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

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## Abstract

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

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

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

## Keywords

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

## Background

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

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

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

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

## 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

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

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

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

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

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

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

## Vitamins

Vitamins are fat-soluble (A, D, E and K) or water-soluble (B complex and C) micronutrients, with different roles in plant metabolism and essential to human health and survival, indeed the human body cannot synthesize these compounds *de novo*, or it can only in insufficient quantities. Vitamins need to be absorbed through different foods and nutritional supplements to fulfil their diverse physiological functions and prevent clinical deficiencies (Fitzpatrick & Chapman, 2020).

Vitamin E (comprising tocopherols and tocotrienols) has been proved to have cholesterol lowering effects (Arya et al., 2019), and its antioxidant and radical scavenging activity has been extensively proved (Fritsche et al., 2017). A sufficient uptake of vitamin E helps to prevent chronic diseases (as cancer, cataracts, and atherosclerosis) and neurological disorders (in particular in elderly people), as well as to reduce infertility (Fritsche et al., 2017; Strobbe et al., 2018). Currently, plenty of QTLs and association mapping studies related to vitamin E have been performed in major crops including barley, maize, rapeseed, rice, soybean, and tomato (Fritsche et al., 2017), but little is known about their gene regulation in fruit trees. In avocado, a metabolomic study on Mexican accessions revealed a higher amount of this compound compared to the control cultivar, suggesting the *VTE3* and *VTE4* genes implied in tocopherols biosynthesis (Peraza-Magallanes et al., 2017). For oil crops, the enhancement of vitamin E in fruits is important in order to prevent oil peroxidation and thus to improve oil quality. In fact, as demonstrated for virgin olive oil, the amount of tocopherol in the final product depends on the quality of the olive fruit, which in turn can change

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

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

Vitamin C or ascorbic acid is one of the most powerful antioxidant vitamins and acts as important ROS scavenger. Besides antioxidant, it exerts plenty of functions in the human body for maintaining a good health. In fact, it is involved in many metabolic and energetic processes, it reduces the cholesterol levels by protecting against atherosclerosis, as well as some types of infections, and can counteract some tumours development. A deficiency of ascorbic acid could lead to anaemia or to the insurgence of scurvy, a disease characterized by neuropathy, spontaneous bleeding, haemolysis, oedema, jaundice, and eventually death (Strobbe et al., 2018). It is known that, among foods, fruits are the best source of this vitamin, and for this reason many studies have been done to explore the genetic variability of different species to identify genotypes suitable for breeding programs aimed at increasing the amount of vitamin C in target cultivars (Surya et al., 2018). These kind of screening were conducted on *Rubus* spp. (Surya et al., 2018), on papaya (Iamjud et al., 2016), on strawberry (Zhong et al., 2017), and on apple (Fang et al., 2017). In this latter study, ascorbic acid concentration in 457 apple accessions was negatively correlated with the expression of three regulatory genes, namely *MdGGPI*, *MdDHAR3-3*, and *MdNAT7-2*. In *Actinidia* spp., the sequencing of a hybrid genotype showed the presence of a QTL on chromosome 26 denoted as qAsA26.1, and the region harbouring this QTL could be considered a supergene for vitamin C determination (McCallum et al., 2019).

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

## Minerals

Unlike vitamins, plants cannot synthesize minerals, but they take up mobile ions from the soil through specific transporters directly into the root plasma membrane, or they synthesize chelating agents able to mobilize minerals absorbed from the soil (Zhu et al., 2013). Human body needs several metals in small 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  $\beta$ -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

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

The overexpression of *PSY* gene has not always been a successful strategy for the modulation of carotenoid content in plant, indeed some researchers have underlined the induction of off-target effects as a consequence of *PSY* ectopic expression. An example is the transgenic tomato overexpressing the *PSY* gene with an increased content of carotenoid, but with dwarf phenotype, which was attributed to a decrease in gibberellin  $A_1$  synthesis, competing in the same metabolic flux (Watkins & Pogson, 2020). Recently, a different gene other than *PSY* was ectopically expressed in apple to increase the level of different carotenoids. In particular, the overexpression of the *DXR* gene from *A. thaliana*, encoding for one of the key enzymes regulating the plastidial isoprenoid pathway, led to an up to 3-fold increase in individual and total carotenoids compared to the non-transgenic control (Arcos et al., 2020). Another important issue in terms of carotenoid fruit biofortification, and in general for nutritional quality enhancement in plants, is the bioavailability of a specific compound, that in the case of carotenoids defines their digested fraction available for human body physiological functions or storage. Some fruit tree species like papaya and citrus fruits are rich of esterified  $\beta$ -cryptoxanthin, which helps in the prevention of osteoporosis and certain kinds of cancers, in addition to its provitamin A function. Although its structure characterized by a unique  $\beta$ -ring can lead to the production of only half the retinol molecules of  $\beta$ -carotene, its esterification allows a higher level of stability and bioavailability for this compound, thus making it another valuable target gene to increase carotenoids in plants (Watkins & Pogson, 2020).

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

## Carbohydrates

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

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

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

## Lipids

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

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

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

## Antinutrients

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

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

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

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

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

## Conclusions and future perspectives

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

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

phytonutrient in a target crop, several aspects need to be taken into consideration. First of all, a deep knowledge on the interconnection among the different metabolic pathways is necessary, because the alteration of one pathway can have unexpected effects on one or more related ones; in this sense, the development of effective bioinformatic and “omics” approaches, especially functional genomic studies, are essential tools for elucidating such pathways (Kumar et al., 2020). In addition, other aspects must be considered, like the bioavailability of the increased nutrient, its effective dose for human diet, as well as the maintenance of the other agronomical traits proper of the target cultivar, which could be negatively affected by the alteration of metabolic fluxes. Further parameters, such as cultivation and production costs, as well as potential allergic responses of the consumer need to be considered (Uncu et al., 2013).

During the last decades a huge number of studies have been published describing the use of biotechnological approaches aimed to the enhancement of healthy compounds in plants; however, the number of released transgenic biofortified crops still represents a very low percentage compared to those obtained by traditional breeding (Garg et al., 2018). Indeed, genetically modified plants are still arising public concern and represent a controversial topic for governmental agencies, especially in Europe. However, the progress made during the last decade in the field of biotechnological techniques have introduced more fast, efficient and precise crop improvement tools, which could arise less biosafety and regulatory concerns, and that could be considered more similar to conventional breeding techniques, like cisgenesis and intragenesis (Capriotti et al., 2020; Limera et al., 2017). An increasing number of studies have been published based on the use of CRISPR/Cas9-mediated genome editing technology for both knock-in and knock-out of target traits also in fruit tree species, and for few of them results showing transgene-free mutants’ regeneration have been reported (Kaur et al., 2020a). Among NBTs, RNAi still represents one of the most exploited biotechnological approaches, especially for metabolically engineered plants, because it can lead to the modulation of a transcript expression without its complete knockout. This represents an important feature when only a reduced expression level of a target gene is necessary. Furthermore, RNAi represents a good compromise to both reduce the risk of off-target effects, and to avoid the introduction of new allergens; indeed, no new protein is introduced through this technique, but the modulation of specific genes expression is mediated by dsRNA molecules expressed in the target plant (Arpaia et al., 2020; Mezzetti et al., 2020).

In EU, the European Court of Justice has recently confirmed that all new NBTs are regulated by the 2001/18 directive on genetically modified organisms (GMOs), because all referring to the use of recombinant DNA technology (Schulman et al., 2020) (Fig. 2). This is negatively impacting on the approval of any new NBTs product in Europe because of the lack of acceptance by the consumers. While in other regions of the world (USA, Canada, Japan, Australia) a regulatory system based on products and not on methods has been adopted, then the approval of any new NBTs product is more facilitated (Eckerstorfer et al., 2019).

A novelty is offered by the exogenous application of dsRNAs on plants, namely spray-induced gene silencing (SIGS), which has been mainly tested as environmental-friendly alternative to traditional pesticides for pests and pathogens control (Taning et al., 2020). Recently, these dsRNA-based products have been classified not as GMOs but as biological pesticides (OECD, 2020). Beside the impact that this technology will have on replacing traditional pesticides, RNA-based compounds for external applications are of high interest to downregulate endogenous genes in plant, opening the possibility to exploit SIGS as a tool also for modulating metabolic pathways in a target crop, without introducing any stable modification in the plant genome (Dubrovina & Kiselev, 2019).

Breeding and biotechnological approaches will strongly contribute to the development of new plants-based biofortified foods, but their diffusion on the market will depend on their acceptance, especially for new products derived from biotechnologies, and their commercial exploitation would also be enhanced through health claims, unfortunately not yet very widespread for fruits enriched with nutritional compounds (Mezzetti et al., 2016).

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## Conflicts of interest

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

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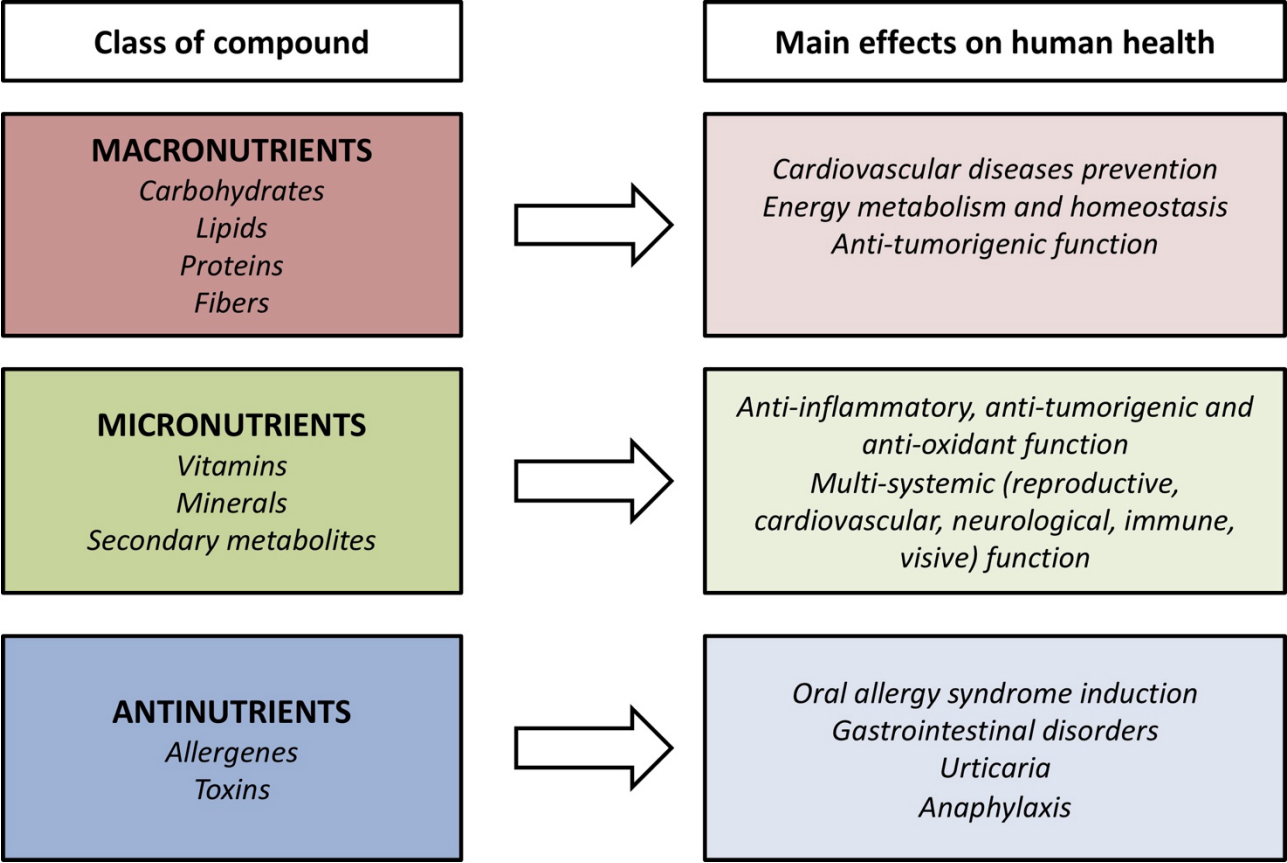
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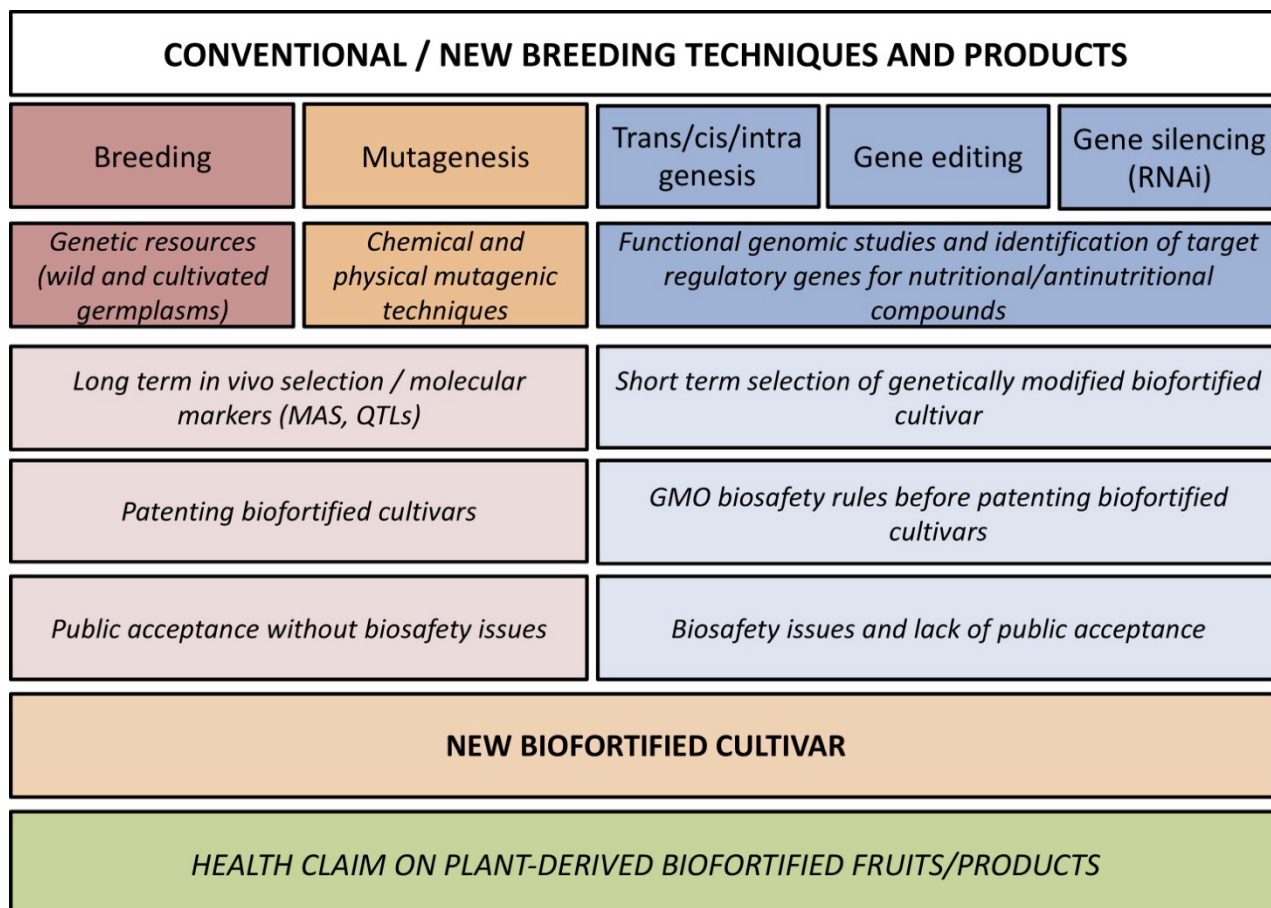
1190 **Figure Captions**

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)
		<i>AaMYB</i> , <i>AabHLH</i>	
	Blood orange	<i>CsRuby</i>	(Naing & Kim, 2018)
	Mangosteen	<i>GmMYB10</i>	(Naing & Kim, 2018)



<b>Flavonols Stilbenes</b>	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> <i>RAP</i> <i>ANR (-)</i> <i>FabHLH3, FabHLH3-delta, FaMYB11 (-), FaMYB9 (-), FabHLH33 (-), FaWD44-1 (-)</i> <i>FaMYB1 (-)</i>	(Giampieri et al., 2018) (Gao et al., 2020) (Fischer et al., 2014) (Hossain et al., 2018)
		<i>Fra a 1, Fra a 3</i>	(Kadomura-Ishikawa et al., 2015)
<b>Flavonols Stilbenes</b>	Pear	<i>PbMYB12b, PbCHSb, PbFLS</i>	(Kaiser et al., 2016; Muñoz et al., 2010)
	Grapevine	<i>STS</i>	(Zhai et al., 2019)
		<i>Vst1</i>	(Kiselev & Dubrovina, 2020) (Kobayashi et al., 2000; Rühmann et al., 2006)
<b>Vitamins</b>			
<b>Vitamin E</b>	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)
<b>Vitamin A</b>	Banana	<i>PSY2</i>	(Paul et al., 2017)
<b>Vitamin C</b>	Apple	<i>MdGGP1, MdDHAR3-3, MdNAT7-2</i> <i>MdHPT1</i>	(Fang et al., 2017) (Seo et al., 2011)
	Kiwifruit	qAsA26.1 (QTL)	(McCallum et al., 2019)
	Strawberry	<i>GGP</i> <i>FanGalUR, FanMDAR, FanGPX, FanGR, FanSODM</i>	(Bulley et al., 2012) (Barbey et al., 2020)
<b>Minerals</b>			
<b>Zinc, Iron</b>	Banana	Ferritin from Soybean	(Kumar et al., 2011)
<b>Terpenes</b>			
<b>Carotenoids</b>	Strawberry	<i>FanPSY, FanZDS</i>	(Barbey et al., 2020)
	Citrus spp.	<i>PSY, ZDS, PDS, HYb, ZEP</i>	(Sugiyama et al., 2014)
	Papaya	<i>LYCB2/E, CCD4, NCED2, AAO3, CyP707A1</i> <i>CpCYC-B, CpLCY-B, CpPDS2, CpZDS, CpLCY-E, CpCHY-B</i>	(Jiang et al., 2019) (Zhou et al., 2019)
	Apricot	<i>LOX 2, CCD1, CCD4</i>	(García-Gómez et al.,

	Grapevine	<i>VvDXS, VvDXR, VvGGPPS, VvPSY, VvCCD</i>	2019)
	Banana	<i>PSY2</i>	(Leng et al., 2017)
		<i>LCY-ε (-)</i>	(Paul et al., 2017)
	Kiwifruit	<i>GGPS, PDS, ZDS, CHX</i>	(Kaur et al., 2020b)
	Apple	<i>AtDXR</i>	(Kim et al., 2010)
	Orange	<i>Csβ-CHX (-)</i>	(Arcos et al., 2020)
			(Pons et al., 2014)
<b>Carbohydrates</b>			
<b>Total soluble sugars</b>	Pear	<i>SUS</i>	(Saito, 2016)
	Apple	<i>MdSWEET15a, MdSWEET9b</i>	(Zhen et al., 2018)
<b>Fructose</b>	Strawberry	<i>MdbHLH3</i> <i>PFP from Giardia lamblia</i>	(Yu et al., 2020)
			(Basson et al., 2011)
<b>Sucrose</b>	Apple	<i>A6PR (-)</i>	(Li et al., 2018a)
	Pear	<i>PbSUT2</i>	(Wang et al., 2016a)
<b>Lipids</b>			
<b>Oleic acid</b>	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)
<b>Linoleic acid</b>	Olive	<i>OeFAD2-2</i>	(Sebastiani & Busconi, 2017)
		<i>FAD7</i>	(Hernández et al., 2017)
<b>Antinutrients</b>			
<b>Allergens</b>	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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