

Enhancing Resistance to Soilborne Pathogens in Strawberry Through Traditional and Genomic-enabled Breeding Approaches



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SUMMARY

Soilborne diseases threaten strawberry (*Fragaria × ananassa*) production worldwide. Standard practices for mitigating economic risks associated with production losses caused by soilborne diseases have been soil fumigation, crop rotation, and natural genetic resistance. Our research focuses on the latter. Historically, growers have relied on the highly effective soil fumigant methyl bromide to control soilborne pathogens. When methyl bromide was banned by a global treaty to protect the ozone layer in 2005 and phased out over the next decade, strawberry growers began adopting alternative production practices. Coincidentally, as fumigation practices changed in conventional production systems, two devastating soilborne diseases were first reported in California: *Macrophomina* in 2005 and *Fusarium* wilt in 2006. The loss of methyl bromide as a solution for controlling soilborne pathogens and challenges associated with alternative production systems have greatly increased the need for the development and deployment of cultivars resistant to multiple soilborne diseases. The research described in this report is part of an ongoing effort to provide the industry with strawberry cultivars carrying the strongest possible natural genetic resistance to multiple soilborne pathogens—especially *Fusarium oxysporum* f. sp. *fragariae*, *Verticillium dahliae*, *Macrophomina phaseolina*, and *Phytophthora cactorum*. Our current research focuses on: (a) understanding the strength and prevalence of resistance to soilborne pathogens in UCD and other public germplasm collections; (b) developing and deploying high yielding long-shelf life cultivars resistant to multiple soilborne and above-ground pathogens; (c) developing a deeper understanding of the genetics of resistance to soilborne pathogens; and (d) accelerating the development of disease resistant cultivars through genomic-enabled breeding.

INTRODUCTION

Soil fumigation with methyl bromide (MeBr), a compound banned in 2005 by a global treaty established to protect the ozone layer, provided nearly complete control of soilborne pathogens in strawberry (*Fragaria × ananassa* Duchesne ex Rozier) for nearly three decades, and was instrumental in the economic expansion of the strawberry industry in the US. Coincidentally, two of the most damaging soilborne diseases appeared for the first time in the aftermath of the MeBr phaseout, *Macrophomina* in 2005 and Fusarium wilt in 2006 (Koike, 2008; Koike et al., 2009; Mertely et al., 2005). Together with Verticillium wilt and Phytophthora crown rot, these diseases are perennially important problems in strawberry production worldwide (Bhat and Subbarao, 1999; Gordon et al., 2006; Maas, 1998). The loss of methyl bromide as a solution for controlling soilborne pathogens, as well as evolving resistance of pathogens to fungicides, has greatly increased the need for the development and deployment of cultivars resistant to pathogens that cause losses in fumigated and non-fumigated production systems in strawberry.

The research described in this report is part of an ongoing effort to provide the industry with strawberry cultivars carrying the strongest possible natural genetic resistance to multiple soilborne pathogens—especially *Fusarium*, *Verticillium*, *Macrophomina*, and *Phytophthora*—and builds on previous efforts to develop disease resistant cultivars in the UCD breeding program. This must be achieved without compromising yield, shelf-life, and other important traits. Our research specifically focuses on: (a) understanding the strength and prevalence of resistance to soilborne pathogens in UCD and other public germplasm collections; (b) developing, advancing, and introducing high yielding long-shelf life cultivars resistant to multiple soilborne and above-ground pathogens; (c) developing a deeper understanding of the genetics of resistance to soilborne pathogens; and (d) accelerating the development of disease resistant cultivars through genomic-enabled breeding. Our long-term goals are to: (a) provide the industry with cultivars resistant to the most damaging diseases in California production systems and (b) develop innovative genomic-enabled breeding approaches for solving disease resistance and other breeding problems in strawberry.

Here we briefly review what has been previously reported and what was previously known about the genetics of resistance to the soilborne pathogens under study. As expected, earlier studies have uncovered a broad spectrum of genetic complexity, from resistance (*R*) genes that confer innate immunity to ‘black box’ phenotypes with complex genetics. Accordingly, the genomic-enabled breeding approaches we and others are implementing in strawberry split along two lines: (a) genetically simple traits where discovered *R*-genes or large-effect quantitative trait loci (QTL) are sufficient to deliver commercially viable resistance via marker-assisted selection (MAS) and (b) genetically complex traits where the discovery and manipulation of individual QTL is insufficient to deliver commercially viable resistance. For the former, our strategy focuses on identifying and manipulating *R*-genes and large-effect QTL via MAS (Noh et al., 2016; Pincot et al., 2018; Roach et al., 2016). For the latter, our strategy focuses on phenotyping across environments and genetic backgrounds, genotyping and phenotyping segregating populations, and testing, validating, and implementing genomic selection (Gezan et al., 2017; Mangandi et al., 2017). Our approaches do not preclude the discovery of large-effect QTL—if such loci are present, there is a high probability that they will be discovered by genome-wide association mapping (GWAS) in genomic selection training populations or by linkage disequilibrium (LD) mapping in segregating populations (Gibson, 2012; Wray et al., 2013).

Fusarium wilt, a soilborne disease caused by the fungal pathogen *Fusarium oxysporum* f. sp. *fragariae*, threatens strawberry production worldwide (Arroyo et al., 2009; Gordon, 2017; Koike et al., 2009; Winks and Williams, 1965). Susceptible genotypes develop vascular wilt symptoms, including stunting, chlorosis, wilting, browning, and dieback. This disease was first discovered on strawberry in California in 2006 and has since become an increasingly serious threat to strawberry production in California and other parts of the world (Henry et al., 2017; Gordon et al., 2016, 2017; Koike et al., 2009; Koike and Gordon, 2015; Paynter et al., 2014; Paynter et al., 2016).

When we started this research in 2015, little had been published and little was known about the genetics of resistance to Fusarium wilt. Two earlier studies had produced conflicting results and reached different conclusions, one claiming resistance was conferred by an *R*-gene (Mori et al., 2005) and the other claiming resistance was quantitative (Paynter et al., 2014). Our initial studies (described below) uncovered a dominant gene (*Fw1*) that confers resistance and numerous potentially novel *R*-genes that confer resistance to Fusarium wilt (Pincot et al., 2018).

Verticillium wilt, a soilborne disease caused by the fungal pathogen *Verticillium dahliae*, threatens strawberry production worldwide (Bhat and Subbarao, 1999; Gordon et al., 2006). *R*-genes conferring resistance to Verticillium wilt have been reported in tomato, potato, cotton, and mint; however, *R*-genes have not been reported and the genetics of resistance appears to be complex in strawberry (Antanaviciute et al., 2015; Bolek et al., 2005; Fradin et al., 2009; Shaw et al., 2003, 2008; Simko et al., 2004; Zhao et al., 2014). While partially resistant cultivars have been reported, insights into the genetics of resistance to Verticillium wilt are lacking (Antanaviciute et al., 2015; Shaw et al., 2008).

Charcoal rot, a soilborne disease caused by the necrotrophic fungal pathogen *Macrophomina phaseolina*, is a recent problem for strawberry growers in the US, with first cases reported in 2005 and spreading thereafter (Gupta et al., 2012; Koike 2008; Koike et al., 2016; Mertely et al., 2005). The risk to production caused by this pathogen appears to be increasing. Little is known about the genetics of resistance to *Macrophomina*. Our preliminary work suggests that the genetics of resistance to this pathogen is complex, not unlike the genetics of resistance to white mold in common bean, a disease caused by the necrotrophic fungus *Sclerotinia sclerotiorum* (Vasconcellos et al., 2017).

Phytophthora crown rot, a soilborne disease caused by the fungal pathogen *Phytophthora cactorum*, has been a long-standing problem in strawberry production. Mangandi et al., (2017) recently reviewed previous work on the genetics of resistance to this pathogen. They identified *FaRPa2*, a dominant *R*-gene conferring partial resistance to Phytophthora. The *R*-gene occurs at low frequency in University of Florida germplasm (Mangandi et al., 2017). The frequency of the *R*-gene in UCD germplasm is unknown. Partially resistant cultivars have been reported; however, insights into the genetics of resistance to Phytophthora are limited in UCD germplasm (Shaw et al., 2008).

To begin addressing questions raised by earlier studies, develop insights into genetics of resistance to soilborne pathogens, and build the foundation for developing the next-generation of disease resistant cultivars, we have been engaged in a series of ongoing breeding and genetic studies. Here we describe the results of those studies, which included: (a) quantifying phenotypic variation for resistance to soilborne pathogens in UCD germplasm and other public germplasm collections; (b) identifying resistant and susceptible cultivars and germplasm accessions; (c) developing a phenotypic database to guide breeding, selection, and cultivar advancement decisions; and (d) developing insights into the genetics of resistance to soilborne pathogens. Over the last year, we heavily focused on investigating the genetics of resistance to Fusarium wilt and Verticillium wilt, initiated studies on the genetics of resistance to *Macrophomina* and *Phytophthora* crown rot, and significantly expanded genomic resources for strawberry, including the development of an octoploid reference genome sequence with chromosome-scale contiguity (Edger et al., 2018b) and a high-density genotyping array with 850,000 single nucleotide polymorphisms (SNPs).

RESULTS & DISCUSSION

Genetics of Resistance to Fusarium Wilt

The results of our initial studies on the genetics of resistance to Fusarium wilt have been published, along with the supporting data, including resistance phenotypes for 565 germplasm accessions in the UCD Strawberry Germplasm Collection (Pincot et al., 2018). The most important results from Pincot et al., (2018), additional unpublished results, and loose ends are highlighted below.

- Through genome-wide association and genetic mapping, we identified a dominant gene (*Fw1*) conferring resistance to AMP132, a virulent isolate from the most common genetic lineage of *F. oxysporum* f. sp. *fragariae* in California (Henry et al., 2017; Pincot et al., 2018).
- The *Fw1* gene was randomly distributed among UCD cultivars and germplasm accessions and traced to cultivars developed in 1935 - 'Solana' (PI 551665) and 'Shasta' (PI 551663).
- The frequency of the *Fw1* gene was low in the UCD germplasm collection (0.18). One-third of 565 UCD germplasm accessions screened for resistance to Fusarium wilt carry the *Fw1* gene—only 3% were homozygous for *Fw1* (Figure 1). Hence, the probability of a random cross segregating for *Fw1* was only 10.9%, which has played a significant role in our parent selection and strategy.
- To confirm and validate the Fusarium wilt resistance phenotypes we observed with artificial inoculation in Davis field experiments, 30 cultivars were screened in a naturally infested field at Monterey Bay Academy, Watsonville, CA (Figure 2). There was complete agreement between the Davis and MBA results.
- Beyond the UCD material (565 accessions), we screened 384 germplasm accessions maintained by the USDA National Plant Germplasm System (NPGS), including heirloom cultivars and wild ecotypes. This work identified numerous additional potentially novel sources of resistance (data not shown). We have several follow up studies underway.
- We identified several novel sources of *R*-genes in our screening of the UCD germplasm collection, including cvs. Wiltguard, Guardian, and Earliglow, and are currently phenotyping and genotyping segregating populations with these sources. We anticipate that this work will uncover novel genes conferring resistance to Fusarium wilt and potentially supply allelic diversity for preemptively addressing the potential for the emergence of new races of the pathogen. We anticipate completing the genetic mapping and validation of at least three novel *R*-genes in 2018-2019.
- While our initial work identified array-based SNPs linked to *Fw1*, we are still in the process of developing individual sub-genome specific high-throughput DNA markers for implementing MAS for *Fw1*. We anticipate completing this work in 2018. This work will be significantly impacted by recently developed and emerging genomic resources (described below).

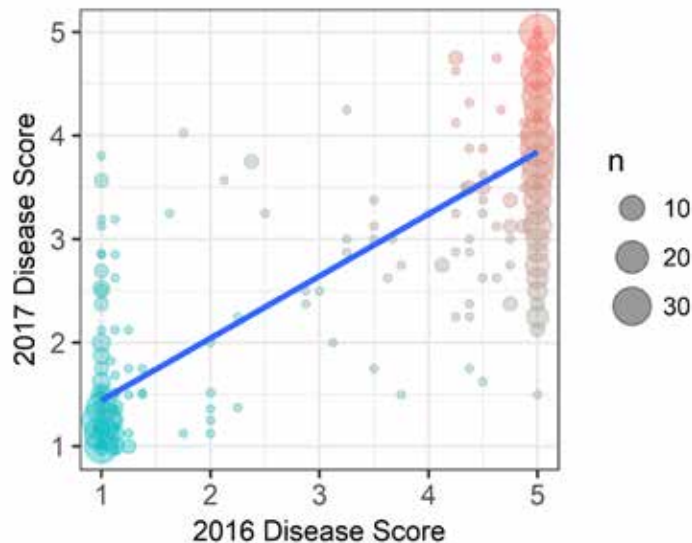


Figure 1. Phenotypic distributions for 565 UCD germplasm accessions screened for resistance to Fusarium wilt in 2016 and 2017 field experiments in Davis, California, where 1 = highly resistant (symptomless) and 5 = highly susceptible (severe symptoms, including dieback). Plants were artificially inoculated with race AMP132 of *Fusarium oxysporum* f. sp. *fragariae*. The phenotypes shown were observed nine weeks post-inoculation in 2016 (x-axis) and 36 weeks post-inoculation in 2017 (y-axis). The phenotypic correlation between years was 0.84 ($p < 0.001$).

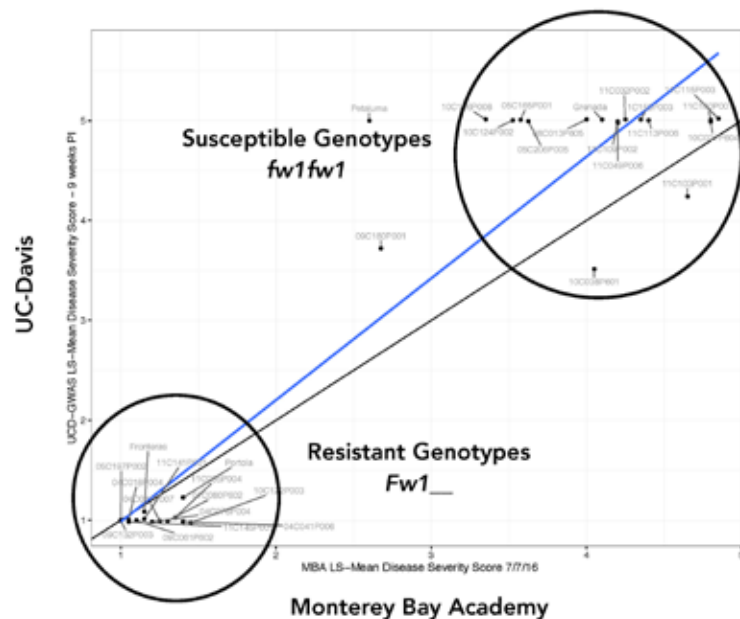


Figure 2. Comparison of Fusarium wilt resistance phenotypes for resistant and susceptible cultivars screened in a naturally infested trial at Monterey Bay Academy, Watsonville, CA (x-axis) and an artificially inoculated trial in Davis, CA, where 1 = highly resistant (symptomless) and 5 = highly susceptible (severe symptoms, including dieback).

Genetics of Resistance to Verticillium Wilt

The design and approach for our studies on the genetics of resistance to Verticillium wilt paralleled those described for Fusarium wilt. The most important results from studies the past two years and a summary of ongoing genetic analyses are highlighted below.

- Last year, 565 UCD and 384 USDA germplasm accessions were screened for resistance to Verticillium wilt in a field study in Davis using artificially inoculated plants. We observed a full range of Verticillium wilt resistance phenotypes and continuous phenotypic distributions among the germplasm accessions tested (Figure 3). The broad-sense heritability for resistance to Verticillium wilt was 0.64.
- Several UCD cultivars were partially resistant. The three most resistant UCD cultivars were 'Palomar' (2.0), 'Grenada' (2.2), and 'Camino Real' (2.4). We identified 81 USDA germplasm accessions that were more resistant than 'Palomar', with resistance scores ranging from 1.0 (highly resistant) to 1.9 (moderately resistant). Of those, 34 had resistance scores between 1.0 and 1.6.
- Thirty of the 960 germplasm accessions screened in Davis (Figure 3) were included in a Verticillium wilt screening study at Cal Poly (data not shown). Disease ratings in the two studies were positively correlated ($r = 0.61$). Most importantly, entries with the strongest Verticillium wilt resistance ratings were identical in both studies.
- While several earlier studies of Verticillium resistance in UCD germplasm have been reported, the genotypes screened in those studies and the underlying phenotypic data were not disclosed (Gordon et al., 2005; Shaw and Gordon, 2003; Shaw et al., 2005; Shaw et al., 2010). Nevertheless, by comparing phenotypes reported in plant patents for UCD cultivars, we determined that the phenotypes we observed were consistent with those previously reported for patented UCD cultivars.
- Interestingly, from the 2016-17 Davis experiment, the mean Verticillium wilt resistance scores for UCD cultivars developed in different decades since 1940 have not substantially changed—on average, cultivars developed in the 1940s and 1960s were slightly more resistant (2.5-2.6) than cultivars developed since 1970 (2.9-3.5).
- When the genome was scanned for the presence of genes conferring resistance to Verticillium wilt, we observed weak GWAS signals (data not shown). These results strongly supported our working hypothesis that the genetics of resistance to Verticillium wilt is complex. While a few marginally significant SNPs were identified, strong signals were not observed anywhere in the genome (data not shown). Our GWAS results were consistent with genetic mapping results reported in a previous study of European germplasm (Antanaviciute et al., 2015).
- To explore the genetics of resistance to Verticillium wilt further, we have initiated genomic prediction studies using phenotypic data collected in 2016-2017 and 2017-2018 (in progress) and will be genotyping a subset of 480 germplasm accessions from the original GWAS population with a SNP genotyping array that is currently under development. We anticipate completing these analyses in 2018-2019.
- To complement the aforementioned genomic prediction study, we developed a factorial population of 565 progeny for pedigree-based QTL mapping and genomic selection studies. This population is currently being phenotyped for resistance to Verticillium wilt and will be genotyped as described for GWAS-480 population. The parents of the factorial diverse UCD and USDA parents with a broad range of resistance to Verticillium wilt.

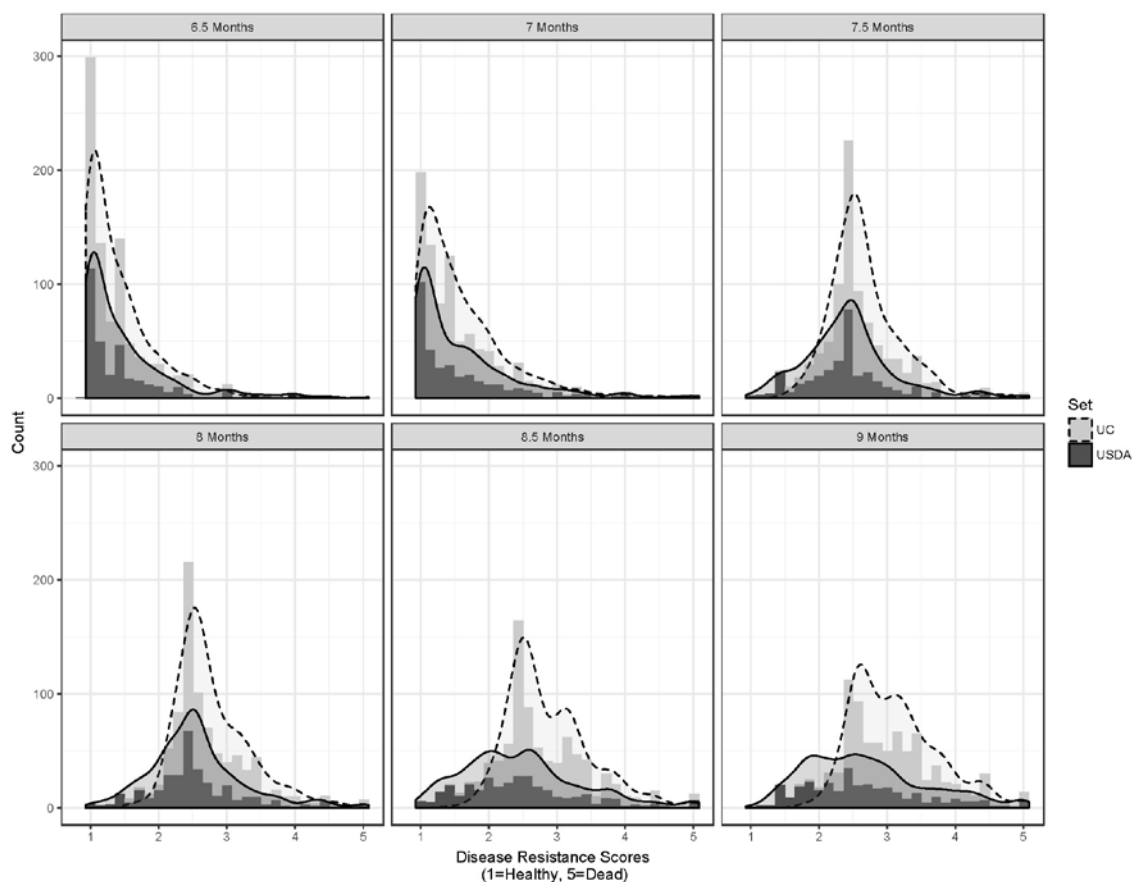


Figure 3. Phenotypic distributions for 565 UCD and 384 USDA germplasm accessions screened for resistance to *Verticillium* wilt in Davis, CA in 2016-2017, using artificially inoculated plants, where 1 = highly resistant and 5 = highly susceptible. Each histogram represents a different post-planting time point.

Genetics of Resistance to *Macrophomina*

Of the four soilborne pathogens we have been studying, *Macrophomina* appears to present the greatest challenge from a breeding and genetics perspective. This conclusion is based on the frequency of germplasm accessions that have exhibited moderate to strong resistance in three separate field experiments.

- Over the last two years, 565 UCD and 384 USDA germplasm accessions were screened for resistance to *Macrophomina* in Davis and Irvine. The phenotypic distributions in both studies were strongly skewed towards the susceptible end of the scale.
- The mean resistance rating was 4.4 and 80% of the germplasm accessions (640/803) tested were highly susceptible (had a disease rating ≥ 4). We only observed one strongly resistant accession (mean disease rating = 1.0): a wild ecotype of *F. chiloensis*, one of the octoploid progenitors of *F. x ananassa*.

- We estimated broad-sense heritability for the entire sample ($n = 803$) and for only those germplasm accessions with disease ratings ≤ 3 ($n = 302$). The former was 0.71, whereas the latter was 0.15. We did this to emphasize that the lower estimate more accurately reflects the underlying genetic reality because including highly susceptible individuals ($n = 501$) in the analysis (essentially dead plants) greatly decreases the non-genetic variance and produces a spurious estimate of heritability.
- From these screening experiments, we have identified several sources of resistance and developed numerous segregating populations for ongoing breeding and genetic studies.

Genetics of Resistance to *Phytophthora* Crown Rot

We initiated work on the genetics of resistance to *Phytophthora* crown rot in the fall of 2017, essentially following the prescription above. Our results-to-date and ongoing studies are highlighted below.

- We screened the GWAS-480 population for resistance to *Phytophthora* in a field study in Davis in 2017-2018 (currently in progress). Our germplasm screening study design was identical to that used for Fusarium wilt (Pincot et al., 2018). We will conduct this study a second year (2017-2018) and anticipate completing GWAS and other statistical analyses in 2018-19. This population, as previously noted, will be genotyped with a greatly enhanced SNP array that is currently being tested.
- Through a collaboration with Seonghee Lee, Vance Whitaker, and Natalia Peres at the University of Florida, we have begun exploring the utility of the *FaRPP2* R-gene in UCD germplasm. The favorable *FaRPP2* allele was present in five of the 10 cultivars screened by Noh et al., (2018). Using the data generated this year, we will examine the effect of this locus on resistance to *Phytophthora* crown rot in UCD germplasm, in addition to scanning the genome for other loci.

Development of an Octoploid Reference Genome Sequence for Strawberry

Here, we describe progress towards the development of resources and platforms to support genomic-enabled breeding in strawberry. Over the last two years, we have collaborated with Patrick Edger (MSU) and several other public-sector scientists to sequence the octoploid ($2n = 8x = 56$) strawberry genome (Figure 4). That work has been completed and produced a near complete, chromosome-scale genome assembly, which is described in a manuscript recently submitted for publication (Edger et al., 2018b). As soon as that paper is accepted, the annotated genome sequence and 100% of the supporting datasets will be released to public databases and will be accessible to the entire research community, e.g., Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>), the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/>), Dryad Digital Repository (<https://datadryad.org/>), and Genome Database for Rosaceae (<https://www.rosaceae.org/>). The development of an octoploid reference genome sequence has been a catalyst for several other advances and ongoing investigations, especially the development of platforms for genotyping DNA variants across the strawberry genome.

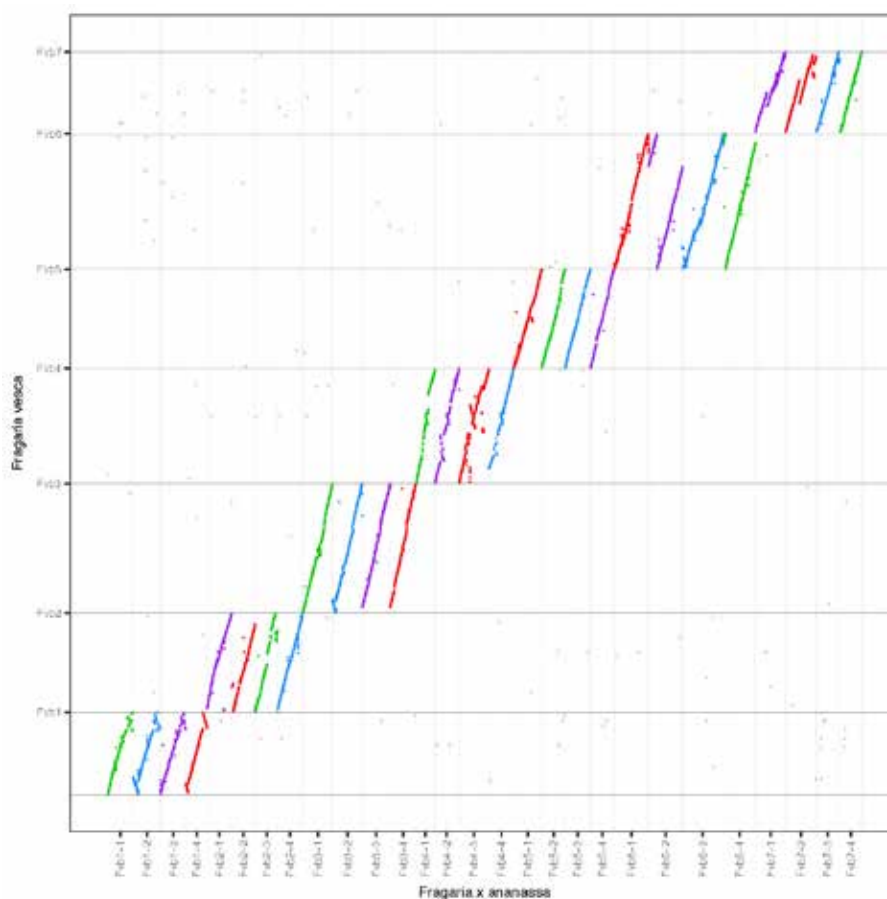


Figure 4. Alignment of diploid *F. vesca* and octoploid *F. x ananassa* reference genome assemblies. There were 28 pseudo-molecules in the latter, equivalent to the 28 chromosome pairs in the octoploid strawberry genome ($2n = 8x = 56$).

Genotyping-by-Sequencing (GBS)

The development of the octoploid reference genome sequence was essential for enabling the application of GBS in strawberry. We developed and completed testing of GBS protocols and a GBS bioinformatics pipeline to build the foundation for ongoing genetic studies and validate the approach for identifying and uniquely mapping DNA variants in the octoploid genome. DNA samples were digested with two commonly used restriction enzyme combinations and processed using a standard GBS protocol (Elshire et al., 2011; Poland et al., 2012). This yielded 1,859,605 to 2,023,367 uniquely mapped DNA variants among the genotypes tested. With stringent filtering of DNA variants, 76,626 to 415,048 single nucleotide polymorphisms (SNPs) were identified among the genotypes tested provided excellent genome-wide coverage and distribution (Figure 5). Because we originally expected challenges with mapping short-read DNA sequences back to the reference genome, the initial GBS experiments were done using 150-bp reads. We are in the process of testing protocols with shorter reads and different multiplexes to maximize throughput and lower genotyping costs for future applications, including genetic mapping of newly discovered Fusarium wilt *R*-genes.

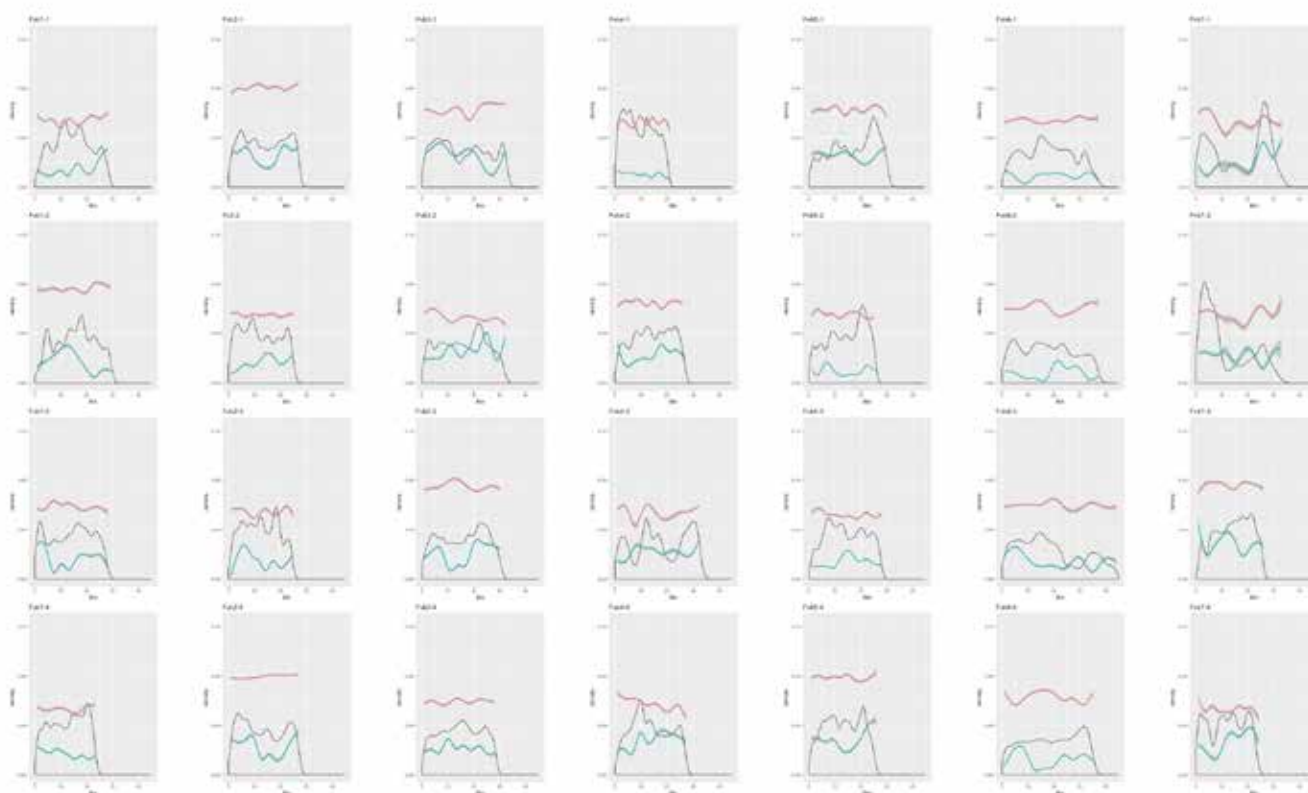


Figure 5. The distribution (black line), coverage (red line), and quality (blue line) of DNA variants identified by GBS using *HindIII-MspI* digested DNA samples of 12 strawberry cultivars. DNA variants were mapped to the octoploid reference genome (Edger et al., 2018b). The 28 chromosome pairs in the octoploid are displayed, with the four sub-genomes in rows and the seven homeologous chromosomes/sub-genome in columns. This work is part of a Ph.D. thesis. Mitchell Feldmann (the student leading this) is still working on the second part of the study and manuscript.

Development of High-Density SNP Genotyping Arrays

The SNP array described by Bassil et al., (2015) was used in the studies described in this report. Without going into great detail, that SNP array has been extremely useful but was developed long before the octoploid genome was sequenced. Starting with the newly developed octoploid reference genome (Figure 5; Edger et al., 2018b), we re-sequenced the genomes of 92 genotypes. We identified 90 million SNPs among elite and wild genotypes and 45 million SNPs among elite genotypes. Using the latter, we identified 850,000 highly sub-genome specific SNPs for inclusion on a new SNP genotyping array (Figure 6). These are predicted to provide 5 to 20 SNPs/gene in 94% of the annotated genes and to cover 97% of the *F. x ananassa* genome. Testing of the new SNP array is currently underway with 384 DNA samples for genotypes provided by several public and private sector scientists. We expect this SNP array to provide genotyping data of unparalleled quality and depth for downstream genetic analyses in strawberry.

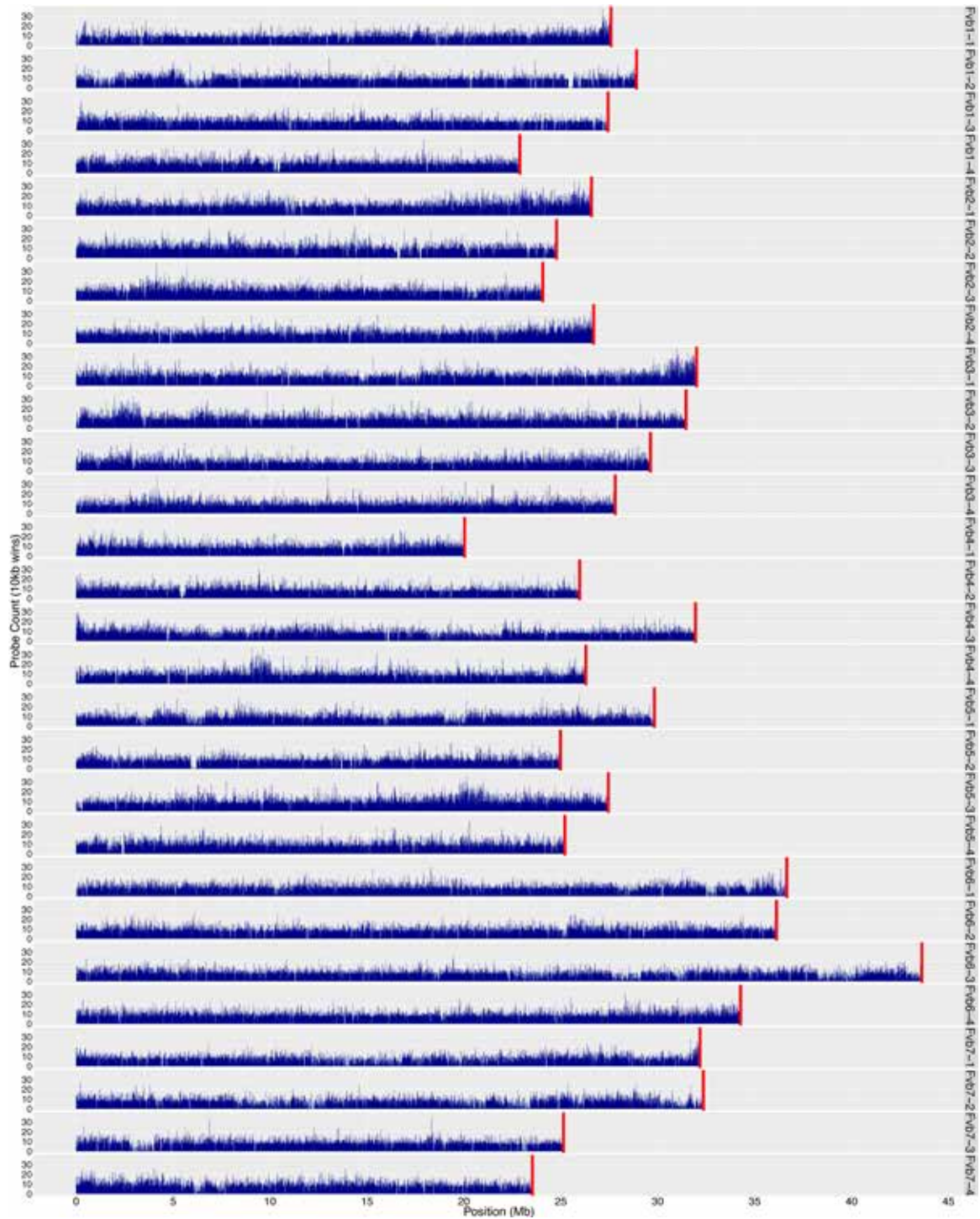


Figure 6. Distribution of 850,000 single nucleotide polymorphism (SNP) probes across the octoploid strawberry genome. The 28 chromosome pairs in the octoploid ($2n = 8x = 56$) genome are displayed in rows with chromosome positions (Mb) along the x-axis. The y-axis displays SNP probe counts.

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