Breeding for Nutritional/Organoleptic Quality

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Contributor: Alessandro Natalini

Due to novel and more demanding consumers' requirements, breeding of vegetable crops confronts new challenges to improve the nutritional level and overall appearance of produce. Such objectives are not easy to achieve considering the complex genetic and physiological bases. Overtime, plant breeders relied on a number of technologies and methods to achieve ever changing targets. F1 hybrid seed production allowed the exploitation of heterosis and facilitated the combination of resistance and other useful genes in a uniform outperforming variety. Mutagenesis and tissue culture techniques permitted to induce novel variation, overcome crossing barriers, and speed up the achievement of true-breeding lines. Marker-assisted selection was one of the milestones in fastening selection, starting from the early '90s in almost all seed companies.

vegetable breeding   quality   cauliflower   tomato   transgenesis   cisgenesis

1. Introduction

Vegetable quality improvement is a global requirement due to current evolution of consumers' demands. In particular, producing vegetables with enhanced nutritional and organoleptic quality is one of the most challenging targets for breeding, facing with climatic changes and the needs for a more efficient production system [1]. High throughput metabolomic, transcriptomic and genomic advances represent useful tools to identify genetic architecture and biochemical pathways and also to predict breeding values for selection and deployment [2].

Among vegetables, tomato and cauliflower, cultivated throughout the world, are important dietary sources of phytochemical and bioactive compounds and represent model systems for fleshy fruit and floral edible parts, respectively [3][4]. While advanced genomic and genetic transformation resources are available in tomato for next-generation precise breeding, the availability of such resources in cauliflower is lower and hence breeding methods are essentially conventional, resulting in slow progress, despite recent interesting novel products [5].

2. Evolution of Breeding for Nutritional and Organoleptic Quality

Investigations on the genes involved in the metabolism and detailed studies of the metabolic pathways are fundamental for plant breeders to develop varieties with improved quality. Since the publication of the Arabidopsis thaliana genome in 2000, next-generation sequencing (NGS) has been applied on several crops, other than model plants. NGS technologies evolved in their sequencing approach from BAC-by-BAC-based sequencing methodologies to whole-genome shotgun with longer read lengths and higher quality of genome sequences [6].

After the release of the inbred tomato cultivar Heinz 1706 as reference genome, several cultivated and wild tomato accessions (e.g., *S. pimpinellifolium*, *S. cheesmaniae*, *S. galapagense*) have been resequenced and compared to the reference [7][8][9][10][11][12]. The aim was to create a pan-genome comprising genomes of cultivated tomatoes and their wild ancestor and progenitors [12], in order to have a more complete characterization of tomato gene function and potential. In these studies, genomic variation of the tested accessions compared to the reference was estimated through mapping of short reads. Several (unknown) loci and highly divergent alleles have been identified, including 4873 additional genes that were absent in the reference genome. Among the (unknown) genes, for example, the tomato lipoxygenase C TomLOXC promoter was identified by Gao and co-workers. TomLoxC has been previously selected against during domestication and conventional breeding. TomLoxC contributes to tomato quality in terms of flavour by catalysing the synthesis of lipid-derived C5 and C6 volatiles and also in terms of nutraceutical value being involved in apocarotenoid (carotenoid derived compounds) production [12]. In particular, genotypes heterozygous for the TomLoxC promoter showed the highest expression in orange-stage fruit and QTL mapping identified TomLoxC as a key gene controlling the content of flavour-associated lipid- and carotenoid-derived volatiles. Furthermore, transgenic tomato fruit, with reduced TomLoxC expression, showed unknown non-enzymatic apocarotenoid pathway [12].

In detail, two flavour molecules are geranylacetone and 6-methyl-5-hepten-2-one (MHO) belonging to the family of apocarotenoids. In GWAS study, Tieman and co-workers [10] identified one associated locus for MHO, four loci for geranylacetone and two loci for both compounds. Comparing the frequency of alleles in wild species and modern accessions, the authors found that allelic combinations for geranylacetone were lost along domestication while two alleles for MHO content were positively selected. In other terms, over cycles of selection, breeders increased MHO levels by enhancing lycopene content and the MHO precursor as well [1][10].

The combination of metabolomics, transcriptomics, and mGWAS is a comprehensive approach to understand the fruit metabolic, and quality changes occurred during domestication and plant breeding [1][13][14]. The QTL approach in biparental populations revealed the genetic architecture of traits related to fruit quality. Nonetheless, QTL mapping does not consider the entire genetic diversity present in germplasm collections; therefore, the GWAS strategy has been used in several instances. Indeed, GWAS allows the screening of a wide range of accessions in order to understand the inheritance of important fruit metabolic traits [1][15][10][14]. GWAS has been performed in hundreds of tomato genotypes to identify genes involved in primary metabolism which includes sugars, acids, and other compounds of tomato flavour. For example, Sauvage and co-authors (2014) identified 44 loci for 19 primary metabolic traits [15].

One of the detected loci linked to soluble solid content is lin5 gene, which encodes for a cell wall invertase that was previously identified in a QTL study for fruit sugar content in silenced tomato plants [16]. Moreover, Tieman and co-authors identified, by means of GWAS, two loci on chromosomes 9 and 11, respectively, that control glucose and fructose contents, respectively. The locus on chromosome 9 corresponded to the lin5 gene linked to an extracellular invertase as emerged in QTL mapping of an F2 population, derived from a cross between a big sized flavourful variety and a small sized flavourless inbred line [10]. Interestingly, the same authors also found a SNP for lin5 associated to an amino acid change in the protein sequence at the position 366 as revealed after molecular

investigations and confirmed with transgenic tomato plants overexpressing lin5 variants [10]. As suggested by Pott and co-workers (2021) [1], breeders, selecting for fruit size, have selected the combination of alleles on chromosome 9 and 11 associated with the lowest sugar content.

As previously mentioned, breeding activities have been oriented also to improve colour and increase antioxidant content, introducing, for example, the alleles responsible for anthocyanin biosynthesis in Sun Black tomato. This genotype is a double mutant line for anthocyanins (Aft Aft/atv atv) obtained after 20 years of breeding activities in which wild tomatoes species have been used in interspecific breeding program as source of Aft and atv genes (accessions LA 1996 for Aft and LA0797 for atv) [17]. In detail, Aft is a dominant gene on chromosome 10 encoding an R2R3-MYB transcription factor responsible for the activation of anthocyanin biosynthesis (absent in WT tomatoes) in presence of light. On the other hand, atv, located on chromosome 7, is a recessive mutation of Atv, which encodes an R3-MYB transcription factor acting as repressor of anthocyanin biosynthesis [18][19].

In tomato, other important qualitative traits are fruit shape and size, being positively selected for larger fruited types since domestication and variably shaped fruit according to the consumers' requirements. A milestone review is the publication of Tanksley for reporting genetic and molecular bases of fruit size and shape variation in tomato [20]. Linked to domestication, the key loci controlling fruit size are: fw1.1, fw2.2, fw3.1, and fw4.1 which positively affected the selection for size from wild berries to the modern tomatoes. The above-mentioned loci can indirectly affect fruit shape under the direct genetic control of three other major loci with a minimal effect on fruit size. The loci for shape are located on chromosome 8 (sun and fs8.1) and on chromosome 2 (ovate) [20]. More recently, based on segregating populations obtained by crossing a plum tomato breeding line (NC 30P) and a grape tomato breeding line (NC-22L-1), QTL analysis and linkage mapping revealed the existence of a QTL on chromosome 10 regulating triangle fruit shape and two possible QTLs on chromosome 12 controlling obovoid shape [21].

Parthenocarpy is the fruit set and growth without fertilization and is a common phenomenon in Angiosperms in sub-optimal growing conditions (e.g., low temperature, low solar radiation, high relative humidity). In tomato, parthenocarpy leads to the absence or low number of seeds, which is a desirable trait for sauce production, and it has been demonstrated to affect fruit quality in terms of flavour (pH, total solid content) [22]. A parthenocarpic tomato mutant line is pat2 that has been introduced in the Severianin variety derived from the interspecific cross *S. lycopersicum* × *S. peruvianum* with contrasting results. Indeed, the expression of pat2 gene is affected by the introgression genetic background and by environmental conditions. Extreme daily and night temperatures and adverse radiation and relative humidity increase the parthenocarpy regulated by pat2 gene [23][24][25]. More recently, researchers demonstrated that Aucsia genes (mainly the DefH9-iaaM gene) regulate auxin synthesis and are responsible for parthenocarpic fruits. Two tomato lines (UC 82), transformed with DefH9-iaaM, were (almost) seedless and showed a higher β-carotene level compared to the control [26].

In Brassica genus, omics approaches have been adopted, not only to investigate pathogen resistance and abiotic stress tolerance (high temperature and high salinity) but also to phenotype and characterize biochemical pathways [27][28][29]. Metabolomic approaches have been used to target primary and secondary metabolites, mainly glucosinolates. These compounds are constituted by a sulphur-linked β-D glucopyranose and an amino-acid

derived chain [28]. Park and co-workers correlated the amount of carotenoid, anthocyanin and phenolic acids to phenotypic variation and metabolic networks of several accessions for purple, white and green coloured cauliflowers [30]. Cauliflower breeding activities were focused on providing hybrids with high carotenoid content [31]. Interestingly, a crossbred between a cytoplasmic male sterile line (marketable white cauliflower) and an inbred line (orange mutant line with small curds) resulted in a marketable orange F1 with high nutritional quality. Indeed, the hybrid showed a 10.8 times higher β-carotene content than the parental white cauliflower [31].

RAPD, ISSR, and AFLP molecular markers have been applied to identify genetic diversity among several cultivars of cauliflower, cabbage, and broccoli [32]. Other useful applications for breeding purposes were the possibility to identify gene introgression among varieties and genetic relationships based on maturity group [32]. Another important milestone in cauliflower is the work of Zhao and co-authors [33]. A specific locus amplified fragments (SLAF) sequencing was used to identify large scale nucleotide polymorphism (SNP) and a high-density genetic map using a segregating population obtained by microspore culture. In this experiment, to design markers, the reference genome of the cabbage *B. oleracea* var. capitata (http://www.ocri-genomics.org/bolbase/ (accessed on 28 April 2021)) has been used. This research identified 81,311 SLAFs, 6815 of which showed polymorphisms between the parents of the segregating population. Interestingly, the distribution of the SLAFs markers was quite homogenous, throughout the reference genome, and therefore, the markers could be usefully applied for breeding purposes [34].

As reported by Zhao and co-authors (2016), genomic studies on *B. oleracea* used, in the past, parental material belonging to different subspecies to develop an intra-specific segregant population in order to identify QTLs, developing genetic maps and markers [33]. As a consequence, the application in terms of breeding was quite limited because the advances did not cover diverse genetic background referred to specific plant organs (e.g., curd in white cauliflower). Indeed, using intra-group parental lines, it has been possible to identify, for example, 13 QTLs associated to the curd-specific traits in white cauliflower [33][35].

As previously reported, the most important system for hybrid seed production is based on Ogura cytoplasmic male sterility, derived from radish and transferred to cauliflower in 1968 by back cross [36]. Dey and co-authors (2017) investigated the influence of Ogura cytoplasm on several qualitative traits related to antioxidant properties [37]. Since the radish chloroplasts of the original Ogura-based cytoplasm showed a negative interaction in the nuclear background of *B. oleracea*, the authors investigated if the substituted chloroplast (through somatic hybridization followed by backcross substitution) Ogura cybrid cytoplasm could still have adverse interaction with nuclear genes in cauliflower. The improved Ogura cybrid cytoplasm was obtained by protoplast fusion and regeneration in several Brassica species [38]. Interestingly, Dey and colleagues evidenced that Ogura cytoplasm is responsible of a reduction in anthocyanin, total chlorophylls, and ascorbic acid content in CMS lines compared to corresponding cauliflower inbred lines. On the other hand, CMS lines showed higher total carotenoid and β-carotene contents compared to inbred lines. This work evidenced that the effect of Ogura cybrid cytoplasm in different nuclear backgrounds is genotype-specific and the possibility to explore in detail the relevant metabolic pathways could fasten breeding to develop improved varieties for quality.

3. Biotechnological Approaches: From Transgenesis to Genome Editing

The combined use of recombinant DNA technology, gene transfer methods, and tissue culture techniques substantially prompted the use of genetic engineering and transformation technologies in vegetables, both in public and private breeding programs, in accordance with local legislative restrictions. Transgenesis has the big advantage to transfer a single-gene without co-transfer undesirable genes unlike conventional breeding based on crossing two parental plants and subsequent back-cross cycles [39].

Among gene transfer methods, that based on Agrobacterium has been widely used on tomato and, to a lesser extent, on cauliflower. Tomato is one of the most used crops for genetic transformation because, due to its features, is often used as a model plant: 24 somatic chromosomes, a relatively small genome size (950 Mb per haploid nucleus), short generation time, easily reproduced by seed and by vegetative propagation, and relatively easy transformation protocols [40].

The first commercial release of recombinant DNA technology was the Flavr Savr tomato, also known as CGN-89564, approved by the Food and Drug Administration (Silver Spring, MD, USA) in 1994. That variety had a remarkable shelf-life due to the introduction of an antisense polygalacturonase gene, but it had also a bland taste, and it was dropped and removed from the market in 1996 [41][42]. In the second half of 1990, tomato Huafan No 1 was released in China; the variety 351N was constituted in the US by Agritope Inc. (Portland, OR, USA) while Monsanto (St. Louis, MO, USA) released 8338 and 5345 and DNA Plant Technology Corp. (Cinnaminson, NJ, USA) commercialized 1345-4 in USA, Japan, Mexico, Canada [42]. The above-mentioned varieties were transformed to increase shelf-life using different gene targets: antisense for polygalacturonase or antisense and gene suppression for enzymes belonging to the ethylene biosynthetic pathway [43]. Subsequently, transgenic tomatoes for improved starch biosynthesis, pectin, and sugar metabolism have been produced, as reported by Barg and co-authors [43].

In around 30 years of tomato genetic transformation, carotenoid and phenolic pathways have repeatedly been the subject of single and multi-gene engineering, as reviewed, for example, in Rosati and co-workers (2000) [44], Long and co-authors (2006) [45], and Giuliano (2014) [46]. Increase in β-carotene synthesis could be achieved by nuclear as well as chloroplast transformation [44][47]. The latter has remarkable advantages: no epigenetic effects leading to gene inactivation, high expression level, possibility of multiple transgene expression in synthetic operons and, being the plastids maternally inherited, reduced risk of transgene dispersal by outcrossing. Wurbs ad co-authors (2007) developed and applied a chloroplast transformation system that introduced in a panel of transgenic and mutant tomato linesa bacterial lycopene β-cyclase gene and led to a fourfold increase in pro-vitamin A content by converting the lycopene to β-carotene [47]. Subsequent investigations on plastid transformation, using both bacterial lycopene β-cyclase and a plant (Narcissus pseudonarcissus) lycopene β-cyclase, demonstrated a different action in tomato plants [48]. Indeed, the bacterial enzyme increased the amount of pro-vitamin A, but did not change carotenoid composition compared to WT. Furthermore, plant lycopene β-cyclase induced (similarly to

the bacterial gene) a conversion of lycopene to β-carotene up to 1 mg/g dry weight of fruit and increased the total amount of carotenoids accumulated in the fruit, suggesting a role as regulatory enzyme [48].

Anthocyanin and flavonoid have also been investigated with transgenic techniques. Orzaez and co-authors (2009) reported that a transgenic tomato line (Del-Ros1) expressing Antirrhinum majus Delila (Del) and Rosea1 (Ros1) transcription factors showed a purple colour and an anthocyanin-rich phenotype [49]. Further investigations demonstrated that Del and Ros1 transformed tomatoes under the control of the fruit-specific E8 promoter produced higher anthocyanins contents than WT but equal to the content in blackberries and blueberries levels. The above-mentioned transgenes enhanced the hydrophilic antioxidant capacity of transformed tomato and resulted in purple colouration of both peel and flesh [50]. Furthermore, both Aft/Aft atv/atv and Ros1-Del transformed purple tomatoes showed, besides an improvement in their nutraceutical value, extended shelf life and lower susceptibility to the postharvest fungus Botrytis cinerea compared to normal tomato [51]. MicroTom plants were transformed using the promoter of the PLI gene because it is a light induced gene, and it is active mainly at skin level. The promoter was able to lead the expression of MYB transcriptional factor Rosea1 together with 35S Delila. The authors suggested that Aft/Aft atv/atv tomatoes and the transgenic tomato lines possess longer storage capability compared to WT due to delay in over-ripening. The higher content in anthocyanins at skin level increases the antioxidant activity and indirectly reduces pathogen spread [51].

Scientific attention has been addressed to stilbene family and mainly to resveratrol, a typical bioactive compound naturally presented in grape (for a review see Giovinazzo et al., 2013) [52]. Giovinazzo and co-authors (2005) produced transformed tomato for a stilbene synthase (StSy) transcriptionally regulated by the cauliflower mosaic virus (CaMV) 35 S promoter [53]. Trans-resveratrol and trans resveratrol-glucopyranoside were produced in tomato tissues and resulting in an increase in ascorbate and glutathione. As a consequence, the total antioxidant level increased compared to WT, but the basal level of tocopherole and lycopene did not change [53].

Using a multiplex approach for metabolic engineering, single, double, triple, and quadruple mutants were obtained, which showed, in comparison with wild type, a higher level of both lycopene and β-carotene. Mutants with homozygous and biallelic mutations could be readily used in breeding. The pink colour of fruits, a trait particularly appreciated in Asian countries, depends on the absence of yellow-coloured flavonoid naringenin chalcone (NarCh) in the fruit peel. Deng and colleagues (2018) reproduced the well-known y mutation, disrupting the SlMYB12 gene by CRISPR/Cas, changing the fruit colour of four elite inbred lines from red to pink [54]. The obtention of homozygous and biallelic mutations already in an elite background made the mutants readily usable. In a proof-of-concept study, Cermák et al. (2015) [55] showed the possibility to accumulate anthocyanin in different tissues of the tomato plant by substituting, through genome editing-driven homologous recombination, the promoter of the MYB transcription factor ANT1, being MYB (MYB–bHLH–WDR protein) complexes responsible for the transcriptional control of the flavonoid biosynthesis pathway [56][57][58].

Increasing the level of the non-proteinogenic amino acid GABA (γ-aminobutyric acid) in tomato fruits could help control blood pressure in hypertensive individuals. The actual level of GABA depends on the function of biosynthetic GAD genes, which have a C-terminal autoinhibitory domain, and on that of genes involved in

catabolism (GABA-T and SSADH). In two independent studies, the GABA content in fruits was increased up to 19-fold that in controls by deleting, by CRISPR/Cas9 technology, the autoinhibitory domain of SlGAD2 and SlGAD3, regularly expressed during fruit development [52], or by a multiplex genome editing approach on five genes (GABA-TP1, GABA-TP2, GABA-TP3, CAT9, and SSADH), involved in GABA metabolism [59]. The "Sicilian Rouge" High GABA variety, edited using the former approach, will be the first genome editing product to be released in Japan (http://www.tomatonews.com/en/japan-breeders-launch-genome-edited-tomato_2_1236.html) (accessed on 28 April 2021).

A range of fruit metabolic phenotypes were obtained by Gago et al. (2017), who adopted the ZFN technology to disrupt the L1L4 gene, encoding one subunit of the heterotrimeric transcription factor Y [60]. In comparison with wild type, fruits of different edited lines showed an increase of some "positive" metabolites (e.g., SSC, fiber, fructose, ascorbic acid, total phenol, β-carotene) as well as a reduction in "negative" ones (e.g., oxalic acid). A "visionary" and futuristic objective was proposed by Rezende Naves and colleagues (2019), who suggested to activate pungency biosynthesis in tomato fruits in order to produce capsaicinoid-accumulating 'Hot' Tomatoes [61]. According to them, that objective could be achieved by the use of transcriptional activator-like effectors (TALEs) and the targeted replacement of promoters of relevant genes.

Organoleptic and nutritional traits in tomato fruits vary dramatically during ripening, which is a multidimensional process involving hundreds of genes with expression regulated by the hormone ethylene, ripening transcription factors, degree of DNA methylation, and several post-transcriptional processes (long non-coding RNAs, RNA-editing of mitochondrial transcripts). Genome-editing approaches in tomato, mostly based on CRISPR/Cas technology, allowed to generate new mutants and pinpoint the role of factors cited above [12][62][63][64][65][66]. The production of novel CRISPR/Cas mutants in genes coding for master regulators, such as RIN, NOR, and CNR, suggested new models of the ripening process, due to the different phenotypes obtained by genome editing compared to the original ones. Thanks to the novel mutants induced by CRISPR/Cas, a clearer picture is now available for rin, once thought to be a loss-of-function mutation, but now considered a gain-of-function one [67][68][69][70]. Those results might have an impact on breeding, since the use in commercial hybrids of the classical rin mutation in heterozygous state (Rin/rin) often resulted in poor flavour and reduced nutritional value [70]. CRISPR/Cas-based genome editing was also used to study the effect of genes involved in fruit softening during ripening. With the aim to slow down the softening process without altering colour, soluble solid content and other aspects of ripening, those studies highlighted the role of PL, encoding a pectate lyase, and the possibility to use such a mutant in tomato breeding [63][71][70]. A result with great potential for tomato breeding was obtained by Yu and colleagues (2017), who, using CRISPR/Cas, introduced viaHDR the mutation alcobaca (alc), responsible for long shelf-life, in a normal background. Fruit colour and other agronomic traits were unaffected [72].

Finally, in a recent paper, *S. pimpinellifolium*, the wild closest ancestor of cultivated tomato, was de novo domesticated by editing simultaneously few major genes. Among them, editing of Lycopene Beta Cyclase (CycB) resulted in 500% improvement of fruit lycopene content compared to *S. lycopersicum* [73].

In cauliflower, Nugent and co-authors (2006) reported nuclear and plastid transformation using PEG-mediated uptake of DNA into protoplasts. Cauliflower has been also transformed using *A. tumefaciens* with isopentenyl transferase (ipt) gene under the control of a senescence-associated gene promoter, pSAG12, isolated from *A. thaliana* [74]. The pSAG12:ipt gene was evidenced to be responsible for delay in leaf senescence, altered synchronous curd initiation, smaller curd size, and higher susceptibility to fungal infection [75]. More recently, Kowalczyk et al. (2018) developed a high efficiency cauliflower transformation method using Rhizobium (=Agrobacterium) rhizogenes in hypocotyl sections of 10-day-old de-rooted seedling [76]. Zhou and co-workers (2008) isolated the Or gene from orange cauliflower and introduced the transgene in potato revealing that the gene controls carotenoid accumulation by inducing the formation of chromoplasts [77].

By contrast with tomato, no reports for genome editing in cauliflower are available, although some enabling studies have been carried out in other vegetable Brassicas [78][79][80][81][82].

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