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Research article

Novel phenotypes related to the breeding of purple-fruited tomatoes and effect of peel extracts on human cancer cell proliferation

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ABSTRACT

The production of anthocyanins in the tomato (*Solanum lycopersicum* L.) fruit is normally absent or poor, but a number of mutants or introgression lines are known to increase anthocyanin levels in vegetative and reproductive tissues. Through conventional breeding, a genetic combination was obtained with the remarkable phenotype of a deep purple fruit pigmentation, due to an accumulation of anthocyanins on the peel. Such a genotype was named Sun Black (SB) as a consequence of its sensitivity to light induction. When characterized for morpho-agronomic traits, SB plants showed increased fertility. Purple fruits displayed an arrangement of the epicarp cells different from normal tomatoes, a feature that could account for different mechanical properties and shelf-life potential. The SB genotype and, to a lesser extent, its single mutant parents showed the capacity to accumulate anthocyanins in the seedling root when grown under light. This phenotype, which was greatly improved by the addition of sucrose to the germination medium, proved to be useful as selection index and gave new insights for in vitro production of anthocyanin extracts. To assess the nutraceutical potential of purple tomatoes, we tested the activity of SB skin extracts on the proliferation of two human cancer cells lines. Cell proliferation was significantly inhibited by SB extract in a dose-dependent manner. When the bioactivity of SB extracts was compared with that of other anthocyanin-containing fruits or vegetables, a significant "Extract*Line" interaction was evidenced, suggesting a crucial role for the extract composition in terms of anthocyanidins and other eventual cell growth-inhibiting compounds.

1. Introduction

Anthocyanins are a group of naturally occurring pigments belonging to the flavonoid family and derived from the phenylpropanoid biochemical pathway [1,2]. They are water-soluble molecules, responsible for the blue, purple, red and orange color of plant organs; more than 500 different compounds have been identified in nature [3]. As with other flavonoids, anthocyanins have important functions in plants, including defence against pathogens and protection against ultraviolet B radiation [4].

Structurally, anthocyanins are the glycosides of anthocyanidins. The aglycone is the flavylium ion (2-phenylbenzopyrilium) showing several hydroxyl and methoxyl substituents on the A and B aromatic rings. Depending on their number and position, some dozen different anthocyanidins have been described in the literature. Among them, the most common are pelargonidin, cyanidin, delphinidin, petunidin, peonidin and malvidin. In fruits and vegetables, anthocyanidins are linked to one or more glycosidic units by alpha or beta linkage in position 3 and sometimes also in positions 5 and 7. The most common sugars are hexoses (glucose, galactose) and pentoses (rhamnose, arabinose and xylose). In addition, anthocyanidins could be acylated with different organic acids such as *p*-cinnamic, caffeic, ferulic and sinapic acid [5].

Being present in foods and vegetables, anthocyanins are components of the human diet. Because of their antioxidant activity, they are considered important phytonutrients contributing beneficial effects leading to the prevention of neoplasias, diabetes,

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Abbreviations: Aft, Anthocyanin fruit; *AN2, ANTHOCYANIN2*; *ANT1, ANTHOCYANIN1*; *atv, atroviolaceum*; CHS, chalcone synthase; FW, fresh weight; GA_3, gibberellic acid; GM, genetically modified; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; SB, Sun Black; WT, wild-type.

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coronary diseases and aging [5,6]. The main sources of anthocyanins in foods are red fruits, principally bilberries, blackberries, blackcurrants, blueberries, strawberries, raspberries, red grapes and red wine among drinks. Discrete amounts of anthocyanins are also present in cereals and vegetables. As a general trend, the content of anthocyanins in a source varies depending on genetic, agronomic and environmental factors such as light, temperature, soil and humidity, but also processing and storage. Data on anthocyanin intake by humans are very variable, depending on the consumption of anthocyanins-rich products [7].

Tomato (*Solanum lycopersicum* L.) does not usually produce anthocyanins in the fruit where the red color is mainly due to the presence of carotenoids such as lycopene and β-carotene. However, some tomato-related wild species do produce anthocyanins in fruits. Thus, alleles conferring varying degrees of anthocyanin pigmentation in the fruit epidermis have been introgressed into cultivated genetic backgrounds, offering the prospect of breeding purple-fruited tomatoes [8,9]. Remarkably, the *Anthocyanin fruit* (*Aft*) allele has been introgressed from *Solanum chilense* (Dunal) Reiche [10]. *Aft* is located on chromosome 10 and behaves as a single dominant gene; its expression gives a patchy purple pigmentation to the tomato fruit, which is due to anthocyanin deposition in the first cell layers of pericarp [8,10].

Aft was shown to co-segregate with two different MYB transcription factor genes, whose sequences are orthologous to *ANTHOCYANIN2* (*AN2*) from *Petunia*, *SlAN2* [8] and *ANTHOCYANIN1* (*SlANT1*) [11]. Although both these genes may be involved in the regulation of anthocyanin synthesis in tomato [8], the most recent report indicates *SlANT1* as the best candidate for representing the *Aft* locus [12].

The recessive *atroviolaceum* (*atv*) allele has been introgressed in tomato from *Solanum cheesmaniae* (L. Riley) Fosberg and, when homozygous, it causes a more intense anthocyanin pigmentation in vegetative tissue. The *S. lycopersicum* allele *Atv* is thus a putative anthocyanin repressor. Anthocyanin deposition in *atv* fruits remains barely detectable [10]. *Atv* is located on chromosome 7 [13] and, to date, no reliable candidate gene has been identified.

A breeding activity aimed at combining different alleles enhancing anthocyanin production in cultivated tomato, recently produced the *Aft_atvatv* combination, which showed the remarkable phenotype of a deep purple pigmentation of the pericarp (Fig. 1a). Such a selection was named Sun Black (SB) with reference to the importance of solar radiation for its expressivity [9]. Accordingly with its dependence on light induction and with its photoprotective function, anthocyanin deposition was limited to the epicarp (Fig. 1b). This tissue specificity in grape has been related to the poor expression of genes of the flavonoid pathway in the flesh compared with the external pericarp [14]. A genetic combination similar to SB has also been described elsewhere [8,15].

Anthocyanin concentration in SB [16] and in the same genetic combination in a different background [8] ranged between 1 and 3 mg/g FW (Table S1). A SB-like line of unreported pedigree showed higher values of total anthocyanins [15]. In parallel with tomato vegetative tissues [17], the anthocyanidin predominantly synthetized by purple tomatoes was petunidin; lower levels of malvidin and delphinidin were found [8,15,16]; similar results were reported in *Aft* (Table S1, [10–12]).

The identity of genes differentially expressed in tomatoes accumulating anthocyanins in the fruit has been studied [11,12,18], paving the way to the comprehension of mechanisms underlying anthocyanin accumulation in these genotypes. These results confirmed the hypothesis that *Aft* and *atv* represent genes encoding regulators of anthocyanin biosynthesis, which act synergistically by activating different sets of genes along the metabolic pathway [18].

To date, little attention has been paid to the description of morpho-physiological aspects of the “Sun Black syndrome” and to aspects directly related to the nutraceutical potential of purple tomatoes. In this work, we describe advances in characterizing the SB trait under these unexplored points of view.

2. Results and discussion

2.1. Morpho-physiological characterization of the Sun Black genotype: fertility aspects

Selection among the progeny of the cross between lines homozygous for *Aft* and *atv* allowed the fixation of tomato lines expressing high anthocyanin pigmentation (Fig. 1a). Pigment accumulation, which started at early stages of fruit development, was limited to the epicarp (the external layer of the pericarp, including cuticle, epidermis and the few cell layers that retain meristematic activity, Fig. 1b). Anthocyanin pigmentation was higher in the portions of the fruit directly exposed to light; about 20% of the epicarp, generally at the stylar pole, was devoid of anthocyanins (not shown).

To investigate the fruiting capacity of high-anthocyanin tomato lines, the fruit set in the wild-type (WT), *atv*, *Aft* and two different SB selections was studied over two years of cultivation. No significant “Genotype*Year” interaction was found. Whereas the WT line showed the lowest fruit set value, the two SB lines had the highest, with more than 30% of the flowers setting fruits (Fig. 2a). Moreover, all the four lines containing “anthocyanin genes” showed the capability to increase ovary weight after 15 d from emasculation in non-pollinated flowers (Fig. 2b), a phenotype which is typical of genotypes expressing parthenocarpic capacities [19].

Fruit weight was not significantly different among the five lines and no differences were found for the number of seeds per fruit (not shown). As a quality trait, soluble solids (°Brix) in the *Aft* parent were higher than in all the other lines (not shown).

The data presented indicated that tomato genotypes with altered anthocyanin accumulation present enhanced fertility. In addition to a higher fruit set and to the capacity of increasing the ovary size in absence of fertilization, in early season cultivations (not included in the data reported above) SB and *Aft* showed a tendency to set seedless fruits. Transcriptomic analysis on fruits of SB and its parental lines, although carried out on mature green fruits, indicated altered expression in SB and *atv* of genes that have been directly involved in the control of fruit set (Table S2 in Ref. [18]), such as *SlAux/IAA9* [19], *SlDEFICIENS* [20] and a cytochrome P450 monooxygenase (A. Mazzucato and F. Ruiu, unpublished data). In addition, 9% of the transcripts differentially expressed between the WT and SB at the mature green stage were represented by genes related to hormone metabolism [18].

This behavior is in agreement with the increasing evidence that flavonoids, at least in some plants, play a crucial role in fertility and sexual reproduction. Consequently, plants with altered flavonoid metabolism present fruit set phenotypes. In tomato, silencing of the chalcone synthase (CHS) gene inhibited pollen tube growth and caused the development of parthenocarpic fruits [21]. Such seedless fruits showed low levels of flavonoids such as naringenin and rutin. Further studies in tobacco indicated the flavonol quercetin as a major regulator of pollen germination and seed and fruit set [22]. Accordingly, evidence was given that flavonoids, such as quercetin, kaempferol and others, inhibit auxin transport and enhance localized auxin accumulation in planta ([23] and refs therein). Thus, metabolic engineering of the flavonoid pathway results in altered expression of several metabolites that may have an impact on the plant fertility, either directly or due to metabolic competition [22,24]. Levels of flavonols higher