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Review Article

Applications of molecular markers in strawberry

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Abstract. This review examines current and potential applications of molecular markers in strawberry (*Fragaria* spp.). The most prevalent uses of markers in strawberry are for analyses of genetic diversity, genetic mapping, and cultivar identification. Cultivar identification is by far the most important use of markers to date for the protection of intellectual property as well as to confirm the identity of nursery stock through multiple cycles of asexual propagation. These applications have been accelerated by continuous discovery of simple sequence repeat markers and the development of multiplexed sets. Mapping studies have confirmed the allopolyploid genome structure of cultivated strawberry and shed light onto genetic events that punctuate its domestication. Though marker-trait associations have been identified for disease resistance, photoperiodic flowering, and sex expression, marker-assisted selection in breeding is currently confined to a few entities in the private sector. Pedigree-based analysis holds promise for identifying additional marker-trait associations and simultaneously mining alleles in multiple genetic backgrounds. Meanwhile, the completion of the diploid strawberry genome sequence promises to accelerate candidate-gene approaches for marker discovery.

Keywords: Candidate gene, genetic diversity, genetic mapping, marker-assisted breeding

1. Introduction

The cultivated strawberry, *Fragaria*×*ananassa* Duchesne ex Rozier ($2n = 8x = 56$), is widely grown throughout the world. Historically it has been utilized as a perennial crop for temperate regions; however, in the last century the strawberry has been adapted to annual production systems and to Mediterranean and sub-tropical growing regions through breeding and cultural manipulations. It is also a species with a fascinating story, originating in the mid-18th century through chance hybridization in European gardens between its octoploid progenitors *F. virginiana* (L.) Miller subsp. *virginiana* collected from North America and *F. chiloensis* Miller subsp. *chiloensis* collected from South America [19]. The vigor of this hybrid was captured by ensuing generations of plant breeders who transformed it into the large-fruited *F.* x *ananassa* that we enjoy today. Excellent nutritional quality and consumer appeal have sustained and grown the market demand for strawberry fruits. Industry and academic personnel are attempting to keep pace with this demand through genetic improvements, advances in production practices, and improved post-harvest handling and are using the many technologies at their disposal.

One of the most important and promising tools for strawberry research and the strawberry industry is that of molecular markers. The development and use of DNA markers and biotechnology in strawberry has been addressed in full or in part by several authors beginning with Hokanson and Maas [42] as well as more recent treatments [29, 63]. Research in this area is progressing rapidly and is expected to accelerate still further since the recent completion

of the genome sequence for the woodland strawberry, the diploid *F. vesca* [68]. Part of the purpose of this article is to present an updated synthesis on DNA marker development. However, the main orientation of this review is toward application, particularly uses of markers that relate to genetic improvement of strawberry. Markers are used in strawberry for diversity and taxonomic characterization, intellectual property protection, mapping and genome analyses, and marker-assisted breeding. These applications, among others, will be discussed and concluded with a treatment of the future of marker technology in strawberry and how this relates to the genetic improvement of this important agricultural crop.

2. Genetic diversity and population structure

The immediate progenitors of the cultivated strawberry, the octoploid species *F. virginiana* and *F. chioloensis*, are abundant in the wild throughout the Americas. *Fragaria chiloensis* is distributed in pacific coastal regions from southern Chile to Ecuador in the southern hemisphere and from central California to British Columbia in the northern hemisphere as well as in Hawaii. By contrast, *Fragaria virginiana* is distributed in the eastern United States and westward to the Rocky Mountains, as well as in southern Canada and Alaska. Both octoploid species are fully inter-fertile with each other and with the cultivated form, and are therefore part of the primary germplasm pool. Understanding these natural populations is important for germplasm characterization and determining breeding utility, and molecular markers have been a useful tool toward this end. Random marker systems such as amplified fragment length polymorphism (AFLP), inter-simple sequence repeat (ISSR), and random amplified polymorphic DNA (RAPD) markers have been used to estimate genetic relationships among strawberry genotypes, with RAPDs perhaps agreeing best with pedigree relationships [24, 25, 52]. The earliest studies of taxonomy and diversity relationships in strawberry were conducted mainly with RAPD markers; though they are poorly reproducible between labs, they have been used to draw general conclusions regarding the structure of wild populations. Porebski and Catling [57] used 12 RAPD primers to determine that North American subspecies *F. chiolensis* subsp. *pacifica* and subsp. *lucida* separated clearly from the South American subspecies *F. chioloensis* subsp. *chiloensis* but were hardly distinguishable from one another at the molecular level. Harrison et al. [38] combined RAPD marker and morphological evaluations to determine that *F. viriginana* subsp. *platypetala* (western Cascade mountains and Olympic Peninsula) is more closely related to North American *F. chiloensis* than to *F. viriginiana* subsp. *virginiana* (east of the Missouri river) and subsp. *glauca* (eastern Rocky Mountains and Black Hills), a relationship not previously determined using morphology alone. A follow-up study, again with RAPD markers, showed that markers distinguished less among the major provenances of North American *F. virginiana* and instead discriminated more among populations within provenances and plants within populations [39], a trend that also holds for *F. chiloensis* accessions from Chile [14]. The authors proposed that for purposes of native germplasm collection and preservation, morphological markers should be used to determine and sample distinct taxonomies and that ecogeographical considerations should help further refine collections. Finally, molecular marker data can help determine the sampling strategy for collections, assuring that enough genetic diversity is present to preserve needed traits. This is important, as it has been demonstrated that genetic architecture of *F. virginiana* populations can vary dramatically due to spatial variations in clonal vs. sexual reproduction [83].

Hokanson et al. [43] built on these studies by evaluating 111 individuals representing all major subspecies and provenances of *F. virginiana* and *F. chiloensis* as well as representative *Fragaria*×*ananassa* cultivars using simple sequence repeat (SSR) markers, which have excellent discriminatory power for diversity analyses [35]. Their data supported the long-held view that the cultivated species derived its *F. chiloensis* background mainly from South American accessions. Interestingly, there was greater allelic diversity in the North American subspecies, suggesting that the South American subspecies were originally derived from North American ancestors. Though there is certainly untapped molecular and phenotypic diversity among South American *F. chiloensis* [7, 14, 36], breeders in search of new germplasm should not overlook North American accessions of *F. chiloensis*. These are timely discoveries, as the narrow genetic base of the cultivated strawberry has been trumpeted for two decades [69] and breeding activity with wild octoploid accessions is enjoying a renaissance [37]. An SSR analysis of 92 strawberry cultivars of diverse origin showed a slight but steady increase in genetic similarity over time based on the decade of cultivar release [33]. In particular, cultivars bred in California/Mediterranean climates had the highest genetic similarity and smallest number of alleles, reflecting inbreeding due to selection within those environments. Reductions of genetic diversity

are expected in the course of breeding, especially within a crop so recently domesticated, but should be carefully monitored using marker technology to ensure that targeted infusions of new alleles are made where and when needed.

3. Cultivar identification

At present, the largest and most widespread use of molecular markers in strawberry is for accurate identification of strawberry genotypes. This is particularly important for preventing misidentification of varieties during nursery propagation, accurately identifying accessions in germplasm repositories, and protection of intellectual property (IP) by strawberry breeders. Initially, random marker systems such as AFLP, ISSR, and RAPD were used for this purpose. Codominantly-scored SSR markers as well cleaved amplified polymorphic sequence (CAPS) markers and their derivatives were later developed from DNA sequence information. Over time, SSR markers have become the preferred marker type for genotype identification in the public and private sectors.

The first important case of IP protection using markers is recorded by Congiu et al. [17] who used RAPD markers to settle a lawsuit regarding unlawful commercialization of a patented strawberry variety. The markers were reproducible enough to be accepted as evidence in court. However, template concentration introduced variability in the banding patterns and had to be carefully monitored. Random marker systems such as AFLP, ISSR, and RAPD have all been used to distinguish among genotypes [3, 24, 31, 47, 75]. However, with increased availability of genomic libraries and expressed sequence tag (EST) databases, SSR markers from wild and cultivated sources have been rapidly developed and adopted for multiple applications [4, 20, 30, 45]. Development of SSR markers will not be limited by a lack of available SSR loci, as a preliminary analysis of *F. vesca* sequences indicates an average density of one SSR per 5 kb [70]. Lewers et al. [50] developed sets of SSRs derived from GenBank and from a genomic library that were highly transferable across *Fragaria* spp. and even had some limited transferability to *Rubus* spp. (raspberry and blackberry). At the writing of this manuscript, 59,800 *Fragaria* ESTs had been deposited in GenBank and are an increasingly useful source for SSR marker development. Over 7,000 unique ESTs from cultivated strawberry have now been published [9]. Thirty-two SSR primer pairs were developed from 'Strawberry Festival' ESTs that were highly polymorphic among 70 cultivars, with an average of 16 alleles per primer pair [5]. These were also highly transferable among *Fragaria* spp., with 100% transferability to *F. virgininana* and *F. chiloensis* and 89% transferable to the diploid species *F. vesca*. While most strawberry SSRs contain di-nucleotide repeats, some contain tri-, tetra-, and even penta-nucleotide repeats [5, 9]. Greater repeat-lengths are desirable because they allow greater size separation among alleles, as well as a reduction in "stutter" bands so commonly associated with SSRs made up of di-nucleotide and even tri-nucleotide repeats. Other sets of SSR primers sets have been highly-transferable among species and across ploidy levels, whether from diploid to octoploid or vice versa [15, 16, 32, 35, 53]. In Japan, SSRs were developed for the express purpose of protecting the IP rights of Japanese strawberry breeders. Four primer pairs from a genomic library of 'Toyonaka' successfully distinguished between 10 important Japanese cultivars [67].

Cleaved amplified polymorphic sequence (CAPS) markers are PCR-based (followed by restriction enzyme digestion) and are typically scored on agarose gels, making them inexpensive and straightforward. Kunihisa et al. [46] surveyed available nucleotide sequences for restriction sites and designed primers to amplify 400–650 bp regions around these sites. The initial PCR using DNA from Japanese cultivars amplified multiple homoeologous alleles, making banding patterns difficult to interpret. To solve this problem, the PCR products were sequenced and subjected to a cluster analysis to identify groups of similar sequences. New primer pairs were chosen to target unique sequence clusters. The resulting CAPS markers amplified from only one subgenome, which was confirmed by diploid inheritance ratios in a test population. Nine improved CAPS markers were used to distinguish between a set of 64 cultivars, demonstrating the usefulness of these markers for cultivar identification.

Gene pair markers could be considered a type of CAPS marker that targets regions between genes of known sequence. The technique was demonstrated in diploid *Fragaria* by designing primer pairs from exons of the adjacent *CDPK1* and *BHLH1* genes [21]. Restriction enzymes were then used to digest the PCR products to generate polymorphic bands. Gene pair markers for this locus were successfully designed from *Rosa*, *Prunus*, and *Rubus* as well, demonstrating the potential of these markers for comparative mapping. There is also potential for use in octoploid strawberry by identifying multiple polymorphisms in each intergenic region which may be associated into haplotypes. The gene pair marker technique relies on the presence of conserved coding regions flanking intergenic

regions that are rich with polymorphisms. The target genes must also be located at relatively short distances from one another, which is often the case in *Fragaria* which possesses a relatively small base genome of approximately 200 Mb [29, 68].

Ruiz-Rojas et al. [59] pursued a creative strategy to identify single nucleotide polymorphisms (SNPs) adjacent to T-DNA insertions in two insertional mutant lines of *F. vesca* and then created CAPS markers to target the polymorphisms. Regions flanking the T-DNA insertions were amplified by High-Efficiency Thermal Assymetric Interlaced PCR (hiTAIL-PCR) and sequenced to discover SNPs. Primers were designed to flank the polymorphisms and restriction endonucleases were chosen that cleaved the site of the SNP in one of the two parents of a diploid mapping population. Derived CAPS (dCAPS) markers were also created where there was no actual restriction site by designing primers that introduced mismatches in the sequence flanking the restriction site during PCR amplification, thereby creating a recognition site for the enzyme to cleave. Mapping these loci showed that the T-DNA insertions occurred close to several presumed strawberry genes [59].

The abundance and transferability of SSR markers across species, combined with high allelic variability and co-dominant scoring, have made this the marker of choice for genotype identification. With over 400 SSR primer pairs developed for *Fragaria* spp. [55, 71], the next logical step was to identify small sets of markers that are the most reproducible across labs and genotyping platforms and can be multiplexed for high throughput. Govan et al. [34] identified 10 primer pairs that could be combined into three multiplexed sets and demonstrated that these markers could readily and reliably distinguish between 56 cultivars of diverse origin and four octoploid species accessions. Brunings et al. [12] built on this data set by surveying these markers in cultivars and breeding selections relevant to the Florida strawberry industry. The markers were highly effective; as few as two SSRs could distinguish between even the most closely related genotypes, including a parent and its self-pollinated offspring. However, some discrepancies in band presence and band size from the original report of Govan et al. [34] emphasizes the fact that primer pairs should be carefully chosen. The authors also recommended that candidates should be tested side-by-side with unknowns under the same reaction conditions and preferably replicated across multiple labs. Most recently, Njuguna [55] identified a reduced set of four SSR markers, three of which were described by Govan et al. [34], to differentiate between strawberry accessions at the National Clonal Germplasm Respository (Corvallis, Oregon). This SSR set differentiated an impressive list of 187 accessions spanning 22 *Fragaria* species. The initial marker screen demonstrated the practical importance of this tool, since potential misidentifications and duplications were discovered in the NCGR collections as a result of the marker screen.

The importance of cultivar identification as an application of molecular markers in strawberry is evidenced by the widespread use of this approach in the public and private sector. Researchers at Foundation Plant Services at the University of California-Davis utilize previously published SSRs [50] to distinguish cultivars and breeding selections relevant to the California strawberry industry [18]. However, private labs handle the bulk of genotyping needs for the industry in the major growing regions of the world. California Seed & Plant Lab processes approximately 600 strawberry samples per year from the United States and 300 samples from other countries including Canada, the UK, Mexico, Australia, and France [P. Randhawa, personal communication]. They also currently utilize published SSR primers, primarily those from Lewers et al. [50]. Approximately 80% of the samples sent to this lab are submitted by nurseries and 20% are submitted by breeding programs. Since strawberries are clonally propagated at least four generations from tissue culture to finished plant, nurseries are highly motivated to ensure that they are selling true-to-type stock. The nursery industry serving the Queensland Australia growing region is particularly proactive in the use of markers, using private labs to test nursery stock at all stages of propagation [L. Ko, personal communication].

4. Linkage mapping and genome structure

Linkage mapping in strawberry has been much more than an academic exercise. Genetic mapping studies have answered important questions about the structure and behavior the diploid and octoploid strawberry genomes and have resulted in the development of many useful markers for other applications. The first published linkage map in strawberry was constructed using an F_2 population from an intra-specific cross of diploid *F. vesca*. The expected seven linkage groups were resolved using RAPD markers [22]. A unique feature of this initial map was that it

contained a large number of codominant RAPD markers. A helpful attribute of these markers was that a non-parental heteroduplex band was observed in addition to the two homoduplex parental bands in heterozygous individuals. It was previously demonstrated that the heteroduplex band can also be generated by mixing DNA of the two parents in the same PCR reaction, a technique referred to as "template mixing" [23]. Screening primers with this technique facilitated the identification of codominant markers for this mapping population. Furthermore, mixing parental and progeny DNA aided the scoring of some markers in which the two parental alleles were difficult to distinguish because the fragments had similar gel mobility.

A second diploid map, now utilized as the reference map for diploid strawberry, was based on a *F. vesca* × *F. bucharica* (previously misidentified as *F. nubicola*) inter-specific cross and is comprised primarily of SSR markers [61]. Development of the FV × FB map (sometimes still referenced as the FV × FN map) has continued unabated, and a bin-mapping strategy was recently employed to further saturate this map with SSR markers from both coding and non-coding regions of the genome [60]. The map was enlarged to 296 markers, including 270 SSR markers and 22 gene-specific markers. The reference map serves as an important source of transferable markers and provides a framework for mapping at the octoploid level. Since the publication of the bin-mapping strategy, additional markers have been added. A total of 320 markers were used to anchor the *F. vesca* genome sequence assembly to the FV × FB map [68]. Of the 272 *F. vesca* sequence scaffolds, 131 were anchored to the map using these markers. An additional 70 sequence scaffolds were anchored to the map using single nucleotide polymorphism (SNP) markers. These markers were generated by Illumina sequencing of restriction digested DNA of the bin set seedlings [68]. In total, approximately 94% (198 Mb) of the entire scaffold sequence was related to the reference map.

The first linkage map in cultivated strawberry was developed by Lerceteau-Köhler et al. [49] by analyzing single-dose markers in a population of diverse parentage ['Capitola' × CF1116 ('Pajaro' × ('Earliglow' × 'Chandler'))]. They resolved 43 linkage groups in the female parent and 43 in the male parent, which was significantly greater than the 28 linkage groups expected for each. Though incomplete, this map served as a starting point for further studies of octoploid chromosome behavior. By analyzing ratios of marker loci in coupling phase vs. repulsion phase, the authors raised the possibility of both disomic and polysomic allele segregation at meiosis. Cytological studies indicate that there could be up to four distinct diploid contributors to the octoploid genome [10], but this subject is still very much under debate. An allopolyploid origin implies pairing within ancestral genomes at meiosis (autosyndetic pairing), which has been indicated by observations of disomic marker segregation ratios in earlier studies [4, 77]. Further refinement of the map of Lerceteau-Köhler et al. [49] combined with comparative mapping using the FV x FB diploid population showed that allele segregation is, indeed, mainly disomic [58]. Furthermore, this study showed high levels of colinearity between the homoeologous linkage groups in the cultivated strawberry and their corresponding diploid linkage groups, indicating that there were no major chromosomal rearrangements during the polyploidization events that gave rise to the octoploid progenitor species and ultimately to *F.* × *ananassa*. A parallel mapping study of a diploid wide cross between *F. vesca* and *F. viridis* also showed no major translocations or other rearrangements disrupting marker order between the species [54]. Indeed, it appears that there is much promise for comparative mapping within and across ploidy levels in *Fragaria*.

These general conclusions about genome structure and chromosome pairing were confirmed by additional octoploid maps using different populations. Sargent et al. [62] resolved 69 linkage groups using the 'Redgauntlet' × 'Hapil' mapping population and found no evidence of polysomic behavior. In terms of transferable SSR and gene-specific markers, this map is one of the most comprehensive to date. In addition, at least one marker from each linkage group could be mapped to the FV × FB diploid map. Spigler et al. [71, 72] were able to develop a high-resolution SSR map of *F. virginiana* that resolved essentially 30 linkage groups in each of the male and female maps. With the exception of one linkage group, all were assigned to one of seven groups which consist of four homoeologous chromosomes each. Furthermore, all homoeologous groups could be associated with their corresponding homoeolog on the diploid reference map.

Now that the allopolyploid nature of the cultivated strawberry is established, breeders and geneticist are able to interpret segregation ratios accordingly, without reference to the complexities of autopolyploid inheritance. Furthermore, many single-dose markers are now available to target the individual sub-genomes, helping navigate the allelic richness of the octoploid species. It is also hoped that these mapping resources will ultimately result in markers linked to traits of interest, which is the topic of the next section.

5. Markers linked to traits of interest

In order for markers to be useful for genetic improvement, marker linkages to important traits, whether major genes or quantitative trait loci (QTL), must be established. In strawberry, several such associations have been found for traits such as disease resistance, photoperiodic flowering, and male and female sterility. These markers have been developed using a variety of methods, including traditional mapping, bulked-segregant analysis (BSA), and candidate gene approaches.

5.1. Disease resistance

The most significant progress has been made in the area of disease resistance. The first marker-trait association in strawberry was for the *Rpf1* locus, a single dominant gene conferring resistance to *Phytophthora fragariae* var. *fragariae*, the causal agent of red stele root rot [40]. Resistance was originally assumed to be polygenic, but the inoculation of isolates to a differential set showed that resistance behaves according to a gene-for-gene model and that at least five different genes are important in resistance [78]. A BSA approach was employed using an F_1 population from the cross Md683 (Rr) × 'Senga Sengana' (rr), segregating 1:1 for resistance. Seven RAPD markers linked to the *Rpf1* locus were identified, the closest of which was 3.0 cM from the gene [40]. Since *Rpf1* was known to confer resistance to at least 16 races of the pathogen and since Md683 was widely used as a source of red stele resistance in US, Canadian, and Scottish breeding programs, these markers held promise for widespread application.

In order to facilitate marker-assisted selection (MAS), SCAR markers were developed from the original RAPD markers [41]. Interestingly, the SCAR-$R1_A$ marker, which is linked with *Rpf1* in coupling, was developed from a RAPD marker (OPO-16C) linked in repulsion. Though both markers are dominantly scored, their simultaneous use would allow the breeder to distinguish heterozygotes. However, care must be taken not to assume that the marker linked in repulsion will be associated with susceptibility in other germplasm. The SCAR-$R1_A$ marker correctly predicted resistance in many cases when applied to a broad range of germplasm. Since assessing red stele resistance phenotypically is time and resource-intensive, is influenced strongly by the environment, and can be complicated by epistatic effects among several resistance genes, these markers are potentially very useful.

Unfortunately, at present there are no reported instances of the use of these markers in breeding. The main reason is that this gene alone is not sufficient for an effective resistance. In Europe, an effective resistance requires the simultaneous presence of resistant alleles at the *Rpf1*, *Rpf2*, and *Rpf3* loci [78, 79, W.E. van de Weg, personal communication]. Likewise, in the United States, cultivars released from the USDA Beltsville breeding program for resistance to *P. fragariae* carried *Rpf1* in combination with *Rpf2*, *Rpf3*, or both [78]. Furthermore, the linkage between SCAR-$R1_A$ and *Rpf1* was broken in the development of the resistant 'Stelemaster' [41], which is an important founder in some breeding programs [W.E. van de Weg, personal communication].

The second major marker-trait association for disease resistance was for *Rca2*, a single gene for resistance to *Colletotrichum acutatum* pathogenicity group 2, which causes anthracnose fruit rot as well as symptoms on other plant parts including leaves and petioles [27]. Lerceteau-Kohler et al. [48] used BSA to identify makers segregating with resistance conferred by *Rca2* and developed two SCAR markers linked to the dominant resistance allele. The Rca2_417 marker resides approximately 0.6 cM from the locus and is resolvable on polyacrylamide gels, whereas the Rca2_240 marker is located at a distance of 2.8 cM on the same side of *Rca2* but is easily visualized on an agarose gel. Since the escape rate for screening by inoculation (5%) was greater than the recombination rate for Rca_240 (< 3%), this marker was determined to be an excellent candidates for MAS. Of course, a condition for the usefulness of both markers is that they must be both present and linked to resistance in the resistant parents used in breeding; there appears to be a good probability of finding such parents, since one or both markers were found present in the majority of resistant cultivars from various breeding programs [48].

A drawback of these markers is that the *Rca2* gene does not confer resistance to *Colletotrichum acutatum* pathogenicity group 1 [27]. Despite this problem, these markers are being used in a limited number of breeding programs. Ciref-CV, an association of strawberry producers in France, supports breeding efforts in southern France where anthracnose can be a serious problem. Breeders associated with this group use the Rca2 markers to choose parents from among available genetic resources [B. Denoyes-Rothan, personal communication]. The breeding pro-

gram of Driscoll Strawberry Associates employs the Rca_240 marker, plus other *Rca2* markers developed in-house, to screen approximately 20% of their seedlings annually [P. Stewart, personal communication].

Since resistance to *C. acutatum* pathogenicity group 1 is controlled by multiple genes, efforts are underway to identify QTL for resistance [28]. In an F_1 mapping population, the distribution of resistance was approximately normal, and multiple QTL were identified, though none had an effect greater than 12.2%. In the same population, QTL for resistance to *Phytophthora cactorum*, the causal agent of phytophthora crown and root rots, were also identified. No genetic correlation between the two resistances was apparent, and no QTL co-localized. Since phytophthora crown rot is an important malady in the major growing regions of the world, including Australia, California [66], Florida, and Spain, robust markers for resistance could have a broad application.

5.2. Photoperiodic flowering

While most strawberries are short-day (SD), meaning that they initiate flower buds under day lengths <14 h, a smaller number of genotypes are considered everbearing (EB) or day-neutral (DN). In their recent review, Stewart and Folta [73] describe these categories in detail and define EB genotypes as those that require long days (>14 h) to initiate and maintain flower production as opposed to DN genotypes which are truly day-length insensitive. However, they are careful to point out that these categories are variable and overlapping and that factors such as temperature, vernalization requirement, and juvenility strongly influence the expression of photoperiodic traits. In *F. vesca*, the DN trait is given by the presence of two recessive alleles at the seasonal flowering locus (SFL) [2]. Analysis of a BC_1 population with ISSR markers yielded bands segregating with SFL that were successfully converted into SCAR markers, one of which had no recombination with the locus. Unfortunately the diploid model system does not translate directly to the octoploids, where repeat flowering appears to be inherited in a dominant fashion [65, 74].

The major genetic source of DN cultivars in the United States was an accession of *F. virginiana* subsp. *glauca* collected from the Wasatch Mountains in Utah by Bringhurst and Voth [11]. Though the inheritance of this trait has been debated for some time, a segregation analysis using germplasm from University of California-Davis confirmed that this source of DN is conferred by a major dominant locus, with modifying effects from minor genes [65]. Weebadde et al. [82] attempted to examine this question by developing a mapping population from a cross of 'Tribute' (DN) × 'Honeoye' (SD) that segregated approximately 1:1 for the DN trait. Eight QTL were described, the most important of which accounted for 36% of variation. Therefore, in this mapping population, DN may not be controlled by a major locus. However, with greater marker saturation of the map, a major QTL might have been identified. Several of the QTL were location specific, which could be due to the influence of temperature and other environmental factors in the expression of this trait.

Sugimoto et al. [74] examined the basis for the EB trait in a cross between 'Ever Berry' (EB) and 'Toyonaka' (SD) which segregated 1:1 for flowering under long days (16 h). In this case, detection of RAPD markers linked to this trait confirmed the presence of a single dominant locus. The closest flanking markers were mapped to 11.8 and 15.8 cM on either side of the gene, which are unfortunately not close enough to be useful in selection. Stewart and Folta opine that the origin of the remontant flowering trait in 'Ever Berry' is likely from a European source as opposed to the *F. virginiana* subsp. *glauca* source [73], which would further limit the utility of these markers for many breeding programs. However, the origin of this trait in 'Ever Berry' is unclear, and it is possible that the single gene detected in this study could be the same one derived from the *F. virginiana* subsp. *glauca* source.

5.3. Male and female sterility

Cultivated strawberries are hermaphrodites, functioning as both males and females. However, *F. virginiana* and *F. chiloensis* are known to contain functional males (female steriles) as well as females (male steriles). Understanding the genetic nature of this phenomenon is important, particularly when using wild species in breeding. The original genetic model developed by Ahmadi and Bringhurst [1] proposed a single locus with alleles 'F', 'm', and 'h' where femaleness was dominant to maleness and hermaphroditism, and where hermaphroditism was also dominant over maleness. To further examine this question, a genetic map of *F. virginiana* was produced using SSR markers which resolved 42 linkage groups [72]. The map was further saturated using approximately 350 SSR primer pairs to resolve essentially 30 linkage groups in each parental map [71]. The linkage map revealed that, in *F. virginiana*, gender

is determined by two closely-linked loci (one for female sterility and the other for male sterility) with limited recombination between them, leading to the hypothesis that this arrangement represents the early form of a sex chromosome. By comparative mapping, the autosomal homoeolog in diploid *Fragaria* was identified as LG6 [71].

5.4. Structural genes

Additional traits have been characterized and associated with markers in diploid *Fragaria* using candidate gene approaches. Deng and Davis [26] used this strategy to further examine the *c* locus, which in its homozygous recessive form results in yellow fruit color in *F. vesca*. They used degenerate primer pairs to target intron length polymorphisms in structural genes associated with pigment production. One functional marker for flavanone 3-hydroxylase (F3H) was linked with no recombination to the *c* locus, showing that F3H is necessary to produce red colored fruit. Sargent et al. [64] followed suit by targeting additional structural genes including anthocyanidin synthase, cellulase, and pyruvate decarboxylase using a similar approach. Primers were designed within exon sequences flanking polymorphic introns, and functional markers were developed for 29 different loci spanning the seven linkage groups.

6. Marker-assisted breeding

The term marker-assisted breeding (MAB) encompasses various uses of markers related to breeding including clone verification and genetic diversity analyses, as well as marker-assisted parent selection (MAPS) and marker-assisted seedling selection (MASS). In an international email survey by Byrne [13] over 100 fruit and ornamental breeders were queried about their use of markers in breeding. While 50% reported using markers of some type, the majority was for cultivar identification, measuring genetic diversity, or taxanomic analyses. Only 3% of respondents indicated that they currently used markers in parent or seedling selection. The author concluded that the lack of marker use in breeding of these crops is mainly from a lack of genetic information about important traits and a dearth of markers associated with those traits that are both closely linked (preferably flanking) and easily scored. Bassil and Lewers [6] conducted a similar email questionnaire for breeders of crops species in the Rosaceae. Both public and private breeding programs indicated that disease resistance and fruit quality were the two most important categories of traits for which they would use MAS. Identifying suitable parents and matching those parents in ideal cross combinations was cited as a desirable use of molecular markers. However, only two breeding programs, both private, responded that they currently use markers for selection. Though multiple marker-trait associations have been identified in cultivated strawberry, the promise of MAB has not been realized in the public sector and appears limited in the private sector. Other than parent and seedling selection for *Rca2* as previously described, the only other reported use of markers in strawberry breeding is, again, by Driscoll Strawberry Associates for selection of the EB trait using markers developed in-house [P. Stewart, personal communication].

Clearly, in order for the promise of MAB to be realized in strawberry, more easily-scored markers tightly linked to economically important traits must be developed. An example of such an economically important trait is resistance to *Phytophthora cactorum*, causal agent of phytophthora crown rot. This disease is prevalent worldwide, and, like many other root diseases, it is difficult to score phenotypically. Studies have indicated that resistance to *P. cactorum* is controlled by more than one gene and has moderate narrow-sense heritability [28, 66]. However, if one or two major QTL could be identified with sufficiently modest genotype by environment interactions and epistatic effects, these could be useful in breeding for a base level of resistance. For most disease resistance traits, complete immunity is rarely required and the prevention of extreme susceptibility is usually sufficient to maintain the market share of a variety.

The question must be asked, if tightly-linked, flanking, easy-to-use markers were available in abundance for strawberry, would the use of these markers be economically advantageous for breeding programs? After all, other technologies such as precision phenotyping and breeding strategies such as the use of mating designs and clonal replicates could also be used to increase genetic gains over time. In their thorough review, Luby and Shaw [51] break down the efficiencies and costs of MAS for fruit breeding programs, using apple, grape, and strawberry at the University of Minnesota as test cases. They concluded that, on an internal cost basis, MAS was least favorable for strawberry due to shorter generation times and lower costs for seedling maintenance. Nevertheless, for a trait

that is simply inherited and very expensive or difficult to phenotype such as a disease resistance trait, MAS may be economically viable, especially if that trait were to provide external financial benefits through increased share in competitive markets [51]. At present, difficult-to-phenotype disease resistance traits that are also known to be simply inherited are not abundant in cultivated strawberry. Perhaps MAS would gain more traction if such traits could be discovered and characterized in wild species and rapidly introgressed into cultivated forms using markers.

When considering the costs of MAS, it must be mentioned that, unlike in Minnesota, much of the strawberry acreage around the world is grown using the annual plasticulture system, which requires high costs per unit of area for land preparation and field maintenance. Some breeding programs for annual systems also produce runner plants of first-year seedlings in summer nurseries for evaluation, as opposed to the original seedlings per se. These factors dramatically increase the cost phenotypic evaluation per seedling genotype and make MAS more financially attractive, especially as the cost of genotyping continues to plummet.

For quantitative traits, Luby and Shaw [51] recommend that MAS will be most economical for traits with heritability < 0.2 and where selection intensity is high, though it is precisely under conditions of low heritability that marker-trait associations become more difficult to establish. Looking at the problem from a different perspective, perhaps markers could be used to transform some complex, low-heritability traits into a set of simply inherited traits. An example of this approach is the work on the *Rpf* genes for *P. fragariae* resistance. What was previously observed as incomplete resistance was dissected into several race-specific resistances conferred by major genes, facilitating the discovery of molecular markers for *Rpf1* [39, 75]. Using another example, Spigler et al. [69, 71] describes small chromosomal duplications in strawberry that can complicate or obscure segregation ratios, as shown by some markers mapping to multiple linkage groups. For instance, the ARSFL_22 marker amplified products from each of the four linkage groups in homoeologous group VI. An allele linked to this locus might segregate as if controlled by four loci, depending on the genotype of the parents, and further complications could be introduced via epistatic interactions among the loci [K. Lewers, personal communication]. In such cases, the breeder could use markers to choose more homozygous parents which would allow better prediction of segregation ratios.

Flavor is a trait with extremely low heritability that is affected by multiple chemical constituents including sugars, organic acids, and volatile compounds which interact with one another in a complex fashion [76, 84]. Volatiles, in particular, are quite expensive to capture and measure. What if the presence of the most important flavor volatiles, some of which are not in cultivated germplasm, could be isolated and tagged using molecular markers? In this case, a complex trait could be simplified by breaking it into its component phenotypes and employing markers to select for those phenotypes.

Another consideration that impacts the potential for MAS in strawberries is the breeding behavior of the crop. Strawberries are characterized by high levels of outbreeding, resultant heterozygosity, and frequent testing of clonal replicates as opposed to the testing of inbred lines. The heterozygosity of cultivated strawberry may be augmented still further, due to recent efforts to directly reconstruct the cultivated strawberry by intercrossing elite clones of *F. virgininana* and *F. chiloensis* [37]. This approach is aimed at maximizing genetic diversity and increases the number of alleles and allelic interactions to be accounted for. Therefore, even if marker-trait associations become established in some breeding programs they may be irrelevant in other sets of germplasm. Therefore, allelic mining at these loci across germplasm sets will be very important [56].

To address these real-world complications, pedigree based analysis (PBA) is being developed as an alternative to traditional mapping [80]. In crops such as strawberry, where a pedigree-breeding approach is followed, the concept of identity by descent (IBD) can be harnessed to relate alleles across the family tree and determine their impact on phenotype. The attractiveness of the approach is that it utilizes materials that breeders have already developed as opposed to mapping populations, and it examines the effects of all available alleles at a locus and in multiple genetic backgrounds simultaneously [80]. Statistical approaches for PBA are being refined and incorporated into the FlexQTL software package [8]. At present, breeding programs for multiple Rosaceous crops, including strawberry, are using this approach and coordinating their efforts under the banner of RosBREED, a project funded by the USDA/NIFA in 2009 (www.rosbreed.org). A major goal of this collaborative project is to help realize the promise of MAB in major crops of the Rosaceae family. By phenotyping pedigreed sets of strawberries at multiple locations using a standardized phenotyping approach, a large data set will be generated that can be analyzed using PBA [44]. Next-generation sequencing (NGS) [81] will be used to generate SNP markers for identifying marker-locus-trait associations. The primary focus of this effort in strawberry will be on fruit traits, which is significant since, despite

the stated potential of MAS for fruit quality from the survey of Bassil and Lewers [6], no markers have been published thus far for fruit traits in cultivated strawberry.

7. Conclusions

At present, the greatest impact of molecular markers in strawberry is for cultivar identification, which has become an important tool for the nursery industry, for germplasm curators, and for breeders for IP protection. This has been greatly facilitated by the rapid increase of SSR markers and subsequent efforts to develop reliable, multiplexed sets that can be used across labs. No doubt, these marker sets will be continually refined and will continue to be used for other purposes including genetic diversity analysis and genetic mapping. Markers have been used to confirm the allopolyploid behavior of cultivated strawberry and show that only small genomic rearrangements have occurred during the domestication of this crop. Further, marker-trait associations for traits such as anthracnose resistance and photoperiodic flowering have been established, and even used in breeding. In order to realize the promise of MAB, particularly in public breeding programs, more and better markers must be generated for economically important fruit quality traits, a goal which will be facilitated by collaborative, PBA approaches.

We can also expect that marker technology will be significantly impacted by the availability of the *F. vesca* genome sequence [68]. Of the 33,263 protein-coding genes discovered in woodland strawberry, approximately 25,000 have already been annotated. Since *F. vesca* is easily transformed and has a short generation time, the functions of candidate genes may be rapidly tested. This will facilitate an increase in the use of candidate gene approaches to tag traits of interest in strawberry as well as other species of the Rosaceae. As NGS technologies dramatically enhance marker discovery and facilitate re-sequencing of breeding lines, marker-assisted breeding will give way to genomics-assisted breeding. If a decade from now multiple genotypes can be regularly and inexpensively sequenced by individual labs, the limiting steps in the process will be analyzing sequence data and associating the sequence data with economically important traits. In other words, the genotype must still be related in a meaningful way to the phenotype. Therefore, strawberry breeders, geneticists, and bioinformatics experts must work together to apply sequencing technologies to the complex genome of the cultivated strawberry in a way that produces tangible genetic improvement, and ultimately a more healthy and satisfying product for the general public.

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