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Improved nutritional quality in fruit tree species through traditional and biotechnological approaches

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Original

Improved nutritional quality in fruit tree species through traditional and biotechnological approaches / Sabbadini, Silvia; Capocasa, Franco; Battino, Maurizio; Mazzoni, Luca; Mezzetti, Bruno. - In: TRENDS IN FOOD SCIENCE & TECHNOLOGY. - ISSN 0924-2244. - 117:(2021), pp. 125-138. [10.1016/j.tifs.2021.01.083]

Availability:

This version is available at: 11566/288046 since: 2024-04-05T16:01:33Z

Publisher:

Published

DOI:10.1016/j.tifs.2021.01.083

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(Article begins on next page)

1 IMPROVED NUTRITIONAL QUALITY IN FRUIT TREE SPECIES THROUGH TRADITIONAL AND
2 BIOTECHNOLOGICAL APPROACHES

3
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18 **Abstract**

19
20 **Background:** The increasing evidences on the relation between diet and human health are driving the
21 consumers toward the choice of foods with high amount of nutrients, considered the molecules responsible
22 for the regulation of several metabolic and biochemical processes, and for the prevention of many chronic
23 diseases. Among food products, fruits represent a natural source of many different nutrients and possess
24 much importance in a balanced diet. The increase of the micro/macronutrient levels and the decrease of
25 antinutrients amount are considered main objectives for the improvement of fruit trees nutritional value.

26
27 **Scope and approach:** This review summarizes many of the attempts made to increase the nutritional quality
28 of fruit tree species during the last decades through the application of traditional and molecular breeding
29 technologies, and the most recent New breeding techniques (NBTs).

30
31 **Key findings and conclusions:** New genotypes with improved nutritional quality have been obtained for
32 different fruit tree species, either by increasing the content of well-known beneficial molecules, such as
33 phenolic compounds, vitamins, and carotenoids, or by decreasing the levels of specific antinutrients. The
34 development of molecular techniques and biotechnological tools allowed the identification and validation of
35 candidate genes involved in the regulation of specific classes of nutritional compounds in fruit trees, and
36 useful to obtain nutritionally improved products. NBTs represent alternative tools to classical breeding
37 techniques, to regulate one or more fruit trees key nutrients in a more quick and precise manner. However,
38 genetic engineering approaches still present public and regulatory concerns that strongly limit their
39 exploitation.

40
41 **Keywords**

42 Biofortification, nutrients, classical breeding, Marker assisted selection, New breeding techniques

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53 **Background**

54 In the modern society consumers are becoming increasingly aware of the relationship between food
55 consumption and health benefits. An increasing number of evidences are indicating that several food
56 components can exert a positive effect on the physiological processes during lifespan. Foods with a high
57 content of bioactive compounds have been demonstrated to have a central role in chronic disease prevention
58 by exerting specific biological activities. Plant-based products represent the most consumed food worldwide,
59 irrespective of culture, location, and economic situation. Therefore, the development of new plant-based food
60 products with improved nutritional properties, through conventional and/or molecular breeding techniques,
61 could have a great impact on the improvement of population health (Mezzetti, 2013; Pott et al., 2019).

62 Plant components of dietary interest are called nutrients and are responsible for the regulation of metabolic
63 and biochemical processes, the repair of body tissues, growth, and energy production. Plant nutrients can be
64 classified into three categories: macronutrients (carbohydrates, lipids, proteins and fibers), micronutrients
65 (vitamins, minerals and secondary metabolites), antinutrients (molecules that negatively influence the
66 bioavailability of macro and micronutrients) (Newell-McGloughlin, 2008) (Fig. 1). The increase of the
67 micro/macronutrients and the decrease of antinutrients levels are considered main objectives of many
68 breeding programs for the improvement of plant nutritional value.

69 Among the plant-based products, fruits, besides their attractive colour, taste and aroma, represent a natural
70 source of many different nutrients and possess much importance in a balanced diet. They usually contain no
71 cholesterol, high amount of water, and low calories associated with the absence of lipids (except for some
72 species, like avocado and olive). Among macronutrients, fruits contain good quantities of carbohydrates, in
73 particular, fructose, which is fermented in the large intestine where it forms small-chain fatty acids useful in
74 the treatment of children constipation. Among micronutrients, vitamins, such as A, B, C and E, represent one
75 of the main healthy compounds, given the high number of positive nutritional properties associated with their
76 consumption (Kaur et al., 2020a). Fruits are also rich in secondary metabolites (e.g. polyphenols, carotenoids,
77 and tocopherols), which possess many healthy properties, including anti-inflammatory and anti-tumorigenic
78 effects. In addition, fruits are characterized by antioxidant activities that significantly contribute to quench
79 the so-called reactive oxygen species (ROS), protecting lipids, proteins and nucleic acids from oxidation, and
80 reducing the insurgence of several related diseases and disorders (Kaur et al., 2020a). Therefore, it is evident
81 that improving the amount of micro and macronutrients in fruit species, as well as reducing the content of
82 undesired compounds, could bring many health benefits to the consumer, also with a lower consumption of
83 dietary fruits.

84 Currently, different strategies have been applied to modify the content of bioactive compounds in foods. The
85 term “biofortification” or “biological fortification” describes a food crop with enhanced nutritional
86 compounds developed through different tools, like agronomic practices (i.e fertilization, plant growth-
87 promoting soil microorganisms), conventional breeding, or biotechnological techniques (Garg et al., 2018).
88 The most accepted method for biofortification is conventional breeding, being a more sustainable and cost-
89 effective system compared to those other two approaches. Many improved varieties have been developed
90 through inter-specific hybridization, using wild species to expand the breeding pool available for the
91 introgression of the characters of interest during the first stages of crossing (Karanjalkar & Begane, 2016).
92 Until now, many attempts for the improvement of nutritional quality in staple crops have been made, while
93 little efforts have been done in fruit tree species. In fact, most of the breeding programs are mainly focused
94 on agronomical and commercial traits, like plant yield and architecture, earliness of production, and
95 resistance to biotic and abiotic stresses (Karanjalkar & Begane, 2016). There are many reasons why
96 researches aimed at improving the nutritional quality of fruit trees have been limited. Fruit qualitative
97 characteristics are usually quantitatively inherited, and the basic knowledge about the metabolism of some
98 compounds of interest is still lacking, due to the fact that it comprises complex interactions among thousands
99 of metabolic pathways. Furthermore, many fruit tree species are characterized by a long juvenile phase that
100 makes breeding process particularly long (e.g. avocado tree can take up to 15 years before flowering, and a
101 recent breeding program on apple took 26 years to release three new commercial varieties) (Migicovsky &
102 Myles, 2017). After this long period, there is the possibility to obtain a progeny that does not express the
103 targeted traits, and consequently, to produce new selections not of interest for the breeders. In addition, when
104 we consider that other external factors such as natural calamities, environmental pressure and pathogens
105 attack could lead to a loss of yield or, in the worst case, of new individuals, it is clear that the breeding of
106 fruit trees for quality improvement is particularly complicated. For all these reasons, the research has been
107 stimulated to find new methodologies and technologies able to amplify the starting gene pool variability, to

108 shorten the breeding processes and facilitate the combination of traits of interest in the new progeny
109 (Karanjalkar & Begane, 2016).

110 In particular, marker-assisted selection (MAS) has emerged as a very promising strategy for improving the
111 breeding selection processes, especially since the complete genome of various fruit species has been made
112 available. This molecular tool can dramatically shorten the number of crossing cycles required to remove the
113 unwanted genes in the backcrossing program.

114 In order to use wild relatives for MAS, the first step is to discover markers for the traits of interest. Linkage
115 mapping (LM) and genome-wide association studies (GWAS) are methods used to analyse the genotype-
116 phenotype relationships (Migicovsky & Myles, 2017). Selection by molecular markers is particularly useful
117 when fruit characteristics are difficult or expensive to recognize or are visible only after a long juvenile
118 period (qualitative traits) (Karanjalkar & Begane, 2016). In addition, MAS can decrease the costs related to
119 the breeding of some perennial plants up to 43% during the first 6-8 years, compared to a traditional breeding
120 program (Edge-Garza et al., 2015).

121 Despite the application of these efficient genomic-based techniques, traditional breeding still presents some
122 limitations, which arise mainly from the procedure followed to obtain a genetically improved plant. Several
123 breeding generations are still necessary to introgress the trait of interest in a local elite cultivar, from a
124 minimum of seven for clonally propagated crops like banana, up to 17 for cross-fertilizing crops like rice
125 (Shimelis & Laing, 2012). In addition, other bottlenecks related to traditional breeding techniques are
126 represented by linkage drag issues and by the limited gene pool where to source the sequences of interest,
127 which is constrained to sexually compatible plants (De Steur et al., 2015; Kanchiswamy et al., 2015).

128 In terms of biofortification, alternative agronomic tools to traditional and molecular breeding are now
129 available, which are represented by genetic engineering approaches including the New breeding techniques
130 (NBTs) (cisgenesis/intragenesis, RNAi, genome editing). These strategies allow the introduction of one or
131 more traits able to improve the biosynthesis, accumulation and/or bioavailability of health-promoting
132 compounds, or the knockdown/knockout of specific genes responsible for the accumulation of undesirable
133 molecules (Uncu et al., 2013). In addition, the accumulation of one or more key nutrients can be targeted to
134 specific edible organs or redistributed between tissues through genetic engineering techniques, which
135 represents an advantage especially for those compounds which are mainly distributed in not edible portions
136 of a crop (Zhu et al., 2013). Till now, transgenic biofortified crops have been mainly characterized by the
137 increase in specific micro/macro nutrients especially in staple crops, with the aim of mitigate the daily diet
138 deficiency of people from developing countries. The most famous example is Golden rice, a variety of rice
139 where provitamin-A pathway was genetically modified to produce β -carotene in the endosperm of the grain,
140 with the aim of addressing vitamin A deficiency in at least 26 countries (Stokstad, 2019). However, this and
141 many other promising transgenic biofortified crops with an effective benefit for the consumer are still facing
142 several commercialization barriers mainly due to the regulatory issues and public concerns linked to
143 genetically modified plants (GMPs) (De Steur et al., 2015).

144 The development and application of NBTs in agriculture should arise lesser biosafety concerns and increase
145 consumer's acceptance if compared with the first generations of GMPs, especially because some of these
146 techniques can be exploited to obtain transgene-free plants (Limera et al., 2017). This is the case of
147 cisgenesis and intragenesis that consist in the introduction of sequences with regulatory regions isolated from
148 sexually compatible species or the plant itself, as done for traditional breeding, avoiding the addition of
149 foreign sequences in the host genome (Schouten et al., 2006). Nowadays, cisgenesis and intragenesis have
150 been applied to a limited group of plant species, especially for the lack of complete genomic information and
151 knowledge on regulatory sequences for many crops. In addition, the identification of transformed events in
152 these kind of transformation systems must rely on the use of cisgenic/intragenic reporter genes, as well as
153 cisgenic/intragenic promoters and selectable markers (Carvalho & Folta, 2017; Krens et al., 2015).

154 Nutritional improvement of plants through biotechnological tools can also be achieved by reducing the level
155 of antinutrients/toxins through the regulation of specific protein coding genes, for example by exploiting
156 RNA interference (RNAi) (Katoch & Thakur, 2013). RNAi is a naturally conserved mechanism in plants,
157 that acts by preventing messenger RNA translation into protein through the action of double strand RNA
158 (dsRNA) molecules which target the silencing of specific transcripts in a sequence-dependent manner
159 (Mezzetti et al., 2020). This technology has been applied for the biofortification of several plant species by
160 reducing the presence of undesired molecules from edible tissues without affecting the level of these
161 compounds in other parts of the plant. For example, cottonseed, which is considered an excellent resource of
162 edible oilseed and a high-quality protein tissue, has been engineered through RNAi methodology to reduce
163 its gossypol content, thus allowing its use for food or as feed, otherwise limited by the presence of this toxic

164 terpenoid (Rathore et al., 2020). Another way to exploit gene silencing to increase specific bioactive
165 compounds consists in the silencing of specific key-enzyme encoding genes within a metabolic pathway,
166 which are known to negatively regulate one or more biosynthetic fluxes (Zheng et al., 2020).
167 The so called “block” strategy mediated by RNAi mechanism, that acts by downregulating up- or
168 downstream enzymes activities limiting specific metabolic flux, can also potentially be obtained through the
169 use of genome editing tools. They have recently been enriched by the type II clustered regularly interspaced
170 short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) system, adapted from the natural
171 immune system against viruses and plasmids of the *Streptococcus pyogenes* (Zheng et al., 2020). This
172 system can lead to single-point mutations, integration or deletions of target genes through the action of an
173 engineered Cas9 protein that can be reprogrammed and guided by a synthetic guide RNA to cleave specific
174 target DNA sequences. The repair step subsequent to the double strand break induced by the two Cas9
175 nuclear domains can be exploited to delete, change or insert a single nucleotide in a target gene or even add a
176 whole new sequence (Kaur et al., 2020a; Limera et al., 2017).
177 All these biotechnological tools can be applied to fruit tree species for many purposes, although the
178 introduction of one or more genes encoding for specific traits, or the expression of RNAi gene constructs, as
179 well as of engineered CRISPR/Cas9 systems, are frequently limited by the availability of efficient *in vitro*
180 regeneration and transformation systems. These protocols are often genotype-dependent and are
181 recommended to be developed from somatic tissues to maintain the genetic uniformity of target clonally
182 propagated plants, like fruit tree species (Ricci et al., 2020; Sabbadini et al., 2019). However, several studies
183 have been carried out during the last decades for the genetic improvement of fruit trees, and NBTs represent
184 an additional tool to be exploited by plant breeders also for the nutritional enhancement of these species
185 (Kanchiswamy et al., 2015).
186 This review will provide an overview of the more recent studies performed on fruit tree species to increase
187 their micro/macro nutrient content (phenolic compounds, vitamins, minerals, carotenoids, carbohydrates and
188 lipids), as well as for reducing antinutritional compounds through both traditional breeding techniques or by
189 the application of genetic engineering strategies, including the more recent NBTs.

190 191 **Phenolic compounds**

192 Phenolic compounds are a large group of plant molecules with function in tissues pigmentation, and in the
193 defence against biotic and abiotic stresses, thanks to their strong antioxidant activity, which also allows the
194 protection of human body from oxidative damages when they are assumed through the diet (Pott et al., 2019).
195 Phenolic compounds have been the most studied secondary metabolites in conventional fruit tree breeding
196 programs as parameter of nutritional quality. A combination of conventional and non-conventional
197 techniques has been used for such studies including inter- and intraspecific crossing, implementation of
198 various -omics technologies and the application of genetic engineering strategies. Plenty of studies are
199 available in literature describing the attempts that have been made to obtain new genotypes with improved
200 phenolics amount, usually exploiting the availability of wild germplasm. Wild genotypes are believed to
201 possess strong antioxidant activity, higher than the cultivated ones, but also among different wild accessions
202 there could be a difference in antioxidant capacity and phenolic content. In many fruit tree species the use of
203 wild accessions to produce genetic diversity and to increase the amount of phenolics in commercial
204 genotypes has become a common strategy, as in persimmon (Ramírez-Briones et al., 2019), papaya (Iamjud
205 et al., 2016), boysenberry (Ryu et al., 2017), saskatoon berry (Lachowicz et al., 2017), blueberry (Yousef et
206 al., 2016), strawberry (Mazzoni et al., 2020), apple (Wang et al., 2018), olive (Pérez et al., 2019),
207 blackcurrant and redcurrant (Stanys et al., 2019).

208 Among phenolic compounds, flavonoids are a major group of bioactive agents which comprises more than
209 9000 bioactive molecules, included chalcones, flavones, flavonols, flavanones, isoflavonoids and
210 anthocyanins, synthesized through the phenylpropanoid pathway (Zhu et al., 2013). In particular,
211 anthocyanins have been associated to a series of potential benefits for human health, included the prevention
212 against certain types of cancer, inflammation, and cardiovascular diseases (Mazzoni et al., 2019). For this
213 reason, these secondary metabolites have been the subject of many studies in which the regulation of their
214 biosynthesis-related genes was tempted, acting mainly at the level of their transcription factors, which
215 include MYB complex, basic helix-loop-helix (bHLH), and WD-repeat proteins (Peng et al., 2020).

216 *MYB* genes have been identified in several fruit tree species, with functions in plant development control and
217 regulation of different bioactive compounds (Allan & Espley, 2018). Most *MYB* transcription factors
218 positively modulate genes involved in flavonoid biosynthetic pathway in several fruit tree species (Table 1).
219 In particular, the apple *MdMYB10* gene, an allele of *MdMYB1/MYBA*, determines the red pigmentation of the

220 Type 1 (red fruit flesh, skin, leaves, and other vegetative tissues) apple. In Type 2 (only red flesh) apples,
221 another MYB transcription factor close to MdMYB10, namely MdMYB110a, is responsible for the red
222 pigmentation of flesh (Wang et al., 2018).

223 Great progress has been made in the application of molecular techniques for the genetic improvement of
224 *Prunus* spp., especially since the high-quality peach genomic sequence has been made publicly available
225 since 2010 (Aranzana et al., 2019). In sweet cherry, skin and flesh colour is related to anthocyanin
226 production, and the major responsible QTL was mapped on linkage group (LG)3 to an interval containing the
227 *PavMYB10* gene. LG3 was also shown to have major genes/QTLs controlling the anther colour in almond ×
228 peach progenies and the skin colour in peach, Japanese plum, and apricot (Aranzana et al., 2019; García-
229 Gómez et al., 2019). 14 QTLs were recently successfully mapped in peach, among them, four QTL clusters
230 were identified on four different LGs with different traits: a QTL for flavonoids accumulation was mapped
231 on LG7 together with the QTL associated to sucrose synthesis. Furthermore, a major QTL for antioxidant
232 capacity, flavonoids and anthocyanins was detected on LG5, indicating the presence of genes involved in the
233 accumulation of nutritional compounds in this region. The candidate genes present in this phytochemical
234 QTL cluster, mapped on LG5, were identified and are the *Prupe.5G105700*, *Prupe.5G105800* (both
235 encoding for MYB-related transcription factors involved in the flavonoid metabolism), and *Prupe.5G116700*
236 [annotated as anthocyanidin reductase ((2S)-flavan-3-ol-forming)], an important regulator of flavonoid
237 biosynthesis in plants (Abdelghafar et al., 2020).

238 Given their high potential for polyphenols content, small fruits called “berries” received particular attention
239 in the development of new genomic-based techniques (Table 1). A metabolomic approach has been applied
240 to identify bioactive compounds in the raspberry population deriving from the crossing of ‘Latham’ x ‘Glen
241 Moy’. The eight main anthocyanins were all mapped on LG1, irrespective of year and type of cultivation,
242 and the candidate genes encoding for bHLH, NAM/CUC2 like protein, and bZIP transcription factors were
243 identified. On the same population, QTLs associated with phenolic pool were detected on LG2, LG3, LG5,
244 and LG6 (Mccallum et al., 2018).

245 The mapping of a wild strawberry *F. vesca* collection allowed the identification of 100 metabolic (m) QTLs
246 for 24 different nutritional compounds. Total anthocyanins were mapped at three different one-year mQTLs
247 (on LG3, LG4, and LG5), while the under-accumulation of the most abundant anthocyanin (pelargonidin-3-
248 glucoside) was mainly linked to a major mQTL on LG5, and the major QTL on LG2 was linked to the over-
249 accumulation of the cyanidin-3-glucoside. Some major mQTLs were also detected for single flavanols
250 (kaempferol on LG1 and LG7, and quercetin on LG2). Stable mQTLs for total and specific flavan-3-ols were
251 mapped on region LG5, while positive major stable QTLs for hydroxycinnamic acid derivatives were
252 mapped on regions LG2 and LG4. Two positive stable QTLs for ellagic acid accumulation were mapped on
253 LG1 and LG4 regions (Urrutia et al., 2015). Hossain et al. (2018) identified, in addition to the well-
254 characterized positive regulator *FaMYB10* gene, other putative key genes involved in anthocyanins
255 biosynthesis in *Fragaria x ananassa* (Table 1).

256 The identification of candidate genes of interest through integrative metabolomics and transcriptomics
257 approaches represents a powerful strategy also for the application of biotechnological tools. The modulation
258 of flavonoid biosynthetic pathway through genetic engineering techniques has been shown to be an efficient
259 alternative strategy to obtain fruits with enhanced nutritional level, and improved health effects (Lin-Wang et
260 al., 2014). As mentioned before, MdMYB10 transcription factor is responsible for anthocyanin accumulation
261 during apple fruit development; this was also confirmed by a research study where the *MdMYB10* gene was
262 overexpressed in apple plants leading to a significant increase in anthocyanin content correlated to a highly
263 pigmented phenotype in the transformed lines (Espley et al., 2007). These genetically modified apples were
264 used in mouse feeding trials, and the results obtained showed that a dietary flavonoid consisting of MYB
265 apples led to a significant decrease in some inflammation markers and modulation of gut microbioma
266 (Espley et al., 2014). The *MdMYB10* gene was also used to develop an *A. tumefaciens*-mediated
267 transformation system for the production of cisgenic apple plants, that were visually identified through the
268 red coloration conferred by anthocyanins accumulation, avoiding the use of transgenic selectable marker
269 genes (i.e antibiotic or herbicide resistance genes), which are often exploited in transgenic vectors to give a
270 selective advantage only to the plant cells that have integrated the sequence of interest (Krens et al., 2015).
271 Recently, the involvement of the *MdWRKY11* gene in the accumulation of anthocyanins after its
272 overexpression in red-fleshed apple has also been suggested, which significantly increased the expression of
273 some MYB transcription factors as well as of structural genes (Liu et al., 2019). The *MYB10* genes from
274 *Fragaria vesca* (*FvMYB10*) and from the cultivated strawberry *Fragaria x ananassa* (*FaMYB10*) were also
275 overexpressed in these two species respectively, producing *F. vesca* fruits with purple skin and red flesh, and

276 an increase in anthocyanin concentration in different plant tissues of both these transgenic species (Kui et al.,
277 2014; Lin-Wang et al., 2014). Among MYB family, repressor of anthocyanin and proanthocyanidin pathway
278 genes have also been characterized in different fruit tree species (Table 1). For example, when the *FaMYB1*
279 gene was overexpressed in strawberry fruit or silenced by an RNAi-construct, a decrease or an accumulation
280 in anthocyanin content were observed in *F. x ananassa*, respectively (Kadomura-Ishikawa et al., 2015).
281 As mentioned above, transcription factors other than MYB, among the ternary MYB-bHLH-WD40 (MBW)
282 complex, have been individuated as positive or negative anthocyanin regulators. In particular, bHLH proteins
283 have been characterized as necessary in flavonoid biosynthesis in different fruit tree species, like apple, kiwi,
284 and grapevine (Allan & Espley, 2018; Wang et al., 2019). As an example, transgenic apple cv. Gala lines
285 were obtained overexpressing the *MdbHLH3* transcription factor, which induced an increase in anthocyanin
286 production in shoots and roots, and a higher transcript level of both the *MdDFR* and *MdUFGT* genes
287 (promoters of the anthocyanin biosynthesis genes) in apple skin was observed (Xie et al., 2012).
288 Other candidate genes have been validated through genetic engineering techniques to improve anthocyanins
289 content in fruit trees. In particular, the overexpression in the octoploid strawberry of the *ANS* gene, which
290 catalyses the synthesis of anthocyanidins, induced an increase in total antioxidant capacity, and higher
291 concentrations of phenolics and flavonoids were observed. The fruit extract collected from the transgenic
292 lines obtained showed a cytotoxic effect on hepatocellular carcinoma (Giampieri et al., 2018).
293 Recently, also the *Reduced Anthocyanins in Petioles (RAP)* gene was characterized in the cultivated
294 strawberry through both overexpression and CRISPR/Cas9-mediated knockout strategies, that determined its
295 role in fruit coloration and anthocyanin accumulation at anthesis (Gao et al., 2020).
296 In addition to flavonoids, stilbenes are another group of plant-derived phenolic compounds, synthesized via
297 the phenylpropanoid pathway, which are known for their health benefits and role in plant defence against
298 pathogens and pests (Kiselev & Dubrovina, 2020). One of the most studied stilbenes is resveratrol, a
299 naturally occurring polyphenol in several plant species like grapevine and blueberries, which has gained
300 particular interest due to its antioxidant, antiangiogenic, and anticancer activities (Kiselev & Dubrovina,
301 2020). One of the key enzymes involved in the stilbene biosynthetic pathway is the stilbene synthase (STS),
302 which has been the focus of several biotechnological approaches to increment the concentration of this class
303 of secondary metabolites in plants, especially in grapevine (Kiselev & Dubrovina, 2020). Some attempts
304 have also been made in other fruit tree species, such as kiwifruit overexpressing the *stilbene synthase* gene
305 (*Vst1*) isolated from different *Vitis* spp., which led to an increase in piceid (resveratrol-glucoside) content
306 (182 µg/g fresh weight in young leaves) in the transgenic lines (Kobayashi et al., 2000). Similar results were
307 observed in genetically modified apple expressing the *Vst1* gene, which led to the accumulation of
308 resveratrol derivatives in fruits (Rühmann et al., 2006).
309 Some biotechnological approaches related to phenolic compounds were also focused on the use of RNAi
310 mechanism for both validating the function of genes of interest, and to redirect specific biosynthetic
311 pathways. In strawberry the downregulation of the *anthocyanidin reductase (ANR)* gene shunted the
312 proanthocyanidin pathway to a premature and ectopic anthocyanin biosynthesis (Fischer et al., 2014).
313

314 **Vitamins**

315 Vitamins are fat-soluble (A, D, E and K) or water-soluble (B complex and C) micronutrients, with different
316 roles in plant metabolism and essential to human health and survival, indeed the human body cannot
317 synthesize these compounds *de novo*, or it can only in insufficient quantities. Vitamins need to be absorbed
318 through different foods and nutritional supplements to fulfil their diverse physiological functions and prevent
319 clinical deficiencies (Fitzpatrick & Chapman, 2020).
320 Vitamin E (comprising tocopherols and tocotrienols) has been proved to have cholesterol lowering effects
321 (Arya et al., 2019), and its antioxidant and radical scavenging activity has been extensively proved (Fritsche
322 et al., 2017). A sufficient uptake of vitamin E helps to prevent chronic diseases (as cancer, cataracts, and
323 atherosclerosis) and neurological disorders (in particular in elderly people), as well as to reduce infertility
324 (Fritsche et al., 2017; Strobbe et al., 2018). Currently, plenty of QTLs and association mapping studies
325 related to vitamin E have been performed in major crops including barley, maize, rapeseed, rice, soybean,
326 and tomato (Fritsche et al., 2017), but little is known about their gene regulation in fruit trees. In avocado, a
327 metabolomic study on Mexican accessions revealed a higher amount of this compound compared to the
328 control cultivar, suggesting the *VTE3* and *VTE4* genes implied in tocopherols biosynthesis (Peraza-
329 Magallanes et al., 2017). For oil crops, the enhancement of vitamin E in fruits is important in order to
330 prevent oil peroxidation and thus to improve oil quality. In fact, as demonstrated for virgin olive oil, the
331 amount of tocopherol in the final product depends on the quality of the olive fruit, which in turn can change

332 according to the genotype (Table 1). With a single crossing generation, it is possible to obtain new genetic
333 variability for the improvement of this compound (Pérez et al., 2019). The genetic variability associated to
334 the total content of vitamin E has also been studied in Turkish and Afghan almond genotypes, identifying
335 some interesting cultivars for future breeding programs aimed at increasing vitamin E (Çelik et al., 2019;
336 Zamany et al., 2017).

337 Vitamin A is important for many functions in human body, for example its deficiency is related to
338 xerophthalmia, a pathophysiological condition that starts with night blindness, and ultimately leads to
339 complete blindness. Vitamin A is also related to the functionality of immune system and its deficiency could
340 lead to a higher child mortality for severe infections as malaria, diarrhoea and measles. Vitamin A deficiency
341 also contributes to the insurgence of anaemia, being involved in the iron metabolism; furthermore, this
342 vitamin is important in the growth process, being necessary in spermatogenesis as well as for proper embryo
343 growth (Strobbe et al., 2018). The geographical and endemic location of vitamin A malnutrition clusters are
344 often characterized by the presence of infectious diseases, poverty, and poor infrastructure that do not assure
345 availability of foods rich in vitamin A. As bananas are key staples in those geographical areas (Africa and
346 South-East Asia), biofortification efforts for vitamin A content in these fruits have been made to ameliorate
347 the nutritional status of these vulnerable populations. The most common conventional breeding strategy for
348 banana is to cross seed-fertile 3x cultivars to 2x accessions that carry the candidate genes, then to select 4x
349 and 2x hybrids from intermediate products, and to cross these hybrids to obtain sterile 3x hybrids with the
350 character of interest. The recent release of the draft genome sequence of *Musa spp.* has allowed genomic
351 studies aimed at identifying candidate genes for nutritional and agronomic traits of interest, and for the
352 optimization of molecular techniques aimed to speed up banana breeding (Amah et al., 2019a).

353 Vitamin C or ascorbic acid is one of the most powerful antioxidant vitamins and acts as important ROS
354 scavenger. Besides antioxidant, it exerts plenty of functions in the human body for maintaining a good health.
355 In fact, it is involved in many metabolic and energetic processes, it reduces the cholesterol levels by
356 protecting against atherosclerosis, as well as some types of infections, and can counteract some tumours
357 development. A deficiency of ascorbic acid could lead to anaemia or to the insurgence of scurvy, a disease
358 characterized by neuropathy, spontaneous bleeding, haemolysis, oedema, jaundice, and eventually death
359 (Strobbe et al., 2018). It is known that, among foods, fruits are the best source of this vitamin, and for this
360 reason many studies have been done to explore the genetic variability of different species to identify
361 genotypes suitable for breeding programs aimed at increasing the amount of vitamin C in target cultivars
362 (Surya et al., 2018). These kind of screening were conducted on *Rubus spp.* (Surya et al., 2018), on papaya
363 (Iamjud et al., 2016), on strawberry (Zhong et al., 2017), and on apple (Fang et al., 2017). In this latter study,
364 ascorbic acid concentration in 457 apple accessions was negatively correlated with the expression of three
365 regulatory genes, namely *MdGGPI*, *MdDCHAR3-3*, and *MdNAT7-2*. In *Actinidia spp.*, the sequencing of a
366 hybrid genotype showed the presence of a QTL on chromosome 26 denoted as qAsA26.1, and the region
367 harbouring this QTL could be considered a supergene for vitamin C determination (McCallum et al., 2019).

368 Vitamins level enhancement in plants through genetic engineering techniques represents a valid alternative,
369 that has been limited to a few crops, like *Arabidopsis* and rice, that do not produce edible fruits, or tomato,
370 that is considered the model of fleshy fruits (Fenech et al., 2019). The overexpression of the strawberry *D-*
371 *galacturonic acid reductase (FaGalUR)* gene in tomato plants led to an increase in L-ascorbic acid also
372 associated to an enhanced tolerance to different abiotic stresses or to complex changes in metabolites
373 (Fenech et al., 2019). Another example of fruit tree-derived gene ectopic expression in model plant is
374 represented by the apple *homogentisate phytyltransferase* gene (*MdHPTI*), which was overexpressed in
375 tomato leading to an increase of tocopherol up to 3.6-fold and 1.7-fold in transgenic leaves and fruits,
376 respectively (Seo et al., 2011). These studies represent some examples of effective strategies to validate
377 candidate genes in model plants useful to obtain economically important fruit trees nutraceutically improved.
378 One of the few examples of fruit tree engineering for vitamins enhancement is represented by transgenic
379 strawberry with increased vitamin C content. In this study, seven independent lines were obtained showing a
380 two-fold increase in ascorbate concentration through the overexpression of the kiwifruit *GDP-galactose*
381 *phosphorylase* gene (*GGP*) (Bulley et al., 2012).

382 383 **Minerals**

384 Unlike vitamins, plants cannot synthesize minerals, but they take up mobile ions from the soil through
385 specific transporters directly into the root plasma membrane, or they synthesize chelating agents able to
386 mobilize minerals absorbed from the soil (Zhu et al., 2013). Human body needs several metals in small
387 amounts, which are used for example as cofactors for several enzymatic reactions or transcription factors.

388 Furthermore, some minerals are of particular interest in the prevention of some diseases. Selenium, for
389 example, is considered protective against certain types of cancer, and its deficiency has been related to
390 pathogenesis of a virus involved in the Keshan's disease. Good levels of zinc in the body have also been
391 associated to normal growth and reproduction, indeed, this compound is involved in the basic functions of
392 the cell (Schneeman, 2001).

393 At our knowledge, no studies have ever been carried out to ameliorate the content of minerals in fruit species
394 through conventional breeding. Only a few comparative studies have been performed to evaluate the mineral
395 content in different genotypes, as in peach, but no concrete research has been carried out aimed at improving
396 breeding programs (Bobis et al., 2017). Most of the studies describing transformed crops with enhanced
397 minerals bioavailability and content through genetic engineering techniques have been carried out on staple
398 crops. Iron-deficiency anaemia is one of the most prevalent micronutrient deficiencies in the world, and iron
399 increase by transformation of staple crops like rice, wheat or grains showed some disadvantages due to the
400 high level of phytic acid (inhibitor of iron adsorption) present in these species, thus alternative target crops
401 should represent a better choice (Kumar et al., 2011). To our knowledge a unique example exists of mineral
402 fortification in fruit tree species, represented by the transgenic banana with a higher level, compared to the
403 wild type control, of both iron and zinc through the overexpression of the soybean ferritin. The results
404 presented in this study showed a 6.32-fold increase in iron accumulation, and a 4.58-fold increase in zinc
405 level detected in the leaves of the transgenic lines. Although no data have been published yet on the increase
406 of both these minerals also in the transgenic fruits of fortified banana, this study showed the possibility of
407 developing functional fruit tree species helping to overcome iron and zinc deficiencies (Kumar et al., 2011).

408 409 **Terpenes**

410 Terpenes is one of the largest class of compounds in fruits and vegetables; among them, the carotenoids
411 family, consisting of carotenes and xanthophylls, is the wider group with more than 600 naturally occurring
412 compounds. In plants, these lipophilic isoprenoids pigments play vital function in the photosynthesis
413 apparatus, in addition to their role as hormones precursors and photooxidative protectors (Zheng et al., 2020).
414 Of all these compounds only a few dozen are common in the human diet, playing essential roles as
415 antioxidant compounds and ROS scavengers, useful to prevent both cardiovascular diseases and some types
416 of cancer, but mainly for being essential dietary precursors of vitamin A (provitamin A). In mammals,
417 provitamin A carotenoids are converted into retinol and other related retinoids that exert an important
418 function in gene regulation and for the vision (Zheng et al., 2020). Recent studies have also suggested a
419 protective role of these compounds against macular degeneration, cognitive function impairment, type-2
420 diabetes, and obesity (Rodriguez-Concepcion et al., 2018).

421 One of the most studied fruit species for its carotenoid content is banana, considered one of the top 10 crops
422 by production, and the main staple food in some countries. Large-scale germplasm studies have been set up
423 on this species to identify genotypes with high levels of provitamin A (Amah et al., 2019b), leading to the
424 release of some new cultivars (Apantu, Bira, Pelipita, Lai, and To'o) in Eastern Democratic Republic of
425 Congo and Burundi (Garg et al., 2018). Other species have also been screened for carotenoid content in new
426 breeding lines, as papaya (Iamjud et al., 2016), and strawberry, where eQTLs were discovered for published
427 alleles of the strawberry *phytoene synthase* (*FanPSY*) and *z-carotene desaturase* (*FanZDS*) genes (Barbey et
428 al., 2020). *Citrus* spp. are among the carotenoid-richest fruit species, so the study of this metabolism in
429 *Citrus* spp. has received particular attention (Table 1). A F1 population from a *Citrus reticulata* intraspecific
430 cross indicated that the carotenoids variation was determined by 17 QTLs on chromosomes 2, 3, 5, 6, and 9.
431 In particular, the QTL on chromosome 2 accounted for more than 20% of the variation of total carotenoid
432 content (Zheng et al., 2019). In three pummelo cultivars, a metabolomic study revealed the involvement of
433 12 differentially expressed genes in carotenoids biosynthesis (Jiang et al., 2019). The carotenoid biosynthetic
434 pathway has also recently been studied in papaya, finding six genes related to carotenoids biosynthesis
435 (Table 1), regulated by the transcription factors CpbHLH1/2 (Zhou et al., 2019). Finally, a recent study on
436 grapevine helped in understanding the carotenoids biosynthesis in this species, indicating that the
437 downregulation of the *VvDXS*, *VvDXR*, *VvGGPPS*, *VvPSY*, and *VvCCD* genes might also be responsible for
438 the decrease of carotenoids amount (Leng et al., 2017). This knowledge gives an important genetic
439 foundation for future MAS into carotenoid fruit tree breeding programs.

440 Metabolic engineering studies have enhanced carotenoids content in different plant species through some
441 main common strategies, which were focused on "pushing" the metabolic flux upstream of a target
442 compound, generally β -carotene, or by "blocking" the expression of genes downstream of a desired
443 compound, and downregulating enzymes responsible for the degradation of carotenoids or competing for the

444 same substrate (Watkins & Pogson, 2020). One of the most popular approaches for the enhancement of
445 carotenoids production has been the overexpression of the *phytoene synthase* gene (*PSY/CRTB*) to obtain
446 higher level of β -carotene (the precursor of pro-vitamin A) in the final target plants (Table 1). As already
447 mentioned, one of the most famous examples of food biofortification through genetic engineering are Golden
448 rice and Golden rice 2, presenting a total level of β -carotene up to 31 $\mu\text{g/g}$ (Paul et al., 2017). A similar
449 strategy has also been applied to obtain the Golden banana. The genetic improvement of domesticated
450 bananas by conventional breeding is hampered by the very low male and female fertility, thus genetic
451 engineering becomes a valid alternative tool. Pro-vitamin A biofortified Cavendish banana has been obtain
452 by overexpressing the *phytoene synthase 2a* (*PSY2a*) gene derived from the Fe'i banana, which is naturally
453 high in β -carotene. One of the transgenic lines obtained reached 55 $\mu\text{g/g}$ dry weight (dw) of β -carotene
454 equivalent (β -CE), compared to the wild-type control with a content between 1 and 4 $\mu\text{g/g}$ dw β -CE (Paul et
455 al., 2017).

456 The overexpression of *PSY* gene has not always been a successful strategy for the modulation of carotenoid
457 content in plant, indeed some researchers have underlined the induction of off-target effects as a
458 consequence of *PSY* ectopic expression. An example is the transgenic tomato overexpressing the *PSY* gene
459 with an increased content of carotenoid, but with dwarf phenotype, which was attributed to a decrease in
460 gibberellin A₁ synthesis, competing in the same metabolic flux (Watkins & Pogson, 2020). Recently, a
461 different gene other than *PSY* was ectopically expressed in apple to increase the level of different carotenoids.
462 In particular, the overexpression of the *DXR* gene from *A. thaliana*, encoding for one of the key enzymes
463 regulating the plastidial isoprenoid pathway, led to an up to 3-fold increase in individual and total
464 carotenoids compared to the non-transgenic control (Arcos et al., 2020). Another important issue in terms of
465 carotenoid fruit biofortification, and in general for nutritional quality enhancement in plants, is the
466 bioavailability of a specific compound, that in the case of carotenoids defines their digested fraction
467 available for human body physiological functions or storage. Some fruit tree species like papaya and citrus
468 fruits are rich of esterified β -cryptoxanthin, which helps in the prevention of osteoporosis and certain kinds
469 of cancers, in addition to its provitamin A function. Although its structure characterized by a unique β -ring
470 can lead to the production of only half the retinol molecules of β -carotene, its esterification allows a higher
471 level of stability and bioavailability for this compound, thus making it another valuable target gene to
472 increase carotenoids in plants (Watkins & Pogson, 2020).

473 The so called “block” strategy, that exploits RNAi mechanism to downregulate genes that negatively impact
474 the accumulation of target carotenoids, has been used to obtain transgenic orange fruits with up to 36-fold β -
475 carotene content in the pulp. This achievement has been possible through the silencing of the *β -carotene*
476 *hydroxylase* (*Cs β -CHX*) endogenous gene, which acts by converting β -carotene into xanthophylls (Pons et al.,
477 2014). The recent discovery and development of the CRISPR/Cas9 biotechnological tool has allowed the
478 precise disruption of specific genes activity involved in carotenoid biosynthesis. For example, a β -carotene-
479 enriched Cavendish banana was obtained through CRISPR/Cas9-based approach targeting the *lycopene*
480 *epsilon-cyclase* gene, showing an increase in β -carotene up to 6-fold (about 24 $\mu\text{g/g}$) compared with the
481 unedited control (Kaur et al., 2020b). Similar studies have also been carried out on sweet orange and
482 kiwifruit as proof of concept, by targeting their endogenous *PDS* gene, leading to a decrease in total
483 carotenoids; these and other recent studies have demonstrated the potential use of CRISPR/Cas9 tool for
484 precise gene knockout in several fruit tree species aimed to their nutritional enhancement (Kaur et al., 2020a).

485

486 Carbohydrates

487 Carbohydrates represent the main energy source in most people's diet and play an important role in energy
488 metabolism and homeostasis. However, this contribution to energy metabolism could lead to weight gain
489 when carbohydrates are consumed in excess (Mann et al., 2007). Consumption of carbohydrates, in
490 particular simple sugars, is commonly not recommended for subjects suffering of diabetes, particularly for
491 the postprandial increases in plasma glucose. Fruit consumption has the advantage to induce lower
492 postprandial increases in plasma glucose when the main simple sugar is fructose. Another benefit from fruit
493 carbohydrates is their high amount in polymeric carbohydrates (e.g. starches and fructans); in particular,
494 fructans (oligosaccharides of fructose) are considered an important ingredient in functional foods because
495 they have been shown to promote a healthy colon while also reducing the incidence of colon cancer (Newell-
496 McGloughlin, 2008). From a sensorial point of view, since the sweetness of fructose and sucrose are higher
497 than that of glucose and sorbitol, fruits containing higher levels of fructose and sucrose are desirable. Thus,
498 the improvements or changes in carbohydrate content represent an important issue for fruits
499 quality/marketability and also for the total calories supplied in the human diet.

500 Sugars (soluble and insoluble) content in plants depends on different factors such as the genotype, ripening
501 stage, and environmental conditions (Uncu et al., 2013). The amount of total carbohydrates and the
502 composition in simple sugars, have recently been investigated in different genotypes of several fruit tree
503 species, indicating which of them could be more suitable for consumption. A F1 population of apple
504 obtained from a crossing in China was useful to detect QTLs related to single sugars: QTL for glucose
505 content was identified on LG4, while QTLs for sucrose, fructose, sorbitol and glucose were mapped on LG3
506 (Ma et al., 2015). Another study analysed 188 apple cultivars to evaluate the role of genes encoding SWEET
507 sugar transporters in fruit sugar accumulation. It was found that the *MdSWEET15a* and *MdSWEET9b* genes
508 were significantly associated with phenotypic variation in the sugar content of fruits (Zhen et al., 2018). In
509 sour cherry, different genotypes showed a different composition in both total carbohydrates and single sugars,
510 with changing amounts of fructose, glucose and, at lesser extent, sorbitol (Proietti et al., 2019). Regarding
511 peach, a recent study on F2 population, deriving from a cross of two highly contrasting accessions, mapped
512 two suggestive QTLs for sugar content on LG3 and LG5. Interestingly, in this study a cluster of QTLs was
513 mapped on LG5 for phytochemical compounds and soluble sugars, underlying the high correlation between
514 these traits, and assuming an important role of sugars in the regulation of phenolic biosynthesis (Abdelghafar
515 et al., 2020). In another study, major QTLs for sugar content in peach were also identified on LG4 and LG6
516 (Cirilli et al., 2016). Overall, these mapping trials suggest that peach sugar content is driven by several QTLs
517 with minor effects, often gathered in clusters, and that the hundreds of genes present within these regions
518 make it hard to detect the gene(s) linked to sugar content. Another *Prunus* species where significant QTLs
519 for soluble sugars content were identified is apricot, in particular, candidate genes involved in D-glucose and
520 D-mannose binding were located on LG4 (García-Gómez et al., 2019). Recently, a preliminary study on
521 papaya crossing population was performed to identify possible QTLs related to the fruit soluble sugar
522 content, revealing the presence of a major QTL on LG7 for fruit sweetness (Nantawan et al., 2019). Sugar
523 content was also investigated in different genotypes of plum (Bobis et al., 2017), and boysenberry (Ryu et al.,
524 2017).

525 In higher plants, the carbohydrates are mainly divided in soluble sugars and starch, and knowledge on this
526 portioning is a prerequisite to apply effective engineering strategies. An increase up to 8-fold in fructose
527 content at the expense of sucrose was observed in transgenic strawberry ripe berries when the *fructose 6-*
528 *phosphate 1-phosphotransferase (PFP)* gene from *Giardia lamblia* was overexpressed (Basson et al., 2011).
529 In a recent work, the overexpression of the transcription factor *MdbHLH3* encoding gene in apple enhanced
530 the accumulation of carbohydrates in transgenic fruits by adjusting their allocation from sources to sinks (Yu
531 et al., 2020). In another work carried out on apple, an RNAi-construct was introduced to suppress the *aldose-*
532 *6-phosphate reductase* gene encoding for a key enzyme for sorbitol synthesis. The transgenic lines obtained
533 showed a decrease in sorbitol concentration and much higher concentration of sucrose supply to fruits (Li et
534 al., 2018a). Sugars are transported for long distances in plants by a family of proteins that acts as sugar
535 carriers, known as “sucrose transporters” (SUT). The pear *sucrose transporter (PbSUT2)* gene has recently
536 been characterized through its overexpression in tomato plants, which showed an increase in sucrose content
537 in the transgenic mature fruits compared to the non-transformed control, and a decrease in other sugars
538 concentration, like glucose and fructose (Wang et al., 2016a).

539 540 **Lipids**

541 Lipids have been recognized as one of the main sources of energy in the human diet, but their amount and
542 quality are fundamental for maintaining a healthy status. In fruits, fatty acids are the most common
543 compounds belonging to lipids, and they are mainly provided through oilseed crop plants. Fatty acids are
544 defined as organic compounds formed by an aliphatic chain and a carboxylic group normally bounded with
545 glycerol-forming acylglycerides, considered necessary for all plant cells, essential for cell division and
546 survival, growth, and development. Saturated fatty acids have been associated to an increased risk of
547 cardiovascular diseases, as atherosclerosis, diabetes, and hypercholesterolemia. Contrarily, unsaturated fatty
548 acids (UFA), as oleic, linoleic and linolenic acids, have been related to be preventive for cardiovascular
549 diseases insurgence, exerting beneficial effects on health (Assefa et al., 2017).

550 The difference in fatty acid patterns is a distinctive characteristic of cultivars within a species, as
551 demonstrated in nuts, which are recognised as oil-rich fruits, but also in this case the content of fatty acids
552 changes according to the species and the cultivar considered, and also on the basis of the geographical
553 provenance. In particular, oleic acid is by far the most abundant fatty acid in hazelnut (about 80% of the total)
554 followed by linoleic, palmitic and stearic acids, while walnut contains the highest concentrations of linoleic
555 and linolenic fatty acids (up to 75% of the total). The study of different nuts genotypes provided information

556 on new genetic sources for the development of new cultivars with promising quality characteristics (Kafkas
557 et al., 2017).
558 Extra virgin olive oil is well known for its high amount of mono-unsaturated oleic acid and di-unsaturated
559 linoleic acid, which in turn derive from the fatty acid pattern of the olive fruit. Olive breeding programs are
560 currently running for the selection of new genotypes with improved agronomic and qualitative characteristics,
561 including fatty acids content. The utilization of germplasm bank as source of genetic variability is a good
562 strategy for olive breeding. The analysis of fruit quality could predict the quality of the oil produced, as
563 several works reported a high correlation for fatty acids composition between the olive fruit and olive oil (De
564 la Rosa et al., 2016). Several studies focused their attention on genes determining fruits and oils traits, and
565 the *OeSAD2* gene was suggested to be the main contributor for oleic acid content in olive fruits, while the
566 *OeFAD2-2* gene is responsible for the linoleic acid content (Hernández et al., 2017). Recently, genomic
567 studies on olive cultivars have been carried out to identify candidate QTLs related to the fatty acids content.
568 A single QTL has been identified on LG20 for oleic acid in the cultivar “Arbequina”, and a QTL for linoleic
569 acid was identified in the same genomic position. The colocalization of mono- and di-unsaturated fatty acids
570 in this location underlines the importance of this region in the fatty acid composition of olive oil, and the
571 *OeSAD2* and *OeFAD2-2* genes were confirmed to be candidate for oleic and linoleic acid content. QTLs for
572 linolenic acid were detected on LG14 and LG19 for “Arbequina”, on LG5 and LG15 for “Picual”, and the
573 *FAD7* gene resulted a good candidate to explain the QTL of linolenic acid (Hernández et al., 2017).
574 In olive, the stearoyl-acyl carrier protein desaturase (SAD) catalyses the first desaturation step leading to
575 oleic acid, and then to linoleic and linolenic acids through further denaturation steps. Therefore, SAD has an
576 essential role in determining the overall UFA content. For this reason, the relationship between SAD-related
577 genes expression and UFA composition has been studied in olive fruits, pointing out that UFA composition
578 could be modified through the transcriptional regulation of SAD-related genes (Hernández et al., 2019).
579 Finally, in palm-oil fruit, the biosynthetic pathway of fatty acid has been investigated, and it was suggested
580 that the *EgWR11-1* gene behaves as a master regulator that activates the expression of oil biosynthetic genes.
581 However, the biosynthesis of oil in palm fruits is the result of the expression of several activators and
582 repressors transcription factors (Yeap et al., 2017).
583 Several biotechnological strategies aimed at incorporating additional fatty acids with healthful attributes, or
584 to modify oil composition, have been applied in different oilseed crops through genetic engineering (Haslam
585 et al., 2016; Rogalski & Carrer, 2011). Among fruit tree species, palm fruit tree has been the focus of several
586 studies for the obtainment of oil palm producing less saturated oil with an increased content of high value-
587 added fatty acids, like oleic acid in the mesocarp at the expense of palmitic content (Masani et al., 2018).
588 Biochemical studies determined two main target enzymes to be regulated for higher oleic acid content, that
589 are the palmitoyl-ACP thioesterase (PAT) and b-ketoacyl-ACP synthase II (KAS II), that have been
590 characterized in model plants and for which different transformation vectors have been designed and
591 constructed with tissue specific promoters (Masani et al., 2018). The progress in regeneration and
592 transformation protocols for this perennial species carried out during the last two decades, and the recent
593 successful sequencing of the oil palm genome will give the opportunity to develop palm oil with modified oil
594 palm lipid metabolism.

595 **Antinutrients**

597 As defence strategy against predators and pathogens, plants produce some bioactive compounds, as
598 resveratrol and glucosinolate, that have been demonstrated to have healthy benefits. However, among these
599 compounds there are also antinutritional metabolites like toxins or allergens, which are considered harmful
600 for human body (Newell-McGloughlin, 2008). Many fruit species contain allergens that cause oral allergy
601 syndrome to consumers, associated with gastrointestinal symptoms, urticaria, and anaphylaxis. The most
602 sensitive individuals to birch tree pollen protein (Bet v 1) are the most subjected to allergy reactions, in fact,
603 many fruit species contain pathogenesis-related proteins homologous to Bet v 1, including Mal d 1 in apple,
604 Pyr c 1 in pear, Pru p 1 in peach, Pru ar 1 in apricot, Pru av 1 in sweet cherry, and Fra a 1 in strawberry
605 (Aranzana et al., 2019; Guarino & Sciarrillo, 2018; Kaiser et al., 2016; Paris et al., 2017) (Table 1). Four
606 classes of allergens have been identified in apple (Mal d 1, Mal d 2, Mal d 3 and Mal d 4), with Mal d 1
607 being the most allergenic (Paris et al., 2017). The amount of Mal d 1 in apple can change according to the
608 cultivar. A reduction of Mal d 1 allergenicity has been observed in genotypes with high polyphenol oxidase
609 activity, which could be enhanced by increasing chlorogenic and caffeic acid in apple genotypes (Kschonsek
610 et al., 2019). A less common but more severe allergy could also be developed in people sensitive to the Mal
611 d 3 protein. Allergen levels in apple are usually not assessed during the breeding process, but some cultivars

612 with low amount of allergens have been identified and reducing the content of these proteins in plants could
613 be the focus of future apple breeding programs (Kaiser et al., 2020).

614 Strawberry is a fruit with potential allergenicity for the consumer, caused by different proteins according to
615 the geographic area. Fra a 3 and Fra a 4 proteins are the mainly responsible for allergies occurring in the
616 Mediterranean area, while Fra a 1 in North and Central Europe. Fruit colour has been correlated with
617 allergen content, therefore the *Fra a 1* and *Fra a 3* genes are linked to the flavonoid biosynthesis. The total
618 elimination of these gene products would lead to colourless strawberries, however, considerable variation in
619 the Fra a 1 content has been described for different red coloured cultivars, suggesting that Fra a 1 and Fra a 3
620 might not be always limiting in the flavonoid biosynthesis (Kaiser et al., 2016). Screening for strawberry
621 accessions with low content of Fra a 1 and Fra a 3 could be useful to identify and breed strawberries aimed at
622 reducing the allergen content.

623 Other species present allergens, like *Actinidia* spp. and *Citrus* spp. (Table 1). In *Actinidia* spp., the protein
624 actinidin (Act 1a) has been recognized for its allergenicity inducing tingling in the oral cavity and
625 anaphylactic reactions. Differences in the content of this protein have been detected in different kiwifruit
626 genotypes, and some of them showing low levels of Act 1 a were suggested as interesting breeding material
627 for low-allergenicity fruits (Kamiyoshihara et al., 2018). The content of allergens is species-dependent, and
628 pummelo was suggested as the species with the lower allergenicity risk, being an interesting breeding source
629 for hypoallergenic citrus fruits (Wu et al., 2016).

630 Among biotechnological tools, RNAi has been used as effective tool for downregulating genes encoding
631 undesirable compounds in several plant species, like gossypol in cottonseed, or phytic acid in wheat (Newell-
632 McGloughlin, 2008; Rathore et al., 2020). RNAi strategy was successfully applied in apple by silencing the
633 *Mal d1* gene. In this study transgenic apple plants showed a significant reduction in *Mal d 1* transcript and
634 many allergic subjects resulted completely tolerant to these fruits (Dubois et al., 2015). In another study the
635 strawberry *Fra a 1* allergen encoding gene was downregulated through transient RNAi-mediated silencing,
636 however the parallel reduction of the *phenylalanine ammonia lyase (FaPAL)* and *chalcone synthase (FaCHS)*
637 flavonoid biosynthesis-related transcripts was observed, leading to white phenotype in the transformed fruits
638 (Muñoz et al., 2010).

639

640 **Conclusions and future perspectives**

641 Over the years, the market demand has changed toward the obtainment of food crops with improved quality
642 and safety, through low environmental impact systems. Several experimental and epidemiological data are
643 confirming that the consumption of fruit and vegetables is related to many health benefits, decreasing the risk
644 of chronic diseases insurgence, and reducing their huge healthcare costs. In addition, the nutritional
645 enhancement of food crops is of increasing interest also as sustainable and promising approach to help in
646 solving the diffused nutrients' deficiencies. Fruit tree species represent an incredible source of biologically
647 active molecules important for the human diet, such as carbohydrates, vitamins, minerals and antioxidant
648 compounds. However, there is no fruit containing all the nutrient constituents and rarely these compounds
649 are uniformly distributed in all plant tissues; in addition, some agricultural practices seem to diminish
650 gradually their level in fruit (Kaur et al., 2020a). Thus, there is the need to work on nutrients maintenance
651 and their target improvement.

652 This review summarized many of the attempts done to increase the nutritional quality of fruit tree species
653 during the last decades through both traditional breeding associated to molecular techniques, like MAS, and
654 the most recent NBTs applied to these crops. The most accepted method for fruit biofortification is
655 conventional breeding, that offers a sustainable and cost-effective alternative to agronomic and transgenic
656 biofortification strategies (Garg et al., 2018). The improvement of fruit characteristics is obtained through the
657 implementation of breeding programs for the traits of interest, and, to set up an effective breeding program,
658 the availability of sufficient genotypic variation is necessary. However, the genetic variation present in the
659 genetic pool of some species is sometimes limited for selected traits of interest. This issue could be solved by
660 the valorisation of wild germplasm and ancient accessions, including them in crossing programs; however, it
661 would take long time and many crossing cycles to introduce the traits of interest into commercial cultivars.
662 The application of molecular techniques, as MAS, to conventional breeding could help in speeding up these
663 selection processes, determining more accurately and in a shorter time the genotypes carrying the genes of
664 interest. However, even if these kinds of molecular techniques significantly optimize the breeding process,
665 the timescale and efforts needed for the biofortification of some fruit tree species remain still relevant, and
666 the application of NBTs represents the most suitable alternative solution (Fig. 2). When considering the use
667 of biotechnological tools for the modulation of a specific biosynthetic pathway and/or for the increase of a

668 phytonutrient in a target crop, several aspects need to be taken into consideration. First of all, a deep
669 knowledge on the interconnection among the different metabolic pathways is necessary, because the
670 alteration of one pathway can have unexpected effects on one or more related ones; in this sense, the
671 development of effective bioinformatic and “omics” approaches, especially functional genomic studies, are
672 essential tools for elucidating such pathways (Kumar et al., 2020). In addition, other aspects must be
673 considered, like the bioavailability of the increased nutrient, its effective dose for human diet, as well as the
674 maintenance of the other agronomical traits proper of the target cultivar, which could be negatively affected
675 by the alteration of metabolic fluxes. Further parameters, such as cultivation and production costs, as well as
676 potential allergic responses of the consumer need to be considered (Uncu et al., 2013).
677 During the last decades a huge number of studies have been published describing the use of biotechnological
678 approaches aimed to the enhancement of healthy compounds in plants; however, the number of released
679 transgenic biofortified crops still represents a very low percentage compared to those obtained by traditional
680 breeding (Garg et al., 2018). Indeed, genetically modified plants are still arising public concern and represent
681 a controversial topic for governmental agencies, especially in Europe. However, the progress made during
682 the last decade in the field of biotechnological techniques have introduced more fast, efficient and precise
683 crop improvement tools, which could arise less biosafety and regulatory concerns, and that could be
684 considered more similar to conventional breeding techniques, like cisgenesis and intragenesis (Capriotti et al.,
685 2020; Limera et al., 2017). An increasing number of studies have been published based on the use of
686 CRISPR/Cas9-mediated genome editing technology for both knock-in and knock-out of target traits also in
687 fruit tree species, and for few of them results showing transgene-free mutants’ regeneration have been
688 reported (Kaur et al., 2020a). Among NBTs, RNAi still represents one of the most exploited biotechnological
689 approaches, especially for metabolically engineered plants, because it can lead to the modulation of a
690 transcript expression without its complete knockout. This represents an important feature when only a
691 reduced expression level of a target gene is necessary. Furthermore, RNAi represents a good compromise to
692 both reduce the risk of off-target effects, and to avoid the introduction of new allergens; indeed, no new
693 protein is introduced through this technique, but the modulation of specific genes expression is mediated by
694 dsRNA molecules expressed in the target plant (Arpaia et al., 2020; Mezzetti et al., 2020).
695 In EU, the European Court of Justice has recently confirmed that all new NBTs are regulated by the 2001/18
696 directive on genetically modified organisms (GMOs), because all referring to the use of recombinant DNA
697 technology (Schulman et al., 2020) (Fig. 2). This is negatively impacting on the approval of any new NBTs
698 product in Europe because of the lack of acceptance by the consumers. While in other regions of the world
699 (USA, Canada, Japan, Australia) a regulatory system based on products and not on methods has been
700 adopted, then the approval of any new NBTs product is more facilitated (Eckerstorfer et al., 2019).
701 A novelty is offered by the exogenous application of dsRNAs on plants, namely spray-induced gene
702 silencing (SIGS), which has been mainly tested as environmental-friendly alternative to traditional pesticides
703 for pests and pathogens control (Taning et al., 2020). Recently, these dsRNA-based products have been
704 classified not as GMOs but as biological pesticides (OECD, 2020). Beside the impact that this technology
705 will have on replacing traditional pesticides, RNA-based compounds for external applications are of high
706 interest to downregulate endogenous genes in plant, opening the possibility to exploit SIGS as a tool also for
707 modulating metabolic pathways in a target crop, without introducing any stable modification in the plant
708 genome (Dubrovina & Kiselev, 2019).
709 Breeding and biotechnological approaches will strongly contribute to the development of new plants-based
710 biofortified foods, but their diffusion on the market will depend on their acceptance, especially for new
711 products derived from biotechnologies, and their commercial exploitation would also be enhanced through
712 health claims, unfortunately not yet very widespread for fruits enriched with nutritional compounds
713 (Mezzetti et al., 2016).

714 715 **Acknowledgments**

716 The authors acknowledge EU financial support through iPLANTA COST Action CA 15223, MIUR-
717 PRIN2017 national program via grant N.20173LBZM2-Micromolecole, “Programma di Sviluppo Rurale
718 della Regione Marche” 2014-2020. Misura 16.1.A.2-Project i.d. 27961 and PRIMA– Partnership for
719 Research and Innovation in the Mediterranean Area 2019-2022 MEDBERRY project.

720 721 **Conflicts of interest**

722 The authors declare no conflicts of interest in relation to this manuscript and state that the opinions expressed
723 are their own and should not be considered to reflect those of any other individuals or organizations.

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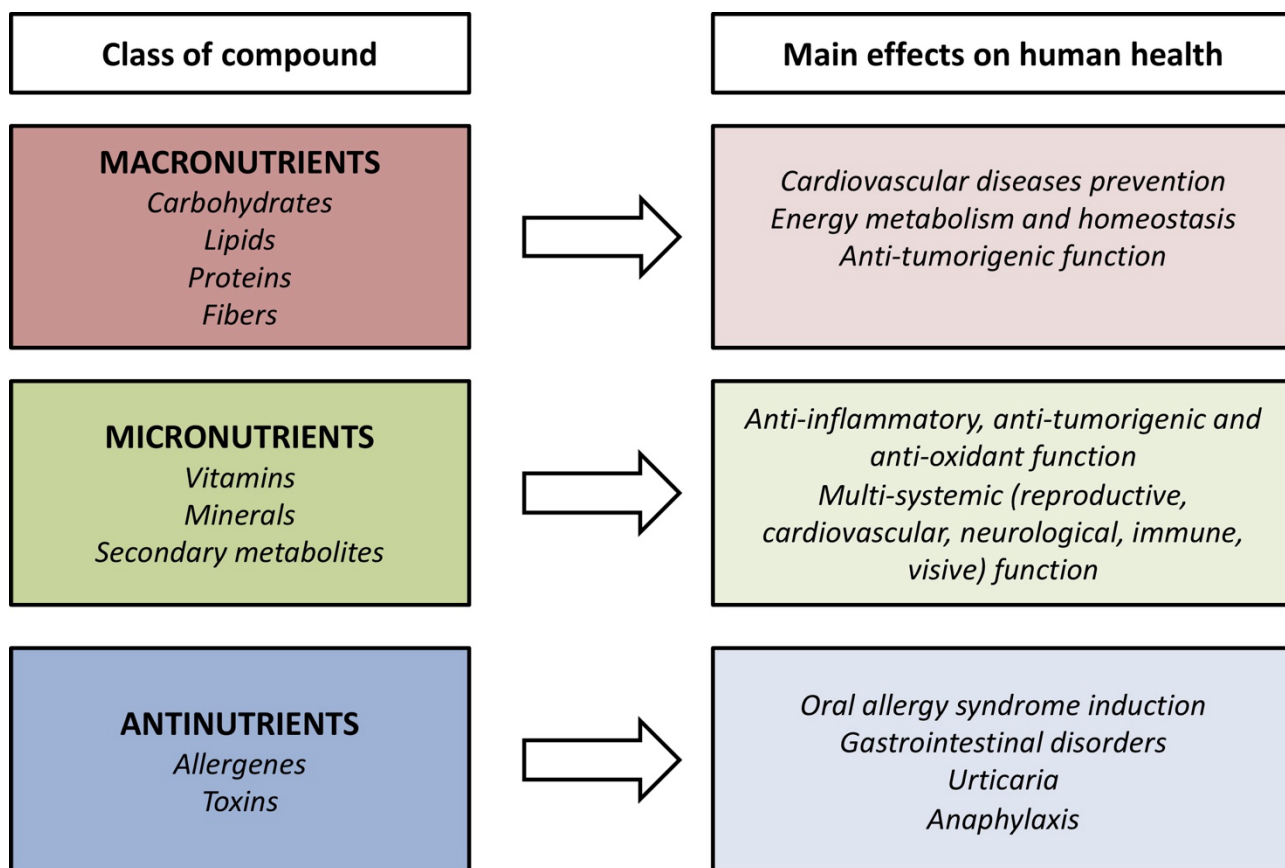
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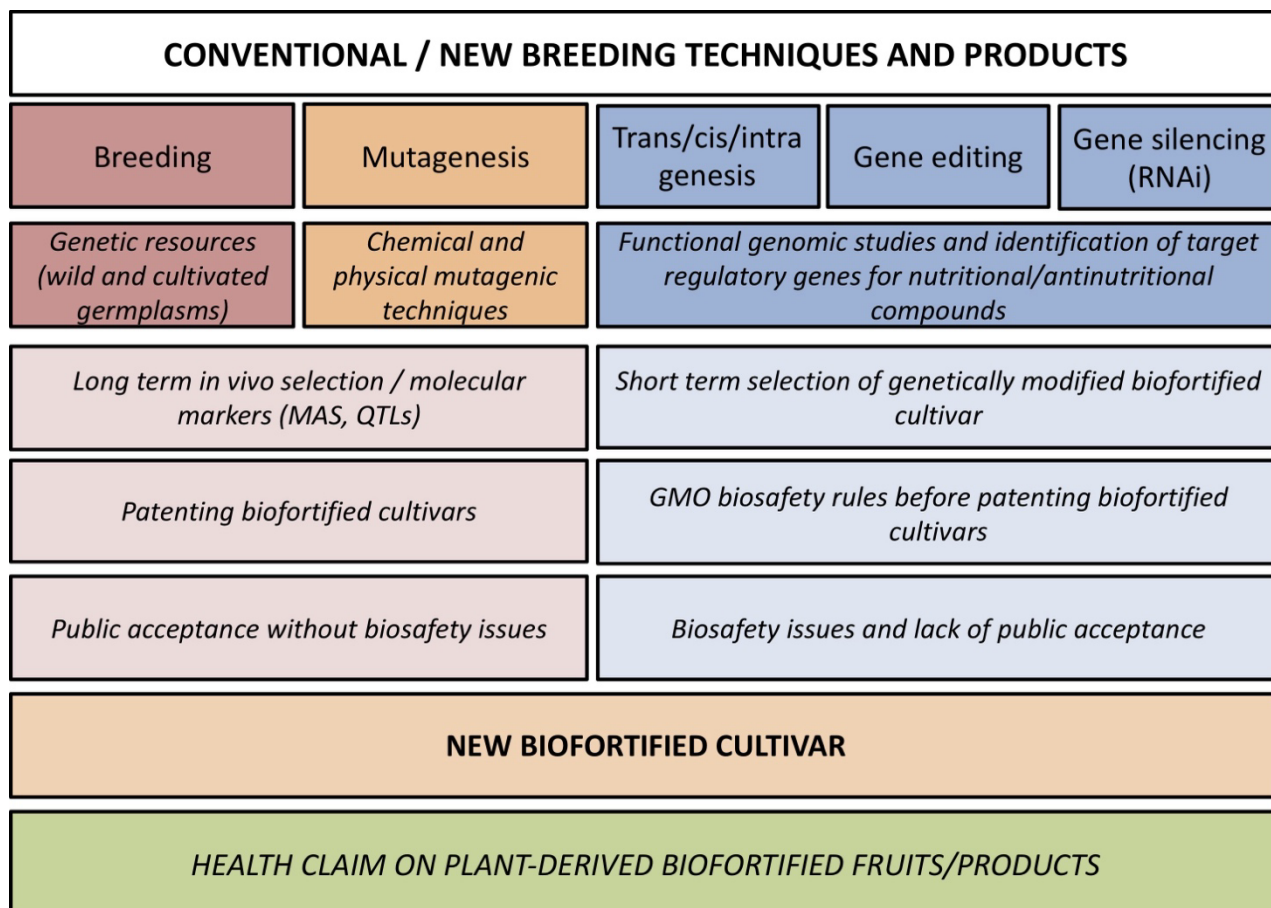
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1190 **Figure Captions**

1191 **Fig. 1.** Plant components of dietary interest and their main health effects.
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1194 **Fig. 2.** Diagram showing different conventional (left part) and biotechnological (right part) strategies applied
 1195 for fruit trees biofortification. For each technique, main advantages, outcomes, public acceptance and
 1196 expected results are reported.
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Table 1. Candidate genes for the improvement of nutritional traits in fruit trees characterized by conventional molecular tools or genetic engineering strategies

Compound	Fruit tree species	Candidate genes	References	
Phenolic compounds				
Flavonoids	Grapevine	<i>VvMYBA1</i> , <i>VvMYBA2</i> , <i>VvMYB4</i> (-)	(Azuma et al., 2008)	
	Pear	<i>PcMYB10</i>	(Brendolise et al., 2017)	
	Apple		<i>PbMYB120</i> (-)	(Song et al., 2020)
			<i>MdMYBA</i> , <i>MdMYB1</i> , <i>MdMYB10</i>	(Espley et al., 2007; Krens et al., 2015; Naing & Kim, 2018)
			<i>MdWRKY11</i>	(Liu et al., 2019)
			<i>MdbHLH3</i>	(Xie et al., 2012)
			<i>MdMYB16</i> (-), <i>MdMYB17</i> (-), <i>MdMYB111</i> (-)	(Liu et al., 2019)
	Bayberry	<i>MrMYB1</i>	(Naing & Kim, 2018)	
	Chinese berry	<i>MrMYB1</i>	(Naing & Kim, 2018)	
	Litchi	<i>LcMYB1</i>	(Naing & Kim, 2018)	
	Kiwifruit		<i>AcMYB110a</i>	(Naing & Kim, 2018)
			<i>MYBC1</i> , <i>WRKY44</i>	(Peng et al., 2020)
			<i>AaF3H</i> , <i>AaLDOX</i> , <i>AaUFGT</i>	(Li et al., 2018b)
Blood orange	<i>CsRuby</i>	(Naing & Kim, 2018)		
Mangosteen	<i>GmMYB10</i>	(Naing & Kim, 2018)		

	Peach	<i>Prupe.5G105700, Prupe.5G105800, Prupe.5G116700</i> <i>PpBL, PpMYB10.1</i> <i>DFR, F3H, F3'5'H, ANS</i>	(Abdelghafar et al., 2020) (Cao et al., 2018) (Qi et al., 2019)
	Blood orange	<i>Cs6g17570, Cs5g31400, Cs9g04810</i>	(Wang et al., 2016b)
	Sweet cherry	<i>PavMYB10</i>	(Aranzana et al., 2019)
	Fig	<i>R2R3-MYB</i>	(Wang et al., 2017)
	Blackcurrant	<i>F3H, F3'5'H</i>	(Li et al., 2019a)
	Blueberry	<i>F3H, F3'5'H, VcUFGALT, VcUFGT73, VcU5GT, VcAPRR2, VcbHLH3, VcWD, VcWD</i>	(Li et al., 2019b)
	Raspberry	<i>bHLH, NAM/CUC2 like protein, bZIP</i>	(Mccallum et al., 2018)
	Strawberry	<i>FvMYB10, FaMYB10, FaMYB5</i>	(Kui et al., 2014; Lin-Wang et al., 2014)
		<i>ANS</i>	(Giampieri et al., 2018)
		<i>RAP</i>	(Gao et al., 2020)
		<i>ANR (-)</i>	(Fischer et al., 2014)
		<i>FabHLH3, FabHLH3-delta, FaMYB11 (-), FaMYB9 (-), FabHLH33 (-), FaWD44-1 (-)</i>	(Hossain et al., 2018)
		<i>FaMYB1 (-)</i>	(Kadomura-Ishikawa et al., 2015)
		<i>Fra a 1, Fra a 3</i>	(Kaiser et al., 2016; Muñoz et al., 2010)
Flavonols	Pear	<i>PbMYB12b, PbCHSb, PbFLS</i>	(Zhai et al., 2019)
Stilbenes	Grapevine	<i>STS</i>	(Kiselev & Dubrovina, 2020)
		<i>Vst1</i>	(Kobayashi et al., 2000; Rühmann et al., 2006)
Vitamins			
Vitamin E	Avocado	<i>VTE3, VTE4</i>	(Peraza-Magallanes et al., 2017)
	Olive	<i>VTE1, VTE2, VTE3, VTE4, VTE5, HPPD, HGGT, GGR</i>	(Georgiadou et al., 2016)
Vitamin A	Banana	<i>PSY2</i>	(Paul et al., 2017)
Vitamin C	Apple	<i>MdGGP1, MdDHAR3-3, MdNAT7-2</i>	(Fang et al., 2017)
		<i>MdHPT1</i>	(Seo et al., 2011)
	Kiwifruit	qAsA26.1 (QTL)	(McCallum et al., 2019)
		<i>GGP</i>	(Bulley et al., 2012)
	Strawberry	<i>FanGalUR, FanMDAR, FanGPX, FanGR, FanSODM</i>	(Barbey et al., 2020)
Minerals			
Zinc, Iron	Banana	Ferritin from Soybean	(Kumar et al., 2011)
Terpenes			
Carotenoids	Strawberry	<i>FanPSY, FanZDS</i>	(Barbey et al., 2020)
	Citrus spp.	<i>PSY, ZDS, PDS, HYb, ZEP</i>	(Sugiyama et al., 2014)
		<i>LYCB2/E, CCD4, NCED2, AAO3, CyP707A1</i>	(Jiang et al., 2019)
	Papaya	<i>CpCYC-B, CpLCY-B, CpPDS2, CpZDS, CpLCY-E, CpCHY-B</i>	(Zhou et al., 2019)
	Apricot	<i>LOX 2, CCD1, CCD4</i>	(García-Gómez et al.,

	Grapevine	<i>VvDXS, VvDXR, VvGGPPS, VvPSY, VvCCD</i>	2019) (Leng et al., 2017)
	Banana	<i>PSY2</i>	(Paul et al., 2017)
		<i>LCY-ε (-)</i>	(Kaur et al., 2020b)
	Kiwifruit	<i>GGPS, PDS, ZDS, CHX</i>	(Kim et al., 2010)
	Apple	<i>AtDXR</i>	(Arcos et al., 2020)
	Orange	<i>Csβ-CHX (-)</i>	(Pons et al., 2014)
Carbohydrates			
Total soluble sugars	Pear	<i>SUS</i>	(Saito, 2016)
	Apple	<i>MdSWEET15a, MdSWEET9b</i>	(Zhen et al., 2018)
Fructose	Strawberry	<i>MdbHLH3</i> <i>PFP from Giardia lamblia</i>	(Yu et al., 2020) (Basson et al., 2011)
Sucrose	Apple	<i>A6PR (-)</i>	(Li et al., 2018a)
	Pear	<i>PbSUT2</i>	(Wang et al., 2016a)
Lipids			
Oleic acid	Olive	<i>OeSAD2</i>	(Sebastiani & Busconi, 2017)
		<i>SAD</i>	(Hernández et al., 2019)
	Oil palm	<i>PAT, KAS II</i>	(Masani et al., 2018)
Linoleic acid	Olive	<i>OeFAD2-2</i>	(Sebastiani & Busconi, 2017)
		<i>FAD7</i>	(Hernández et al., 2017)
Antinutrients			
Allergens	Apple	<i>Mal d 1, Mal d 2, Mal d 3, Mal d 4</i>	(Dubois et al., 2015; Paris et al., 2017)
	Peach	<i>Pru p 2.04, Pru p 4.01, Pru p 7, Pru p 2.01, Pru p 3, Pru p 4.02, Pru p 2.02, Pru p 2.03</i>	(Aranzana et al., 2019)
	Strawberry	<i>Fra a 1</i>	(Guarino & Sciarrillo, 2018; Muñoz et al., 2010)
	Kiwifruit	<i>Act 1 a</i>	(Kamiyoshihara et al., 2018)
	Citrus spp.	<i>Cit s 1.01, Cit s 2.01, Cit s 3.01</i>	(Wu et al., 2016)

(-) = negative regulator

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