to expand (Francoz *et al*. 2015). Although the changes to cell wall enzymes during the growth of plant organs and under the influence of different environmental effects have been widely investigated (reviewed by Tenhaken 2015), the literature contains very few reports investigating their role in the regulation of fruit expansion. Our previous results confirmed that an increase in cell wall associated peroxidase activities may play a role in restricting fruit growth rate and in the control of fruit maturation during the exposure of tomato plants to different degrees of drought and irrigation (Savić *et al*. 2008).

Different metabolic changes occur during tomato fruit growth and development. New sophisticated analyses such as transcriptomics, proteomics, and metabolomics contribute to the understanding of metabolites turnover during tomato fruit development (Karlova *et al*. 2014). Similar to gene expression, profiling proteomics offers the opportunity to examine and classify protein accumulation during tomato developmental processes. The results of Faurobert *et al*. (2007a) provided an overview of the main cherry tomato proteome variations during precise stages of fruit growth and ripening related to proteins associated with carbohydrate metabolism, photosynthesis and respiration, amino acid metabolism, secondary metabolism, and vitamin and lipid metabolism. Previously, we conducted proteome analyses to investigate the influence of the effects of partial rootzone drying (PRD) on metabolic changes in tomato fruit (Marjanović *et al*. 2012). The identified proteins were associated with carbon and amino acid metabolism, cell wall composition, stress defence and antioxidative defence. In general, they indicated that slower metabolic flux in PRD fruits may be the cause of the slower growth rate when compared to fully-irrigated fruits. Recently, Szymanski *et al*. (2017) demonstrated that proteomic profiling of tomato fruits within two tissues and five developmental stages cover 83% of all predicted enzymatic reactions in the metabolic network of the tomato.

Proteomics is of special importance as a tool for understanding fruit ripening. Rocco *et al*. (2006) carried out the proteomic analysis of two tomato ecotypes (regional and commercial) during ripening. Approximately 57% overlapping gel coordinates were detected, showing that a relatively large number of proteins were ecotype-specific. The proteins identified during the maturation processes were mainly associated with physiological processes such as defense, stress, redox control, carbon metabolism, energy metabolism, and cell signaling.

Although proteomic research is currently widely used, it is surprising that there is no data examining the metabolic changes that occur during the growth and development of the fruits of tomato mutants. Therefore, a proteomic approach was used in the presented study to analyze the molecular mechanism of mutant *flacca* fruit development and to compare it with a similar experimental pattern already tested in the fruits of wild type (Marjanović *et al*. 2012). We expect these results, as well as other morphological, physiological and biochemical measurements, to improve the understanding of how the reduced concentration of the ABA hormone affects the growth, development and final size of tomato fruits.

MATERIAL AND METHODS

Plant material and physiological analysis. Tomato (*S. lycopersicum*) mutant *flacca* was used for the experi-

ment which was carried out in a phytotron chamber (the photoperiod was 14 h; light intensity at plant level 300 $\mu molm^{-2}s^{-1}$, temperature 25/18°C and relative humidity 70%). The seeds of *flacca* (*flc*; genetic background Ailsa Craig) were provided by GIBIS/I, Gatersleben, Germany. The tomato seeds were germinated in the substrate Postground X (Klasmann-Deilmann, Germany) in styrofoam containers. In the phase of fully developed seventh leaves, the plants were transplanted into pots with a volume of 20 l which were filled with 11 kg of Postground H substrate and irrigated daily to maintain field capacity, with volumetric soil water content (θ) of 36%. The measurement of volumetric soil water content was done by a TDR probe (Time domain reflectometer, TDR100, TRASE, Soil Moisture Equipment Corp., USA).

During the experiments, the following parameters were measured as indicators of the growth and development of the tomato fruits: fruit diameter, number of fruits, the fresh and dry weight of the ripe fruits. Samples were collected and measured from at least 20 plants. The dynamics of the phenological phases during the fruit development were monitored according to the BBCH scale (Meier 2001).

Biochemical analysis. For the biochemical analysis, fruit pericarps were collected in different stages of fruit development, and in relation to the beginning of flowering. Pericarps were collected from at least 20 different samples. The selection of fruit samples was based on the similar size of the equatorial diameter of the fruit measured with a digital caliper (Carl Roth, Germany). Pericarp samples were taken from the equatorial part of the selected fruits (Thompson *et al.* 1998). The samples were immediately frozen in liquid nitrogen, stored at -80°C, and prior to analysis were ground to a fine powder in pre-chilled steel cylinders in a mixer mill. The dynamics of peroxidase activity and ABA content in the fruit pericarps were measured at different stages of fruit development (15 and 20 days post-anthesis (dpa) - the phase of intensive cell growth and rapid increase in cell volume, 30 dpa - at the beginning of the fruit ripening stage, and 45 dpa - the phase when the accumulation of carotenoids in the fruits begins) and are presented in Fig.1.

Cell wall-associated peroxidase activity was determined by a modified guaiacol test (Chance & Maehly 1955). Enzyme activity (ionic cell wall-associated peroxidase) was measured by a guaiacol test. This method is based on the spectrophotometric measurement of yellow colouration, derived from tetra guaiacol, which occurs as a product of the reaction between guaiacol (H-acceptor) and H_2O_2 (H-donor) under the action of peroxidase. A total of 200 µl of phosphate buffer (20 mM PBS pH 5.5) with guaiacol 0.56% (v/v), 19 µl 20 mM phosphate buffer (pH 5.5), 1 µl of sample and 40 µl 0.03% H_2O_2 was added to each well of microtiter plate. The microtiter plates were incubated at 25°C. The reaction lasted for 10 minutes from the addition of H_2O_2 to the samples, and then absorbance was measured by an ELISA spectrophotometer (Tecan, Sunrise) at 470 nm. The measured peroxidase activity was calculated using the equation of curve and expressed in horseradish peroxidase equivalent units (HRPEU)/g fresh weight of the sample. The equation of curve was obtained as previously described, except that instead of the sample solution, horseradish peroxidase with similar activity (0.004 IU) was used. The change in absorbance was recorded at 470 nm at 25°C, for every 2 min. over 20 minutes.

Fig. 1. Photography of *flacca* fruits in the examined stages of fruit development.

The concentration of the abscisic acid hormone was determined by the ELISA test (Asch 2000) based on the immunological reaction of the highly specific monoclonal antibody (MAC 252) with abscisic acid (ABA) assuring that there was no cross reaction between the antibodies and other substances in the test sample (Quarrie *et al.* 1988). Microtiter-well plate was coated with ABA, which competes with the ABA in the sample for binding to the limited amount of primary antibody (MAC 252). The amount of bound primary antibody is inversely proportional to the amount of abscisic acid in the sample. Then, an enzyme labelled the secondary antibody was added, which binds to the primary antibody. After the immunological reaction, staining was carried out with p-nitrophenyl phosphate, and the absorbance of the solution was read in the ELISA reader at 405 nm. The value is inversely proportional to the concentration of ABA, which was calculated by using the standard (calibration) curve obtained by measuring a series of standard solutions of known ABA concentration (±).

Proteomic analyses. Proteomic analyses of the fruit pericarps were conducted at 30 dpa, the stage close to the beginning of the ripening processes. Pericarps were collected from at least 15 different fruits, immediately frozen in liquid N and stored at -80°C. Prior to protein extraction, the samples were ground to a fine powder in pre-chilled steel cylinders by a mixer mill. All procedures for protein extraction by the phenol method (Faurobert *et al.* 2007b), separation (by two-dimen-

sional electrophoresis), analysis (by LC-MS/MS mass spectrometry) and identification (using the SGN tomato unigene database) were previously described in Marjanović *et al*. (2012).

Statistical analysis. Descriptive statistics were used to analyse the investigated morphological and biochemical parameters and the results were presented as the mean values and standard error (±) from 20 different samples. For protein quantification, Progenesis SameSpot software version 3.0 was used to detect varying spots using one-way analysis of variance (ANOVA) on normalized spot volume from the gel repeats with $p < 0.01$ and $q < 0.015$. Proteomic analyses were carried out from the fruit pericarps of at least 15 different samples. In order to assess the different effects between *flacca* and the wild type, a one-way ANOVA model was applied using R software version 2.11.1 (The R Project for Statistical Computing; http://www.r-project.org/), with significance levels of $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

RESULTS AND DISCUSSION

Fruit growth analysis. Analyses and comparisons of the results of the *flacca* fruit growth parameters (Table 1) with the literature data for wild type Ailsa Craig showed that both the *flacca* diameter (1.3-1.6 times) and fruit weight (2-3 times) were smaller than in the wild type (Rančić *et al*. 2010). A comparison of the results of *flacca* fruits with those obtained for wild type fruits grown in the same experimental conditions (Marjanović *et al*. 2012) showed that mature mutant fruits were smaller (by about 24%), as well as in number (by 30%) and had a lower final yield (by 76%) compared to the wild type. Nitsch *et al*. (2012) demonstrated that several ABA mutants (*notabilis, flacca* and double mutants *notabilis/flacca*) had reduced fruit size in comparison with the wild type which was caused by the smaller size of the pericarp cells. These results also indicated that ABA promotes tomato fruit growth *via* a positive effect on cell expansion.

The final size of the fruit is determined by the rate and duration of cell enlargement. Therefore, we also analyzed the time course of the fruit growth rate which is presented by two growth curves on the same graph (Fig. 2a, b). The first curve was obtained when the tomato fruits diameters vs. time were fitted to the second order of regression. The obtained curve followed a sigmoidal pattern of growth, similar to that reported by Monselise *et al*. (1978). The second symmetrical, bell-shaped curve was obtained by fitting the changes in the fruit growth rate (FGR) vs. time with a third order of regression line. The time course of fruit diameter changes indicated that after a short initial growth phase (cell division), the exponential phase of growth continued leading to cell elongation and a rapid increase in fruit diameter (Fig. 2a). The fruit diameter results showed that the intensive fruit growth rate started after 7 to 10 dpa and reached a plateau between 40 and 50 dpa.

The presentation of the FGR showed that the period of intensive fruit growth (more than 1.1 mm/day) was between 13th and 28th dpa (Fig. 2b). A comparison with the results of Marjanović *et al.* (2012) showed that the maximal FGR in *flacca* (1.3 mm/day at 13th dpa) was obtained 4 days earlier than in Ailsa Craig fruits and that the maximal FGR value was significantly lower compared to Ailsa Craig (1.7 mm/day at 17th dpa). After the phase of rapid cell elongation, the fruit growth rate declined between 35 and 45 dpa, and continued until the fruits reached their final size. These results indicated that lower maximal FGR values resulted in smaller *flacca* fruits in comparison to the wild-type fruits. Also, following the fruit phenological phases we noticed a slower fruit development in *flacca*, since the fruit ripening phase was completed within 65 days of anthesis, namely delayed for 6 days compared to the wild type which could be caused by the slower fruit growth rate.

Tomato fruit size depends on a combination of cell number and cell size, which are determined by cell division and expansion (McAtee *et al*. 2013). The histological and cytological analysis of fruit development in tomato wild type cv. Alisa Craig and ABA-deficient mutant *flacca* showed that the total number of pericarp cells increased up to 20 days post- anthesis in both genotypes, suggesting a similar period of cell division. However, the final fruits were about three times smaller in *flacca* compared to the wild type, which is related to the reduction in pericarp cell number and cell size (Pećinar *et al*. 2019). This study, conducted in similar growing conditions, may indicate that the smaller fruit in the *flacca* mutant observed in our experiment may be the result of both the reduced cell number in the pericarp as well as the reduced cell elongation process.

Peroxidase activity. During the fruit development, the cell division and cell expansion phases imply the regulation of the cell wall metabolism, which have a direct influence on the fruit firmness and texture. It is known that cell wall peroxidase enzymes are involved in modifying the cell wall structure by reducing cell wall expansion through the oxidation of phenols and forming diphenol bridges between polymers. The measurements of the cell wall-associated peroxidase activity in the pericarp of the *flacca* mutant (Fig. 3a) indicated a trend of increased activity during fruit development from 15 dpa (0.37 HRPEU/g FW) until 45 dpa when maximal activity was recorded (1.63 HRPEU/g FW).

Several studies have shown the role of cell wall-associated peroxidases in the regulation of cell growth of different plant organs and species (Bacon *et al.* 1997; Thompson *et al.* 1998; Jovanović *et al.* 2004). Metabolic changes during tomato fruit development affect the

Table 1. Investigated parameters of *flacca* mutant fruits. Reported values are mean ± SE

Fruit diameter (mm)	Fruit number /plant	Fruit FW (g)	Fruit DW (g)	Fruit FW /plant (g)	Fruit DW /plant (g)
37.73±1.54	30.17±1.51	17.26±1.27	1.38±0.24	521.57±46.14	41.59±7.71

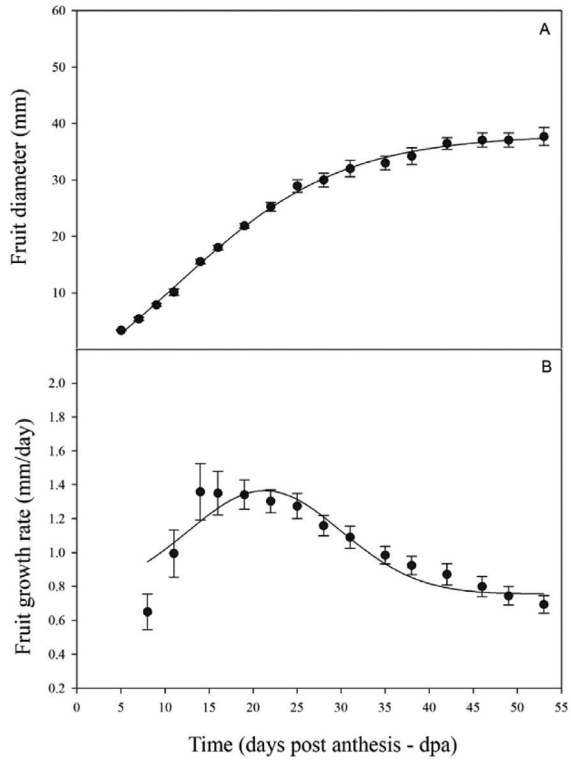

Fig. 2. Fruit growth dynamics of tomato mutant *flacca* (fruit diameter – A; fruit growth rate – B).

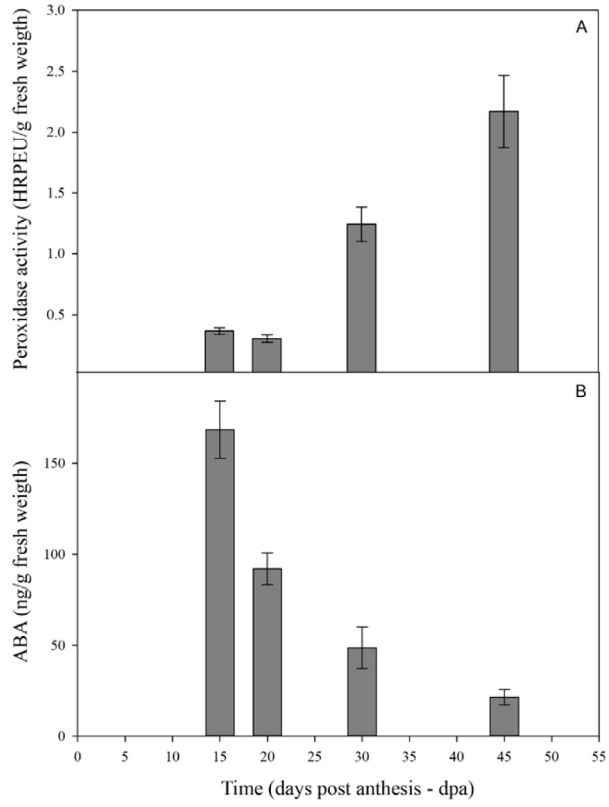

Fig. 3. Cell wall peroxidase activity (A) and ABA content (B) in the pericarp during tomato fruit development.

formation of different peroxidase isoforms in the cell wall, and some of them play an important role in the regulation of tomato fruit growth by catalysing the stiffening of the fruit exocarp in the latter stages of fruit development and limiting further fruit growth (Andrews *et al*. 2002; Vicente *et al*. 2007). In our experiment the increased ionically bound cell wall peroxidase activity in the *flacca* mutant corresponds to the cessation of fruit growth and the beginning of the ripening phase. This is in agreement with the literature data. The results of Savić *et al*. (2008) indicated that cell wall peroxidase activity increases during the deceleration of tomato fruit growth and during maturation. Also, the study of the effects of PRD on tomato fruit growth in cultivar Ailsa Craig showed that the increase in the activity of cell wall-associated peroxidase in the tomato fruit pericarp coincided with the end of cell growth and the beginning of the ripening process thus indicating that this enzyme may regulate tomato fruit maturation (Marjanović *et al*. 2015).

ABA accumulation in fruits. Tomato fruit growth and development are under the control of different hormones where abscisic acid plays an important role (Kumar *et al*. 2014). The measurements of the abscisic acid content in the fruit pericarp of the tomato *flacca* mutant (Fig. 3b) indicated that ABA concentration decreased from the beginning of intensive cell growth - 15 dpa (168.37 ng/g FW) until the breaker stage (45 dpa - 21.48 ng/g FW). In the period between 20 dpa and 45 dpa, the growth rate of the *flacca* fruits begins to decrease sharply which corresponds to the decline of ABA content.

The accumulation of ABA in different parts of the fruit and at different growth stages indicates the specific

Table 2. Identified proteins in the pericarp of different tomato genotypes (Ailsa Craig and *flacca*).

Spot No°[a]	Accession No°[b]	Accession No°[b]	Protein identification	PS TargetP[c]	Ailsa Craig[d] *	*flacca*[d]	Genotype effect[e]
1	SGN-U578193	P26300	Enolase	Cyt	4.9	3.7	*
2	SGN-U577646	Q40546	Pyruvate kinase isozyme G	cTP	0.8	0.4	***
3	SGN-U579289	Q41135	50 kDa ketoacyl-ACP synthase	cTP	11.6	6.7	**
4	SGN-U578195	P29000	Acid beta-fructofuranosidase	Cyt	3.1	2.3	ns
5	SGN-U580213	Q8LK04	Glyceraldehyde 3-phosphate dehydrogenase	Cyt	15	12.7	**
6	SGN-U579393	P26300	Enolase	Cyt	5.9	8.1	**
7	SGN-U575371	Q9FR11	Pyruvate dehydrogenase	cTP	2.1	1	***
8	SGN-U566720	Q5XMB8	Cytosolic acetoacetyl-coenzyme A thiolase	Cyt	11.1	5.8	***
9	SGN-U578979	Q8GT30	Dihydrolipoyl dehydrogenase	mTP	3.8	2.5	**
10	SGN-U577224	B9SHB0	Alcohol dehydrogenase	cTP	9	11.1	**
11	SGN-U315784	Q9FS27	Cytosolic cysteine synthase	Cyt	18.1	13.7	ns
12	SGN-U577991	P43281	S-adenosylmethionine synthetase	Cyt	14.8	8.3	**
13	SGN-U571120	A7YVW1	ACTCH P5CDH1	Cyt	2.1	1.2	*
14	SGN-U580783	P43282	S-adenosylmethionine synthetase	Cyt	3.3	2.2	**
15	SGN-U577702	Q40143	Cysteine proteinase 3	mTP	11.5	6.2	*
16	SGN-U584866	A0FH76	EBP1	Cyt	2.6	1.3	***
17	SGN-U565096	Q9XG77	Proteasome subunit alpha type-6	Cyt	7.4	6	*
18	SGN-U573205	Q9FES7	Sulfur	cTP	6.7	10.1	**
19	SGN-U569024	Q2PYY1	Protein transport SEC13-like protein;	Cyt	3.9	2.3	**
20	SGN-U569332	B9MYQ8	Predicted protein;	Cyt	3.1	1.5	***
21	SGN-U577869	P05495	ATP synthase subunit alpha	SP	9.7	7.5	ns
22	SGN-U568784	O49949	Magnesium dependent soluble inorganic pyrophosphatase	Cyt	4.4	3.7	*
23	SGN-U578510	A7WPL2	Putative cinnamyl alcohol dehydrogenase	Cyt	13.6	8	***
24	SGN-U566162	B6ZN57	prolyl 4-hydroxylase	SP	4.1	2.7	**
25	SGN-U577151	Q6IV07	UDP-glucose:protein transglucosylase-like protein	Cyt	3.4	1.7	***
26	SGN-U574142	D2D2Z3	UDP-L-rhamnose synthase	Cyt	5.1	3.4	***
27	SGN-U580784	Q3I5C4	Cytosolic ascorbate peroxidase	Cyt	30.1	23.1	**
28	SGN-U578588	Q7YK44	Superoxide dismutase	cTP	4.5	5.7	ns
29	SGN-U578449	Q52QQ4	Ascorbate peroxidase	Cyt	16.1	12.1	***
30	SGN-U581258	Q201Z4	Thioredoxin	Cyt	9.8	6.2	***
31	SGN-U583863	Q7XAV2	Superoxide dismutase [Cu-Zn]	SP	12.9	10.1	*
32	SGN-U581590	Q43779	Superoxide dismutase [Cu-Zn]	Cyt	54.8	42.9	*
33	SGN-U580023	P23322	Oxygen-evolving enhancer protein	cTP	14.8	27.5	*

34	SGN-U577430	B9RBP6	Heat shock protein 70	Cyt	9.8	6.9	**
35	SGN-U579203	O81535	Annexin p35	Cyt	16.8	13.3	***
36	SGN-U581281	P29795	Oxygen-evolving enhancer protein 2	cTP	13	19.6	***
37	SGN-U566348	B9HK69	Predicted protein	cTP	3.2	2.2	**
38	SGN-U580030	Q84QE4	Putative chloroplast thiazole biosynthetic protein	cTP	10.5	7.1	***
39	SGN-U566913	B9MVM3	Predicted protein	Cyt	5.6	3.9	***
40	SGN-U570828	B9SQB9	Ran-binding protein	Cyt	0.6	0.3	**

[a] Spot number.
[b] Number under which proteins are registered in the Sol Genomic Network / UniProt database.
[c] Localisation of the peptide in a cell, on the basis of TargetP software: cTP - chloroplast transit peptide, mTP - mitochondrial peptide, Cyt - cytoplasm, SP - secretory protein.
[d] Quantitative values of the identified proteins.
[e] ANOVA results for genotype effects: ns, non-significant effect; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
* The data for Ailsa Craig were reproduced from Marjanović *et al.* (2012) with the permission of publisher Mary Ann Liebert, Inc.; New Rochelle, NY.

roles of ABA in the reproductive development of tomatoes. According to Gillaspy *et al.* (1993), endogenous ABA concentration in tomato fruits reaches itsd maximum during the cell growth phase. High ABA concentrations in the tomato pericarp, as well as in the axis and locule were found in the early growth stages (around 19 dpa) when tomato fruits have a high growth rate, and then tend to decrease towards the end of this phase (Kojima *et al.* 1993). Our results related to the fruit growth rate and ABA concentration in this phase are in agreement. The investigation of ABA content in the tomato pericarp of Ailsa Craig grown under different irrigation treatments also showed a declining trend during the tomato development until the end of the cell growth phase without any significant differences between differently treated plants (Marjanović *et al.* 2015).

The study of different ABA-deficient tomato mutants has served to gain new insights into the role of the main hormones which regulate fruit growth and expansion. The analysis of the hormones in the pericarp of tomato young fruits in the stage corresponding to maximal cell expansion showed that auxin levels in the ABA-deficient mutants (*flacca* and *notabilis*) were not correlated with the reduced fruit size when compared to the wild type. Also, the size of the pericarp cells in the *not/flc* double mutants was smaller than in the wild types, thus indicating that ABA promotes cell expansion in wild type tomato fruits (Nitsch *et al.* 2012). According to these results, the lower concentration of ABA responsible for the small fruit size of *flacca* and *notabilis* mutants were accompanied by higher ethylene evolution rates. Literature data indicated that ABA-deficient tomato mutants *notabilis* and *flacca* produce 1.6–2.3-fold more ethylene than wild types (Hussain *et al.* 2000). In our experiment, the average concentration of ABA in the *flacca* fruits was 49% of the concentration of ABA measured in the wild type fruits of Ailsa Craig (Marjanović *et al.* 2015). Although we did not measure the ethylene levels in the fruit, it could be assumed that the antagonistic interaction levels between ABA and ethylene could also explain the size of the *flacca* fruits in our experiment, which was significantly lower than in wild type Ailsa Craig.

Our results also showed that the increase in cell wall peroxidase activity coincides with the reduction in ABA concentration as well as with declined fruit growth rate and the beginning of the fruit ripening stage (Fig. 3a, b). According to the literature, peroxidase activity can be associated with the hormonal status of plants. The results of Lin & Kao (2001) show that elevated ABA concentrations preceded an increase in the peroxidase activity in the roots of rice plants. Also, the suppression of the *NCED1* gene in the ABA biosynthetic pathway results in the down-regulation of some ripening-related cell wall genes, as well as in an increase in the tomato fruit firmness (Sun *et al.* 2012). On the other hand, the results of Andrews *et al.* (2000) suggest the possibility that increases in peroxidase activity during fruit ripening was induced by ethylene. The comparison of the temporal dynamics of cell wall peroxidase activity and ABA content in our experiment indicated an opposite trend during the fruit development. To our knowledge, there is no data in the literature on the effects of the relationship between ABA and peroxidase on tomato fruit growth. However, since it is well known that ABA reduces cell wall extensibility (Kutschera & Schopfer

1986), it could be assumed that ABA affects cell wall peroxidase activity to cause cell wall stiffening, a process which coincides with the cessation of cell expansion and fruit growth.

Proteomic analysis. Several proteomic studies have been carried out on tomato (Faurobert *et al*. 2007a; Manaa *et al*. 2011; Osorio *et al*. 2011), but there are still limited data for proteomics related to tomato mutants (Rohrmann *et al*. 2011; Kilambi *et al*. 2013). In our proteomic analysis 40 protein spots were classified into several metabolic categories: carbon metabolism, amino acid metabolism, protein translation, processing and degradation, energy metabolism, cell wall related, oxidative stress, stress defence and heat shock proteins, and 4 proteins with unknown functions (Table 2; Fig. 4).

The analysis of the identified proteins was done by comparing the proteomic data for the *flacca* mutant with the proteomic data for the wild type Ailsa Craig, which was grown under the same experimental conditions (Marjanović *et al*. 2012) as the *flacca* mutant. The results showed significantly different variation in abundance between genotypes and the majority of proteins were down-regulated in the *flacca* mutants compared to the wild type (Table 2). The expression of proteins associated with carbon metabolism was dominant in the proteomic analysis, which is in accordance with the literature. Xu *et al*. (2013) showed that in the proteome profile of the fruit pericarp from different tomato genotypes, proteins of primary metabolic processes represented the largest class of proteins (mostly enzymes involved in sugar metabolism), followed by proteins of macromolecular metabolic processes and then a subgroup related to stress responses (chaperonins and proteasome proteins).

Most of the identified proteins from the carbon metabolism group were down-regulated in *flacca* compared to the wild type (Fig. 5). These are proteins whose main function lies in glycolysis which is an important metabolic process for growing tissues, since their intermediates are substrates for the synthesis of amino or fatty acids, but also for secondary metabolites such as isoprenoids and phenylpropanoids (Voll *et al*. 2009). The down-regulation of enzymes such as pyruvate kinase isoenzyme G (spot 2) and glyceraldehyde-3-phosphate dehydrogenase (spot 5) in the *flacca* mutant compared to the wild type indicated lower metabolic flux in the glycolysis, which is important for ATP production and the fruit ripening process. Among all the proteins involved in carbon metabolism, enzyme pyruvat dehydrogenase (spot 7) showed the highest reduction in abundance (about 52%), which could indicate that both energy and the main precursor for lipid biosynthesis are available in much lesser amounts in mutants than in wild types. Together with dihydrolipoyl dehydrogenase (spot 9) these enzymes are an important part of the multi-enzymatic complex which converts pyruvate to Acetyl Co-A and links glycolysis as a cytoplasmatic process with the TCA cycle.

During tomato fruit development some enzymes involved in the recycling of hexose-P exhibit high activity during the late elongation phase, reaching their maximum at the green mature stage (Quinet *et al*. 2019). The maintenance of metabolic processes is important for fruit growth and depends on the optimal supply of carbon substrates for growing cells. Also, sugars are important in tomato fruits for the generation of turgor pressure in order to promote cell expansion (Kanayama 2017), and they also act as signal molecules regulating fruit development and metabolism. The down-regulation of the enzyme fructofuranosides (spot 4) involved in the hydrolysis of sucrose into monosaharides glucose and fructose could also be responsible for the slower *flacca* fruit growth rate and its smaller size compared to the wild type fruits.

Proteomic profiling of the microsomal fraction of the tomato fruit in the mature green stage (MG30) corresponding to the end of cell expansion and the start of ripening showed an increased abundance of proteins involved in glycolysis and carbohydrate metabolism, amino acid biosynthesis and the catabolic process, the synthesis of precursor metabolites and energy, as well as proteins involved in cell wall synthesis and remodeling (Pontiggia *et al*. 2019). Among the proteins involved in glycolysis several enzymes showed much higher abundance including enolase. In our proteome analysis two spots identified as enolase represented the same protein. Enolase (spot 1) was down-regulated, while enolase as spot 6 was up-regulated in *flacca* compared to the wild type. Although other enzymes of glycolysis were down-regulated, the high level of one enolase isoform (spot 6) might reflect the high demand for piruvate for *de novo* syntheses in tissues. Enzyme activity profiles in young tomato fruits are characterized by the high catalytic capacities of enzymes involved in glycolysis (including enolase) and the tricarboxylic acid cycle. These results indicated that rapid growth in these phases was supported by high glycolytic flux (Biais *et al*. 2014).

Alcohol dehydrogenases (ADH) are enzymes of carbon metabolism which could have different biochemical properties related to distinct metabolic roles. They are involved in fermentation and facilitate the interconversion between aldehydes and alcohols, but also have a role in the regulation of fruit maturation and aroma production during fruit ripening (Speirs *et al*. 1998). The study of the microsomal proteome of tomato fruit pericarp showed that some forms of alcohol dehidrogenase are present only in mature green fruits, while others are present in the red ripe stage, which indicates their different role in fruit development (Pontiggia *et al*. 2019). Overexpression of this enzyme at the red ripe stage in tomato fruit development is related to the accumulation

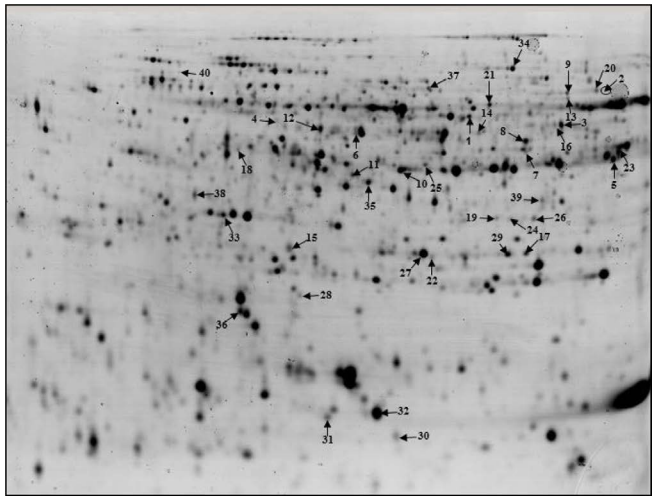

Fig. 4. Two-dimensional electrophoresis gel of tomato pericarp proteins

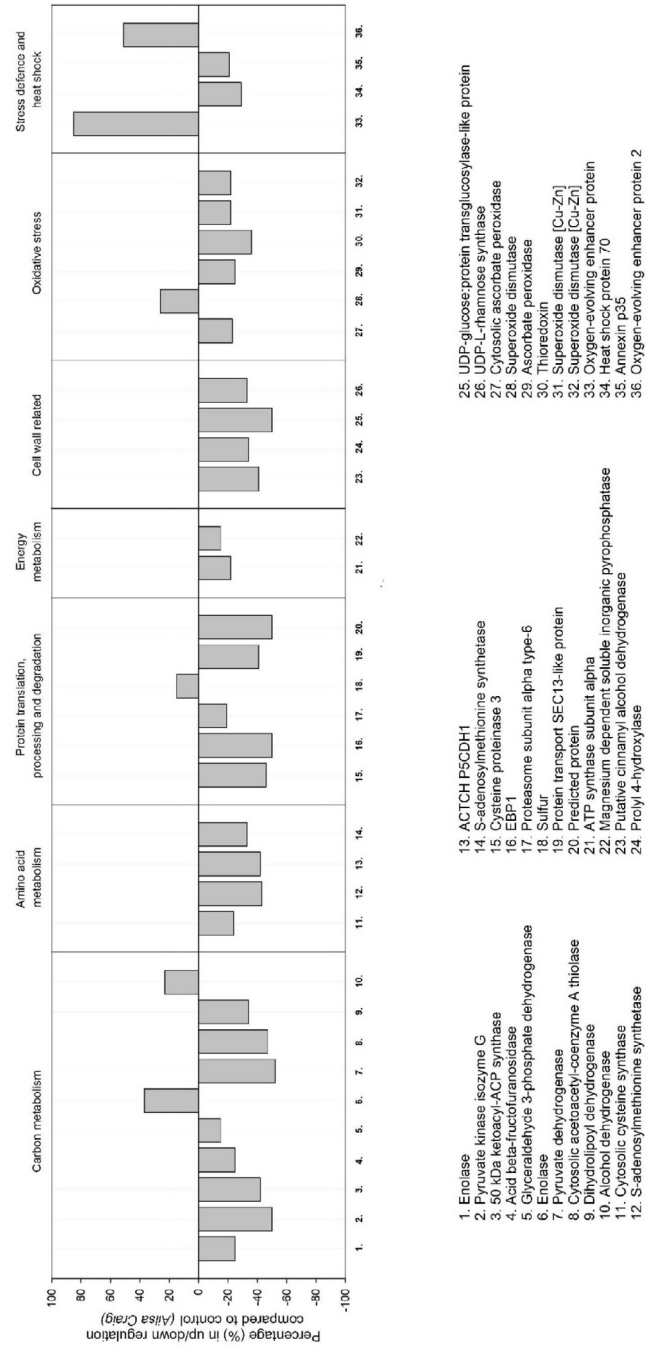

Fig. 5. Protein expression in the pericarp of the *flacca* tomato fruits compared to the wild type (Ailsa Craig) grown under the same experimental conditions and cultivated at the same time.

of flavor volatiles in tomato fruits (Claudius & Ludivine 2017).

In our experiment, alcohol dehydrogenase (spot 10) was up-regulated in *flacca* by 23% compared to the wild type (Table 2). Singh *et al*. (2010) found that expression patterns of ADHs differed during specific stages of development and ripening of mango fruit. Expression analysis also indicated that mango ADHs were responsive to ethylene, but regulated differently by ABA, in a positive or negative manner. Based on these results, it could be speculated that overexpression of alcohol dehydrogenase in the *flacca* mutant could be indirectly related to ABA-deficiency.

Some enzymes involved in fatty acid metabolism were down-regulated in *flacca*. Acetoacetyl-coenzyme A thiolase (spot 8) catalyses the condensation of two acetyl-CoA to form the 4-C acetoacetyl-CoA (Dyer *et al*. 2009). Ketoacyl-ACP synthase (spot 3) is involved in the synthesis of fatty acids, catalysing the successive elongation of a growing acyl chain with C2 units (Siggaard-Andersen *et al*. 1991). Together with the aforementioned proteomic changes associated with enzymes primarily involved in sugar metabolism, it may be concluded that the fruit of the *flacca* mutant has reduced metabolic flux, which may explain their slower growth rate and size compared to wild type Ailsa Craig.

Metabolite profiling on the fruit of the tomato cultivar Micro-Tom and hormone mutants demonstrated that ABA had a dominant effect on the regulation of primary metabolism in tomato fruit, while ethylene plays an important role in the transition from primary to secondary metabolism (Li *et al*. 2019). In that study the sugar contents decreased in the pericarp of the ABA-deficient *notabilis* mutant, thus supporting the results of Bastías *et al*. (2014) about the effect of ABA on sugar metabolism. A similar trend was observed for the content of organic acids, other intermediates of the TCA cycle and mostly for the amino acids in the *not* mutant. All these findings indicated that ABA limited carbon sources, which could be responsible for reduced fruit growth and size as well as for the quality of ABA-deficient tomato fruits.

All proteins related to amino acid synthesis and metabolism were down-regulated in *flacca* compared to

the wild type (Fig. 5). Cytosolic cysteine synthase (spot 11) is involved in the biosynthesis of amino acids and glutathione, as well as other secondary metabolites. Isoenzymes of S-adenosylmethionine-synthetase (spots 12 and 14) catalyse the biosynthesis of ethylene precursors, but this enzyme is also involved in the phenylpropanoid pathways related to components in the cell wall. Literature data report that more abundant isoenzymes of S-adenosylmethionine-synthetase were found in the MG30 stage, which is consistent with the dramatically increased ethylene production which occurs in the breaker stage (Pontiggia *et al*. 2019). The down-regulation of these isoenzymes in our study could imply the delay of the beginning of the ripening process in the *flacca* mutant, but also had a negative effect on the synthesis of lignin and other secondary metabolites in the *flacca* fruits.

Six proteins involved in the processes of protein synthesis or degradation were identified (Fig. 5). Cysteine proteinase 3 (spot 15), commonly known to participate in the catabolic metabolism of proteins, was down-regulated in *flacca,* which is in accordance with the determined slower fruit development. Similar expression was found for growth-regulating protein EPB1 (spot 16) which serves as the link between the biosynthesis of ribosome and cell proliferation (Squatrito *et al*. 2004), and proteasome subunit alpha type-6 (spot 17) as part of the ATP-dependent multienzyme proteinase complex. The comparison of the metabolite profiles of primary and secondary metabolites in the tomato pericarp in the mature green stage indicated the reduced synthesis of Ser-Gly-Cys and degradation of amino acids in tomato high pigment mutant hp1 in contrast to wild type Ailsa Craig (Rohrmann *et al*. 2011). The down-regulation of the enzymes in our experiment implicated slower protein metabolism in the tomato *flacca* fruits in order to preserve the necessary energy.

It is well known that photosynthesis remains active in green fruits and can produce up to 20% of the fruit photosynthetates (Pesaresi *et al*. 2014). Although the plastids of the mature-green tomato exocarp retain their chloroplastic structure with high chlorophyll content, at the same time they gain the capacity to ripen and respond to ethylene (Barsan *et al*. 2012). In our experiment only one protein, sulfur (spot18), which is part of the subunit of Mg-chelatase which catalyses the biosynthesis of chlorophyll is up-regulated in the *flacca* mutants (51% compared to the wild type) (Table 2). It could be assumed that in this stage of fruit development the *flacca* mutant still has an active photosynthetic process which slows down the transition of chloroplasts to chromoplasts and delays the beginning of the ripening stage.

A study of the proteome profiles from the pericarp of hp1 mutant and wild type (cv. Ailsa Craig) fruits showed that the transcript levels for ABA biosynthetic genes were down-regulated in hp1 compared to the wild type (Kilambi *et al*. 2013). These findings suggest the relation between low ABA content and increases in chloroplast number and size, as previously reported for the hp3 ABA-deficient mutant (Galpaz *et al*. 2008). The hp1 mutation also exhibits decreased ethylene production and delayed ripening in addition to increased fruit firmness (Wang *et al*. 2019). All these findings highlight the role of ABA in fruit growth and development, which was especially reflected in the phenotypic response of mutants with ABA deficiency.

Fruit growth is the result of an increase in cell volume, which requires the addition of energy as well as the biosynthesis of cell wall components. Both energy-related proteins were down–regulated in the *flacca* mutants, ATP synthase subunit alpha as a secretory protein (spot 21) which participates in ATP synthesis, and magnesium-dependent soluble inorganic pyrophosphatase (spot 22) which catalyses the hydrolysis of inorganic phosphate (Ppi) into orthophosphate (Pi) and provides energy for a variety of anabolic processes. Such a response is in accordance with the already observed trend of reduced enzyme synthesis in carbon and protein metabolism in *flacca* fruits.

The cell expansion phase also implies the regulation of cell wall metabolism, which directly influences fruit firmness and texture. Several cell wall related proteins are down-regulated in the *flacca* pericarp. Cinnamyl alcohol dehydrogenase (spot 23) and UDP-glucose: protein transglucosylase-like protein (spot 25) are involved in the formation of cell wall lignans, while UDP-L-rhamnose synthase (spot 26) plays a role in the synthesis of the cell wall pectin polysaccharide. Prolyl-4-hydroxylase (spot 24) participates in the formation of 4-hydroxyproline in peptide sequences of cell wall glycoproteins. The changes in these proteins could be related to the smaller size of the *flacca* fruits or the slower expansion phase observed in the pericarp in the mature green stage. Also, it is well known that phenylpropanoid metabolism and its components are under hormonal control. Research into crosstalk during fruit ripening indicated that one of the pathways of the induction of phenylpropanoids is dependent on abscisic acid (Vighi *et al*. 2019), so ABA deficiency could be the potential cause for such a response observed in our experiment.

During the ripening of climacteric fruits, reactive oxygen species (ROS) and oxidative stress are produced as a result of increased fruit respiration, as well as fruit color changes due to the transformation of chloroplasts into chromoplasts (Muñoz & Munné-Bosch 2018). The conversion of chloroplast to chromoplast leads to a substantial change in the antioxidant apparatus, with a reduction in the activities of SOD, CAT, and most of the enzymes associated with the ascorbate–glutathione cycle. Proteomic analysis showed significant changes in of antioxidant enzymes during tomato fruit growth. Most antioxidative proteins were down-regulated, ascorbate

peroxidases APX (spots 27 and 29) and cytosol superoxide dismutase SOD (spot 32), with the exception of superoxide dismutase (spot 28) which was up-regulated (Fig. 5).

During the initial stages of tomato fruit ripening, the antioxidant system effectively removes ROS and thus prevents the negative effects of oxidative stress. However, during the later stages of maturation, the activity of antioxidant enzymes decreases, resulting in damage to membrane systems as a consequence of oxidative stress (Mondal *et al*. 2004). The exogenous application of ABA during tomato ripening promoted the activities of different antioxidative enzymes and the expressions of the major genes involved in the phenylpropanoid pathway were up-regulated (Tao *et al*. 2020). Also, high levels of ABA stimulate the generation of ROS and subsequently affect the expression of many genes involved in the antioxidant defense system (Mou *et al*. 2015).

Biochemical and histological characterization of ABA tomato mutants indicated different activity of antioxidative enzymes in the leaves at 40 dpa (Monteiro *et al*. 2012). They found an increase in ascorbate peroxidase (APX) in *not* mutant, while the activity of superoxide dismutase (SOD) isoform III in leaves of *sit*, and CAT activity in both *sit* and *not* mutants, were reduced. On this basis, it could be assumed that ABA has different effects on the expression and activity of some antioxidative enzymes and their isoforms, which could explain the results in our experiment. In addition, high levels of superoxide dismutase isoform (spot 28) indicate an active photosynthetic process in the chloroplasts and the incomplete transition of chloroplasts to chromoplasts thus delaying the beginning of the ripening stage of the *flacca* mutant.

The group of stress defence and heat shock proteins included oxygen-evolving enhancer proteins (spots 33 and 36) related to the photochemical reactions of photosynthesis and involved in the water-splitting apparatus within photosystem II. The transition from chloroplast to chromoplast in tomato fruit ripening was generally associated with loss of photosynthetic activity and the presence of proteins involved in chlorophyll degradation, while the activity of oxygen-evolving enhancer proteins depended on different isoforms (Bian *et al*. 2011). In our experiment, the up-regulation of these proteins in the *flacca* fruits compared to the wild type could be related to the prolonged photosynthetic activity of the *flacca* mutant at this stage of fruit development and the delayed ripening phase.

HSPs are proteins involved in various cellular processes by supporting the maintenance of protein structure (Sung *et al*. 2001), but can also be modulated during plant development as well as by a wide range of environmental stresses. The down-regulation of heat shock protein 70 (HSP70), (spot 34) in our experiment could be related to the different function of HSP isoforms during the transition to fruit development stages or to ABA deficiency. Annexin p35 (spot 35) belongs to a multifunctional family of proteins which bind phospholipids and are involved in exocytosis stimulated by Ca^{2+} (Bassani *et al*. 2004). Faurobert *et al*. (2007a) showed the increased expression of annexin p34 in tomato in the period from 7 to 21 dpa which correlates with the expansion of the cell wall. Since the proteomic analyses in our study were conducted at 30 dpa, at the stage close to the beginning of ripening processes, it could be assumed that the down regulation of this enzyme in the *flacca* mutant compared to the wild type could be in relation to the role of ABA in the regulation of cell expansion and the cessation of fruit growth.

CONCLUSION

To our knowledge, this is the first study to analyze the link between ABA deficiency and the growth and development of the fruit tomato *flacca* mutant. Fruit growth analysis indicated that the slower fruit growth rate of the *flacca* fruits was reflected in reduced fruit size as well as prolonged fruit development compared to the wild type. The time course of cell wall-associated peroxidase activity in the fruit pericarp of the *flacca* mutant implicated an increasing trend towards the cessation of fruit growth and the beginning of the ripening phase, which is opposite to the dynamics of ABA content changes and thus indicated their opposite relationship during the fruit development. Our proteomic analysis revealed the down-regulation of most proteins from the groups of carbon and amino acid metabolism, protein translation and processing, energy metabolism, and cell wall processes in the *flacca* fruits compared to the wild type. This indicates that the reduced metabolic flux in the *flacca* fruits may explain their slower growth and smaller final size compared to the wild type. Some of the proteins were up-regulated in the *flacca* mutant such as alcohol dehydrogenases which could be indirectly related to ABA deficiency, taking into account the dominant effect of ABA on the regulation of primary metabolism in tomato fruits. Also, these findings indicated that ABA limited carbon sources which could be responsible for reduced fruit growth and the size of tomato ABA-deficient fruits. The up-regulation of the sulfur protein and oxygen-evolving enhancer proteins involved in chlorophyll biosynthesis and photochemical reactions implicated that the *flacca* fruit maintains active photosynthesis and delays the beginning of the ripening stage. The majority of the antioxidative proteins were down-regulated, with the exception of an isoform of superoxide dismutase which implied a different effect of ABA on the production of ROS and the activity of some antioxidative enzymes. The down-regulation of stress defence proteins such as HSP and anexin p35 in the *flacca* mutant compared to the wild type could be related to the

role of ABA in regulating cell wall expansion and the cessation of fruit growth.

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REZIME

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Biohemijski i proteomički pristup u analizi rastenja plodova mutanta paradajza

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Da bi se procenili efekti nedostatka ABA na rast ploda paradajza, ABA mutant *flacca* je gajen u optimalnom vodnom režimu zemljišta i urađene su različite analize, uključujući morfološke (broj, prečnik i biomasa ploda), fiziološke (trajanje rasta i brzina rasta ploda), biohemijske (akumulacija ABA, enzimska aktivnost peroksidaze ćelijskog zida) kao i proteomičke analiza. Analiza rastenja plodova pokazala je da je sporija brzina rastenja i razvoja plodova uticala na manju veličinu plodova *flacca* u odnosu na plodove divljeg tipa. Poređenje aktivnosti peroksidaze ćelijskog zida i sadržaja ABA u eksperimentu ukazalo je na njihov antagonistički odnos tokom razvoja ploda. Proteomičkom analizom utvđena je smanjena biosinteza većine enzima iz grupe metabolizma ugljenih hidrata i aminokiselina, sinteze i degradacije proteina, energetskog metabolizma i metabolizma ćelijskog zida u plodu *flacca* u poređenju sa divljim tipom. To je ukazalo na smanjen intenzitet metabolizma, koji je uticao na sporiji rast i razvoj ploda i redukovao veličinu ploda ABA mutanta. Ova otkrića, takođe, ukazuju na to da ABA ograničava izvore ugljenih hidrata, što bi mogao biti razlog za smanjeni rast plodova i veličinu plodova paradajza sa nedostatkom ABA. Povećana regulacija proteina sumpora i proteina koji pripada grupi odbrane od stresa, u plodovima *flacca*, uticala je na održavanje fotosinteze u kasnoj fazi rastenja, što usporava prelazak u fazu sazrevanja. Većina antioksidativnih proteina i proteina odbrane od stresa bila je smanjena u plodovima *flacca*, što bi moglo biti povezano sa ulogom ABA u aktivnosti različitih izoformi antioksidativnih enzima, kao i u kontroli rastenja ćelijskog zida i prestankom rasta ploda.

Ključne reči: ABA, peroksidaza ćelijskog zida, *flacca* mutant