Compendium of Plant Genomes
Series Editor: Chittaranjan Kole

Timo Hytönen · Julie Graham · Richard Harrison
Editors

The Genomes of Rosaceous Berries and Their Wild Relatives

Compendium of Plant Genomes

Series editor

Chittaranjan Kole, Raja Ramanna Fellow, Department of Atomic Energy, Government of India, Kalyani, India

Whole-genome sequencing is at the cutting edge of life sciences in the new millennium. Since the first genome sequencing of the model plant Arabidopsis thaliana in 2000, whole genomes of about 70 plant species have been sequenced and genome sequences of several other plants are in the pipeline. Research publications on these genome initiatives are scattered on dedicated web sites and in journals with all too brief descriptions. The individual volumes elucidate the background history of the national and international genome initiatives; public and private partners involved; strategies and genomic resources and tools utilized; enumeration on the sequences and their assembly; repetitive sequences; gene annotation and genome duplication. In addition, synteny with other sequences, comparison of gene families and most importantly potential of the genome sequence information for gene pool characterization and genetic improvement of crop plants are described.

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The Genomes of Rosaceous Berries and Their Wild Relatives

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This book series is dedicated to
my wife Phullara, and our children Sourav,
and Devleena

Chittaranjan Kole

Preface to the Series

Genome sequencing has emerged as the leading discipline in the plant sciences coinciding with the start of the new century. For much of the twentieth century, plant geneticists were only successful in delineating putative chromosomal location, function, and changes in genes indirectly through the use of a number of 'markers' physically linked to them. These included visible or morphological, cytological, protein, and molecular or DNA markers. Among them, the first DNA marker, the RFLPs, introduced a revolutionary change in plant genetics and breeding in the mid-1980s, mainly because of their infinite number and thus potential to cover maximum chromosomal regions, phenotypic neutrality, absence of epistasis, and codominant nature. An array of other hybridization-based markers, PCR-based markers, and markers based on both facilitated construction of genetic linkage maps, mapping of genes controlling simply inherited traits, and even gene clusters (QTLs) controlling polygenic traits in a large number of model and crop plants. During this period, a number of new mapping populations beyond F_2 were utilized and a number of computer programs were developed for map construction, mapping of genes, and for mapping of polygenic clusters or QTLs. Molecular markers were also used in studies of evolution and phylogenetic relationship, genetic diversity, DNA-fingerprinting, and map-based cloning. Markers tightly linked to the genes were used in crop improvement employing the so-called marker-assisted selection. These strategies of molecular genetic mapping and molecular breeding made a spectacular impact during the last one and a half decades of the twentieth century. But still they remained 'indirect' approaches for elucidation and utilization of plant genomes since much of the chromosomes remained unknown and the complete chemical depiction of them was yet to be unraveled.

Physical mapping of genomes was the obvious consequence that facilitated development of the 'genomic resources' including BAC and YAC libraries to develop physical maps in some plant genomes. Subsequently, integrated genetic–physical maps were also developed in many plants. This led to the concept of structural genomics. Later on, emphasis was laid on EST and transcriptome analysis to decipher the function of the active gene sequences leading to another concept defined as functional genomics. The advent of techniques of bacteriophage gene and DNA sequencing in the 1970s was extended to facilitate sequencing of these genomic resources in the last decade of the twentieth century.

As expected, sequencing of chromosomal regions would have led to too much data to store, characterize, and utilize with the then available computer software could handle. But development of information technology made the life of biologists easier by leading to a swift and sweet marriage of biology and informatics, and a new subject was born—bioinformatics.

Thus, evolution of the concepts, strategies, and tools of sequencing and bioinformatics reinforced the subject of genomics—structural and functional. Today, genome sequencing has traveled much beyond biology and involves biophysics, biochemistry, and bioinformatics!

Thanks to the efforts of both public and private agencies, genome sequencing strategies are evolving very fast, leading to cheaper, quicker, and automated techniques right from clone-by-clone and whole-genome shotgun approaches to a succession of second generation sequencing methods. Development of software of different generations facilitated this genome sequencing. At the same time, newer concepts and strategies were emerging to handle sequencing of the complex genomes, particularly the polyploids.

It became a reality to chemically—and so directly—define plant genomes, popularly called whole-genome sequencing or simply genome sequencing.

The history of plant genome sequencing will always cite the sequencing of the genome of the model plant *Arabidopsis thaliana* in 2000 that was followed by sequencing the genome of the crop and model plant rice in 2002. Since then, the number of sequenced genomes of higher plants has been increasing exponentially, mainly due to the development of cheaper and quicker genomic techniques and, most importantly, development of collaborative platforms such as national and international consortia involving partners from public and/or private agencies.

As I write this preface for the first volume of the new series 'Compendium of Plant Genomes,' a net search tells me that complete or nearly complete whole-genome sequencing of 45 crop plants, eight crop and model plants, eight model plants, 15 crop progenitors and relatives, and three basal plants is accomplished, the majority of which are in the public domain. This means that we nowadays know many of our model and crop plants chemically, i.e., directly, and we may depict them and utilize them precisely better than ever. Genome sequencing has covered all groups of crop plants. Hence, information on the precise depiction of plant genomes and the scope of their utilization is growing rapidly every day. However, the information is scattered in research articles and review papers in journals and dedicated Web pages of the consortia and databases. There is no compilation of plant genomes and the opportunity of using the information in sequence-assisted breeding or further genomic studies. This is the underlying rationale for starting this book series, with each volume dedicated to a particular plant.

Plant genome science has emerged as an important subject in academia, and the present compendium of plant genomes will be highly useful both to students and teaching faculties. Most importantly, research scientists involved in genomics research will have access to systematic deliberations on the plant genomes of their interest. Elucidation of plant genomes is of interest not only for the geneticists and breeders, but also for practitioners of an array of plant science disciplines, such as taxonomy, evolution, cytology,

physiology, pathology, entomology, nematology, crop production, biochemistry, and obviously bioinformatics. It must be mentioned that information regarding each plant genome is ever-growing. The contents of the volumes of this compendium are therefore focusing on the basic aspects of the genomes and their utility. They include information on the academic and/or economic importance of the plants, description of their genomes from a molecular genetic and cytogenetic point of view, and the genomic resources developed. Detailed deliberations focus on the background history of the national and international genome initiatives, public and private partners involved, strategies and genomic resources and tools utilized, enumeration on the sequences and their assembly, repetitive sequences, gene annotation, and genome duplication. In addition, synteny with other sequences, comparison of gene families, and, most importantly, potential of the genome sequence information for gene pool characterization through genotyping by sequencing (GBS) and genetic improvement of crop plants have been described. As expected, there is a lot of variation of these topics in the volumes based on the information available on the crop, model, or reference plants.

I must confess that as the series editor, it has been a daunting task for me to work on such a huge and broad knowledge base that spans so many diverse plant species. However, pioneering scientists with lifetime experience and expertise on the particular crops did excellent jobs editing the respective volumes. I myself have been a small science worker on plant genomes since the mid-1980s and that provided me the opportunity to personally know several stalwarts of plant genomics from all over the globe. Most, if not all, of the volume editors are my long time friends and colleagues. It has been highly comfortable and enriching for me to work with them on this book series. To be honest, while working on this series I have been and will remain a student first, a science worker second, and a series editor last. And I must express my gratitude to the volume editors and the chapter authors for providing me the opportunity to work with them on this compendium.

I also wish to mention here my thanks and gratitude to the Springer staff, Dr. Christina Eckey and Dr. Jutta Lindenborn in particular, for all their constant and cordial support right from the inception of the idea.

I always had to set aside additional hours to edit books besides my professional and personal commitments—hours I could and should have given to my wife, Phullara, and our kids, Sourav and Devleena. I must mention that they not only allowed me the freedom to take away those hours from them but also offered their support in the editing job itself. I am really not sure whether my dedication of this compendium to them will suffice to do justice to their sacrifices for the interest of science and the science community.

Kalyani, India Chittaranjan Kole

Preface

Raspberries and strawberries are two of the major rosaceous soft fruit crops grown today with a global value in excess of $2bn worldwide. In recent years, these crops have lagged behind major agricultural staples in the genomic tools and resources available to them and comparatively little is known about the genomic architecture of berry crops and their wild relatives. This book, over 14 chapters, attempts to synthesize current research efforts into both wild relatives and rosaceous berry crops and highlight some of the most exciting developments in soft fruit breeding and genomics.

Chapter 1 gives an overview of the current economic importance of strawberry, the largest and most important rosaceous soft fruit crop, and charts production growth globally. It highlights the need for breeding solutions to the current high labour costs for harvesting, which is likely to play an increasing role globally in affordable horticultural produce. Chapter 2 places berry crops into their broader phylogenetic context and highlights the diversity in both *Fragaria* and *Rubus* families that remains largely unexplored in modern breeding.

Chapter 3 details the genomic resources available for the perennial model *Fragaria vesca*, detailing some of the genomic tools that have now arisen for crop species as a result of this more fundamental research. In the same vein, Chap. 4 focusses on the perennial cycle which has been elucidated to a fine level of detail in *Fragaria vesca* and is now being used to understand control of flowering in the octoploid strawberry. Similarly, studies of fruit quality using both near isogenic lines and other multiparental or association populations provide a promising mechanism to understand components of secondary metabolism which can then be used in crop species to improve quality. Disease resistance, again a key component of crop breeding programmes, often relies on families of genes involved in the perception of non-self or modified self. In Chap. 6, knowledge of the complement of some of the more common families of resistance genes is charted for *Fragaria* and *Rubus* genomes.

Moving into the crop systems, the remaining chapters all focus upon one or more aspects of rosaceous berry genomics. Carrying on the theme of disease resistance, Chap. 7 reviews the current state of research into diseases of the octoploid strawberry, describing the QTLs that are known for bacterial, fungal, and oomycete diseases. Chapter 8 does the same but for fruit ripening and fruit quality QTL. Chapters 9–12 all deal with cutting-edge approaches

to breeding and genetics in the octoploid strawberry. Chapter 9 gives a comprehensive overview of the genotyping tools available for the octoploid strawberry and points to the use of new technologies for more cost-effective and rapid genotyping. Chapter 10 details progress on the octoploid sequence, utilizing some of the latest techniques such as 10X Chromium to untangle this complex crop genome. Chapter 11 provides both a historical perspective and future look towards the potential of genetic transformation in strawberry, highlighting the potential for genome editing as a tool for crop improvement. Finally, Chap. 12 details the use of genomic selection, a predictive breeding method in the octoploid. Taken together these techniques are set to revolutionize berry breeding in the coming decade.

Chapters 13 and 14 highlight the progress made in *Rubus* genomics and trait identification in recent years, with a report of the black raspberry genome in Chap. 13, and an overview of the biology of fruit development in red raspberry in Chap. 14.

It is hoped that this book gives an overview of the current status of berry genomes and highlights the enormous potential that this sector has to embrace modern technologies for crop improvement. We would like to thank each author for giving up their valuable time and for their efforts. We would also like to thank the series editor, Prof. Chittaranjan Kole, the editorial team at Springer for their assistance and also Angela Chapple for administrative assistance.

East Malling, UK Richard Harrison
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Contents

1 **The Economic Importance of Strawberry Crops** 1
David Simpson

Part I The Genomes of Crop Wild-Relatives

2 **Phylogenetics of *Fragaria*, *Rubus* and Related Taxa** 11
Maria Kinga Sobczyk

3 **Genomic Resources for the Woodland Strawberry (*Fragaria vesca*)** 25
Elizabeth I. Alger, Marivi Colle and Patrick P. Edger

4 **Control of Flowering in Strawberries** 35
Elli A. Koskela and Timo Hytönen

5 **Fruit Quality and the Use of Near-Isogenic Lines for Functional Characterization in *Fragaria vesca*** 49
Maria Urrutia and Amparo Monfort

6 **The NB-LRR Disease Resistance Genes of *Fragaria* and *Rubus*** 63
Leon van Eck and James M. Bradeen

Part II The Genomes of Rosaceous Berries

7 **Disease Resistance in Polyploid Strawberry** 79
Charlotte F. Nellist

8 **Fruit Ripening and QTL for Fruit Quality in the Octoploid Strawberry** 95
Delphine M. Pott, José G. Vallarino, Sonia Osorio and Iraida Amaya

9 **Genotyping Tools for the Octoploid Strawberry** 115
Robert Vickerstaff

10 **Current Status of Octoploid Strawberry (*Fragaria* × *ananassa*) Genome Study** 129
Sachiko N. Isobe, Kenta Shirasawa, Soichiro Nagano and Hideki Hirakawa

11 The History and Current Status of Genetic Transformation in Berry Crops 139
Elena Palomo-Ríos, Miguel A. Quesada, Antonio J. Matas, Fernando Pliego-Alfaro and José A. Mercado

12 Genome-Assisted Breeding in the Octoploid Strawberry 161
Sujeet Verma, Luis F. Osorio, Seonghee Lee, Nahla V. Bassil and Vance M. Whitaker

13 Sequence and Analysis of the Black Raspberry (*Rubus occidentalis*) Genome 185
Robert VanBuren, Doug Bryant, Jill M. Bushakra, Kelly J. Vining, Sergei Filichkin, Patrick P. Edger, Erik R. Rowley, Henry D. Priest, Todd P. Michael, Michael Dossett, Chad E. Finn, Nahla V. Bassil and Todd C. Mockler

14 Developmental Transitions to Fruiting in Red Raspberry 199
Julie Graham and Craig Simpson

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1 The Economic Importance of Strawberry Crops

David Simpson

Abstract

Strawberries are produced commercially in 76 countries. China is the largest producer and the top five producing nations also include USA, Mexico, Turkey and Spain. Production continues to increase, particularly in Asia, North and Central America, and North Africa with a matching increase in demand in many parts of the world. The development of the strawberry industry in California in the twentieth century was followed by rapid expansion of local industries in many other parts of the world including the Mediterranean region, Central and South America, Australia and China. In all of these regions, it was possible to identify the areas where a combination of short days with warm or mild temperatures made it possible to produce high yields over a long season. Plant breeding has had a very significant role in increasing the geographical adaptation of strawberries. The most notable achievement has been to transform the crop from a plant with a short season of production and a modest yield of small, soft berries to a highly productive plant capable of cropping over a long period with large, firm berries suitable for shipping over long distances.

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Berries have been grown for food for over 2000 years and before that would have been an important part of the human diet when harvested from the wild. Commercial production of berries began to become important in the early part of the nineteenth century following the breeding of improved cultivars of the cultivated strawberry (*Fragaria* × *ananassa*), while raspberries followed several decades later in the century after the hybridisation of the European and North American sub-species of the red raspberry (*Rubus idaeus*). Both types of berry became important crops initially in western Europe and North America but were characterised by short seasons of only a few weeks. During the twentieth century, production gradually spread to many other parts of the world and berry crops steadily increased in their economic importance, particularly in the post-war period when improved cultivars and new agronomic practices resulted in an extension of the production season.

1.1 Strawberries

Worldwide production of strawberries exceeded 7.7 million tonnes in 2013, following an increase of 142% over the previous 20 years (FAOStat),

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with commercial production in 76 countries covering all continents except Antarctica. China is the largest producer, with 3 million tonnes in 2013, and the top five producing nations also include USA, Mexico, Turkey and Spain. Strawberries are popular with consumers in nearly all parts of the world and the steady increase in global production had come about due to the adaptability of the species, which can be grown in anything from cool temperate to sub-tropical conditions, and the ingenuity of growers, scientists and agronomists in developing a variety of production systems to adapt to local conditions.

Breeding has been hugely significant in the success of strawberries. The first hybrids of *F.* × *ananassa* were produced in European gardens in the late eighteenth century, but breeding began in earnest in the ninetieth century and the cultivar 'Keens' Seedling', released in 1821, can be considered to be the first 'modern' strawberry. In nearly 200 years of subsequent breeding, the most notable achievement has been to transform the strawberry from a plant with a short season of production and a modest yield of small, soft berries to a highly productive plant capable of cropping over a long period with large firm berries suitable for shipping over long distances. A notable example of this has been the progress achieved in the last 70 years by the breeding programme at the University of California. Cultivars were developed that both extended the range of the production area within the state and greatly increased the length of the production season. A comparison of the cultivars released in 1945–1966 with those released 1993–2004 showed yield, fruit size and firmness increased by factors of 2.4, 1.7 and 1.9, respectively (Shaw and Larson 2008). These cultivars have helped California establish its dominant position in US production, where it currently supplies 88% of the market for fresh and frozen strawberries, and the value of the California strawberry crop was approximately $1.6 billion in 2015 (California Department of Food and Agriculture). Furthermore, the example from the development of the industry in California led to similar expansion of local industries in many other parts of the world including the Mediterranean region, Central and South America, Australia and China. In all of these regions, it was possible to identify areas where a combination of short days with warm or mild temperatures made it possible to grow the UC cultivars successfully, demonstrating the great adaptability of strawberry plants. Most countries also have local breeding programmes, developing cultivars that are specifically adapted for the climatic conditions and the market, but there are now also large international breeding companies such as Driscoll's who have breeding programmes covering many countries across six continents.

The fact that strawberries are produced in 76 countries is an indication of their popularity with consumers worldwide but also demonstrates their value to many different local economies. Despite great advances in extending shelf life and cool chain marketing, the fresh berries remain a perishable crop that needs to be produced close enough to the market to avoid excessive transport costs. This is in contrast to many tree fruit crops, which can be successfully stored for long periods and thus shipped worldwide, resulting in production being focussed in regions that are geographically suited to produce high yields at a relatively low cost per unit. The situation is different with perishable berry crops because there is often a good economic rationale for production even in marginal areas. Harvesting of dessert strawberries is currently all done by hand, and this makes the crop labour intensive, particularly during the harvest season. In turn, this provides employment and benefits the local economies in the production regions. In some countries local labour is readily available for harvesting, but in others, such as North America and Western Europe, there is a heavy reliance on migrant labour with the workers often staying on the fruit farms in temporary accommodation for the duration of the season.

1.1.1 Global Production

Between 2004 and 2013, the world production of strawberries increased by 41% (Table 1.1),

Table 1.1 Strawberry production in the main growing regions (tonnes)

Country or region	2013	10-year trend (%)
World	7,739,622	+41
China	3,005,304	+61
USA	1,360,869	+36
European Union	1,125,552	+2
Mexico	379,464	+114
Turkey	372,498	+140
Egypt	254,921	+143
South Korea	216,803	+7
Russia	188,000	−9
Japan	160,237	−19
Morocco	145,233	+37

Source FAOStat

indicating both the growing economic importance of the crop and the growing demand from consumers. Mexico, Turkey and Egypt are the three countries to have shown the greatest increase over this period, and this demonstrates a trend for production to shift to countries that have climates offering long growing seasons combined with readily available labour at relatively low cost and proximity to large domestic or export markets.

1.1.1.1 North and Central America

For many decades, the USA was the world's largest producer of strawberries before being overtaken by China in 1994. Production is focussed in two states that have an ideal climate and soils for strawberries with California producing 88% of the total crop and Florida 9%. Jointly, these two states are able to supply the US market year-round. The domestic market is very strong with per capita consumption almost doubling over the last two decades due to consumers having increased awareness of the health aspects of eating berries coupled with an improved year-round availability. Annual consumption was 4.5 kg per person in 2014 (USDA-NASS) of which 3.6 kg was fresh fruit and 1.4 kg frozen. From 1995 to 2014 the US population increased by 20% to 319 million (US Census Bureau), which combined with the increased consumption resulted in the market increasing by 132%. Over the same period production increased by 107%, with most of the balance provided by imports from Mexico who supply over 99% of the imported fresh fruit and 82% of frozen strawberries. The USA is a net importer of strawberries but still exports 11% of its production, with Canada being the main market for fresh and frozen berries and Japan also receiving significant volumes of frozen.

California and Florida both have breeding programmes in the public and private sectors. These programmes have been very successful in developing cultivars that have been successful not only in these two states but in many other regions throughout the world, thus providing an important income stream from plant or fruit royalties returned to the breeders. In particular, cultivars released from the programmes at the Universities of California and Florida are grown throughout the world and have often been used to start large-scale strawberry production in regions that have not previously grown strawberries on a significant scale. These cultivars then often form the base germplasm for new breeding programmes designed to develop cultivars with improved local adaptation.

Strawberry growing in Mexico has increased greatly in the twenty first century, and it is now the world's third biggest producer. The main strawberry growing regions are Michoacan and Guanajuato in central Mexico and Baja

California in the northwest (Wu et al. 2012). Mexico typically exports 30–45% of its crop, making it the world's third largest exporter of fresh strawberries, and most of it is shipped to the USA. The season in Mexico commences earlier than California, thus providing a good export opportunity, but the seasons overlap considerably, and the rapid recent increase in Mexican imports has led to an oversupply of strawberries on the US market in some years. Most of the cultivars grown in Mexico originate from California and are a mixture of proprietary cultivars and those developed by the University of California.

1.1.1.2 European Union

Strawberries are produced in 26 countries within the European Union (EU) but Spain, Poland and Germany account for 58% of the production (FAOStat), while the leading nine countries produce 90% of the total (Fig. 1.1). Average per capita consumption is 5 kg (Binard 2016), slightly higher than the USA (4.5 kg), but this has remained fairly constant, and consequently the production has only increased by 2% over the 10 years to 2013 (Table 1.1). There is a high level of trade between countries within the EU, amounting to 37% of total production, and the two largest producers export high volumes. Spain has 28% of EU production at 312,500 tonnes but 83% of this is exported, mainly as fresh berries during the period February to May when the main market is in northern Europe, particularly Germany, France and the UK. Poland has 17% of EU production and is also a large exporter, but their 80% of the production is for the processing sector, with exports mainly as frozen berries which are used as the raw material for jams, dairy products and other processed foods.

Germany, France and the UK are the main importers within the EU market, and each of these also has a significant volume of home production. These countries represent strong

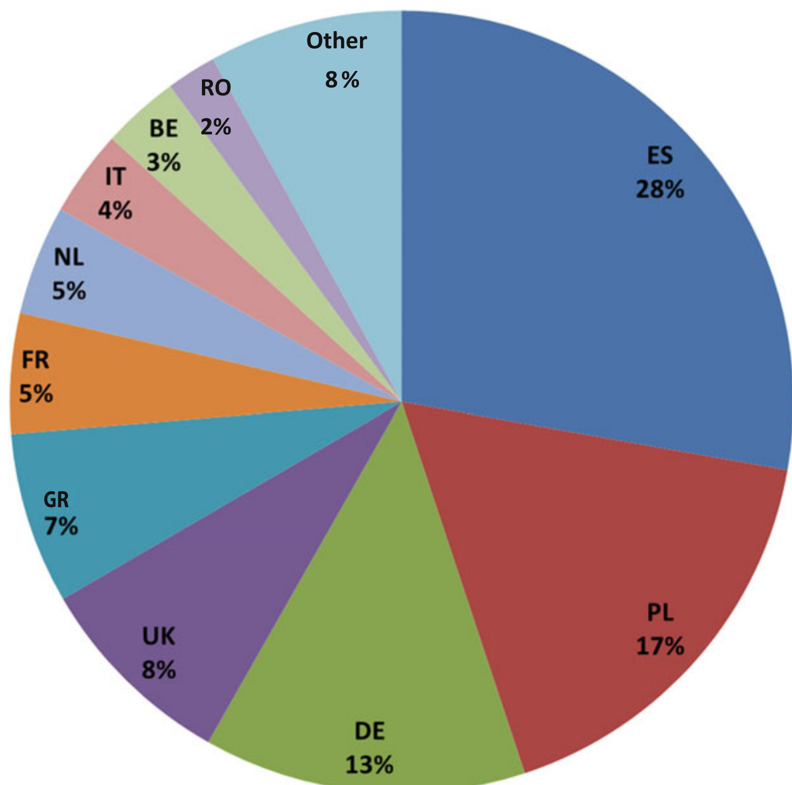

Fig. 1.1 Strawberry production in the European Union. The contribution of each country to the total production volume (tonnes) is shown (FAOStat 2013)

markets, which have been growing, but the per capita consumption is still below average for France and the UK at 3.8 and 3.5 kg per annum, respectively. Imports to and exports from the EU are relatively small compared to the internal trade. In 2013, imports of c. 29,000 tonnes were equivalent to less than 3% of the EU production with most fruit coming from Morocco and Egypt who can supply the market during the winter, before the Spanish season commences. Exports outside the EU account for 6% of total production, with the main markets being other European countries that are not members of the EU (Binard 2016).

There are many strawberry breeding programmes in the EU, in both the public and private sectors. All of the main producing countries have at least one significant programme and in many countries there are several. In the Mediterranean countries, production is based on low chill cultivars that will crop over a long period in late winter and early spring. Cultivars developed by the local programmes compete with those bred in California or Florida, and there is strong competition between the many programmes, resulting in a high turnover of cultivars. The cultivars grown in southern Europe are not adapted in the north, where the colder winters favour cultivars with a higher chilling requirement. Large, successful breeding programmes in France, Holland and the UK have produced most of the cultivars grown in northern Europe, but some cultivars from North America have also been successful, notably day-neutral types.

1.1.1.3 Asia

China is by far the world's largest producer of strawberries, but South Korea and Japan are also big producers in East Asia. Chinese production increased by 61% in the 10 years to 2013 and is now more than the combined output from North America and Europe. China is such a large country that it encompasses a wide range of different climates, but most strawberry production is concentrated in winter and spring, using low-chill short-day cultivars. However, demand outside this period has recently led to an increase in summer and autumn production using day-neutral cultivars. The main production regions are in the Liaoning, Sichuan, Shandong, Anhui and Hebei provinces (Zhang pers. comm.), which covers a geographical range from 30°N to 42°N, thus providing for a long season of production. Most of the crop is grown under protection from polytunnels or traditional Chinese greenhouses. These growing regions provide good proximity to the large cities and centres of population in central, eastern and north China, and 95% of the production is for the fresh market. Chinese consumers prefer strawberries with a high sugar content combined with low background acidity, and consequently the cultivars bred in Japan are preferred to those originating from the USA or Europe, where a different sugar:acid balance is preferred. Cultivars developed abroad currently dominate in China but in recent years there has been a significant increase in breeding activity, in both the public and private sectors, and successful Chinese-bred cultivars are increasing their market share.

Japan has a long history of strawberry growing, dating back to the 1880s, and it became a major producer from the 1950s. FAO statistics began in 1961, and from then until 2000 Japan was consistently in the top three strawberry producing countries worldwide. However, in the twenty-first century Japanese production has declined and it currently ranks ninth in the world, having been overtaken by some of the countries that have rapidly increased their production such as China, Mexico, Turkey and Egypt. The decline in production is attributed to demographic changes, such as ageing farmers and lack of successors, combined with lower per capita consumption (USDA GAIN report 2015), which reduced by 30% from 2000 to 2013. Nevertheless, strawberries remain a very popular fruit in Japan and, with negligible exports, virtually all the production is of fresh berries that are consumed within the country. In the 1960s, Japan developed a forcing technique for strawberries that was particularly well adapted for their climate (Yoshida 2013), and currently the growers are almost exclusively using Japanese-bred short-day cultivars that were developed for this system. The main season is from December to

May with virtually all production in glass or plastic greenhouses. This winter production is concentrated on the islands of Honshu and Kyushu, and in addition, there is a small amount of summer production on Hokkaido using ever-bearing and day-neutral cultivars. Imports of fresh strawberries to Japan are on a small scale, but in 2014 over 3000 tonnes were imported from the USA from July to November and mainly used in the confectionery trade. Imports of frozen berries are much larger, at over 30,000 tonnes, with China being the main source.

Strawberry growing in South Korea shows similarities with Japan as it has a large production of fresh berries of which 98% is consumed domestically. However, unlike Japan, the production in South Korea is increasing, with a growth of 7% in the 10 years to 2013. Around 97% of strawberries are grown in greenhouses with the peak production period being from January to March. Most of the production is in the central and southern regions of the country with Gyeongsangnam-do and Chungcheongnam-do provinces producing 66% while Jeollanam-do and Jeollabuk-do provinces produce around 20%. Until 2005, Japanese-bred cultivars were dominant, but in the last 10 years this situation has changed dramatically with now over 80% of production using cultivars bred in South Korea. The cultivars show similarities to those from Japan, being low-chill short-day types that are adapted to forcing and have a high sugar content combined with low acidity.

In west Asia, Turkey is the main strawberry producer. Strawberries have been grown in Turkey since the nineteenth century, but modern strawberry production is considered to have started in the 1980s when high-yielding cultivars were imported from California. There has been approximately a tenfold growth in production since that time, with an increase of 140% and in the 10 years to 2013 (Table 1.1). The main growing areas are the Mediterranean, Aegean and Marmara regions with the season covering winter and early spring (Kafkas 2017). Around 80% of production is under protection from low or high tunnels with the focus on high-quality fresh berries, but towards the end of the season the fruit is harvested for freezing and other industry uses. Approximately 90% of the fruit is consumed domestically with the remainder exported, mainly as fresh berries with the main markets being eastern Europe and Ukraine. Some Turkish-bred cultivars are grown, but this is on a fairly small scale, and most of the production is using cultivars from Florida, California and Australia.

1.1.2 Future Prospects

Strawberry production and consumption have increased dramatically in the twenty-first century, and this trend looks set to continue. China, for example, has experienced a huge increase in consumption of fresh strawberries running in parallel with strong economic growth, and it is likely that this trend will be repeated in other countries with rapidly developing economies, such as Brazil and India. Developments achieved through breeding and innovative agronomic systems have made fresh strawberries available throughout the year, so they are no longer considered a seasonal crop by consumers. Their popularity continues to increase in most countries because they are considered to be a healthy food that is affordable, pleasant to eat and requiring minimal preparation. The challenge facing producers will be to meet the increasing demand in a sustainable and cost-effective way. The largest cost in production is harvesting, which for all dessert berries is done by hand and this presents a challenge in countries where agricultural wages are high. Many countries rely on migrant labour for harvesting, but this can bring problems with availability of staff, provision of accommodation and political issues. Unsurprisingly, there has been a trend for production to move to countries where labour costs are lower, although these are typically further away from the main markets and long-distance shipping of perishable berries can be problematic and expensive. A possible solution to this problem could be provided by robotic harvesting, particularly for strawberries grown in glasshouses

or permanent polytunnels. Prototype harvesters have already been developed and tested in Japan, China, USA and Europe (Feng et al. 2012; Hayashi et al. 2010), but it is likely that if this technology becomes widespread then new cultivars will be required that are better adapted to the new production systems.

There is undoubtedly huge scope for further genetic improvement of strawberries to deal with the many challenges and opportunities facing growers who are striving to increase production and improve quality to satisfy an increasing market worldwide.

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Part I

The Genomes of Crop Wild-Relatives

2 Phylogenetics of *Fragaria, Rubus* and Related Taxa

Maria Kinga Sobczyk

Abstract

Fragaria L. and *Rubus* L. are two Rosaceae genera with strikingly different levels of taxonomic diversity (20 vs. ~500 species, respectively), yet with similar processes likely at play in their evolution—polyploidisation, hybridisation and apomixis. Each genus contains important soft fruit crops—garden strawberry in *Fragaria*, red raspberry and blackberries in *Rubus.* Furthermore, congeneric wild species have been used as a source of agronomically beneficial alleles. Both *Rubus* and *Fragaria* display well-supported monophyly, but resolution below the subgenus level in *Rubus* and beyond the two main clades in *Fragaria* is limited. More comprehensive sampling of plant genomes at a population level combined with more sophisticated models of phylogenetic inference is required for complete detangling of the reticulate genealogies in *Fragaria* and *Rubus*.

2.1 Introduction

Rosaceae is a monophyletic family in the core eudicot order Rosales, comprised of approximately 3000 species split over 88 genera and four classically circumscribed subfamilies (Maloideae, Amygdaloideae, Rosoideae, Spiraeoideae) which have been described on the basis of joint evidence of chromosome numbers and fruit type (Schulze-Menz 1964; Morgan 1994; Potter et al. 2002, 2007). However, recent genome-wide molecular analyses posit the merger of Maloideae, Amygdaloideae, and Spiraeoideae into Amygdaloideae and the split of the former Rosoideae into Rosoideae and Dryadoideae (Xiang et al. 2017). The cultivated perennial soft fruit species are concentrated in the subfamily Rosoideae, in addition to the roses (genus *Rosa*). Rosoideae features such species of notable economic importance to humans as: garden and woodland strawberry (*Fragaria* × *ananassa* and *Fragaria vesca*), red raspberry (*Rubus idaeus*), blackberries (e.g. *Rubus laciniatus*) and hybrid berries such as loganberry (*Rubus* × *loganobaccus*). Rosoideae occupies a basal position in the Rosaceae chloroplast and nuclear loci phylogeny from Potter et al. (2007). In this classification, the Rosoideae subfamily is split into three tribes: *Sanguisorbeae, Potentilleae* and *Colurieae* (Fig. 2.1). *Fragaria* belongs to the *Fragariinae* subtribe in the *Potentilleae*, while *Rubus* and

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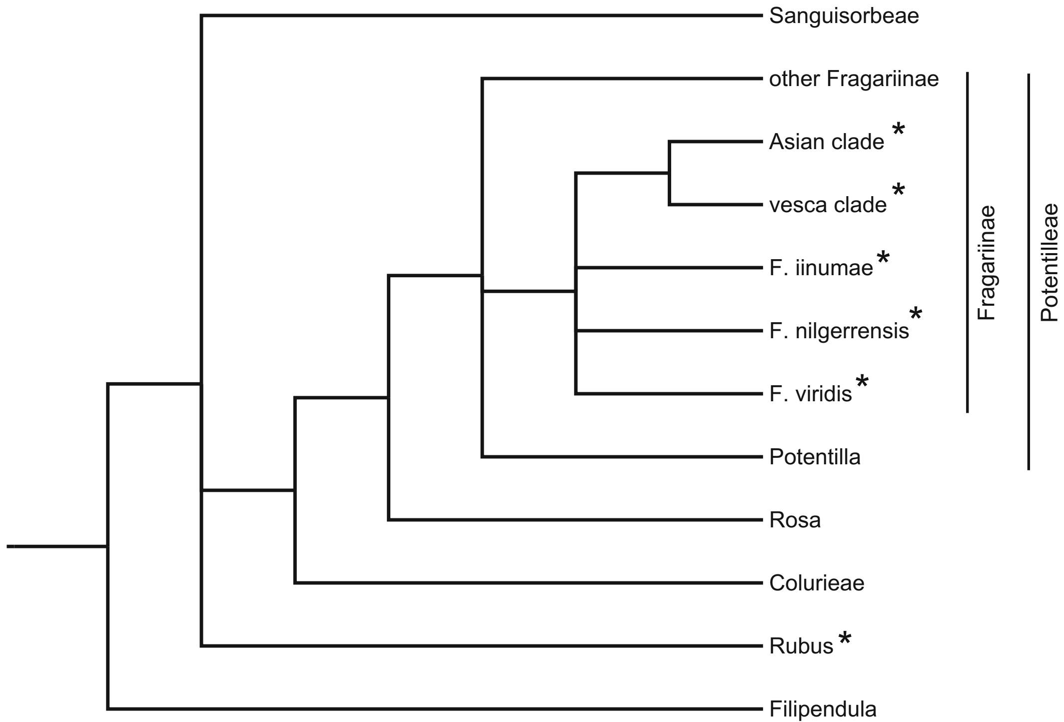

Fig. 2.1 Unrooted cladogram showing the well-supported phylogenetic relationships between different taxa of subfamily Rosoideae. Tips containing *Fragaria* and *Rubus* are marked with an asterisk (*). Unresolved placement of *F. iinumae*, *F. nilgerrensis* and *F. viridis* is depicted as a polytomy, after Potter et al. (2007)

Rosa are not placed within any tribe. Fossils of Rosaceae date to at least 90 Mya, with the crown group estimated to have arisen ~100 Mya at the boundary of Early and Late Cretaceous (Xiang et al. 2017). Later in Neogene, Rosaceae begins to appear in Asian geological record (DeVore and Pigg 2007).

Nowadays, Rosoideae is found widely distributed in the Northern Hemisphere. Rosoideae genera represent a range of species richness levels; while only 20 species have been described in *Fragaria* (Potter et al. 2000), *Potentilla* (Eriksson et al. 1998) and *Rubus* (Alice and Campbell 1999) and contain up to ~500 and ~750 circumscribed species, respectively, with species aggregates often used for taxonomic reference. Limited phylogenetic resolution frequently seen between different Rosoideae species is presumed to be due to a fairly recent and rapid radiation-like diversification. This hypothesis is supported by the remarkable phenotypic diversity displayed by members of the clade, with limited presence of taxonomically important synapomorphies and repeated evolution of traits such as plant growth habit and fruit type (Potter et al. 2007). For instance, species in the Rosoideae typically bear achene/achenetum fruits with the exception of drupetum in *Rubus*; however, this character is not a synapomorphy and has evolved multiple times. Species- and even genus-level barriers to gene flow are low in Rosoideae with complex role of polyploidisation (ancestral $x = 7$ or 9) and hybridisation events in the origin of its species (Illa et al. 2011). This has resulted in taxonomic complexity and provided fertile ground for a number of studies regarding their phylogenetic histories (Dickinson et al. 2007; Wang et al. 2016).

2.2 Fragaria

2.2.1 Introduction

The phylogeny of at least 20 wild species (Table 2.1) contained in the genus *Fragaria* L. is still not entirely understood due to frequent presence of complex polyploidisation events—nearly half of *Fragaria* species are polyploid (Folta and Davis 2006; Hummer et al. 2011). However, it is not for the lack of effort, as in fact, the second oldest phylogenetic network known is featured in Duchesne's *Natural History of Strawberries* (1766) and depicts a now confirmed relationship between polyploids *F.* × *ananassa* and *Fragaria chiloensis* and *Fragaria virginiana* (Morrison 2014). In terms of modern molecular phylogenies, *Fragaria* has been the subject of well-sampled studies based on (1) the nuclear ribosomal internal transcribed spacer (ITS) region and chloroplast *trn*L intron and *trn*L–*trn*F spacer, (2) nuclear *GBSSI* and *DHAR* genes (Rousseau-Gueutin et al. 2009), (3) nuclear *ADH-1* gene (DiMeglio et al. 2014), (4) chloroplast genome (Njuguna et al. 2013), (5) combined chloroplast genome, mitochondrial exome

Table 2.1 Taxonomic diversity of genus *Fragaria*, after Folta and Davis (2006), Liston et al. (2014)

Division	Species	Ploidy	Distribution
"*vesca*" clade	*F. vesca* L. subsp. *californica* Staudt	2*x*	California, Southern Oregon coast
	F. vesca L. subsp. *americana* Staudt	2*x*	N. America, east of Rocky Mountains
	F. vesca L. subsp. *bracteata* Staudt	2*x*	N. America, west of Rocky Mountains
	F. vesca L. subsp. *vesca* Staudt	2*x*	Europe to Siberia
	F. mandshurica Staudt	2*x*	Northeast Asia
	F. bucharica Losinsk.	2*x*	Western Himalaya
	F. x *bifera*	2*x*	France, Germany
	F. orientalis Losinsk.	4x	Northeastern Asia
	F. moschata Duchesne	6x	Western Eurasia
	F. iturupensis Staudt	8*x*/ 10*x*	Iturup Island of the Kuriles
	F. chiloensis Duchesne	8*x*	Western N. America, Chile, Argentina, Hawaii
	F. virginiana Duchesne	8*x*	N. America
	F. × *ananassa* Duchesne	8*x*	cultivated
	F. cascadensis Hummer	10*x*	Oregon, USA
"Asian" clade	*F. daltoniana* J. Gay	2*x*	Eastern Himalayas
	F. nipponica Makino	2*x*	Japan
	F. nubicola Lindl.	2*x*	Eastern Himalayas
	F. pentaphylla Losinsk.	2*x*	Northern and Southwest China
	F. chinensis Losinsk.	2*x*	China
	F. corymbosa Losinsk.	4*x*	Northern China, Russian Far East
	F. gracilis Losinsk.	4*x*	Northwest China
	F. moupinensis Cardot	4*x*	Southwest China
	F. tibetica Staudt & Dickoré	4*x*	Tibet
Unplaced	*F. iinumae* Makino	2*x*	Western Japan, eastern Russia
	F. nilgerrensis Schltdl. ex J. Gay	2*x*	Central and Southeast Asia
	F. viridis Duchesne	2*x*	Western Eurasia

and nuclear SNP markers (Govindarajulu et al. 2015), (6) 276 low-copy nuclear gene transcript sequences (Qiao et al. 2016), (7) 24 low-copy nuclear genes (Yang and Davis 2017) and (8) 257 low-copy nuclear genes (Kamneva et al. 2017).

The monophyly of *Fragaria* is well-supported (Potter et al. 2000; Lundberg et al. 2009). The sister genus to the *Fragaria* clade, *Potentilla* encompasses many ornamental plant species (Fig. 2.1). The most recent common ancestor of *Potentilla* and *Fragaria* has been estimated at 12.1–38.8 Mya (Njuguna et al. 2013)—possibly even up to 50 Mya as in Xiang et al. (2017), while the last common ancestor of all extant 27 *Fragaria* species and subspecies is estimated at between 1 and 8 Mya (Njuguna et al. 2013; Qiao et al. 2016). The young divergence of the clade placing it in the Pliocene–Pleistocene is corroborated by circumstantial evidence, such as low genetic differentiation observed within wild populations of *Fragaria* species, such as *F. chiloensis* and *F. virginiana* and the conservation of synteny (Hokanson et al. 2006; Rousseau-Gueutin et al. 2008).

The ploidy of *Fragaria* species ranges from diploids ($x = 7$, $n = 14$ species), such as the model species *F. vesca* and *Fragaria iinumae*, tetraploids (e.g. *Fragaria orientalis*), one hexaploid species (*Fragaria moschata*) and four octoploids of a complex allopolyploid origin (e.g. the cultivated strawberry *F.* × *ananassa*), and two decaploids—*Fragaria iturupensis* and recently described *Fragaria cascadensis* in Western Oregon, previously assigned to *F. virginiana* (Hummer 2012). Each species, with the exception of *F. iturupensis* (both octo- and decaploid), maintains a constant ploidy within species (Staudt and Olbricht 2008; Hummer et al. 2009).

Fragaria species from within and across the ploidy levels tend to produce vigorous hybrid offspring, such as diploid *Fragaria* × *bifera* (*F. vesca* × *Fragaria viridis*) (Njuguna et al. 2013), *Fragaria* × *bringhurstii* Staudt—pentaploid progeny of the octoploid *F. chiloensis* and the diploid *F. vesca* (Bringhurst and Khan 1963), suggesting limited divergence (Noguchi et al. 2002; Sargent et al. 2004; Bors and Sullivan 2005a, b). This conjecture is supported by so far poor resolution of the *Fragaria* phylogenetic tree based on traditional gene markers.

2.2.2 Overview of *Fragaria* Phylogeny

In earlier studies drawing on chloroplast DNA sequences (Harrison et al. 1997) and the nuclear ITS region (Potter et al. 2000), few well-supported monophyletic species clades were recovered, including *F. iinumae,* which occupies a single clade in the Sargent (2005) phylogeny of diploid *Fragaria* species. Furthermore, the Asian diploid *Fragaria* species occupy another distinct strongly supported clade according to Sargent (2005).

Building on that, both nuclear (Rousseau-Gueutin et al. 2009; DiMeglio et al. 2014; Qiao et al. 2016) and chloroplast (Njuguna et al. 2013) phylogenies lend strong support (>95% bootstrap support) to the presence of two main lineages in the genus: the "Chinese" clade made up of five diploid (*Fragaria pentaphylla*, *Fragaria daltoniana*, *Fragaria chinensis*, *Fragaria nipponica*, *Fragaria nubicola*) and four tetraploid species (*Fragaria moupinensis*, *Fragaria tibetica*, *Fragaria gracilis*, *Fragaria corymbosa*) found in the Sino-Himalayan region, except for *F. nipponica* with its range in Japan, Sakhalin and the Kurils (Staudt and Olbricht 2008); and the "*vesca*" clade which comprises 11 species from diploid to decaploid (diploid *F. vesca* subspecies—ssp. *vesca*, *americana*, *bracteata*, *californica*, *F.* × *bifera*, *Fragaria bucharica*, *Fragaria mandshurica,* tetraploid *F. orientalis*, hexaploid *F. moschata*, octoploid *F.* × *ananassa*, *F. chiloensis*, *F. virginiana* and octo-/decaploid *F. iturupensis*), distributed in North and South America, boreal Eurasia and Hawaii. In addition, the exact position of three diploid species (Eurasian *F. viridis*, Japanese *F. iinumae* and Southeast Asian *Fragaria nilgerrensis*) is not resolved yet (Fig. 2.1; Table 2.1).

2.2.3 Origin of Polyploid *Fragaria* Species

The existence of a strongly supported *F. vesca* clade containing polyploid species in addition to diploid *F. vesca* and *F. bucharica* and *F. viridis* is an indicator of reticulate evolution across ploidy levels. Indeed, the interesting question of the origin of polyploid *Fragaria* species has been the focus of a number of recent studies utilising an array of phylogenetics and population genetics methods.

The origin of the octoploids has been placed at somewhere between 0.37 and 2.05 Mya (Njuguna et al. 2013). Many *Fragaria* diploid and polyploid species are known to produce functional unreduced gametes, making gametic nonreduction the most likely mechanism of chromosome number doubling in polyploid *Fragaria* (Dickinson et al. 2007; Bringhurst and Gill 1970). The evolutionary history behind the origin of various polyploids in *Fragaria* appears complex, with various scenarios considered in the past, and no definitive sequence of events established for most species, in particular the cultivated strawberry *F.* × *ananassa.* While the actual event, which resulted in creation of this octoploid hybrid—crossing of two American octoploid species: South American *F. chiloensis* and North American *F. virginiana,* is historically well-documented and can be traced back to a botanic garden in Paris in mid-1700s, the origin of the parental species remains unresolved (Liston et al. 2014; Sargent et al. 2015). Due to the overall similarity and small size of *Fragaria* chromosomes, classical cytological methods have not been helpful in determining the karyotypic origin of the polyploids (Folta and Davis 2006).

First phylogenetic studies based on sequencing of nuclear low-copy genes suggested that one octoploid subgenome had been provided by an ancestral form of *F. vesca* or *Fragaria mandshurica*, while a second subgenome donor had been *F. iinumae* (Rousseau-Gueutin et al. 2009; DiMeglio et al. 2014), together forming a 2A,2A′,2B,2B′ genome structure, likely directly produced by a cross between two auto-polyploids of the two species (Bringhurst 1990). Lately, a new method (POLiMAPS—Phylogenetics Of Linkage-Map-Anchored Polyploid Sub-genomes), combining targeted sequencing of regions anchored in the *F. vesca* linkage map, painted a more complex picture (2Av,2Bi, 2B1,2B2) (Tennessen et al. 2014). One genome is hypothesised to have come from a *F. vesca*-like ancestor (Av), one from a *F. iinumae*-like ancestor (Bi) and two subgenomes (B1, B2) from a *F. iinumae*-like autotetraploid. Following the formation of the octoploid species, gene flow from the *F. vesca*-like subgenome appears to have resulted in homogenisation of the other three subgenomes. However, a recent study involving a novel class of markers—haploSNPs (Sargent et al. 2015)—does not support the origin of the B1, B2 subgenomes in a *F. iinumae*-like autotetraploid but rather in one or two more unknown diploid ancestors—possibly related to *F. viridis, F. bucharica* or *F. mandshurica* (Kamneva et al. 2017; Yang and Davis 2017)—which had formed a hexaploid with *F. iinumae* (2Bi, 2B1, 2B2) and subsequently introgressed with a diploid *F. vesca*-like species (2Av). However, comparison of the nuclear genome with mitochondrial and chloroplast genomes shows phylogenetic incongruence between the two organellar genomes and implies organelle capture at some point during the origin of the octoploids (Govindarajulu et al. 2015). In addition to Mahoney et al. (2009) and Njuguna et al. (2013), Govindarajulu et al. (2015) provide evidence that *F. vesca* subsp. *bracteata* was the chloroplast donor in the octoploid strawberries, while both *F. vesca* subsp. *bracteata* and *F. iinumae* were the sources of the mitochondrial genomes, which subsequently recombined (Mahoney et al. 2009; Njuguna et al. 2013; Govindarajulu et al. 2015). This is not surprising as the origin of the octoploids necessitates at least three interspecific hybridisation events, which could have been influenced by differential maternal and paternal inheritance of the organellar genomes.

The other octoploid (as well as decaploid) species, *F. iturupensis*, share the same plastid donor with *F. chiloensis* and *F. virginiana*, as the

three form a clade sister to *F. vesca* ssp. *bracteata* in a chloroplast phylogeny (Njuguna et al. 2013). Otherwise, little is known about this obscure endemic to Iturup Island in the Kuril archipelago (Staudt and Olbricht 2008). A study by Wei et al. (2017) utilising a newly constructed linkage map based on target-enriched sequencing shed more light on the final recognised octoploid species—*F. cascadensis*, which might share common origin with *F. iturupensis* in Beringia in the Pleistocene. Maximum likelihood phylogenies and topology tests in the study proved in agreement with an "ancient hybrid speciation" hypothesis featuring extinct taxa: paternal *F. iinumae*-like diploid progenitor and maternal octoploid progenitor ancestral to extant octoploid strawberries.

Consistent signal has been obtained regarding the origin of remaining polyploids in the "vesca" clade: the tetraploid *F. orientalis* and hexaploid *F. moschata* (musk strawberry), which may form a polyploid series (Harrison et al. 1997; Njuguna et al. 2013). Nuclear phylogeny points to allotetraploid origin of *F. orientalis* from *F. vesca* and *F. mandshurica* and indel mapping in the chloroplast spacers as well as the *Adh* gene phylogeny for *F. viridis* to be the third progenitor of *F. moschata* (Lin and Davis 2000; Rousseau-Gueutin et al. 2009; DiMeglio et al. 2014). The most comprehensive analysis of origin of lower-order *Fragaria* polyploids to date confirms the ancestry hypotheses above (Kamneva et al. 2017).

Kamneva et al. (2017) analysis involved identification of potential hybridisation events from 257 gene tree topologies with consensus network analysis followed by their evaluation using two maximum likelihood frameworks. It provided first insight into so far unknown phylogenetic history of tetraploids in the "Chinese" clade (*F. corymbosa*, *F. gracilis*, *F. moupinensis, F. tibetica*). Previously proposed diploid–tetraploid series of species within the "Chinese" clade (*F. pentaphylla* → *F. tibetica*, *F. chinensis* → *F. gracilis*/*F. corymbosa*, *F. nubicola* → *F. moupinensis*) put forward on the basis of matching geographical distribution, and some morphological similarities (Staudt and Dickoré 2001; Staudt 2008) have received only limited support in the current phylogenetic studies (Kamneva et al. 2017; Yang and Davis 2017). Instead, *F. corymbosa*, *F. gracilis*, *F. moupinensis* (Kamneva et al. 2017) are all shown to share *F. pentaphylla* and *F. chinensis* parentage. In the case of *F. tibetica, F. pentaphylla* and *F. nubicola* form the most likely ancestor pair.

2.3 Rubus

2.3.1 Introduction

Rubus L. is a highly heterozygous genus, with ploidy ranging from di to dodeca amongst its at least 430–500 species, which are taxonomically controversial due to morphological diversity, widespread hybridisation, polyploidisation (60% of species) with mixed infraspecific ploidy and apomixis (Jennings 1988; Meng and Finn 2002; Vamosi and Dickinson 2006; Evans et al. 2007; Wang et al. 2010). The classic taxonomy of *Rubus* by Focke (1914) partitioned *Rubus* into 12 subgenera (Jennings 1988; Sochor et al. 2015; Wang et al. 2016). The three largest subgenera, *Eubatus* Focke (=*Rubus L.,* 132 species), *Idaeobatus* (117 species), *Malachobatus* (115 species), contain the bulk of the species diversity. All of the other nine subgenera (*Anoplobatus*, *Chamaebatus*, *Chamaemorus*, *Comaropsis*, *Cylactis*, *Dalibarda*, *Dalibardastrum*, *Lampobatus*, *Orobatus*) contain less than 20 species, each (Table 2.2).

In the genome-wide low-copy gene phylogeny of the family Rosaceae, Rubeae tribe (containing exclusively *Rubus* L.) diverged very early around 75 Mya in Late Cretaceous (Xiang et al. 2017). So far, lack of dated paleobotanical sources and variable mutation rate have prevented any serious attempts at further dating in *Rubus* (Sochor et al. 2015). Nevertheless, coalescent analysis and ecological niche modelling suggest that the species range shifts associated with the climate change during the Last Glacial Maximum have increased the rate of hybridisation in the genus and possibly speciation (Mimura et al. 2014).

Table 2.2 Taxonomic diversity of genus *Rubus*, after Alice and Campbell (1999), Morden et al. (2003), Wang et al. (2016)

Subgenus	Species no.	Representative species	Ploidy
Anoplobatus Focke	6	*R. ribesoideus* Matsum., *R. odoratus* L.	2*x*–3*x*
Chamaebatus Focke	5	*R. calycinus* Wall. ex D. Don, *R. nivalis* Douglas, *R. pectinellus* Maxim.	2*x*, 6*x*
Chamaemorus Focke	1	*R. chamaemorus* L.	8*x*
Comaropsis Focke	2	*R. geoides* Smith.	4*x*, 6*x*
Cylactis Focke	14	*R. fockeanus* Kurz, *R. arcticus* L., *R. saxatilis* L., *R. lasiococcus* A. Gray	2–3*x*, 4*x*
Dalibarda Focke	5	*R. pedatus* Smith, *R. gunnianus* Hook.	2*x*
Dalibardastrum Focke	4	*R. tsangorum* Hand.-Mazz., *R. amphidasys* Focke ex Diels, *R. tricolor* Focke, *R. nepalensis* (Hook.f) Kuntze	4*x*, 6*x*
Idaeobatus Focke	117	*R. idaeus* L., *R. coreanus* Miq., *R. niveus* Thunb., *R. ellipticus* Smith, *R. occidentalis* L., *R. hirsutus* Thunb.	2*x*, 3*x*, 4*x*
Lampobatus Focke	10	*R. alpinus* Macfad., *R. parvus* Buch., *R. moorei* F. Muell.	4*x*
Malachobatus Focke	115	*R. buergeri* Miq, *R. assamensis* Focke, *R. parkeri* Hance, *R. lineatus* Reinw.	4*x*, 6*x*, 8*x*
Orobatus Focke	19	*R. nubigenus* Kunth, *R. roseus* Poir.	6*x*
Rubus L. (=*Eubatus* Focke)	132	*R. fruticosus* L., *R. caesius* L., *R. ursinus* Cham. et Schltdl., *R. cuneifolius* Pursh.	2*x*–12*x*
		R. leucodermis Douglas ex Torr. & A. Gray, *R. laciniatus* Willd., *R. armeniacus* Focke.	

While *Rubus* is distributed worldwide, most of its species are concentrated in the Northern Temperate Zone (Kalkman 2004). *Idaeobatus* is widely found across Europe, North America, Asia and South Africa (Jennings 1988). Subgenus *Rubus* is chiefly confined to Europe as well as North and South America, and *Malachobatus* centre of diversity is located in Asia. In particular, southwestern China is a key biodiversity hotspot for *Rubus*, with 139 endemic species (Yü et al. 1985; Lu and Boufford 2003). The taxonomic system adopted in China is similar to Focke's but differs in placement for a number of species (Lu 1983; Yü et al. 1985). Nearly, 200 *Rubus* species are split into eight subgenera but can mostly be found in *Idaeobatus* and *Malachobatus* (Lu 1983; Wang et al. 2016).

The domesticated species are concentrated in the subgenera *Idaeobatus* and *Rubus,* and the two can be distinguished by the separation of mature fruits from the receptacle found only in *Idaeobatus*. *Idaeobatus* species include the familiar crops: European red raspberry (*R. idaeus* subsp. *idaeus*), North American red raspberry (*R. idaeus* subsp. *strigosus* Michx) and the black raspberry (*Rubus occidentalis*). Blackberries, such as *Rubus ursinus* and *R. laciniatus,* are placed in the subgenus *Rubus* (Thompson 1995). Arctic berries include cloudberry (*Rubus chamaemorus*), found in the European boreal region and which hold potential for domestication (Korpelainen et al. 1999). Many introduced cultivated *Rubus* species, such as *Rubus armeniacus* (Armenian blackberry) and *Rubus fruticosus,* have become noxious invaders (Caplan and Yeakley 2010; Clark et al. 2013).

The first studies looking at *Rubus* taxonomy from the perspective of modern molecular phylogenetic methods were undertaken using (1) *ndh*F nuclear gene (Howarth et al. 1997),

(2) ITS region (Alice and Campbell 1999), (3) ITS region and chloroplast *trn*L–*trn*F spacer (Yang and Pak 2006), (4) three AFLP markers (Miyashita et al. 2015), (5) ITS region, cpDNA (Sochor et al. 2015), (6) three chloroplast (*rbc*L, *rpl20–rps12*, *trn*G–*trn*S) and three nuclear (ITS, *GBSSI-2, PEPC*) loci (Wang et al. 2016). These studies provide firm support for the monophyly of the genus.

2.3.2 Evolutionary Processes Important for *Rubus* Evolution

2.3.2.1 Hybridisation

The major evolutionary force in generating species diversity in *Rubus* is hybridisation, as shown by crossing and genotyping experiments (Kraft et al. 1995; Clark and Jasieniuk 2012; Sochor et al. 2015). In Europe, only four diploid (*Rubus ulmifolius, R. canescens, R. incanescens, R. sanctus*) and two tetraploid sexual species (*Rubus caesius*, *R.* ser. *Glandulosi*) of subgenus *Rubus* contrast with 744 polyploid, highly apomictic, variously classified taxa with low overall genetic diversity (Sochor et al. 2015; Šarhanová et al. 2017). cpDNA and ITS phylogenetic analyses indicate a reticulate evolutionary history of the group, with polyploids descendent from four extant and two unknown/extinct diploids, also involving unidirectional gene flow from the *Idaeobatus* subgenus.

Hybridisation between the *Idaeobatus* and *Rubus* subgenera as well as across ploidy levels occurs widely in nature and has been documented through cytology, crossing experiments, morphological similarities and molecular analysis of the ITS region (Alice and Campbell 1999; Alice et al. 2001). For example, in Europe, the tetraploid blackberry *R. caesius* (European dewberry, subgenus *Rubus*) hybridises with a diploid raspberry *R. idaeus* (subgenus *Idaeobatus*). It has been proposed that crosses between the two gave rise to a number of species, such as *Rubus maximiformis* and *Rubus picticaulis* (Alice et al. 2001). Another example of gene flow between distantly related species in *Rubus* is the origin of the hexaploid–dodecaploid *R. ursinus* from hexaploid Hawaiian endemic *Rubus macraei* (subgenus *Idaeobatus*) and an unknown species in the *Rubus* subgenus (Alice and Campbell 1999; Morden et al. 2003).

Ease of hybridisation in the genus has been employed by utilising the gene pool of wild species in breeding programs. For instance, *Rubus coreanus* and *Rubus crataegifolius* have been a source of traits such as high yield and resistance to pest and disease for cultivated raspberry. Moreover, commercial hybrids have been created using species from different subgenera—the loganberry (*R.* x *loganobaccus*) being the result of a cross between octoploid blackberry *R. ursinus* and diploid raspberry *R. idaeus* (Keep et al. 1977; Briggs et al. 1982; Gu et al. 1995; Clark et al. 2011).

2.3.2.2 Polyploidisation

Polyploidy is not evenly distributed in *Rubus*. While *Idaeobatus* is mostly diploid, subgenera *Chamaebatus*, *Malachobatus*, *Dalibardastrum, Orobatus* and *Comaropsis* contain only polyploids (Thompson 1997; Naruhashi et al. 2002). Meiotic pairing behaviour and rDNA distribution patterns in *Malachobatus* tetraploids are consistent with their allopolyploid origin (Wang et al. 2015). Polyploidisation has played a particularly important role not only in the evolutionary history of the subgenera, which are exclusively polyploid but also in largely polyploid blackberries (subgenus *Rubus*) (Alice and Campbell 1999). Up to dodecaploid (12*x*) species have been found in *Rubus*, with mode fixed at tetraploidy (Thompson 1997).

Frequent polyploidisation and facultative apomixis in *Rubus* led to low levels of intraspecific genetic variation (Amsellem et al. 2001; Dickinson et al. 2007). In fact, it has been put forward that the species concept should only apply to *Rubus* passing the hard criteria of sexual reproduction and diploidy, due to high frequency of apomictic biotypes of varied ancestry with limited geographical range (less than 50 km in diameter) (Weber 1996). For that reason, up to 900–1000 species had been described in the

genus (Thompson 1997) but a number of species have now been culled down to a total of ∼500 species grouped into infrageneric ranks, such as section and series (Šarhanová et al. 2017).

2.3.3 Overview of *Rubus* Phylogeny

2.3.3.1 Challenges in *Rubus* Taxonomy and Phylogeny

Extensive phenotypic plasticity and homoplasy in *Rubus* make use of morphological characters in reconstructing the genus taxonomy unreliable and led to polyphyletic lineages being classified by Focke as distinct subgenera (Nybom and Schaal 1990; Alice and Campbell 1999). However, at the same time extensive hybridisation and introgression in the genus make a given single gene tree not necessarily reflect the species tree due to stochastic sampling (Degnan and Rosenberg 2009). In the future, whole genome phylogenies will allow us to revisit the phylogenetic relationships within and between different subgenera.

Current phylogenetic analyses in *Rubus* have only so far provided good resolution at the subgenus level, but not yet at the species level, especially in the key sections *Idaeobatus* and *Malachobatus* due to insufficient variation and multiple reticulation events (Wang et al. 2016). The latter could also contribute to instances of incongruence observed between chloroplast and nuclear data detected in subgenus *Idaeobatus* in a study of Chinese *Rubus* (Wang et al. 2016).

Only one phylogenetic study has sampled all 12 *Rubus* subgenera (56 species in total) so far. In Alice and Campbell (1999), low variability in the nuclear ITS region permitted resolution just around the subgenus level and supports monophyly of subgenus *Orobatus,* and tentatively, *Rubus* and *Malachobatus*. The three main clades recovered (bootstrap support >90%) consisted of (1) *Comaropsis*, *Dalibarda*, *Lampobatus* species in the Southern Hemisphere, (2) subgenus *Rubus* with *Rubus alpinus* of subgenus *Lampobatus*, (3) remaining subgenera, including *Idaeobatus*.

Species in the nine smaller subgenera formed clades with species from the big subgenera (*Idaeobatus*, *Malachobatus*) and amongst themselves, with variable bootstrap support and polytomies, further underscoring the need for more revision in *Rubus* taxonomy and new molecular phylogenies to discern the origin of its species, in particular polyploids. Initial such proposals include move of *Rubus trifidus* from subgenus *Anoplobatus* to *Idaeobatus* (Alice and Campbell 1999; Yang and Pak 2006). Additional sampling of all species in the nine smaller subgenera is likely to flag up even more inconsistencies between molecular phylogenies and classical taxonomies. For instance, Alice and Campbell (1999) sampled four out of ten *Lampobatus* species, and one of them—*R. alpinus*—appears more closely related to the *Rubus* subgenus, while *Rubus gunnianus* of subgenus *Dalibarda* and *Rubus geoides* of subgenus *Camaropsis* allied with the other three *Lampobatus* species (Alice and Campbell 1999).

2.3.3.2 Asian *Rubus*

A recent phylogenetic study of Chinese *Rubus* featured the most comprehensive sampling of subgenera *Idaeobatus* (63 species) and *Malachobatus* (36 species) (Wang et al. 2016). It recovered *Idaeobatus* as polyphyletic with at least four independent clades, and interestingly, correlating with leaf shape morphological variation. On the other hand, *Malachobatus* appears monophyletic, with *Rubus tsangorum* and *Rubus amphidasys* of sect. *Dalibardastrum* and *Rubus peltatus* of subgenus *Idaeobatus* nested. Morphologically and karyotypically, heterogeneous three species of subgenus *Cylactis* form a polyphyletic group, and the two polyploid species in subgenus *Dalibardastrum* might have originated from sections *Moluccani* and *Stipulosi* of subgenus *Malachobatus*.

Idaeobatus

The main Chinese *Idaeobatus* lineage consists of species in sections *Thyrsidaei*, *Idaenthi*, *Pileati*, *Wushanenses*, *Stimulantes*, *Pungentes* with individual sections also polyphyletic and not

supporting the classical taxonomy. The second clade consisting of sections *Rosaefolii, Leucanthi, Corchorifolii*, as well as some species from *Stimulantes* and *Pungentes*, is only present in the cpDNA tree but not nuclear gene tree. In particular, sections *Stimulantes* and *Pungentes* appear particularly taxonomically challenging, with *Rubus ellipticus* (*Stimulantes*), *Rubus pinfaensis* (*Pungentes*) forming a third strongly supported clade in *Idaeobatus*. Lastly, the sections *Alpestres* and *Peltati* are related to subgenus *Malachobatus*, forming either a mixed clade (section *Peltati*) or positioned as sister to *Malachobatus* (section *Alpestres*).

Malachobatus

The taxonomy of *Malachobatus*, comprised of tetra-, hexa-, octo-, tetradeca-ploids is problematic, with, e.g. sections *Moluccani*, *Elongati*, *Sozostyli* not recovered as monophyletic groups in any phylogeny (Thompson 1997; Naruhashi et al. 2002; Wang et al. 2008, 2016). Certain polyploids in the subgenus *Malachobatus* may share common origin, through forming a polyploid series out of hybrids between *Rubus pentagonus* of subgenus *Idaeobatus* and species in the subgenus *Cylactis*. Such hypothesis is offered by the placement of the polyploids in *Malachobatus* and three *Cylactis* members as a sister group to *R. pentagonus,* in addition to 45S rDNA genomic in situ hybridisation (GISH) results (Wang et al. 2015). Furthermore, in Alice et al. (2008) *R. pentagonus* clustered with two *Malachobatus* polyploid species (*Rubus calophyllus*, *Rubus lineatus*) and the three species show taxonomically informative morphological similarities.

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Genomic Resources for the Woodland Strawberry (*Fragaria vesca*)

3

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Abstract

Fragaria vesca is one of the putative diploid progenitors and donor of subgenome A in the octoploid genome of the economically important cultivated strawberry (*Fragaria* × *ananassa*). With its small genome size, short generation time, and well-established transformation system, *F. vesca* is an ideal model species for *Fragaria* and other rosaceous crops. The *F. vesca* genome was first sequenced in 2011 and since then, with the availability of other molecular genetics and genomic resources, the assembly and annotation of the draft genome were further improved. The *F. vesca* draft genome has been an invaluable resource to the strawberry research community, with numerous studies using it as reference genome to identify candidate genes related to agronomically important traits. It has led to the generation of a 90 K array, metabolic pathways database, and various other new genetic resources. The strawberry genome also has been used in comparative genomics studies that elucidated the relationship among members of the Rosaceae family and identified genes and genomic signals across several rosaceous species. Altogether, the discoveries made with this genome will provide potential ways to improve cultivated strawberry and expand on our understanding of the Rosaceae family.

3.1 Introduction

The allo-octoploid strawberry (*Fragaria* × *ananassa*) is one of the economically significant crops in the Rosaceae family, with the strawberry industry in the USA valued at $2.9 billion (USDA NASS 2017). The USA is the top producer of strawberries in the world, accounting for nearly a third of total strawberry production. Strawberries are mainly sold for fresh market consumption, with fresh market and processing accounting for 81 and 19% of strawberry production, respectively (USDA NASS 2017). In addition to its economic importance, strawberry is emerging as a powerful model system to investigate how plant genomes have and continue to be shaped by both natural and artificial selection. Furthermore, the *Fragaria* genus includes species with various ploidy levels, from diploid to decaploid (Liston et al. 2014), making it an excellent system for examining consequences of polyploidization thus further increasing the scientific importance of this species.

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Cultivated strawberry was developed from a cross between two wild octoploid species, *Fragaria virginiana* and *Fragaria chiloensis*. Without an octoploid genome ($2n = 8x = 56$; Bombarely et al. 2010), genetic analysis of the cultivated strawberry (*F.* × *ananassa)* has been difficult to accomplish. It has been proposed that the various subgenomes comprising the octoploid genome can be traced to contemporary diploid species. *Fragaria iinumae*, *Fragaria nubicola*, and *Fragaria vesca* are often suggested as potential diploid progenitors, with *F. vesca* being the most widely studied and used as a model system for studying cultivated strawberry. To date, a draft genome of *F. vesca* is available and serves as a reference genome for *Fragaria* species. Here, we review the *F. vesca* genome sequenced by the Strawberry Genome Project in 2011 (Shulaev et al. 2011), and the research conducted with it and the improvements made to the genome since then.

3.2 Pre-genome Era

During the past two decades, research targeting a diverse array of important traits in *Fragaria* species has been gaining momentum, and molecular genetics and genomics resources have developed rapidly. Molecular markers such as AFLP, RAPD, SCAR, and SSR were developed to examine the genetic diversity in strawberry and construct linkage maps for comparative genomics studies and marker-assisted breeding. Quantitative trait loci (QTL) mapping studies have described marker-trait associations for different traits such as sex expression (Spigler et al. 2008, 2010; Goldberg et al. 2010), flowering habit (Kaczmarska and Hortynski 2002; Albani et al. 2004; Sugimoto et al. 2005), and disease resistance (e.g., Haymes et al. 1997; Guérin et al. 2003; Denoyes-Rothan et al. 2005). In addition, the combination of linkage studies and candidate gene approaches have resulted in the identification of genes involved in the regulation of flower and fruit development, fruit quality, and yield and response to biotic and abiotic stresses in strawberry (Deng and Davis 2001; Ruiz-Rojas et al. 2010; Zorrilla-Fontanesi et al. 2011). Also, the development of transformation and in vitro regeneration systems for strawberry made functional analysis of some of the candidate genes possible (Haymes and Davis 1998; Wang et al. 2004; Oosumi et al. 2006; Husaini and Abdin 2008).

Despite the significant progress in characterizing some of the underlying genetic factors influencing important agronomic traits in strawberry, the process has been slow, expensive, and resource intensive. The scarcity of genomic resources for *Fragaria* has hindered research efforts over the past decade, especially when compared to other economically important members of the Rosaceae family, such as pear and apple, with well-developed physical maps and draft genome sequences (Velasco et al. 2010). Initial insight into *Fragaria* genome content came from the analysis of ~1.0 Mb of *F. vesca* gene space generated through Sanger sequencing of 17–44 kb DNA pieces captured in thirty randomly selected fosmids (Pontaroli et al. 2009). A total of 184 protein-coding genes were predicted to occupy the ~1 Mb assembled insert. Based on this initial gene prediction, it was estimated that *F. vesca* genome contains ~30,500 genes. Transposable elements (TEs) were also identified and long terminal repeats (LTR) *copia-* and *gypsy-like* were the the most abundant TEs. Sequencing of additional 20 gene-targeted *F. vesca* fosmids (30–52 kb inserts) revealed gene sequences that are potentially involved in flower and fruit development and disease resistance in *Fragaria* (Davis et al. 2010). Despite this additional genomic resource, information on *Fragaria* genome remained lacking. Thus, to improve the genomic resources for strawberry, the Rosaceae research community leveraged the decreasing cost of sequencing to generate a draft genome sequence for strawberry. Although it would have been ideal to sequence the commercially relevant octoploid *F.* × *ananassa*, the complexity of its genome and lack of more advanced computational tools at that time made it quite challenging. Thus, *F. vesca*, the diploid woodland strawberry and a subgenome contributor to the octoploid genome, was chosen as the first reference genome (Shulaev et al. 2011).

3.3 Post-genome Era: The Woodland Strawberry—*Fragaria Vesca*

In the 1300s, the small-fruited woodland strawberry, *F. vesca*, was considered an ornamental plant and used as ground cover in gardens across Europe (Hummer and Hancock 2009). However, interest in the economic value of its fruits grew over time and by mid-sixteenth century, woodland strawberry was grown on nurseries and fields for commercial production (Wilhelm and Sagen 1972). Both white or yellow- and red-fruited *F. vesca* are distributed in Europe and Asia but only *F. vesca* subsp. *vesca* was able to establish in North and South America (Liston et al. 2014). The sequenced *F. vesca* "Hawaii 4" was collected in a volcanic area in Hawaii but was also found growing in South American Andes. Based on molecular phylogenetic analyses, diploid *Fragaria* species were classified into three groups, with *F. vesca* grouped together with *Fragaria viridis* and *Fragaria mandshurica* while *F. iinumae* is considered monophyletic, and the remaining diploids falling into the third group (Rousseau-Gueutin et al. 2009; Njuguna et al. 2013). Aside from being a subgenome contributor to the octoploid strawberry, *F. vesca* was also proposed as one of the parents of the tetraploid species, *Fragari orientalis* (Rousseau-Gueutin et al. 2009), and the hexaploid, *Fragaria moschata* (Staudt 1959; Potter et al. 2000). *F. vesca* "Hawaii 4" genome size is estimated to be ~250 Mb (Shulaev et al. 2011) which is 1.8x bigger than Arabidopsis but 2x and 3x smaller than the sequenced diploid pear and apple, respectively (Velasco et al. 2010; Wu et al. 2013). In addition to being a likely ancestor of the higher ploidy *Fragari*a species and having a small genome size, *F. vesca* also is easy to propagate, has a short generation time and well-established transformation systems, making it an ideal model species for the rosaceous crops (Oosumi et al. 2006; Shulaev et al. 2011).

The genome of the yellow-fruited, inbred line *F. vesca* "Hawaii 4" (accession number PI551572; $2n = 2x = 14$) was sequenced using three short-reads sequencing platforms, Roche 454, Illumina/Solexa, and SOLiD (Shulaev et al. 2011). The assembly, using Celera Assembler version 5.3, generated 3263 scaffolds with N50 = 1.36 Mb, of which 272 scaffolds represents 95% of the 240 Mb estimated genome size. Due to unavailability of a dense linkage map or well-developed physical map, the assembled genome sequence was anchored to a linkage map with 390 genetic markers. This linkage map was developed using an F_2 population derived from a cross between *F. vesca* 815 and *F. nubicola* 601 (Sargent et al. 2008). Out of the 272 scaffolds, 204 scaffolds (~94% of the genome size) was anchored to the map. Given the limited genomic resources to help improve the genome, scaffold alignment and orientation errors existed in the assembled v1.0 draft genome. In 2011, an updated version of the draft genome (v1.1) was released with a revised pseudomolecule assembly due to changes made in the orientation and position of some of the contigs.

In these versions of the draft genome (both v1.0 and v1.1), a total of 34,809 gene models were annotated using an ab initio gene prediction software, GenMark-ES+, in which 25,050 genes have protein domains and have homologs in other plant species (Shulaev et al. 2011). Gene ontology annotation indicated that majority of these genes are involved in "molecular function," "catalytic activity," and "biological processes." Unique gene cluster analysis suggested that ~55% of the annotated genes aligned to 9,895 gene families (derived from gene sequences of rice, Arabidopsis, grape, and strawberry) wherein ~7% of these gene families are unique to strawberry. During annotation, RNA genes including transfer RNA, ribosomal RNA, spliceosomal RNA, small nucleolar RNA, and microRNA were also identified. Annotation of repetitive sequences and transposable elements in *F. vesca* genome revealed 576 TE exemplars which occupy ~22% of the genome. Among the transposable elements, the most abundant ones are the LTR retrotransposons and miniature inverted repeats (e.g., CACTA). Compared to other plant species, *F. vesca* has relatively low number of transposable elements (e.g., LTRs)

and this is suggested as one of the reasons for the relatively small genome size (Shulaev et al. 2011).

With the goal of further improving the current version of the annotation and with increasing availability of genomic resources for *F. vesca*, the group of Darwish et al. (2015) re-annotated the *F. vesca* draft genome v1.1. Using the MAKER pipeline and a combination of other resources including reference guided and de novo assembled transcripts from 25 different tissue types of *F. vesca*, trained ab initio predictors (SNAP and Augustus), gene predictions from the first version of the annotation, and protein sequences from different plant species, a second-generation annotation (TowU_Fve) was generated (v1.1a2). In this second version of the annotation, 2286 more genes were identified, of which ~1600 gene models contain a protein domain and have homologs in other plant species. In addition, the draft genome was also annotated in 2015 by an automated pipeline in NCBI, the NCBI Eukaryotic Genome Annotation Pipeline. NCBI's annotation predicted 27,288 gene models wherein around 88% was protein coding. The set of proteins of the annotated coding genes was searched against the *Arabidopsis* known RefSeq proteins and out of 24,056 genes (88%), 21,576 genes were found to have homologs. The discrepancy in the number of annotated genes between Darwish et al. (2015) and NCBI is possibly due to differences in the annotation pipeline used and version of the genome that was annotated.

The completion of the *F. vesca* draft genome provided an invaluable resource for the strawberry research community. The reference genome has been used to improve our knowledge of strawberry in a variety of ways, such as through comparative genomics, transcriptome, and metabolite profiling studies. The genome has also been used to expand our understanding of the Rosaceae family, which includes several other important crops like apple, cherry, and peach. Furthermore, as a subgenome contributor to the octoploid genome, the *F. vesca* draft genome can also serve as a reference genome to assist in the analysis and annotation of the octoploid *Fragaria* genome and related species.

Transcriptome studies utilizing the strawberry genome have targeted a wide range of important traits in strawberry. For example, Kang et al (2013), through a reference-guided transcriptome assembly, created transcriptome profiles for five different stages in early strawberry fruit growth to examine the role of auxin and gibberellin (GA) in fruit and receptacle development. Their findings showed auxin and GA receptors were more highly expressed in the receptacle tissue, suggesting the achene is the source of auxin and GA while the receptacle is the affected tissue. Strawberry is unique because the fleshy edible portion that many call the "fruit" is actually an enlarged receptacle tissue while the true fruit is the dry achenes on the receptacle surface (Kang et al. 2013). The same group also conducted a transcriptome study on flower development in strawberry by examining different floral tissues from various developmental stages (Hollender et al. 2014). Their study revealed that several meristem regulators, such as *LOST MERISTEM* and *WUSCHEL*, were potential hub genes in the developing receptacle network due to their high number of connecting edges. In addition to studies that examined underlying genetic factors regulating fruit development, other groups have used the annotated genome to uncover genes associated with abiotic stress response. As an example, Wang et al. (2017) scanned the *F. vesca* genome for potential basic leucine zipper (bZIP) genes involved in drought and heat stress and candidate genes were identified through expression analysis (Wang et al. 2017).

In addition to gene expression projects, the genome has been used to identify and compare metabolic pathways with the creation of FragariaCyc (Naithani et al. 2016). FragariaCyc is a database of curated metabolic pathways in strawberry. As of 2016, the database contained 66 super-pathways, 488 unique pathways, 2348 metabolic reactions, 3507 enzymes, and 2134 compounds (Naithani et al. 2016). FragariaCyc also allows researchers to compare different metabolic pathways to one another, upload and analyze expression data using the Omics Viewer tool, and compare pathways across multiple plant

species. FragariaCyc continues to be updated based on current research in strawberry, making it a valuable resource to the strawberry community.

The *F. vesca* genome was also used to develop 90 K Axiom® SNP array for cultivated strawberry (Bassil et al. 2015). For the strawberry array to be effective for the cultivated octoploids, it must contain a large number of SNP markers distributed across all four subgenomes. To accomplish this, 19 octoploid *Fragaria* accessions and one diploid were sequenced and mapped to the *F. vesca* reference genome. Mapping revealed 36,140,217 unique variants in the sequenced genomes at over 10,619,615 coordinate sites in the *F. vesca* genome (Bassil et al. 2015). After quality checking and filtration, 95,063 of these variants were included as markers on the array (Bassil et al. 2015). The development of this SNP array provides a useful tool for marker-assisted breeding in cultivated strawberry.

The completion of the *F. vesca* genome added to the growing number of Rosaceae genomes available for analysis. To date, the apple (*Malus* × *domestica*), pear (*Pyrus bretschneideri; Pyrus communis*), Chinese plum (*Prunus mume*), peach (*Prunus persica*), and black raspberry (*Rubus occidentalis*) genomes have been assembled (Velasco et al. 2010; Wu et al. 2013; Chagné et al. 2014; Zhang et al. 2012; Verde et al. 2013; VanBuren et al. 2016). With the increasing number of available reference genomes in the Rosaceae family, the Rosaceae phylogeny was reconstructed by examining 124 rosaceous species (Xiang et al. 2016). The study generated 115 new transcriptomic datasets and utilized available transcriptomic and genomic datasets, including the sequenced genomes (excluding black raspberry, which was not yet completed). Phylogenetic analysis was conducted to date the emergence of different tribes and genera and identify potential whole genome duplications that may have contributed to the evolution and diversification of fruit in Rosaceae. The black raspberry and strawberry genomes are largely collinear with several large-scale rearrangements (VanBuren et al. 2016). The relationship between black raspberry and peach was more complex, with each black raspberry chromosome mapping to several different peach chromosomes (VanBuren et al. 2016). These and other analyses made possible by Rosaceae genomes will help continue to expand our knowledge of the history of this economically important family.

3.4 Current Status of the *F. vesca* Genome

In 2014, Tennessen et al. released an updated version of the *F. vesca* genome. Improvements were made based on linkage maps generated from *Fragaria vesca* ssp. *bracteata* (Fvb) individuals. Three linkage maps were used to update the genome, with two of the linkage maps generated based on an outcross between two individuals collected from a single population in Mary's Peak, Oregon (Tennessen et al. 2013). Based on the resulting progeny and the DNA sequences of the parents, distinct linkage maps were created for the maternal parent (MRD30), a male-sterile individual, and the paternal parent (MRD60), a hermaphrodite (Tennessen et al. 2013). The third map was generated from the F1 of a selfed *F. vesca* ssp. *bracteata* collected from the Lincoln National Forest, Cloudcroft, NM (Tennessen et al. 2014).

The maternal parent from Oregon was sequenced at low coverage on the Illumina GAIIx using 80 bp single end sequencing (Tennessen et al. 2013). The paternal parent, the offspring from the cross, and the selfed offspring were sequenced on the Illumina HiSeq 2000 with 101 bp paired end sequencing at Oregon State University (Tennessen et al. 2013). The resulting reads were then quality filtered and mapped to the *F. vesca* reference genome along with two scaffolds missing from the released genome. Biotinylated RNA baits for polymorphic sites identified between the parents based on reference mapping were designed and used for effective linkage mapping (Tennessen et al. 2013).

Linkage maps were created using OneMap in R and after manual correction, seven linkage groups were generated, as expected for *F. vesca*.

The polymorphisms used to create the linkage maps were placed in linear order and then compared to the *F. vesca* ssp. *vesca* reference genome (FvH4). The new linkage map assembly was used as evidence to rearrange and, in some cases, break up the scaffolds of the reference genome to create a more accurate assembly. In the comparison of the updated Fvb assembly and the reference genome, 44 interchromosomal translocations, 40 intrachromosomal translocations, 39 inversions, and 18 placements of unmapped *F. vesca* regions were found (Tennessen et al. 2014). Based on these translocations, 39 splits were made in the 246 FvH4 scaffolds and two additional scaffolds were added. The 287 new scaffolds created were rearranged to match the Fvb maps. The new assembly, known as the *F. vesca* v2 genome, contained 208.9 Mb where 207.0 Mb were assembled into 7 pseudochromosomes and 1.9 Mb were left unassembled. Some of the rearrangements may be true differences between the two subspecies, but most were likely due to assembly. For example, if rearrangements were as common as the comparison between the Fvb linkage map and the FvH4 genome suggests, then more rearrangements would have been observed between the three Fvb genomes. Also, most of the rearrangements showed the entire scaffolds were misplaced, which is more likely to be an assembly error rather than a true rearrangement (Tennessen et al. 2014).

The amount of unassembled sequence was decreased with the Tennessen update, but 1.9 Mb of the scaffold sequence remained unassembled (Tennessen et al. 2014). In addition to the unassembled sequences, there are likely errors in the assembly due to the limitations of the sequencing and assembly methods used. Recently, the genome was re-sequenced using long-read single-molecule real-time Pacific Biosciences sequencing (Edger et al. 2018). With this method, 2.5 million reads were generated, spanning 19.4 Gb with an average N50 length of 9.2 kb. The raw reads were assembled using the Canu assembler and polished through two rounds with Quiver (Koren et al. 2017; Chin et al. 2013). High coverage Illumina data was aligned to the PacBio assembly and error corrected using Pilon (Walker et al. 2014). The final error corrected assembly contained 61 contigs with an N50 length of 7.9 Mb, spanning 219 Mb. A high-resolution, two-enzyme optical map from Bionano Genomics was then used to anchor the contigs to pseudomolecules. First, the contigs were scaffolded with the BsqQI map and then the resulting assembly was used as a reference for the BssSI map. After the BioNano and PacBio data was combined, the assembly spanned 220.8 Mb across 31 scaffolds with an N50 length of 36.1 Mb. With this method, over 99.8% of the assembly was anchored to the seven pseudomolecules and ~24.96 Mb of new sequence was added to the genome, known as the *F. vesca* v4 genome (Edger et al. 2018). When the v2 and v4 genomes were compared, numerous large-scaled scaffolding errors were found in each chromosome (Fig. 3.1). Annotation for the new assembly was completed using the MAKER-P annotation pipeline, previously published *F. vesca* transcriptome data, *Arabidopsis thaliana* protein sequences, and the UniprotKB database. The annotation identified 28,588 gene models, which included 1496 genes not annotated in the previous genome annotation (Edger et al. 2018). The majority of these new genes were identified in gaps that were in previous versions of the genome. This improved genome, along with the vast amount of genomic data and resources already available for strawberry, will help accelerate strawberry research.

3.5 Future Research

Due to decreasing sequencing cost and availability of more advanced sequencing platforms and bioinformatics tools, construction of a pan-genome for *Fragaria* species is becoming feasible. Pan-genome formation involves the comparison of genomes from different genotypes within a species to establish a core genome (genes shared by all genotypes) and a disposable

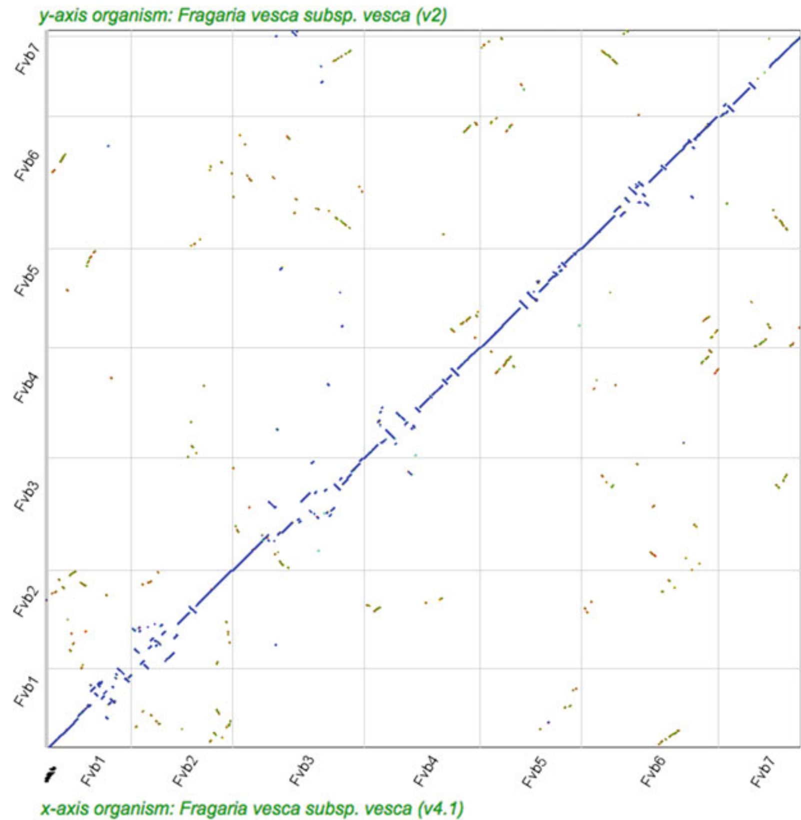

Fig. 3.1 Macrosyntenic plot comparing the v2 (*y*-axis) and v4 (*x*-axis) *Fragaria vesca* genome assemblies at the chromosome level (Fvb1–Fvb7) (Edger et al. 2018). Syntenic "orthologous" regions are shown within diagonal boxes comparing each chromosome (e.g., Fvb1 v2 and Fvb1 v4) while other syntenic blocks between different chromosomes are retained duplicated regions from an ancient polyploid event unique to eudicots. Negatively sloped and repositioned sections represent misassembled and incorrectly scaffolded regions in v2

genome (genes found in a subset of genotypes). Pan-genomes will serve useful to the broader research community to better understand the underlying genetic architecture that encodes various traits. Similarly, breeders can utilize the pan-genome by selecting genotypes with beneficial genes for use in breeding projects. Insights into the evolution of the *Fragaria* genus will increase the resources available for researchers and provide a framework for similar studies in other Rosaceae genera.

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4 Control of Flowering in Strawberries

Elli A. Koskela and Timo Hytönen

Abstract

Strawberries (*Fragaria* sp.) are small perennial plants capable of both sexual reproduction through seeds and clonal reproduction via runners. Because vegetative and generative developmental programs are tightly connected, the control of flowering is presented here in the context of the yearly growth cycle. The rosette crown of strawberry consists of a stem with short internodes produced from the apical meristem. Each node harbors one trifoliate leaf and an axillary bud. The fate of axillary buds is dictated by environmental conditions; high temperatures and long days (LDs) promote axillary bud development into runners, whereas cool temperature and short days (SDs) favor the formation of branch crowns. SDs and cool temperature also promote flowering; under these conditions, the main shoot apical meristem is converted into a terminal inflorescence, and vegetative growth is continued from the uppermost axillary branch crown. The environmental factors that regulate vegetative and generative development in strawberries have been reasonably well characterized and are reviewed in the first two chapters. The genetic basis of the physiological responses in strawberries is much less clear. To provide a point of reference for the flowering pathways described in strawberries so far, a short review on the molecular mechanisms controlling flowering in the model plant Arabidopsis is given. The last two chapters will then describe the current knowledge on the molecular mechanisms controlling the physiological responses in strawberries.

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4.1 The Yearly Growth Cycle

Strawberries are small perennial plants that reproduce both sexually through seeds and clonally via runners. Strawberry stem is called rosette crown that consists of short internodes produced from the apical meristem. Each node harbors one trifoliate leaf and an axillary bud. Axillary buds may develop into runners, which are elongated stems with two long internodes followed by a daughter plant, into axillary leaf rosettes called branch crowns or remain dormant. The apical meristem may keep on producing new nodes or develop into a cymose inflorescence. When the apical meristem turns into an

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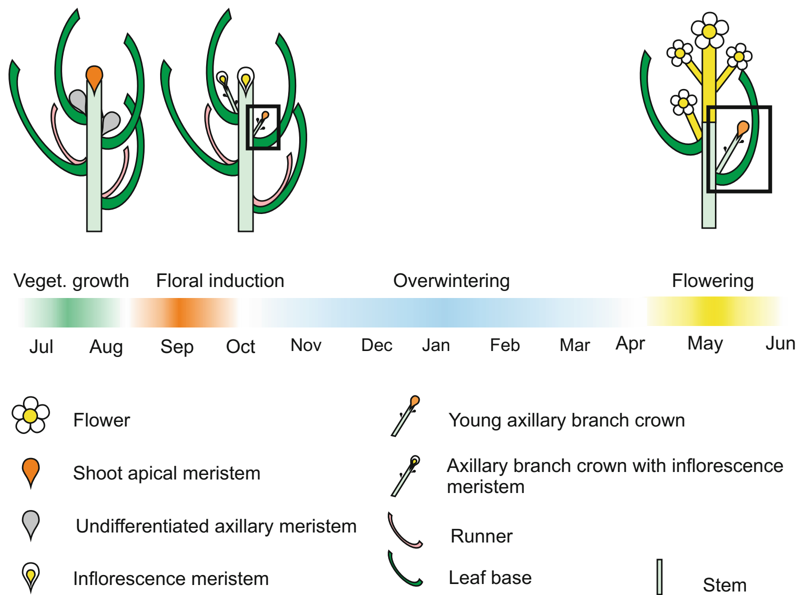

Fig. 4.1 Plant structure and typical seasonal growth cycle of strawberries grown in temperate zone. During the summer months, strawberries grow vegetatively and axillary buds produce runners, whereas in autumn, young axillary buds differentiate into branch crowns. Floral induction occurs in autumn, and apical meristems of main and branch crowns develop into inflorescence meristems. Inflorescence development is completed in the following spring and summer when plants flower and produce fruits. The axillary buds that were too young to be induced to flower the preceding autumn (highlighted with black rectangles) grow out as new branch crowns, thus completing the perennial life cycle

inflorescence meristem, the uppermost axillary bud develops into a branch crown that sustains vegetative growth in a sympodial fashion (Fig. 4.1; Darrow 1966).

The fate of the apical and axillary meristems is dictated by seasonal changes in photoperiod and temperature. During the summer months, strawberries grow vegetatively and axillary buds differentiate into runners. In autumn, axillary buds develop into branch crowns instead of runners, and the apical meristems of the main crown, and bigger branch crowns form terminal inflorescences. Short days (SDs) in autumn also cause an overall halt in vegetative development, and the plants enter a state of semi-dormancy characterized by shorter petiole length and stunted growth (Sønsteby and Heide 2011). Winter chilling is needed to break this dormant state, and in the following spring, inflorescences initiated the preceding autumn complete their development, and the next seasonal cycle follows (Fig. 4.1; Darrow 1966).

The seasonal growth cycle described above holds true for both the diploid woodland strawberry *Fragaria vesca* (L.) and its cultivated octoploid relative *Fragaria* × *ananassa* (Duch.), although exceptional environmental responses have been described in both species (Sect. 1.2). In both species, flowering is induced in SDs at cool to medium temperatures and inhibited by long days (LDs) and high temperature. Because of the close resemblance of physiological responses, it has long been speculated that the molecular regulatory networks controlling environmental responses may be similar in the two species (e.g., Battey et al. 1998). Indeed, this view recently gained empirical evidence when Koskela et al. (2016) showed that some of the core genes involved in photoperiod and temperature-regulated flowering responses function similarly in the two strawberry species. Due to these similarities, it is feasible to present findings on diploid and octoploid strawberry physiology and molecular pathways side-by-side.

In some cases, this is also necessary, as physiological responses have been examined at a greater depth in octoploid than in diploid strawberries, while molecular studies into flowering regulation have mostly focused on the diploid *F. vesca*.

To understand flowering, i.e., generative development, it is important to comprehend vegetative development. These two processes are often interrelated; for instance, the number of flowering-competent apical meristems in strawberry depends on the number and age of branch crowns on the plant and the timing of branch crown formation (Hytönen et al. 2004). Because of the interdependency between vegetative and generative development, this review aims to give a thorough picture of the effects that the seasonally changing environment imposes on the yearly growth cycle of strawberries. The latter chapters will then describe the current knowledge on the molecular mechanisms controlling these physiological responses.

4.1.1 Summer

Long days and high temperature during summer promote vegetative development, i.e., the differentiation of axillary buds into runners. Vegetative vigor is also manifested by increased petiole length (Sønsteby and Nes 1998; Heide and Sønsteby 2007). The wild diploid strawberry accessions studied to date stay in the vegetative stage when the daylength exceeds 16–17 h (Heide and Sønsteby 2007). However, the studied accessions originate from Norway, where the days are very long during summer. It is quite likely that more Southern accessions continue vegetative development under shorter photoperiods.

High temperatures of above 17–20 °C are considered optimal for runner production (Battey et al. 1998). Indeed, when diploid strawberry is subjected to cool temperature (11 °C), runner production ceases within a few weeks (Fig. 4.2). Also in cultivated strawberry, 16-h photoperiod and temperatures above 18–24 °C enhance both runner formation and petiole elongation (Heide 1977; Bradford et al. 2010), although different

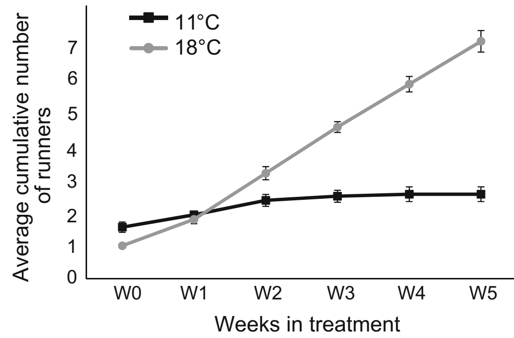

Fig. 4.2 Average cumulative number of runners at different temperatures. Runner-propagated plants of seasonal flowering *F. vesca* were grown in LDs at either 11 or 18 °C for five weeks. Error bars denote the standard error of the mean (n = 16 and 10 for 11 and 18 °C treatments, respectively)

cultivars may exhibit significant differences both in their overall runnering capacity and in their responses to photoperiod (Sønsteby and Nes 1998). On the other hand, SDs strongly suppress runner formation in most studied octoploid strawberry cultivars and also in the octoploid progenitor species *Fragaria virginiana* and *Fragaria chiloensis* (Serçe and Hancock 2005; Bradford et al. 2010).

4.1.2 Autumn

As the days get shorter and temperature lower toward autumn, runner formation ceases and the uppermost axillary buds develop into branch crowns. A prolonged exposure to SDs and/or cool temperature induces flowering, and the shoot apical meristem develops into an inflorescence meristem. Also, the apical meristems of the oldest branch crowns may form inflorescences, while the younger branch crowns remain vegetative and continue the typical life cycle of a perennial plant during the next growing season (Hytönen et al. 2004; Albani and Coupland 2010).

In the diploid strawberry, flower induction is triggered by integration of photoperiodic and temperature signals. Temperature is perhaps the more important factor, as it sets the limits for photoperiod-induced flowering. In most *F. vesca*

accessions, high temperature (>21 °C) inhibits flowering under all photoperiods, whereas cool temperature (10 °C) induces flowering photoperiod-independently. At intermediate temperatures (13–18 °C), flowering is promoted by SD conditions (Heide and Sønsteby 2007; Rantanen et al. 2015). Because of the strong interaction between temperature and photoperiod, the critical photoperiod for flower induction varies depending on temperature. At 15 °C, the critical photoperiod is between 16 and 17 h, whereas at 18 °C photoperiods shorter than 16 h are required to induce flowering (Heide and Sønsteby 2007; Rantanen et al. 2015). The wild strawberry accessions studied so far require at least four weeks of inductive light conditions for floral induction, with a longer exposure resulting in more rapid flowering (Heide and Sønsteby 2007). However, our results indicate that flower induction occurs earlier at cool temperatures (E. Koskela, T. Toivainen, and T. Hytönen, unpublished).

Seasonally flowering octoploid strawberry cultivars show a similar trend in their flowering responses to environmental conditions, although the critical temperatures and photoperiods vary from cultivar to cultivar. In most cultivars, flowering is inhibited by high temperatures of above 22 °C, and cool temperatures (10–12 °C) induce flowering independently of the photoperiod. At intermediate temperature range, flowering is promoted by SDs (Heide 1977; Bradford et al. 2010). The relative importance of the flowering pathways (photoperiod vs. temperature) differs between cultivars; some cultivars respond exclusively to short photoperiod, with no flower induction taking place in LDs even at cool temperature (Sønsteby and Nes 1998; Sønsteby and Heide 2006; Verheul et al. 2007). In other cultivars, flowering occurs photoperiod-independently at temperatures as high as 18 °C (Heide 1977; Bradford et al. 2010).

4.1.3 Winter

In addition to floral induction, autumnal environmental conditions bring about additional physiological changes in strawberries. An exposure to SDs and mild temperature for an extended period of 10 to 15 weeks has been shown to induce a state of semi-dormancy in both diploid (Sønsteby and Heide 2011) and octoploid strawberries (Darrow and Waldo 1934; Guttridge 1985; Sønsteby and Heide 2006). Semi-dormant plants have shorter petioles and smaller leaves than nondormant plants and are slow to resume growth when returned to LDs (Jonkers 1965). For example, semi-dormant plants of cultivar Elsanta resumed regular growth vigor after approximately one month in LDs (Lieten 1997).

The most effective dormancy-breaking treatment is subjecting the plants to several weeks of chilling, i.e., temperatures between −2 and 6 °C (Guttridge 1985). However, the chilling requirement is genotype-specific, and genotypes adapted to higher latitudes have longer periods of dormancy than genotypes adapted to more Southern conditions (Sønsteby and Heide 2011). The dormancy-inducing effect of SDs can also be averted by simultaneously subjecting the plants to low temperature at below 6 °C that constantly counteracts the dormancy-inducing effect of SDs (Lieten 1997; Sønsteby and Heide 2006, 2011). Dormant strawberries are not only slow to resume growth, but they also have lower pollen fertility, produce more misshapen fruits and lower yield than nondormant strawberries (Lieten 1997). Therefore, dormancy release by chilling is an important aspect from the point of view of practical crop production, and the progressing global warming may cause problems in the future, especially for growers in areas of mild winters.

4.1.4 Spring

Environmental conditions that favor vegetative development also promote inflorescence outgrowth and floral development in spring. According to studies on wild diploid strawberry accessions carried out under controlled conditions, photoperiod in spring has no effect on the number of flowers; however, longer days speed up flower development and thus have a mild

promoting effect on the timing of flowering (Heide and Sønsteby 2007). Also in the octoploid strawberry, LDs advance floral development in the spring (Sønsteby and Heide 2006).

The effect of temperature on the rate of flower and fruit development has not been studied in the diploid strawberry. Experiments with several octoploid strawberry cultivars suggest that warm spring temperatures speed up both flower and fruit development resulting in earlier cropping. However, there is a negative relationship between temperature and total yield; with rising temperature, a smaller proportion of flowers produce fruit, and the average fruit size is also smaller (Le Mière et al. 1998; Manakasem and Goodwin 2001).

4.2 Exceptional Environmental Responses in *Fragaria*

Although the environmental responses described above hold true for most genotypes of diploid and octoploid strawberries, both species contain genotypes with exceptional responses. One of the best studied exceptional responses is the everbearing character, which has been described in both species. Everbearing strawberries are capable of initiating flowers throughout summer, contrasting the seasonal flowering habit of the SD-induced strawberries. Moreover, everbearing strawberries tend to produce less runners than SD strawberries, and many diploid everbearing genotypes do not produce runners at all (Brown and Wareing 1965).

Flowering in the everbearing diploid strawberry genotypes is promoted by LDs. A study by Mouhu et al. (2009) demonstrated that flowering in the diploid accession Hawaii-4 occurs earlier under LDs than SDs at 18 °C, and this difference becomes more clear at higher temperature of 22 °C (Rantanen et al. 2014). Similar results were reported by Sønsteby and Heide (2008a), who showed also that LDs are almost obligatory for flower induction at 27 °C in two reportedly non-runnering diploid strawberry cultivars. Interestingly, the same authors also noted sporadic runner formation in these two cultivars at high temperature.

Everbearing octoploid strawberry cultivars have often been classified either as everbearers or day-neutrals, especially in the older literature. This distinction was based on the origin of the trait; everbearers originate from old cultivars with a LD flowering response, whereas the day-neutral trait is from a more recent introduction of *F. virginiana* ssp. *glauca* germplasm into the cultivated strawberry (Durner et al. 1984). More recently, it has been shown that both everbearing and day-neutral octoploid strawberries are actually obligatory LD plants at high temperature (27 °C), quantitative LD plants at intermediate temperatures and day-neutrals only at temperatures below 10 °C (Nishiyama and Kanahama 2002; Sønsteby and Heide 2007). It must be noted, however, that true day-neutrality has been described in a population of *F. virginiana* ssp. *glauca* from Utah which is considered the main source of the everbearing trait in modern-day everbearing cultivars (Sønsteby and Heide 2008b). Therefore, the origin of the photoperiodic response in modern everbearing cultivars is unknown.

Runnering is enhanced by high temperatures in both diploid and octoploid everbearing strawberries (Sønsteby and Heide 2007; 2008a). The effect of photoperiod is more difficult to interpret, and there seem to be differences between cultivars; some studies have reported that LDs promote runner production in octoploid everbearing cultivars (Manakasem and Goodwin 2001; Bradford et al. 2010), whereas other workers have described SDs to strongly enhance runnering (Sønsteby and Heide 2007). In a diploid everbearing strawberry accession Hawaii-4, SDs strongly promote runnering (K. Mouhu, S. Samad, E. Koskela, and T. Hytönen, unpublished). In general, conditions that do not favor floral induction appear to enhance runner production. In contrast to runner production, everbearing genotypes tend to produce more branch crowns than SD strawberries, thus

providing a greater number of crown apical meristems for inflorescence initiation. The higher number of branch crowns produced by ever-bearing genotypes results in higher yield potential (Camacaro et al. 2002), but on the other hand decreases the number of axillary buds that could develop into runners, which limits their utility for commercial production (Camacaro et al. 2002; Dale et al. 2002; Sønsteby and Heide 2007; Bradford et al. 2010).

Another exceptional environmental response is the vernalization requirement characterized in an arctic diploid strawberry accession from Northern Norway. This accession does not flower if exposed to SDs, even when the temperature is as low as 9 °C (Heide and Sønsteby 2007). The same authors tested also whether this arctic Alta accession could be induced to flower if exposed to 2 °C for 5–15 weeks. However, even after a 15week exposure, only a proportion of the plants (38%) flowered. It is noteworthy that the Alta accession does flower after overwintering in field conditions, albeit considerably later than the other tested Norwegian accessions (Heide and Sønsteby 2007). These data indicates that the Alta accession requires a prolonged period at exceptionally cold conditions to fulfill the vernalization requirement. Physiological and molecular characterization of the accession is underway.

4.3 Genetics of Flowering

Physiology of flowering is well studied in strawberries, but the genetic basis of the physiological responses is much less clear. Genetic control of flowering has been most extensively studied in the annual model plant Arabidopsis, in which both endogenous and environmental pathways regulate flowering (Bouché et al. 2016). Although Arabidopsis and strawberry are distantly related, many genetic components that regulate flowering in Arabidopsis have also been identified in strawberry (Mouhu et al. 2009). It is therefore likely that similar genetic mechanisms could control at least some aspects of reproduction in the two species.

4.3.1 Genetics of Flowering in Arabidopsis

Arabidopsis in an annual plant in which flowering is promoted by LDs and high temperature. The photoperiodic flowering response in Arabidopsis is controlled by a rhythmic expression output generated by the circadian clock and involving the action of dozens of genes (Bouché et al. 2016). The CCT domain transcription factor *CONSTANS* (*CO*) is one of the rhythmically regulated genes, and it is required for the LD-induced flowering in Arabidopsis (Putterill et al. 1995; Sawa et al. 2007). At the protein level, CO stability is regulated by light; the protein is rapidly degraded in dark, and thus only *CO* expressed during the light period leads to accumulation of CO protein (Andrés and Coupland 2012).

The circadian regulation of CO is active in leaf vascular tissues, whereas inflorescences are initiated at the shoot apical meristem. The mobile signal linking these tissues is FLOWERING LOCUS T (FT; Koornneef et al. 1991; Corbesier et al. 2007), which is a member of the CETS protein family (CEN, TFL1, and FT; Pnueli et al. 2001). *FT* expression is upregulated in LDs directly by CO (Tiwari et al. 2010), and the translated FT protein is actively exported from the phloem companion cells to sieve elements and transported to the shoot apex (Liu et al. 2012).

Members of the CETS protein family do not have DNA binding domains, but they do form complexes with other proteins (Yeung et al. 1999; Pnueli et al. 2001). In the shoot apical meristem, FT forms a complex with a bZIP transcription factor FD and 14–3–3 proteins (Pnueli et al. 2001; Abe et al. 2005; Wigge et al. 2005) to upregulate downstream target genes, including the MADS-box transcription factor *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*; Samach et al. 2000) and the meristem identity genes *APETALA1* (*AP1*; Abe et al. 2005), and *FRUITFUL* (*FUL*; Teper-Bamnolker and Samach 2005). The expression of the meristem identity genes is considered a sign of irreversible commitment to flowering (Hempel et al. 1997).

In Arabidopsis, FT is involved also in the ambient temperature regulation of flowering. Changes in ambient temperature have an effect on chromatin conformation at the *FT* locus, with low temperature making the locus less accessible to transcription factors (Kumar and Wigge 2010). One of these transcription factors is PHYTOCHROME INTERACTING FACTOR4 (PIF4), a bHLH transcription factor that activates *FT* transcription and promotes flowering in SDs at high temperature (Kumar et al. 2012).

Another pathway that regulates flowering as a response to ambient temperature involves the MADS-box transcription factors SHORT VEGETATIVE PHASE (SVP) and FLOWERING LOCUS M (FLM). The floral repressor SVP is degraded at high temperature (Lee et al. 2013). FLM can be spliced in alternative ways in a temperature-dependent manner; at low temperature, the prevailing splice form is the flowering-repressive FLM-β. Higher temperatures cause an increase in the relative amount of the splice variant FLM-δ, which is impaired in DNA binding (Posé et al. 2013). FLM-β and FLM-δ compete for forming heterodimers with SVP; at low temperature, the most abundant complex is SVP-FLM-β, which actively prevents flowering. However, at high temperature the prevailing protein complex is SVP-FLM-δ, which cannot bind to DNA and thus cannot repress the expression of the target genes. The derepressed target genes, including at least *SOC1* and possibly *FT*, can therefore promote flowering as a response to high temperature (Lee et al. 2013; Posé et al. 2013).

The shoot apex is a convergence point for both floral-promoting and inhibitive factors. One of the factors with a repressing effect on flowering is TERMINAL FLOWER1 (TFL1; Shannon and Meeks-Wagner 1991), which belongs to the same CETS protein family as FT (Kobayashi et al. 1999). Similarly to FT, also TFL1 is able to form complexes with FD and 14–3–3 proteins (Abe et al. 2005; Wigge et al. 2005; Hanano and Goto 2011; Ho and Weigel 2014). The floral-promoting or inhibiting effect of FT or TFL1, respectively, is hypothesized to be caused by differential recruitment of coactivators, possibly TCP transcription factors (Ho and Weigel 2014).

The abovementioned pathways have been characterized in the annual plant Arabidopsis. However, the components of especially the photoperiodic pathway are fairly conserved across land plants. Homologs of *CO* and *FT* have been shown to regulate the photoperiodic responses in, e.g., the short-day monocot rice (Hayama et al. 2003), and *FT* homologs seem to have roles as universal floral promoters in, e.g., cucurbits (Lin et al. 2007), barley (Yan et al. 2004), and many perennial species including poplar (Böhlenius et al. 2006) and apple (Tränkner et al. 2010). There is also some evidence for *FT* playing a part in ambient temperature responses in, e.g., chrysanthemums (Nakano et al. 2013) and Satsuma mandarin (Nishikawa et al. 2007), as in these species the ambient temperature-induced flowering correlates with increased transcription of *FT*-like genes.

4.3.2 Genetics of Flowering in the Diploid *F. Vesca*

The molecular control of flowering in the diploid strawberry was truly begun to be deciphered after the publication of the *F. vesca* genome in 2011 (Shulaev et al. 2011). In 2012, two groups independently utilized genetic mapping to show that the switch from a seasonal flowering phenotype to an everbearer is brought about by a small deletion in a gene encoding the strawberry ortholog of *TFL1* (*FvTFL1*; Iwata et al. 2012; Koskela et al. 2012). *TFL1* homologs repress flowering in many plants, including the Rosaceae family crop species rose (Iwata et al. 2012), apple (Kotoda et al. 2006) and pear (Freiman et al. 2012).

Also in strawberry, *FvTFL1* functions as a floral repressor, whose downregulation is required for floral induction (Koskela et al. 2012). *FvTFL1* expression responds to both photoperiod and temperature; cool temperature downregulates the gene, high temperature causes an increase in its transcript level, and at intermediate temperatures, LDs upregulate and SDs

silence *FvTFL1* (Fig.4.3; Koskela et al. 2012; Rantanen et al. 2015). In the seasonal flowering *F. vesca* background, the decrease in *FvTFL1* mRNA level concurs with upregulation of the meristem identity genes, *F. vesca AP1* (*FvAP1*) and *FUL* (*FvFUL*), and results in flowering (Mouhu et al. 2009; Koskela et al. 2012; Rantanen et al. 2015).

FvTFL1 is regulated by the photoperiodic pathway through the action of *F. vesca FT1* (*FvFT1*) and *SOC1* (*FvSOC1*). Both genes are upregulated by LDs, and functional evidence from transgenic plants indicate that FvFT1 acts at least partially through upregulation of *FvSOC1* (Mouhu et al. 2013). Overexpression of *FvSOC1* in seasonal flowering *F. vesca* background leads to upregulation of *FvTFL1*, resulting in repression of flowering, whereas the RNAi silencing of *FvSOC1* leads to continuous flowering in LDs. Thus, activation of the photoperiodic *FvFT1*–*FvSOC1* pathway represses flowering in seasonal flowering *F. vesca* accessions in LDs by upregulation of the floral repressor *FvTFL1* (Koskela et al. 2012; Mouhu et al. 2013). Under SDs, however, *FvFT1* is rapidly downregulated (Rantanen et al. 2014, 2015), whereas the downregulation of *FvTFL1* and flower induction occur much slower (Heide and Sønsteby 2007; Koskela et al. 2012). Therefore, additional unknown factors are likely needed to downregulate *FvTFL1* in SDs.

Interestingly, altering *FvTFL1* expression level in the seasonal flowering *F. vesca* background did not change patterns of vegetative development, i.e., runner and branch crown formation (Koskela et al. 2012). These results seem to be at odds with physiological studies, which have indicated that flowering and runner formation are almost mutually exclusive processes (reviewed in, e.g., Hytönen and Elomaa 2011).

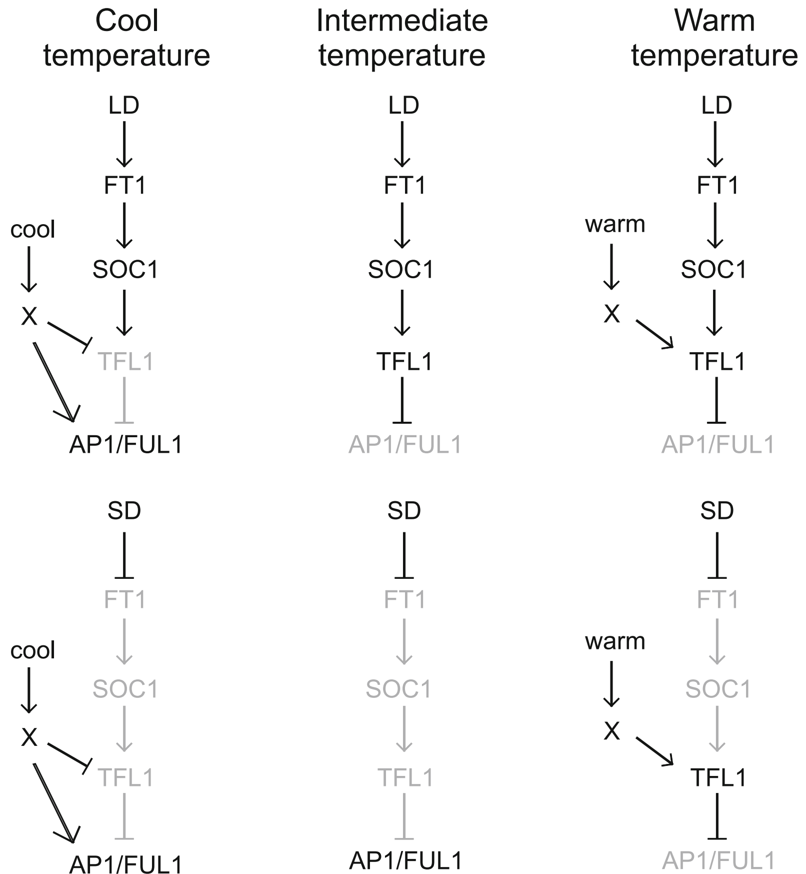

Fig. 4.3 Diagram of environmentally regulated flowering pathways in woodland strawberry. The upper row depicts genetic pathways that are active (in black) or inactive (in gray) in LD conditions at cool, intermediate or warm temperature. The lower row depicts the activity of the pathways under SD conditions. Temperature regulates the expression of *FvTFL1* and the meristem identity genes *FvAP1* and *FvFUL1* through unidentified genetic factors marked by 'X' in the diagram

However, this can be explained by the observations by Mouhu et al. (2013), who showed that overexpressing the photoperiodic pathway gene *FvSOC1* results in increased runner production whereas silencing the gene promotes axillary bud development into branch crowns. The changes in *FvSOC1* expression also alter the expression of many gibberellin (GA) biosynthetic and signaling pathway genes, and GA is needed for the differentiation of axillary buds to runners (Hytönen et al. 2009; Mouhu et al. 2013). Thus, *FvSOC1* and *FvTFL1* are components of the photoperiodic pathway, in which *FvTFL1* controls the onset of flowering and *FvSOC1* regulates both flowering via *FvTFL1* and aspects of vegetative development via the GA pathway.

Early physiological studies have suggested that inhibition of flowering and promotion of vegetative vigor in LD-grown strawberries could be caused by a hormone that is produced in leaves and transported to the shoot apex (Thompson and Guttridge 1959). The same workers also demonstrated that the effect of LDs could be mimicked by GA application (Thompson and Guttridge 1959; Guttridge and Thompson 1964). As the upregulation of *FvSOC1* leads to upregulation of the GA pathway, and *FvSOC1* is upregulated by *FvFT1* (Mouhu et al. 2013), it can be speculated that the LD-induced mobile 'hormone' could be FvFT1. The FvFT1 protein is likely produced in leaf vascular tissue and transported to the shoot apex, where it activates *FvSOC1* and results in increased vegetative vigor. In seasonal flowering strawberries, the floral-promoting effect of FvFT1 is counteracted by increased *FvTFL1* expression, which inhibits flowering (Koskela et al. 2012).

In everbearing accessions with mutated *FvTFL1*, the photoperiodic *FvFT1–FvSOC1* pathway is activated in LDs similarly to SD *F. vesca* accessions. However, the outcome of the pathway is reversed, as the mutation at *FvTFL1* causes a frameshift and renders the protein product non-functional (Koskela et al. 2012). In everbearing accessions, the LD-dependent upregulation of the putative mobile floral promoter *FvFT1* leads to upregulation of the meristem identity genes *FvAP1* and *FvFUL1* and causes early flowering (Koskela et al. 2012; Rantanen et al. 2014). A similar genetic basis for continuous flowering has been described in rose (*Rosa* × *wichuriana*), in which a retrotransposon insertion blocks *TFL1* transcription (Iwata et al. 2012).

The temperature-regulated pathway in strawberry is not as well characterized as the photoperiodic pathway. However, functional data strongly suggests that *FvTFL1* regulation has a central role in temperature-mediated responses. As described earlier, *FvTFL1* expression responds to changes in temperature, with higher temperatures increasing the transcript level of the gene, whereas *FvFT1* mRNA expression is reduced at high temperatures. Moreover, the upregulation of *FvTFL1* by high temperature is independent of the activity of the photoperiodic *FvFT1–FvSOC1* pathway, as high temperature upregulates *FvTFL1* also in plants with silenced *FvSOC1* (Rantanen et al. 2015). The factor that activates *FvTFL1* at high temperature remains unknown, but is a topic of active ongoing research. Thus, the ambient temperature-regulated pathway in strawberries appears to be very different from the pathway described in Arabidopsis, in which the temperature regulation of *FT* controls flowering time (Bouché et al. 2016).

How exactly FvTFL1 represses flowering in the diploid strawberry has not been directly studied, but results from other species provide strong cues as to its mode of action. As discussed earlier, it has been demonstrated that Arabidopsis FT and TFL1 can compete for binding to the bZIP transcription factor FD expressed in the shoot apical meristem (Abe et al. 2005; Wigge et al. 2005). Studies in several distantly related plant species, including tomato (Pnueli et al. 2001) and rose (Randoux et al. 2014) have indicated that the same competitive mechanism is active in a wide range of plant species. Therefore, it is likely that the same dynamic balance of FT and TFL1 controls the downstream responses also in strawberry.

In addition to strawberries, TFL1 maintains the vegetative status of the meristems also in several other Rosaceous species, including apple

(Flachowsky et al. 2012), pear (Freiman et al. 2012), and rose (Iwata et al. 2012). However, divergent spatial and temporal expression patterns of *TFL1* homologs can bring about different phenological outcomes. For example, apple *TFL1* (*MdTFL1-2*; Mimida et al. 2009) was recently described as having a role in limiting fruit set in apple trees with heavy fruit loads. In the study of Haberman et al. (2016), it was shown that variations in *MdTFL1-2* expression level correlated with the amplitude of alternate bearing of different apple cultivars and was related to different fruit loads on a tree. Since TFL1 seems to be a general floral repressor in Rosaceae, it is a target of active ongoing research. Studies in a Rosaceae model *F. vesca* are paving the way for molecular understanding of its regulation.

As discussed in Sect. 1.1.3, dormancy and dormancy release by chilling are important physiological events that have large effects on the next year's yield potential in strawberries. The molecular basis for these physiological responses have not been studied in detail in strawberries, but results from other Rosaceous species suggest that a group of DORMANCY-ASSOCIATED MADS (DAM; Bielenberg et al. 2008) transcription factors may contribute to the cessation and inhibition of growth in dormant perennial trees (Bielenberg et al. 2008; Saito et al. 2013). In peach, the loss of a cluster of six *DAM* genes abolishes the response to dormancy-inducing conditions (Bielenberg et al. 2008), and the expression patterns of three of these genes in peach buds support a role in growth cessation and onset of dormancy (Li et al. 2009). A similar trend in the seasonal expression patterns of *DAM* genes has been described also in Japanese pear; the expression of *DAM* genes increases during autumn and reaches the maximum level at the time of the deepest dormancy in November, after which *DAM* expression is gradually reduced (Saito et al. 2013). Other studies have indicated that *DAM* expression is likely regulated by CBF transcription factors, miRNAs, and epigenetic mechanisms (Leida et al. 2012; Niu et al. 2016; Saito et al. 2015). The onset of dormancy could be very well regulated by DAM transcription

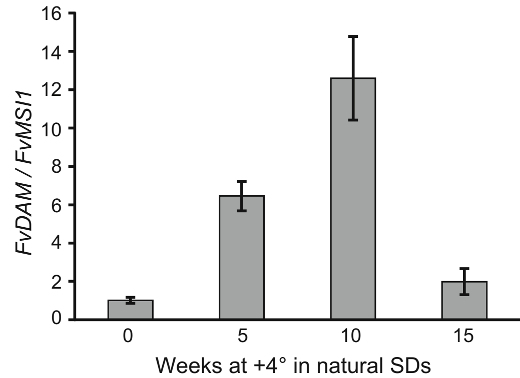

Fig. 4.4 Accumulation of *FvDAM* mRNA as a response to 4 °C under natural SD conditions. Plants of the Norwegian vernalization-requiring accession Alta were grown in the mentioned conditions, and shoot apex samples were collected at 0, 5, 10, and 15 weeks. Error bars denote standard deviation between biological replicates (n = 3)

factors in strawberries as well; *DAM* expression level increases gradually as a response to SDs and cool temperature, after which there is a sudden drop in *DAM* mRNA (Fig. 4.4).

Given the vast distribution of *F. vesca* around the Northern Hemisphere, it is to be expected that the species contains a range of different environmental responses. Indeed, accessions collected from Southern Europe are able to upregulate *FvFT1* at shorter photoperiods than more Northern accessions, and they have shorter critical photoperiod for flower induction (S. Kymäläinen, T. Toivainen, and T. Hytönen, unpublished). Whether *FvTFL1* or other flowering-time genes are differentially regulated in accessions of different latitudinal origins awaits further inspection.

4.3.3 Genetics of Flowering in the Octoploid Strawberry

Recent studies in the octoploid strawberry have taken advantage of the genetic data gained from the work with the diploid strawberry. As described above, *FvFT1* and *FvSOC1* are involved in the regulation of LD-dependent photoperiodic flowering in the diploid

strawberry. Several independent reports (Nakajima et al. 2014; Nakano et al. 2015; Koskela et al. 2016) have shown that the octoploid strawberry homolog of *FT1* (*FaFT1*) is expressed in leaf tissue exclusively in LD conditions. Moreover, the diurnal expression pattern of *FaFT1* is similar to that of *FvFT1* (Koskela et al. 2016), rendering support for the view that the two genes have the same LD-dependent function in the two species. The octoploid strawberry homolog of *SOC1* (*FaSOC1*) mostly shows similar expression patterns to *FvSOC1*, although cultivar-dependent differences have also been reported; whereas Koskela et al. (2016) consistently found high levels of *FaSOC1* expression only in LDs in the five studied cultivars, Nakano et al. (2015) were not able to detect such clear-cut differences between photoperiodic treatments in the Japanese cultivar Nyoho. Therefore, strawberry cultivars may have differences in the photoperiodic regulation of *FaSOC1* expression, but further experiments are needed to examine how these differences correlate with flowering time.

As *FvTFL1* is a major regulator of flowering responses in the diploid strawberry, it has been of great interest to elucidate whether the octoploid strawberry homolog (*FaTFL1*) has a similar function as a floral repressor. In the diploid strawberry, *FvTFL1* is photoperiodically regulated by the *FvFT1*–*FvSOC1* pathway, with LDs activating the pathway and leading to repression of flowering through the upregulation of *FvTFL1* (Koskela et al. 2012; Mouhu et al. 2013; Rantanen et al. 2014). At the gene expression level, similar responses to environmental conditions have been reported in several octoploid strawberry cultivars; *FaTFL1* is activated in LDs and also by high temperature (Koskela et al. 2016). However, there are cultivar-dependent differences in *FaTFL1* regulation, as some cultivars show age-dependent *FaTFL1* downregulation independently of the photoperiod (Nakano et al. 2015; Koskela et al. 2016).

It was recently reported that *FaTFL1* is indeed a floral repressor also in the octoploid strawberry, as silencing the gene in the SD cultivar Elsanta leads to flowering under LD conditions (Koskela et al. 2016). In line with the function of FvTFL1 in the diploid strawberry (Koskela et al. 2012), silencing *FaTFL1* did not have any effect on vegetative development, nor did it change *FaSOC1* expression (Koskela et al. 2016). Thus, regulation of flowering in diploid and octoploid strawberries appears to be conserved at the molecular level, at least in the case of the photoperiodic and temperature-regulated flowering pathways.

Although all the available data indicates that *FaTFL1* is an important regulator of flowering in the octoploid strawberry, it is not the gene behind the everbearing character in cultivars. Several QTL mapping studies have shown that the everbearing trait resides in octoploid strawberry LGIV and also has a negative effect on runner formation (Gaston et al. 2013; Castro et al. 2015; Honjo et al. 2015), whereas *FaTFL1* is located on LGVI and does not directly affect vegetative development (Koskela et al. 2016). A recent report by Perrotte et al. (2016a) suggested that the octoploid strawberry homolog of *FT2* (*FaFT2*) is located within the QTL mapping window and could be a candidate for the observed phenotypes in everbearing octoploid strawberries. Indeed, the FaFT2 protein has been shown to possess all the hallmark features required for a floral-promoting function (Nakano et al. 2015). However, *FaFT2* expression pattern suggests that *FaFT2* acts downstream of *FaAP1* and has a role in late stages of floral development (Nakano et al. 2015). This is also supported by the finding that its *F. vesca* ortholog *FvFT2* exhibits the highest expression level in flower buds (Koskela et al. 2012). The identity of the gene causing the everbearing trait in the octoploid strawberry thus still remains a mystery. In any case, considering the data presented above, the gene must either silence *FaTFL1* or act downstream of it.

Experiments using octoploid strawberry have also revealed a locus that affects flowering trait not characterized in the diploid strawberry. Longitudinal analysis of flowering data of everbearing strawberries has shown that there is a QTL on LGIII that affects the number of successive flowering phases and the intensity of

flowering (Perrotte et al. 2016b). This QTL also has an effect on branch crown formation, and the authors suggested that genes related to the GA pathway might present good candidate genes for the QTL (Perrotte et al. 2016b). Since the number of branch crowns affects the number of inflorescences in strawberry (Hytönen et al. 2004), further studies are needed to test if the QTL on LGIII increases the intensity of flowering indirectly through increased branching.

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5 Fruit Quality and the Use of Near-Isogenic Lines for Functional Characterization in *Fragaria vesca*

Maria Urrutia and Amparo Monfort

Abstract

Strawberries (*Fragaria* genus) can be found in various versions of ploidy, with the most popular being the octoploid commercial varieties. Studies of the diploid strawberry are able to shed light into the genetic control of valuable traits in plants of all ploidy levels, and their simple genetics offer many advantages from a scientific perspective. Crossing populations have been a valuable tool for genetic studies of *Fragaria vesca*. A near-isogenic line (NIL) collection of diploid strawberries has been developed between *F. vesca* Reine de Valles (RV) as the recurrent parent and *Fragaria bucharica* (FB) as the donor parent. Studying the varying phenotypic traits of the NIL collection can reveal information about the effects of the introgressions. The phenotyping of a whole mapping population allows identification of QTL and major genes for more than a hundred traits and metabolic compounds. Description of functional compounds of ripe berries provides an interesting global insight in strawberry fruit nutritional composition.

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5.1 Genetic Resources in Diploid *Fragaria* and *Fragaria vesca* NIL Collection

Fragaria vesca has many advantages to be used as a model species for the Rosaceae family. It presents high collinearity with cultivated octoploid strawberry (Rousseau-Gueutin et al. 2008; Tennessen et al. 2014) and highly conserved syntenic blocs with crop trees from the *Rosacea* family like apples (*Malus* × *domestica*) and peach (*Prunus persica*) (Illa et al. 2011). In addition, diploid strawberry (*F. vesca*) tools and resources have bursted in the last few years. The genome sequencing of the *F. vesca* Hawaii-4 was a milestone for strawberry research community (Shulaev et al. 2011). An improved assembly has been generated using *F. vesca* spp. *bracteata* genetic map (Tennessen et al. 2014), and a new annotation version based on transcriptomic data from five different fruit tissues at five developmental stages has become available (Darwish et al. 2015). Furthermore, whole genome re-sequencing data of highly inbred accessions of *F. vesca* var. Hawaii4, var. Rügen and var. Yellow Wonder has been published, providing thousands of SNPs variants between *F. vesca* varieties and revealing that even advanced inbreds retain extensive heterozygous areas (Hawkins et al. 2016). Besides, a high throughput genotyping platform, Istraw90 (Affimetrix, CA, USA), developed based on genomic data of

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six diploid accessions including three *F. vesca*, one *Fragaria mandshurica* and two *Fragaria iinumae*, and 19 octoploid accessions including 15 *F.* × *ananassa*, three *Fragaria chiloensis,* and one *Fragaria virginiana*, have been recently released (Bassil et al. 2015). Although it might seem less relevant, diploid strawberry presents advantageous physical and physiological traits for laboratory working conditions. Its short intergenerational period allows several (typically 3–4) seed to seed cycles a year, and its small size permits cultivation in pots in small greenhouses and growth chambers. Furthermore, most accessions are self-compatible and develop runners, which facilitate self-pollination and clonal propagation of plant materials.

Taking together, the outstanding scientific advantages that *F. vesca* presents in the context of the *Rosaceae* family and the boost of genetic and genomic resources during the last 20 years make *F. vesca* as an excellent model for both basic and applied research. In this scenario, genetic resources reflecting *Fragaria* genus diversity, including mapping populations and newly developed genetic tools, will contribute to fully explore the economic potential of the genus.

5.1.1 Mapping Populations

Mapping populations are tools for qualitative and quantitative genetics that aim to facilitate detecting genetic regions accounting for the observed variability in a phenotypic trait. In diploid strawberry, there are well-established F_2 mapping populations. The first reported diploid strawberry linkage map was generated from an F_2 population derived from a cross between *F. vesca* spp. *vesca* var. "Baron Solemacher," a non-runnering European cultivar, and *F. vesca* spp. *americana* "W6" collected in the wild (Davis and Yu 1997). It was built using 75 randomly amplified polymorphic DNA (RAPD) markers spanning over 445 cM. These markers were subsequently used in two new F_2 populations to map locus "c" that is responsible for the yellow strawberry fruit color. *F. vesca* var. "Yellow Wonder," an alpine European variety producing yellow fruits, was crossed with *F. vesca* "DN1C" and *F. nubicola* "FRA520" (that was probably *F. bucharica*) (Deng and Davis 2001). Later, an F_2 population from a cross between *F. vesca* "FDP815" and *F. bucharica* "FDP601" allowed the construction of the current reference linkage map in *F. vesca* (Sargent et al. 2004, 2006a). This map was first built using 78 markers (68 SSRs, three morphological traits, six gene-specific markers and one sequence-characterized amplified region) covering 448 cM and later enhanced with 109 additional SSRs evenly distributed among the seven *Fragaria* chromosomes, which reduced the map size to 424 cM with an average marker interval of 2.3 cM. It has been revised at least three more times by Sargent et al. (2006b, 2008, 2011) who mapped 24 new gene-specific markers, and 103 and 194 SSRs, respectively, and by (Ruiz-Rojas et al. 2010) who added 74 cleaved amplified polymorphism markers (CAP). This collection has been extensively used for gene mapping among the *Fragaria* community (Sargent et al. 2006b).

5.1.1.1 Mutant Population

Mutant populations are useful tools for reverse genetics. Phenotypic changes induced by mutation help to identify and functionally characterize causal genes. In diploid strawberry, two main mutant collections have been developed. In the first place, an efficient transformation protocol and a T-DNA mutant collection were developed using *F. vesca* "Hawaii-4" (National Clonal Germplasm Repository accession PI 551572) by (Oosumi et al. 2006). Although insertion regions and candidate genes can be identified in T-DNA mutants of interest (Oosumi et al. 2010; Ruiz-Rojas et al. 2010), this method is unsuitable to produce a large-scale knock-out population, as it requires an independent transformation event for each mutant. In the second place, transposon tagging for insertional mutagenesis, which allows to develop multiple transgenic lines from a single-transformation event, was developed in Hawaii-4 and gave rise to a new mutant collection (Veilleux et al. 2012).

5.1.1.2 Near-Isogenic Lines

NIL collections: definition

Near-Isogenic Line (NIL) collections are mapping populations based on introgression lines. Each line contains a single homozygous introgression from a donor parent (usually an exotic accession) while its genetic background is identical—near isogenic—to a recurrent parent (typically an elite variety), and all lines together cover the whole donor genome in overlapping introgressions. NIL collections are conceptually genomic libraries of the donor parent in the recurrent parent background (Eshed and Zamir 1994).

Development of a NIL collection

In order to build a NIL collection, an F_1 individual is backcrossed with the recurrent parent, and the resulting BC_1 generation is recursively backcrossed for n generations to obtain single introgression of desired genetic size. BC_n individuals are self-pollinated to fix homozygous lines. Classical approaches have laid on large BC_1 populations, where individuals with low introgression number could be selected, to speed up selection process and reduce the number of generations needed to fully develop a NIL collection. This approach is assisted by thorough genotyping with molecular markers in each generation and selection criteria include (1) In BC_1: select the minimum number of individuals harboring the minimum number of introgressions possible per individual that together cover twice the genetic background with overlapping introgressions. (2) In BC_2 to BC_n: Lines with single introgression are selected for self-pollination. The minimum number of lines harboring the minimum number of introgressions (>1) that together cover the genetic background is selected for next backcrossing generation until single-introgression lines covering the whole genome are reached. (3) Single-introgression lines are self-pollinated one or more times to get completely homozygous introgressions. High-density genotyping in BC_2 followed by selection criteria laying on the same principles mentioned above can be used to accelerate NIL selection (Barrantes et al. 2014; Perpiñá et al. 2016)

NIL collection uses

Insertion of wild alleles and phenotypic variability into the elite germplasm is one of the ultimate objectives of NIL collections. NILs usually share >95% of their genomes with the recurrent parent, which normally is an elite variety; this allows a rapid transference of the beneficial wild alleles to the commercial material and facilitates crop improvement. In addition, it reduces infertility problems and unwanted deleterious interactions typically associated to wide crosses with wild species (Barrantes et al. 2016; Zamir 2001). Recent examples of wild alleles introgressions into elite material are the round-shaped melons in the cv. Piel de Sapo background (Díaz et al. 2014) or the improved yield in modern wheat breeds across environments with introgressions from wild accession (Merchuk-Ovnat et al. 2016).

QTL mapping is among the most extended uses of NIL collections due to their special genetic structure. QTL mapping using NILs is conceptually simplified as all significant difference between an isogenic line and the recurrent parent is attributed to the introgressed fragment. In addition, their high isogenicity isolates QTL avoiding epistatic effects and facilitates the identification of minor QTL. Due to the theoretical immortality of NILs, that can be indefinitely replicated by self-pollination, identical collections can be tested in different environments, through several years or under different (biotic or abiotic) stress conditions or treatments, which increases phenotyping accuracy and facilitates the estimation of environmental effects and genotype x environment-interactions (Monforte et al. 2001). Some examples of QTL mapped using NILs include QTL affecting yield, antioxidant capacity, lycopene content, and fruit-quality traits in tomato (Ashrafi et al. 2011; Barrantes et al. 2016; Eshed and Zamir 1995; Rousseaux et al. 2005) and QTL associated to yield potential in rice (Fujita et al. 2013).

Fine QTL mapping, candidate gene identification, and positional cloning are also facilitated by NIL collection with the development of sub-NILs. Lines harboring QTL of interest can

be backcrossed with the recurrent parent to generate new recombinants in the introgressed region. The phenotyping of this progeny (or their self-pollinated offspring if the exotic allele is recessive) narrows down the QTL position and typically allows identification of candidate gene which could be ultimately isolated and characterized. Some bibliographic examples of fine mapping are the dissection of brix, yield, and fruit shape QTL in tomato (Monforte and Tanksley 2000a) and the fine mapping of a QTL involved in climacteric ripening in melon (Vegas et al. 2013). In some cases, resolution reaches to gene level as in a Brix QTL in tomato (Fridman et al. 2000).

Other reported research using NIL collections include studies on the genetic basis of heterosis (Fernández-Silva et al. 2009; Melchinger et al. 2007), genetics of metabolism (Di Matteo et al. 2013; Schauer et al. 2006), transcriptomes and their correlation with metabolism (Lee et al. 2012) and enzyme activity (Steinhauser et al. 2010).

NIL collection examples

Dozens of NIL collections have been developed in the last twenty years since the first one was reported in middle 90s by Eshed and Zamir (1994, 1995) who introgressed *Lycopersicon pennellii* in the genetic background of cultivated tomato. Others have been developed using different combinations of parents in tomato (Barrantes et al. 2014; Monforte and Tanksley 2000b) in other crop species like barley (von Korff et al. 2004), maize (Pea et al. 2009), rice (Tian et al. 2006), melon (Eduardo et al. 2005; Perpiñá et al. 2016), lettuce (Jeuken and Lindhout 2004), diploid strawberry (Urrutia et al. 2015), and in the model species *Arabidopsis thaliana* (Fletcher et al. 2013; Keurentjes et al. 2007; Koumproglou et al. 2002) among others.

***F. vesca* NIL collection**

Recently, the first NIL collection of Rosaceae family was developed in woodland strawberry and it was proven to be a useful genetic tool for the *Fragaria* genus (Urrutia et al. 2015).

Characteristics and parents of F. vesca NIL collection *F. vesca* NIL collection was developed from an interspecific cross between *F. vesca* and *F. bucharica* (Fig. 5.1). The recurrent parent is *F. vesca* var. Reine des Vallées, an alpine self-fertile accession (Arulsekar and Bringhurst 1981) that belongs to the same subspecies of the sequenced woodland strawberry *F. vesca* spp. *vesca* var. "Hawaii 4" (Shulaev et al. 2011). It is a non-runnering robust variety producing large red fruits with an intense aroma that is cultivated for commercial purposes. The donor parent is *F. bucharica* FDP601, a self-incompatible species

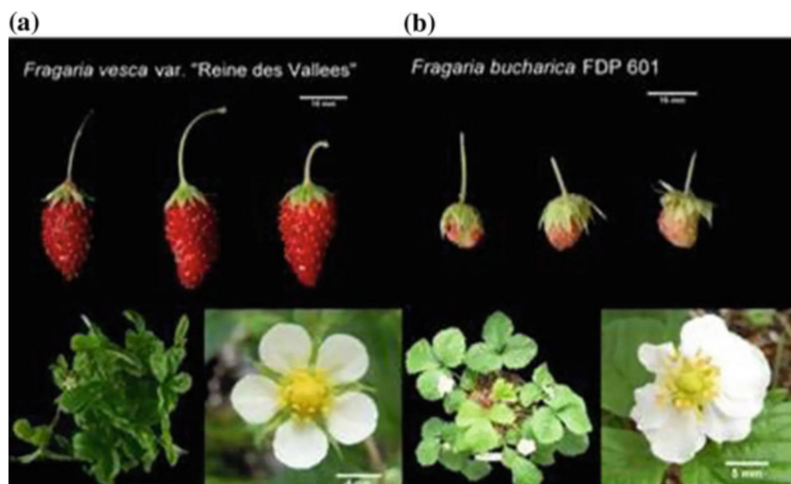

Fig. 5.1 Fruit, plant, and flower phenotypes of the recurrent (*F. vesca*) and donor (*F. bucharica*) parents of diploid *Fragaria* NIL collection. **a** *F. vesca* var. "Reine des Vallées." **b** *F. bucharica* FDP 601

native to the Himalaya (Boskovic et al. 2010). It has no commercial interest as it produces tiny fruits with few seeds, if any, but has interesting traits for breeding including its runnering habit and its resistance to oidium. The two species can be crossed to produce fertile offspring as they are phylogenetically close (Rousseau Gueutin et al. 2009), and they have been used to produce genetic reference map (Sargent et al. 2004), but more importantly, they present significant phenotypic differences at morphological, metabolic, and phenological level.

F. vesca NIL collection comprises 39 homozygous and 2 heterozygous NILs (Fig. 5.2) covering 522 cM and 192.7 Mb (96 and 92% of the genome in genetic and physical distance, respectively). The collection includes 5 NIL harboring introgressions of a whole chromosome, and the whole genome is covered with a reduced set of 9 NILs (Urrutia et al. 2015, 2016). Overlapping introgressions define 37 bins with an average size of 14 cM, with 4–8 lines representing each chromosome. The NIL collection has been genotyped using 72 SSRs that allow to define cM size of introgressions and 1510 single nucleotide polymorphisms (SNPs) issued from IStraw90® genotyping array (Bassil et al. 2015) that permit to calculate the size of introgressions in Mbp (Table 5.1). The entire *F. vesca* genome is covered with overlapping introgressions summing a total of 210 Mb. Physical introgression sizes, without considering whole chromosome introgressions, range from 1 Mb (0.4% of genome) to 31 Mb (15% of genome) with an average size of 16 Mb (7.6% of total coverage). Genome fragments identified by overlapping regions or bins have an average size of roughly 5 Mb (2.3% of genome), ranging from 0.16 to 29.30 Mb (0.07–13% of total coverage).

Agronomical and metabolite QTL A phenotypic analysis of NIL population has allowed to map 16 stable QTL for agronomical traits (Urrutia et al. 2015) including three QTL for fruit shape, four QTL for flowering time, and flowering habit including the previously identified locus for seasonal flowering habit on LG6:30–39 cM containing *FvTFL1* (Koskela et al. 2012). In addition, two QTL for floral stem length, one for plant dwarfism, one for petal color, one for petal number among others have been identified, and the location of the runnering locus (R) has been confirmed on LG2:39–47 cM.

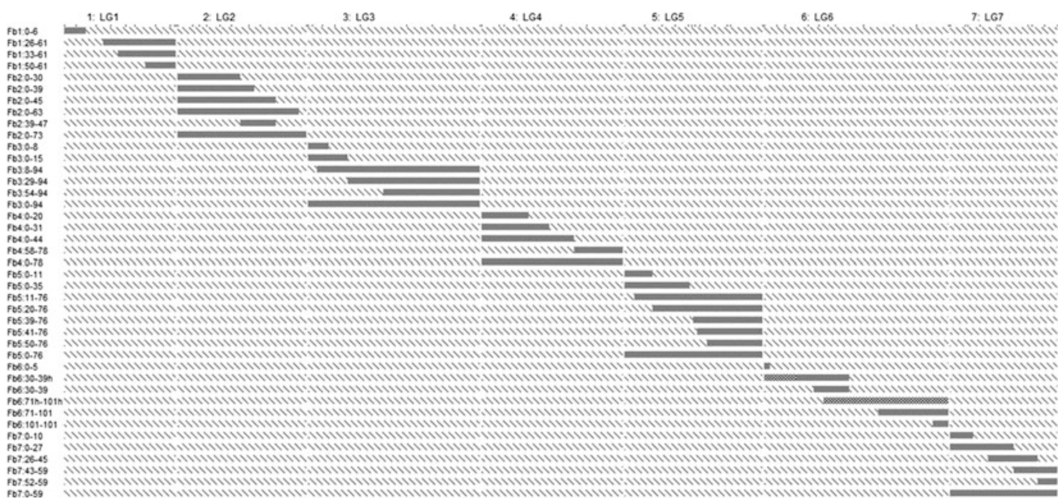

Fig. 5.2 *F. vesca* NIL collection genotypes. Backward slashed and gray areas represent genetic regions from the recurrent and donor parent, respectively. Dotted areas correspond to heterozygous regions (modified from Urrutia et al. 2015)

Table 5.1 NIL introgressions in genetic (cM) and physical distances (Mb)

	n	Mb	cM
NILs	39 + 2	192.7	522
Minimum number NILs	9		
Average introgression size		16	32.9
Average BIN size		4.9	14.2
Smallest introgression		1	3.2
Smallest BIN		0.2	3.2

Targeted metabolomics for sugars (HPLC) and polyphenols (LC-MS) applied overripe fruits of the NIL collection allowed to map 83 stable QTL for nutritional characters including five QTL for sugar (glucose and fructose) accumulation and 76 QTL for 18 out of 22 identified polyphenols (Urrutia et al. 2015, 2016). The same approach targeting volatile compounds with important organoleptic value is showing promising results (Urrutia personal communication).

These promising results reveal the huge potential of the *F. vesca* NIL collection for QTL mapping regarding agronomic and/or molecular traits. With new genomic data available in *F. vesca*, namely the re-sequenced and annotated genome (Darwish et al. 2015; Hawkins et al. 2016; Shulaev et al. 2011; Tennessen et al. 2014), this genetic resource is very powerful for the identification of candidate genes, and it opens avenues for future genetic studies in diverse areas exploring different developmental stages, tissues, susceptibility to biotic or abiotic stress, transcriptomics, and added-value commercial traits among others.

5.2 Fruit Quality in Strawberry

5.2.1 What Is Fruit Quality in Strawberry: From Producers' to Consumers' Point of View

Plant and especially crop phenotypes are evaluated and selected in terms of quality from a human perspective. This quality definition can be very wide and is guided by economic profit and product marketability. In general, most desirable traits are linked to yield ratio, biotic and abiotic stress tolerance and production period. In the case of fruit crops, traits related to overall fruit appearance including uniformity, size, shape, and color are also taken into account. In the last years, new traits are entering into play. Recent interest in nutrition, phytochemicals, and the lack of taste of some new fruit varieties have moved forward research on healthier and more flavorful fruits (Folta and Klee 2016; Whitaker et al. 2013).

Commercial strawberry cultivars have reached high-quality standards in agronomical parameters and are characterized by large, uniform, firm, and intense red fruits (Folta and Klee 2016; Stewart 2011; Whitaker et al. 2013). However, the main factors contributing to consumer satisfaction are sweetness and flavor intensity which are directly related to sugars and specific volatile profiles (Bruhn et al. 1991; Schwieterman et al. 2014). In addition, strawberries are highly recommended in a healthy diet as they are rich in health-beneficial phytochemicals, such as high polyphenol and vitamin C content.

Wild strawberry (*F. vesca* varieties) has minor economic and commercial importance. However, it has much to offer to get deeper insights in strawberry fruit organoleptic and phytochemical quality traits. The scope of this section is to summarize current knowledge on this topic.

5.2.1.1 Organoleptic and Nutritional Fruit Quality

Color

The characteristic intense bright red color of ripe strawberries is very appealing for humans and

animals. However, white strawberries are also naturally present and co-exist with red varieties in the wild. Diploid white accessions include the sequenced *F. vesca* spp. *vesca* var. Hawaii-4 (Shulaev et al. 2011), var. Yellow Wonder, var. White Solemacher, var. Pineapple Crush and var. White Soul. Some *F. vesca* red varieties are var. Reine des Vallées, var. Baron Solemacher, var. Rügen and var. Alexandria. The color of the berries (C) was long ago described as a monogenic trait and mapped to a region at the end of LG1 (Brown and Wareing 1965; Deng and Davis 2001). A causal candidate gene, flavanone 3-hydroxylase, was proposed but direct conclusive evidence remained elusive until a recent study has revealed that a single nucleotide polymorphism producing a non-synonymous change (Try to Ser) in FvMYB10 (located in Fvb1: 15, 405, 782–15, 407, 498 bp) is responsible for changes in pigmentation between red and white diploid strawberry accessions (Hawkins et al. 2016).

Antioxidants: Polyphenol content

Diet rich in vegetables and fruits is known to promote health and reduce the risk of certain diseases such as cancer, diabetes, obesity, or cardiovascular diseases. The role of plant phytochemicals in disease prevention and treatment, and mainly the role of plant polyphenols, having antioxidant and anti-inflammatory properties and involved in regulation of cell survival pathways, has been extensively reviewed in experimental and animal models (Amararathna et al. 2016; Braakhuis et al. 2016; Shankar et al. 2016; Zhou et al. 2016). Strawberry, and especially woodland strawberry, is an important natural source of polyphenols (Scalzo et al. 2005; Wang et al. 1996). The potential health benefits of strawberry intake include prevention of inflammation, cardiovascular diseases, obesity, metabolic syndrome, certain cancers, and neurodegenerative diseases (see Giampieri et al. (2015) for a thorough review on this topic). Polyphenols also contribute to major organoleptic traits such as color, sensitivity to enzymatic browning, bitterness, and astringency.

Polyphenols are a large class of plant secondary metabolites deriving from the phenylpropanoid pathway and classified in four main groups flavonoids, phenolic acids, lignans, and stilbenes; flavonoids and phenolic acids being the most abundant phenolics in ripe strawberry fruit. A recent study identified a total of 67 phenolic compounds in *F. vesca* (var. "Rüegen" and "Yellow Wonder") including anthocyanins, flavonols, flavan-3-ols, proanthocyanidins, ellagic acid, and its derivatives (Sun et al. 2014). Comparing the phenolic profiles of the two previous mentioned woodland strawberry accessions with the cultivated strawberry (*F.* × *ananassa* var. "Fort Laramie"), it was seen that *F. vesca* profile is more complex and diverse. It shows a distinctive accumulation pattern of polyphenols and includes compounds that were not detected in *F.* × *ananassa* such as peonidin 3-O-glucoside, peonidin 3-O-malonylglucoside, taxifolin 3-O-arabinoside, and methylellagic acid glycosides (Sun et al. 2014).

Flavonoids, the most abundant polyphenols in ripe fruit of woodland strawberry, can be divided into three main subgroups: anthocyanins, flavan-3-ols, and flavonols. Anthocyanins, whose chemical structure consists of six aglycones (anthocyanidins) that differ in the number of hydroxyl groups on their B-ring and their methylation pattern, are responsible for red mature berries pigmentation. They are very abundant in red-fruited accessions, but undetectable in white-fruited *F. vesca* like "Yellow Wonder" (Table 5.2) (Muñoz et al. 2011; Sun et al. 2014; Urrutia et al. 2016; Zhang et al. 2015). Most abundant anthocyanin in diploid strawberry fruits is pelargonidin-3-glucoside followed by cyaniding 3-glucoside, but other pelargonidin, cyanidin and peonidin-glucosides and malonylglucosides are also accumulated (Muñoz et al. 2011; Sun et al. 2014; Urrutia et al. 2016). In comparison with commercial strawberry (*F.* × *ananassa*), *F. vesca* anthocyanin pattern is more diverse and accumulates higher concentrations of cyanidin 3-glucoside at ripe stage (Fig. 5.3). Differences in expression rates of a Flavonoid 3′-hydroxylase (F3′H) during fruit

Table 5.2 Relative phenolic accumulation in *Fragaria vesca* accessions

Chemical families	(poly)-phenolic compounds	*F.vesca* RV	*F.vesca* YW
Anthocyanins		0.83	0
	Pelargonidin-3-glucoside	0.5	–
	Pelargonidin-3-glucoside-malonate	0.115	–
	Cyanidin-3-glucoside	0.375	–
Flavonols		0.08	0.5
	Kaempferol-glucoside	0.075	0.07
	Kaempferol-glucuronide	0.7	0.75
	Kaempferol-coumaryl-glucoside	0.08	0.07
	Quercetin-glucoside	0.11	0.055
	Quercetin-glucuronide	0.035	0.055
Flavan-3-ols		0.055	0.4
	Procyanidin B1	0.2	0.22
	Procyanidin B3	0.165	0.155
	Catechin	0.22	0.225
	(epi)catechin dimers iso1	0.17	0.16
	(epi)catechin dimers iso2	0.215	0.225
	(epi)afzelechin-(epi)catechin dimers	0.03	0.02
Flavanones		0.015	0
	Eriodictyol iso1	0.785	0.81
	Eriodictyol iso2	0.215	0.19
Hydroxycinnamic.ac.deriv		0.02	0.07
	p-Coumaroyl-glucose ester	0.155	0.1
	p-Coumaroyl-glucoside	0.025	0.01
	Cinnamoyl-glucose ester	0.665	0.51
	Feruloyl-glucose ester	0.085	0.27
	Caffeoyl-glucose ester	0.07	0.12
Ellagic acid	Ellagic acid	0.005	0.03

Percentage contribution of chemical families and specific metabolites to total phenolics content in harvests for RV and YW (modified from Urrutia et al. 2016)

development and ripening between the two species are responsible for this divergence in anthocyanins composition (Thill et al. 2013). QTLs for cyanindin 3-glucoside and pelargonidin 3-glucoside-malonate have been mapped to regions LG2:45–63 cM and LG5:41–50 cM, respectively (Urrutia et al. 2016).

Diploid strawberry fruit flavan-3-ols comprises monomers, oligomers, and polymers of (epi)catechin and (epi)afzelechin units, called proanthocyanidins (PAs) (Muñoz et al. 2011; Sun et al. 2014; Urrutia et al. 2016). PAs are relatively abundant polyphenols in strawberry; however, a study by Buendía et al. (2010) pointed out that their content might be underestimated by traditional phenolic-extraction methods (methanolic extractions). Their analysis, focusing on PAs quantification by acid-catalyzed depolymerization in presence of a nucleophile and HPLC separation revealed higher concentrations than anthocyanins in octoploid strawberry. QTL for several PAs was mapped to a

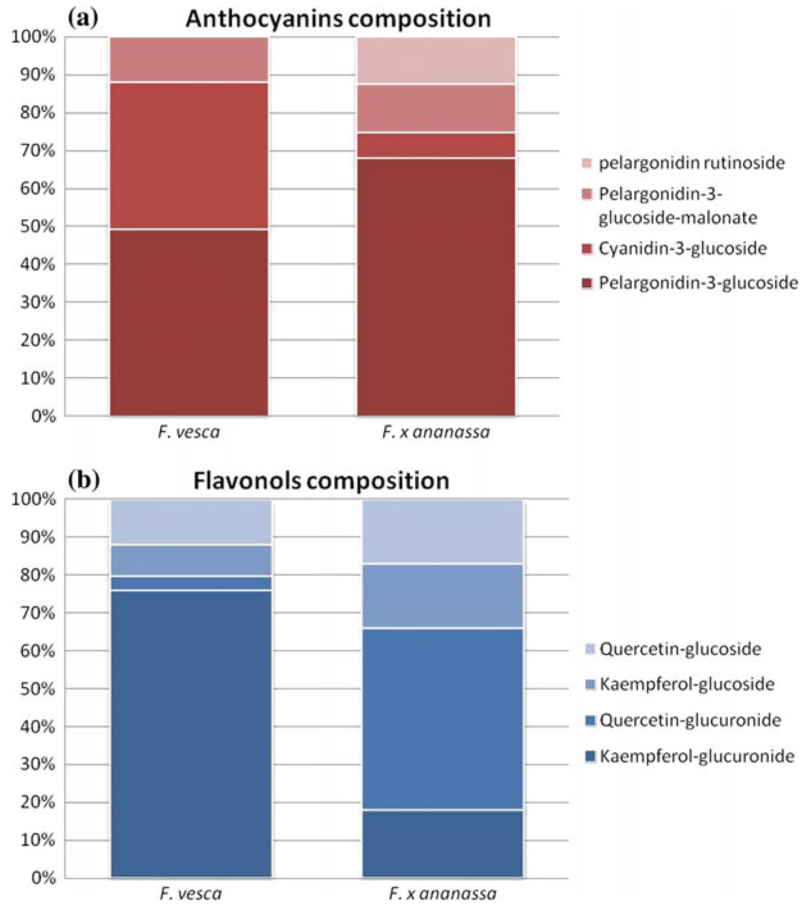

Fig. 5.3 Anthocyanin and flavonol comparison between *F. vesca* and *F.* × *ananassa*. Anthocyanin (**a**) and flavonol (**b**) percentual composition in *F. vesca* and *F.* × *ananassa*. *F. vesca* data extracted from Urrutia et al. (2016) and *F.* × *ananassa* data extracted from Ring et al. (2013)

region at the beginning of LG5:0–11 cM (Urrutia et al. 2016).

Flavonols are present in many fruits but usually accumulated only in small quantities. Their chemical structure includes two phenyl (A and B) and one heterocyclic rings (C). Substitution patterns in the B-ring and nature and position of sugar moieties provide structural diversity to this group. Kaempferol conjugates (kaempferol-glucuronide and kaempferol-glucoside) are the major flavonols in *F. vesca*, but kaempferol-coumaryl-glucoside and quercetin derivatives are also detected (Muñoz et al. 2011; Urrutia et al. 2016). In contrast, *F.* × *ananassa* preferentially accumulates quercetin conjugates (Aaby et al. 2007; Buendía et al. 2010; Ring et al. 2013). QTL explaining the accumulation of kaempferol and quercetin conjugates were mapped on LG1:26–61 cM and LG2:63–73 cM, respectively, and a QTL for kaempferol-coumaryl-glucoside accumulation was located on LG7:43–59 cM (Urrutia et al. 2016).

Phenolic acids comprise hydroxybenzoic and hydroxycinnamic acid derivatives. The most abundant in *F. vesca* berries is cinnamoyl-glucose ester followed by *p*-coumaroyl-glucose ester. Other detected phenolic acids include *p*-coumaroyl glucoside, caffeoyl-glucose ester, feruloyl-glucose ester, ellagic acid and its hydrolyzable tannins (Muñoz et al. 2011; Sun et al. 2014; Urrutia et al. 2016). The *F. vesca* NIL collection, allowed to map a QTL controlling cinnamoyl-glucose ester accumulation on LG2:0–30 cM and two co-locating QTL explaining coumaroyl-glucose ester and *p*-coumaroyl glucoside content on LG4:9–20 cM (Urrutia et al. 2016). Genes and enzymes involved in phenylpropanoid and flavonoid

Table 5.3 Principal strawberry aroma contributors

Chem. family	Volatile compound	Aroma description
Esters	Butyl acetate	Fruity, thinner
	Butyl butanoate	Fruity pineapple
	Ethyl butanoate	Fruity
	Ethyl hexanoate	Fruity, strawberry, apple
	E-2-hexenyl acetate	Fruity-green
	Z-3-hexenyl acetate	Fruity-green
	Hexyl acetate	Fruity apple cherry flowery
	Methyl 2-aminobenzoate	Woodland strawberry-like, spicy-aromatic, flowery
	Methyl 2-methylpropanoate	Fruity
	Methyl butanoate	Fruity
	Methyl cinnamate	Spicy, nut
	Methyl hexanoate	Fruity
	Myrtenyl acetate	Herbaceous pleasant, refreshing, sweet
Aldehydes	E-2-hexenal	Herbaceous, green notes, fatty
	Z-3-hexenal	Herbaceous, green leaf-like, grassy
Alcohols	Eugenol	Spicy, nutmeg
	Z-3-hexen-1-ol	Herbaceous, green notes
Furans	Furaneol	Sweet, caramel, strawberry, green
	Mesifurane	Sweet, caramel, burnt
	Terpinen-4-ol	Spicy, turpentine
Ketones	2,3-butanedione	Buttery, caramel, vanilla
Lactones	g-decalactone	Fruity, peach-like
Terpenes	Linalool	Flowery, sweet
	Nerolidol	Rose, apple, green

Modified from Schieberle and Hofmann (1997), Ulrich et al. (1997, 2007), Jetti et al. (2007), Olbricht et al. (2008)

biochemical pathways during fruit development have been characterized in *F.* × *ananassa* (Almeida et al. 2007; Carbone et al. 2009; Halbwirth et al. 2006; Schulenburg et al. 2016).

Volatiles

From a human point of view, fruit aroma is a quality trait that has a major impact on flavor and consumers' preference (Schwieterman et al. 2014). It is composed by a complex and diverse mixture of volatile compounds having in common a lipophilic nature, a low molecular weight (<300 Da) and a high vapor pressure that can be released into the atmosphere in absence of diffusion barriers (Dudareva et al. 2006; Pichersky et al. 2006). Their substrates and synthesis pathways are very diverse but can be mainly classified as terpenoids, phenylpropanoids/benzenoids, fatty acid derivatives, and amino acids derivatives; their chemical structures include esters, terpenes, ketones, lactones, aldehydes, and alcohols (Dudareva et al. 2013; Granell and Rambla 2013; Schwab et al. 2008). These compounds account only for about 1% of known secondary metabolites and contribute for only 10^{-7}–10^{-4}% of the fresh fruit weight (Dudareva et al. 2004; Berger 2007; Brückner

and Grant Wyllie 2008); yet some of them can be perceived by human's olfactory system and are decisive for fruit quality perception. Volatile profiles are very diverse and dynamic being dependent on numerous factors such as species and variety, maturity stage, postharvest treatment, and analysis technique (Granell and Rambla 2013). However, volatile production generally increases with ripening (Goff and Klee 2006).

Strawberries are appreciated for their flavor and aroma. More than 360 volatiles have been detected in strawberry extracts (Latrasse 1991); however, only around 20 of them actually contribute to strawberry smell adding fruity, flowery, herbaceous green, sweet caramel, and/or spicy notes (Table 5.3) (Jetti et al. 2007; Olbricht et al. 2008; Schieberle and Hofmann 1997; Ulrich et al. 1997, 2007). In general, high ester concentration is associated with pleasant strawberry aroma and the refreshing impression associated to *Z*-3-hexenal which has a very low odor threshold (Ulrich et al. 1997). Woodland strawberry (*F. vesca*) aroma is remarkably different from cultivated (*F.* × *ananassa)* species being more intense, flowery, and aromatic (Ulrich et al. 1997, 2007). Distinctive wild strawberry aroma is added by the semi-volatile, methyl 2-aminobenzoate (also known as methyl anthranilate), that is present in most *F. vesca* accessions and absent in most commercial varieties (Ulrich et al. 1997, 2007), the low degree of inheritability of this compound might explain why it has been lost through breeding process (Olbricht et al. 2008). In addition, different ester pattern between the two species also contributes to distinctive smells (Dong et al. 2013).

Although diversity among *F.* × *ananassa* varieties is narrower compared to *F. vesca*, significant differences in aroma patterns including extreme quality phenotypes have been detected between *F.* × *ananassa* cultivars or among *ad hoc* mapping populations (Schwieterman et al. 2014; Zorrilla-Fontanesi et al. 2012).

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The NB-LRR Disease Resistance Genes of *Fragaria* and *Rubus*

6

Leon van Eck and James M. Bradeen

Abstract

Plants possess sophisticated surveillance and response systems against potential pathogens. In most cases, the genes underlying plant disease resistance encode nucleotide-binding leucine-rich repeat (NB-LRR) proteins. While the single gene nature of NB-LRR genes makes them widely accessible for plant improvement, the potential for rapid pathogen adaptation and, thus, reduced resistance durability is high. Few disease resistance genes of known function have been cloned in the Rosaceae but several have been mapped, with associated markers available for marker-aided selection. In strawberry, resistance to red stele root rot (*Phytophthora fragariae* var. *fragariae*), anthracnose (*Colletotrichum acutatum*), and angular leaf spot (*Xanthomonas fragariae*) have all been the targets of genetic mapping. In *Rubus,* gene *H* conditions pubescence in raspberry is associated with resistance to gray mold (*Botrytis cinerea*), spur blight (*Didymella applanata*), cane blight (*Leptosphaeria coniothyrium*), and cane spot (*Elsinoë veneta*). It is unclear whether pubescence acts as a preformed physical barrier to infection or if gene *H* is physically linked to NB-LRR genes conditioning the various resistances. Resistance to *Raspberry bushy dwarf virus* has been genetically mapped and markers associated with resistance to the aphids *Amphoromphora idaei* and *Amphorophora agathonica,* vectors of important raspberry viruses, have been identified. Candidate gene approaches including PCR-based methods for generating resistance gene fragments hold some potential for development of markers useful in strawberry and raspberry breeding. Finally, the availability of whole genome sequences from *Fragaria* and *Rubus* species enables in silico discovery of NB-LRR genes and visualization of evolutionary relationships and physical genome distribution, a focus of research in our research laboratory.

6.1 Introduction: Disease Resistance and NB-LRR Genes

Plants lack the specialized cells that compose the adaptive immune system of animals. Nevertheless, the plant innate immune system has evolved a large repertoire of sophisticated mechanisms for detecting and responding to pests and pathogens. Plant innate immunity is multi-tiered. At the most

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basal level, receptors at the plasma membranes of plant cells, known as Pattern Recognition Receptors (PRRs), recognize molecular features of invading microbes, known as pathogen-associated molecular patterns (PAMPs), and initiate intracellular signaling cascades that end in a defense response, known as PAMP-Triggered Immunity (PTI). The PAMPs that elicit PTI are generally slow-evolving and widely conserved essential microbial components, such as bacterial flagellin or fungal chitin (Dodds and Rathjen 2010; Zipfel 2014).

Pathogens have evolved a range of effector proteins to manipulate their host and suppress PTI responses. The most well-characterized bacterial effectors are delivered via the Type III Secretion System (Chisholm et al. 2006; Jones and Dangl 2006). Fungal and oomycete effectors are likely delivered from haustoria to the host cytoplasm via exocytotic membrane trafficking (Panstruga and Dodds 2009). The next tier of plant immunity directly or indirectly recognizes the presence of these effectors and is termed Effector-Triggered Immunity (ETI). The downstream effects of PTI and ETI are not discrete; rather, ETI accelerates and amplifies the effects of PTI, usually taking the form of a hypersensitive cell-death response at the site of infection (Jones and Dangl 2006). Successful ETI requires the presence of both a specific effector encoded by the genome of the pathogen and a corresponding resistance (*R*) gene product encoded by the plant genome. This genotype-specific interaction was first characterized in the work of Harold Flor (1955) as a gene-for-gene relationship between resistant flax (*Linum usitatissimum*) cultivars and isolates of flax rust (*Melampsora lini*) carrying the corresponding factors conditioning avirulence.

Harboring gene products that allow for their detection imposes selective pressure on microbial populations to avoid recognition, and virulent strains of microbes are able to suppress or evade *R* gene-dependent ETI. Plant populations, in turn, evolve new *R* gene specificities. This continued co-evolutionary arms' race between plants and pathogens has resulted in the proliferation of disease resistance genes in plant genomes.

The vast majority of plant *R* genes encode cytoplasmic surveillance proteins of the NB-LRR family. NB-LRR proteins are characterized by the presence of a Nucleotide-Binding domain, the NB-ARC (Nucleotide-Binding shared by human Apaf-1, plant R, and nematode Ced-4 proteins). In the most simplistic model, the NB-ARC acts as a molecular switch to activate intracellular signaling (Van der Biezen and Jones 1998). Additionally, NB-LRR proteins exhibit a C-terminal Leucine-Rich Repeat (LRR) domain, which mediates protein–protein interactions (Kobe and Kajava 2001).

NB-LRRs can be further classified based on prominent domains for dimerization at the N-terminal. A major distinction is the presence or absence of an N-terminal Toll/Interleukin-1 Receptor (TIR) domain (Bernoux et al. 2011). Non-TIR NB-LRRs may possess a Coiled-Coil (CC) domain at the N-terminal instead (Maekawa et al. 2011). Figure 6.1 is a schematic representation of two NB-LRR proteins from *Fragaria vesca* that exemplify these groups: gene34870-v1.0-hybrid encodes a TIR-type NB-LRR, whereas gene04430-v1.0-hybrid encodes a non-TIR NB-LRR with an additional CC domain.

The NB-ARC domain contains several structural motifs that facilitate the formation of a nucleotide-binding pocket, of which the P-loop, Kinase-2, GLPL, and RNBS-A–D (Fig. 6.1) are among the most conserved (Takken et al. 2006). By contrast, the LRR domain is highly variable (being involved in pathogen recognition and therefore under positive selection), with varying repeat configurations and non-canonical LRR motifs (Takken and Goverse 2012). A complex fold stabilized by electrostatic interactions between the NB-ARC and the LRR, assisted by the CC or TIR domains, secures the NB-LRR in the inhibited position in the absence of its corresponding effector. The perception of an effector by the LRR induces a hair-trigger series of fast conformational changes resulting in the ATP-binding state of the NB-ARC domain and subsequent activation of signaling.

Some pathogen effectors are directly bound by NB-LRR proteins, as in the direct binding of the

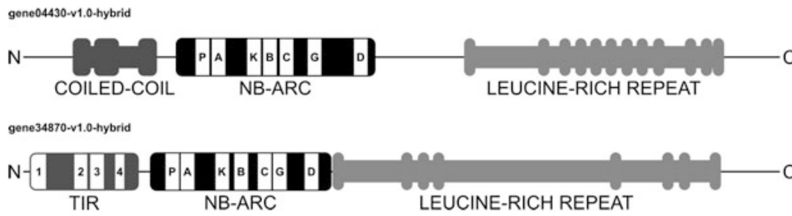

Fig. 6.1 Proteins encoded by the *F. vesca* genome illustrate the two main types of NB-LRR proteins, differentiated by the presence or absence of a TIR domain at the N-terminal. Both genes encode NB-ARC and LRR domains, but gene34870-v1.0-hybrid encodes an additional TIR domain with four conserved internal motifs. The other, gene04430-v1.0-hybrid, is an example of the non-TIR class of NB-LRR and encodes an additional Coiled-Coil domain at the N-terminal. The NB-ARC domain contains several conserved motifs: P, P-loop. A, RNBS-A. K, Kinase-2. B, RNBS-B. C, RNBS-C. G, GLPL. D, RNBS-D. The LRR domain is distinguished by the presence of several repeating leucine-rich motifs

flax rust AvrL567 effector proteins by the flax R proteins L5, L6, and L7 (Dodds et al. 2006). Instead of detecting effector presence directly, other R proteins may detect effectors indirectly via their action on other plant proteins. For example, no direct interaction has been demonstrated between the *Arabidopsis thaliana* NB-LRR Rpm1 and the *Pseudomonas syringae* effector protein AvrRpm1. Instead, Rpm1 detects disruption of the AvrRpm1 target, a plant protein called RIN4, which is itself involved in regulating plant defense (Mackey et al. 2002). This molecular surveillance of effector activity is often called the "guard model." The "decoy model" is a variation of this, in which R proteins detect disruption of bait proteins that mimic effector targets. The tomato (*Solanum lycopersicum*) protein Pto is a kinase that acts as a mimic of other plant kinases: The true effector targets for the *P. syringae* effector AvrPto. In turn, Prf is a tomato NB-LRR that monitors the binding of AvrPto to Pto (Ntoukakis et al. 2014). Pto and Prf are bound together in a complex; other NB-LRR proteins also form multimeric complexes with the plant proteins they guard, often stabilized via the CC or TIR domains. A subset of NB-LRR proteins has taken the decoy model to the next level, incorporating additional protein sensor domains homologous to effector targets at the C-terminal of the NB-LRR itself (Cesari et al. 2014). The RRS1 NB-LRR of *A. thaliana* is an example of such an integrated decoy, since it includes a C-terminal WRKY domain, used to detect *P. syringae* effectors, such as PopP2, that evolved to interfere with plant WRKY transcription factor function during pathogenesis (Sarris et al. 2015).

Developing varieties of Rosaceous berries resistant to disease forms part of the mandate of berry breeding programs around the world. *R* genes constitute a key resource for meeting the needs of the berry industry in the twenty-first century, and identifying natural sources of resistance for deployment in plant improvement programs will increasingly focus beyond primary germplasm to also include wild relatives in the secondary gene pool (Boyd et al. 2013). To date, the identification and introgression of *R* genes in *Fragaria* and *Rubus* have been challenging, but new genomic tools may expedite breeding for pest and pathogen resistance.

6.2 Genetics: Mapping and Markers for Disease Resistance in *Fragaria* and *Rubus*

For Rosaceous berries in particular, the search for NB-LRR genes encoding resistance against pests and pathogens has been arduous. For many diseases of Rosaceous berries, only a single or perhaps a few *R* genes have been intentionally deployed in commercial cultivars (Table 6.1). Single *R* genes are likely to be less durable than multiple, pyramided *R* genes, since changing a single effector specificity presents a much lower

Table 6.1 Summary of known *R* genes and their closest markers

Genus	Pathogen/pest	*R* gene	Donor	LG	Markers	Reference
Fragaria	*Phytophthora fragariae* var. *fragariae*	*Rpf1*	*F.* × *ananassa* "Md683"	6	SCAR-R1	Haymes et al. (2000)
		Rpf2	*F.* × *ananassa* "Aberdeen"			Van de Weg (1997)
		Rpf3	*F.* × *ananassa* "Aberdeen"		E36M59H	Haymes et al. (1998)
		Rpf4				Sasnauskas et al. (2007)
		Rpf6			E39M51B	Haymes et al. (1998)
	Colletotrichum acutatum	*Rca2*	*F.* × *ananassa* "Dover"		STS-Rca2_240	Lercetau-Köhler et al. (2005)
	Xanthomonas fragariae	*FaRXf1*	*F. virginiana* US4808	6D	HRM6D_33.083	Roach et al. (2016)
Rubus	*Raspberry bushy dwarf virus*	*Bu*	*R. idaeus* "Glen Clova"		rasp_N_gene_1202	Stephens et al. (2016)
	Amphorophora idaei	A_1	*R. idaeus* "Baumforth A"	3	RU103a	Sargent et al. (2007)
		A_2	*R. idaeus* "Chief"			Sargent et al. (2007)
		A_3	*R. idaeus* "Chief"			Sargent et al. (2007)
		A_4	*R. idaeus* "Chief"			Sargent et al. (2007)
		A_5	*R. idaeus* "Chief"			Sargent et al. (2007)
		A_6	*R. idaeus* "Chief"			Sargent et al. (2007)
		A_7	*R. idaeus* "Chief"			Sargent et al. (2007)
		A_8	*R. strigosus* L518			Sargent et al. (2007)
		A_9	*R. strigosus* L518			Sargent et al. (2007)
		A_{10}	*R. occidentalis*			Sargent et al. (2007)
	Amphorophora agathonica	Ag_1	*R. idaeus* "Lloyd George"			Dosset and Finn (2010)
		Ag_2	*R. strigosus* (eastern Canada)			Dosset and Finn (2010)
		Ag_3	*R. strigosus* (eastern Canada)			Dosset and Finn (2010)
		Ag_4	*R. occidentalis* ORUS 3778	6	S99_32802	Bushakra et al. (2012)
		Ag_5	*R. occidentalis* ORUS 3817		SCAR-R1	Dosset and Finn (2010)

evolutionary hurdle to the pathogen. Thus, deployment of single gene resistance increases the risk of virulent, resistance-breaking strains developing in the field. When breeding for resistance, the plant breeder's task is in essence to manipulate the molecular arms' race between plants and their diseases in favor of the plant.

A gene-for-gene relationship between differentially resistant strawberry cultivars and isolates of the red stele root rot pathogen *Phytophthora fragariae* var. *fragariae* was first defined in the late 1980s (Van de Weg 1989), and through cultivar–race interaction studies, five distinct *R* genes were identified (Van de Weg 1997) (Table 6.1). *Rpf1* was the first of these, identified in the *F.* × *ananassa* clone "Md683." By the 1990s, Randomly Amplified Polymorphic DNA (RAPD) markers had become an important tool for gene mapping and molecular plant breeding. Bulk Segregant Analysis (BSA) of an "Md683" (*Rpf1*/*rpf1*) × "Senga Sengana" (*rpf1*/*rpf1*) F_1 population was used to develop seven RAPD markers linked to the *Rpf1* locus (Haymes et al. 1997). This part of the *F.* × *ananassa* genome exhibits disomic segregation, facilitating the construction of the first molecular linkage map for a disease resistance trait in cultivated octoploid strawberry. The linkage map for *Rpf1* was subsequently improved using a combination of RAPDs and Amplified Fragment Length Polymorphism (AFLP) markers (Haymes et al. 1998), and Sequence Characterized Amplified Region (SCAR) markers (Haymes et al. 2000; Sasnauskas et al. 2007). Linkage maps for *Rpf2*, *Rpf3*, and *Rpf6* have also been constructed (Haymes et al. 1998; Hokanson and Maas 2010).

Strong resistance to pathogenicity group 2 of *Colletotrichum acutatum*, a causative agent of anthracnose in strawberries, was identified in the cultivars "Sequoia" and "Dover" (Denoyes and Baudry 1995). A subsequent inheritance study across multiple strawberry genotypes designated this resistance *Rca2*, a single-dominant gene that also exhibits a disomic segregation pattern (Denoyes-Rothan et al. 2005). In an approach similar to that taken for mapping *Rpf1*, BSA of a "Capitola" (*Rca2*/*rca2*) × 'Pajaro' (*rca2*/*rca2*) F_1 population was used to associate AFLP markers with the resistance trait (Lerceteau-Köhler et al. 2005), and two of the AFLP markers were successfully converted to SCAR markers (Table 6.1). *Colletotrichum* resistance has also been observed in *F. vesca*, *Fragaria moschata*, *Fragaria chiloensis,* and *Fragaria virginiana*, although it is not yet clear whether this is due to the presence of homologs of *Rca2* in these species (Denoyes-Rothan et al. 2005).

Resistance to *Xanthomonas fragariae*, the causative agent of Angular Leaf Spot (ALS) in strawberry, was first identified in the wild strawberry accessions US4808 and US4809 (Maas et al. 2002). Since no commercial cultivars of *F.* × *ananassa* with resistance to ALS were available, these two wild accessions were used to introgress the trait into breeding material. However, since US4808 is an accession of *F. virginiana* collected from the wild in Minnesota in 1986, and US4809 is the progeny of a cross between *F. virginiana* SG-26, collected in Georgia, and *F.* × *ananassa* "Earliglow," the genetics of ALS resistance remained unclear. To improve matters, Roach et al. (2016) developed a complex multi-family population derived from both US4808 and US4809, to assay for the inheritance of resistance and aid in Quantitative Trait Locus (QTL) identification. They observed not only 1:1 segregation (implying single gene resistance) and high broad-sense heritability of the trait, but also identified a large-effect QTL at the distal end of linkage group 6D. Collectively, these data suggested the presence of a single-dominant gene for ALS resistance, which they named *FaRXf1* (Roach et al. 2016). Using a combination of Single Nucleotide Polymorphism (SNP) markers developed from the multi-family population, Roach and colleagues reduced the genomic region delimiting the QTL to 520 Kbp. Several candidate NB-LRRs are found in this region, along with other genes with possible resistance function, such as serine-threonine protein kinases.

Surprisingly, one of the most important genes conferring disease resistance in *Rubus* is not a member of the NB-LRR family, but instead controls trichome development. Gene *H* confers

cane pubescence in raspberry and is therefore likely involved in preformed, passive defenses because of its strong effect on morphology. It is associated with resistance to gray mold (*Botrytis cinerea*), spur blight (*Didymella applanata*), cane blight (*Leptosphaeria coniothyrium*), and cane spot (*Elsinoë veneta*) (Williamson and Jennings 1992). Although it has been suggested that the resistance inherited with cane pubescence may in fact be due to multiple *R* genes closely linked to the *H* locus (Graham et al. 2006), NB-LRR genes conditioning resistance to these pathogens have not yet been described.

Resistance against the *Raspberry bushy dwarf virus* (RBDV) is conditioned by the single-dominant gene *Bu,* first identified via segregation analysis in the *Rubus idaeus* cultivar "Glen Clova" (Jones et al. 1982). The first attempt to develop markers associated with *Bu* used a modified BSA approach, with different resistant and susceptible genotypes derived from the resistant "Newburg" (Ward et al. 2012). The SCAR and Cleaved Amplified Polymorphic Sequence (CAPS) markers were subsequently tested using a "Nootka" (*Bu*/*bu*) × WSU1499 (*bu*/*bu*) population segregating for *Bu*. Since the most promising CAPS marker, BC615_553_*Alu*I, did not segregate with the trait in some genetic backgrounds, additional markers were sought using a comparative analysis with the assembled diploid *F. vesca* genome (Shulaev et al. 2011). A strawberry scaffold harboring sequence orthologous to the CAPS marker was recovered and scrutinized for likely candidate *R* genes. The best candidate exhibited homology to the tobacco *N* gene, which affords resistance to the *Tobacco Mosaic Virus* (TMV) and encodes a TIR-NB-LRR (Whitham et al. 1994). From this candidate, a new SCAR marker, raspN_gene_1202, was derived that more faithfully segregates with the resistance trait across multiple genetic backgrounds (Ward et al. 2012). In a follow-up study (Stephens et al. 2016), SCAR marker BC615_553 was located to scaffold 00152 from a Genotyping By Sequencing (GBS) dataset generated for the RBDV resistant "Heritage" cultivar (Ward et al. 2013). Similar to the findings of Ward et al. (2012) in *F. vesca*, two *N*-like NB-LRR genes were found within a distance of 55 kbp from BC615_553 on the *R. idaeus* "Heritage" scaffold (Stephens et al. 2016). Of the SNP-based markers developed from these *Bu* candidates, RubRgeneP8 was the most consistent at discriminating resistant and susceptible cultivars. However, it is still not clear whether one of the two NB-LRRs is in fact the *Bu* gene.

Aphid resistance genes often encode proteins of the NB-LRR family. Both the *Mi-1* gene in tomato for resistance to the potato aphid (*Macrosiphum euphorbiae*) (Vos et al. 1998) and the *Vat* gene in melon for resistance to the cotton-melon aphid (*Aphis gossypii*) (Dogimont et al. 2014) encode non-TIR NB-LRR proteins. The two aphid species of major importance to raspberry growers, *Amphorophora idaei* in Europe, and *A. agathonica* in North America, are of concern mainly because they act as vectors for various viruses underlying the Raspberry Mosaic Disease (RMD) complex, including the *Raspberry leaf mottle virus* (RLMV), *Raspberry leaf spot virus* (RLSV), *Black raspberry necrosis virus* (BRNV), and *Rubus yellow net virus* (RYNV). Work to identify *Rubus* germplasm resistant to aphids was already underway by the mid-twentieth century. Briggs (1965) proposed a gene-for-gene relationship between specific virulent biotypes of *A. idaei* and resistant *R. idaeus* cultivars carrying the A_1, A_2, and A_3 genes. Breeding programs in Europe focus on the A_1–A_{10} genes for resistance to *A. idaei*, whereas their counterparts in North America largely rely on the Ag_1–Ag_5 genes for resistance to *A. agathonica* (Dossett and Finn 2010). Although compartmentalized as such by breeding programs in different parts of the world, these two gene series may be at least partially allelic. Based on their pattern of single-dominant inheritance and biotype specificity, raspberry aphid resistance genes are likely to encode NB-LRRs (Lightle et al. 2012), although none have been characterized to date. A combination of AFLP and Simple Sequence Repeat (SSR) markers was developed for the A_1 gene of *R. idaeus* from a "Malling Jewel" (a_1/a_1) × "Malling Orion" (A_1/a_1) F_1 population (Sargent et al. 2007). The AFLP markers mapped A_1 to linkage group 3, flanked

by the co-dominant SSR RU103a and the AFLP aE40MCAAN107. *Ag_4* is an *A. agathonica* resistance gene identified in ORUS 3778, a wild accession of *R. occidentalis* from Maine, USA (Dossett and Finn 2010). This accession was used to develop further resistant black raspberry germplasm, including ORUS 4305, an ORUS 3021 (ag_4/ag_4) × ORUS 4153 (Ag_4/ag_4) F_1 population that segregates 1:1 for *Ag_4* (Bushakra et al. 2015). A linkage map was developed for mapping population ORUS 4305 using a combination of GBS and 26 SSR markers previously developed in *Rubus*. This allowed *Ag_4* to be mapped to linkage group 6 close to marker S99_32802 (Bushakra et al. 2015).

6.3 Genomics: Approaches to Candidate Resistance Gene Identification in *Fragaria* and *Rubus*

The octoploidy of cultivated strawberry has made it particularly difficult to associate molecular markers with disease resistance genes. Marker development has been much more successful for loci such as *Rpf1* and *Rca2* that exhibit disomic inheritance despite the octoploid genomic background (Haymes et al. 1997; Denoyes-Rothan et al. 2005). Instead of using molecular markers to identify the *R* loci underlying particular resistance phenotypes, reverse genetics aims to understand the function of *R* genes identified via genomic or transcriptomic approaches. Disease resistance research in both *Fragaria* and *Rubus* has increasingly adopted reverse genetics methods to discover and characterize potentially useful *R* genes.

In the mid-1990s, PCR-based methods emerged for the amplification of disease resistance gene sequences from plant DNA, with initial reports from potato (Leister et al. 1996), soybean (Yu et al. 1996; Kanazin et al. 1996), *A. thaliana* (Aarts et al. 1998), and maize (Collins et al. 1998). Since the NB-ARC domain has motifs such as the P-loop, Kinase-2, and GLPL that are highly conserved among NB-LRR genes (Fig. 6.1), degenerate PCR primers designed to target those motifs will amplify a set of related sequences, called Resistance Gene Analogs (RGAs), fragments derived from putative genes with predicted *R* gene function.

In the first attempt to assay the *R* gene composition of strawberry, RGAs were isolated from six *Fragaria* × *ananassa* cultivars, *F. vesca*, *F. chiloensis*, and *Potentilla indica* using degenerate PCR primer sets designed to target the P-loop and GLPL motifs of the TIR-NB-LRR *N* gene of *Nicotiana tabacum* and the CC-NB-LRR *RPS2* gene of *A. thaliana* (Martínez Zamora et al. 2004). Of the 51 clones with high homology to known *R* gene sequences, only 28 had uninterrupted ORFs, and only 22 sequences were considered unique, with pairwise amino acid identity below 97%. All but one of the sequences was of the TIR-type and displayed sequence similarity with the *N* gene. Despite this limited sampling of *R* gene diversity, phylogenetic analysis allowed identification of seven distinct RGA families in strawberry.

Seven sets of degenerate primers designed based on known Rosaceae RGAs were successfully employed to amplify 75 RGAs from *R. idaeus* “Latham” (Samuelian et al. 2008). In a combination of forward and reverse genetics approaches, the sequences of these RGAs were used to inform PCR-based marker development. The resulting CAPS and SCAR markers were placed on a genetic linkage map developed using a (“Titan” × “Latham”) × “Titan” BC_1 population segregating for resistance to the root rot pathogen *Phytophthora rubi* (Pattison et al. 2007). The RGA fragment 9_Ri_14–36 mapped to a previously identified QTL for *P. rubi* resistance (Samuelian et al. 2008).

The same set of degenerate primers used by Samuelian et al. (2008) were used to identify 47 RGAs from five genotypes of Andean blackberry (*Rubus glaucus*) differing in resistance to anthracnose (*Colletotrichum* spp.) (Afanador-Kafuri et al. 2015). Phylogenetic analysis of these 47 RGAs and others from other Rosaceae species revealed clusters of closely related *Rubus* RGAs exclusively associated with anthracnose-resistant genotypes. While this correlation is intriguing, genetic mapping of specific RGAs

relative to phenotypic resistance is required to verify co-association.

Putative *R* genes effective against a specific pathogen can also be identified by using degenerate primers to amplify RGAs from cDNA instead of genomic DNA; ensuring analysis is limited to the subset of NB-encoding sequences that are truly expressed. With this modified approach, a set of 23 RGAs were amplified from the crown rot (*Phytophthora cactorum*) resistant *F. vesca* accession "Bukammen," but not the susceptible genotype FDP821 (Chen et al. 2016). Subsequent real-time PCR analysis of the pathogen responsiveness of these RGAs highlighted RGA109 as a good candidate for further characterization as a potential *P. cactorum* resistance gene.

Li et al. (2013) identified 94 candidate *R* genes in *F. vesca* by performing a BLAST search against *F. vesca* whole genome shotgun reads deposited in NCBI, using the conserved NB-ARC sequences of 22 partial NB-LRR cDNAs from *F.* × *ananassa* as query. They subsequently cloned 36 partial NB-encoding cDNAs. Real-time PCR primers targeting these RGA transcripts were used to quantitatively evaluate the RGA responses of resistant and susceptible *F. vesca* ecotypes to inoculation with *Colletotrichum gloeosporiodes*. RGA loci responsive to *C. gloeosporiodes* infection might be appropriate targets for marker development.

6.4 Bioinformatics: Genome-Wide Analyses of NB-LRRs in *Fragaria* and *Rubus*

Complete genome sequences for a growing number of plant species are becoming available thanks to the rapid advances in high-throughput sequencing methods. Concomitant improvement of bioinformatic tools has also facilitated genome sequence analysis, including *R* gene discovery in silico.

The first genome sequenced for a Rosaceous berry was that of *F. vesca* (Shulaev et al. 2011), with revision v2.0.a1 published soon after (Tennessen et al. 2014). This was followed by draft genomes for the octoploid *F.* × *ananassa,* and four wild *Fragaria* species, *Fragaria iinumae*, *Fragaria nipponica*, *Fragaria nubicola*, and *Fragaria orientalis* (Hirakawa et al. 2014), enabling infrageneric comparative analyses. The first *Rubus* to be sequenced is *Rubus occidentalis* (VanBuren et al. 2016), chosen for its particularly low level of heterozygosity compared to other *Rubus* species (Bushakra et al. 2012).

As a model species with a small genome (211.7 Mb in the v2.0.a1 assembly), *F. vesca* has played an important role in comparative analyses of NB-LRR genes across the Rosaceae. Estimates of the *R* gene repertoire of *F. vesca* vary with different gene calling methods and annotation criteria, from as low as 144 (Zhong et al. 2015) to as high as 346 (Jia et al. 2015). However, both of these studies reported a smaller number of NB-LRR genes in *F. vesca* than in other Rosaceous species—such as apple, pear, and peach—with larger genomes and more recent genome duplication events. Evidence from comparative analysis of the black spot (*Diplocarpon rosae*) resistance locus *Rdr1* in rose (*Rosa* spp.) with its orthologous loci in apple, peach, and *F. vesca* suggests an extensive history of gene duplication for NB-LRRs in the Rosaceae, both before and after speciation events (Terefe-Ayana et al. 2012). By different estimates, a quarter (Arya et al. 2014) to a half (Jia et al. 2015) of NB-LRR genes in *F. vesca* arose via recent tandem duplication events. To date, no in silico analysis of the complete NB-LRR repertoire of the *F.* × *ananassa* genome has been reported.

The annotated genome sequences for *F. vesca*, *F.* × *ananassa, F. iinumae*, *F. nipponica*, *F. nubicola*, *F. orientalis*, and *R. occidentalis* are available on the public Genome Database for Rosaceae (www.rosaceae.org; Jung et al. 2014). To estimate *R* gene number and composition in these seven species, we scrutinized predicted peptide sequence data sets for the presence of NB-ARC domains by searching against the Pfam protein family database, using an E-value threshold of $\leq 1 \times 10^{-5}$ (pfam.xfam.

org; Finn et al. 2014). We subsequently annotated LRR and TIR domains with additional Pfam searches, and Coiled-Coil domains were predicted with COILS (Lupas et al. 1991). Results are summarized in Fig. 6.2. As expected for a highly heterozygous octoploid, *F.* × *ananassa* exhibited the most NB proteins, with 576, whereas only 84 NB proteins were recovered from the *R. occidentalis* predicted peptide data set. We determined 198 NB sequences in *F. vesca*, which was within the range of previous estimates (Jia et al. 2015; Zhong et al. 2015). The proportion of TIR-containing NB sequences in *F. vesca* was 18%, which compared favorably with the 16% reported by Zhong et al. (2015). To investigate the genomic organization of putative *R* genes, we plotted the mapping positions of NB-encoding sequences on the linkage groups of *F. vesca* (Fig. 6.3). Of the 198 NB sequences, a total of 194 were successfully mapped to linkage groups, while the remaining four mapped to unassembled scaffolds. Contrary to the findings of Li et al. (2013), LG6 exhibits the most NB-encoding genes, with 48 loci, and LG2 the least, with 16 loci. In agreement with previous reports (Li et al. 2013; Jia et al. 2015), NB-encoding genes are unequally dispersed across the *F. vesca* genome, occurring both as single loci and in clusters, particularly in the distal portions of chromosomes.

To explore the phylogenetic diversity in NB sequences across the seven *Fragaria* and *Rubus* species, we aligned the NB-ARC domains of all 2131 proteins using the MUSCLE multiple sequence alignment tool (Edgar 2004). This alignment was used to construct an approximately maximum-likelihood tree with FastTree (Price et al. 2009). The resultant unrooted phylogenetic tree is divided into two major clades (Fig. 6.4) composed of TIR-NB-LRR and non-TIR NB-LRR sequences. No species-specific sub-clades were observed for *Fragaria*, suggesting the preservation of highly related genes between highly related species. There is

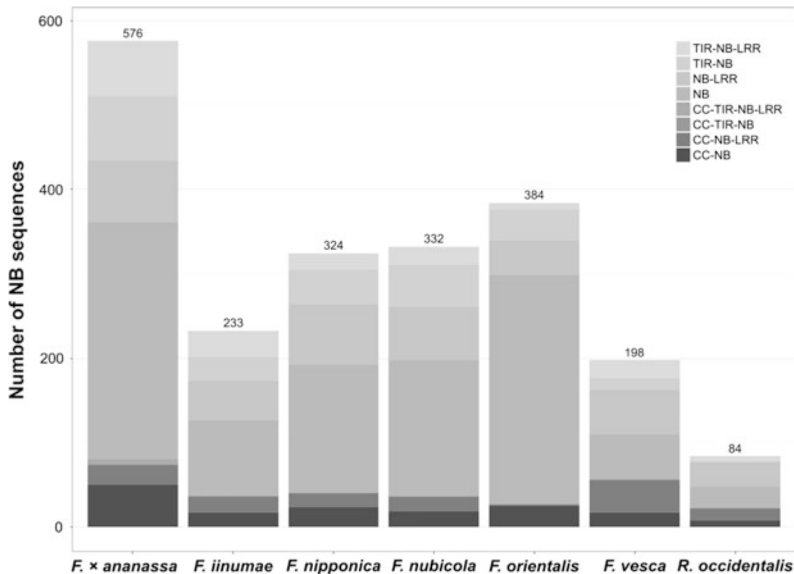

Fig. 6.2 A summary of Nucleotide-Binding domain-encoding genes identified from the publicly available genome sequences of various Rosaceous berry species. The total number of sequences identified per genome is indicated at the top of each bar. The highly heterozygous octoploid strawberry (*F.* × *ananassa*) genome yielded the most NB genes and the homozygous black raspberry (*R. occidentalis*) genome the least. The proportions of NB sequences with various configurations of additional domains are indicated by different shades (see inset key). No additional domains were identified for the majority of NB-encoding genes

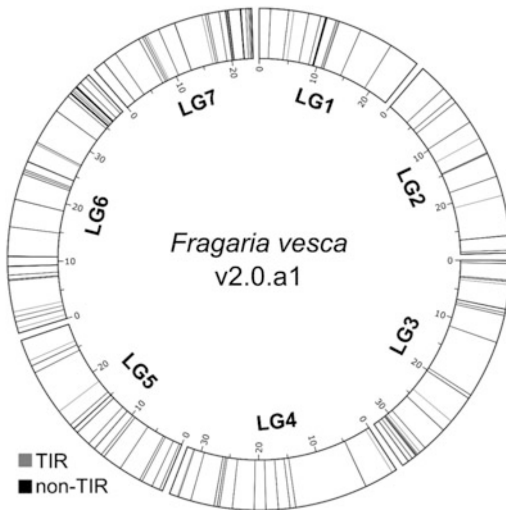

Fig. 6.3 Distribution of putative *R* genes within the genome of *F. vesca*. The seven linkage groups are arranged in a circle (LG1–7), with the positions of 194 NB-encoding sequences indicated by lines. Four NB sequences mapped to scaffolds not assembled into linkage groups (not shown). Sequences also encoding a TIR domain are represented by gray lines, and genes encoding no TIR domain by black lines. Putative *R* genes are unequally dispersed across the genome, either arranged as single loci or as clusters, often at the distal ends of chromosomes

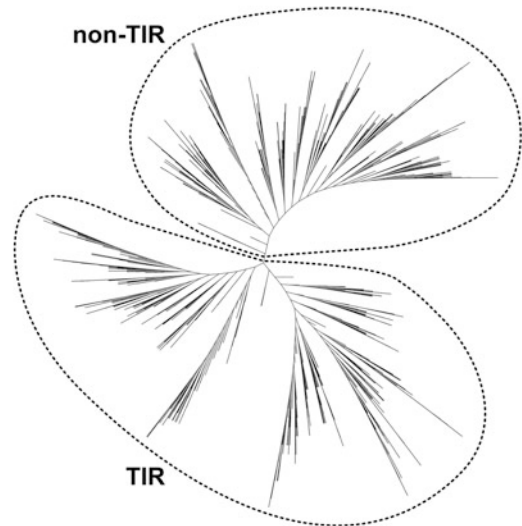

Fig. 6.4 Cladogram of putative R proteins encoded within the genomes of *F. vesca*, *F.* × *ananassa, F. iinumae*, *F. nipponica*, *F. nubicola*, *F. orientalis*, and *R. occidentalis*. The cladogram is based on a multiple sequence alignment of 2131 NB-ARC peptide sequences and constructed in FastTree using the approximately maximum-likelihood method. Two large groups corresponding to TIR and non-TIR are highlighted with dotted lines

therefore much untapped potential for the transfer of useful NB-LRR genes from wild relatives to cultivated strawberry.

As genome sequencing efforts expand to include more wild species and also the resequencing of multiple genotypes within a species, so too will our understanding of the true extent of the *R* gene space for Rosaceous berries. This *R* gene space constitutes a precious resource for plant breeders that are currently little understood and underutilized. With the cultivation of Rosaceous berries comes the continual threat of new, virulent strains of pathogens arising in the field. Wide-scale deployment of NB-LRR genes in response to these threats demands development of new informatics tools to identify useful genetic diversity within the primary and secondary gene pools of *Fragaria* and *Rubus*, sequence-based mining of gene bank collections, and improved breeding strategies for accelerated *R* gene introgression.

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Part II

The Genomes of Rosaceous Berries

7 Disease Resistance in Polyploid Strawberry

Charlotte F. Nellist

Abstract

The cultivated strawberry (*Fragaria* × *ananassa* Duchesne) is grown globally, from subarctic regions through to subantarctic regions and is host to a vast array of economically important disease-causing pathogens. Diseases are a significant limiting factor to crop production worldwide, resulting in high costs caused not only by yield losses but also by the expense of pesticide applications to try to prevent or manage the diseases. Traditionally, the major strategy for disease control in strawberry production relied heavily upon pre-plant fumigation and chemicals. The withdrawal of many of these active chemicals, including fungicides and soil fumigants, is increasing the challenges in strawberry production, resulting in a rise of occurrences and severities of some once well-controlled diseases. There is an absence of commercial cultivars that possess high levels of resistance to multiple pathogens. Breeding for disease resistance is a high priority for many breeding programmes across the world. This chapter will discuss some of the major diseases of the cultivated strawberry, the current status of resistance and future prospects.

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7.1 Introduction

The cultivated strawberry, *Fragaria* × *ananassa* Duchesne ($2n = 8x = 56$), is an accidental hybrid between two wild octoploid species, *Fragaria chiloensis* and *Fragaria virginiana*, which occurred approximately 250 years ago, making it one of the youngest crop species (Darrow 1966). *F. chiloensis* and *F. virginiana* are very closely related and thought to have originated from a common octoploid ancestor (Harrison et al. 1997). The cultivated strawberry is an allopolyploid outbreeder with a genome comprised of four comparable homeologous sets of diploid chromosomes, which is considered to have arisen from as many as four different diploid ancestors, including *Fragaria vesca* and *Fragaria iinumae* (Hummer et al. 2011; Tennessen et al. 2014). The octoploid genome is estimated to be 698 Mb, 80% of the size of quadrupling the diploid genomes (~200 Mb each) (Hirakawa et al. 2014). The model diploid species, *F. vesca* (the woodland strawberry; $2n = 2x = 14$), in comparison has a relatively small genome size of ~ 240 Mb and has been used a model plant for the *Rosaceae* genus, due to this and its amenability to functional studies (Shulaev et al. 2011).

F. × *ananassa* is cultivated globally, from subarctic regions through to subantarctic regions, and is host to a vast array of economically important disease-causing pathogens. Diseases

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are a significant limiting factor to crop production worldwide, resulting in high costs caused not only by yield losses but also by the expense of pesticide applications to try to prevent or manage the diseases. Traditionally, the major strategy for disease control in strawberry production relied heavily upon pre-plant fumigation and chemicals. Methyl bromide was widely used as a pre-plant soil fumigant because of its broad-spectrum effect on soil-borne diseases, weeds, nematodes and insects. It had been central to the high plant density, continuous cropping system used in strawberry production for over forty years (Ajwa et al. 2004). However, due to its ability to deplete ozone, it was identified as a substance of concern and a phase-out programme was initiated. Under the Montreal Protocol, it was banned in developed countries from 2005 and in developing countries from 2015. The withdrawal of methyl bromide along with other active chemicals, including fungicides and soil fumigants, is increasing the challenges in strawberry production, resulting in a rise of occurrences and severities of some once well-controlled diseases (Maas 2014). There is an absence of commercial strawberry cultivars that possess high levels of resistance to multiple pathogens. Breeding for disease resistance is a high priority for many breeding programmes across the world (Faedi et al. 2002). This chapter will discuss some of the major diseases of the cultivated strawberry, the current status of resistance and future prospects; see Table 7.1 for a summary of the diseases and their presence across the world.

7.2 Fungal Diseases

Fungal pathogens are the most significant strawberry pathogens in terms of causing yield losses. Fungal diseases can affect all parts of the plant, including roots, crowns, leaves, flowers and fruit, with many genera of fungal pathogens able to cause disease on strawberry, including *Botrytis*, *Colletotrichum*, *Fusarium*, *Macrophomina*, *Podosphaera* and *Verticillium*. *Mucor* spp. and *Rhizopus* spp. are also well known for causing post-harvest rot in fruit (Maas 1998). Recently, a new fungal genus, *Neopestalotiopsis*, has been correlated with multiple diseases in strawberry. *Neopestalotiopsis* spp. are primarily thought of as opportunistic pathogens that affect stressed plants. *Neopestalotiopsis clavispora* (previously known as *Pestalotiopsis clavispora*) has been identified in association with mortality in strawberry plants, causing crown rot disease (Chamorro et al. 2016). *Neopestalotiopsis longisetula* (previously known as *Pestalotiopsis longisetula*) has been coupled with causing leaf spot disease in strawberry (Rodrigues et al. 2014). *Neopestalotiopsis iranensis* sp. nov. and *Neopestalotiopsis mesopotamica* have been connected with fruit rot in strawberry (Ayoubi and Soleimani 2016).

7.2.1 Powdery Mildew

Powdery mildew (*Podosphaera aphanis*, syn. *Sphaerotheca macularis* f. sp. *fragariae*) was one of the first diseases to be described on strawberry and is still seen as one of the most problematic diseases of perennial production worldwide (Maas 2004). Yield losses of up to 60% have been reported in the USA (Horn et al. 1972). Leaves, petioles, calyces of flowers and fruit are all susceptible to mildew growth. High humidity and relatively cool temperatures, along with short day or low light intensity, facilitate growth and rapid spread of the disease (Maas 2004). Powdery mildew is a particular problem on strawberries in protected or closed culture systems (Maas 1998). *P. aphanis* is an obligate biotrophic airborne fungus, surviving only within the living tissue of strawberry. Powdery mildew infection has very distinguishing symptoms, initial symptoms include infected leaves curling upwards, with white patches of mycelium developing on the underside of leaves, and in extremely susceptible cases this can cover the whole of the lower surface. Discolouration of the leaf may be observed, with purple/reddish blotches occurring on the lower leaf surface. In severe cases, necrosis may also be observed resulting in eventual leaf death. Most yield losses are the result of infected flowers and fruit.

Table 7.1 Distribution and availability of genomic resources and resistance markers for a selection of diseases of the cultivated strawberry, *Fragaria* × *ananassa*

Disease	Causal agent	Presence/significant problem?				Reference genome available	Resistance identified?
		Europe	North America	Asia	Australia		
Grey mould	*Botrytis cinerea*	X	X	X	X	GenBank: AAID00000000 (Amselem et al. 2011)	
Anthracnose/ black spot	*Colletotrichum acutatum*	X	X			GenBank: LUXP00000000 (from pepper) (Han et al. 2016)	Yes, *Rca2* (Denoyes-Rothan et al. 2005; Lerceteau-Köhler et al. 2005)
	Colletotrichum gloeosporioides		X	X		GenBank: AMYD00000000.1 (from avocado)	
Fusarium wilt	*Fusarium oxysporum* spp. *fragariae*		X	X	X		Yes, *Fw1* (Pincot et al. 2018)
Leaf blotch	*Gnomonia comari*	X			X		
Charcoal rot	*Macrophomina phaseolina*		X		X	GenBank: AHHD00000000 (from jute) (Islam et al. 2012)	
Mucor fruit rot	*Mucor* spp.	X					
Leaf spot	*Mycosphaerella fragariae*		X				
Black root rot	*Pythium* spp., *Fusarium* spp., *Rhizoctonia* spp. and several nematodes		X		X		
Crown rot	*Phytophthora cactorum*	X	X			GenBank: PRJNA380728 (from European beech)	Yes, polygenic (Denoyes-Rothan et al. 2004; Mangandi et al. 2017)
Red core	*Phytophthora fragariae*	X	X			GenBank: JHVZ00000000.3 and MWJK00000000 (Gao et al. 2015; Tabima et al. 2017)	Yes, gene-for-gene, *Rpf1* (Haymes et al. 1997), *Rpf2*, *Rpf3*, *Rpf6* (Haymes et al. 1998) and *Rpf4* (Rugienius et al. 2006
Powdery mildew	*Podosphaera aphanis*	X		X			
Rhizopus fruit rot	*Rhizopus* spp.	X	X				

(continued)

Table 7.1 (continued)

Disease	Causal agent	Presence/significant problem?				Reference genome available	Resistance identified?
		Europe	North America	Asia	Australia		
Verticillium wilt	*Verticillium dahliae*	X	X			GenBank: ABJE00000000.1 (from lettuce) (Klosterman et al. 2011)	Yes, polygenic (Antanaviciute et al. 2015)
Angular leaf spot	*Xanthomonas fragariae*	X	X			GenBank: CP016830.1-CP016835.1 (Henry and Leveau 2016)	Yes, single dominant gene, *FaRXf1* (Roach et al. 2016)

Chemical control of powdery mildew requires routine and regular sprays with both fungicides and potassium bicarbonate throughout the growing season.

Commercial strawberry cultivars vary in their susceptibility to *P. aphanis* (Nelson et al. 1995; Nelson et al. 1996). Resistance to powdery mildew in strawberry is polygenic and found to be highly heritable (Nelson et al. 1995; Liang and Lin 2014). It has also been noted that different genes may confer resistance under different intensities of disease pressures (Nelson et al. 1995). Ontogenic (age-related) resistance is also observed in strawberry leaves and fruits in response to *P. aphanis* (Asalf et al. 2014). Studies into the development of resistance in leaves found that as the strawberry leaves expanded and unfolded, ontogenic resistance suppressed pathogen establishment and growth; in the oldest leaves, all the pre- and post-penetration processes were inhibited (Asalf et al. 2016). Resistance to powdery mildew has also been investigated and noted in the wild relatives *F. chiloensis* and *F. virginiana* (Hancock et al. 2002; Luby et al. 2008), and immunity has been reported in the hexaploid species *Fragaria moschata* (Maas 1998).

Powdery mildew diseases are caused by different fungal species in the family *Erysiphaceae*. In other crops, resistance to powdery mildew has been provided by an alternative approach to the traditional use of dominant resistance genes (*R*-genes), which recognise pathogen avirulence genes (*Avr*) and are based on gene-for-gene resistance. *R*-gene-mediated resistance can be overcome relatively quickly as the pathogen is under selection pressure to evolve to overcome the single (or multi-gene) resistance. On the other hand, resistance based on susceptibility genes (*S*-genes) is thought to be recessively inherited and able to provide durable, broad-spectrum resistance through loss-of-function mutations, which limit the ability of the pathogen to cause disease (Pavan et al. 2010). *S*-genes were first discovered in barley (*Hordeum vulgare* L.) in 1942, in response to the powdery mildew pathogen of barley, *Blumeria graminis* f. sp. *hordei* (*Bgh*) (Jørgensen 1992). Barley possesses the *Mildew Locus O* (*MLO*) gene, and the protein is the prerequisite for successful colonisation by the mildew fungus, *Bgh*. *MLO*-based resistance is achieved through the mutation or knockout of the *MLO* gene, resulting in the prevention of the mildew fungus from penetrating host epidermal cells, inhibiting the pathogen from forming haustorium and stopping infection. *MLO* mutants have effectively been used in European barley agriculture for over 25 years, with no evidence of the resistance being broken by *Bgh*. This type of resistance is similar to non-host resistance, resistance against all non-adapted pathogens. Our understanding of non-host resistance is limited; however, it is considered the most durable and prevailing prevention of plant disease. *MLO* homologs have been investigated in *F. vesca,* and 17 have been identified in the diploid species (Pessina et al. 2014). Further work is required to investigate if knockdown of *MLO* genes could reduce susceptibility to powdery mildew in the cultivated strawberry.

7.2.2 *Verticillium* Wilt

Verticillium wilt disease is caused by the soil-borne fungal pathogen *Verticillium dahliae* Klebahn. The pathogen is extensively distributed and has a wide host range, including many crop species and numerous weed species, although some host range specificity does exist (Bhat and Subbarao 1999). *Verticillium* wilt caused devastating losses in California before the 1960s, when the use of chloropicrin and methyl bromide as soil fumigants became the standard practice (Paulus 1990). Periods of environmental stress, such as drought or high temperatures bring on the first symptoms of the disease (Maas 2004). Often one of the first symptoms observed is the dark brown necrosis on the petiole base. *V. dahliae* compromises the vascular system of the plants causing the wilting of the plant, the outer leaves wilt first, and the inner leaves can become stunted but visibly healthy until the plant dies. *V. dahliae* produces persistent resting spores, microsclerotia which can survive in the soil for many years.

Varying levels of resistance to *V. dahliae* have been identified in accessions of the octoploid species of *F.* × *ananassa*, *F. chiloensis* and *F. virginiana* (Maas et al. 1989; Shaw et al. 1996; Vining et al. 2015). Resistance to *Verticillium* wilt in *F.* × *ananassa* is quantitative; polygenic and the quantitative trait loci (QTLs) appear to behave in an additive manner (Shaw et al. 1996; Antanaviciute et al. 2015). Antanaviciute et al. (2015) identified a total of 11 QTLs (*RVd1*-*RVd11*) in the *F.* × *ananassa* 'Redgauntlet' × 'Hapil' population. The simple sequence repeat (SSR) or microsatellites markers associated with the individual QTL were found to be plentiful in the cultivated strawberry germplasm investigated, demonstrating that clear genetic gain in resistance to *V. dahliae* is possible through marker-assisted breeding (Antanaviciute et al. 2015).

7.2.3 *Fusarium* Wilt

Fusarium wilt (also known as *Fusarium* yellows) of strawberry is caused by the fungus *Fusarium oxysporum* f. sp. *fragariae* (Fof); it was first reported in South East Queensland, Australia, in 1962 (Winks and Williams 1965). Fof is pathogenic to only strawberry and poses a serious risk to commercial strawberry production across the world, with strawberry crop losses as high as 50% reported (Maas 1998). Symptoms of the disease are characteristic of other wilt diseases caused by *F. oxysporum*, starting with stunting of younger leaves, development of lesions on the petioles and necrosis of the roots, leading to rapid wilting, total collapse and eventually death of the plant. Fof is a soil-borne pathogen and enters the plant through the root cortex; it disrupts the vascular system of the plants, blocking the translocation of water and minerals, ultimately resulting in the wilting of the plant (Fang et al. 2012a). When observed in the field, these symptoms are very similar to other fungal diseases causing sudden plant collapse, such as *Macrophomina phaseolina*, *Verticillium* spp. and *Colletotrichum* spp.; therefore, isolation of the pathogen is required for accurate identification (Maas 2014).

Variation in host susceptibility has been observed, and commercial cultivars with varying levels of resistance have been identified (Kim et al. 1982; Takahashi et al. 2003; Herrington et al. 2007).[1] Resistance has also been identified in wild clones of *F. chiloensis* (Dávalos-González et al. 2006). Resistance to Fof is thought to be caused by an obstruction of pathogen growth and colonisation both on the plant's root surface and within host tissue, restricting Fof to the epidermal layer ensuring the vascular system is maintained (Fang et al. 2012a). Comparisons between the proteomic response of resistant and susceptible plants to Fof have been investigated, and potential key molecular components have been identified (Fang et al. 2013). Research is also being carried out to try and understand the mechanisms underlying Fof virulence, to help

[1] A genome-wide association study recently led to the identification of a single dominant resistance gene (*Fw1*), with the strongest resistance candidates being Toll/interleukin-1 receptor nucleotide-binding site leucine-rich repeat (TIR NBS-LRR) encoding genes (Pincot et al. 2018).

provide data for more effective strategies to manage *Fusarium* wilt (Fang and Barbetti 2014). Population studies of isolates of Fof based on comparisons between random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RLFP) of ribosomal DNA (rDNA) and vegetative compatibility groups (VCGs) have shown that isolates are genetically different and can be split into distinct clades (Nagarajan et al. 2004, 2006).

7.2.4 Anthracnose

Anthracnose diseases of strawberry cause severe problems for growers and propagators globally (Maas 2014). Anthracnose is caused by two species complexes of the hemibiotroph *Colletotrichum*, *Colletotrichum acutatum* and *Colletotrichum gloeosporioides*. In addition to strawberry, the host range of these pathogens is very wide and so inoculum sources are ubiquitous (Maas 2014). The disease can affect any part of the plant, causing leaf spots, fruit rots, crown rots and in some cases, it can cause lesions on the petioles and stolons. *C. acutatum* is the principal cause of anthracnose fruit rot, and *C. gloeosporioides* is the primary cause of Colletotrichum crown rot. *C. acutatum* was first identified as a strawberry pathogen in 1983, in California, USA (Smith and Black 1986). It is the predominant cause of anthracnose disease in strawberry in the UK and Europe. The *C. gloeosporioides* complex (which includes *Colletotrichum fragariae*) is only found occasionally in Europe; however, it is widespread in the USA and China.

Commercial strawberry cultivars vary in their susceptibility to anthracnose diseases (Mangandi et al. 2015). Differences in virulence of *C. acutatum* isolates have been observed, enabling the separation of isolates into two groups, based on their pathogenicity on strawberry (Denoyes and Baudry 1995). Resistance to pathogenicity group 1 was found to be quantitative, and five QTLs were identified in the cultivated strawberry (Denoyes-Rothan et al. 2004). Intermediate levels of resistance to pathogenicity group 2 were found to be quantitative and controlled by multiple minor genes (Denoyes-Rothan et al. 2005). In the same study, a single dominant gene, *Rca2*, was identified in controlling high-level resistance to pathogenicity group 2 and is widespread in the germplasm from both Europe and the USA (Denoyes-Rothan et al. 2005). Two sequence characterised amplified regions (SCAR) markers for *Rca2*, STS-Rca2_417 and STS-Rca2_240 have been developed for use in marker-assisted breeding (Lerceteau-Köhler et al. 2005; Whitaker 2011). The use of STS-Rca2_417 enabled 81.4% accuracy for predicting resistant/susceptible genotypes in the 43 cultivars studied (Lerceteau-Köhler et al. 2005). Pyramiding *Rca2* with the QTL for resistance to pathogenicity group 1 in commercial cultivars should provide broad-spectrum control of *C. acutatum* (Denoyes-Rothan et al. 2004, 2005; Lerceteau-Köhler et al. 2005).

Resistance to Colletotrichum crown rot, caused by *C. gloeosporioides* in the cultivated strawberry, appears to be race-non-specific and quantitative (MacKenzie et al. 2006). Resistance has been also investigated in accessions of both *F. chiloensis* and *F. virginiana*, with varying levels of resistance observed (Mangandi et al. 2015). In the same study, they also identified high levels of resistance in some advanced selections, providing promising material for the Florida breeding programme (Mangandi et al. 2015).

7.2.5 Botrytis Rot/Grey Mould Rot

Botrytis fruit rot (also known as grey mould rot) is a serious problem worldwide and can be one of the most destructive pathogens in strawberry production (Maas 1998). It is caused by the airborne fungal necrotrophic pathogen *Botrytis cinerea* Pers. Fr., which can colonise the leaves, crown, petals, flower stalks and fruit caps of strawberry plants, making it very difficult to control. It is responsible for substantial pre- and post-harvest yield losses, being not only an issue in production but also during the storage and transit of fruit. Yield losses of up to 55% have

been observed in fields untreated with fungicides (Daugaard 1999). Humidity has the biggest impact on disease development, in low humidity, infections can be contained to discrete locations, and however, in high humidity, infections can spread rapidly.

Resistance to *B. cinerea* is reported to be quantitative and of low combining ability; it is noted that there has been limited success in breeding and selecting resistant cultivars resistant to this economically important pathogen (Maas 2004). Bestfleisch et al. (2014) investigated resistance and identified several partly resistant genotypes, three *F.* × *ananassa* cultivars 'Diana', 'Joerica' and 'Kimberly', along with an accession of *F. virginiana*, 'Wildmare Creek' and *F. vesca* subsp. *bracteata*. Resistance has also been evaluated in wild accessions of *F. chiloensis,* and diversity was observed between accessions (González et al. 2009). The use of the transgenic expression of defence genes from other plant species has been successful. Vellicce et al. (2006) expressed a chitinase gene, *ch5B* from *Phaseolus vulgaris* (common bean), and found it correlated with high levels of resistance to *Botrytis* rot in the cultivated strawberry.

7.2.6 Charcoal Rot

Charcoal rot, caused by *Macrophomina phaseolina,* is an important re-emerging disease of strawberry, reported to affect the leaves, stolons, roots and crowns of the cultivated strawberry (Maas 2014). *M. phaseolina* has a very broad host range, infecting many economically important crops. Charcoal rot was first identified on strawberry in 1958 in Illinois, USA, and since then has been reported across the world (Tweedy and Powell 1958; Maas 2014). Over the last decade, there have been increasing incidences reported in California, USA (Koike et al. 2016). *M. phaseolina* causes stunting, wilting and death in strawberry; the symptoms are easily confused with those of *Verticillium*, *Fusarium* and *Phytophthora* (Maas 2014).

Limited studies have been performed investigating resistance to charcoal rot. In Australia, Fang et al. (2012b) investigated seven cultivars and identified 'Albion' as the most resistant cultivar and 'Camarosa' as the most susceptible to charcoal rot. Recently, in California, Sànchez et al. (2016) identified significant differences in the susceptibility of strawberry cultivars; 'Florida Fortuna' was found to be the most susceptible and 'Florida Festival', 'Amiga' and 'Naiad' were described as the least susceptible. Further work is required to explore these tolerance/resistance mechanisms.

7.3 Oomycete Diseases

Oomycete pathogens of strawberries include *Phytophthora* spp. and *Pythium* spp., both in the family *Pythiaceae*. Both genera can infect a wide range of plant species, causing serious damage in both ornamental and agricultural crops. *Phytophthora* are destructive hemi-biotrophic pathogens, and the two pathogens on strawberry are *Phytophthora cactorum* and *Phytophthora fragariae*. *Pythium* are necrotrophic pathogens. Black root rot is a disease complex, involving *Pythium* spp., *Fusarium* spp., *Rhizoctonia* spp. and nematodes such as the root lesion nematode *Pratylenchus* spp. In Japan, black root rot is known as stunt disease and *Pythium ultimum* has been associated with the disease (Watanabe et al. 1977).

7.3.1 Crown and Leather Rot

Phytophthora cactorum (Lebert and Cohn) Schröeter is the causative agent of two diseases in strawberry: strawberry leather rot disease and strawberry crown rot disease. Strawberry leather rot disease, affecting fruit, was first reported in 1924 in several southern states of the USA (Rose 1924), and strawberry crown rot disease was first discovered in northern Germany in 1952 (Deutschmann 1954). Both diseases can cause economic losses in strawberry production globally; in 1981, reports from commercial farms in Ohio described crop losses from leather rot of 20–30% (Ellis and Grove 1983), and in Norway, there have been reports of plant losses of up to

40% caused by crown rot (Stensvand et al. 1999). Amplified fragment length polymorphism (AFLP) analysis of *P. cactorum* isolates of crown rot and leather rot shows they are distinctly different from each other and from *P. cactorum* isolated from other hosts (Eikemo et al. 2004).

Strawberry plants infected with crown rot develop initial symptoms frequently during in hot periods, when plants are stressed. Plants can often appear stunted, and the youngest leaves are usually the first to wilt, spreading to the rest of the leaves, eventually resulting in the collapse and death of the plant. Red-brown lesions can be observed within the crown, when removed from the soil and split longitudinally. It is extremely challenging to reliably differentiate crown necrosis caused by *P. cactorum* from that produced by *Colletotrichum* spp., this is a particular problem during the latter stages of the disease. In susceptible cultivars, *P. cactorum* is able to maintain a latent infection for several months when the plants are not under stress, leading to its undetected spread through propagation material, causing problems in both soil and substrate systems. Sexually produced oospores are the primary source of inoculum; these are the resting spores that can persist in the soil or infected plants for many years.

Leather rot can infect fruit at any growth stage. Immature green berries develop brown margins, spreading to the whole fruit leading to the 'leathery' texture (Ellis and Grove 1983). The disease is much harder to visually detect on mature red berries as there is only slight colour change (Ellis and Grove 1983). Affected berries produce unpleasant smells, the disease alters taste of fruit, and this is a substantial problem in the processing industry as even low levels of infection are reported to result in off-flavoured jam (Ellis and Grove 1983).

Commercial cultivars vary in their susceptibility to *P. cactorum* (Eikemo et al. 2003; Gołębniak et al. 2006; Shaw et al. 2008; Schafleitner et al. 2013). No correlation has been found between resistance to crown rot and resistance to leather rot (Eikemo and Stensvand 2015). In *F. vesca,* a single major gene locus was identified on the proximal end of linkage group 6, named *Resistance to Phytophthora cactorum 1* (*RPc-1*). *RPc-1* was identified as spanning 69 potential plant disease resistance genes, including multiple different classes of resistance gene (Davik et al. 2015). Toljamo et al. (2016) observed the expression of these potential resistance genes in *P. cactorum* inoculated *F. vesca* Hawaii 4 roots. Within the locus, NBS-LRR gene 101297569 was identified as being a strong candidate along with highly expressed L-type-lectin-RLKs (receptor-like kinases) 101310048 and 101309756, as they were significantly upregulated (Toljamo et al. 2016). Both types of genes have been associated with resistance to many pathogen species (Chisholm et al. 2006). Further characterisation is required to fully understand the resistance mechanisms against *P. cactorum*.

Studies have identified resistance to crown rot in the octoploid strawberry, and it appears to be under polygenic control (Denoyes-Rothan et al. 2004; Shaw et al. 2006, 2008). A total of five putative QTLs for resistance to crown rot were identified in the *F.* × *ananassa* 'Capitola' × 'CF1116' progeny, with the QTL effects ranging from 6.5 to 10.2% (Denoyes-Rothan et al. 2004). Recently, a major QTL was identified on linkage group 7D, named *Fragaria* × *ananassa Resistance to Phytophthora cactorum 2* (*FaRPc2*) (Mangandi et al. 2017). Mangandi et al. (2017) highlight that not only is this major QTL located on a different linkage group to the resistance identified in *F. vesca*, which would be located on 6A under the octoploid convention (as described van Dijk et al. 2014), but that it is also located on the least similar subgenome to *F. vesca*, indicating that there are multiple sources of resistance to *P. cactorum* in *Fragaria*.

7.3.2 Red Core Root Rot

Red core root rot, also known as red stele root rot, is a serious disease of strawberry roots

caused by the oomycete *Phytophthora fragariae* Hickman. The pathogen is only pathogenic to strawberry; symptoms of the disease include wilting of the plant, the dying off of the tips of the roots, described as 'rat's tails', the red discolouration of the stele of the roots and eventually leading to the total collapse of the plant and death. *P. fragariae*, like *P. cactorum*, is able to sustain a latent infection in propagation material, when plants are not under stress. It is a quarantine pathogen in Europe, the European and Mediterranean Plant Protection Organization (EPPO) appointed its 'A2' status, and it is recognised that the pathogen is present in Europe but it is imperative to prevent its spread further. The genome of *P. fragariae* has been sequenced and assembled, with a total size estimated at 73.68 and 76 Mb with a G + C content of 53.25 and 53.35%, respectively (Gao et al. 2015; Tabima et al. 2017).

In the octoploid strawberry, a gene-for-gene model is reported to explain the interaction of the cultivated strawberry with *P. fragariae* (van de Weg 1997a). The original proposed model described five interacting resistance genes (*R1-R5*) with five pathogen avirulence factors (*Avr1-Avr5*) (van de Weg 1997a). The resistance genes are reported to have varying effects. *Resistance to Phytophthora fragariae race 2* (*Rpf2*) is controlled by a single dominant gene, providing resistance close to immunity (van de Weg 1997b). Resistance conferred by *Rpf1* and *Rpf3*, to *P. fragariae* races 1 and 3, respectively, provide dominant, incomplete resistance (van de Weg 1997c). Markers for *Rpf1*, *Rpf2*, *Rpf3* and *Rpf6* have been developed and tested (Haymes et al. 1998). Resistance has also been explored in other polyploids, *F. chiloensis* (Daubeny and Pepin 1965) and *Fragaria virginiana* ssp. *glauca* (formally known as *Fragaria ovalis*); however, at the time their use in breeding programmes had ceased since their progeny had little immediate commercial value (van de Weg 1997c). Since then further work on interspecific crosses of *F. ananassa* 'Tristar' × *F. chiloensis* 'Del Norte' are reported to potentially contain four resistance genes: *Rpf1*, *Rpf2*, *Rpf3* and *Rpf4* (Rugienius et al. 2006). In comparison, the diploid *F. vesca* is reported to be universally susceptible to *P. fragariae* (van de Weg 1997a). Findings in raspberry identified a dominant two-gene model in response to the closely related *Phytophthora rubi* (Cooke et al. 2000; Pattison et al. 2007).

7.4 Bacterial Diseases

Few bacterial pathogens are reported to cause disease in the cultivated strawberry (Maas 1998). Cauliflower disease is caused by the bacterium *Corynebacterium fascians* (Tilford) Dawson in combination with its vectors, the nematodes *Aphelenchoides fragariae* and *Aphelenchoides ritzemabosi* (Crosse and Pitcher 1952). Both bacterial wilt disease and angular leaf spot (ALS) disease are caused solely by bacteria, *Pseudomonas solanacearum* (Goto et al. 1978) and *Xanthomonas fragariae* (Kennedy and King 1962), respectively. More recently, two new bacterial pathogens have been observed on strawberry, *Xanthomonas arboricola* pv. *fragariae*, causing bacterial leaf blight (Janse et al. 2001), and *Erwinia pyrifoliae* (Wenneker and Bergsma-Vlami 2015).

7.4.1 Angular Leaf Spot

The most economical important bacterial disease of the cultivated strawberry is ALS disease caused by *Xanthomonas fragariae* (Kennedy and King 1962). It is a comparatively new disease of strawberry, first described in 1960 in the USA but has since spread and is reported worldwide. *X. fragariae* is reported to only infect *Fragaria* species (Maas et al. 2002). The classical symptoms of the disease are the angular water-soaked foliar lesions, which are translucent when viewed under light along with the presence of distinctive bacterial ooze under conditions of relatively high humidity (Maas 2004). Marketable yield losses of around 8% have been reported in Florida, USA (Roberts et al. 1997). Due to its economic

importance and the fact it can be transmitted through asymptomatic nursery plants, the European and Mediterranean Plant Protection Organization (EPPO) has listed *X. fragariae* as an 'A2' quarantine pathogen (CABI 1997), and all exports of strawberries must maintain high phytosanitary standards in order to exclude *X. fragariae* and restrict the spread of the disease.

There is no effective chemical control of *X. fragariae* available; copper formulations or acibenzolar-s-methyl have been reported to reduce disease pressure but can be toxic to plants and so not a suitable option (Roach et al. 2016). Currently, there are no commercial cultivars reported with high levels of resistance to *X. fragariae* (Roach et al. 2016). Resistance to the four clades of *X. fragariae* has been observed in wild accessions of polyploid strawberry, US4808 and US4809 (Maas et al. 2000, 2002). Initial reports described the segregation of resistance as being controlled by two or three unlinked loci (Hartung et al. 2003; Lewers et al. 2003; Jamieson et al. 2013). Further research has identified a single dominant allele at a single locus controlling the resistance (Jamieson et al. 2014; Roach et al. 2016). Roach et al. (2016) propose the inconsistency between research could be due to the loss of susceptibility alleles at unknown loci in the latter stages of the backcrossing programme, when introgressing the trait into elite octoploid strawberry germplasm. Roach et al. (2016) identified a large effect, highly heritable QTL at the distal end of linkage group 6D, termed *FaRXf1* (*Fragaria* × *ananassa* Resistance to *Xanthomonas fragariae* 1). FaRXf1 provides resistance to all four clades of *X. fragariae*, indicating the resistance is not isolate-specific (Roach et al. 2016).

7.5 Viral and Phytoplasma Diseases

The cultivated strawberry is hosted to more than 20 viral species, transmitted by insect and nematode vectors. In many commercial cultivars, clear symptoms are not observed, but rather a loss of vigour, stunting of the plant and lower yields can be attributed to infection (Maas 1998). Aphids are the most prolific vectors, and the four most economically important viruses affecting strawberry are strawberry mild yellow-edge virus (SMYEV), strawberry crinkle virus (SCV), strawberry mottle virus (SMoV) and strawberry vein-banding virus (SVBV) (Maas 1998). Viruses are often found in complexes in strawberries, and this can lead to severer declines in plant health and result in the unmarketability of fruit. Differences in tolerance levels of cultivars exist in response to viral diseases; however, research into resistance is lacking.

Phytoplasmas are phloem-restricted, cell-wall-less prokaryote pathogens transmitted by insect vectors, and there are more than 12 phytoplasmas associated with disease in strawberry (Maas 1998). Symptoms of phytoplasmal diseases include plant stunting, leaf yellowing, fruit deformation, fruit loss and plant death (Maas 1998). Control of strawberry phytoplasmal diseases has been problematic due to the shortage of control measures and host resistance/tolerance (Zhao et al. 2004).

7.6 Conclusions and Future Prospects

Plant pathogens threaten sustainable agriculture, global food security and cause substantial crop losses annually. Breeding for disease resistance is an environmentally efficient way of controlling plant diseases. However, resistance can often be broken down after only a few years in the field if based on a single *R*-gene, as with the case in South-Eastern Australia where a major gene resistance to Blackleg (*Leptosphaeria maculans*) in *Brassica napus* broke down within three years of commercial production (Sprague et al. 2006). Durable disease resistance can be achieved through the pyramiding or stacking of multiple resistance genes in single cultivars, including, where available, major genes (race-specific *R*-genes), minor/partial genes (quantitative) and even stacking genes of small effect, using genome selection (Poland and Rutkoski 2016).

Combining quantitative resistance with *R*-gene-mediated resistance can enhance the longevity of the *R*-gene (Brun et al. 2010). The durability of resistance is directly related to the selection pressure on a pathogen's avirulence genes; a cultivar whose resistance is the accumulation of numerous genes with partial effects should exert little selection pressure on any single avirulence gene, leading to only a low level of disease developing for many years (Pedersen and Leath 1988). This is in comparison with cultivars which only rely on a single major resistance gene and exert a much greater selection pressure on the pathogen's single avirulence gene to overcome the resistance. Combining or stacking multiple genes into a single cultivar is not a simple task. The use of molecular markers and genome-wide studies to screen large numbers of progeny has vastly improved and reduced the cost; however, it is still a lengthy process that involves multiple crosses. Understanding the genetic diversity and variation in an isolate's pathogenicity is highly important to fully comprehend host–pathogen interactions as it provides a deeper understanding of fundamental resistance processes and plant immunity.

The genome of the wild diploid, *F. vesca,* has been sequenced, and genes associated with disease resistance have been identified, allowing functional gene studies within *Fragaria* (Shulaev et al. 2011). The efforts of several groups are now focused on trying to sequence the more complicated allo-octoploid heterozygous cultivated strawberry, *F.* × *ananassa*. The availability and affordability of long-read sequencing technologies, such as single-molecule real-time sequencing from Pacific Biosciences (English et al. 2012), Oxford Nanopore Technologies (Laver et al. 2015) and 10X Genomics (Coombe et al. 2016), as well as optical mapping (Levy-Sakin and Ebenstein 2013) and Dovetail long-range mate pair sequencing (Putnam et al. 2016), are enabling improved exploration and annotation of this complex genome. Other recent technologies such as the highly informative and high-throughput Affymetrix IStraw90 Axiom® SNP (single nucleotide polymorphism) genotyping array have integrated the unique properties of the allo-octoploid *F.* × *ananassa* genome to facilitate QTL discovery and marker-assisted breeding (Bassil et al. 2015). The latter version of the array, the Axiom® IStraw35 384HT, is a smaller, cheaper version, based on mapped IStraw90 SNP probes from multiple groups across the world (Verma and Whitaker 2016; Verma et al. 2017).

The utilisation of the wild octoploid relatives *F. virginiana* and *F. chiloensis*, which are fully interfertile with the cultivated strawberry species *F.* × *ananassa,* provides attractive alternative options for the investigation and introgression of desirable traits such as disease resistance. The development of a resistance gene enrichment sequencing (RenSeq) technique opens up the possibility of rapidly identifying resistance genes from cultivated and wild strawberry, as demonstrated in potato by identifying resistance genes to *P. infestans* from wild distantly related species (Jupe et al. 2013; Witek et al. 2016). This approach enables the selective sequencing of resistance gene analogues such as NBS-LRR, both Toll/interleukin-1 receptor (TIR)-NBS-LRR (TNL) and non-TNL/coiled-coil NBS-LRR (CNL), as well as RLK, and receptor-like proteins (RLP), all of which, have been associated with plant *R*-gene candidates and facilitate the production of high-density *R*-gene genetic maps.

The use of transgenic plants could reduce the length of time taken to combine different resistances (to the same pathogen and different pathogens); however, there is still widespread unacceptance in the general public over genetically modified organisms (GMOs). The use of new targeted genome editing may see things change, as techniques such as the clustered regularly interspaced short palindromic repeats (CRISPR/Cas) system enable the alteration of plant genomes, which are indistinguishable from those produced by conventional breeding and chemical or physical mutagenesis (Belhaj et al. 2015). The United States Department of Agriculture (USDA) has recently ruled that two crops, a variety of mushroom and a variety of corn, produced using the CRISPR/Cas system

are not going to be regulated the same as traditional/conventional GMOs (Waltz 2016a, 2016b). We are on the cusp of a genomic revolution; by combining some or all of these technologies and studying both the pathogen and strawberry, it will enable the deployment of durable disease resistance in polyploid strawberry.

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8 Fruit Ripening and QTL for Fruit Quality in the Octoploid Strawberry

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Abstract

Fruit development and ripening is a unique developmental process to flowering plants that ensures the propagation of seeds and plant survival. In addition, fruits are an essential part of human diet. In particular, strawberry is a rich source of nutraceuticals such as vitamin C, folate and phenolic compounds. Strawberry production and breeding is becoming an extremely competing area of economic development worldwide. Cost of production in many countries is increasing due to a number of challenges such as rising labour costs, pest control or water availability. One way to increase competiveness is increasing fruit quality of new strawberry cultivars. Amazing advances have been made in our knowledge of the different metabolic pathways that take place in the final stages of fruit development and that lead to a flavourful and ripe strawberry fruit. Similarly, different genes involved in gene regulation during ripening have been discovered and characterized. In parallel, the discovery of *loci* responsible for natural variation among strawberry germplasm is producing a growing amount of DNA markers that after validation could be used in accelerating the selection of new cultivars with improved fruit quality. This chapter summarizes main advances in the study of fruit ripening in the octoploid strawberry and QTL controlling fruit quality traits.

8.1 Fruit Development and Ripening in Strawberry

Fruit ripening is a complex and coordinated developmental process that leads to the irreversible development of a soft and edible ripe fruit. Fleshy fruits have been classified as climacteric or non-climacteric based on the production of a characteristic burst of respiration and concomitant production of the hormone ethylene that induces the transcription of genes that will result in ripening (Giovannoni 2004; Seymour et al. 2013). Strawberry is a non-climacteric fruit since it does not exhibit a peak in respiration and ethylene production during ripening (Given et al. 1988). It is considered a false fruit, as the berry results from the development of the flower receptacle in which the real fruits are embedded,

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the achenes (Fig. 8.1). Each achene contains a single seed and a hard pericarp and is attached to the receptacle by vascular strands (Perkins-Veazie 1995).

Fruit development in cultivated strawberry (*Fragaria* × *ananassa*, Duch.) can be divided into four phases (Gillaspy et al. 1993): (i) fruit set, which consists in flower opening (anthesis), fertilization and development of the ovary; (ii) fruit growth by cell division, which is accompanied by seed and early embryo formation; (iii) a second phase of fruit growth, which is maintained mainly by an increase in cell volume in which the embryo passes through a maturation phase; and (iv) ripening (Fig. 8.1). Visually, strawberry fruit growth and maturation can be divided into six different stages: small green, medium green, big green, white, turning and red (Fait et al. 2008). The development of fruit from anthesis to the red stage encompassed a period of approximately 30 days and is strongly influenced by auxin, which positively effects the initial growth phase of the receptacle. Later in fruit development, auxin levels decrease and the ripening process is induced (Given et al. 1988).

Ripening involves softening of fruit tissues by cell wall degrading enzymatic activities to facilitate seed dispersal. In addition to softening, other important changes associated with ripening include colour (loss of green and increase of non-photosynthetic pigments), accumulation of sugars, a decline in organic acids and variation in many volatile compounds that provide the characteristic flavour (Aharoni and O'Connell 2002). The majority of these changes contribute to increasing interest and palatability to animals. Strawberries are highly appreciated for their aroma, which results from a complex combination of volatile organic compounds (VOCs). More than 360 VOCs have been identified in strawberry varying among different species within *Fragaria* and displaying a strong developmental and environmental regulation (Schieberle and Hofmann 1997; Ulrich et al. 1997, 2007; Olbricht et al. 2011; Ulrich and Olbricht 2013; Schwieterman et al. 2014).

Metabolism during fruit development involves the conversion of high molecular weight precursors to smaller compounds that help to the development of viable seeds. Primary metabolites, mainly sugars, organic and amino acids, play a significant role in the overall flavour and nutritional characteristics of fruits (Fig. 8.2). The sweetness of fruits is the central character determining fruit quality, and it is determined by the total sugar content and by the ratios among those sugars. During ripening, the accumulation of the major sugars, sucrose, glucose and fructose, is evident (Hancock 1999; Fait et al. 2008). Organic acids are other intermediate metabolites important as flavour components, either by themselves, because the organic acid-to-sugar ratio defines quality parameters at harvest time in fruits, or as precursors of other secondary metabolites. The main organic acids are the TCA intermediates citrate, malate, as well as quinate

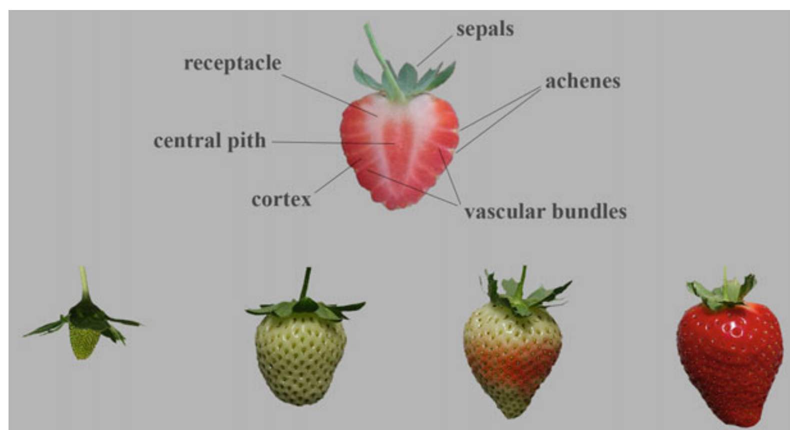

Fig. 8.1 Strawberry fruit morphology and development. The developmental stages shown here are, from left to right, green, white, turning and red

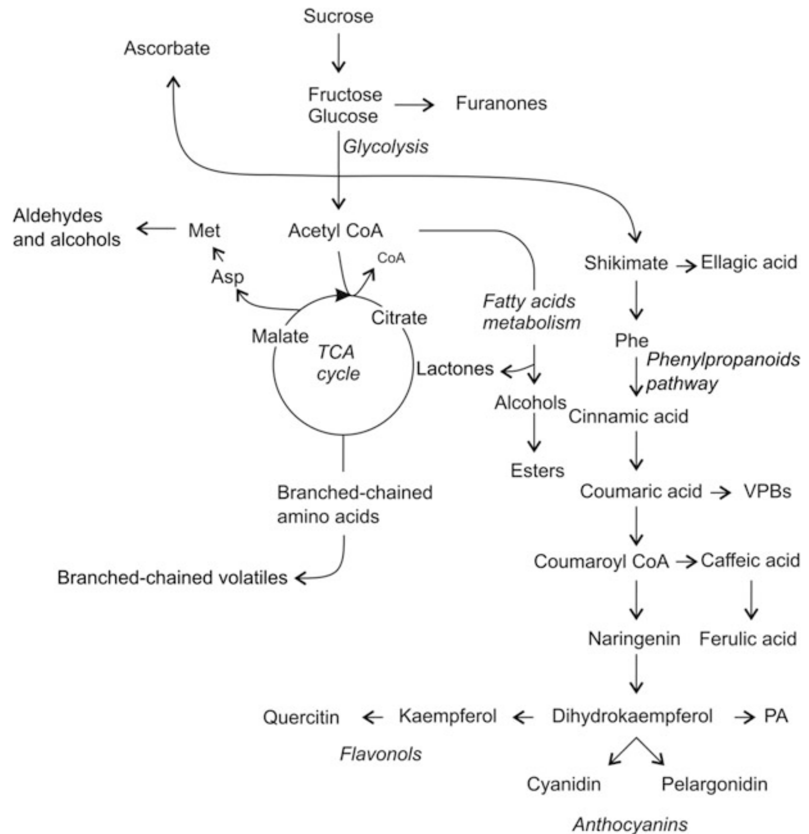

Fig. 8.2 A schematic overview of connections between primary metabolism and the major secondary metabolic pathways of strawberry fruits (adapted from Tohge et al. 2014). VPBs, phenylpropanoids and benzenoids; PA, proanthocyanidins

(Moing et al. 2001). Interestingly, levels of both citrate and malate were also highly correlated with many important regulators of ripening in an independent study that was focused on early fruit development (Mounet et al. 2012). The main class of secondary metabolites in strawberry is phenolic compounds, which are responsible for the colour and flavour of the fruit (Fig. 8.2). They also provide protection against biotic and abiotic stresses (Aaby et al. 2005, 2007). During early stages, flavonoids, mainly condensed tannins, accumulate to high levels and provide an astringent flavour (Almeida et al. 2007). When fruits begin to ripen, other flavonoids such as anthocyanins and cinnamic and coumaric acid derivatives accumulate to high levels (Lunkenbein et al. 2006a). Many of these phenolic compounds including flavonoids are considered important antioxidants with beneficial properties for human health such as in prevention of cancer and cardiovascular diseases (Alvarez-Suarez et al. 2011; Mazzoni et al. 2016).

Fait et al. (2008) and Zhang et al. (2011) analysed the composition of primary and secondary metabolites in achenes and receptacles separately during the six stages of fruit development and ripening. The analysis highlighted a metabolic shift between the first three stages (small green, medium green and big green) and the later stages (white, turning and red) in either organ. Changes in receptacle probably reflect the metabolic activity of the fruit, while the pattern of metabolite changes in achenes suggests the accumulation of storage and protective compounds as well as precursors for hormonal and secondary metabolites.

8.2 Hormonal Control of Fruit Ripening

In both climacteric and non-climacteric fruits, the dramatic changes occurring during fruit ripening must be tightly regulated by plant hormones (Giovannoni 2004). In climacteric fruits, the role of ethylene in ripening has been known for more than fifty years. Different studies have studied this hormone during the ripening process of non-climacteric fruits because it has been described a little increase of ethylene during ripening (Iannetta et al. 2006). However, in spite of many efforts, no results have been obtained that can demonstrate a clear relationship. The expression of two genes involved in softening of strawberries (*expansin* and *cellulase*) seems to be ethylene insensitive (Civello et al. 1999). On the other hand, the expression of other ripening-related genes in strawberry (*pectin methyl esterase* and *β-galactosidase*) was modified by treatments with ethylene (Castillejo et al. 2004; Trainotti et al. 2001). Interestingly, this dual effect of ethylene has also been found in climacteric peach fruits where the role of this hormone can either be positive or negative according to different genes (Trainotti et al. 2003). Even if strawberry has been classified as a non-climacteric fruit, low levels of ethylene have been detected during fruit development: it is relatively high in green fruits, decrease in white fruits and increase again in the red stage (Perkins-Veazie et al. 1996; Iannetta et al. 2006). Interestingly, strawberry mutants with reduced sensitivity for ethylene present alterations in fruit ripening, such as modification in flavonoid biosynthesis, pectin metabolism and volatile biosynthesis (Merchante et al. 2013). In addition, downregulation of the ethylene biosynthesis-related and ethylene signalling genes, *FaSAMS1* and *FaCTR1*, inhibits fruit colouring (Sun et al. 2013).

In strawberry, auxin is produced in the achenes and controls growth and ripening of the receptacle (Given et al. 1988; Manning 1994; Davies et al. 1997; Trainotti et al. 2005). Auxin levels are low at flowering, rise rapidly by the small green stage and then decline as fruit growth continues (Symons et al. 2012). In the first stages of fruit development, auxin is responsible for the expansion of the receptacle and at the same time prevents ripening (Given et al. 1988). The levels of auxin-responsive genes (*Aux/IAA* genes) are very high at early stage of fruit development, decrease sharply at ripening stage and might play a negative role in regulating fruit ripening (Liu et al. 2011). A transcriptomic analysis showed that auxin activates the expression of genes involved in cell proliferation and growth and represses genes related to ripening (Medina-Puche et al. 2016). Thus, as fruit ripens, a decrease of the levels of auxins activates the expression of ripening-related genes. Furthermore, exogenous applications of auxin delay fruit ripening and repress the expression of many ripening-related genes (Given et al. 1988; Manning 1994; Bustamante et al. 2009; Rosli et al. 2009; Symons et al. 2012). By contrast, the expression of ripening-specific genes is accelerated following the removal of the achenes, which are a source of endogenous auxin (Aharoni et al. 2002; Harpster et al. 1998). However, detailed studies on the content, synthesis and signalling of this hormone in different fruit parts at different developmental stages are lacking.

As a consequence of the prominent role of auxin in the development and ripening of strawberry fruit, less attention has been paid to possible roles of other plant hormones in these processes such as gibberellins (GAs) and abscisic acid (ABA). Endogenous gibberellins have been identified in strawberry immature fruits, including the bioactive forms GA_1 and GA_3 (Blake et al. 2000). It has been reported that application of GA_3 to ripening fruits caused a significant delay in the development of the red colour (Martinez et al. 1996). Also, external application of GA_3 was able to modify the expression of genes such as *FaGAST,* which encodes a protein involved in cell enlargement and final fruit size (de la Fuente et al. 2006) and *FaXyl*, encoding a β-xylosidase (Bustamante et al. 2009). It has been suggested that auxin regulates the levels of GA through controlling the expression of gibberellin 3-oxidase, which catalyses the final step in the synthesis of the bioactive form of GA

(Csukasi et al. 2011). Interestingly, the highest content of GA is detected in the white receptacle and coincides with the highest expression of *FaGAMYB*, a MYB transcription factor which is the target of two members of the miR159 family, whose mature transcript levels are at their lowest at the white stage. One of them, *FaMIR159a*, is downregulated by GA treatment (Csukasi et al. 2012). When *FaGAMYB* is silenced, the expression of several genes responsible for important metabolic changes associated with ripening, such as anthocyanin and sugar accumulation, is affected and maturation of the receptacle is delayed. This result could indicate a possible indirect role of GA in ripening, in addition to its role in the growth of the receptacle (Vallarino et al. 2015). Furthermore, it was suggested that *FaGAMYB* connects GA and ABA signalling pathways during ripening. When *FaGAMYB* is silenced, lower expression levels of *FaNCED1* and *FaNCDED2* are observed, resulting in a decrease of ABA levels and indicating that *FaGAMYB* could act upstream of ABA (Vallarino et al. 2015).

The key role of abscisic acid (ABA) during ripening has been described recently, and it has been shown that auxin and ABA interact to control the development and ripening process (Chai et al. 2011; Jia et al. 2011). There are two increases of ABA content during fruit development: one from big green to white stage and the other, much more noticeable, from turning to red stage (Jia et al. 2011; Ji et al. 2012). ABA content in achenes is much higher than in the receptacle; therefore, both auxin and ABA may be produced in achenes and transported to other tissues, such as the receptacle. Expression of genes encoding key enzymes in the synthesis of ABA, such as *FaNCED1*, is under the negative control of auxin. Indeed, expression of *FaNCED1*, *FaNCDED2* and *FaCYP707A1*, a key gene involved in the degradation of ABA, is enhanced in de-achened big green fruits, which are not able to reach normal size and start ripening before fruits treated with synthetic auxin. On the contrary, when *FaNCED1* is downregulated, fruits are unable to ripen and remain uncoloured. This phenotype can be rescued by the application of exogenous ABA (Ji et al. 2012).

ABA signal can be perceived by multiple receptors, including ABAR/CHLCH (magnesium chelatase H subunit) and the PYR/PYL/RCAR family (Shen et al. 2006; Santiago et al. 2009). The downregulation of these genes results in the same phenotype, with uncoloured and unripe fruits that cannot be rescued by treatment with exogenous ABA (Chai et al. 2011; Jia et al. 2011). Ayub et al. (2016) demonstrated that exogenous ABA increases the expression of both *FaPYR1* and *FaCHLH*. ABA-induced fruit ripening is mediated through the repression of *FaSnRK2.6*, which has been shown to be a negative regulator of fruit development (Han et al. 2015).

Recent studies indicate that sugars, especially sucrose, function as important signals in the regulation of fruit ripening, through the control of ABA levels (Jia et al. 2013). Fruit growth and development are closely correlated with a change in sucrose content. Exogenous sucrose and its non-metabolizable analogue, turanose, induce ABA accumulation in fruit and accelerate ripening. When the accumulation of sucrose in the fruit is blocked, by downregulation of *FaSUT1*, a decrease of both sucrose and ABA is observed, and ripening is arrested. This result could indicate that sucrose may be a signal upstream of ABA signalling (Jia et al. 2013).

8.3 Transcriptional Regulators

Even if the number of studies is more limited, some transcription factors (TF) have been associated with different pathways involved in the ripening process, such as flavonoid biosynthesis or aroma production. For instance, Aharoni et al. (2001) characterized an R2R3 MYB protein homologue, FaMYB1, which plays a role in the control of the expression of genes directly related to the biosynthesis of anthocyanins and the flavonol quercetin (lower end of the flavonoid pathway). Another R2R3 MYB protein, FaMYB10, has been described as a general regulator in the flavonoid/phenylpropanoid pathway

during ripening. In fact, it has been shown that the silencing of *FaMYB10* affects the synthesis of anthocyanins (Medina-Puche et al. 2014). Moreover, the function of this TF is conserved across the *Rosaceae* family (Telias et al. 2011; Hawkins et al. 2016; Jin et al. 2016; Zhai et al. 2016). Also, FaMYB10 controls the expression of another R2R3 MYB TF, *FaEOBII*, which is present in the ripe receptacle and regulates the production of the volatile eugenol (Medina-Puche et al. 2015). The expression of *FaEOBII* is repressed by auxins and activated by ABA in parallel to the ripening process. Other TF involved in the flavonoid pathways is *FaSCL8*, which downregulation represses many genes of the flavonoid pathway (Pillet et al. 2015). Other TFs, FaMYB9/FaMYB11, FabHLH3 and FaTTG1, have been described to play a role in the control of proanthocyanidins (PA), which are the main class of flavonoids present in the unripe receptacle (Schaart et al. 2013).

In a recent study, transcription factor ABA-stress-ripening (ASR), which is involved in the transduction of ABA and sucrose signalling pathways, was isolated and analysed in the non-climacteric strawberry and the climacteric tomato (Jia et al. 2016). The expression of the *ASR* gene was influenced not only by sucrose and ABA, but also by jasmonic acid (JA) and indole-3-acetic acid (IAA), and these four factors were correlated with each other during fruit development. This study provided new evidence on the important role of ASR in cross-signalling between ABA and sucrose to regulate tomato and strawberry fruit ripening.

8.4 Key Metabolic Pathways During Fruit Ripening

8.4.1 Fruit Size and Softening

The primary cell wall is composed of numerous polymers, which vary in structure somewhat between species, but eight polymeric components (cellulose, three matrix glycans composed of neutral sugars, three pectins rich in D-galacturonic acid and structural proteins) are usually present. The metabolic changes during ripening include alteration of cell structure involving changes in cell wall thickness, permeability of plasma membrane, hydration of cell wall, decrease in the structural integrity and increase in intracellular spaces (Redgwell et al. 1997). Ripening is also usually accompanied by a reduction in cell turgor, due to increasing concentration of solutes in the cell wall space and to wall loosing (Shackel et al. 1991).

In strawberry, the reduction of firmness starts at the transition from the white to the red mature stage (Perkins-Veazie 1995). The main mechanism responsible for tissue softening is pectin depolymerization and solubilization (Huber 1984; Nogata et al. 1996; Rosli et al. 2004). In fact, the pectin-soluble fraction increases from 30% in unripe fruit to 65% in ripe fruit (Huber 1984), the middle lamella is extensively degraded (Perkins-Veazie 1995), and cells appear separated by a considerable intercellular space and reduced cell-to-cell contact area (Redgwell et al. 1997). Several cell wall-related genes expressed during receptacle ripening are inhibited by auxin (Trainotti et al. 2001; Benítez-Burraco et al. 2003; Harpster et al. 1998; Martínez et al. 2004; Molina-Hidalgo et al. 2013; Paniagua et al. 2016). Among cell wall hydrolases, pectin-degrading enzymes are mostly implicated in fruit softening such as pectate lyases (PL) and polygalacturonases (PG) (Benítez-Burraco et al. 2003; Youssef et al. 2013). Three varieties of strawberry with contrasting fruit firmness differ in the expression pattern of two PG-related genes, indicating that these genes significantly contribute to pectin solubilization (Villarreal et al. 2008; Molina-Hidalgo et al. 2013). Other enzymes such as rhamnogalacturonate lyases and FaRGlyase1 have been shown to be involved in the degradation of pectins present in the middle lamella between parenchymatic cells of the receptacle (Schols et al. 1990). Also, a putative β-galactosidase, FaβGal4, could be involved in

pectins solubilization, since *FaβGal4* downregulation results in fruits that are on average 30% firmer than controls (Paniagua et al. 2016).

The regulation of fruit size is clearly far more complex, many genes are expected to be involved, and the process is less studied in strawberry. Two GAST-like genes, *FaGAST1* and *FaGAST2,* have been shown to play a role in the control of strawberry size in the early stages of fruit development (de la Fuente et al. 2006; Moyano-Cañete et al. 2013). Both genes have a similar expression pattern, showing two peaks of expression at the medium green and red stages. Cell division stops at the end of small green stage, and therefore, it has been suggested that *FaGAST* genes could be involved in the decrease of growth rate. In addition, transgenic lines overexpressing them produce significantly smaller fruits than control plants (de la Fuente et al. 2006; Moyano-Cañete et al. 2013).

8.4.2 Allergens

The reactivity to strawberry is most probably an epiphenomenon because of primary sensitization to birch allergen Bet v 1 rather than a direct sensitization resulting from strawberry exposure, as allergy against birch pollen is often accompanied by adverse reaction to fresh fruit due to specific IgE cross-reactivity to Bet v 1. (Karlsson et al. 2004). Fra a 1 strawberry proteins show homology to Bet v 1, and a natural white-fruited mutant was found to be free from Fra a 1 allergen and tolerated by individuals affected by allergy (Hjernø et al. 2006). When *Fra a 1* is silenced, several key enzymes of the anthocyanin biosynthesis pathway are also reduced, indicating that Fra a 1 proteins have an essential function in pigment formation in strawberry fruit (Hjernø et al. 2006; Muñoz et al. 2010, Griesser et al. 2008; Casañal et al. 2013). The isoform Fra a 1.02 is highly expressed in ripe fruit and is identified as the prominent Bet v 1-like allergen by stimulation index value in skin prick test (Franz-Oberdorf et al. 2016).

8.4.3 Vitamins

Strawberry is a rich source of ascorbic and folic acids, two important nutrients in human diet (Tulipani et al. 2008). Different pathways have been proposed for the biosynthesis of ascorbic acid in plants, even if the prevalence of these pathways in different tissues and developmental stages is still unknown (Davey et al. 2000; Jain and Nessler 2000; Valpuesta and Botella 2004; Cruz-Rus et al. 2011). One of them, the mannose/galactose pathway seems to be responsible for ascorbic acid biosynthesis in green fruit, as two genes encoding enzymes of this pathway are downregulated as fruit ripening proceeds from green to red stages (Cruz-Rus et al. 2011). In ripe fruit, synthesis of ascorbic acid can occur using galacturonic acid as initial substrate, as its levels correlate well with the expression of a D-galacturonate reductase, an enzyme catalysing one step of this pathway (Agius et al. 2003; Cruz-Rus et al. 2011).

Folate or folic acid is also an abundant micronutrient in strawberry fruit, with an average content in the range of 20–25 mg/100 g fresh weight (Tulipani et al. 2008; Giampieri et al. 2012). Currently, the mechanism of folic acid synthesis regulation is not well understood (Hanson and Gregory 2011). Transcriptomic analysis indicates that ABA may play a regulatory role in folic acid homoeostasis, as genes responsible for this process were downregulated in ABA-treated receptacles (Li et al. 2015).

8.4.4 Colour, Anthocyanins and Phenylpropanoids

Phenolic compounds, the main class of secondary metabolites in strawberry fruits, are essential constituents of human diet for their strong antioxidant and anti-inflammatory activities, which may reduce sensitivity to oxidative stress (Tulipani et al. 2009; Mazzoni et al. 2016). Flavonoids are the most represented class of phenols in strawberries and include

anthocyanins, which are responsible for the pigmentation of fruits, proanthocyanidins and flavonols, the most abundant being quercetin and kaempferol. Other phenolic acids frequently detected in strawberry are glucose derivatives of cinnamic, caffeic, ferulic and sinapic acids (Hanhineva et al. 2011). During ripening, a shift from the accumulation of the astringent proanthocyanidin polymers to coloured anthocyanins occurs (Fait et al. 2008). The amount of phenylalanine is very high at the early stages of development as it serves as a precursor for proanthocyanidins (Fig. 8.2), and its amount rises again at the very last stage of maturation enabling the synthesis of anthocyanins (Halbwirth et al. 2006).

Two types of genes are required for the biosynthesis of flavonoids: the structural genes encoding enzymes and the regulatory genes that control their transcription (Winkel-Shirley 2001; Pombo et al. 2011). Phenylalanine ammonia lyase (PAL) is the first enzyme of the phenlypropanoid pathway, catalysing the conversion of phenylalanine to trans-cinnamic acid. *FaPAL6* gene expression was only detected in red strawberry fruit, even if PAL activity was detected at all ripening stages, suggesting that it belongs to a gene family in strawberry. The higher *FaPAL6* expression and activity detected in ripe fruit in the cultivar Camarosa could be associated with enhanced anthocyanin accumulation (Pombo et al. 2011). Furthermore, Song et al. (2015) performed a quantitative proteomic study in green, white and red stages of receptacle, showing that the protein abundance of several enzymes of the flavonoid and anthocyanin synthesis increases in fruit of more advanced ripeness. They also identified several isoforms of these enzymes, such as five PAL, in which abundance differs among the different ripening stages. Chalcone synthase (CHS) catalyses the formation of naringenin, the precursor for several flavonoids, and is regarded as a point of control in the flow between the flavonoid pathway and the other competing directions of the phenylpropanoid pathway (Winkel-Shirley 2001; Verhoeyen et al. 2002). The expression of the CHS gene in fruit is developmentally regulated and associated with colour accumulation (Aharoni et al. 2002; Manning 1998; Lunkenbein et al. 2006a).

The main pigments in strawberry fruit are pelargonidin 3-O-glucoside (92%) and cyanidin 3-O-glucoside (4%). The first stable product of the anthocyanin pathway is formed when a glycosyltransferase attaches a sugar to the hydroxyl group on the anthocyanidin aglycone. Griesser et al. (2008) showed that FaGT1, a glycosyltransferase, is involved in the synthesis of anthocyanin in the ripe receptacle, its silencing causing a decrease of colour and pelargonidin in the fruits.

8.4.5 Flavour: Sugars, Acids and Volatile Compounds

Flavour is the sum of a large set of primary and secondary metabolites, perceived and measured by the taste and olfactory system (Klee, 2010). Strawberry flavour can be defined as the overall sensory quality perceived by humans: sugars, acids and volatiles (taste and aroma), texture and firmness (tactile sensation) and pigments (vision) (Schwieterman et al. 2014).

Sugars, organic acids and their ratio play a key role in taste perception of strawberries. Furthermore, sugars are not only important in determining sweetness, but also as precursors for aroma compounds, antioxidants and pigments (Vandendriessche et al. 2013). Glucose is the predominant sugar at all developmental stages, and total sugar content increases approximately 1.5-fold from white to red stages, while the most abundant acid is citrate (Fait et al. 2008; Basson et al. 2010). Combined sugar and acid content and sugar-to-acid ratio increase during ripening but are also strongly affected by genetic and environmental factors (Basson et al. 2010; Ornelas-Paz et al. 2013). Invertase activity is higher in white and turning fruits in comparison with green fruits, leading to a diminution of sucrose and increase of glucose and fructose (Bood and Zabetakis 2002; Basson et al. 2010).

More than 350 volatile compounds have been described in strawberry, having one of the most

complex fruit aromas (Zabetakis and Holden 1997; Bood and Zabetakis 2002; Schwab et al. 2008). Volatile organic compounds (VOCs) can be classified according to their chemical classes, being furanones, lactones, esters, aldehydes and alcohols the dominating aroma compounds (Jetti et al. 2007; Schwab et al. 2009). Green fruits are characterized by high levels of aldehydes and alcohols, some of them showing high negative correlations with ripeness (Jetti et al. 2007; Burdock and Fenaroli 2009). Aldehyde abundance decreases during ripening, but does not disappear completely, contributing with green notes to the final aroma (Jetti et al. 2007). Esters are the most abundant class of VOCs in ripe strawberry fruits, providing sweet fruity notes associated with pineapple, bananas or apple (Jetti et al. 2007; Burdock and Fenaroli 2009). The last step of volatile ester synthesis is catalysed by alcohol acyltransferases (AAT), using different alcohols as substrates (Wyllie and Fellman 2000). Two *AAT* genes have been characterized in cultivated strawberry, and their expression increased from the white stage throughout fruit ripening, correlating with the total content of esters, thus suggesting that this gene family could encode important enzymes contributing to fruit aroma (Aharoni et al. 2000; Cumplido-Laso et al. 2012). Both genes encode AAT with enzymatic activity for different short-chain alcohols in the presence of acetyl-CoA. Furthermore, downregulation of *FaAAT2* expression by agro-infiltration of fruits resulted in a significant reduction of different esters (Cumplido-Laso et al. 2012). The concentration of two furanones, furaneol and mesifurane, increases during ripening and has been shown to contribute notably to the caramel-like, sweet, floral and fruity aroma of ripe strawberry (Pérez et al. 1996; Jetti et al. 2007). Two genes, *FaQR* and *FaOMT*, important for their biosynthesis have been characterized (Lunkenbein et al. 2006b; Raab et al. 2006). Lactones are another important volatile group contributing to fresh peachy aroma and increasing the perception of sweetness in the fruit (Ulrich et al. 2007; Schwieterman et al. 2014; Ulrich and Olbricht 2016). Interestingly, the concentration of γ-decalactone varies greatly among cultivars with very high levels in some and undetectable in other varieties (Larsen et al. 1992; Jetti et al. 2007; Olbricht et al. 2008). FaFAD1, a fatty acid desaturase, has been proposed to be responsible for its synthesis, and the deletion of the gene in some genotypes can explain the absence of γ-decalactone in their fruits (Sánchez-Sevilla et al. 2014; Chambers et al. 2014). Sequestered volatile compounds, such as glucosylated derivatives, may be an important pool of non-volatile precursors in many fruits. Nine ripening-related UDP-glucosyltransferases (UGTs) have been functionally characterized in strawberry, and one of them has been shown to catalyse the glucosylation of furaneol (Song et al. 2016).

8.5 QTLs Controlling Fruit Quality Traits in Octoploid Strawberry

8.5.1 Challenges of QTL Mapping in a Complex Polyploid

The majority of agronomical and fruit quality traits are quantitative and by definition show continuous variation due to polygenic inheritance and environmental influences. The identification of quantitative trait loci (QTL) controlling important quality traits and the development of markers linked to these QTLs are allowing marker-assisted breeding in many crops (Collard and Mackill 2008). *F.* × *ananassa* is an allo-octoploid species ($2n = 8x = 56$) originated from the hybridization between two wild octoploid species, *Fragaria chiloensis* and *Fragaria virginiana* (Darrow 1966). The polyploid nature of strawberry imposes important challenges for genetic studies; each trait can be controlled by up to 4 homoeologous gene series (homoeoalleles). Homoeoalleles are located at orthologous positions that belong to the different sub-genomes that compose the polyploid species (Lerceteau-Köhler et al. 2012). Analysis of coupling/repulsion phases has suggested the prevalence of disomic behaviour in the cultivated strawberry, despite the possible existence of residual levels of polysomic segregation

(Lerceteau-Köhler et al. 2003; Rousseau-Gueutin et al. 2008). These and other early results are supported by the latest phylogenomic studies that suggested the cytological formula AABBB′B′B″B″, which includes one sub-genome related to *Fragaria vesca* and three B sub-genomes more related to *Fragaria iinumae* (Tennessen et al. 2014).

Unravelling complex traits involve the development of linkage maps, QTL mapping and/or association mapping (or linkage disequilibrium mapping) (Collard and Mackill 2008). Although no association studies have been reported in octoploid strawberry yet, a number of biparental populations have been reported for strawberry, derived from different crosses such as 'Capitola' × CF1116 (Lerceteau-Köhler et al. 2003; Rousseau-Gueutin et al. 2008), 'Tribute' × 'Honeoye' (Weebadde et al. 2008), 'Redgauntlet' × 'Hapil' (Sargent et al. 2009), 232 × 1392 (Zorrilla-Fontanesi et al. 2011), 'Dover' × 'Camarosa' (Ring et al. 2013) and 'Delmarvel' × 'Selva' (Castro and Lewers 2016). Some of these populations have already been used for QTL mapping. An important resource that facilitates the identification of candidate genes underlying QTL in *F.* × *ananassa* is the available *F. vesca* genome sequence (Shulaev et al. 2011). Comparative mapping analyses between the diploid reference genome (and/or genetic maps) and the octoploid genetic maps have shown high macrosynteny and colinearity levels between *Fragaria* genomes, enabling the identification of genes in the octoploid by colocalization in the corresponding diploid genome sequence (Rousseau-Gueutin et al. 2008; Sargent et al. 2009; Tennessen et al. 2014; Sánchez-Sevilla et al. 2015). However, only one of the four sub-genomes of strawberry has been derived from a *F. vesca* ancestor and it is thus expected that additional and/or unrelated *loci* are present in the octoploid species. The availability of a reference genome of the octoploid strawberry in the near future will be a more suitable tool for the search of underlying genes in QTL regions.

8.5.2 QTL Studies for Fruit Quality in Cultivated Strawberry

The first article describing the identification of QTLs for fruit quality traits has been conducted in an F1 population derived from two strawberry selections, 232 and 1392, contrasting in agronomical and fruit quality traits (Zorrilla-Fontanesi et al. 2011). A total of 33 QTLs were detected in 1–3 years controlling agronomical traits such as yield or fruit size and fruit quality traits such as soluble solids content (SSC), ascorbic acid, titratable acidity (TA), colour and firmness. Twelve QTLs (36.4%) were stable over 2 or all 3 years. The phenotypic variation explained by the detected QTLs was generally less than 20%, indicating that all analysed traits were complex and quantitatively inherited. Different QTL clusters were detected, some expected such as for anthocyanins and colour parameters, but also detected for ascorbic acid and acidity in linkage group (LG) IV-2 or for anthocyanins and acidity in LG V-2. Strawberry is particularly rich in ascorbic acid, but its content varies widely among cultivars (Ariza et al. 2015). Three QTLs explaining a total of 45% of variation in this trait were identified by the study of Zorrilla-Fontanesi et al. (2011). Candidate genes related to ascorbic acid biosynthesis or recycling were identified in the confidence interval of each of these QTLs (as well as for other QTLs) and could serve as a starting point for further studies. For example, the gene *FaEXP2* encoding for a fruit-specific expansin was identified within a QTL for fruit firmness in LG VII-1. An apple expansin, Md-Exp7, has been associated with a QTL controlling firmness on Malus LG1 (Costa et al. 2008).

A total of 87 QTLs for 19 quality traits, including fruit size, firmness, colour, sugars, organic acids and anthocyanins, were detected in an F1 population derived from the cross between cv. Capitola and the breeding line CF116, differing in fruit quality traits and flowering habit (Lerceteau-Köhler et al. 2012). The percentage of

variance explained by each QTL ranged from 5 to 17%. Twelve traits were analysed for three consecutive years, and among them, 16 of the 60 QTL (27%) were detected at least in 2 years. Cluster of QTLs for different traits were also observed, as for example clusters for sugar- and acid-related traits were observed on the homoeologous group (HG) VI. The non-random distribution of QTLs across the chromosomes may reflect pleiotropic effects of one *locus* or the presence of tightly linked genes. QTL clusters often mimicked the level of correlation observed between the traits. As observed in the population 232 × 1392, the QTLs explained low-to-moderate percentages of phenotypic variation for a given trait, most probably explained by multiple *loci* controlling fruit quality traits.

In the study of Lerceteau-Köhler et al. (2012), 23% of the QTLs were detected at likely homoeologous locations and thus considered as homoeo-QTLs. Similarly, homoeo-QTLs were also detected in the study of Zorrilla-Fontanesi et al. (2011). In the cultivated octoploid strawberry, each locus can be represented up to four times in the genome as homoeologous *loci*, each presenting two homologous alleles. A number of homoeo-QTLs could be detected the same year, suggesting that several copies of the gene underlying the QTL are functional. The detection of some other homoeo-QTL was year-dependent. Therefore, changes in allelic expression could take place in response to environmental changes.

A recent study using a third F1 population derived from the cross ‘Delmarvel’ × ‘Selva’ detected a number of QTLs controlling the content of total anthocyanins, total phenolics, antioxidant capacity, TA and SSC (Castro and Lewers 2016). A total of 27 QTL for fruit quality traits were detected, and the phenotypic variation explained by each QTL ranged from 4.8 to 10.7%. Colocations between anthocyanins and antioxidant capacity or total phenolics were detected in different LGs. These colocations were supported by high correlation coefficients between the three traits, suggesting that selecting for one of them such as total phenolics may be useful for indirect selection of fruits with higher antioxidant capacity. However, this should be studied for each trait in detail as other studies have shown a competition of different phenolics pathways for common substrates (Ring et al. 2013).

Three traits were common between the three previously discussed QTL studies: anthocyanins, SSC and TA (Zorrilla-Fontanesi et al. 2011; Lerceteau-Köhler et al. 2012; Castro and Lewers 2016). A number of QTLs for each of these traits were identified in approximately the same location on the same HGs, suggesting that common *loci* are controlling the variation in multiple genetic backgrounds (Table 8.1). As examples, two QTLs for SSC were detected in the three populations in the middle part and the upper arm of LGs belonging to HG V and VI, respectively. Similarly, a QTL for TA was detected in the three analyses in the lower part of one LG of HG IV. To properly compare QTL positions, linkage maps should be saturated with common markers between populations and with sub-genome specific markers such as the haplo-SNPs described by Sargent et al. 2016.

The 232 × 1392 population was also profiled for VOCs by GC-MS, and 70 QTLs controlling the variation of 48 different compounds were detected (Zorrilla-Fontanesi et al. 2012). Among them, 35 (50%) were stable over two or all three years. With the exception of HG II, clusters of QTLs were detected in all the HGs, indicating linkage or most probably the pleiotropic effect of one *locus* over different related VOCs. Clusters of QTL for different esters and alcohols were commonly found, and all these VOCs showed high correlation between them indicating the presence of a single *locus* at each position involved in the biosynthesis or regulation of all the biosynthetically related compounds. The percentage of phenotypic variation explained by each QTL ranged from 14.2 to 92.8%. This high proportion of major QTL suggests that variation in strawberry fruit aroma is regulated by a limited set of *loci* with a high effect rather than by multiple *loci* with reduced effects, in contrast to the two previous studies (Zorrilla-Fontanesi et al. 2011; Lerceteau-Köhler et al. 2012). Natural variation in the content of two key VOCs,

Table 8.1 Quantitative trait loci (QTL) controlling the content of anthocyanins, soluble solids content and titratable acidity reported for strawberry

		Zorrilla-Fontanesi et al. (2011)			Lerceteau-Köhler et al. (2012)			Castro and Lewers (2016)		
Trait	HG	QTL	Marker	R^2 (%)	QTL	Marker	R^2 (%)	QTL	Marker	R^2 (%)
Anthocyanins	I	–	–	–	ANTH-Ia-f	ccaa280	6	–	–	–
	II	*antII-M.6*	CFVCT027-131	11.7	ANTH-IIa-f	BFACT002	6	Antho1_II-D-4	AW061432-249	7
	III	***antIII-F.1***	**BFACT036-159/130**	**10.3**	**ANTH-IIIa-f**	**tcta277**	**8**	–	–	–
	V	*anthV.M2*	ChFaM044-226	9.2–24.8	–	–	–	Antho3_V-S-3	EMFn184-245	8.4
	VI	–	–	–	**ANTH-VIa-m/f**	**gata170/caag162**	**8.0–17.0**	**Antho2_VI-D-4**	**BFACT010-246**	**5.8**
					ANTH-VIb-f	**ccaa278**	**7**	**Antho1_VI-D-3**	**EMFv104-117**	**10.7**
Soluble solids content	**II**	***sscII-F.1***	**Fvi11-302/310**	**17.6**	–	–	–	**SSC2_II-D-5**	**CO817772-249**	**5.6**
	III	–	–		SSC-IIIa-m/f	EMFv004	9	–	–	–
	V	***sscV-M.4***	**ChFaM269-445**	**11.6**	**SSC-Va-f**	**tcaa355**	**8**	**SSC2_V-D-1**	**BFACT005-157**	**7.9**
	VI	***sscVI-M.3***	**cct/aca-146**	**10.7–12.7**	**SSC-VIa-m/f**	**EMFv006**	**6.0–8.0**	**SSC1/2_VI-S-3**	**Fvi20-143**	**8.1–8.9**
Titratable acidity	I	–	–	–	TA-Ia-m	tgaa197	9	–	–	–
	II	–	–	–	TA-IIc-m	tgtg270	7	–	–	–
	III	–	–	–	TA-IIId-m	caaa263	7	–	–	–
	IV	***taIV-F.2***	**ChFaM023-153/171**	**7**	**TA-IVa-f**	**tggx136**	**7**	**TA3_IV-D-3**	**FxaAGA02N04C-192**	**7.7**
	V	***taV-M.2***	**ChFaM106-144 - ChFaM109-150**	**12.1–18.1**	**TA-Vb-m/f**	**gaaa310/cttg195**	**7.0–12.0**	–	–	–
	VI	–	–	–	–	–	–	TA2/3_VI-D-4	ARSFL7-245/258, BFACT010-246	7.9–8.2

QTLs that locate in similar positions on the same homoeology group in different populations are highlighted in bold

mesifurane and γ-decalactone, is controlled by major genes as one QTL controlling 42–67.3% and above 90% of total variation was detected, respectively. A combination of metabolomics and expression studies in the parental and contrasting F1 progeny lines resulted in the identification of *FaOMT* as the gene controlling natural variation in mesifurane content in strawberry (Zorrilla-Fontanesi et al. 2012). An indel of 30 bp in the promoter of this gene was identified in progeny lines and fully cosegregates with both the presence of mesifurane and high expression of *FaOMT* in the ripe receptacle.

γ-decalactone is the most abundant lactone in red ripe fruit, which provides 'peachy' notes in strawberry (Douillard and Guichard 1989; Ménager et al. 2004). This lactone was detected at high level in the parental line 1392 but not in 232, and the presence of the volatile in fruits was inherited in half of the progeny lines. The gene controlling the variation was mapped to the bottom of LG III-2 (Zorrilla-Fontanesi et al. 2012). A novel approach combining genome-wide RNA-seq analysis to a bulk segregant analysis identified the fatty acid desaturase *FaFAD1* as a key gene controlling γ-decalactone content in strawberry (Sánchez-Sevilla et al. 2014). In parallel, another group using complementary approaches in a different segregating population identified the same gene required to synthesize γ-decalactone in fruits (Chambers et al. 2014). Both studies provided evidences that *FaFAD1* was essential, as different lines with a deletion of this gene were not able to accumulate the VOC.

Markers in genes *FaOMT* and *FaFAD1* have been developed and are able to predict the phenotype with 100% accuracy within these mapping populations. Validation of the predictive capacity of these markers in a wider and diverse collection of germplasm has resulted in above 91% accuracy for both gene markers (Cruz-Rus et al. 2017), indicating that they could be used for efficient and reliable implementation in breeding programs (see Chap. 12).

As described above (Sect. 8.4.4), anthocyanins, flavonoids and phenylpropanoids are the major phenolic compounds that accumulate in ripe strawberry (Fait et al. 2008; Tulipani et al. 2008) and play important roles in fruit pigmentation and protection against abiotic and biotic stress. Ring et al. (2013) coupled an examination of the transcriptome by microarray analysis with metabolite profiling of different strawberry genotypes to reveal genes whose expression levels correlated with altered phenolic composition. Within the differentially expressed ESTs, a putative peroxidase expressed in ripe fruit and roots, *FaPRX27*, was identified and enzymatic assays indicated that FaPRX27 could be involved in lignin biosynthesis. Using two different mapping populations, QTL controlling different phenolic compounds and flavonoids were identified in the same region where *FaPRX27* is located, and also associated with a QTL for fruit colour (Ring et al. 2013). Genetic analyses were extended by functional analyses using transient expression by agro-infiltration of fruits. The results highlighted a competition between lignin biosynthesis and anthocyanins and fruit colour development.

In another study using the same oligonucleotide-based strawberry microarray platform, a *rhamnogalacturonate lyase* gene (*FaRGlyase1*) induced during fruit ripening was functionally characterized (Molina-Hidalgo et al. 2013). Expression of *FaRGlyase1* was positively regulated by ABA and negatively by auxins, and the protein shown to be involved in the degradation of pectins present in the middle lamella between parenchymatic cells. The gene *FaRGlyase1* was mapped in the population 'Dover' × 'Camarosa' and shown to colocalize with a QTL controlling fruit firmness in LG 1B (Molina-Hidalgo et al. 2013). Taken together, the results indicated that *FaRGlyase1* could play an important role in fruit softening during ripening and post-harvest life.

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9 Genotyping Tools for the Octoploid Strawberry

Robert Vickerstaff

Abstract

Genotyping is the process of determining which set of alleles an individual has at a set of loci in its genome. An expanding set of techniques have become available for genotyping the cultivated, octoploid strawberry, beginning in the early 2000s. Despite the complexity of an allopolyploid genome, it has never the less proven possible to obtain reliable genotypic data from strawberry. With the decreasing cost of sequencing and other related technologies, more genotyping methods will continue to be developed and are likely to become integrated into research and breeding programs, even following the expected release of a comprehensive genome sequence in the near future. This chapter explores a range of genotyping techniques and covers the additional procedures sometimes required to make them function for the cultivated strawberry, providing a detailed look at genetic mapping as a key use of genotyping data.

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9.1 Introduction

Genotyping is the process of determining which sequence variant or allele is present at one or more loci in the genome of an individual, in order to reveal information about its genetic makeup, and as such is fundamental to a range of important practises within plant science and horticulture. These include anything from verifying the cultivar or parentage of a particular plant, to constructing genetic maps to inferring the population structure or evolutionary history of a species. Historically, genotyping was only feasible for a limited number of loci and gave incomplete, fragmentary coverage of the genome. With advances in technology, sufficient loci can now be probed to give a much more complete picture, and for a larger number of samples. The role of genotyping is changing due to the decreasing cost and increasing quantity and quality of DNA sequence data now becoming available, and the likelihood is that in future every plant breeding and research program will work with near complete information about the genomes of their cultivars. Once enough key parental individuals have had their complete genome sequence worked out, it is generally not necessary to independently perform complete sequencing on all new progeny since these can only inherit their DNA from a limited number of known parental types. Given genotype information from a limited number of loci in progeny

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genomes, their complete genetic make up can easily be inferred. In this way, for the foreseeable future a role will remain for techniques to quickly and cheaply ascertain the alleles present at a selected set of loci within plant genomes, thus allowing far more samples to be studied in parallel than would be the case were the complete DNA sequences of each to be read out in full. Conversely, if complete genome sequences are not required, such as for crop wild relatives, then methods are now available to collect DNA sequence information from just the locations of interest, such as genes suitable for phylogenetic analysis or for disease resistance.

The complex allopolyploid genome (2n = 8x = 56) of the cultivated strawberry (*Fragaria* × *ananassa*), consisting of essentially four diploid genomes combined in the same nucleus, makes the application of genotyping techniques less straightforward since most standard methods will, if used unmodified, tend to confound the homeologous alleles present across the four sub-genomes. An additional complication is the lack of viable inbred lines due to the inbreeding depression observed in the octoploid when selfing is used, such that the plants being genotyped are always heterozygous across large parts of the genome, possessing two alternative alleles for each sub-genomic locus, and this precludes the use of traditional F2 and backcross mapping populations. A comprehensive octoploid genome sequence, which is not currently available, would make many genotyping methods easier and more informative, and the delay in achieving a genome also stems from the technical challenges presented by a heterozygous, polyploid genome. With the many promising novel sequencing technologies now becoming available an octoploid genome sequence is expected to be completed in the near future, but until then the genome of the closely related *Fragaria vesca* (Shulaev et al. 2011; Tennessen et al. 2014) will remain a very useful substitute.

It is now fairly clear that *F.* × *ananassa* behaves in a disomic, diploidised manner during meiosis such that, after some additional work during analysis, genotype data can in practise generally be processed using software tools designed for outbreeding diploid species rather than needing an entirely different class of algorithm, as is the case for autopolyploids.

9.2 Genotyping for Genetic Mapping

A key use for genotyping is the construction of genetic maps, also known as linkage maps, whereby, in the case of *F.* × *ananassa*, generally two distinct heterozygous individuals are crossed to produce a significant number of progeny known as an F1 mapping population, typically from 50 up to one or two hundred. Although cultivated strawberry cannot be inbred to produce pure, homozygous lines, it can be clonally propagated, meaning that once a cross has been performed, the parental and progeny genotypes can be maintained indefinitely. This allows a potentially unlimited number of experiments to be performed on plants with the same genotypic makeup, and also allows for the generation of additional progeny from the same cross to expand the population size if required. To build a map genotype data are collected, using a suitable genotyping technology, from as many loci as possible from both parents and the progeny, which are then used to infer the position of those loci across the chromosomes using appropriate software. Genetic maps have historically provided the first genome-wide information about plant genomes, well before it was feasible to produce complete genome sequences, and have been used to compare synteny between related species by utilising shared markers. By measuring the phenotypes of key traits in the same progeny, it is possible to map the location of genes controlling them (Zorrilla-Fontanesi et al. 2011), so-called quantitative trait loci (QTL). Genetic maps are also routinely used to scaffold genome sequences together into complete, whole chromosome scale pseudo-molecules, as was done with the *F. vesca* genome (Shulaev et al. 2011), utilising the superior range of linkage information to complement the shorter range information obtained from direct sequencing of DNA fragments.

The following sections introduce some of the fundamental principles of genetic mapping, beginning from the simplest case of a backcross between two inbred parents, and leading up to the F1 cross applied to octoploid strawberry, in order to provide the context for later detailed discussion of the various genotyping technologies available.

9.2.1 The Backcross

The simplest of the traditional mapping population designs is the backcross, where two different inbred parental cultivars are crossed to produce one F1 hybrid individual, which is then crossed back to one of the parents to produce the BC1 progeny, which form the mapping population. Assuming the parents are diploid, we can represent the genotype of parent 1 at a generic locus as AA and that of parent 2 at the same locus as BB, to indicate that they are homozygous but possess distinct alleles. The F1 will have a heterozygous genotype AB at every locus where the parents differ. Furthermore, the F1 will have all the A alleles from parent 1 in *coupling phase* with each other on the same chromosome and all the B alleles from parent 2 in coupling phase on the other homologous chromosome. Assuming the BC1 progeny is produced by crossing parent 1 with the F1 hybrid; each locus will always receive allele A from parent 1, and can receive either allele A or B from the F1 parent, due to random chromosome segregation, giving only two possible genotypes, AA and AB. During meiosis in the F1 crossing over will occur between the homologous chromosomes, resulting in some gametes containing a mixture of A and B alleles on the same chromosome. When we examine the genotypes of two loci in the BC1 progeny, we can easily count the number of progeny showing evidence of recombination simply as those that have different genotypes either A_1A_1, A_2B_2 or A_1B_1, A_2A_2 (where the subscript indicates locus 1 or locus 2). The fraction of progeny where the two loci have different genotypes is known as the *recombination fraction* and is an indication of whether the loci are present on the same chromosome and if so how far apart they are in terms of genetic distance. This is the basis for creating linkage maps which position the markers on linkage groups corresponding to the chromosomes.

9.2.2 The F2

The F2 mapping population again uses two distinct inbred parents and the initial cross produces the heterozygous F1 hybrid, but now the F1 is selfed to produce the F2 progeny population. In this way, the F1 again has genotype AB at all loci where the parents differ, but the F2 loci can be any one of three genotypes: AA, AB or BB. Working out whether the genotypes at two loci in the progeny indicate a recombination has taken place between them is slightly more complex than in the backcross case. Firstly, twice as many crossovers can be detected since both parents of the F2 are heterozygous, unlike the backcross where one parent of the BC1 was homozygous. Secondly, some information is missing in the genotype data of the progeny even if the genotyping technology used can clearly distinguish all three possible genotypes: if the progeny is of genotype AB, it is not clear which of the maternal and paternal gametes provided which of the two alleles. As a consequence, when comparing two loci if both have genotype AB it is not clear whether zero or two recombinations have taken place. In practise, this can be inferred using a standard expectation maximisation (EM) algorithm (van Ooijen and Jansen 2013).

9.2.3 The F1 for a Diploid Species

Where inbred lines cannot be used as population parents, it is possible to create maps using heterozygous, non-inbred parents. The F1, or outcross, mapping population consists of two different heterozygous parents crossed together to produce multiple F1 progeny, which act as the mapping population directly. This creates a more complex situation genetically, even considering a diploid species, since less is generally known in

advance about the genotypes of the parents, unless the grandparental plants are also available for genotyping. For the BC and F2 populations, we were able to use the allele symbols A and B to denote that the allele originated from parent 1 or parent 2, respectively. The total number of alleles could not be greater than two since both parents were inbred. For heterozygous parents, there could be up to four alleles at one locus. It is also unknown in advance what the phase of the parental alleles is, such that when comparing the genotypes between two parental loci, if both are heterozygous and from the same chromosome, it is generally not known which pairs of alleles are in coupling phase together on the same homolog. For biallelic loci, there are three possible informative configurations of parental alleles: AB × AA, AA × AB or AB × AB, which is to say that either parent 1 can be heterozygous, or parent 2, or both. If both are homozygous, for either allele, then the locus is not informative since crossovers cannot be detected. For multi-allelic loci, there are many more possibilities (Ooijen and Jansen 2013; Maliepaard 2000).

The simplest method to make a linkage map for this population type, known as the two-way pseudo-testcross, is to split the markers into those that are only informative in the maternal and paternal parents, and treat these as two separate maps, each one much like a backcross. The difference is that the phase of the markers is not known in advance, unlike a true backcross where parent 1 crossed with the F1 always has a segregation type of AA × AB where the A alleles of all loci are in coupling phase with each other. In the pseudo-testcross, even if the genotypes at a single locus are in the same configuration AA × AB it is not known whether the A allele in the heterozygous parent is in coupling or repulsion phase with the A allele at another locus. In practise, it is possible to impute this missing information by initially assuming coupling phase, calculating the recombination fraction, and then inferring the presence of repulsion phase where the recombination fraction is greater than 0.5. The genotypes could then be recoded as a standard backcross and a linkage map made using standard mapping software.

The loci where both parents are heterozygous, AB × AB, cannot be fully utilised using the two-way pseudo-testcross. If a progeny has a genotype of AB, it is not known which parent provided which allele (similar to the case of the standard F2 cross from inbred parents), and these genotype values must be treated as missing data in the present case. For progeny with AA or BB genotypes, it is clear which allele came from which parent, and therefore these genotypes can be recoded and used within both maternal and paternal maps. Since on average half of the progeny will be heterozygous, this type of locus will have 50% missing data when used in this manner. In order to fully utilise the genotype data, a dedicated outcross mapping program is required which is capable of making use of the heterozygous calls from the AB × AB loci.

An alternative mapping population type is available for outbreeding species, namely the S1. In this case, a single heterozygous parent is selfed to produce S1 progeny which are used as the mapping population. This situation is largely equivalent to the F1 population except that all loci will be segregating as the AB × AB type.

9.2.4 The F1 for Octoploid Strawberry

The octoploid strawberry is now known to behave disomically during meiosis (Rousseau-Gueutin et al. 2008), which is to say that the same pairs of chromosomes form up at each meiosis, such that the two most *F. vesca*-like will always pair with each other, the two most *Fragaria iinumae* like with each other and so on. This means that, in terms of the recombination events which are the basis of genetic mapping, *F.* × *ananassa* behaves like a diploid with 28 pairs of chromosomes. This is in contrast to an autopolyploid where, if the chromosomes form into bivalent pairs, they do not always pair in the same combinations. There would also be the possibility of forming multivalent groupings in which more than two chromosomes participated at once. Fortunately, these complexities do not need to be considered for *F.* × *ananassa*.

The remaining difficulty presented is to obtain genotype information from single sub-genomic loci without confounding it with the three similar loci likely to be present in the homeologous chromosomes. The octoploid genome is approximately 80% of the size that would be expected if the diploid *F. vesca* genome were simply multiplied by four. In the absence of a complete octoploid genome sequence, we can therefore reasonably assume that a large proportion of loci have remained duplicated as two or more homeologous copies since the origin of the octoploid clade containing the cultivated strawberry. Genotyping technology works by recognising variations within specific sequence motifs, such as restriction enzyme cleavage sites, PCR primer binding sites or SNPs within otherwise conserved regions. If the local DNA sequences are very similar or identical between homeologous sites, then the genotyping technology may be unable to distinguish them directly.

The solution is generally to use some combination of genotyping techniques which can be targeted to single loci together with data filtering steps which remove markers which do not show the ratio of segregating alleles expected for a single locus. When genotype data are from a mapping population, provided the population size is large enough, testing for the expected Mendelian segregation ratios is a powerful way to exclude markers segregating at more than one locus. For example if a marker detects only the presence/absence of the B allele, and is specific to a single locus, then if the parental genotypes are AB × AA or AB × AB this would lead to a presence:absence ratio of 1:1 or 3:1, respectively, which would produce a mappable marker provided the mapping software supported dominant marker types (where the genotypes AB and BB have not been distinguished). The 1:1 and 3:1 ratios would also result if the genotyping technology was not sub-genome specific provided all but one of the homeologs happened to be homozygous for allele A. This latter case can be represented symbolically as ABAAAAAA × AAAAAAAA or ABAAAAAA × ABAAAAAA, respectively. If the genotyping system can detect allele dosages, then a single locus with parental genotypes AB × AB would obviously give progeny genotypes AA, AB and BB in the ratio 1:2:1. The polyploid equivalent of a single segregating locus among four homologs ABAAAAAA × ABAAAAAA would also give the same 1:2:1 ratio provided the dosage of allele B could be detected correctly as 0, 1 or 2 in the presence of multiple A alleles. However, there is a possible problem with this case. Consider the following parental genotypes ABAAAAAA × AAABAAAA, where the locus is heterozygous in both parents but on different sub-genomes. Here, the ratios of 0, 1 and 2 doses of allele B are of the expected Mendelian ratios, but the marker will show linkage to different linkage groups in the maternal and paternal maps and may falsely join the two homeologous linkage groups together if a combined map is made unless it is filtered out. This problem is avoided if only single-dose markers are used. Consider also crossing the parental genotypes BBABAAAA × BBBBAAAA, which would produce the same two genotypes in the progeny in a ratio of 1:1. If the genotyping technology can detect the change in dosage of the two alleles, then it can correctly signal that there are two genotypes involved in the ratio expected for a locus heterozygous in one parent. However, it is possible the two dosage levels might be misread as 2 and 3 copies, respectively, (or 4 and 5 copies) of the B allele instead of the actual 3 and 4 copies, which would suggest that the two parent genotypes are BBBBAAAA × BBBBBAAA, and consequently the marker would be misclassified as segregating in the wrong parent. In this case, provided that the majority of markers are classified correctly, it is simple to test for linkage between other maternal and paternal markers and infer whether the locus is in fact segregating in the maternal or paternal parent.

The most robust way to filter the markers is to choose only individual alleles which are present in one parent in a single dose and absent from the other parent entirely, and which segregate in a 1:1 ratio in the progeny, so-called single-dose markers (Wu et al. 1992). This would automatically exclude both the problematic cases presented above. Those where it was difficult to

determine which parent was heterozygous would be excluded as neither would lack the allele. Loci heterozygous in two or more sub-genomes would also be excluded on the basis that progeny were also not showing the expected 1:1 ratio. This robust filtering comes at the expense of reducing the number of usable markers. An alternative approach is to accurately assess the doses of all the alleles in order to make full use of all the segregation occurring (van Dijk et al. 2012), although this approach is necessarily more complex.

9.2.5 Genetic Mapping and Variant Calling Software

Software for creating genetic maps does not always explicitly support the outcross (F1) or selfing (S1) type of crosses generally used with *F.* × *ananassa*. The two-way pseudo-testcross approach can be used to separate the markers into maternally and paternally informative sets and map these separately using most standard mapping software (Grattapaglia and Sederoff 1994) such as MSTmap (Wu et al. 2008). This approach has the disadvantage that markers shared between the maternal and paternal maps are not fully utilised (van Ooijen and Jansen 2013). Software which explicitly supports outcross mapping includes JoinMap 4.1 (Van Ooijen 2011), TMap (Cartwright et al. 2007), Onemap (Margarido et al. 2007) and LepMap (Rastas et al. 2013).

Raw reads generated from whole genomic DNA, genotyping-by-sequencing or target capture protocols are generally aligned to a reference sequence, presently *F. vesca* being the best available option. A variant caller is then generally used to identify loci where the aligned reads differ from the reference sequence. BWA (Li 2013) is a commonly used read mapping tool, which outputs files which can then be fed into a variant caller such as SAMtools (Li et al. 2009), however, in order to correctly call variants from octoploid genomic DNA reads aligned to the diploid *F. vesca* a custom Python or Perl script maybe necessary.

9.3 Key Genotyping Technologies

9.3.1 AFLP Markers

The first genetic map of cultivated strawberry (Lerceteau-Köhler et al. 2003) used amplified fragment length polymorphism (AFLP) markers (Vos et al. 1995), which use a combination of restriction enzymes, polymerase chain reaction (PCR) and gel electrophoresis to generate and size a set of DNA fragments as a set of bands on the gel. The presence or absence of each band is dependent on short sequence variations adjacent to the corresponding restriction enzyme cut sites, detected by the primers. 113 progeny of an F1 cross between Capitola and CF1116 where genotyped and a map constructed using the two-way pseudo-testcross method using single-dose restriction fragments, giving a maternal map of 235 markers and a paternal map of 280. The genetic map was built using MAPMAKER (Lander et al. 1987) and JoinMap 2.0 (Stam 1993).

An advantage of the AFLP genotyping system is that it is 'open', in that it does not require detailed knowledge of the genome sequence prior to genotyping, since it relies only on finding restriction enzymes and PCR primer pairs which produce usable polymorphic bans. In this way, it is capable of discovering suitable loci as part of the genotyping process itself. A corresponding disadvantage is that, owing to the lack of detailed tailoring of primer sequences to specific target sites, these markers have proved unreliable when transferred to other mapping populations or species. A single primer pair can generate multiple usable markers, for example, Zorrilla-Fontanesi et al. (2011) report a total of 77 AFLP markers from 6 primer pairs. This class of marker continued in use after the advent of microsatellite markers, due to their ability to help produce better map saturation.

9.3.2 SSR Markers

The lack of transferability of markers between crosses and closely related species was resolved

by the introduction of microsatellite or simple sequence repeat (SSR) markers (James et al. 2003). In this system, PCR primer pairs are used to create amplicons specifically targeted to span tracts of short tandem repeats, which, due to their high mutation rate, are likely be polymorphic for the repeat number (in fact only a small number of markers should be enough to uniquely identify most cultivars). Gel electrophoresis is then used to separate the amplified fragments by size, such that a single amplicon can yield multiple distinguishable alleles. Monomorphic fragments can be used for calibration between different samples (van Dijk et al. 2012), so that absolute sizes can be estimated and compared. The high variability means that the homeologous loci across the four sub-genomes of the octoploid have a good chance of having at least one allele that is specific to a single sub-genome, and therefore can easily be mapped as a single-dose fragment presence/absence marker. The higher specificity of the primer pairs compared to AFLP markers allowed them to be designed in the diploid *F. vesca* but then be transferred to target the homologous loci in *F.* × *ananassa*, for example Monfort et al. (2006) reported 41 SSR markers developed from an *F. vesca* genomic DNA library, 94% of which also produced one or more amplified band in *F.* × *ananassa*. By comparing the order of shared SSR markers between octoploid maps and a reference map from a cross between the closely related diploid species *F. vesca* and *F. bucharica* (Sargent et al. 2009; Rousseau-Gueutin et al. 2008), it was possible to determine for the first time that diploid and octoploid genomes were largely collinear and showed few large-scale rearrangements (Sargent et al. 2012). The amplification efficiency of SSRs derived from the *F. vesca* genome was also used to identify which of the four octoploid sub-genomes was likely to be the most closely related to it (van Dijk et al. 2014). With increases in marker density the inversion on linkage group 2D with respect to *F. vesca* was identified, and the resolution of an octoploid map was sufficient to suggest that the version 1.1 *F. vesca* genome sequence contained assembly errors (van Dijk et al. 2014). An integrated SSR map from multiple mapping populations managed to achieve a total of 4474 markers (Isobe et al. 2013), including 3746 markers designed from expressed sequence tags (ESTs) generated from *F. vesca*. The loci were not evenly spread between the 28 linkage group, with the number of loci ranging from 18 to 131. As noted by the authors, it is likely that the use of a large proportion of *F. vesca* derived primers may have resulted in a bias towards more markers on the most *vesca*-like sub-genome.

9.3.3 The IStraw90 Microarray

The Affymetrix Axiom IStraw90 genotyping array is a SNP microarray designed by the international RosBREED consortium as the first high-throughput genotyping platform for octoploid strawberry (Bassil et al. 2015). Its design was only possible following the release of the genome sequence of the diploid progenitor *F. vesca* (Shulaev 2011). The diploid genome was constructed using a combination of three sequencing technologies: Roche 454, Illumina and Life Technologies SOLiD for a combined coverage of 39x of the 240 Mb genome. The resulting scaffolds were assembled into full chromosome scale pseudo-molecules using a genetic map of a cross between *F. vesca* and *Fragaria bucharica* (initially misidentified a *Fragaria nubicola*) consisting of 76 progeny, the majority of markers being SSRs (Sargent et al. 2006, 2008). The assembly has since been improved by remaking the pseudo-molecules using higher density genetic maps constructed for *F. vesca* ssp. *bracteata* (Tennessen et al. 2014).

Following the initial publication of the diploid genome, work began on designing a microarray for genotyping the octoploid strawberry (Bassil et al. 2015). Genomic sequences from 19 octoploid accessions were generated and aligned to the diploid reference genome and SNPs present in the octoploid were identified. An initial 36 million sequence variations were filtered to identify those suitable for conversion into sub-genome specific SNP probes on the Axiom

microarray. The challenge was to distinguish between variations between different sub-genomes, referred to as homeologous sequence variants (HSVs), and the variations occurring within individual sub-genomes which were candidates for sub-genome specific SNPs. Filtering steps included ensuring that the variation was present in at least two accessions and also absent from another two, such that the variant would not be a fixed variation between sub-genomes, but a true SNP. The two main classes of marker designed for the microarray were those which detected variants that appeared to be specific to a single sub-genome already (these consisted of three sub-categories: biallelic SNPs, multi-allelic SNPs and indels), and those which could be linked to a single sub-genome by utilising an adjacent HSV, a so-called technical ploidy reduction strategy. The latter class, referred to as haploSNPs, were designed so that the probe sequence used by the microarray would only be able to hybridise to the SNP location provided the nearby HSV was of a particular variant only present in a single sub-genome. The HSVs utilised for this purpose were HSV–SNPs and HSV-indels adjacent to the true SNP and HSV insertions containing the true SNP itself (Table. 9.1).

The Affymetrix Axiom microarray platform utilises approximately 30 base probe sequences which hybridise to the complementary sequence in the genomic DNA of the sample being genotyped. Fluorescently labelled oligomers then hybridise with the genomic DNA on the bases immediately after the end of the probe sequences, the colour of the fluorescence indicating information about the first base position after the probe. There are two colours available, so that the signal given indicates A or T using one colour and C or G using the other. The final output for each marker is the signal intensity for each of two possible alleles, called A and B. The Affymetrix Power Tools software can then be used to convert from the raw intensity signals into final genotype calls. This works by performing cluster analysis on a batch of multiple samples, where for each marker the A and B intensity values are plotted on the x- and y-axes of a graph, respectively. For a biallelic SNP where the samples contain all three possible genotypes, AA, AB and BB, the intensity, values will tend to form three clear clusters when plotted in this manner. If the samples contain only two of the three possible genotypes only two clusters result, but it is usually possible to infer what the genotypes are from their location in intensity space. For those markers where the probe sequences are hybridising to loci from two or more sub-genomes, it is still often possible to produce usable genotype calls provided that only one of the loci is polymorphic. For example, if two sub-genomic loci are hybridising and only three genotypes, AAAA, ABAA and BBAA are present, the plot will still show three clusters much like the biallelic case, except that their positions will be shifted further along the A-allele intensity axis. The same situation applies to the cases where three or four sub-genomic loci are hybridising,

Table 9.1 Breakdown of the SNPs included in the Affymetrix Axiom IStraw90 array by category

	SNP	mSNP	Indel	SNP–SNP	Indel-SNP	Insertion-SNP	Other
Markers	63,263	1761	9528	7092	1177	2843	9399
Passed QC	9186	222	886	1928	148	154	85
% Passed	14.5	12.6	9.3	27.2	12.6	5.4	0.9

Non-haploSNP-based markers: SNP—biallelic SNP, mSNP—multi-allelic SNP, indel—non-haploSNP indel; haploSNP-based markers: SNP–SNP, Indel-SNP, Insertion-SNP; other—SNPs based on *F. iinumae* or speculative codon-based SNP. Passed QC—number of markers which passed stringent quality control in a test set of 306 *F.* × *ananassa* samples. (Adapted from: Bassil et al. 2015, Table 4)

provided only one locus is polymorphic, the cluster analysis process can generally identify the three clusters and call the genotypes of the polymorphic locus correctly. Where three clear clusters are present, the assignment of genotypes to them is relatively straightforward, and consequently those markers showing this pattern should be treated as the most reliable. Where only two clusters are present, indicating that one of the three possible genotypes is absent, and given the additional variation introduced by differences in effective ploidy level, it is possible that the clusters maybe assigned incorrect genotypes. As an example, we have found that, in an F1 mapping population from the *F.* × *ananassa* cross Redgauntlet × Hapil, 11,587 usable markers were obtained from the APT software including 6389 showing only two clusters, of which 241 were subsequently found through linkage analysis to have been classified as heterozygous in the wrong parent (i.e. classified as heterozygous in Redgauntlet where in fact linkage showed them to be heterozygous in Hapil, or vice versa), which amounts to an error rate of 2% of the total usable markers, or 3.8% of the two-cluster markers.

Sargent et al. (2015) report a linkage map created using the IStraw90 array to genotype 86 progeny of the *F.* × *ananassa* F1 cross Darselect × Monterey. Figure 9.1 shows the number of markers per linkage group, broken down by sub-genome. It can be seen that the most *F. vesca*-like sub-genome does not always have the largest number of markers, as might have been expected due to ascertainment bias introduced by the microarray design process having relied on alignment of sequences to the *F. vesca* genome.

9.3.4 Diversity Arrays Technology

Sanchez-Sevilla et al. (2015) report the development of two genotyping methods for the octoploid strawberry produced in conjunction with Diversity Arrays Technology Pty Ltd. The first is a DArT microarray (Jaccoud et al. 2001) containing a total of 603 markers, which were used to genotype 62 diverse strawberry cultivars in order to perform a diversity analysis. The DArT microarray was created using a pair of restriction enzymes (PstI and TaqI) chosen to produce an optimal range of restriction fragment sizes when tested on 6 of the strawberry accession. Digested genomic DNA from all 62 accessions was PCR amplified and then used to create clones. Individual clones were isolated, DNA was again PCR amplified and then attached to the microarray to create the hybridisation probe arrays. Genomic DNA from the 62 samples was fluorescently labelled and hybridised to the arrays. In this way, the microarray markers were derived from the same set of samples that were being genotyped as part of the array development process, which should minimise ascertainment bias. The platform provides fewer markers than the IStraw90 microarray, but a greater number of accessions were used in its development, and it provided sufficient markers to perform a diversity analysis of the 62 accessions.

The second genotyping method utilised was the related DArTseq method (Sansaloni et al. 2011), which includes the use of restriction enzyme digestion, but then proceeds to sequence the resulting genomic DNA fragments on the Illumina platform, in a similar way to the genotyping-by-sequencing (GBS) methodology (Elshire et al. 2011). Again pairs of restriction enzymes were tested to produce the best fragment size distribution for the subsequent protocol steps, with PstI and MseI being chosen. DArTseq was applied to the parents and 94 F1 progeny of the cross ‘232’ × ‘1392’. After the digestion step adapters were ligated such that in the subsequent PCR step only fragments terminated with a PstI and an MseI overhang were amplified, before being sequenced on the Illumina GAIIx platform. The proprietary DArTsoft14 software was used to convert the raw reads into marker calls [in a comparable manner to the Stacks software (Catchen et al. 2013)], by grouping identical reads and clustering based on a given maximum number of mismatches. These marker calls were further filtered to identify single-dose alleles present in only single parent and segregating in a 1:1 ratio in progeny, and those heterozygous in

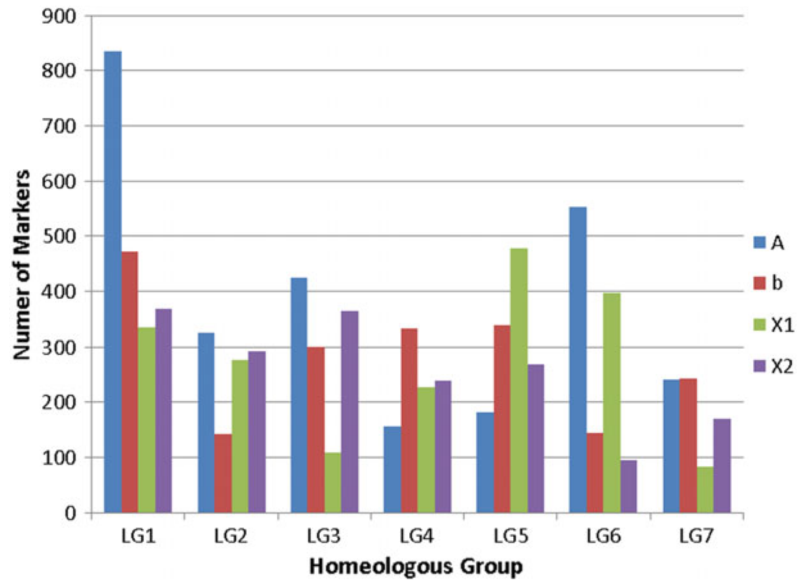

Fig. 9.1 Number of markers per linkage group in the genetic map of the F1 cross Darselect × Monterey. A—*F. vesca*-like sub-genome; b—the most *F. iinumae* like sub-genome; X1, X2—the two remaining sub-genomes. (From: Sargent et al. 2015, Table 1)

both parents and showing a 3:1 ratio in progeny. The total of 4242 DArTseq markers was passed to the final mapping step along with 408 previously produced SSR markers. The final map was created using JoinMap 4.1 (Van Ooijen 2011).

9.3.5 Double-Digest RAD

Davik et al. (2015) report a genetic map constructed from 145 F1 progeny of the cross Sonata × Babette created using the double-digest restriction-site associated DNA (ddRAD) method (Peterson et al. 2012). Similar to the DArTseq method, this involves digestion with a pair of restriction enzymes, EcoRI, and MspI. After adapter ligation, fragments were size selected using a Pippin Prep to around 400 bp before being sequenced using an Illumina MiSeq. The sequence data were processed using Stacks (Catchen et al. 2013) requiring a minimum of 5 reads per stack with a maximum of 4 mismatches between candidate alleles and minimum of 240 bases read length. Candidate loci were required to be present in both parents and at least 100 offspring and were filtered for segregation distortion using a chi-squared test at the 5% significance level. The map was constructed using JoinMap 4.1. This resulted in a map containing a total of 907 markers spread across 31 linkage groups, indicating that the map was not completely saturated, since at least one chromosome was represented by more than one linkage group. A comparison to the *F. vesca* genome suggested the map spanned the majority (approximately 79%) of the *F.* × *ananassa* genome.

9.3.6 Target Capture

Tennessen et al. (2013, 2014) report genetic maps of the diploid *F. vesca* ssp *bracteata* and the two octoploid progenitor species of the cultivated strawberry *Fragaria virginiana* and *Fragaria chiloensis* constructed using a target capture approach. This method relies on custom designed probes, in this case RNA baits were synthesised using the MYbaits system of Mycroarray, to hybridise with the targeted loci in genomic DNA extracted from the samples, much like a microarray system, except that the hybridisation happens in solution. The bound baits are then collected using a biotin mediated magnetic bead system, before sequencing using the Illumina platform. The custom baits were designed to target variations found in a pair of

bracteata plants and their F1 mapping population (Tennessen et al. 2013), which were discovered using low coverage genome sequencing of the parents and progeny, mapped to the *F. vesca* genome, and then passed through a variant calling pipeline based on BWA (Li 2013) and SAMtools (Li et al. 2009). 6575 variants were targeted with three baits each (6376 SNPs and 199 indels of 1–2 bps), targets were chosen to be at least 100 bps from any other variable sites, and at least 10 kb from other target sites. A linkage map was constructed for this F1 cross as well as another population created using the progeny from selfing another *bracteata* individual. The same baits were also used to capture DNA from two octoploid F1 mapping populations (Tennessen et al. 2014), one from a cross between two unrelated *F. virginiana* parents and another from unrelated *F. chiloensis* parents. Captured DNA samples were sequenced using the Illumina platform and reads were aligned to the *F. vesca* genome assembly using BWA. Variants were called using customer Perl scripts due to the lack of a standard tool to call reads from an octoploid mapped to a diploid genome. Loci were excluded if they had a read depth of less than 32 and were selected for mapping where the minor allele had a frequency of approximately 1/8th, comparable to a single-dose restriction fragment, and showed the expected Mendelian segregation. Maps were made using the R package Onemap (Margarido et al. 2007). The *F. virginiana* 67 progeny F1 population yielded 4469 markers, including 594 heterozygous in both parents, whereas the *F. chiloensis* 42 progeny F1 population gave 3322 markers including 780 heterozygous in both parents.

9.4 Future Genotyping Trends

While SSR markers will likely retain a role for scoring smaller numbers of loci and samples, the current trend is towards SNP-based genotyping. SNPs have a lower mutation rate, but are more common and likely more evenly distributed across the genome. They are also more suitable for high-throughput fully automated analysis. The SNP-based technologies already tested in cultivated strawberry reviewed in Sect. 3 have shown the feasibility of overcoming the difficulties of a heterozygous allopolyploid genome. The IStraw90 microarray has demonstrated successful SNP calling using carefully designed probes. The DArTseq- (Sánchez-Sevilla et al. 2015) and ddRAD (Davik et al. 2015)-based maps have shown SNPs can be called from an open genotyping system using sequence reads directly without first being aligned to a reference genome. The target capture maps of *F. virginiana* and *F. chiloensis* (Tennessen et al. 2013) successfully called SNPs from captured reads aligned to the *F. vesca* genome in place of the octoploid, and using capture probes which were originally designed as diploid markers within *F. vesca,* and therefore had not been explicitly selected to avoid confounding homologous loci, a processes which was instead performed in silico postcapture. With increasing knowledge of the octoploid genome sequence these sequencing-based approaches to SNP calling are likely to become more efficient.

Genotyping-by-sequencing approaches should be better suited to the discovery of novel variation, but require careful optimisation of the complexity reduction steps and the level of multiplexing in order to get reproducible markers. Microarray-based genotyping suffers from ascertainment bias, but has so far produced the largest number of markers of any method. Target capture appears to be a good compromise between the two extremes as it allows for precise tailoring to the regions of interest, but requires less detailed design, as shown by the successful capture of octoploid SNPs using baits designed for *F. vesca*. Where extended haplotype information is required, such as to determine the complete allele sequences of long genes, target capture can be combined with one of the long read technologies such as Pacific Biosciences PacBio RS II or Sequel System, or Oxford Nanopore Technologies MinION sequencing. The full range of available SNP genotyping methods, reviewed in (Kim et al. 2016) have yet to be reported in strawberry, but having validated the basic approach, it is likely that further

techniques will now be tried and adapted to the needs of strawberry, and the most suitable for the various applications of genotyping will emerge.

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10 Current Status of Octoploid Strawberry (*Fragaria* × *ananassa*) Genome Study

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Abstract

The complex structure of the polyploid genome has inhibited advances in genomics and genetic analysis in polyploid species. Octoploid strawberry (*Fragaria* × *ananassa*) is allopolyploidy species ($2n = 8x = 56$) with an estimated genome size of 1C = 708–720 Mb. The recent study reported by Tennessen et al. (2014) suggested that the genome of *F.* × *ananassa* consisted of each one pair of *F. vesca*-like and *Fragaria iinumae*-like genomes and two other pairs of subgenomes. Therefore, while the genome sequences of *F. vesca* have played an important role, the whole genome sequences of *F.* × *ananassa* are also essential for a more detailed and thorough understanding in studies about *F.* × *ananassa*. The construction of high-quality subgenome-specific reference sequences in *F.* × *ananassa* has been a long-dreamt goal, due to its potential for analyzing the expression of previously unexplored genes, such as in the evolution in *Fragaria* species, and for accelerating molecular breeding. In this chapter, we review the recent results of large-scale genome and transcriptome analyses related to genome sequence dissection in *F.* × *ananassa*.

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10.1 Introduction

Polyploidization often induces increases in plant size and stress tolerance that contribute to plant domestication, and the polyploidization process is thus considered highly beneficial to human life. However, the complex structure of the polyploid genome has inhibited advances in genomics and genetic analysis in polyploid species. Although recent advances in genome sequencing technologies have drastically changed the field of genome study and led to the publication of a large number of whole genome sequences of plant species (Michael and VanBuren 2015), in the case of polyploidy species, the number of published genome sequences has been limited due to the difficulty of sequence assembly. Those few polyploidy sequences that have been published include octoploid strawberry (*Fragaria* × *ananassa;* Hirakawa et al. 2014), hexaploid bread wheat (The International Wheat Genome Sequencing Consortium 2014), tetraploid oilseed rape (Chalhoub et al. 2014), tetraploid upland cotton (Li et al. 2015) and tetraploid mustard (Yang et al. 2016). In most of polyploidy species, the genomes of ancestor diploid species were sequenced as references prior to the dissection of genomes of the targeted

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polyploid species (D'Hont et al. 2012; Wang et al. 2012; Ling et al. 2013; Bertioli et al. 2016). This was the approach used for *F.* × *ananassa*: as an ancestral reference for the sequencing of this polyploid species, the whole genome sequences of possible diploid ancestor, *F. vesca,* were published by Shulaev et al. (2011) and greatly contributed to the advances of genomic and genetic analyses. The whole genome sequence data and gene annotations of *F. vesca* were later revised after the first publication (Tennessen et al. 2014; Darwish et al. 2015).

F. × *ananassa* is an octoploid species ($2n = 8x = 56$) with an estimated genome size of 1C = 708–720 Mb (Akiyama et al. 2001; Davis et al. 2007). It was generated in the sixteenth century by an artificial crossing between *Fragaria chiloensis* and *Fragaria virginiana* in Europa (Yanagi and Noguchi 2016). The history of *F.* × *ananassa* is not long, but the evolutionary origin and genome structure have not been fully determined. The first allo-autopolyploidy structures were proposed by Federova (1946, AABBBBCC) and Senanayake and Bringhurst (1967, AAA'A'BBBB). Years later, the allopolyploidy structure (AAA'A'BBB'B') was suggested by Bringhurst (1990) based on isozyme segregation. In the current century, support for the allopolyploidy model has been provided by the segregation patterns of CPAS markers (Kunihisa 2011) and SSR markers (Isobe et al. 2013). More recently, Tennessen et al. (2014) proposed the AvAvBiBiB_1B_1B2B2 model according to the results of targeted DNA capture and sequencing by using next-generation sequencing (NGS) technology. The new model indicates that the genome of *F.* × *ananassa* consisted of a single subgenome of each of the *F. vesca*-like ancestor (Av) and *F. iinumae*-like ancestor (Bi), and two subgenomes (B1 and B2) derived from an *F. iinumae*-like autotetraploid. The results also suggested the possibility that *F.* × *ananassa* included *F. vesca*-dislike sequences on the genomes. Therefore, while the genome sequences of *F. vesca* have played an important role, the whole genome sequences of *F.* × *ananassa* are also essential for a more detailed and thorough understanding in studies about *F.* × *ananassa*. In this chapter, we review the recent results of large-scale genome and transcriptome analyses related to genome sequence dissection in *F.* × *ananassa*.

10.2 Deep Sequencing of the Genomes of *F.* × *ananassa*

Hirakawa et al. (2014) performed de novo whole-genome sequencing of a Japanese strawberry variety, "Reikou" by using the Illumina and Roche 454 sequencing platforms. The genome size of *F.* × *ananassa* was estimated to be 692 Mb. Their report was the first published study on de novo whole-genome sequencing in a polyploidy species. At the time of their work, assembly algorithms for discriminating multiple heterozygous genomes were not well established. Therefore, they constructed a virtual reference genome (haploid genome), which integrated the genome sequences of homoeologous chromosomes, by eliminating heterozygous bases in the process of sequence assembly (Fig. 10.1). A total of 2.6 Gb of Roche 454 reads were assembled by Newbler 2.7 (heterozygotic mode), whose algorithm was based on Overlap Layout Consensus (OLC), and GapCloser 1.10. As the result, a total of 7,609,454 scaffolds were generated with 101.2 Mb in length, which was approximately half the length of the haploid genome. To supply the missing sequences, a total of 277 Gb of Illumina reads, including 300–600 bp of insertion paired-end (PE) reads and 2 Kb of insertion mate pair (MP) reads, were additionally obtained and assembled by SOAPdenovo v1.05 and GapCloser 1.10. The base algorithm of SOAPdenovo was De Bruijn graph (DBG) approach and did not eliminate heterozygous bases. To eliminate the heterozygosity, the Illumina scaffolds generated by SOAPdenovo v1.05 were re-assembled by Newbler 2.7 after the removal of sequences that showed significant similarity to the 454 scaffolds. A total of 1,153,521 Illumina-specific scaffolds were re-assembled by Newbler 2.7 in the heterozygotic mode and

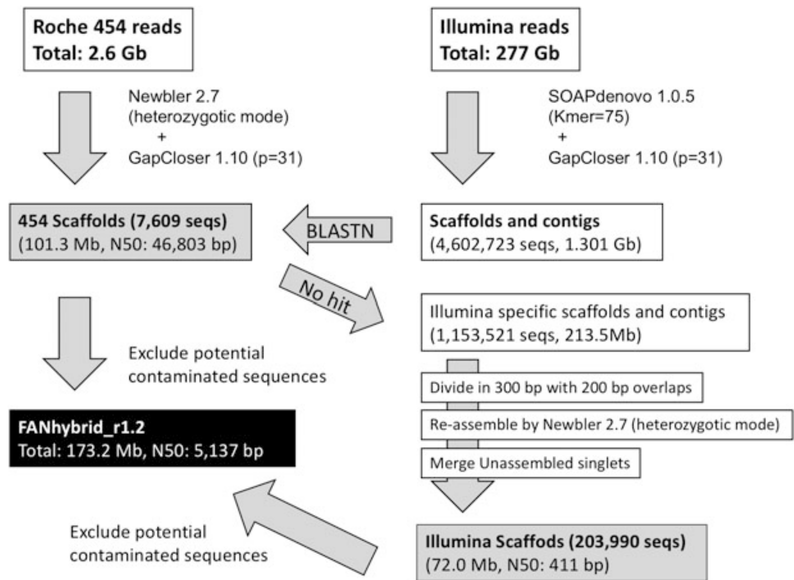

Fig. 10.1 Strategy of sequencing and assembly of the reference genome of *F.* × *ananassa* (FANhybrid_r1.2, Hirakawa et al. 2014)

generated 203,930 sequences comprising 72.0 Mb. The 454 scaffolds and Illumina-specific scaffolds were integrated and qualified as a reference genome for *F.* × *ananassa* (FANhybrid_r1.2). FANhybrid_r1.2 consisted of 212,588 sequences, comprising a total of 173.2 Mb with an N50 length of 5137 bp.

In parallel, Illumina PE reads were obtained for four wild *Fragaria* species, *F. iinumae* (diploid), *Fragaria nipponica* (diploid), *Fragaria nubicola* (diploid), and *Fagus orientalis* (tetraploid), to make a comparison among the genomes of *Fragaria* species. De novo assemblies by SOAPdenovo v1.05 and GapCloser 1.10 were performed for each Illumina PE reads and generated scaffolds with a total length of 200–214 Mb in each species. The assembled genome sequences of the four wild *Fragaria* species (FII_r1.1, FNI_r1.1, FNU_r1.1, FOR_r1.1) as well as Illumina scaffolds of *F.* × *ananassa* (FAN_r1.1) and *F. vesca* genome sequences (v1.1) were mapped onto the reference genome of *F.* × *ananassa* (FANhybrid_r1.2) to establish the subgenomic structure of *F.* × *ananassa*. Repetitive sequences were detected in each assembled genome sequence. The 18 rRNA, and 300 tRNA genes encoding, and a total of 45,377 gene sequences were predicted based on FANhybrid_r1.2. The generated genome assembly sequences, gene models of *F.* × *ananassa,* and the four wild *Fragaria* species are available at the Strawberry GARDEN (http://strawberry-garden.kazusa.or.jp) and the GDR database (https://www.rosaceae.org/species/fragaria/all).

10.3 Genomic Retrotransposons

According to the deep sequencing analysis by Hirakawa et al. (2014), 0.3–0.6% of the genome of *F.* × *ananassa* is occupied by long terminal repeat (LTR) retrotransposon sequences. Retrotransposons are mostly inactive, but are sometimes activated under stress conditions, including tissue culture. In strawberries, micropropagation is commonly performed during seedling production, and thus the somaclonal variation occurring under tissue culture is a crucial issue for seedling management. Ma et al. (2008) considered that the activation of transposon elements (TEs) was a possible cause of somaclonal variation, and isolated Ty1–copia and Ty3–gypsy unique sequences from the RNAs of tissue culture calluses. Southern dot blot hybridization showed high copy numbers of TEs in the *F.* × *ananassa* genome (2875 in the Ty1-copia group

and 348 in the Ty3-gypsy group), but no transcriptionally active sequences were isolated in reverse transcriptase (RT) genes. Later, the research group isolated active RT gene sequences from strawberry plants grown on different phytohormone levels (Ma et al. 2010).

LTR retrotransposons are also used in molecular markers by using polymorphisms caused by insertion of retrotransposon sequences. Monden et al. (2014) performed a screening of LTR retrotransposon families using the combination of an Illumina sequencing platform and a comprehensive LTR library based on the primer binding sites (PBSs) adjacent to the 5′ LTR. The insertion sites of LTR sequences were compared among seven strawberry cultivars and *F. vesca*, resulting in the screening of LTR families that showed high insertion polymorphisms among closely related cultivars. The results suggested that the insertion of the screened LTR families occurred quite recently.

10.4 Cytoplasmic Genome Sequences

Cytoplasmic DNA, i.e., chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA), were used in phylogenetic analysis of genus *Fragaria*. Honjo et al. (2009) investigated variation of the two non-coding region of cpDNA sequences in 75 *F.* × *ananassa* accessions and seven wild relatives and identified *F.* × *ananassa*-specific haplotype sequences. Njuguna et al. (2013) performed whole-genome sequencing of cpDNA in 21 *Fragaria* species with Illumina sequencing platforms and revealed geographic segregation of chloroplast haplotypes in *F. vesca* and sexual system evolution to the two octoploid founders of *F.* × *ananassa*.

Mahoney et al. (2010) reported that *F. iinumae* is possible mtDNA donor of the ancestral octoploid species of *F.* × *ananassa*, *F. chiloensis,* and *F. virginiana* by comparison of sequences of the mitochondrial maturase R (matR) gene in two diploids, two octoploids, and one decaploid *Fragaria* species. More recently, Govindarajulu et al. (2015) performed comparative analysis of mitochondrial exome, chloroplast, and nuclear genome sequences in the five wild *Fragaria* species including two octoploids and three diploids, and identified potential breakage in linkage disequilibrium of cytoplasmic genomes. These studies suggest that whole cytoplasmic genome sequences of *F.* × *ananassa* accessions would provide further understanding in variations of functional genes and evolutional history in *F.* × *ananassa*.

10.5 RNA-Seq Analysis

Several whole-transcriptome shotgun sequencing (RNA-Seq) analyses in *F.* × *ananassa* have been reported over the past several years. Amil-Ruiz et al. (2013) identified four reference genes, *FaRIB413, FaACTIN, FaEF1α, and FaGAPDH2*, for transcript normalization in *F.* × *ananassa* defense responses from the thirteen potential preselected genes by comparing the gene expression profiles associated with different tissues, cultivars, biotic stresses, ripening and senescent conditions, and salicylic acid/methyl jasmonate (SA/JA) treatments.

Most of the RNA-Seq analyses in *F.* × *ananassa* were performed to identify gene expression in fruit-related traits for their agricultural importance. Chambers et al. (2014) and Sánchez-Sevilla et al. (2014) concurrently reported identification of the *FaFAD1* gene, which encodes an omega-6 fatty acid desaturase based on RNA-Seq analysis of BC_1 progeny derived from “Elyana” and “Mara des Bois” (Chambers et al. 2014) and the “232” ×“1392” F_1 mapping population (Sánchez-Sevilla et al. 2014). The *FaFAD1* gene expression level was significantly correlated with the presence and absence of γ-decalactone, a volatile compound that conferred a peach flavor note to strawberry fruits. Both studies obtained Illumina transcript reads from ripe fruits and performed differential gene expression (DGE) analysis with *F. vesca* gene sequences as a reference. In addition, Sánchez-Sevilla et al. (2014) used *F.* × *ananassa* assembled transcript sequences as a reference for the calculation of Fragments Per Kilobase of

exon per Million fragments mapped (FPKM). Although the materials and a part of the approach were different between the two reports, the identified gene sequence was identical.

The cultivars “Elyana,” “Mara des Bois,” and their progeny were further used for the identification of flavonoid pathway genes (Pillet et al. 2015). As in the previous study (Chambers et al. 2014), Illumina transcript reads were obtained from the ripe fruits, and DGE analysis was performed with the *F. vesca* gene sequences. Based on correlation analysis in the expression profiles within a set of phenylpropanoid genes, the authors hypothesized that the genes whose expressions were positively correlated with at least four flavonoid pathway genes were functionally connected to the pathway. Fifteen transcription factors were selected according to this hypothesis, and three candidate genes, *FaTCP11*, *FaPCL1-like,* and *FaSCL8*, were subjected to functional analysis during transient expression by agro-infiltration. The results of the transient expression suggested that the three genes had roles in the synthesis of flavonoids.

Chen et al. (2016) performed de novo assembly and gene functional annotation based on 10.78 Gb Illumina transcript reads. The assembled 44,457 unigenes were annotated based on databases such as the NCBI non-redundant protein sequences (NR) and nucleotide sequences (NT) databases, the Kyoto Encyclopedia of Genes and Genomes (KEGG), and the Gene Ontology (GO) and euKaryotic Ortholog Groups (KOG) databases. With the assembled unigenes, transcriptome profiling was reported in responses to exogenous auxin (IAA) and abscisic acid (ABA) in harvested strawberry fruits by DGE analysis.

10.6 MicroRNA (miRNA) Identification

MicroRNAs (miRNAs) regulate post-transcriptional or transcriptional gene expression and have received much attention for their roles in gene control. miRNA sequences in *F.* × *ananassa* were first reported by Han et al. (2014). A total of 34 candidate miRNA sequences were determined based on RNAs extracted from the roots, stems, leaves, flowers, and fruits of the strawberry variety “Sweet Charlie.” The gene expression analysis by qRT-PCR and functional analysis revealed that the 34 miRNAs were involved in the growth, development, and physiological processes of *F.* × *ananassa.*

Larger-scale identification of miRNAs was reported by Xu et al. (2013). Two small RNA (sRNA) libraries were constructed from strawberry fruits stored for 0 and 24 h at 20 °C and sequenced using the Illumina Solexa platform. The obtained sRNA sequences were mapped onto the known plant miRNAs in miRBase (19.0), and 88 known mature miRNA sequences were determined. In addition, 1224 novel miRNA sequences were identified by BLAST search against the strawberry genome sequences registered in the GDR database (www.rosaceae.org). Differential expression analysis of the miRNA libraries revealed that six known miRNA families and six predicted candidates were involved in regulating fruit senescence.

10.7 Challenges in the Development of High Quality, Subgenome-Specific Reference Sequences

Most of the genomic and genetic analyses in polyploids have been performed using methodologies developed for diploid species. Thus, the interactions between homoeologous genes have generally been ignored. Nonetheless, the discrimination of homoeologous sequences is essential for genomic and genetic studies in polyploid species, and several initiatives to overcome this difficult issue have recently been started (Glover et al. 2016).

Song et al. (2016) developed a tool for improving genome assembly with RNA-Seq reads, Rascaf, and applied it to a re-assembly of the *F.* × *ananassa* and the four wild *Fragaria* Illumina scaffolds developed by Hirakawa et al. (2014). The short contigs were joined based on determination of the most likely paths in an exon

block graph on short contigs with 70 M PE RNA-Seq reads. In the case of *F.* × *ananassa* (FAN_r1.1), a total of 5866 novel connections were found, while 5267–10,147 connections were identified in the four wild *Fragaria* genomes (FII_r1.1, FNI_r1.1, FNU_r1.1, FOR_r1.1).

High-density linkage maps are important resources for the discrimination of homoeologous genomes, and the IStraw 90 K Axiom® array developed as part of the RosBREED project (https://www.rosbreed.org/) has greatly contributed to the map construction (Bassil et al. 2015). SNPs for the array were discovered among 26 Fragaria species, including 16 *F.* × *ananassa* lines and 10 accessions of wild species. A total of 6594 SNPs were mapped onto the first linkage map developed by Bassil et al. (2015). The probe sequences of the IStraw 90 K Axiom® array were derived from the *F. vesca* genome (v1.0), and therefore, the *F. vesca* genome sequences were anchored onto the *F.* × *ananassa* genome via the linkage map. High-density linkage maps were also constructed by digest restriction-associated DNA sequencing (ddRAD-Seq; Davik et al. 2015) and diversity array technology (DarT; Sánchez-Sevilla et al. 2015). Sequences derived from the *F.* × *ananassa* genome were directly anchored onto the maps, because both the ddRAD-Seq and DArT array approach used *F.* × *ananassa* genome sequences for SNP identification.

A high-density linkage map also contributed to the prediction of the genome structure of *F. ananassa*. Sargent et al. (2016) constructed a linkage map with 8407 SNPs with the IStraw 90 K Axiom® and predicted the ancestor genomes of each linkage group by using haploSNPs as indicators of ancestral genomes. The SNP alleles on the linkage map were compared with the corresponding *F. vesca* and *F. iinumae* alleles, and the haploSNPs were categorized as follows: YY (matching both *F. vesca* and *F. iinumae*), YN (matching *F. vesca* but not *F. iinumae*), NY (matching *F. iinumae* but not *F. vesca*), and NN (not matching either *F. vesca* or *F. iinumae*). Based on the location of the classified haploSNPs, Sargent et al. made the novel hypothesis of an *F.* × *ananassa* genome structure, AA, bb, XX, X-X, where A, b, and X-X represent the *F. vesca*-like, *F. iinumae*-like, and bivalent pairing of homologues, respectively.

The haplotype phasing was not well considered on the primary stage of whole-genome sequencing study due to a lack of efficient technologies for discrimination of maternally and paternally derived sequences. However, the recent progress in NGS and computational technologies has enabled the construction of phased data at feasible cost (Tewhey et al. 2011). The phasing technologies have been developed mainly for diploid species, but they are also applicable for the discrimination of subgenome sequences. For example, Aguiar and Istrail (2013) applied a HapCompass framework for polyploid genome assembly. Though the developed assembly algorithms were more complex than those for diploid phasing due to a large number of possible haploid combinations, their demonstration indicated that the accuracy of SNP phasing in the polyploid genome was increased when the amount of input data was increased.

Genome sequencing technology is no longer used only in research fields. Indeed, it has become a popular concept in society and is widely applied in the field of human health. The number of genome-sequencing and genome-assembly services' providers has increased along with the increasing demand. NRGene, a bioinformatics service provider, developed the DenovoMAGIC program (http://nrgene.com/products-technology/denovomagic/), which assembles Illumina reads into long and phased sequences. The efficiency of DenovoMAGIC in strawberry genome assembly was recently confirmed by Shirasawa et al. (2017). *F.* × *ananassa* (cv. "Reikou") Illumina reads generated from PE, MP, and 10X Genomics libraries (https://www.10xgenomics.com/) were assembled by DenovoMAGIC3.0. The total length of the assembled sequences was 1406 Mb and consisted of 32,715 sequences and N50 of 3.9 Mb (Table 10.1). When the haplotype phases of assembly genomes were compared with those identified in the linkage map of the S_1 population

Table 10.1 Summary of assembly statistics in the *F.* × *ananassa* genome cv.'Reikou'

Sequence ID	FANhybrid_r1.2	FAN_r1.1	–	–
Number of sequences	211,588	625,966	7563	32,715
Total length (bp)	173,229,572	697,765,214	1,108,561,665	1,406,451,310
Average length (bp)	819	1115	146,577	42,991
Max length (bp)	348,406	51,398	14,978,214	17,025,478
N50 length (bp)	5137	2201	3,886,453	3,887,271
A	47,398,175	201,607,452	326,578,512	417,844,835
T	47,314,079	201,232,679	326,728,402	417,689,936
G	29,454,229	127,672,886	210,940,335	269,940,552
C	29,542,534	127,932,068	211,028,295	270,047,128
n	0	0	4,396,000	1,680,000
N	19,520,555	39,320,129	28,890,121	29,248,859
GC% (GC/ATGC)	38.4	38.8	39.2	39.3
Input reads	Roche 454: 2.6 Gb Illumina (PE + MP) 277 Gb	Illumina (PE + MP) 277 Gb	Illumina (PE + MP) 328 Gb	Illumina (PE + MP) 328 Gb Illumina (10X) 100 Gb
Assembler	Newbler: 2.7 SOAPdenovo 1.0.5 GapCloser 1.10	SOAPdenovo 1.0.5 GapCloser 1.10	DenovoMAGIC 3.0	DenovoMAGIC 3.0
References	Hirakawa et al (2014)	Hirakawa et al (2014)	Shirasawa et al (2017)	Shirasawa et al (2017)

of "Reikou", 40 mis-assemblies were found in the scaffolds. The quality of generated scaffolds was high and is expected to contribute to pseudomolecule construction.

Polyploidy species are still a large unexplored area in genetics and genetic studies. Wang et al. (2016) recently reported on the epigenetic and transcriptomic changes caused by interspecific hybridization and genome doubling in *Fragaria* species. The construction of high-quality subgenomespecific reference sequences in *F.* × *ananassa* has been a long-dreamt goal, due to its potential for analyzing the expression of previously unexplored genes, such as in the evolution in *Fragaria* species, and for accelerating molecular breeding. The reports reviewed in this chapter suggest that this dream may soon come true.

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11 The History and Current Status of Genetic Transformation in Berry Crops

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Abstract

Cultivated strawberry (*Fragaria* × *ananassa*) and *Rubus* berries, raspberry and blackberry, are small fruits highly appreciated by consumers and worldwide cultivated. The polyploidy nature of these species, their high heterozygosity, and the lack of natural resistance to several pests are the main constraints to the development of improved genotypes by conventional breeding. In recent years, genetic transformation has demonstrated to be a powerful tool to overcome these limitations. In this chapter, the current state of genetic transformation technology in *Fragaria* and *Rubus* spp. is reviewed. The feasibility of strawberry to regenerate in vitro has allowed the development of efficient transformation protocols for both cultivated and the wild strawberry *Fragaria vesca*. Important traits such as photoperiod requirements for flowering, fungal tolerance, biotic stress tolerance, and fruit shelf life have been manipulated in transgenic strawberry plants through the introduction of different genes. Furthermore, tools for the development of intragenic plants, containing chimeric genes from the own species and devoid of marker genes, have been generated. By contrast, the recalcitrance of *Rubus* tissues to regenerate in vitro has impeded the development of robust transformation protocols in these species, although a few number of studies have successfully obtained transgenic plants carrying genes of interest. Main achievements, limitations, and future prospects of genetic transformation in both genera are discussed.

11.1 Introduction

Rosaceous berries are characterized by their delicious sensorial attributes and attractive appearance. In addition, these fruits are rich in antioxidants and other bioactive compounds with health promoting properties, which make them important components of our diet to prevent various diseases (Hannum 2004; Szajdek and Borowska 2008). Among them, the octoploid strawberry (*Fragaria* × *ananassa*, Duch.) is the most valuable and worldwide cultivated berry. Strawberry has also a high interest among researchers, particularly in the Rosaceae community. In fact, the wild strawberry *F. vesca* has

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been adopted as a model for genomic investigations within Rosaceae due to its small genome, sequenced in 2011, small herbaceous structure, short generation time, and robust transformation systems. Additionally, strawberry is being used as a model for physiological and molecular studies on non-climacteric fruit growth and ripening. Conversely, *Rubus* spp. berries have lower economic importance than strawberry, but their world production and cultivation area have been continuously increasing since 1980. This genus is one of the most complex taxonomic group, due to its polyploidy genome and high tendency for interspecific hybridization. It includes 12 subgenera, but most species are divided into two categories: raspberry and blackberry subgenera, *ideaus* and *ursinus*, respectively, with many hybrids between both of them (Gough 2008).

Strawberry breeding programs have been implemented in many countries to address main problems of this crop, e.g., yield, pest, and disease resistance and fruit quality in terms of size, flavor, and shelf life (Graham 2005; Quesada et al. 2007). Although these programs have been quite successful, strawberry breeding is hampered by the octoploid nature of this species, its high heterozygosity, and its narrow germplasm base. In fact, most genes in modern cultivars derive from a few nuclear and cytoplasmic sources (Sjulin and Dale 1987; Dale and Sjulin 1990). Genetic transformation could provide a means to solve some of the limitations found with conventional breeding. Even more, as strawberry is propagated vegetatively, genes can be integrated directly into the genome of elite cultivars without the need of further breeding. The amenability of strawberry tissues to regenerate in vitro has allowed that protocols for the genetic transformation of strawberry have been established for many commercial cultivars. Even more, traits of great interest to the strawberry industry, such as photoperiod requirements for flowering, fungal tolerance, or postharvest shelf life, have successfully been improved through genetic transformation. Despite these achievements, no transgenic strawberry genotype has been commercialized yet, probably due to the poor consumer's acceptance of transgenic fruits.

Pest resistance, enhanced fruit quality, adaptation to mechanical harvesting, and low chilling requirements for tropical and subtropical regions are the main objectives of *Rubus* breeding programs (Skirvin et al. 2005). In these species, breeding is hindered by polyploidy, apomixis, pollen incompatibility, and poor seed germination (Skirvin et al. 2005). Contrary to strawberry, the lack of reliable systems for in vitro shoot regeneration has impeded progress in the development of the genetic transformation technology in *Rubus*.

In this chapter, major advances and limitations of the genetic transformation technology in *Fragaria* and *Rubus* are reviewed. Constraints to the commercialization of transgenic berries and future research lines are also discussed.

11.2 Strawberry (*Fragaria* spp.)

11.2.1 In Vitro Tissue Culture

In vitro plant regeneration is an essential prerequisite to implement a successful transformation protocol. Each species has its own specific requirements, and for some genotypes, this is the major bottleneck for their genetic improvement via genetic transformation. In general, strawberry can be easily manipulated in vitro and efficient protocols for micropropagation and plantlet regeneration have been established for many cultivars of both cultivated and wild strawberries.

Adventitious shoot regeneration has commonly been used in transformation experiments (Mercado et al. 2007a; Quesada et al. 2007; Husaini et al. 2011), although strawberry can also be efficiently regenerated through somatic embryogenesis (Wang et al. 1984; Donnoli et al. 2001; Biswas et al. 2007; Husaini and Abdin 2007; Husaini et al. 2008; Kordestani and Karami 2008). An extensive range of explants, generally obtained from stocks of micropropagated material derived from the aseptic culture of runner meristems, can be used for shoot

organogenesis, e.g., leaf disks, petioles, stipules, stem tissue, runners, mesophyll protoplasts, cotyledons, roots, anthers, ovaries, immature embryos, and sepals (Mercado et al. 2007a; Husaini et al. 2011). Among these explants, leaf tissue has the greatest regeneration capacity, yielding regeneration rates high enough to undertake a transformation program with a guarantee of success. However, some commercial cultivars are highly recalcitrant and show a restricted ability to regenerate shoots in vitro, independently of the explant used (Passey et al. 2003). Moreover, Passey et al. (2003) found indications of a genetic linkage for in vitro recalcitrant behavior when comparing the regeneration rates of several strawberry cultivars, suggesting that a strong genetic component determines the success of adventitious regeneration.

Besides the explant type, other factors, such as hormonal balance, basal medium, and incubation conditions, can affect the regeneration rate. Most protocols use Murashige and Skoog (1962) (MS) mineral solution although other formulations with lower ionic strength, e.g., N30K, are better for regeneration and micropropagation of some cultivars (López-Aranda et al. 1994; Barceló et al. 1998). Strawberry tissues require a medium supplemented with cytokinin and auxin for successful regeneration. A combination of the cytokinin 6-benzylaminopurine (BA) with one of the following auxins at lower concentration, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), or 2,4-dichlorophenoxyacetic acid (2,4-D), is commonly used (Nehra et al. 1989; Sorvari et al. 1993; Barceló et al. 1998; Finstad and Martin 1995). The combination of BA and IBA is the most used, but it is prone to induce callus proliferation from leaf disks (Husaini and Srivastava 2006). On the other hand, the auxin 2,4-D should be avoided at elevated concentrations since it has been reported that produces high rates of somaclonal variations (Nehra et al. 1992).

Thidiazuron (TDZ) has been shown to be very effective in the regeneration of woody species (Huetteman and Preece 1993) due to its cytokinin activity and as modulator of endogenous auxin levels. In strawberry, TDZ alone or in combination with IBA has been successfully employed in the regeneration of some genotypes (Passey et al. 2003; Hanhineva et al. 2005; Qin et al. 2005a; Landi and Mezzetti 2006; Zakaria et al. 2014); however, according to Debnath (2005, 2006), this growth regulator exerts an inhibitory effect in the elongation of shoots.

The physical factor that has been more studied in the regeneration of strawberry tissues is the light/dark incubation. Dark incubation reduces the exudation of phenolic compounds from explants that can be highly toxic to the plant tissue (George 1993). Along this line, Landi and Mezzetti (2006) reported a stimulatory effect of continuous darkness on shoot regeneration of several *Fragaria* genotypes. In the cv. 'Chandler,' a two-week pretreatment in the dark followed by transfer to a 16-h photoperiod of 40 µmol m^{-2} s^{-1} was the optimum light regime (Barceló et al. 1998). Light quality also influences strawberry regeneration. Qin et al. (2005a) observed that red and green plastic films enhanced the regeneration of 'Toyonaka' leaf disks when compared with control fluorescent lamps, while blue and yellow films reduced organogenesis. The enhanced effect of red and green plastics seemed to be related to an increase in the activity of antioxidant enzymes and in the endogenous content of cytokinins and gibberellins. Tian et al. (2003) also reported a key role of antioxidant activities and hydrogen peroxide in the morphogenetic response of strawberry callus, and treatments that increase antioxidant activities, such as the addition of silver nitrate to the regeneration medium, enhanced shoot regeneration (Qin et al. 2005b). The effect of light quality in strawberry regeneration deserves further investigations since spectral characteristics of LED lights, whose use is increasingly being used in in vitro growth chambers nowadays, can differ significantly from fluorescent lights.

In most cases, a solid medium, gelled with agar or phytagel, is employed for strawberry regeneration and micropropagation. As an alternative method, Hanhineva et al. (2005) developed a temporary immersion bioreactor system in which the leaf explants are alternatively exposed

to liquid medium and air. The main advantages of this system were the reduction of the material handling and the labor costs. This system has been adapted for the regeneration of transgenic strawberry plants, cv. 'Jonsok' (Hanhineva and Kärenlampi 2007).

11.2.2 Genetic Transformation

Genetic transformation of *F.* × *ananassa* and *F. vesca* was first reported in the 1990s, using in all cases *Agrobacterium tumefaciens* as the gene delivery system (James et al. 1990; Nehra et al. 1990; El Mansouri et al. 1996). Since then, a number of protocols for transformation of many strawberry cultivars, including breeding lines and most popular genotypes, have been reported. As strawberry explants are easily infected and transformed by *A. tumefaciens*, this method has been the most employed, although some studies using direct gene transfer systems have also been reported. Independently of the system employed, the transformation efficiencies are generally low, between 1 and 10%, and highly genotype dependent (Husaini et al. 2011).

11.2.2.1 *Agrobacterium*-Mediated Transformation

In the *Agrobacterium* transformation system, explants precultured in the appropriate medium are infected with a diluted *A. tumefaciens* culture, co-cultivated for 2–3 days with the bacteria, and finally transferred to the regeneration medium supplemented with a selection agent and an antibiotic to eliminate bacteria. Different steps of the transformation process are shown in Fig. 11.1. Source and type of explant, *A. tumefaciens* strain, preinduction of explants prior to bacterial inoculation, and the selection procedure are key factors determining the success of the transformation system. The transformation

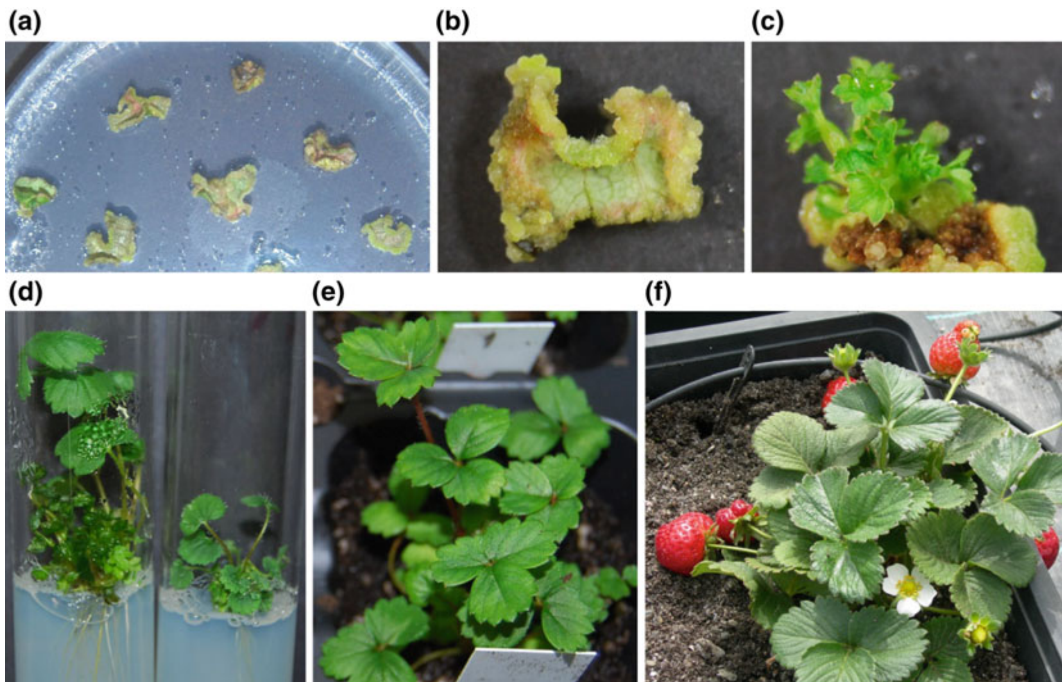

Fig. 11.1 Different stages of the *Agrobacterium*-mediated transformation process in *F.* × *ananassa*, cv. Chandler. **a** Aspect of the leaf disks two weeks after *Agrobacterium* inoculation, cultured in a selection medium supplemented with kanamycin. **b** A detailed view of a leaf disk producing callus in the presence of kanamycin. **c** Explant regenerating shoots after 4 weeks of selection. **d** Micropropagation of transgenic shoots. **e** Acclimatization of transgenic plants. **f** Transgenic plant growing in the greenhouse

efficiencies reported in strawberry using *A. tumefaciens* varied between 0.1% in the cv. 'Rapella' (James et al. 1990) to 100% in *F. vesca* 'Alpine' (Oosumi et al. 2006). This variability cannot be attributed exclusively to the genotype or to the regeneration ability of the cultivar but to the different transformation protocols employed. Main factors affecting transformation rate via *Agrobacterium* infection are discussed below.

Explant Type Most transformation experiments in cultivated strawberry and *F. vesca* have been performed using leaf disks as explants (James et al. 1990; Nehra et al. 1990; El Mansouri et al. 1996; du Plessis et al. 1997; Martinelli et al. 1997; Barceló et al. 1998; Puite and Schaart 1998; Wawrzynczak et al. 2000; Alsheikh et al. 2002; Ricardo et al. 2003; Gruchala et al. 2004; Pantazis et al. 2013; Zakaria et al. 2014; Haddadi et al. 2015). Other tissues have also been employed for strawberry transformation, e.g., petioles (James et al. 1990; Alsheikh et al. 2002), stipules (Monticelli et al. 2002; Chalavi et al. 2003), stem sections from the base of in vitro proliferating plantlets (Graham et al. 1995; Mathews et al. 1995a, Mathews et al. 1998). Few studies have analyzed the effect of explant type on transformation efficiency. James et al. (1990) found that petioles from cv. 'Rapella' were more efficiently transformed than leaf disks despite their lower regeneration capacity. In *F. vesca* and *F. v. semperflorens*, by contrast, leaf disks yielded higher transformation rates than petioles (Alsheikh et al. 2002). In cvs. 'Tristam' and 'Totem,' the use of stem tissue from the base of in vitro plants increased significantly the number of transgenic plants recovered when compared with leaf explants (Mathews et al. 1998). The source of explant tissue has also been demonstrated to be important. Leaf disks of juvenile origin, derived from in vitro grown seedlings, yielded better results than adult material, probably due to their higher regeneration potential (Barceló et al. 1998).

Preinduction The presence of a large number of cells competent for both regeneration and transgene integration is one of the most important factors in plant transformation systems (Birch 1997). The incubation of mechanically wounded explants in the regeneration medium for a short period prior to *Agrobacterium* inoculation is the most effective trigger for shifting cells into the competent state. The beneficial effect of preculturing strawberry explants for a period of 7–10 days has been demonstrated (Barceló et al. 1998; Husaini 2010). However, a 4-day preculture reduced transformation rates in 'Camarosa' (Haddadi et al. 2015).

***Agrobacterium* virulence** Several *A. tumefaciens* strains, from the less virulent LBA4404 to the supervirulent strains AGL0 and derivatives EHA100 and EHA105, have been successfully employed in strawberry transformation (Husaini et al. 2011), but unfortunately, comparative studies of the effect of strain virulence on transformation efficiency are scarce. The addition of the phenolic compound acetosyringone to the bacterial culture medium, at 50–150 µM, is a common practice to increase *Agrobacterium* virulence, improving strawberry transformation rate (Alsheikh et al. 2002; Gruchala et al. 2004; Zakaria et al. 2014; Haddadi et al. 2015).

Selection of transformed explants Selectable marker genes that confer resistance to antibiotics or herbicides are the most common in plant transformation, including *Fragaria* spp. When using these systems, the choice of an adequate selection strategy is critical for the recovery of transgenic plants; a selection level excessively high can be deleterious even for transgenic cells, but a selection pressure too low can lead to the recovery of escapes (false transgenics) or chimeras (plants containing transgenic and non-transgenic sections). Although the antibiotic hygromycin and the herbicide phosphinothricin have been employed in some studies (Mathews et al. 1995a; Oosumi et al. 2006; Folta et al. 2006), most binary vectors used in strawberry transformation contain the *neomycin phosphotransferase II* (*nptII*) gene for kanamycin selection of *Agrobacterium* inoculated explants (Quesada et al. 2007; Husaini et al. 2011). Generally, strawberry tissues are highly sensitive

to kanamycin, and shoot organogenesis is impaired at kanamycin levels higher than 50 mg/l (El Mansouri et al. 1996; Barceló et al. 1998; Gruchala et al. 2004; Qin et al. 2011; Haddadi et al. 2015; Reis and Ayub 2015). However, some genotypes may be highly resistant to this antibiotic and higher selection levels and/or different selection strategies are needed to recover true transgenics. Thus, Schaart et al. (2004) used 150 mg/l kanamycin for the selection of transgenic tissue, cv. 'Calypso.' In the cv. 'Totem,' Mathews et al. (1998) found that a high percentage of plants recovered in a medium supplemented with 25 mg/l kanamycin were chimeras. To avoid this problem and recover pure transgenic material, Mathews et al. (1998) developed an iterative regeneration protocol in which explants from primary transgenics were subjected to three regeneration cycles, increasing the kanamycin level from 40 mg/l in the first round to 80 mg/l in the third cycle. Besides genotype, the frequency of chimerism seems to depend also on the type of explant, being stipules more susceptible to regenerate chimeric tissues (Chalavi et al. 2003). The lack of rooting ability or improper rooting in the presence of selection is the most evident sign of chimerism in putative transgenic shoots (Mathews et al. 1998; Qin et al. 2011).

Several studies have found that the delay of the selection phase after *Agrobacterium* infection for a short period of time improved the transformation efficiency. This preselection phase varied from 2 to 10 days (Nehra et al. 1990; Barceló et al. 1998; Husaini 2010; Haddadi et al. 2015). However, a long delay in the application of the selection pressure, e.g., 3 weeks, decreased transformation efficiency (Mathews et al. 1995a).

The use of visual selection markers instead of phytotoxic products has received little attention in strawberry. Oosumi et al. (2006) combined the use of green fluorescent protein (GFP) with hygromycin selection to increase screening efficiency in several genotypes of *F. vesca*. More recently, Kortstee et al. (2011) found that anthocyanin production induced by the ectopic expression of a mutant allele of the transcription factor *MYB10* from apple could be used as selectable marker in the absence of antibiotic selection. The expression of this gene under the control of its natural promoter induced the accumulation of anthocyanin in shoots regenerated from leaf disks of cv. 'Calypso.' However, not all regenerated shoots containing the transgene showed anthocyanin accumulation and very high levels of anthocyanin accumulation induced the death of the shoot (Kortstee et al. 2011).

Besides the selective agent, the selection medium must be supplemented with a bactericidal antibiotic to eliminate *Agrobacterium*. The choice of this antibiotic from the narrow range of chemicals generally used for this purpose is not trivial, since most of them, besides their phytotoxic effect, have growth regulator-like activity and can affect the regeneration capacity of the explants (Padilla and Burgos 2010). James et al. (1990) reported that the use of cefotaxime at a concentration of 100 mg/l significantly increased the percentage of petiole and leaf disk explants regenerating shoots. By contrast, Hanhineva and Kärenlampi (2007) and Qin et al. (2011) found that this antibiotic had a negative effect on shoot regeneration in cvs. 'Jonsok' and 'Toyonaka,' respectively. Timentin, a mixture of ticarcillin and clavulanic acid, showed lower phytotoxic effects than carbenicillin or cefotaxime, even at high concentrations, in the transformation of 'Camarosa' leaf disks (Haddadi et al. 2015).

11.2.2.2 Direct Gene Transformation Systems

Direct gene transformation systems are especially suitable for species recalcitrant to *Agrobacterium* infection; as discussed above, this is not the case of strawberry. Thus, few studies have been devoted to the development of these transformation methods in *Fragaria*. Nyman and Wallin (1992a) reported the transformation of leaves- and petioles–protoplasts by electroporation, obtaining a transformation frequency of 1–5 10^{-4} selected calli per plated protoplast. Although this transformation strategy can be successful, the use of protoplasts should be avoided due to the high frequency of

chromosomal alterations and somaclonal variations observed in the regenerated plants (Nyman and Wallin 1992b). Wang et al. (2004) transformed strawberry calli obtained from anther cultures by particle bombardment and regenerated several transgenic lines expressing a late embryogenesis abundant protein gene for salt tolerance.

Using a different approach, Cordero de Mesa et al. (2000) developed a novel transformation method for leaf disks of cv. 'Chandler' combining *Agrobacterium* infection and biolistic. In this system, gold microprojectiles were coated with *Agrobacterium* cells, instead of the naked plasmid, and used to bombard leaf explants. The transformation frequency increased from 7% using the standard *Agrobacterium* protocol to 20% with the combined method. The higher efficiency of this transformation system could be more related to the fact that the bombardment allows *Agrobacterium* to reach a higher number of regenerating cells in the explant rather than to an enhanced bacterial infection rate due to the microwounding of the explant.

11.2.2.3 High-Throughput Transformation Systems for Functional Genomics

Although a large number of both cultivated and wild strawberry genotypes have been transformed, in general, the efficiency is low and the procedures take a long time to generate a stable transgenic plant ready to be analyzed in the greenhouse. These constraints hamper functional genomics studies in *Fragaria*. As an example, the generation of a T-DNA mutant population in *F. vesca* large enough to disrupt all the genes in the genome would comprise at least 255,000 independent transformation events (Oosumi et al. 2006). Several studies developed high-throughput *Agrobacterium* transformation systems aimed to be used as valuable tools for gene function studies in *Fragaria*. Oosumi et al. (2006) described a transformation protocol for several accessions of *F. vesca* that yielded 100% transformation efficiency and required 14–15 weeks to be completed. In this system, inoculated leaf explants, developing callus in the presence of hygromycin, were dissected into secondary and tertiary explants. GFP expression in calli was used to identify transgenic leaf segments with apparent independent cellular origin. Several populations of pCAMBIA T-DNA mutants (Oosumi et al. 2010) as well as *Ac/Ds* transposon mutants (Veilleux et al. 2012; Pantazis et al. 2013) of *F. vesca* accession PI 551572 have been obtained using this procedure. The transposon tagging system could potentially create an unlimited number of transposon insertion mutants from a relatively small number of initial transformants. Zhang et al. (2014) used a similar transformation approach to introduce RNAi constructs in *F. vesca* 'Hawaii 4,' using GFP and kanamycin selection instead of hygromycin. Unexpectedly, plant regeneration in this genotype occurred through somatic embryogenesis rather than adventitious shoot organogenesis, and approximately a 17% of transgenic plants, independently of the gene construct, showed leaf morphology alterations related to tetraploidy.

In cultivated strawberry, Folta et al. (2006) obtained the genetic line LF9, derived from a seed of self-pollinated 'Strawberry Festival,' that exhibited rapid organogenesis in vitro, completing the transformation cycle, from explant to plant in soil, in about 60 days. This genotype has been used for the functional analysis of *PpMlo1* gene from *Prunus persica*, involved in powdery mildew resistance (Jiwan et al. 2013).

11.2.2.4 In Planta Transient Transformation

Transient transformation techniques are increasingly being used in gene functional studies as a mean to overcome main drawbacks of the traditional stable transformation systems. Transient transformation does not require regeneration of transformed cells, and the expression of the target genes can be analyzed shortly after DNA delivery, accelerating research time. Spolaore et al. (2001) reported for the first time the transient expression of GUS gene in strawberry fruit, by infiltration of an *Agrobacterium* suspension. Since then, many studies have used agroinfiltration for the functional characterization of genes, mainly related to the fruit ripening process

(Schawb et al. 2011; Guidarelli and Baraldi 2015). Fruit developmental stage at the time of bacterial injection seems to be a key factor in the success of the transient transformation (Hoffmann et al. 2006). Appropriate controls should be employed when using this technique since *Agrobacterium* injection induces wounding and pathogen responses that could mislead interpretation of data; e.g., Yeh et al. (2014) found that lignin content, fruit firmness and expression of the peroxidase gene *POD27* were increased after fruit agroinfiltration. Virus-induced gene silencing using vectors based on the tobacco rattle virus has also been employed in *F.* × *ananassa* plants (Tian et al. 2015) and fruits (Li et al. 2013).

11.2.3 Use of Genetic Transformation to Improve Selected Traits

11.2.3.1 Fungal Resistance

Fungi are among the most important strawberry pathogens in terms of economic losses. More than 50 different genera can affect strawberry cultivars, although the major diseases are caused by *Phytophthora fragariae* (red stele disease), *Verticillium* spp. (*Verticillium* wilt), *B. cinerea* (gray mold), and *Colletotrichum* spp. (anthracnose) (Maas 1998; Garrido et al. 2011). As a nearly global practice, the control of fungal and other strawberry pathogens is based on the soil sterilization with fungicides prior to planting, most of these chemicals exerting negative effects to the environment. For some fungal diseases, e.g., *Verticillium* wilt or anthracnose, completely resistant genotypes have not yet been found within the strawberry germplasm (Shaw et al. 1996; Casado-Díaz et al. 2006). Thus, the improvement of strawberry tolerance to fungal pathogens by means of genetic transformation is one of the most prominent research areas to make this crop more environmental friendly and sustainable.

Asao et al. (1997) obtained transgenic plants, cv. 'Toyonaka,' overexpressing a chitinase gene from rice. These plants showed less disease symptoms when infected with *Sphaeroteca humulis* without affecting fruit yield in the field (Asao et al. 2003). A chitinase from *Solanum chilense* (formerly *Lycopersicon chilense*) was also used by Chalavi et al. (2003) to enhance resistance to *Verticillium dahliae*. Vellicce et al. (2006) transformed cv. 'Pajaro' with a chitinase from *Phaseolus vulgaris*, a glucanase or a thaumatin-like protein, both from *Nicotiana tabacum*, or combination of these genes. Transgenic plants overexpressing the chitinase showed enhanced tolerance to *B. cinerea* when detached leaves were inoculated with the pathogen. The other genes assayed did not show a successful resistant response. By contrast, the expression of a *thaumatin II* gene from *Thaumatococcus daniellii* induced a high level of resistance to *B. cinerea* in cv. 'Firework' (Schestibratov and Dolgov 2005).

In general, antipathogenic genes from fungal or bacterial origin have proven to be more powerful than their plant counterparts in conferring pathogen resistance (Dana et al. 2006; Shakhbazau and Kartel 2008). Along this line, chitinases from *Trichoderma harzianum* such as CHIT42 or CHIT43 have successfully been used to increase fungal tolerance in many species (Shakhbazau and Kartel 2008). In strawberry, cv. 'Camarosa,' the ectopic expression of CHIT42, did not induce a clear tolerance to *Colletotrichum acutatum* (Mercado et al. 2007b). By contrast, the transformation with a *Trichoderma* β-1,3-glucanase gene increased tolerance to crown root diseases caused by *C. acutatum* and *Rosellinia necatrix* (Mercado et al. 2015). Plant fitness was affected in these transgenic plants, showing a reduced vigor and fruit set. This negative effect could be due to the interference of the high chitinase activity with cell growth and pollination or to the constitutive activation of pathogen response pathways.

RolC from *Agrobacterium rhizogenes* has been used to improve plant growth and tolerance to pathogens in several species. Its role in plant development is not known, but it seems to be related to an increase in auxin sensitivity. Landi et al. (2009) assessed the effect of this gene in strawberry, cv. 'Calypso.' Transgenic plants showed higher yield than control and an enhanced tolerance to *Phytophthora cactorum*.

As an alternative to the ectopic expression of antifungal proteins, a few studies have assessed the effect of modifying the expression of genes involved in the pathogen response pathway or in the synthesis of natural products to improve tolerance. The antisense silencing of *Mlo* gene in strawberry using the homologue from *Prunus* conferred resistance to powdery mildew (Jiwan et al. 2013). It is known that this gene is a negative regulator of the plant defense response. Silva et al. (2015) overexpressed the *AtNPR1* gene, a key positive regulator of the systemic acquired resistance response, in *F. vesca*. Transgenic plants showed enhanced resistance against a broad range of fungal (*C. gloeosporioides*, *C. acutatum*, and *Podosphaera aphanis*) and bacterial (*Xanthomonas fragariae*) pathogens. As observed with the expression of the *Trichoderma* glucanase by Mercado et al. (2015), transgenic plants were shorter and most of them did not produce runners and fruits, apparently due to the constitutive activation of the pathogen response. Both studies emphasize the need of alternative strategies to avoid these side effects, such as the use of promoters weaker than *CaMV35S* or chemical- or pathogen-inducible promoters that could activate transgene expression when the pathogen interacts with the plant. Finally, Hanhineva et al. (2009) failed to get resistance to *B. cinerea* when transforming with a stilbene synthase gene from grape. This enzyme produces the phytoalexin resveratrol, but this metabolite was not detected in the leaves of transgenic plants.

11.2.3.2 Insect and Virus Resistance

Few studies have been devoted to introduce insect or virus resistance genes in strawberry. James et al. (1992) and Graham et al. (1995) obtained transgenic plants expressing a cowpea proteinase inhibitor gene. The product of this gene interacts with proteinases, inhibiting proteolytic activity and interfering with the development of many Lepidopteran and Coleopteran larvae (Watt et al. 1999). James et al. (1992) did not found any positive effect of the transgene expression in the resistance to vine weevil larvae (*Otiorhynchus sulcatus* F.). By contrast, Graham et al. (1997a, 2002) reported that transgenic plants were less damaged than controls when subjected to the same pathogen under glasshouse and field conditions. A lectin gene from *Galanthus nivalis*, a different anti-insect protein, has also been introduced in strawberry although transgenic plants failed to show resistance to weevil larvae (Graham 2005). Regarding virus resistance, Finstad and Martin (1995) transformed strawberry plants with the coat protein gene from strawberry mild yellow edge potexvirus. The viral protein was stably expressed in the plants, but the assessment of virus tolerance was not reported.

11.2.3.3 Herbicide Tolerance

Strawberry plants tolerant to the most common herbicides glufosinate and glyphosate have been obtained. In the first case, du Plessis et al. (1997) introduced the *phosphinothricin acetyl transferase* gene into cv. 'Selekta.' Field evaluation of the transgenic plants revealed that four out of the 22 lines resistant to glufosinate ammonium resembled the characteristics of control, non-transformed, plants. Resistance to glyphosate, the active compound of the herbicide Roundup, has been obtained in 'Camarosa' through the introduction of the *EPSP synthase* gene from CP4 strain of *A. tumefaciens* (Morgan et al. 2002). After the application of commercial levels of Roundup in the nursery, transgenic lines showed a range of tolerance to the herbicide, from complete tolerance to death.

11.2.3.4 Abiotic Stress Tolerance

Strawberry is very sensitive to soil salinity, reducing fruit yield and fruit quality (Dziadczyk et al. 2003; Saied et al. 2005). To increase resistance to salt and drought stress, Wang et al. (2004) introduced a late embryogenesis abundant protein gene from barley, LEA3, in cv. 'Toyonaka.' Although its function is largely unknown, it has been suggested that LEA-type proteins participate in ions sequestration, macromolecules, and membranes stabilization and as water-binding molecules. Transgenic lines cultured in vitro in the presence of 50 or 100 mM NaCl for 7 days showed lower levels of wilting

than control plants (Wang et al. 2004). More recently, Husaini and Abdin (2008) used an osmotin gene from *N. tabacum* to induce salt resistance in cv. 'Chandler.' This protein belongs to the pathogenesis-related protein family PR-5, and its expression is induced by both abiotic stress and microbial infection. Plants of some osmotin expressing lines grew better in vitro than their controls under salinity (Husaini and Abdin 2008) and drought (Husaini et al. 2012) stresses, but their growth rate was slightly lower under non-stressing conditions.

Despite strawberry being tolerant to cold temperatures, freezing can be detrimental to strawberry production, especially if this stress coincides with flowering and fruit set. Several approaches have been followed to achieve freezing tolerance in this species. Owens et al. (2002) obtained two transgenic lines overexpressing the cold-induced transcription factor CBF1 from *Arabidopsis*. CBF transcription factors mediate expression from a regulon involved in cold acclimation (Gilmour et al. 1998). Leaf disks of transgenic plants showed an improvement on the freezing tolerance, but no freeze tolerance was observed in the fruit receptacle. A different transcription factor that regulates stress-related genes, *RdreB1BI*, has been introduced into strawberry under the control of the stress inducible promoter *rd29A* (Wang et al. 2014). The proteomic analysis of these transgenic lines suggested that cold tolerance induced by *RdreB1BI* was related to an increased photosynthesis and to the accumulation of defense-related proteins (Gu et al. 2013). Houde et al. (2004) introduced the dehydrin gene, *wcor410*, from wheat. Dehydrins are a different class of LEA proteins, hypothetically involved in the stabilization of plasma membrane during dehydration associated with freezing stress. Transgenic lines showed a 5 °C reduction in the threshold temperature of freezing damage in leaves when plants were previously cold acclimated. However, this improvement in freezing tolerance was not observed either in roots or in non-acclimated plants, suggesting that WCOR410 protein needs to be activated by another factor to confer full freeze protection.

11.2.3.5 Fruit Yield and Quality

One of the aims of most strawberry breeding programs is to extend the cropping season by breeding for early, late, or everbearing cultivars, adapted to specific climatic conditions. However, the physiology of flowering in this species is complex and photoperiod and temperature interact in the control of floral induction. Recently, it has been demonstrated that the silencing of the floral repressor *FvTFL1* eliminates the short-day photoperiod requirements for flowering in *F. vesca* (Koskela et al. 2012) and *F.* × *ananassa* (Koskela et al. 2016), converting short-day genotypes in everbearing genotypes. The modulation of the expression of this gene is a promising approach to manipulate flowering and runnering in strawberry.

Strawberry fruit is perceived by consumers as one of the most inconsistent commodities in the marketplace, and therefore, increasing fruit quality parameters are main objectives of biotechnological breeding programs. One of the main problems of this fruit is the reduced postharvest shelf life due to its rapid softening, usually accompanied with pathogen infection. Traditionally, strawberry has been considered as a non-climacteric fruit, although some evidences suggest that ethylene could participate in its ripening (Iannetta et al. 2006). Then, the reduction of ethylene biosynthesis could increase strawberry postharvest shelf life as observed in other climacteric fruits. To this purpose, Mathews et al. (1995a) transformed strawberry with the *S-adenosylmethionine hydrolase* from T3 bacteriophage, a gene that reduces the level of ethylene precursors. Unfortunately, the effect of ethylene reduction on fruit ripening was not reported.

An alternative strategy to reduce softening consists in the silencing of genes encoding enzymes involved in cell wall degradation, since it is generally accepted that fruit softening is mainly associated with cell wall disassembly (Brummell and Harpster 2001; Mercado et al. 2011). This approach has been really fruitful, and transgenic plants cv. 'Chandler' producing fruits with a reduced softening rate and extended postharvest shelf life have been obtained when

downregulating genes encoding a pectate lyase (Jiménez-Bermúdez et al. 2002; Youssef et al. 2009), a polygalacturonase (Quesada et al. 2009), or a β-Galactosidase (Paniagua et al. 2016). The highest increment in fruit firmness at the ripe stage was detected with the silencing of the polygalacturonase gene, and, apparently, the transgenic manipulation did not modify other fruit quality parameters (Posé et al. 2015), although pectate lyase and, especially, β-galactosidase silenced fruits were slightly smaller than non-transformed fruits. The silencing of cell wall modifying genes could also be useful for the fruit processing industry. Strawberry jam prepared with pectate lyase silenced fruits contained higher amount of berry pieces, one of the main parameters of jam quality, and transgenic juices were more viscous (Sesmero et al. 2007, 2009). Cellulase genes have also been assessed as a mean to increase fruit firmness in strawberry with negative (Woolley et al. 2001; Palomer et al. 2006; Mercado et al. 2010) and positive (Lee and Kim 2011) results.

The deciphering of the molecular pathways responsible for color and aroma of strawberry fruits is also an active research area. A few studies have demonstrated that both traits can be modified by transgenesis. Lunkenbein et al. (2006a) downregulated a chalcone synthase (CHS) gene to analyze its role in pigment accumulation in ripe fruit. A 95% reduction in the CHS expression was required to affect pigmentation. However, the silencing of this gene resulted in an unpredictable biochemical phenotype, with modifications in the level of metabolites downstream and upstream the CHS branching point. Anthocyanin content in fruit has also been modified by the silencing of anthocyanidin reductase (Fischer et al. 2014). One of the most important components of strawberry aroma is 4-hydroxy-2,5-dimethyl-3(2H)-furanone. This compound is methylated during the ripening process to 2,5-dimethyl-4-methoxy-3(2H)-furanone (DMMF) by a *O*-methyltransferase enzyme. Lunkenbein et al. (2006b) downregulated this gene obtaining a near-total depletion of DMMF in several lines, without affecting the levels of other volatile compounds.

For the biotechnological manipulation of fruit carbohydrate content, Park et al. (2006) downregulated an ADP-glucose pyrophosphorylase small subunit gene, an enzyme that catalyzes a key step in starch biosynthesis. The amount of soluble sugar increased, while starch decreased in these transgenic fruits. Basson et al. (2011) expressed a fructose 6-phosphate 1-phosphotransferase (PFP) gene from *Giardia lamblia* in cv. 'Selekta' to modulate carbon partitioning in fruit. Despite the fact that PFP activity was significantly higher in some transgenic lines, total sugar contents were not affected.

11.3 *Rubus* spp.

11.3.1 In Vitro Tissue Culture

Rubus spp. plant regeneration is highly genotype dependent; although regeneration protocols have been developed for some selections, there is not an efficient method applicable to a wide range of genotypes. Generally, adventitious organogenesis is more efficient in blackberry genotypes (Graham et al. 1997b). Vujović et al. (2014) found a high organogenesis capacity with all in vitro treatments assayed using the blackberry genotype 'Čačanska Bestrna,' whereas the raspberry cv. 'Meeker' showed very low regeneration capacity. Furthermore, Mezzetti et al. (1997) observed a genotype effect in type of explant and the response to hormonal treatments between blackberry and raspberry cultivars, but also between two blackberry cultivars. It has been postulated that raspberry genotypes showing leaf chlorosis in vitro are recalcitrant to adventitious regeneration from leaves (Zawadzka and Orlikowska 2006).

Leaves and petioles from in vitro grown plants are the explants more commonly used in *Rubus* spp. adventitious regeneration (Swartz et al. 1990; Turk et al. 1994; Tsao and Reed 2002; Zawadzka and Orlikowska 2006; Lazic and Ruzic 2007; Gupta and Mahalaxmi 2009; Vujović et al. 2014). Meng et al. (2004) found higher regeneration efficiencies and higher number of regenerated shoots using leaves in

comparison with petioles. Similarly, Mezzetti et al. (1997) found a better organogenesis response using leaves in 'Hull Thornless' blackberry and 'Autumn Bliss' raspberry; however, the blackberry cv. 'Chester' showed higher regeneration rates using petioles. Similar results were obtained by Mathews et al. (1995b) in three red raspberry and Gajdošová et al. (2015) in two blackberry cultivars, although the latter used petioles attached to the base of leaves. Heh Ran et al. (2008) compared the use of leaves, petioles, and leaves with attached petioles in Korean black raspberry (*R. coreanus* Miq.), the last type of explant showing the highest regeneration percentage. On the other hand, Cousineau and Donnelly (1991) did not find any significant difference between all explants tested in 'Comet' red raspberry. As an alternative explant, Fiola et al. (1990) used detached cotyledons to obtain adventitious organogenesis in blackberry and hybrids with raspberry.

MS formulation is generally used for induction of adventitious shoot regeneration, although some genotypes have proven to regenerate better using N_6 (Chu et al. 1975) than MS mineral solution (Turk et al. 1994; Zawadzka and Orlikowska 2006). Regarding hormonal balance, BA alone or in combination with IBA has successfully been used in *Rubus* spp. shoot induction (McNicol and Graham 1990; Swartz et al. 1990; Owensy de Novoa and Conner 1992; Turk et al. 1994; Graham et al. 1997b; Mezzetti et al. 1997; Tsao and Reed 2002), although many authors found the use of TDZ, alone or in combination with auxins, more effective (Fiola et al. 1990; Cousineau and Donnelly 1991; Turk et al. 1994; De Faria et al. 1997; Graham et al. 1997b; Zawadzka and Orlikowska 2006; Lazic and Ruzic 2007; Heh Ran et al. 2008; Debnath 2010). Meng et al. (2004) and Gajdošová et al. (2015) used TDZ during the first weeks to induce adventitious regeneration, but later, this cytokinin was replaced by BA to avoid excessive shoot proliferation. Vujović et al. (2014) observed that regeneration of blackberry 'Čačanska Bestrna' in the presence of TDZ decreased when it was used in combination with auxin. Graham et al. (1997b) also found an inhibitory effect of 2,4-D on shoot regeneration in the presence of TDZ. Forchlorfenuron (CPPU), a synthetic compound with cytokinin-like activity, was employed by Millan-Mendoza and Graham (1999) to improve organogenesis in recalcitrant red raspberry cultivars. However, the cytokinin zeatin did not prove to be effective, showing low or not regeneration efficiency (Graham et al. 1997b; Mezetti et al. 1997).

Rubus spp. adventitious regeneration is approached in light conditions. The effect of a dark period in this process has been studied by Fiola et al. (1990) and Cousineau and Donnelly (1991). Both studies failed to find any enhancement effect of a dark pretreatment in the regeneration efficiency. However, Meng et al. (2004) used a pretreatment of 3 weeks in darkness before transferring the explants to the regeneration medium under light conditions.

11.3.2 Genetic Transformation

Only a few number of reports attempted *Rubus* spp. genetic transformation, all of them through *A. tumefaciens* inoculation. Genotype and the antibiotic selection are the most critical factors determining the success of the process.

Explant Type and *Agrobacterium* Virulence
Leaves have been used as explants in transformation studies performed by Hassan et al. (1993) and De Faria et al. (1997). Kokko and Kärenlampi (1998) found that internodal stems segments were better explants for transformation than leaves and petioles, obtaining a transformation rate in the range 15–27% with this explant versus 1% when using leaves. Similar results were obtained by Millan-Mendoza and Graham (1999), who did not regenerate any shoot using leaves but obtained a regeneration percentage of 32% using internodal stems. Other authors found more suitable the use of petioles (Mathews et al. 1995b; Mezzetti et al. 2004) or flag petioles (Súkeníková et al. 2015) as explants for *Agrobacterium* inoculation.

The *A. tumefaciens* strain most widely used in *Rubus* spp. transformation is LBA4404 (De Faria et al. 1997; Millan-Mendoza and Graham 1999;

Mezzetti et al. 2004; Súkeníková et al. 2015). More virulent strains have also been used, e.g., EHA105 (Mathews et al. 1995b), EHA101 (Kokko and Kärenlampi 1998), and C58 (Hassan et al. 1993). Súkeníková et al. (2015) studied the efficiency of different *A. tumefaciens* strains, LBA4404, AGL0, and C58, on blackberry transformation, selecting LBA4404 as the most appropriate for this material.

The inoculation time of the explants with the *A. tumefaciens* culture varied between 10 and 60 min in all published works, but there is no study regarding its effect on transformation efficiency. The same occurs for co-cultivation time, which is generally established between 2 and 4 days in darkness.

Selection of Transformed Explants The antibiotic kanamycin as selection agent has been employed by many authors (Fiola et al. 1990; Hassan et al. 1993; De Faria et al. 1997). In general, a severe growth limitation takes place at 10 mg/l, and 40 mg/l completely inhibits explant growth. However, the use of kanamycin above 40 mg/l (De Faria et al. 1997; Kokko and Kärenlampi 1998) yielded very low transformation efficiencies (0.25–8.1%), independently of the explant or *Agrobacterium* strain used. Millan-Mendoza and Graham (1999) used kanamycin at lower concentrations, 10 mg/l; however, although they obtained an adequate regeneration efficiency of stem explants after inoculation with *Agrobacterium*, few shoots survived the whole selection process and rooted in medium with kanamycin, obtaining a final transformation efficiency of 10%. In nectaberry (*R. arcticus* L.) transformation, Kokko and Kärenlampi (1998) used kanamycin at 50 mg/l, obtaining an efficiency between 1.5 and 7.6%, which proved a lower sensitivity of this genus to this antibiotic. Mathews et al. (1995b) attempted the genetic transformation of three raspberry cultivars, using hygromycin at 10 mg/l as selective agent. The transformation rate varied greatly among genotypes, from 0.9% in 'Chilliwalk' to 49.6% in 'Meeker.' Súkeníková et al. (2015), using two different binary vectors, compared the effect of both kanamycin and hygromycin at 10 mg/l as selective agents in blackberry transformation. They found a high tissue sensitivity to both antibiotics, but specially to kanamycin; i.e., no transgenic shoot was recovered in the presence of this antibiotic. Additionally, these authors came up to the conclusion that the transgenic shoots recovered were chimeric, due to the necessity of using nest PCR to confirm their transgenic nature. Mathews et al. (1995b) also observed that shoots recovered in the selection medium were chimeric, but after repetitive regeneration events in selection medium using explants from these primary shoots, transgenic plants of homogeneous nature were obtained. By contrast, Millan-Mendoza and Graham (1999) proved that every raspberry transgenic plant rooting in the presence of kanamycin was PCR-positive and non-transgenic escapes were not found.

11.3.3 Use of Genetic Transformation to Improve Selected Traits

To date, only three studies have used genetic transformation to improve *Rubus* spp. Mathews et al. (1995b) introduced the *S-adenosylmethionine hydrolase* gene under the control of the wound and fruit-specific promoter *E4* from tomato, in several raspberry cultivars to control fruit ripening. Millan-Mendoza and Graham (1999) used the pROK binary vector harboring the coat protein gene of Raspberry Bushy Dwarf Virus (RBDV) for virus resistance. In both cases, the analysis of transgenic plants was not reported. The expression of RBDV coat and movement proteins in transgenic raspberry plant for virus tolerance has been patented (Beachy et al. 1999; Martín et al. 2003). In an attempt to increase fruit yield, Mezzetti et al. (2004) transformed raspberry and strawberry with the *iaaM* gene from *Pseudomonas syringae*, an auxin-producer gene, under the control of the *DefH9* promoter from snapdragon, which

regulates expression in placenta/ovules. A higher number of inflorescences and flowers was observed in the transgenic lines from both species, resulting in greater yields.

11.4 Future Approaches for the Commercialization of Transgenic Berries

Many different traits have successfully been modified in strawberry through genetic transformation, demonstrating the usefulness of this technology for improving sustainable production and fruit quality. A search in the USA and EU databases of field tests of genetically modified (GM) crops yielded 59 notifications for transgenic strawberries since 1994, 50 in the USA (Information System for Biotechnology 2016) and 9 in the EU (Joint Research Centre 2016). Fungal resistance, product quality, and herbicide tolerance are the phenotype categories most studied (Fig. 11.2), reflecting the main concerns of the strawberry industry. However, no transformed strawberry cultivar has been commercialized yet. Regarding *Rubus* spp., the poor development of transgenesis in these species makes it difficult that GM raspberry or blackberry could reach the commercialization phase in the near future.

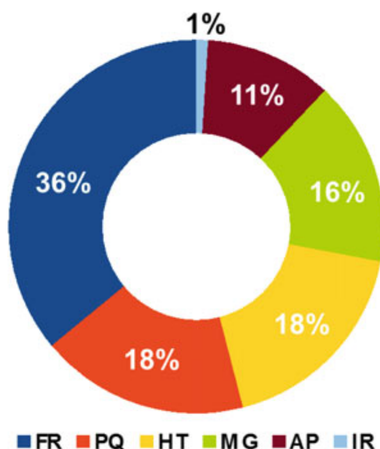

Fig. 11.2 Field trials of transgenic strawberry plants (*Fragaria* × *ananassa* Duch.) performed in the USA and Europe during the period 1994–2016. FR: fungal resistance, HT: herbicide tolerance, PQ: product quality, MG: marker gene, AP: agronomical properties, IR: insect resistance

Legislations of GM plants require very expensive risk assessment tests to compliance with environmental and human health safety, e.g., assessment of the rates of gene flow between GM crops and wild relatives, the potential for transferred material to cause an adverse effect in the new genetic background, or an assessment of substantial equivalence (determining whether the GM crop is as safe as its traditional counterpart in terms of agronomical and compositional traits, safety and nutritional equivalence) (Cockburn 2002). Information about environmental risk assessment in transgenic strawberry is scarce. Asao et al. (2003) reported that the culture of transgenic strawberries with enhanced tolerance to fungal pathogens exerted the same effect on other plants and microflora than a non-transgenic plant. Similarly, Landi et al. (2009) and Husaini et al. (2012) did not find any negative impact of transgenic expression of a *RolC* and an osmotin gene, respectively, in the symbiotic association with root mycorrhizas. As regard to gene flow, Quesada et al. (2007) found that the transfer rate of the kanamycin resistant gene from transgenic strawberries to non-transformed plants under greenhouse conditions was extremely low, in spite of growing control plants under a high transgenic plant density. Although limited, these few reports suggest that the environmental risks of field release of transgenic strawberries would be quite low.

Besides the strict GM regulation, the negative public attitude toward genetic modification is one of the most important obstacles to the commercialization of transgenic fruits. A survey about the attitude of consumers toward genetically modified strawberries performed in Norway, Denmark, and the UK showed that it was, in general, rather negative; however, in front of different hypothetical modifications, consumer acceptance increased when introduced traits were beneficial for consumers, e.g., lower requirements for pesticides, healthier fruits (Schaart et al. 2011). Furthermore, most respondents

found more acceptable the use of genes from the same species.

The genetic modification using DNA sequences from the same species or cross-compatible species is called cisgenesis, the newly introduced DNA is a natural genome fragment including its untranslated regions and regulatory elements (Schouten et al. 2006), or intragenesis, the newly introduced DNA is a chimeric gene created by combining coding sequences and regulatory elements from different genes of the same species (Rommens et al. 2007). Both technologies require that transgenic plants obtained have to be devoid of unnecessary gene sequences such as selectable marker genes or vector backbone sequences. In strawberry, Schaart et al. (2004) developed a recombinase-based system for the production of marker-free transgenic plants. They constructed a binary vector containing an inducible recombinase gene and a bifunctional selectable marker, created by the combination of *nptII* gene for kanamycin selection and *codA* gene that confers sensitivity to 5-fluorocytosine, flanked by recombinase sites. Chemical induction of recombinase activity through the application of dexamethasone induced recombinase activity, leading to the excision of the undesired fragment. This vector was used to generate intragenic strawberry plants expressing the polygalacturonase inhibiting protein, *FaPGIP*, for *B. cinerea* resistance (Schaart et al. 2011). The selectable marker gene was successfully removed although intragenic plants failed to show fungal resistance. Using a similar approach, Untergasser et al. (2012) developed the plasmid pHUGE-Red which allowed the transfer of large DNA sequences, up to 8 genes, and the recovery of marker-free plants in several species, including strawberry. The integration of vector backbone sequences could also be a problem when trying to generate marker-free plants since several researches reported a high frequency of integration of this undesired DNA into strawberry (Morgan et al. 2002; Abdal-Aziz et al. 2006).

Genome editing tools, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guide DNA nucleases, allow the modification of a gene of interest without involving foreign DNA. As a result, plants generated with these systems may be outside the scope of current GMO regulations and therefore may be considered as non-transgenic genetically altered plants (Nagamangala Kanchiswamy et al. 2015; Araki and Ishii 2015). These editing tools are currently being applied to an array of fruit crop species, although some studies are mostly proof of concept. Recently, Zhou et al. (2018) reported for the first time the genome-editing of wild strawberry by CRISPR/Cas9. Even if few studies have been reported for *Fragaria* or *Rubus*, genome edition is certainly the most promising approach for the biotechnological improvement of these species.

Independently of the transformation or genome editing method of election, there is a common need for a well-curated biological database for both molecular information and phenotypical information. *Fragaria* and *Rubus* are already present in the Rosaceae Genome Database (GDR; http://www.rosaceae.org; Jung et al. 2014) with abundant resources for genome and genotype. However, the phenotype data available is way behind the molecular, even though some efforts are made to include the different transgenic lines already available for strawberry in a custom biological database (http://www.gefpdb.net; Pineda et al. 2013). These databases, and the associated toolkits, would facilitate the modification of new traits of interest by providing the starting point for gene selection, genome site identification, vectors design, and germplasm management.

11.5 Conclusions

Efficient *A. tumefaciens* transformation protocols have been established for many strawberry commercial cultivars, and they have been applied to improve important traits like flowering requirements, fruit quality, and fungal resistance. Genetic transformation of *Rubus* cultivars has also been accomplished, but the low regeneration

ability of this species and, consequently, low transformation efficiency have prevented an extensive use of genetic transformation in functional genomics or biotechnological breeding in this crop. Despite some successful achievements in both berries, the process is still far from being a routine technique and several aspects should be investigated. The search for novel transformation strategies, less genotype dependent, would be desirable to overcome the low transformation efficiency and reproducibility, especially in *Rubus*. Additionally, genetic transformation technology should be expanded to wild species other than *F. vesca*. Tissue-specific and inducible regulatory sequences from the own species to drive transgenes are necessary not only to generate cisgenic or intragenic plants, but also to achieve a better control of transgene expression, avoiding undesired pleiotropic effects due to constitutive transgene expression. In line with this, few studies have been devoted to analyze the occurrence of somaclonal variations in transgenic strawberry plants, despite this process being a source of unintended effects. Studies on the stability of transgenes in the field and risks assessments are also important areas for future research. Finally, the development of successful genome editing protocols in these species would be a major advance that could accelerate the commercialization of genetically engineered berries in the near future.

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12 Genome-Assisted Breeding in the Octoploid Strawberry

Sujeet Verma, Luis F. Osorio, Seonghee Lee, Nahla V. Bassil and Vance M. Whitaker

Abstract

The application of genomic information to the breeding of allo-octoploid ($2n = 8x = 56$) cultivated strawberry (*Fragaria* × *ananassa*) has increased rapidly in the last five years. These advances have been fueled by technological improvements in high-throughput genotyping and genome sequencing, as well as concerted efforts to develop DNA tests for routine use in breeding. Genome-wide and subgenome-specific markers have advanced availability of DNA tests for major loci, as well as the development and validation of genomic selection methodology for complex traits in strawberry. Eight DNA tests for fruit quality and disease resistance loci are fully or partially in the public sphere. Genome-wide predictions have delivered genetic gain efficiencies for parent selection larger than 50% of conventional methods but without the need for phenotypic information. Meanwhile, the construction of haploblocks and haplotypes allows increased understanding of genome structure as it relates to breeding applications. With octoploid sequence assemblies merely months away and the development of gene editing technologies, precision manipulation of genes may shape the future of strawberry genetic improvement.

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12.1 Introduction

In past decades, genome-assisted breeding in the allo-octoploid ($2n = 8x = 56$) cultivated strawberry (*Fragaria* × *ananassa*) has lagged behind other fruit crops. Yet in the last five years, marker-assisted breeding (MAB) in strawberry has increased rapidly due to technological advances in strawberry genomics and collaborative efforts by the international strawberry research community. Critical contributions have been made by the RosBREED project, funded by the US National Institute of Food and Agriculture (NIFA) via the Specialty Crop Research Initiative (SCRI), with the goal of bridging the chasm between genomics discoveries and breeding application in Rosaceae crop species. This project has spurred the development and validation of whole-genome genotyping platforms and multiple DNA markers linked to traits of interest, which will be described in detail later in this chapter. A study by RosBREED in 2010

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found that 57% of strawberry breeders surveyed had not yet attempted the use of markers or other genomics-based tools. By 2014, the proportion of strawberry breeders giving the same response had dropped to 29% (Michael T. Coe, personal communication).

The practical impact of genomic information and tools on strawberry breeding is shaped by the strawberry breeding process. Strawberry, like many other clonally propagated fruit crops, has a typical breeding cycle (Fig. 12.1) in which many full-sib crosses are made each year, usually avoiding crosses among full-sibs and often avoiding crosses among half-sibs to reduce inbreeding depression. Several thousand seedlings are evaluated visually during a single season (in the case of annualized strawberry production such as in California, Florida, southern Spain) or in multiple seasons in some growing systems in temperate climates. Subsequently, selected seedlings are clonally propagated via runners to conduct replicated trials of a smaller number, perhaps a few hundred, of advanced selections. Objective phenotypic measurements of individuals are often first taken at the clonal testing stage. In annualized production systems, if parents can be reliably chosen during the first year of clonal testing, the breeding cycle can be completed in as little as three years from cross to cross (Fig. 12.1).

When MAB is applied at the stage of parent pool selection or for the selection of specific cross combinations, we refer to this as marker-assisted parent selection (MAPS). When applied to select seedlings within or across families, we refer to this as marker-assisted seedling selection (MASS). While genomic information can assist strawberry breeding in other ways, such as to determine if clones are true-to-type or to assess genetic diversity, in this chapter we focus primarily on selection activities that are designed to directly increase genetic gains. These gains can come about in a number of ways, for instance by reducing the time required for the breeding cycle, by increasing selection intensity, or by selecting more accurately than it is possible via phenotyping alone.

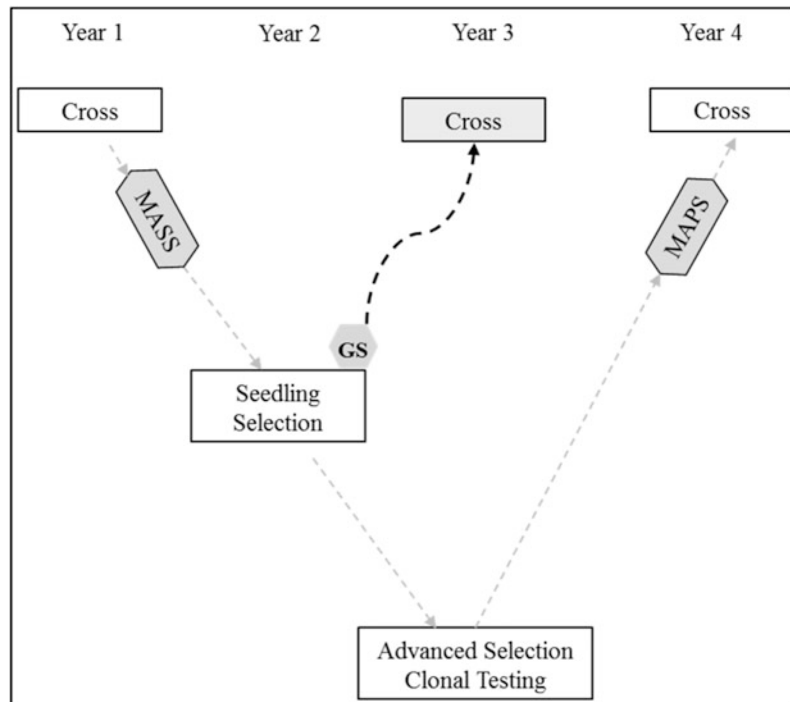


Fig. 12.1 An example of a strawberry breeding cycle incorporating marker-assisted seedling selection (MASS) and marker-assisted parent selection (MAPS) for major loci and genomic selection (GS) for parent selection one year earlier than conventional pedigree-BLUP (PBLUP) selection

In the following sections, advances in genetics and genomics that have breeding relevance are reviewed, from linkage mapping and quantitative trait locus (QTL) mapping to the development high-throughput marker systems and the validation of specific DNA tests linked to traits of economic interest. We conclude with a discussion of genomic selection (GS) in strawberry, a predictive method using genome-wide markers to predict performance for complex, quantitative traits.

12.2 Discovering and Exploiting Major Loci

12.2.1 Linkage Mapping and Genome Structure

The first linkage map in *Fragaria* was constructed for *F. vesca*, using random amplification of polymorphic DNA (RAPD) markers, generating the expected number of seven linkage groups (LG) with a total length of 445 cM (Davis and Yu 1997). The first simple sequence repeat (SSR) linkage map was developed in *F. vesca* × *F. nubicola* with 68 markers and a total map distance of 448 cM (Sargent et al. 2004). Since then, diploid maps were further enhanced by adding gene-specific and other marker types (Sargent et al. 2016).

The first genetic map for *F.* × *ananassa* was constructed by Lerceteau-Köhler et al. (2003). A total of 30 LGs for the female and 28 LGs for the male were obtained with an average length of 1550 cM. In 2009, Sargent et al. developed a genetic map using a cross between *F.* × *ananassa* cultivars, 'Redgauntlet' and 'Hapil' and compared it to the diploid *Fragaria* reference map in order to develop molecular tools to facilitate genetic studies and marker-assisted breeding in octoploid strawberry. The map was composed of 315 markers: 218 SSRs, 11 gene-specific, and 86 amplified fragment length polymorphisms (AFLPs) and RAPDs spanning 3116 cM, with additional SSR markers added later (Sargent et al. 2011). Further improvements were made by van Dijk et al. (2014) who developed an allele dose-based SSR map which distinguished subgenomes on the basis of level of divergence from *F. vesca*. The technique used is termed Microsatellite Allele Dose Configuration and Establishment (MADCE), deriving genotype configurations using the proportion of amplified alleles over the total number of allowed alleles. Homoeologue letter A was assigned to the subgenome with the highest amplification efficiency (fewest null alleles), and homoeologue letter D was assigned to the subgenome with the lowest efficiency (many null alleles) (van Dijk et al. 2014). In addition, the authors identified signatures of breeding such as homozygous regions and regions containing inversions.

With the accessibility of the 'woodland strawberry,' *F. vesca* ($2n = 2x = 14$), whole-genome sequence (Shulaev et al. 2010), the worldwide strawberry research community collaborated on the development of high-throughput genome scanning capability. Thus, Bassil et al. (2015) under the umbrella of the RosBREED project and in collaboration with Affymetrix, Inc., developed the IStraw90 Axiom® single nucleotide polymorphism (SNP) array. Using several ploidy reduction strategies, subgenome-specific SNP markers were placed on this 90K array. Although this array is commercially available to the worldwide community, cost per sample (80–105 USD) is limiting for some applications. In order to reduce the price of scanning, the Axiom® IStraw35 array (Verma et al. 2016b), a 384-pin array, was developed in 2016 as a lower cost version of the IStraw90. In order to determine the most useful IStraw90 SNP probes for the new array platform, the international community provided lists of polymorphic and/or mapped SNPs to generate a final design of 38,506 SNP probes contributed from the University of Florida (UF); Wageningen University and Research (WUR) Center, Netherlands; East Malling Research (EMR), UK; University of New Hampshire (UNH); Instituto Andaluz de Investigación y Formación Agraria y Pesquera (IFAPA), Spain; National Institute of Agriculture Research (INRA), Bordeaux, France; and Institut de Recerca i Tecnologia Agroalimentaries (IRTA) Centre de Recerca en

Agrigenomica (CRAG), Spain. Per sample cost for the new array in the USA is currently 50 USD. Documentation of SNP probes on the IStraw35 and the corresponding probes from the IStraw90 are available online (http://gcrec.ifas.ufl.edu/faculty/dr-vance-m-whitaker/research/).

Availability of high-throughput genotyping techniques such as target capture hybridization, genotyping by sequencing (GBS), and the IStraw90 Axiom® SNP array have contributed to considerable progress in understanding the allo-octoploid genome of the cultivated strawberry. Tennessen et al. (2014) used a novel approach called Phylogenetics Of Linkage-Map-Anchored Polyploid Subgenomes (POLiMAPS) and target capture hybridization genotyping of *F. vesca* subsp *bracteata*, *F. chiloensis,* and *F. virginiana* populations in order to gain a better understanding of the evolutionary origins and dynamics of the octoploid strawberry subgenomes. They suggested that one of the four subgenomes originated with the diploid cytoplasm donor *F. vesca*, one with the diploid *F. iinumae*, and two with an unknown ancestor close to *F. iinumae*. They also generated an improved assembly of the reference diploid genome, *Fragaria vesca* ssp. *bracteata* (Fvb). Using the 'haploSNP' category of ploidy-reduced markers from the IStraw90 SNP array, Sargent et al. (2016) placed 14 of the 28 homoeologues into discrete subgenomes based on affinity toward *F. vesca* (A) and *F. iinumae* (B) and proposed a subgenomic formula of the form AA, bb, X–X, X–X where X–X represents homoeology and bivalent pairing without invoking subgenome integrity. Recently, Mahoney et al. (2016) utilized the IStraw90 array and GBS to develop a high-density linkage map of the ancestral diploid strawberry, *F. iinumae*, which should also aid in understanding the genome of octoploid strawberry and should help facilitate the development of the next generation of genotyping tools.

The array also facilitated an explosion of SNP-based linkage mapping, leading to breeding applications. At the University of Florida (UF) strawberry breeding program, three genetic maps using three different mapping populations were developed. The initial genetic map utilized the 'Holiday' × 'Korona' (H × K) population of 75 individuals (Bassil et al. 2015; Eric van de Weg, personal communication). Essentially, the H × K SNP map was modified to maximize polymorphism in UF germplasm, resulting in 3814 high-quality SNPs and following the denotation of subgenomes and orientation of linkage groups according to van Dijk et al. (2014). This was the map utilized for quantitative trait locus (QTL) analyses for disease resistance and other traits (Roach et al. 2016a; Mangandi et al. 2017). Subsequently, UF generated two additional maps using two different mapping populations: (1) FL_08-10 × FL_12.115-10 (N = 165) and (2) 'Treasure' × 'Winter Dawn' (N = 91) (Verma, unpublished). A total of 14,332 SNPs and 13,502 SNPs were included in the FL_08-10 × 12.115-10 and 'Treasure' × 'Winter Dawn' genetic maps, respectively. The FL_08-10 × 12.115-10 genetic map is being used extensively for analyses of disease resistance QTLs (Anciro et al. 2016).

12.2.2 QTL Discovery and Validation

Mapping of QTL determines the association of marker alleles with genetic loci conferring observed phenotypic variation. An essential requirement for a reliable QTL mapping project is a high-resolution genetic map. In this regard, it is hard to understate the importance that the SNP array development has been for genetic analyses in the octoploid cultivated strawberry. A second technological necessity for large-scale QTL analysis is software that can efficiently merge large amounts of phenotypic and genotypic data for both biparental and multiparental populations. To this end, FlexQTL™ software has been a powerful strawberry genetics tool in recent years. This software is based on Bayesian statistics and simulates Markov chain Monte Carlo (MCMC) iterations for QTL mapping in pedigree-linked populations (Bink et al. 2014). In this section, we review QTL studies that have identified loci that have been exploited for genetic improvement, or that have sufficiently

large phenotypic effects to be considered for use in genetic improvement of the octoploid cultivated strawberry.

In the last two decades, numerous QTLs for strawberry fruit quality traits and a few for reproductive traits were detected across diverse breeding material worldwide. Lerceteau-Köhler et al. (2012) were the first to map QTLs in octoploid strawberry, detecting several QTLs involved in fruit quality traits (Fig. 12.2). Most of the QTLs they detected explained between 10% and 17% of the phenotypic variation for the corresponding trait. Castro and Lewers (2016) also conducted QTL analysis for fruit quality traits, validating some previous results of Lerceteau-Köhler et al. (2012). The soluble solids content (SSC) QTLs detected by Lerceteau-Köhler et al. (2012) were located on LG IIIa, Va, and VIa, with the EMFv006 SSR marker closely associated with the SSC QTL on LG VIa. Castro and Lewers (2016) also detected three QTLs for SSC that were located on LG VI-S-3 homoeolog group VI (HG-VI), LG II-D-5, and LG-V-D-1. Using the van Dijk et al. (2014) genetic map for comparison, the SSC QTL on LG VIA may be common between these two studies. In addition, Castro and Lewers (2016) also detected a QTL for SSC/TA ratio and titratable acidity (TA) on LG VI-D-4 (HG-VI) close to the BFACT010 SSR marker. BFACT010 is located upstream of EMFv006 on the H × K genetic map (Eric van de Weg, personal communication) and appears to be associated with phenotypic variation for SSC and TA.

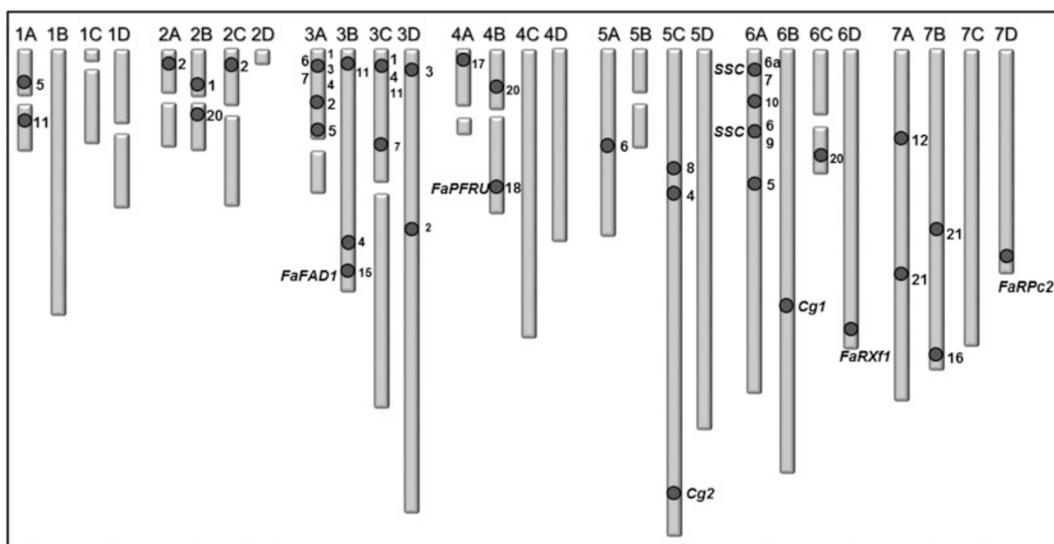


Fig. 12.2 Overview of QTLs detected in the octoploid cultivated strawberry *Fragaria* × *ananassa*. The map presented above represents the FL_08-10 × 12.115-10 genetic map developed at the University of Florida (Verma unpublished). The QTL positions represent approximate relative positions of QTLs from multiple studies. *Fruit diameter(FD)*-1, *fruit length(FL)*-2, *FD/FL*-3, *firmness*-4, *anthocyanin*-5 (Lerceteau-Köhler et al. 2012); *soluble solids content*-6 (Lerceteau-Köhler et al. 2012; Castro and Lewers 2016); *UF_SSC*-6a, *glucose*-7 (Lerceteau-Köhler et al. 2012); *fructose*-8, *sucrose*-9 (Lerceteau-Köhler et al. 2012); *antioxidant capacity*-10 (Castro and Lewers 2016); *pH*-11 (Lerceteau-Köhler et al. 2012); *phenolics*-12 (Castro and Lewers, 2016), resistance to Phytophthora crown rot (PhCR) due to *Phytophthora cactorum* (*FaRPc2*)-13 (Verma et al. 2016a; Mangandi et al. 2017); resistance to angular leaf spot resistance due to *Xanthomonas fragariae* (*FaRXf1*)-14 (Roach et al. 2016a, b); *FaFAD1*-15 (Chambers et al. 2014; Sánchez-Sevilla et al. 2014); *mesifurane* (*FaOMT*)-16, *LOX* (Lipoxygenase)-17 (Zorrilla-Fontanesi et al. 2012); remontancy (*FaPFRU*)-18 (Gaston et al. 2013; Castro et al. 2015; Verma et al. 2016a; Perrotte et al. 2016); resistance to Colletotrichum crown rot due to *Colletotrichum gloeosporioides* (*Cg1* and *Cg2*)-19 (Mangandi 2016; Anciro et al. 2016); *fruit weight*-20 (Verma et al. 2016a); *titratable acidity*-21 (Verma, unpublished)

At the University of Florida, a high-density H×K SNP genetic map, specific to UF material, is being utilized to conduct QTL analysis for fruit quality and disease resistance traits (Verma et al. 2016a). A QTL associated with SSC (UF_SSC) was detected near BFACT010, possibly upstream of the LG VIa SSC QTL detected by Lerceteau-Köhler et al. (2012) (Verma unpublished). BFACT010 and EMFv006 were placed at 12.6 and 42.5 cM, respectively on LG 6A (van Dijk et al. 2014). Interestingly, a QTL for glucose was detected close to the UF_SSC QTL and a QTL for sucrose was detected close to the SSC QTL near EMFv006 by the Lerceteau-Köhler et al. (2012) study. These two QTLs detected in different germplasm sets explained similar proportion of the phenotypic variation at about 10%. This suggests that two different QTLs for SSC on LG 6A may be variable/expressed in different genetic backgrounds, with one influencing glucose and the other influencing sucrose.

When using the H × K SNP genetic map in the pedigree-linked RosBREED strawberry germplasm set, we also detected an SSC QTL on LG 6A close to EMFv006 (Verma et al. unpublished), at a similar location to that found by Lerceteau-Köhler et al. (2012) (Fig. 12.2). The RosBREED strawberry germplasm set was phenotyped in Oregon and Michigan in 2011 and 2012 and is comprised of 26 families, mainly representing the Oregon and Michigan breeding programs such as: 'Honeoye,' 'Tribute,' 'Fort Laramie,' 'Seascape,' 'Puget Reliance,' 'Totem,' 'Earliglow,' and a few selections.

A locus controlling remontancy (also known as day-neutrality or perpetual flowering) is another outstanding example of a trait explored by several researchers. Gaston et al. (2013) detected a major QTL on LG IVb-f controlling perpetual flowering (*FaPFRU*) in *F.* × *ananassa* in a cross between 'Capitola' and CF1116. The presence of this major QTL supported results from other studies that used the *F. virginiana* ssp. *glauca* originating from the Wasatch Mountains in Utah, USA (Powers 1954), that is heavily used in the UC Davis breeding program (Hancock 1999). Detection of a single major QTL or multiple QTLs seems to depend upon how many genes were introgressed from other Wasatch ancestral sources (Powers 1954; Hancock et al. 2002). Gaston et al. (2013) determined that a single dominant allele in one subgenome controls the transition from short-day to remontancy in this source. Dominant mutations are very common in polyploid species in order to reduce functional redundancy (Doyle et al. 2008; Feldman et al. 2012) with Albani et al. (2004) finding remontancy to be dominant as well. Castro et al. (2015) also detected a major QTL in a cross between 'Honeoye' and 'Tribute' on LG IV-T-1. They analyzed data collected from five different locations, and a major QTL was mapped on the same LG IV-T-1 for all five locations. The marker ChFaM148-184T was highly associated with this QTL, which explained 32.4% of the total phenotypic variation. The RosBREED strawberry germplasm dataset currently is being analyzed for remontancy, and initial results suggest a major QTL at the bottom of LG 4A (Verma et al. unpublished). As of now, we believe the *FaPFRU* locus and the QTL detected on LG IV-T-1 for day-neutrality and runnering is likely the same as was detected in the RosBREED strawberry germplasm set.

QTLs have been detected for other fruit quality and reproductive traits and include: fruit diameter, fruit length, firmness, anthocyanin, phenolic compounds, gamma-decalactone and mesifurane (strawberry aroma compounds), fruit weight and titratable acidity (Zorrilla-Fontanesi et al. 2012; Lerceteau-Köhler et al. 2012; Sánchez-Sevilla et al. 2014) (Fig. 12.2).

A number of major and minor QTLs for disease resistance have also been described in octoploid strawberry. Disease resistance traits have been a significant focus for strawberry, due to the importance of disease management in strawberry production and the fact that disease resistance loci often have large effects. The first report of a marker-trait association for disease resistance in strawberry was from Haymes et al. (1997), which identified 7 RAPD markers linked to the *Rpf1* gene for resistance to *Phytophthora fragariae*, the causal agent of red stele root rot. Later, two sequence-characterized amplified

region (SCAR) markers closely associated with the *Rpf1* gene were found to be linked with resistance to *P. fragariae* (Haymes et al. 2000). The *Rca2* gene is a major locus, at which a dominant allele confers resistance to *Colletotrichum acutatum* pathogenicity group 2 (Lerceteau-Köhler et al. 2005). Also, Antanaviciute et al. (2015) identified several QTLs associated with resistance to *Verticillium dahlia* in the cultivated strawberry.

At the University of Florida, an effort was initiated in 2014 to design trials to identify QTLs for resistance to crown and root rot diseases caused by *Colletotrichum gloeosporioides* and *Phytophthora cactorum*, and to angular leaf spot caused by *Xanthomonas fragariae*. For the crown and root rot studies, clonal replicates of more than 1100 seedlings from 139 full-sib families arising from more than 60 parents were evaluated during two consecutive seasons in control inoculated field experiments. Individuals were genotyped with the IStraw90 Axiom® SNP array, and FlexQTL™ software was used to conduct pedigree-based QTL analysis. Two QTLs, *Cg1* and *Cg2,* for resistance to *C. gloeosporioides* were detected and validated in two consecutive years (Mangandi 2016; Anciro et al. 2016) (Fig. 12.2). While *Cg1* explained ~26% of phenotypic variation for mortality due to crown rot caused by this pathogen, *Cg2* was minor and explained ~5% of the total phenotypic variation (Fig. 12.2). A major QTL for resistance to *P. cactorum* or phytophthora crown rot, *FaRPc2*, was detected and validated in two consecutive years (Verma et al. 2016a; Mangandi et al. 2017). This locus explained up to 35% of the phenotypic variation in mortality due to infection with *P. cactorum*. In a separate study, a large-effect QTL was detected for resistance to bacterial angular leaf spot caused by *X. fragariae* (*FaRXf1*) which segregated 1:1 indicating control by a single dominant allele (Roach et al. 2016a, b).

12.2.2.1 Haploblocking and Haplotyping

Advances in marker technology are now facilitating deeper investigations of the genetic architecture behind traits of interest. Defining haploblocks and haplotypes and their associated phenotypic effects are important goals of the latest iteration of the RosBREED project. Haploblocks are chromosomal regions which have lower probabilities of recombination in the middle and higher probabilities at the borders in a given germplasm set and are considered as regions of limited haplotype diversity. SNP marker alleles in close proximity constituting a haploblock are highly correlated, resulting in reduced number of SNP haplotypes within a block (Patil et al. 2001). Haplotypes are defined as unique combinations of marker alleles observed in a population and transmitted to the next generation in blocks due to linkage disequilibrium (Gabriel et al. 2002; Voorrips et al. 2016). The SNP haplotypes within a given haploblock are called 'haplotype group' (Fig. 12.3).

Haploblocking and haplotyping have significant value in genetic improvement of strawberry, as they can be used to help describe allelic diversity in a region controlling a trait of interest. Here we present *FaRPc2,* a recently discovered QTL associated with resistance to Phytophthora crown rot at the bottom of LG 7D (Mangandi et al. 2017), as an example (Fig. 12.3). Haploblocking was performed for LG 7D using the four-gamete method implemented in the Haploview software (Barrett et al. 2005), and phasing of marker alleles was performed with FlexQTL™ (Bink et al. 2014). The analysis revealed that the locus spans the last two haploblocks on LG 7D. A recombination hotspot (rhs1) was identified between the last two haploblocks. In order to capture haplotypes within complex pedigrees, haplotyping was performed using a set of 19 SNP markers that fell under the QTL confidence interval region defined by FlexQTL™. These 19 SNP markers resulted in five major haplotypes (H1–H5) in the UF strawberry germplasm. Analysis of variance indicated that haplotypes H2 and H3 were associated with resistance, and the other three were associated with susceptibility against Phytophthora crown rot. These five haplotypes represented more than 95% of the haplotype diversity within this breeding material. In this example, haploblocking and haplotyping were very

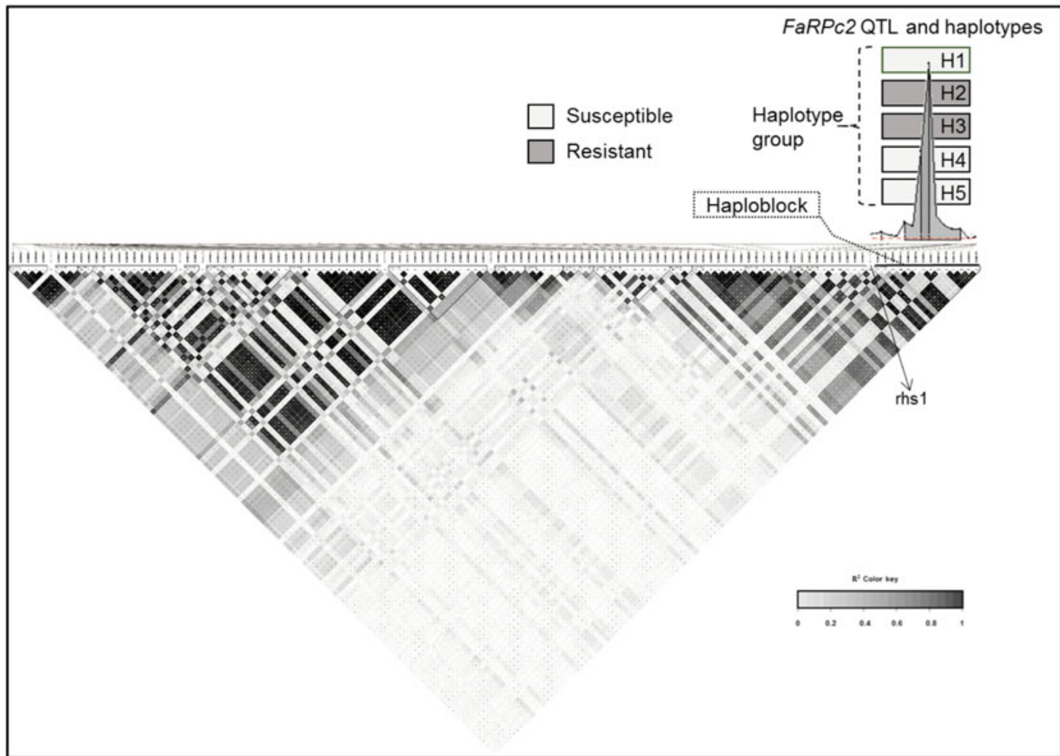


Fig. 12.3 Pairwise linkage disequilibrium (LD) (r^2) and haplotype block estimation for LG 7D of cultivated strawberry using Haploview software. The arrow for rhs1 represents a recombination hot spot between the last two haploblocks on LG 7D

valuable approaches for describing allelic diversity and guiding approaches for selection at this locus.

12.2.3 Candidate Gene Approaches

In addition to QTL mapping approaches to identify important loci, candidate gene approaches have also been used in the cultivated strawberry. One of the primary examples is *FaFAD1* which controls variation for a flavor volatile called γ-decalactone. This compound has been described as 'fruity,' 'sweet,' and 'peachy' in strawberry, but only half of the surveyed cultivars can produce this compound in their ripe fruit. Chambers et al. (2014) and Sánchez-Sevilla et al. (2014) applied both candidate gene-based and map-based approaches to identify and characterize the region associated with the production of γ-decalactone. Sánchez-Sevilla et al. (2014) detected a locus controlling γ-decalactone production at the bottom of LG III-2 on the integrated '232' × '1392' linkage map (Fig. 12.2). On the other hand, Chambers et al. (2014) used an F1 progeny from a cross between 'Elyana' (a γ-D producer) and 'Mara de Bois' (a γ-D non-producer) and identified that transcript accumulation of the candidate *FaFAD1* is absent in non-producers. Chambers et al. (2014) then confirmed clear segregation of presence and absence of *FaFAD1* with producers and non-producers.

Furanone 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF, furaneol) imparts a caramel-like, sweet aroma to strawberry and is frequently accompanied by its methyl ether 2,5-dimethyl-4-methoxy-3(2H)-furanone (DMMF, mesifurane) (Pyysalo et al. 1979; Larsen and Poll 1992; Pérez et al. 1996; Raab et al. 2006). Mesifurane

is a key flavor compound in strawberry fruit. This can occur at a high concentration of up to 55 mg kg^{-1} fresh weight (Larsen and Poll 1992) and low odor threshold (10 ppb in water) in some cultivars or selections (Schieberle and Hofmann 1997). The strawberry protein *O*-methyltransferase (FaOMT) controls variation in mesifurane content via the methylation of furaneol using S-adenosyl-L-Met as methyl donor (Wein et al. 2002; Lunkenbein et al. 2006). Zorrilla-Fontanesi et al. (2012) detected several QTLs that cosegregated with volatile candidate genes. One of those was *FaOMT* (*O*-methyltransferase) which controls variation in mesifurane content in strawberries. The *FaOMT* was mapped to the bottom of LG VII-F.1 and was associated with the ChFaM160 SSR marker. However, the QTL controlling mesifurane content was mapped at the same position but on another LG of the same homeologous group VII-F.2 (Fig. 12.2). The authors confirmed that an *FaOMT* homoeolog on LG VII-F.2 was the gene controlling variation in mesifurane content, which explained phenotypic variation of 43–67%.

12.3 DNA Marker Systems

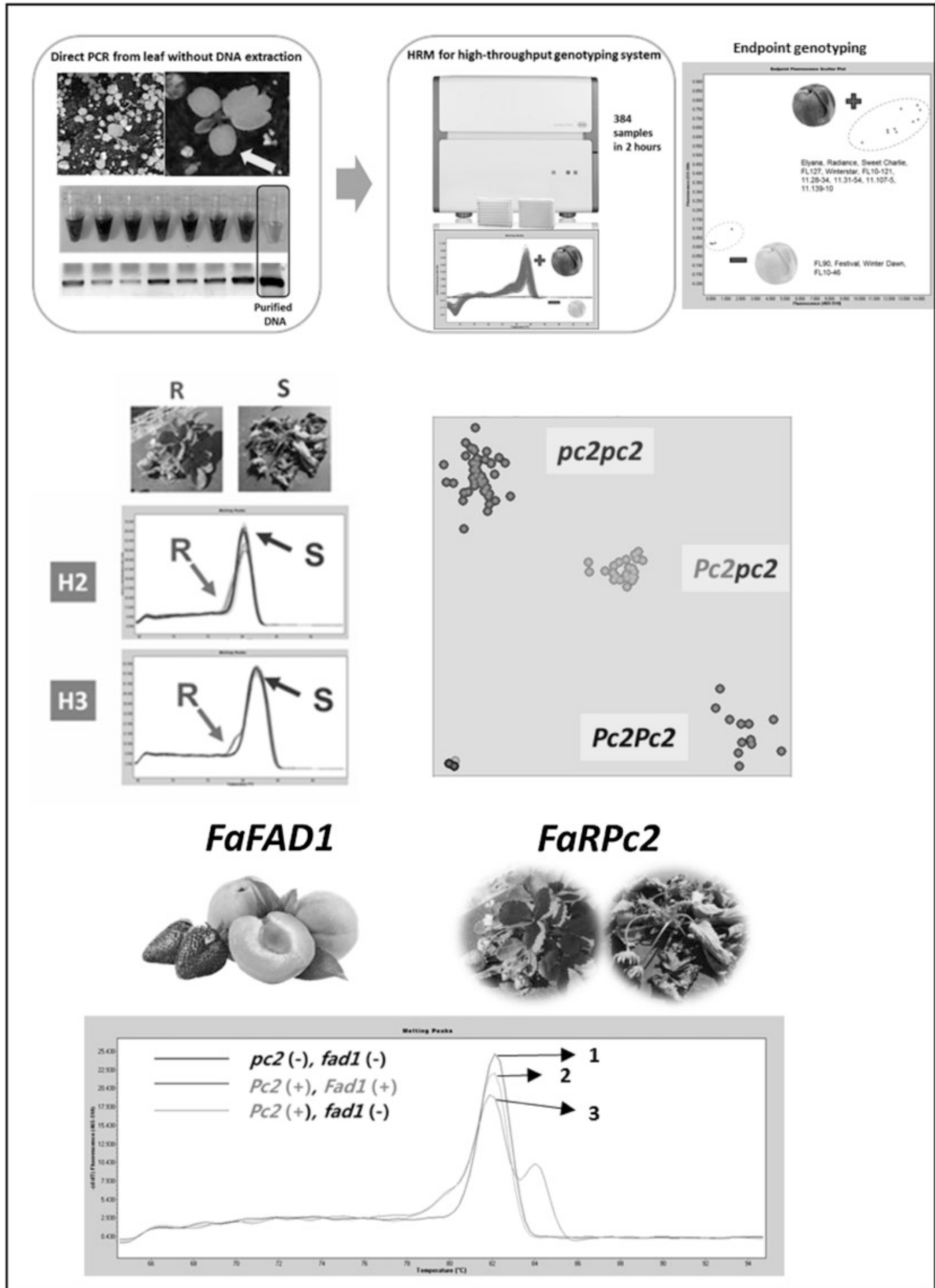
The recent and significant breakthroughs in genome sequencing and genome-wide marker scanning in strawberry have opened up new avenues in strawberry that have been routine in other species for years. Yet the allo-octoploid constitution of the cultivated strawberry must be carefully navigated when designing marker systems in this crop. In this section, we focus on DNA markers that are useful for routine application in breeding for major loci, which we also refer to as DNA tests.

12.3.1 DNA Markers in Allo-Octoploid Cultivated Strawberry

DNA marker technology in strawberry breeding has developed rapidly over the past two decades. A number of DNA marker types such as restriction fragment length polymorphism (RFLP), AFLP, RAPD and inter-simple sequence repeat (ISSR) markers were utilized in the past (Hubbard et al. 1992; Harrison et al. 1997; Morales et al. 2011). However, these marker systems have suffered from numerous limitations in terms of technical labor, cost, degree of polymorphism, transferability, and reproducibility. Therefore, sequence-characterized marker systems such as SSR and SNP have become the markers of choice for strawberry breeding applications.

The first strawberry SSR markers were developed from wild diploid accessions of *F. vesca* and *F. viridis* (Sargent et al. 2003, 2011; James et al. 2003). The transferability of SSRs to the octoploid cultivated strawberry was evaluated and transferable SSRs were identified (Davis et al. 2006; Monfort et al. 2006). SSRs were also developed directly in the octoploid strawberry (Shimomura and Hirashima 2006). SSR markers developed in diploid strawberry are highly transferable (up to 90%) to octoploid strawberry (Davis et al. 2006; Zorrilla-Fontanesi et al. 2012). Following the availability of advanced sequencing technologies and the *F. vesca* genome sequence, approximately 4500 SSR markers including expressed sequencing tag (EST)-SSRs (*F. vesca* and *F.* × *ananassa*) were developed in the octoploid strawberry and used for constructing linkage maps (Sargent et al. 2012; Isobe et al. 2013). Simple sequence repeat markers are effective for mapping due to their abundance in the genome, high level of polymorphism, and ability to detect many alleles across subgenomes.

The advent of second- and next-generation sequencing technologies has enabled high-throughput methods for SNP identification and analysis in wild and cultivated strawberries. However, there are a few drawbacks to using SNPs. Useful SNP markers that are closely linked to target traits are a very small percentage of the total available polymorphisms in allo-octoploid strawberry. Construction of haplotypes is often necessary in order to develop SNP-based DNA markers for a given trait. Also,


Direct PCR from leaf without DNA extraction
Purified DNA
HRM for high-throughput genotyping system
384 samples in 2 hours
Endpoint genotyping
Elyana, Radiance, Sweet Charlie, FL127, Winterstar, FL10-121, 11.28-34, 11.31-54, 11.107-5, 11.139-10
FL90, Festival, Winter Dawn, FL10-46
R
S
H2
H3
pc2pc2
Pc2pc2
Pc2Pc2
FaFAD1
FaRPc2
Melting Peaks
pc2 (-), fad1 (-)
Pc2 (+), Fad1 (+)
Pc2 (+), fad1 (-)
1
2
3
Temperature (°C)

◀ **Fig. 12.4** A high-throughput marker system: **a** direct PCR from strawberry leaf tissues and HRM analysis for *FaFAD1*. Strawberry seedlings were grown for two weeks, and one cotyledon (or one leaf disk, 3 mm diameter) was harvested and boiled in an extraction buffer, which was used directly in PCR (Noh et al. 2016). Tube #1 to #7 (left to right) are from randomly collected leaf samples of γ-decalactone producing accessions, and #8 is purified DNA from Florida cultivar Sweet Charlie (γ-decalactone producer); **b** detection of *FaRPc2* by HRM and KASP markers in resistant accessions. Two unique melt curves represent resistance (R) or susceptibility (S) at *FaRPc2* for two different resistant haplotypes (H2 and H3). A KASP marker for H3 at *FaRPc2* distinguishes three marker genotypes; and **c** Multiplex for *FaFAD1* and *FaRPc2*. Arrow 1: absence of *FaRPc2* and *FaFAD1* desirable alleles; Arrow 2: presence of *FaRPc2* and absence of *FaFAD1*; Arrow 3: presence of *FaRPc2* and *FaFAD1*

the transferability of SNP markers is low between strawberry species or genetically diverse breeding germplasm. Another disadvantage is that array technology is biased toward the panel of genotypes used to detect polymorphisms and is an expensive process. Genotype-by-sequencing can discover several million SNPs at lower per sample cost within the genotypes of interest, but this approach requires significant computational capabilities and suffers from the challenges of missing data.

12.3.2 Application of Marker Systems in Strawberry Breeding

DNA markers associated with traits in octoploid strawberry have increased dramatically in recent years. Currently, SSRs and SNPs are widely applied for marker-assisted selection (MAS) in strawberry breeding programs (Zorrilla-Fontanesi et al. 2012; Darwish et al. 2015; Sánchez-Sevilla et al. 2015). Although SSRs are highly polymorphic, robust, and reliable for octoploid strawberry, they are not an appropriate high-throughput genotyping platform for large breeding populations due to cost (3.5 USD per data point) and limited throughput. More importantly, in the octoploid strawberry, a single SSR marker often produces more than five alleles that are amplified from multiple subgenomes. The number of subgenome specific SSRs was approximately 20% of all the SSRs developed by Kazusa DNA Research Institute for Japanese strawberry breeding (Sachiko Isobe, personal communication). The use of multi-allelic SSRs for high-throughput MAS for large populations can be complicated. Therefore, SSRs have limitations for high-throughput applications as well as for fine mapping and positional cloning of genes of interest.

For effective MAS in screening large seedling populations for multiple target traits, cost-effective and high-throughput genotyping platform are critical. Unfortunately, laborious and expensive DNA extraction procedures have been required to achieve sufficient DNA quality in recalcitrant species such as strawberry. When combined with gel-based marker systems, current genotyping methods are becoming a bottleneck for large breeding populations. Alkaline (NaOH) DNA extraction has been successfully used for many important crops (Xin et al. 2003; Wang et al. 1993; Alexander 2016). A rapid DNA extraction method and high-throughput genotyping platforms were recently developed (Noh et al. 2016) for effective MAS in strawberry and other Rosaceae fruit crops.

As shown in Fig. 12.4, a rapid extraction system in strawberry combined with high-resolution melting (HRM) analysis has been successfully applied in strawberry breeding at UF. The entire procedure from sample collection to rapid DNA extraction to genotyping for a 96-well PCR plate takes about 2 h. HRM detects genetic variations such as SNPs and insertions/deletions without the need for agarose gels (Simko 2016). HRM technology is a cost-effective (about 0.20 USD per data point), high-throughput, and user-friendly marker system for deployment in breeding programs. Recently, SNP-based high-throughput HRM markers have been developed for disease resistance and fruit quality traits and are detailed in section four. One limitation of HRM analysis is that crude DNA directly from leaf tissues may

not be suitable for high-throughput HRM genotyping, especially when the melting temperature (T_m) difference between markers is less than 0.5 °C.

Other types of SNP detection methods such as molecular beacons, whether probe-based or TaqMan-based, are also suitable for high-throughput MAS in strawberry breeding. For probe-based SNP genotyping, including endpoint genotyping and Kompetitive Allele-Specific PCR (KASP), any instrument designed for SNP genotyping should be flexible across methods. To develop endpoint and KASP markers, the specific SNP at the target region must be identified and confirmed by sequencing prior to designing allele-specific probes. This system requires two sets of probes labeled with fluorescein and costs approximately 250 USD for each probe. Thus, it is important to validate the specific SNP at the target region, PCR conditions, and genotype determination prior to ordering probes. These two marker systems are very sensitive, robust and provide easy data analysis, making them powerful tools for high-throughput MAS. One limitation for probe-based allele-specific markers is that multiplex genotyping is not feasible. On the other hand, HRM is a post-PCR analysis and does not require sequence validation of the target region. HRM markers (about 6 USD per marker) can detect any known and/or unknown SNP or small indel (2–10 bp) present in the targeted region. Figure 12.4 shows a DNA marker system based on crude DNA extracts combined with HRM, endpoint genotyping, and KASP assays that is suitable for marker-assisted seedling selection in strawberry. We propose that, in the future, SNP-based HRM and allele-specific markers such as endpoint genotyping and KASP are routinely used for MAS in strawberry breeding programs worldwide.

12.4 DNA Tests for Major Loci

When a substantial proportion of the phenotypic variance for a trait of interest is controlled by one or few major loci, MAS at these loci is often a desirable strategy. As was mentioned above, several major loci segregate in a single subgenome and are suitable for MAS in the cultivated octoploid strawberry. For many of these loci, high-throughput DNA tests have been developed. Some of the DNA tests are already used widely in multiple breeding programs, others have recently been validated and are used in one or few breeding programs, and some are in the process of validation by one or more breeding programs. These tests are detailed in Table 12.1.

The volatile compound γ-decalactone is associated with 'peach-like' aroma in strawberry fruit. Using a metabolomics approach combined with RNAseq, Chambers et al. (2014) identified the fatty acid desaturase gene (*FaFAD1*) essential to its biosynthesis. Meanwhile, Sánchez-Sevilla et al. (2014) discovered the same locus on LG 3 by combining transcriptome analysis with a map-based approach. Interestingly, about half of cultivars tested were non-producers of this volatile and had a deletion of this gene. Thus, a functional SCAR marker was developed in the upstream region of the gene that amplifies a 500 bp product when the gene is present and does not amplify when the gene is absent. Amplification of a nearby marker (BFACT045) provides a positive control band for visualization by gel electrophoresis. The marker was found to correlate perfectly with the presence/absence of γ-decalactone as measured by GC/MS, whether in segregating seedling populations, cultivars, or wild species accessions. In the same study, an SSR marker was also developed 11 kb upstream of *FaFAD1*, and a 205 bp product was detected only in producers.

While the ability to produce γ-decalactone is governed by this locus, when the gene is present the amount of volatile produced is highly environmentally influenced. Thus, the marker functionally and perfectly predicts the ability to produce the volatile, but not the amount produced at a given harvest time point. When commercial germplasm was surveyed for γ-decalactone production, it was found that many of the best-flavored varieties from the University of Florida, including 'Sweet Charlie,' 'Florida Elyana,' and Sweet Sensation® 'Florida127'

Table 12.1 Eight DNA tests currently in use or in development for use in cultivated strawberry breeding

Trait	Locus	DNA test	Test status	Test references
γ-decalactone	*FaFAD1*	Gel-based, SSR	Validated, widely used	Chambers et al. (2014), Sánchez-Sevilla et al. (2014)
		HRM	Validated, used in RosBREED	Noh et al. (2016)
Mesifurane	*FaOMT*	Gel-based	Validated, used in RosBREED	Zorrilla-Fontanesi et al. (2012), Iraida Amaya (personal communication)
Remontancy (perpetual flowering)	*FaPFRU*	SSR	Validated, used in RosBREED	Gaston et al. (2013), Perrotte et al. (2016)
Resistance to *Colletotrichum acutatum* group 2	*Rca2*	SCAR	Validated, used by multiple programs	Lerceteau-Köhler et al. (2005)
Resistance to *Phytophthora cactorum*	*FaRPc2*	HRM, KASP	Validated, in use by UF	Mangandi et al. (2017)
Resistance to *Phytophthora fragariae*	*Rpf1*	SSR	Validated by Eric van de Weg and RosBREED	Eric van de Weg (personal communication), Mathey (2013)
Resistance to *Xanthomonas fragariae*	*FaRXf1*	HRM	Validation in progress	Roach et al. (2016a)
Soluble solids content	LG 6A	SSR	Validation in progress	Salinas et al. (in preparation)

contained high amounts of the compound (Schwieterman et al. 2014). Beginning in 2014, the University of Florida breeding program began using the gel-based functional marker for MASS.

Because the gel-based marker limited throughput for MASS, high-resolution melting (HRM) markers were later developed (Noh et al. 2016). Several HRM primer pairs were developed in the *FaFAD1* region that are capable of detecting the presence/absence of the gene. In particular, GDHRM5 has been the most accurately scored marker in the UF breeding program's MASS efforts and can be multiplexed with HRM markers for other traits. However, a codominant marker would still be desirable to provide enhanced accuracy of scoring and to distinguish between homozygous and heterozygous producers. A target region capture sequencing for the *FaFAD1* genomic region is underway to detect the gene-specific polymorphisms and development of codominant marker.

Mesifurane was recently mapped and colocalized with the candidate gene *FaOMT* on LG VII (Zorrilla-Fontanesi et al. 2012). The detected QTL for mesifurane explained from 42 up to 67.3% of the phenotypic variance, indicating a strong effect of this locus on total mesifurane variation. Sequence analysis identified 30 bp in the promoter of this *FaOMT* homoeolog that fully cosegregated with both the presence of mesifurane and the high transcript accumulation of *FaOMT* during ripening. An agarose gel-based marker was developed and identifies the functional allele (248 bp) and the non-functional allele (217 bp after 30 bp deletion), thus resulting in the ability to identify mesifurane-producing and non-producing cultivars.

Remontancy, also referred to as perpetual flowering or day-neutrality, is a very important trait in commercial strawberry, particularly in the central coast of California and other summer production regions. The transition from short-day to remontant flowering habit appears to be mostly controlled at the *FaPFRU* locus in the UC Davis source of day-neutrality (Gaston et al. 2013).

This single dominant locus confers the ability to initiate flowers under long days at permissive temperatures. Further fine mapping of *FaPFRU* revealed an SSR marker (Bx215) that is tightly linked to the locus (Perrotte et al. 2016). Validation of this marker in RosBREED germplasm revealed that more than 90% of individuals not containing the 129 bp SSR allele at Bx215 did not flower under long days. Results were more mixed for those individuals having at least one copy of the 129 bp SSR allele, most likely due to the effects of high temperatures in the testing environment (manuscript in preparation). It is expected based on the published results that the Bx215 marker should predict remontant flowering habit quite accurately in the UC Davis source given an environment with permissive temperatures below 21 °C. Since this is considered a qualitative trait, in theory this DNA test should account for most of the genetic variation present for the trait. This test is being evaluated for predictiveness in multiple environments and breeding programs including: Michigan State University in East Lansing, MI; University of Florida GCREC in Wimauma, Florida; Washington State University in Mount Vernon, Washington; and the USDA-ARS-HCRL in Corvallis, Oregon.

A number of DNA tests for disease resistance in strawberry have been developed in the last decade. Some of the first developed are linked to the *Rca2* locus which confers resistance to *C. acutatum* pathogenicity group 2 (Lerceteau-Köhler et al. 2005). A dominant allele at this locus confers resistance, and two SCAR markers in the region were developed by converting AFLP markers. The easiest to use is the *Rca2-240* marker, which amplifies a 240 bp fragment and can be visualized via agarose gel-based electrophoresis. While the authors have had some reports of the use of this marker or other markers developed in this region (personal communications) for use in breeding, the marker has not been highly predictive in University of Florida germplasm. This is likely due to either recombination between the marker and *Rca2* in certain pedigrees, the presence of different pathotypes of *C. acutatum* in different regions, separate genetic control of vegetative resistance (which was the focus of the original study) and fruit resistance, and/or the possible presence of other loci conferring resistance in different germplasm sources. More work is needed to elucidate the genetic architecture of resistance to this pathogen, as well as to develop additional marker resources for use in breeding.

A second resistance locus of interest is the *FaRPc2* locus, conferring resistance to root and crown rot caused by *P. cactorum* (Mangandi et al. 2017). A pedigree-based QTL analysis located this region to the bottom of linkage group 7D and indicated that most of the heritable genetic variation for resistance is controlled at that locus. In all, about 35–40% of the phenotypic variation for resistance seems to be controlled by *FaRPc2*. While it is likely that additional resistance loci are functioning in other germplasm sets (Richard Harrison, personal communication), *FaRPc2* is the major determinant of resistance in University of Florida germplasm and apparently in UC Davis germplasm as well. This is not surprising, as there are strong pedigree linkages between the germplasm of these two breeding programs. HRM markers have been developed in the QTL region that can detect two different SNP haplotypes (H2 and H3) associated with resistance segregating in different pedigrees (Noh unpublished). These markers can be multiplexed together and were first used in the University of Florida breeding program for seedling selection in 2016. Recently, KASP marker for the resistance haplotype, H3, has been developed and tested for the selection accuracy of the marker within UF breeding germplasm.

The *Rpf1* gene has been reported to confer incomplete resistance from resistance factor R1 to *P. fragariae* var. *fragariae*, the causal agent for red stele in strawberry (van de Weg 1997). Existing markers include the RAPD OPO-8A located 1.7 cM from the *Rpf1* gene and the SCAR-R1 marker that flanks the locus at 3 cM (Haymes et al. 2000). Crossover between *Rpf1* and either one of these markers is believed to cause the loss of the gene or marker in some cultivars (Haymes et al. 2000). Recently, a microsatellite simple sequence repeat (SSR) marker was developed and found to

detect presence of R1 with a 99% marker-trait association in the tested strawberry samples (Eric van de Weg personal communication). This marker was not found in either *F. chiloensis* or *F. virginiana* expressing this resistance source (Mathey 2013). Analysis of 56 *F.* × *ananassa* individuals with clear disease resistance scores detected marker presence in 17 of 18 individuals that had the R1 source of resistance and absence in 32 of 38 accessions that were susceptible to this source (Mathey 2013). Based on the resultant marker-trait association of 87.5% (49/56), this SSR marker appeared useful for predicting R1 resistance in the cultivated *F.* × *ananassa* strawberry.

Resistance to the major bacterial disease of strawberry, angular leaf spot disease caused by *X. fragariae*, was recently attributed to a dominant allele at a single locus on linkage group 6D of octoploid strawberry (Roach et al. 2016a). Since the trait is highly heritable, with broad-sense heritability above 0.7, DNA tests developed for this locus are expected to achieve a 60% disease reduction from homozygous susceptible individuals to individuals containing at least one copy of the resistant allele. Multiple HRM DNA tests were developed in the region (Roach et al. 2016a supplementary material). While one detects two main SNP alleles, another detects five different HRM curves. Validation of these markers is still in process, but results so far have indicated some recombination between the locus and the markers in inoculated seedling populations at the University of Florida. Development of additional markers is in progress, with the hope of developing more robust markers that will also function across germplasm sets.

Since overall liking of strawberry fruit is most greatly influenced by sweetness intensity (Schwieterman et al. 2014), developing a DNA test that can predict sweetness is desirable. The soluble solids content (SSC) in strawberry mostly consists of sugars (80–90%) (Perkins-Veazie 1995) and has been used as an indicator of sweetness. An SSR marker, EMFv006, was located close to a stable quantitative trait locus (QTL) for SSC in the 'Capitola' × CF1116 population on LG VI (Beatrice Denoyes, personal communication; Lerceteau-Köhler et al. 2012). QTL analysis in 609 strawberry individuals representing worldwide cultivars, selections, and breeding and mapping populations in 2011 and 2012 (Mathey 2013; Salinas 2015) confirmed the presence of a stable low-effect QTL near the EMFv006 marker on LG VI (Jason Zurn and Sujeet Verma, personal communication). We are further testing association of this marker with SSC in various germplasm sets, but so far no association has been consistently detected between the allele states of EMFv006 and the SSC phenotype suggesting the marker may not be useful for MAS in its present state. The sequence of the alleles from the marker is being further evaluated to determine if polymorphism exists and can be converted to a useful DNA test.

In summary, DNA tests for eight different traits in cultivated strawberry have been briefly described here. Four are for quality-related traits and four are for disease resistance traits. While markers for specific flavor volatiles serve breeders by targeting discrete elements of a complex volatile mix, markers for disease resistance can replace expensive and cumbersome disease screens. It is important to note that the tests reviewed here are, to some degree, in the public realm. Private breeding programs have also reported the use of or experimentation with these DNA tests, as well as additional tests developed in-house. For example, Driscoll's has reported the routine use of a test for a fruit volatile compound not reviewed in this chapter and a test that predicts crop timing (Phil Stewart, personal communication). In the future, whether public or private, we anticipate a continued increase in the number and use of DNA tests, particularly for fruit quality and disease resistance traits.

12.5 Genome-Wide Prediction

Marker-assisted breeding in most crops has been oriented toward major loci and the development of DNA tests for those loci. A much more

difficult task is to explain the genetic variation behind complex quantitative traits which are affected by many loci, each with small effects. Until the end of the twentieth century, pedigree-based prediction of breeding values (BV) for complex quantitative traits, using mixed models, was the standard in animal breeding and in some tree and plant breeding programs. Then came the theoretical paper of Meuwissen et al. (2001), which proposed genomic selection (GS) as a methodology for utilizing increasing amounts of genome-wide markers for the prediction of genomic breeding values (GBV).

In GS, we assume that at least some of the genome-wide molecular markers will be in linkage disequilibrium (LD) with QTLs affecting the trait of interest. The method is based on a reference population where the individuals are phenotyped and genotyped for the trait of interest, and a prediction model is built by simultaneously estimating marker effects across the genome for each individual. Next, untested individuals from a validation population are genotyped, their molecular information is included in the model, and their GBV or total genotypic values are predicted. The untested individuals are ranked by their GBV, and the best individuals are used either as parents for the next generation of breeding or for deployment. In this method there is no concern with determining the significance of QTL or characterizing any specific loci, only the prediction of performance for complex traits.

The practical applications of GS in animal breeding have been widely published (Hayes and Goddard 2010; Daetwyler et al. 2013; Meuwissen et al. 2016) and due to their successes several GS simulations and empirical studies have been carried out testing this approach on trees (Grattapaglia et al. 2011; Resende et al. 2012; Isik et al. 2016), tree fruits (Wong and Bernardo 2008; Kumar et al. 2012; Iwata et al. 2013), agronomic crops (Bernardo and Yu 2007; Zhong et al. 2009; Heffner et al. 2009), and other horticultural species (Würschum et al. 2013; Duangjit et al. 2016; Slater et al. 2016). The most important benefits of GS versus traditional pedigree-based breeding value prediction are (1) a higher prediction accuracy of GBV because Mendelian sampling of each individual can be estimated more accurately than by pedigrees and (2) a reduction of the breeding cycle of long-generation species by predicting the GBV of untested individuals.

In the previous section, DNA tests for major loci in strawberry were reviewed. However, several yield traits and quality traits of commercial importance in strawberry breeding programs are controlled by hundreds or perhaps even thousands of genes of small effects, following the infinitesimal model (Meuwissen et al. 2016), and both QTL and genome-wide association studies (GWAS) approaches are unable to explain all of the genetic variation for these traits. Therefore, GS has value for prediction of complex quantitative traits, with specific benefits to strawberry breeding programs potentially including: (1) more accurate BV estimation by using marker relationships in place of pedigrees, (2) reducing testing effort by eliminating partially or completely the establishment of certain field experiments, and (3) earlier use of predicted parents in the breeding cycle.

12.5.1 Testing Genomic Selection Methodology in Strawberry

In order to establish GS methodology for strawberry and determine its potential value in strawberry breeding, a series of replicated trials was conducted in consecutive years at the Gulf Coast Research and Education Center in Wimauma, Florida (Gezan et al. 2017). The breeding cycle at UF is completed in as little as three years beginning with the crossing of selected parents from the previous year in a partial diallel mating design to generate around 60 full-sib families for seedling testing (Fig. 12.1). In the next year, germinated seedlings from this population are screened using DNA tests for major loci and the retained seedlings clonally propagated by runners and visually evaluated. At the end of the season, runners and crowns of the best-selected individuals are clonally multiplied and one year later

planted in the advanced selection trials, as a replicated clonal single-plant plot trial for statistical evaluation and in an un-replicated multiple-plot trial, for visual evaluation.

A total reference population of 1628 individuals from the seedling trials and advanced selection trials from two consecutive seasons, 2013 and 2014, were phenotyped and genotyped with the IStraw90® Axiom® SNP array. In all four trials, we evaluated early marketable yield (EMY, g/plant), total marketable yield (TMY, g/plant), average fruit weight (AWT, g/fruit), soluble solids content (SSC, %), and total unmarketable (cull) fruit (TC, %). After quality control measures were implemented, approximately 17,000 SNP loci were utilized for genomic selection analysis. GS was tested with several methods, including one parametric method Genomic BLUP (GBLUP) (VanRaden 2008), four Bayesian methods BayesB (Gianola 2013), BayesC (Habier et al. 2011), Bayes ridge regression (BRR) (Hoerl and Kennard 1970), Bayesian LASSO (BL) (Park and Casella 2008), and one semi-parametric method, Bayesian reproducing kernel Hilbert spaces (RKHS) (Gianola et al. 2006). The GS standard statistical model can be described as: $y_i = u + \sum_{j=1}^{p} x_{ij} B_J + e_i$, where y_i is the phenotype, u is the phenotypic mean, x_{ij} is the marker effect of the ith individual, B is the regression coefficient of the jth marker, and e is the residual effect. The GBV is calculated as the sum of marker effects for an individual across the genome.

The performance of the GS methods was evaluated by estimating the predictive ability (PAB) and prediction accuracy (ACC) of each trait in a true validation, using the trials from one year as training populations and those from next year as testing populations. The ACC of GBVs is affected by three types of information: LD, co-segregation, and genetic relationships which impact the persistence of GS across generations (Habier et al. 2013). Parent selection efficiency estimates for each trait and advanced clonal test were obtained by calculating the ratio of genetic gains obtained when GS is performed in an incomplete scenario versus a complete scenario. The incomplete scenario was based on a GS model, for a given trait, using true validation to predict GBV for another test to be planted the next year. The complete scenario consisted of building the model in the latter year with both the phenotypic and marker information, representing the best scenario for selection and ranking in a practical situation when the maximum information is available.

Partial results from true validation, using trials from different years, are presented in Table 12.2. The prediction ability of pedigree-based BLUP (PBLUP) was lower than every GS method for all traits except for average fruit weight, and BayesB showed similar or better PAB for all traits than the other GS models. A similar trend was observed for ACC but higher differences were found between BayesB and PBLUP, illustrating the benefits of GS in breeding value prediction. The ACC of BayesB was on average higher across traits than other GS models and substantially greater for the yield traits EMY, TC, and TMY. Differences in ACC among traits may be explained by the influence of the trait's heritability on this parameter (Estaghvirou et al. 2013), and by the genotype by environment interaction (G $\times$ E), between trials and years which was higher for EMY and TMY than for the other traits.

Published reports on true validation from horticultural plants are scarce, and most GS plant studies have been conducted using cross-validation, a method that uses the same trials as training and testing populations. The true validation results of PAB and ACC (Table 12.2) were within the range of other published estimates of tree fruit and plant species (Gouy et al. 2013; Kumar et al. 2015; Duangjit et al. 2016; Michell et al. 2016). In simulations and some experimental datasets, little differences among GS models have been found (Resende et al. 2012; Daetwyler et al. 2013; Isik et al. 2016). However, in an empirical study involving barley, maize, and wheat, for 10 GS models and 18 traits, there were similarities and differences in prediction accuracy among the models (Heslot et al. 2012). The superiority of BayesB over other models has been demonstrated when the

Table 12.2 Predictive ability (PAB), prediction accuracy (ACC), and selection efficiency (%), for pedigree-based BLUP (PBLUP), BayesB, and the average of five additional GS models built from independent trials established in consecutive years

		PAB			ACC		Selection Efficiency (%)	
Trait	PBLUP	BayesB	Mean[a] other models	PBLUP	BayesB	Mean other models	PBLUP	BayesB
AWT	0.49	0.49	0.49	0.55	0.61	0.61	60	74
EMY	0.29	0.32	0.30	0.42	0.63	0.59	23	34
SSC	0.43	0.44	0.44	0.63	0.73	0.72	58	53
TC	0.32	0.35	0.34	0.16	0.40	0.38	25	55
TMY	0.31	0.35	0.30	0.51	0.75	0.63	14	46
Mean	0.37	0.39	0.37	0.45	0.62	0.59	36	52

AWT average weight (g/fruit), *EMY* early marketable yield (g/plant), *SSC* soluble solids content (Brix%), *TC* proportion of total culls (%), *TMY* total marketable yield (g/plant)

[a]The models are GBLUP, BayesC, Bayes ridge regression, Bayesian LASSO, and Bayesian reproducing kernel Hilbert Spaces

genetic architecture of the trait is controlled by few QTLs (Daetwyler et al. 2010; de los Campos et al. 2013). Moreover, high BayesB estimates have been attributed to its ability to capture not only QTLs in LD with markers but also genetic relationships between the training and testing populations (Habier et al. 2007; Zhong et al. 2009; Clark et al. 2011). In our study, LD estimates across the genome showed strong pairwise marker associations even when LD was corrected for relatedness (Gezan et al. 2017).

The efficiency of parent selection obtained by selecting the top 10% individuals suggest that for most traits near or above 50% efficiency can be realized with the use of predictions from GS models based only on marker information (Table 12.2). Moreover, large differences between the GS model and PBLUP were found for all traits except SSC, indicating the benefits of integrating this tool in the breeding program.

In summary, the use of genomic selection for complex quantitative traits in the strawberry breeding program is a robust tool and provides: (1) higher genetic gains for parent selection compared to traditional pedigree-BLUP selection and (2) should allow the UF strawberry breeding program to increase genetic gains by predicting some superior parents one year earlier than in the normal breeding cycle (Fig. 12.1).

12.5.2 Future Prospects for Genomic Selection

The excellent progress made in the application of genomic selection in animal sciences and plant breeding has opened a new frontier for the exploration and use of new approaches and strategies in short generation species and minor species. In strawberry breeding, there is a wide set of options to increase genetic gains. In the UF strawberry breeding program, this may include several approaches to application of GS as well as several research avenues for the future:

1. As we accumulate GS data over time, we will test models aggregating data from multiple cycles of selection to predict genetic values of the subsequent cycle, an approach that has provided improved genomic prediction accuracies in rye, *Secale cereale L.* (Auinger et al. 2016).
2. The prediction of varieties for deployment requires building GS models that account for additive, dominance, and epistatic effects. Previous experiences have indicated small to moderate increases of prediction accuracy for single-trait GS models (Sun et al. 2014; Kumar et al. 2015).

3. One of the aims when using GS in a breeding program is the reduction of genotyping costs in the training population to increase selection intensity and genetic gains in the test population. Genotyping using exon sequence capture, based on a lower number of markers, has demonstrated similar predictive abilities when compared to denser SNP array models, providing a cost-saving option for genotyping large populations (Santos et al. 2016).
4. Within family selection has proven to be a cost-effective strategy in breeding programs with large families (Lillehammer et al. 2013). The approach increases genetic gains compared to both conventional breeding and GS applied to the whole training population, by using a combination of traditional family breeding value estimation and low marker density genotyping of the tested population.
5. Exploring the potential of multi-trait GS models for genetically correlated target traits and hard-to-measure traits could potentially increase prediction accuracies for low-heritability traits in multi-trait analysis versus single-trait analysis (Calus and Veerkamp 2011; Hayashi and Iwata 2013; Guo et al. 2014).

12.6 Looking Ahead

Recent and rapid advances in genome-enabled breeding of strawberry can be linked to important technological and methodological advances in genomics. However, an important goal still on the horizon is a high-quality sequence assembly for the octoploid cultivated strawberry. Assembling and distinguishing the four subgenomes of cultivated strawberry will facilitate breeding progress on multiple fronts by enabling next-generation SNP arrays with better coverage and less subgenome bias, by allowing more accurate functional annotation of regions of interest, and by facilitating more efficient gene cloning. This will lead to the discovery and characterization of additional genomic regions governing economically important traits and the development of functional markers targeting candidate genes in those regions. As the cost of genotyping continues to decrease, low-density SNP genotyping combined with marker imputation methods should enable genomic selection in strawberry seedling populations.

From a broad perspective, the quest of geneticists and breeders is to associate phenotypic variation with its underlying biological causes and then to track those causes in a precise way for genetic improvement. Much has been made of the missing heritability of traits in many species that cannot be accounted for by detected QTL. It is here that we must look not only to DNA sequence variation in unique coding regions, but also to copy number variation, gene regulatory variants, epigenetic variation, post-transcriptional modifications, translation rate and the like (Pai et al. 2015), not to mention the proteome and metabolome. In one example from octoploid strawberry, a correlation network analysis was applied to RNAseq data from two parents and their segregating progeny to identify transcription factors modulating flavonoid biosynthesis (Pillet et al. 2015). What is particularly exciting is not just the increase in knowledge from such studies but the increasing power to leverage that knowledge by making targeted modifications to the genome. The future in this regard lies with the clustered regularly interspaced short palindromic repeats (CRISPR)—CRISPR associated protein 9 (Cas9) system. Commonly known as a ‘gene editing’ system, CRISPR-Cas9 allows not just targeted editing of a few base pairs but even site-specific gene replacements and insertions (Li et al. 2016). In other words, gene editing will likely be used in strawberry in the near future to replace undesirable alleles with desirable alleles at a given locus, or to insert a protein-coding sequence not previously present, in the genetic background of the breeder’s choosing.

A word of caution: no technology or method can overcome a lack of available phenotypic

and/or genetic diversity. This is particularly important in regard to climate change, as breeding populations do not adapt as well as natural populations to changing environmental conditions (Schmitt and Fournier-Level 2016). Therefore, as new approaches are implemented in strawberry breeding, it is critical to maintain efforts in pre-breeding and germplasm development, harnessing genetic variation as the fuel for selection. Infusions of new germplasm will result in a continual supply of new traits and new loci to target for genetic improvement.

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13 Sequence and Analysis of the Black Raspberry (*Rubus occidentalis*) Genome

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Abstract

The US Pacific Northwest is the primary production region of black raspberry, and this high-value specialty crop has been underutilized for several decades. Black raspberries contain high levels of anthocyanins and other bioactive compounds, which has sparked a renewed interest in breeding programs and cultivation. Despite this potential, black raspberry stands have seen a marked decline that many attribute to disease pressures and only three new cultivars have been released over the last 20 years. Here we discuss the available genomic resources for black raspberry, including the recently released draft genome. These resources will expedite marker-assisted improvement of raspberry with applications across the Rosaceae family. The 243 Mb black raspberry genome was sequenced using an Illumina-based whole genome shotgun sequencing approach, and a chromosome-scale assembly was generated using a high-density genetic map. Black raspberry is the sixth genome to be sequenced in the Rosaceae facilitating in-depth comparative genomics across the family. Black raspberry and the diploid wild strawberry (*Fragaria vesca*) are largely collinear with some lineage-specific structural rearrangements. The genome has 28,005 genes which is comparable to other Rosaceae species and includes a number of recently duplicated genes which may be related to domestication. Gene expression atlases during fruit ripening and *Verticillium* inoculation provide insights into ripening and disease resistance,

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respectively. Together the resources discussed here will provide tools for the improved understanding and breeding of Rosaceae crops.

13.1 Introduction

The genus *Rubus* is large and diverse (Thompson 1995; Alice 2002). Among the 700–800 species, three are significant crops: red raspberry (*Rubus idaeus* L.), black raspberry (*Rubus occidentalis* L.), and blackberry (*Rubus* sp. L.). Red and black raspberries are diploid ($2n = 2x = 14$) and belong to the subgenus *Idaeobatus*. Blackberry is tetraploid and belongs to the subgenus *Rubus*. The native range of black raspberry spans from New Brunswick to North and South Carolina and west to Kansas and Nebraska where it typically grows along disturbed areas and forest openings (Hitchcock and Cronquist 1973). The US Pacific Northwest grows most black raspberries, with 668 hectares earning growers US $6.8 million (Anonymous 2015) making it a lucrative minor fruit crop. Recent studies into the health benefits of dark-colored fruits and berries in particular suggest that black raspberries are especially high in bioactive compounds (Seeram et al. 2001; Stoner et al. 2005; Ash et al. 2011; Montrose et al. 2011).

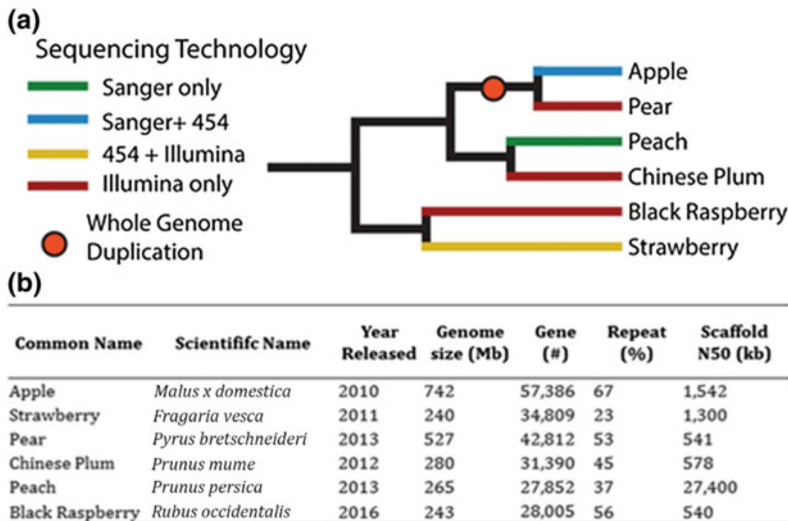
An attractive feature of black raspberry is its combination of unique flavor and anthocyanin profiles (Dossett et al. 2008; Tulio et al. 2008; Dossett et al. 2011) and bioactive compounds (Zikri et al. 2009; Zhang et al. 2011). Interest in breeding improved cultivars has been stimulated by these characteristics, yet progress has been slow. Formal breeding of black raspberry began in the late 1800s and in 1925, there were nearly 200 named cultivars, including some with yellow fruit (Hedrick et al. 1925). Breeding programs arose in Iowa, Maryland, North Carolina, with most breeding focused in Geneva, New York. Early breeding efforts were focused on crossing red raspberry and black raspberry to create ‘purple’ raspberries showing variation in production timing (Jennings 1988). By the 1970s, most of these cultivars had been lost and the handful remaining had low phenotypic and genetic diversity (Weber 2003), believed by Ourecky (1975) to be the result of cultivars with the same genotype being grown under different names. Ourecky’s supposition was confirmed by Dossett et al. (2012) who showed that cultivars Bristol, Cumberland, Munger, New Logan, Plum Farmer, and Shuttleworth in the National Clonal Germplasm Repository collection had identical genetic fingerprints at 20 loci. Since 1975, only three black raspberry cultivars have been released, compared to 189 red raspberry and 75 blackberry cultivars released from 1994–2014 (Bushakra et al. 2015a; Galletta et al. 1998). None of the relatively new black raspberry cultivars have been widely planted. Adding to the narrow gene pool of elite cultivars is high homozygosity (Dossett et al. 2012) of the species in general. In 2006, a search for wild black raspberry germplasm was conducted along its native range and led to the discovery of untapped genetic diversity including three sources of aphid resistance (Dossett and Finn 2010). The discovery of new germplasm led to the desire to better understand the genetics and genomics of this crop and to develop mapping populations and new selections that include one or more sources of aphid resistance.

As a member of the Rose family (Rosaceae), black raspberry is part of an economically important group of specialty crops including apple (*Malus* × *domestica* Borkh.), pear (*Pyrus* spp.), stone fruits (*Prunus* spp.), strawberry (*Fragaria* × *ananassa* Duch. ex Rozier), and roses (*Rosa* spp.). The genus *Rubus* has an estimated 750 species (Thompson 1995; Alice 2002), making it the largest and most complex in the family. Rosaceae, as a family, has ample genetic resources available. Currently, draft genomes are available for apple (Velasco et al. 2010), peach (Verde et al. 2013), pear (Wu et al. 2013a, b; Chagné et al. 2014), strawberry (Shulaev et al. 2011), and Chinese plum (Zhang et al. 2012), with other Rosaceae crop genomes on the horizon. The genomic resources publically available for black raspberry include the genome sequence of selection ORUS 4115-3 (VanBuren et al. 2016), RNA sequences (RNA-seq) from different tissues (VanBuren et al. 2016), a

saturated genetic linkage map of population ORUS 4305 (Bushakra et al. 2015a), a genetic linkage map of a 'purple' raspberry population designated 96395S1 × 'Latham' (Bushakra et al. 2012), and genomic- and expressed sequence tag (EST)-derived SSR markers (Dossett et al. 2010a; Jung and Main 2013; Bushakra et al. 2015b). The wealth of genetic and genomic resources will allow for detailed comparisons across the Rosaceae to better understand the evolution of this complex family and to explore traits of agronomic interest. The genome sequence of black raspberry will be another useful tool for Rosaceae breeders and researchers.

13.2 Assembling the Black Raspberry Genome

Apple (*M.* × *domestica*) was the first genome to be sequenced from the Rosaceae, and it provided an excellent foundational resource for the fruit breeding community (Velasco et al. 2010). The apple genome was sequenced using a whole genome shotgun approach with high-quality Sanger reads and incorporation of the newly released 454 pyrosequencing. The relatively long length of Sanger reads (300–1000 base pairs) and the low error rate produced a series of gold standard reference genomes including *Arabidopsis* (The Arabidopsis Genome Initiative 2000), rice (IRGS 2005), and *Brachypodium* (Vogel et al. 2010). The high per base pair cost of Sanger sequencing combined with a low throughput made it economically unfeasible to sequence most specialty crop genomes such as those in the Rosaceae. The advent of next generation sequencing (NGS) technologies significantly lowered the cost of genome sequencing, facilitating the completion of over 150 plant genomes to date (Michael and VanBuren 2015). The woodland strawberry (*Fragaria vesca*), pear (*Pyrus bretschneideri*), and Chinese plum (*Prunus mume*) genomes were sequenced using only NGS technologies with much lower costs than the Sanger sequencing-based peach (Verde et al. 2013) and apple (Velasco et al. 2010) genome projects (Fig. 13.1a, b). Black raspberry was the sixth Rosaceae genome to be sequenced



Common Name	Scientififc Name	Year Released	Genome size (Mb)	Gene (#)	Repeat (%)	Scaffold N50 (kb)
Apple	*Malus x domestica*	2010	742	57,386	67	1,542
Strawberry	*Fragaria vesca*	2011	240	34,809	23	1,300
Pear	*Pyrus bretschneideri*	2013	527	42,812	53	541
Chinese Plum	*Prunus mume*	2012	280	31,390	45	578
Peach	*Prunus persica*	2013	265	27,852	37	27,400
Black Raspberry	*Rubus occidentalis*	2016	243	28,005	56	540

Fig. 13.1 Summary of Rosaceae genome projects. Six Rosaceae genomes have been sequenced to date. The tree represents subfamily phylogenetic relationships; apple and pear in Maloideae, peach and Chinese plum in Amygdaloideae, black raspberry and strawberry in Rosoideae. **a** Phylogeny and sequencing technology of sequenced Rosaceae genomes. **b** Summary statistics of select genome features

(VanBuren et al. 2016), and several other Rosaceae genome projects are planned or currently underway.

The black raspberry selection ORUS 4115-3 was chosen for genome sequencing because of its apparent tolerance to Verticillium wilt (VW, *Verticillium dahliae* Kleb.), a soilborne fungal disease and a leading cause of stand decline in commercial fields in Oregon (Dossett 2011). ORUS 4115-3 has low residual within-genome heterozygosity, which simplified genome assembly and resulted in a higher quality genome sequence. High within-genome heterozygosity was a major challenge in the pear and apple genome projects and remains an issue in sequencing clonally propagated species (Michael and VanBuren 2015). The black raspberry genome was sequenced using an Illumina whole genome shotgun approach with genome anchoring using a high-density genetic map. Eight Illumina paired-end libraries with insert sizes ranging from 165 base pairs (bp) to 4700 bp were sequenced. The 1.15 billion reads collectively span 118 Gb and represent 325x coverage of the 293 Mb genome (Meng and Finn 2002). The wide distribution of insert sizes allowed assembly across complex repeat regions that exceeded the 100 bp length of the raw Illumina reads. The ALLPATHS (Butler et al. 2008) algorithm was used for genome assembly. The final assembly contains 9245 contiguous sequences (contigs) in 2226 scaffolds spanning 243 Mb or 83% of the estimated 293 Mb genome size. The scaffold N50 length is 353 kilobase pairs (kb), and half of the assembly is contained in the largest 178 scaffolds. The assembly quality of black raspberry is similar to other Illumina-based Rosaceae draft genome assemblies including pear (Wu et al. 2013a, b) and Chinese plum (Zhang et al. 2012) which have scaffold N50s of 540 and 577 kb, respectively (Fig. 13.1b).

The scaffold level assembly had a well-assembled gene space but the fragmented nature was insufficient for quantitative trait locus mapping, marker-assisted breeding, or genome selection approaches which require a chromosome-scale assembly. The black raspberry scaffolds were therefore assembled into seven pseudomolecules representing the haploid chromosome number using a high-density genetic map. The genetic map was derived from an F_1 population of 115 plants from the cross ORUS 3021-2 (female) × ORUS 4153-1 (male) (Bushakra et al. 2015a). The genetic map was constructed using a genotyping by sequencing (GBS) approach with incorporation of simple sequence repeat (SSR) markers. The maternal genetic map (ORUS 3021-2) has a total of 1195 markers across seven linkage groups with a total map size of 1294 centimorgans (cM) and an average distance between markers of 1.1 cM. The paternal genetic map (ORUS 4153-1) has lower marker density with 516 markers across seven linkage groups with a total map size of 950 cM and an average distance between markers of 1.84 cM. The lower marker density in the paternal genetic map is likely the product of low within-genome heterozygosity in ORUS 4153-1. The marker order in the genetic maps were used to order and orient scaffolds into a chromosome-scale assembly. A minimum of two markers per scaffold was required for anchoring, and at least three markers were required for orientation. Synteny with the strawberry (Shulaev et al. 2011; Tennessen et al. 2014) and peach (Verde et al. 2013) genomes was used to resolve ambiguities or inconsistencies in scaffold ordering between the two parental maps. The two genetic maps were largely in agreement, and ambiguities were found for less than 10% of the scaffolds. In total, 626 scaffolds were anchored to the seven black raspberry chromosomes, collectively spanning 203 Mb or 84.5% of the assembly (Fig. 13.2). The pseudochromosome sizes range from 22.7 Mb for chromosome 1–41.7 Mb for chromosome 6. The black raspberry genome assembly and annotation are available for download on the Genome Database for Rosaceae (https://www.rosaceae.org/).

The chloroplast and mitochondrion genomes were also assembled from the Illumina WGS reads. The black raspberry chloroplast genome was assembled into a single 151,783 bp contig using the reference-guided ‘alignreads’ pipeline

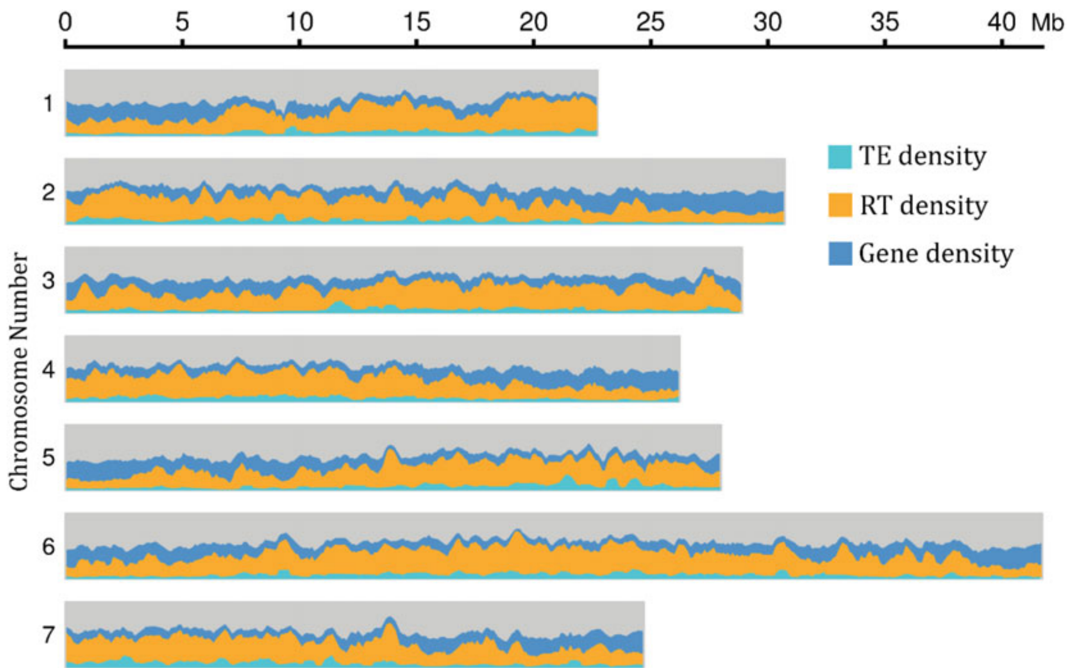


Fig. 13.2 Landscape of the black raspberry genome. Transposable element (TE), retrotransposon (RT), and gene density are plotted for each of the seven black raspberry chromosomes

(Straub et al. 2011) with the woodland strawberry (Shulaev et al. 2011) chloroplast sequence as a reference. The black raspberry mitochondrion genome was assembled into four scaffolds collectively spanning 449 kb. This size is similar to other Rosaceae mitochondrion genomes such as apple which is 396,947 bp (Velasco et al. 2010). The chloroplast genome contains 88 protein-coding genes, four ribosomal RNA genes, and 40 tRNAs. The black raspberry chloroplast is 3802 bp smaller than the strawberry chloroplast with five additional tRNAs as well as the protein-coding gene *infA*. The *infA* gene encodes translation initiation factor 1 and has been lost from most studied rosid chloroplast genomes (Millen et al. 2001), although there is evidence of its retention in the Rosaceae species *Prinsepia utilis* Royle (Wang et al. 2013). Compared to the apple chloroplast (Velasco et al. 2010), black raspberry has 8285 fewer base pairs. Like strawberry, the atpF group II intron is absent from black raspberry.

The black raspberry genome was annotated using a training set of transcripts and the genome annotation pipeline from MAKER (Cantarel et al. 2008). A set of 33,783 reference-guided and 71,622 de novo-assembled transcripts were generated using RNA-seq from tissue of young leaf, *Verticillium*-inoculated and un-inoculated root, green fruit, red fruit, ripe fruit, and cane tissue of the cultivar Jewel. These transcripts were clustered into 29,460 representative sequences that were used for input into MAKER producing a preliminary set of 32,300 putative gene models. Gene models with no functional annotation based on InterProScan and BLASTP database queries were removed leaving a final set of 28,005 protein-coding genes. These results are similar to the number of protein-coding genes found in strawberry (34,809 genes), peach (27,852 genes), and Chinese plum (31,390 genes), yet less than that found in pear (42,812 genes) and apple (57,386 genes). Gene density in the black raspberry genome is highest toward the ends of chromosomes and lowest in the pericentromeric regions and is inversely correlated with retrotransposon and DNA transposon density (Fig. 13.2).

13.3 Genome Evolution and Comparative Genomics Across the Rosaceae

Repetitive element composition varies extensively across plant genomes with ranges from ~3% in the compact 82 Mb *Utricularia* genome (Ibarra-Laclette et al. 2013) to over 90% in the 17 Gb hexaploid wheat genome (Mayer et al. 2014). Repetitive elements are major drivers of genome evolution and play a role in gene regulation and the evolution of new traits (Bennetzen 2000). Repetitive elements in the black raspberry genome were identified using structure- and homology-based approaches. Repeats collectively span 136 Mb or 56% of the black raspberry genome. This proportion of repeats is higher than most sequenced Rosaceae genomes except for apple where it was 67%. Rosaceae genomes with lower proportion of repeats include the largely collinear strawberry (23.0%), peach (37%), Chinese plum (45%), and pear (53%) (Fig. 13.1b). These differences may in part be due to sensitivity and thoroughness of repeat identification and level of completeness of the respective genome assemblies. For instance, woodland strawberry and black raspberry have similar genome sizes and collinearity but have a twofold difference in annotated repeats. The most abundant repeat types in black raspberry are the long terminal repeat (LTR) retrotransposons and the *Copia* and *Gypsy* LTR families which account for 10.6 and 11.6% of the genome, respectively. LTRs are distributed non-randomly across the genome with peaks near the centromeric regions and an inverse correlation with gene density (Fig. 13.2). Long interspersed elements (LINES) are unusually abundant with 27,506 elements accounting for 4.1% of the genome. This is tenfold higher than other sequenced Rosaceae genomes and higher than most plant genomes in general (Michael and VanBuren 2015). DNA transposons comprised 8.1% of the genome, with the *Helitron* family of terminal inverted repeat (TIR) the most abundant with 10,118 elements accounting for 4.1% of the genome.

Plant centromeres are characterized by tandem arrays of highly repetitive satellite DNA that can exceed several Mbs in length. The array lengths, distribution, and sequence composition vary greatly between species with little evolutionary conservation. Black raspberry centromeric repeats have a base monomer length of 303 bp with higher order structures of dimers (606 bp), trimers (909 bp), tetramers (1212 bp), and pentamers (1515 bp). The 303 bp centromere repeat in black raspberry is longer than most plant genomes which typically have centromeric array lengths between 150 and 200 bp, though this is highly variable (Melters et al. 2013). The 303 bp repeats are distinct from the 167 and 140 bp centromere repeats found in the strawberry (Shulaev et al. 2011) and pear (Wu et al. 2013a, b) genomes with little sequence conservation. Centromeric repeats are typically tightly clustered and localized within the centromeric regions with very few elements found outside of these regions. Centromeric repeats in black raspberry are found in large arrays on chromosomes 1 and 2 which likely correspond to the centromere. Surprisingly, arrays are also widely dispersed across entire chromosomes with 36,225 centromeric repeats found in 558 genome scaffolds. These 558 scaffolds collectively span over 30% of the genome. These arrays extend into relatively gene-rich regions including the entirety of scaffold 1 which is over 2.5 Mb in length with a normal gene density. Similar patterns of dispersed centromeric repeats are found in plants with holocentric chromosomes (Melters et al. 2012) suggesting centromeres may be highly complex in black raspberry.

The base chromosome number for diploids in the Rosaceae is $n = 7$, 8, or 9, and most agronomically important species have small genomes ranging in size from 200 to 400 Mb. Apple and pear are exceptions to this rule with a shared, recent whole genome duplication event, a subsequent chromosome fusion ($n = 17$) and larger genome sizes of 742 and 527 Mb, respectively. This WGD event in apple and pear complicates comparative genomics within the Rosaceae so peach and strawberry were used for detailed

comparisons with black raspberry. Each genomic region in black raspberry is orthologous to a single region in peach and strawberry suggesting that there has been no lineage-specific whole genome duplication event after the core eudicot-wide gamma hexaploidization (Bowers et al. 2003) (Fig. 13.3a). Black raspberry and strawberry share 15,533 pairs of collinear genes in 573 syntenic blocks while black raspberry and peach share 16,307 collinear genes in 611 syntenic blocks. The remaining genes have either undergone structural rearrangements from their shared ancestral state or are lineage-specific. The seven base chromosomes of black raspberry and strawberry are largely collinear with the exception of several large-scale chromosomal rearrangements (Fig. 13.3a). Despite the high degree of macrosynteny, strawberry and black raspberry have numerous gene level rearrangements though gene spacing is generally conserved (Fig. 13.3b). Peach and black raspberry are more distantly related, and chromosome level comparisons between black raspberry and peach (n = 8) are complex. Each chromosome in black raspberry corresponds to regions in several peach chromosomes. For instance, black raspberry LG1 corresponds to regions from peach chromosomes 1, 7, and 8, and LG3 corresponds to peach chromosomes 4 and 6 (Fig. 13.3a). This is largely consistent with previous findings in strawberry (Shulaev et al. 2011) and markers from a black raspberry genetic map (Bushakra et al. 2012). Despite the large-scale rearrangements, gene spacing is generally highly conserved between species (Fig. 13.3c).

Black raspberry, red raspberry, and other *Rubus* species can hybridize, suggesting a high degree of genome similarity. Synteny between black raspberry and other *Rubus* species is generally conserved based on common markers in linkage maps of red raspberry (Woodhead et al. 2010) and tetraploid blackberry (Castro et al. 2013). Black raspberry was largely congruent with red raspberry with 55 of the 63 matched markers occurring on the same linkage group. However, fewer than half of the 56 surveyed markers between blackberry and black raspberry occurred on the same linkage group. Most notably, the majority of the shared markers on blackberry LG7 from Castro et al. (2013) align to LG2 in black raspberry, and five markers from blackberry LG2 align to black raspberry LG7. These differences can be surveyed in more detail as additional genomic resources for *Rubus* become available.

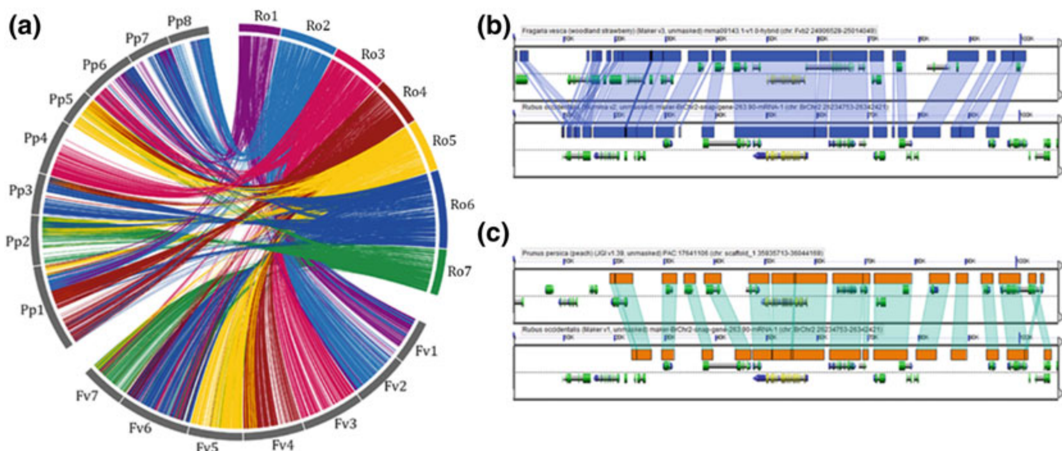


Fig. 13.3 Comparative genomics across the Rosaceae. **a** Circos plot (Krzywinski et al. 2009) showing macrosynteny between the black raspberry (Ro1–7), peach (Pp1–8), and strawberry (Fv1–7) chromosomes. Each connecting line represents a syntenic gene pair. **b** Microsynteny between strawberry (top) and black raspberry (bottom). Connections indicate conserved, orthologous sequences. **c** Microsynteny between peach (top) and black raspberry (bottom)

13.4 Signatures of Domestication in the Black Raspberry Genome

Black raspberry is one of the most recently domesticated fruit crops with targeted breeding efforts originating in the northeastern USA in the 1880s (Jennings 1988). Though the domestication history is short, the black raspberry genome should contain signatures of human selection for desirable fruit quality and disease resistance traits. Gene duplications provide the raw material for evolutionary innovation and have essential roles in the evolution of new traits (Ohno 1970). For instance, a retrotransposon-mediated gene duplication in tomato is responsible for the elongated fruit shape trait developed during domestication (Xiao et al. 2008), and a tandem array of disease-resistance genes confers nematode resistance in soybean (Cook et al. 2012). Gene duplications can occur *en masse* via whole genome and segmental duplications or singly via small-scale duplications. Small-scale duplicates (SSDs) can arise during uneven crossing over in meiosis or through retrotransposon-mediated duplication. SSDs originating from uneven crossing over typically occur in tandem gene arrays with upwards of 25 gene copies at a single locus. Retrotransposon-mediated duplication typically moves new genes to other regions of the genome. Newly duplicated genes are free to evolve new function and contribute to emergent traits such as yield increases, quality improvement, or even aesthetic appeal during plant domestication. Very recent SSDs (with synonymous substitution rate $K_s < 0.0001$) may be related to human domestication traits and older SSDs ($K_s < 0.4$) are lineage-specific and likely occurred after the divergence of black raspberry from red raspberry and other *Rubus* species.

Black raspberry has 4295 SSDs corresponding to 1770 ancestral copies, which is slightly lower than, but of similar proportion (18% of genes) to that of other Rosaceae genomes. Most SSDs are single events: 1339 ancestral genes are present in two copies, 265 in three copies, 99 in four copies, and 67 in five or more copies. The largest tandem array has 17 duplicated gene copies. Of these, 240 SSDs occurred very recently (with $Ks < 0.0001$) and 3468 SSDs that are older. Recent SSDs are enriched for gene ontology (GO) terms related to metabolism, fruit development, and seed development suggesting selection during domestication. Recent SSDs are largely distributed among KEGG pathways involved in fructose and mannose metabolism and the pentose phosphate pathway. There are two tandem duplicates of chalcone synthase (*CHS*), which is the first committed step of flavonoid biosynthesis. Black raspberry fruits have high levels of anthocyanins, particularly cyanidin-3-rutinoside and cyanidin-3-xylosylrutinoside that contribute to their dark color and distinguish them in part from red raspberries (Dossett et al. 2010b). This pair of tandem gene duplicates occurred after divergence from red raspberry and may contribute to the high levels of anthocyanins observed in black raspberry.

Newly duplicated genes can retain their ancestral function after duplication, partition their ancestral function between the new and old gene copies (subfunctionalization), or diverge and develop new functions (neofunctionalization) (Ohno 1970). The available RNA-seq-based expression profile was used to assess the fate of tandem gene duplicates (TD) in black raspberry. Over 56% (991) of the TD clusters are differentially expressed (DE) in all of the surveyed tissues, and 89% (1575) are DE in at least one tissue. In addition, 15% (646) of TD clusters have a putatively non-functional copy, with reads per kilobase per million mapped reads (RPKM) < 1 in all tissues, and 3.8% (164) of TD clusters had no detectable expression. This suggests that the majority of TDs have undergone subfunctionalization or regulatory neofunctionalization, may be involved in domestication or fruit quality-related traits, and are key targets for further crop improvement.

13.5 Additional Genomic Resources for Black Raspberry

Genomic resources for black raspberry were greatly limited until the last several years when a wealth of resources became available. This includes detailed gene expression atlases from a series of ripening fruits and plants inoculated with *Verticillium*, a metabolic network, and several high-density genetic maps. These resources are currently being leveraged for improving disease resistance and fruit quality traits.

Black raspberry fruits are high in anthocyanins, phenolics, and antioxidants (Wang and Lin 2000; Moyer et al. 2001; Dossett et al. 2008). Gene expression changes during fruit ripening were surveyed using RNA-seq. Three fruit development stages were collected (green, red, and ripe fruit) to identify genes related to fruit ripening and anthocyanin accumulation (VanBuren et al. 2016). Overall, 4446 genes were differentially expressed between green and red tissue, 9694 between green and ripe tissue, and 8376 between red and ripe tissue. Genes upregulated during fruit ripening (green vs. red and red vs. ripe) are enriched in GO terms related to hydrolase activity, cell wall degradation, sugar transport, anthocyanin accumulation, and others. The enzymes in the anthocyanin biosynthesis pathway were annotated, and gene copies contributing to accumulation in fruit were identified based on upregulation in ripening fruit compared to the other black raspberry tissues. Two functional copies of the gene encoding *CHS* were highly expressed in ripe fruit with RPKM of 10,019 and 18,342 compared to ranges of 105–907 in other tissues. Similar patterns of ripe fruit-specific expression were observed in genes encoding chalcone isomerase, flavanone 3-hydroxylase, flavonoid 3′-hydroxylase, leucoanthocyanidin dioxygenase, and anthocyanidin 3-O-glucosyltransferase.

Verticillium wilt is the leading cause of stand decline in commercial black raspberry fields (Dossett 2011). Gene expression changes between *Verticillium*-inoculated and non-inoculated (wild-type) 'Jewel' roots were surveyed to identify genes associated with disease response (VanBuren et al. 2016). Analysis of the RNA-seq data identified 147 DE genes. A comparison of these DE genes against the NCBI GenBank Database identified eight genes with apparent homology to known or candidate genes involved in disease resistance (R). Of the eight R-like genes, seven were upregulated 1.5–4.4-fold and one was downregulated 2.4-fold in inoculated plants relative to control plants. Twelve of the differentially expressed genes have homology to transcription factors. Seven of these genes were upregulated 1.5–3.1-fold, and five were downregulated 1.5–2.4-fold in inoculated versus control plants. The DE genes between *Verticillium*-inoculated versus control root tissues constitute a set of candidate genes for *Verticillium* response in black raspberry. Among the upregulated genes are homologs of *Arabidopsis* pest and pathogen resistance genes including *PDF1.4* (reviewed by Thomma et al. 2002), *HSPRO2* (Murray et al. 2007), and two mitogen-activated protein kinase genes *MEKK1* (Yan et al. 1994) and *NPK1* (Nakashima et al. 1998). A homolog of powdery mildew resistance gene *MLO3* (Freialdenhoven et al. 1996) was among the downregulated genes, suggesting pathogen-specificity is invoked in black raspberries inoculated with *Verticillium*.

Metabolic networks are powerful tools for modeling agronomically important metabolic pathways such as sugar and starch biosynthesis, photosynthesis, and secondary compound biosynthesis pathways. Each node in a metabolic network represents an enzyme, and edges or connections represent a shared metabolite between those enzymes (de Oliveira Dal'Molin et al. 2010). Metabolic networks are available for other Rosaceae crops including FragariaCyc, a strawberry network constructed using the Pathway-Tools platform (Naithani et al. 2016). A metabolic network for black raspberry was constructed using pairwise genomic alignments of black raspberry with *Arabidopsis thaliana* and *Vitis vinifera* genomes (VanBuren et al. 2016) with the software package QUOTA-ALIGN (Tang et al. 2011). The black raspberry metabolic network has 4774 nodes (enzymes) with

39,005 edges and encompasses most biochemical pathways including photosynthesis, core anabolic and catabolic processes, and various signaling and stress pathways. Comparisons among different plant networks are now possible and may shed light on variations in specific biosynthesis pathways or responses to biotic and abiotic stresses.

13.6 Conclusions and Future Prospects

Specialty crops like black raspberry are underutilized and often suffer from disease susceptibility, variable yields, and restrictive growing conditions stemming from their low or eroded genetic diversity and limited breeding efforts (Mayes et al. 2011). Despite these issues, specialty crops have great potential for increasing crop diversity and global food security and many have high-value market demand. Black raspberries are prized for their flavor and nutritional qualities yet production has declined since the early 1900s, and only three new cultivars have been released over the last 20 years. Black raspberry cultivars suffer from narrow genetic diversity stemming from the limited gene pool used in the elite germplasm and lack of breeding progress (Dossett et al. 2008, 2012). This narrow gene pool has hindered attempts to increase yield, quality, and disease resistance. High-quality genomic resources provide a foundation for modern crop improvement programs. In the short-term, the reference genome will move black raspberry into the modern breeding era through the development of SSR and SNP-based markers for marker-assisted breeding. A high-quality genome serves as a reference for future genotyping by sequencing-based mapping projects as well as for identifying genes underlying disease- and fruit quality-related QTL. The genome is also a foundation to survey diversity in cultivated and wild black raspberry accessions for designing future crosses and implementing genome selection. Orthology across the Rosaceae crops can be established using comparative genomics approaches to help translate discoveries from one species into another.

Additional genomic resources such as gene expression atlases and a metabolic network are suitable for long-term improvement projects. Differential expression under *Verticillium* inoculation has already identified candidate genes related to disease resistance of this devastating pathogen. Black raspberry fruits are high in anthocyanins and other bioactive compounds. Leveraging gene co-expression and metabolic networks may help to improve nutritional quality. Together these genomic resources will pave the way for future improvement and potentially global commercialization of black raspberry.

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14 Developmental Transitions to Fruiting in Red Raspberry

Julie Graham and Craig Simpson

Abstract

Climate change is impacting soft fruit crops. In raspberry, uneven bud break, greater variability in time to fruit ripening and crumbly fruit are already in evidence. Understanding the developmental process and how the environment impacts will be crucial in sustaining the industry in this changing climate against a background of biotic stresses. This chapter reviews regulation of processes leading to flowering time and fertilisation, developing fruit, ripening, colour, flavour and size. Recent developments of genomic and transcriptome tools which will have a significant role in breeding of the next generation of raspberry fruit are considered.

14.1 Introduction

Red raspberry has seen a significant increase in consumer demand while facing considerable grower challenges. Many of these challenges are associated with the fruit developmental process. For example, growers, breeders and propagators are seeing an increased occurrence of crumbly fruit, a lack of evenness of bud break and shifts in ripening period across seasons. Understanding the developmental process and how the environment impacts on it will be crucial in sustaining the industry in a changing climate and a background of increasingly aggressive biotic stresses. This chapter will review what is known about regulation of processes leading to flowering time and fertilisation, developing fruit, ripening, colour, flavour and size. It will also consider explanations for the increasing occurrence of crumbly fruit, a serious threat to consistent fruit harvest. The chapter will further review the recent development of genomic and transcriptomic tools which will have a significant role in breeding of the next generation of raspberry fruit.

14.2 Life Cycle

Raspberry bears short-lived woody shoots on a long-lived perennial root system bearing juvenile and mature shoots (canes) simultaneously on an individual plant. In biennial-fruiting cultivars, the canes have a two-year life cycle contrasting with primocane cultivars (annual fruiting) which complete the cycle of vegetative growth, flowering and fruiting in a single season (Jennings 1988). Low temperatures and/or short days are required for flowering in biennial cultivars, whereas the annual-fruiting raspberries initiate

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flower buds under the high temperature and long-day conditions of summer. With the absence of dormancy in a certain proportion of the initiated buds, primocane varieties flower in the first year unlike the biennial-fruiting cultivars where floral initiation is accompanied by growth cessation and bud dormancy. Dormancy release requires exposure to low temperatures or winter conditions, thus conferring a two-year life cycle upon the shoots. Shoots originating from root adventitious buds in biennial cultivars are juvenile and must develop 15–20 leaves before they can be induced to flower. In contrast, the juvenile phase is absent in annual-fruiting cultivars, which respond to a vernalisation treatment at the five-leaf stage (Sonsteby and Heide 2011).

14.3 Bud Break

Timing of bud break is of significant economic importance influencing realised fruit yields. Bud break in raspberry crops is often uneven with many of the sub-apical buds remaining in a dormant state (White et al. 1998). The control of bud break is a key ecological factor in woody perennial plant survival and is dependent upon exposure to a particular duration of cool temperatures (chilling) to release dormancy, followed by an appropriate temperature to permit growth in the spring (Rallo and Martin 1991). Such a mechanism prevents premature bud burst during warmer winter days that could result in subsequent frost damage. Three temporally overlapping stages of dormancy have been distinguished (Lang et al. 1987). In paradormancy, growth suspension is maintained by plant tissues outside of the dormant meristem, often by the apical bud. Endodormancy is controlled from within the bud itself and is characterised by a requirement for sustained exposure to low, near freezing temperatures before active growth can resume. Ecodormancy is where growth is prevented by environmental factors, e.g. low temperatures. Current theories of dormancy suggest that endodormancy is followed by a period of ecodormancy, where buds are held in a dormant state until temperatures rise in the spring allowing growth resumption (Lang et al. 1987; Faust et al. 1997; Arora et al. 2003). Raspberry exhibits a high degree of paradormancy caused by apical dominance, which is manifested in the typical unbranched form of the canes (Måge 1975). White et al. (1998) carried out experiments on intact canes, single nodes and cane trisections after different lengths of chill units to determine whether the buds were in an endodormant or paradormant state. Buds on the lower parts of the intact canes remained in a dormant state long after buds from higher up the intact cane and the single nodes from all parts of the cane had emerged from the deepest phase of endodormancy. No significant differences were apparent in the trisected cane portions in bud break levels throughout the experiment when compared with the single nodes. A period of secondary dormancy was also observed in the intact canes not seen in the single nodes or the trisected canes which indicates that treatments which reduce paradormancy may also minimise the risk of secondary dormancy. Methods which overcome paradormancy in protected crops therefore might include tipping (removal of the cane apex), horizontal training methods, more efficient chilling methods and chemical treatments. Phenological models of endodormancy release and subsequent bud break, based on the relationship between climate and periodic plant growth, have been formulated (Fuchigami and Wisniewski 1997). This requirement is being met sporadically with ongoing climate warming leading to erratic bud break and less consistent fruit yields being reported (Jennings pers comm). An understanding of the molecular and cellular basis of signals that control the processes of dormancy induction or dormancy release in woody perennial plants remains elusive (Dennis 1994; Chao 2002). Hormonal and environmental signals exert their effect on the induction, maintenance and release of dormancy, via activation and repression of diverse gene activities (reviewed in Olsen 2003). In order to understand the regulation of dormancy transition at the level of gene expression, Mazzitelli et al. (2007) measured bud burst in a growth-permissive environment following exposure to chilling

(4 °C cold storage). A microarray approach was then used to follow changes in gene expression that occurred. Sequence analysis of over 5000 clones, in many cases, enabled their functional categorisation and the development of hypotheses concerning the mechanisms of bud dormancy release.

14.4 Flowering Control

The timing of flowering is a key component of plant adaptation, affecting geographical distribution and geographical range. There is now evidence that red raspberry flowering time has shifted in response to changes in climate (Fitter and Fitter 2002; Amano et al. 2010). To understand this important change in flowering time requires a detailed understanding of the regulatory processes. Many plant species, including raspberry, are unable to respond to the florally inductive cues until they have reached a certain developmental stage. They have a juvenile phase which only responds to a prolonged period of cold (vernalisation) after a certain stage of development. Sonsteby and Heide (2014) determined the conditions necessary for full dormancy release and concluded that 20 or more weeks of chilling at temperatures near freezing were required for full dormancy release and promotion of flowering along the entire length of the cane. Much is known about the genetic pathways regulating flowering, and recent advances have now shed light on the mechanisms underlying vegetative phase change (Wu et al. 2009). The transition to flowering is regulated by a range of environmental and physiological cues (Fornara et al. 2010; Pin and Nilsson 2012; Song et al. 2012) that need to be fully understood in perennial crops. Flowering time variation is highly relevant to yield, quality and environmental considerations as flowering at the appropriate time ensures best use of the available growing season, promoting sustainability and reducing the need for inputs. Activity of CONSTANS (CO) a key component in leaves of the photoperiodic pathway accumulates under long-day conditions and activates transcription of FLOWERING LOCUS T (FT) (Simon et al. 2015) which interacts with bZIP transcription factors (Abe et al. 2005; Cao et al. 2016) activating a cascade of downstream genes leading to flowering. This basic flowering process is impacted by a number of autonomous and stress-related signals. For example, the MADS box FLOWERING LOCUS C (FLC) and short vegetative phase proteins (SVP) form a complex to repress flowering until the plant is exposed to the appropriate level of cold. The small non-coding RNA, miRNA156, acts by repressing members of the SQUAMOSA PROMOTER BINDING LIKE (SPL) family of transcription factors. Flowering repression is lost as miRNA156 is reduced as the plant ages (Wang 2014; Wu et al. 2009). The plant thus requires both down-regulation of miRNA156 and FLC for flowering to occur. In raspberry, RiMADS_01 was identified as a potential candidate affecting vernalisation (Graham et al. 2009a). The gene is similar to SVP modulating the timing of the developmental transition to flowering phase in response to temperature (Lee et al. 2007), and in colder seasons, RiMADS_01 was associated with earlier flowering. An SPL homolog has also been identified in raspberry within a QTL associated with floral transition. In terms of raspberry life cycle, the difference between annual and biennial cultivars is in whether floral initiation is linked to the induction of bud dormancy or whether floral initiation is followed by direct flower development. Although this is genetically determined, it is a plastic trait under environmental control. Thus, at low temperatures and short photoperiods, the majority of initiated buds also enter dormancy in annual-fruiting cultivars, with tip-flowering as a result (Heide and Sonsteby 2011).

14.5 Flower Morphology

Raspberry flowers have five sepals and petals, the sepals persisting until the fruit is ripe. Flower parts develop during active growth processes, and then, growth is arrested until pollination and fertilisation occurs, initiating fruit development.

Major genes have been identified with a role in modifying the number and size of the sepals. The recessive gene sx_3 (Keep 1964) induces either an extra whorl of sepals with the normal number of petals but fewer anthers, to two extra whorls of sepals where petals and anthers are completely suppressed or just a few anthers present. Another gene L_1 gives very large sepals with lobes contracted to a narrow point (Jennings 1988). In raspberry, the sepals reflex away from the fruit, but in other *Rubus* spp., they close after pollination until the fruit is ripe. Raspberry stamens arise in whorls of 60–90 with the numbers of both stamens and styles affected by ploidy, genotype and major genes (Jennings 1988). Most raspberries are hermaphrodite (genotype *FM*), but some male (*fM*) and some female (*Fm*) genotypes and sterile genotypes (*fm*) where recessive genes *f* and *m* suppress development of the female and male parts (Jennings 1988) occur. Styles arise spirally on the terminal part of the receptacle and determine the size and shape of the fruit. Mezetti et al. (2004) reported results from transgenic raspberry plants expressing the DefH9-iaaM auxin-synthesising gene. DefH9-iaaM plants had an increased number of flowers per inflorescence and an increased number of inflorescences per plant resulting in an increased number of fruits. The weight and size of transgenic fruits were also increased. The DefH9-iaaM gene is expressed in the flower buds. The total IAA (auxin) content of young flower buds expressing the DefH9-iaaM gene was increased in comparison with non-transgenic flower buds.

14.6 Fertilisation

The current model of fruit set implies that ovary growth is blocked before pollination and that auxin is a key regulator of ovary growth de-repression at fruit set (Goetz et al. 2007; Pandolfini et al. 2007). Following pollination in raspberry, there is a period of rapid growth due to cell division. This is followed by a period of slow growth during which the embryo develops and the endocarp becomes hardened, until finally cell enlargement results in a period of rapid growth. Other phytohormones (gibberellin, cytokinin, brassinosteroids, ethylene, and abscisic acid) play a role in fruit initiation and development (Schwabe and Mills 1981; Vriezen et al. 2008). The carpel has an ovary with two ovules one of which usually aborts. Fertilisation usually occurs the day after pollination, and the endosperm nucleus begins to divide a day later. The egg cell however does not begin to divide until the fourth day after pollination. Initially, the whole system behaves as a unit with the embryo, endosperm, testa and endocarp growing alongside each other (Topham 1970). Common to many fleshy fruits, once raspberry fruit growth has started, it is largely independent of the seed as the seed becomes dormant (McAtee et al. 2013). Raspberry fruits are aggregates of all the drupelets formed after fertilisation from each ovary from the same flower adhering to a common receptacle. In effect, each drupelet is a complete fruit and the control of drupelet formation must be co-ordinated in the aggregate fruit. The cohesion of the drupes depends on the entanglement of epidermal hairs. In some *Rubus* spp., wax on the outside of the epidermal cells also plays a role. A good set of drupelets and optimum early development of the seed appear to depend on the interactions between the gametes of the two parents. The maternal genotype has significant influence especially on timing of embryo sac differentiation where late differentiation is associated with low drupelet set (Jennings 1988). Self-incompatibility (gene *S*) is common among the diploid *Rubus* spp but domesticated forms are self-compatible due to mutation where the pollen of cultivated forms has changed to a self-compatible state.

14.7 Fruit Ripening

Raspberry fruits ripen from 30 to 60 days after pollination with variations in duration to ripe fruit dependant on genotype and environmental conditions. Fruits are generally divided into climacteric, which show a rise of respiration and ethylene formation at the beginning of ripening,

while non-climacteric fruit lacks this increase and other phytohormones may have a greater role to play. Reports vary on the classification of raspberry ripening as climacteric (Iannetta et al. 1999) or non-climacteric (Perkins-Veazie and Nonnecke 1992; Zheng and Hrazdina 2010). Evidence suggests that fruit ripening and abscission are controlled by ethylene from the recepticle and respiration continues even after the fruit is picked (Iannetta et al. 2000; Fuentes et al. 2015). Many raspberry cultivars differ in the amount of ethylene they produce with the ease of fruit abscission related to those cultivars producing higher amounts (Jennings 1988). But, there is no strong evidence of a large climacteric peak of respiration and ethylene at the start of raspberry fruit ripening. Auxin treatment delays fruit ripening in strawberry and grape, supporting a role for auxin in non-climacteric fruit and over-expression of an *F*-box auxin receptor in tomato enhanced fruit softening through upregulation of cell wall-degrading enzymes (Davies et al. 1997; Aharoni et al. 2002; El-Sharkawy et al. 2016). A microarray transcriptomic analysis of ripening strawberry recepticles challenged with phytohormones found auxin-regulated genes led to recepticle fruit growth and development and abscisic acid (ABA)-regulated genes involved in ripening (Medina-Puche et al. 2016). ABA and pyrabactin applied directly to raspberry fruit after fruit set did not alter fruit development and ripening, but doubled vitamin C content in fruit (Miret and Munné-Bosch 2016). Ethylene formation therefore may have a minor role in raspberries that may be co-ordinated with auxin and ABA formation as part of the mechanism that regulates timing of ripening in different fruit species (Trainotti et al. 2007; McAtee et al. 2013; Tadiello et al. 2016).

As part of the ripening process, fruits progressively lose firmness associated with fruit expansion when the thin-walled mesocarp cells become distended resulting in a loss of skin strength, the separation of the drupelets from the receptacle and a breakdown of cell walls in the mesocarp (Sexton et al. 1997; Vicente et al. 2007; Zheng and Hrazdina 2010). Abscission layers form in tissues as the fruit ripens at the point of attachment of each drupelet to the receptacle. A number of key enzymes have been shown to significantly impact on the degree and speed of the fruit-softening process; β-galactosidase and expansin genes act early in the ripening process and restrict or control the activities of other ripening-related hydrolases including polygalacturonases (PG), pectin-methylesterases (PME), endo-1,4-β-glucanases, xyloglucan endotransglycosylases and pectate lyases (Sexton et al. 1997; Iannetta et al. 2000, Jimenez-Bermudez et al. 2002; Costa et al. 2008; Santiago-Domenech et al. 2008; Uluisik et al. 2016). Studies on fruit softening have shown an absence of change in hemicellulosic polymers and an increased solubility of cell wall polyuronides accompanied by depolymerisation. Pectic compounds, therefore, seem to be the cell wall polymers undergoing extensive modifications during raspberry ripening (Stewart et al. 2001; Vicente et al. 2007).

Ripening stages across different years and environments from a raspberry mapping population were examined to identify QTLs for the overall ripening process, as well as for the time to reach each stage. QTLs were identified across four chromosomes for ripening and the time to reach each stage. A MADS box gene RiMADS, Gene H and several raspberry ESTs were associated with the QTLs (Graham et al. 2009a). It was interesting that Gene *H*, known to be associated with cane morphology, was also associated with a slowing down of ripening across all stages (Graham et al. 2006, 2009a). Sequencing of the Gene *H* region (McKenzie et al. 2015) identified a DIVIA like Myb transcription factor (Werewolf) which has been shown to be a post-transcriptional regulator of FT (Seo et al. 2011). Ripening-related QTL and underlying genes have also been identified for anthocyanin production (Kassim et al. 2009) colour development (McCallum et al. 2010) and volatile production (Patterson et al. 2013). Recently, fruit softening was examined using both qualitative scoring and quantitative scoring of fruit firmness, length, mass and resistance to applied force to identify QTL in a raspberry mapping population (Latham × Glen Moy). QTLs were located

primarily on linkage group (LG) 3 with other significant loci on LG 1 and LG 5 which showed mostly additive effects between the two parents (Simpson et al. 2016). The expression of key genes that underlie these QTLs, with roles in cell wall solubility, water uptake, polyamine synthesis, transcription and cell respiration, showed variable expression patterns across fruit development. Highly significant positive and negative correlations between genes supported precise regulation of different cell processes throughout raspberry fruit development. Variable timing in expression was also found in some genes between soft (Latham) and firm (Glen Moy) cultivars (Simpson et al. 2016).

14.8 Fruit Colour

Considerable fruit colour variation exists in different raspberry varieties, and this influences consumer perception of ripeness. Although the genetics of the anthocyanin pathway is well documented, the regulatory controls of both quantitative and qualitative variations of fruit anthocyanin content are less advanced (Castellarin and Di Gaspero 2007). This is an issue for fruit breeding, which is a lengthy and costly process in woody perennials (Graham and Jennings 2009). Anthocyanin consists of an aglycone (cyanidin and pelargonin) with a varying number of sugar residues attached. The concentration of anthocyanins in raspberry is known to be determined by the interplay of a number of genes. The recessive allele of gene *t* results in a low anthocyanin concentration. Homozygous *tt* interacts with a recessive inhibitory gene *i* to produce apricot coloured fruits, while gene *P* prevents *tt* from completely blocking anthocyanin synthesis (Jennings 1988). In the field, anthocyanin concentrations vary as a result of genotype, varying seasonal sunlight variation and as a result of growth under polytunnels (Kassim et al. 2009; Mazur et al. 2014). The final expression of fruit colour may be attributed to varying proportions of individual co-pigments and to pH, which has a significant influence on the colour of anthocyanin solutions (Jennings 1988). Co-pigmentation allows the formation of complex interactions between pigments and colourless compounds, which enhance colour intensity. Several compounds may act as co-pigments including flavonoids, alkaloids, amino acids, polysaccharides, metals, organic acids, nucleotides and other anthocyanins (Castaneda-Ovando et al. 2009). The enzymes involved in flavonoid biosynthesis, which includes anthocyanins and flavonols, are well characterised (Jaakola 2007). Flavonols are derived from dihydroflavonols by flavonol synthase (FLS) (Nielsen et al. 2002). Following flavonoid synthesis in the cytoplasm, anthocyanins and proanthocyanins are transported to the vacuole where they can be permanently stored. This transfer is facilitated by a glutathione S-transferase (GST), the gene for which has been identified in many plant species including *Petunia hybrida* (AN9 gene) (Mueller et al. 2002) and maize (Bz2 gene) (Alfenito et al. 1998). GST is involved in the last genetically defined step in anthocyanin biosynthesis by adding a glutathione onto anthocyanins such as cyanidin-3-glucoside. Major structural genes (F3’H, FLS, DFR, IFR, OMT, GST) and transcription factors (bZIP, bHLH, MYB) influencing flavonoid biosynthesis have been identified, mapped and shown to underlie QTL for quantitative and qualitative anthocyanin composition. Measures of individual anthocyanins mapped to the bHLH gene on LG 1 and a bZIP gene on LG 4 (Kassim et al. 2009; Bushakra et al. 2013), whereas colour and total anthocyanins mapped to different overlapping QTL on LG 2, LG 3, LG 4 and LG 6 (McCallum et al. 2010). Chalcone synthase (PKS1 and PKS5) genes mapped to LG 7 and did not underlie the anthocyanin QTLs identified (Kassim et al. 2009). Transcription factors related to the C1 and R genes, which belong to the MYB and bHLH family, respectively, have also been shown to regulate the flavonoid accumulation pathway in other plant species including apple (Espley et al. 2007) and grape (Lijavetzky et al. 2006). Other MYB genes regulate other flavonoid genes and a MYB12 gene from Arabidopsis has been shown to regulate flavonol and caffeoylquinic acid synthesis when expressed in

tomato fruit (Luo et al. 2008). In raspberry, two MYB genes underlie a QTL on LG 3 with a major impact on fruit ripening, importantly at the transition from the green to the green/red stage (Graham et al. 2009a, and these are implicated in the expression of fruit colour. Gene families like aquaporins may also have a role in colour as these are water channel proteins capable of transporting water and small molecules across cellular membranes. Tonoplast intrinsic proteins have been shown to act as water channels expressed predominantly within storage tissues. As glucose is accompanied by the transport of water, these genes are strong candidates for quantitative differences relating to the storage and transport of sugar molecules (Martinoia et al. 2000). McCallum et al. (2010) identified aquaporins on LG 2 in a QTL for colour scores and total anthocyanin measures (McCallum et al. 2010).

14.9 Fruit Composition

The composition of raspberry fruit is a key factor in taste. Raspberry fruits are mainly water with about 9% soluble solids. Pectins make up 0.1–1.0% of the soluble fraction, but this amount decreases with ripening due to hydrolysis. The main sugars are glucose, fructose and a smaller amount of sucrose. A typical ripe raspberry fruit will contain 5–6% sugar. Citric acid is the second largest component of the soluble fraction; raspberries contain very little malic acid, but at least ten other acids in trace amounts. The amount of acid in the fruit increases early in development and then decreases as the fruit begins to ripen. Flavour is central to quality in soft fruit and is determined by the content and ratios of sugars and acids as well as the volatiles. In raspberries, the two main flavour attributes sweetness and sourness (Harrison et al. 1999; Brennan and Graham 2009) vary with season and environment where flavour excellence relates to weather conditions (Jennings pers comm). Little work has been carried out in raspberry to correlate sensory evaluations with fruit composition. A study by Zait (2012) aimed to understand the association between sugars and acids and sweetness and sourness perceptions. Data from Patterson et al. (2013) on volatiles content and from Kassim et al (2009) on anthocyanin content was correlated with sensory data to develop a preliminary flavour model. Sweetness, sourness and flavour intensity traits were not adequately explained by singular contributions of either sugars or acids content, but through synergistic relationships between all flavour metabolites. Raspberry fruit accumulates the phenylpropanoid p-hydroxyphenylbutan-2-one (raspberry ketone) with content correlated with those of anthocyanins and soluble solids. Although only a small proportion of total volatiles, it has been reported to be a key determinant of raspberry flavour (Larsen et al. 1991). Fruit flavour volatile contents are generally continuous traits which are found to display a normal pattern of distribution which may be controlled by several genes of small effect or one or two genes conferring a large effect, or a combination of both. In the study of Kassim et al. 2013, raspberry volatile production appears to be significantly influenced by environmental as well as complex genetic factors and this work has provided a basis from which to proceed towards identifying the important variables contributing to desirable flavour/aroma characters at the genetic level. The demonstration that the concentrations of volatiles change across seasons and environments coupled with the shift or loss of significant QTL significantly associated with volatiles content across seasons and environments highlights the complex regulatory nature of volatile regulation.

In raspberry, the high levels of polyphenols and their distinct phytochemical profile have been associated with health benefits in humans (Rao and Snyder 2010; McDougall and Stewart 2012; Burton-Freeman et al. 2016). Levels of polyphenols are under genetic control subject to modulation by environmental factors such as temperature, sunlight and rainfall and can vary from season to season (Mazur et al. 2014). A number of tools have been developed for the rapid spectrophotometric quantification of specific compound classes such as total polyphenols, total anthocyanins and total

antioxidant capacity in fruit (Nwankno et al. 2012). Chromatographic methods have been developed for the analysis of key quality components such as individual sugars and organic acids (Nwankno et al. 2012), ascorbic acid (Walker et al. 2006), individual polyphenols and anthocyanins (Stewart et al. 2007) and volatiles (Patterson et al. 2013). A GC/MS method has been adapted from potato profiling (Hancock et al., 2014) to profile berry fruit metabolites, allowing the identification and quantification of a range of sugars, organic acids, amino acids, fatty acids, fatty alcohols and phytosterols. A study of ten different cultivars grown over three seasons in Western Norway found the main phenolic compounds in raspberry fruit were ellagitannins and anthocyanins with Cyanidin-3-sophoroside the most abundant anthocyanin (Mazur et al. 2014). An evaluation of the antioxidants in 14 different raspberry cultivars during fruit ripening identified the dominant antioxidants as ellagitannins, anthocyanins and proanthocyanidin-like tannins (Beekwilder et al. 2005). During the ripening process, some anthocyanins were newly produced, while others, like cyanidin-3-glucoside, were already present early in fruit development. The level of tannins, both ellagitannins and proanthocyanidin-like tannins, was reduced strongly during fruit ripening. Among the 14 cultivars, major differences were observed in the levels of pelargonidin-type anthocyanins and some proanthocyanidin-type tannins and the content of ellagitannins varied approximately threefold (Beekwilder et al. 2005). These findings suggest that the content of individual health-promoting compounds varies significantly in raspberry, due to both developmental and genetic factors. Large variation among 64 genotypes analysed for soluble sugars, titratable acids, pH and phenolic compounds was observed, highlighting the breeding and health potential within the germplasm (Weber et al. 2008). Using a mapping population, total phenol content (TPC) and total anthocyanin content (TAC) in ripe fruit was examined over five seasons under two environments to examine variability (Dobson et al. 2012). Corresponding measurements of antioxidant capacity using FRAP and TEAC were highly correlated with TPC over the entire dataset. The subset of anthocyanin content was genotype-dependent and also correlated with TPC, but the proportion of different anthocyanin compounds contributing to total phenolic pool varied from progeny to progeny. QTL was identified on linkage groups 2, 3, 5 and 6. The QTL that specifically influences TPC is of particular interest to boost the antioxidant capacity of raspberry fruits, which is often related to their bioactivities.

14.10 Fruit Enlargement

Fruit size is a key quality attribute due to its effect on yield and picking costs. Raspberry genotypes show a wide variation in fruit size, cell number and/or volume, which may be due to a number of genetic, environmental and growth management factors. Cells increase in volume by uptake of water and loosening and extension of the cell wall through the action of expansins and cell wall hydrolysing enzymes (Cosgrove 2016). Gibberellins are regulators of cell expansion and applied to grapes in the early stage of seedless berry development to increase berry size (Fortes et al. 2015). In raspberry, mutation of a major gene designated L_1 was identified in a large fruited 'Malling Jewel' mutant which resulted in an increase in both drupelet number and size. The gene itself however proved to be unstable mutating back to its normal-sized form (Jennings 1988). In tomato, a single major QTL for fruit weight was identified on chromosome 2, in close proximity to a cloned fruit weight gene *fw* 2.2 (Frary et al. 2000; Zygier et al. 2005). Some recent work has been carried out in raspberry to understand the basis of the genetic control of fruit size. QTL was identified on three linkage groups in the reference 'Glen Moy' by 'Latham' mapping population (Graham et al. 2004), across four seasons and three environments. Candidate genes for auxin, ethylene and specific size regulatory genes (fruit weight) and transcription factors were identified (McCallum et al. 2010). Initial analysis has found one marker explained 23% trait variation. In a *Rubus*

parvifolius × Tulameen cross one QTL was identified for fruit size (Molina-Bravo et al. 2014).

14.11 Disorders

In relation to fruit development, the disorder known as 'crumbly' fruit has become a serious problem in the raspberry industry. Drupelets are generally reduced in number and greatly enlarged or, in the case of small reductions, cohere imperfectly such that fruit readily crumbles when picked (Daubeny et al. 1967; Jennings 1988). There have been a number of causes suggested for the crumbly condition including infection with certain viruses (Jennings 1988; Murant et al. 1974; Daubeny et al. 1978). A genetic cause was demonstrated when the crumbly phenotype appeared in virus-clear mother plants (Jennings 1988). The cultivar 'Latham' can show a crumbly phenotype and this is thought to be due to a mutation of the dominant allele at a heterozygous gene locus, causing plants to become homozygous for a deleterious recessive gene (Jennings 1967b). From a 'Latham' self, Jennings (1967b) demonstrated that seedlings obtained could be classified into three groups: normal, crumbly and sterile. Studies have also shown that extensive tissue culturing of plants may increase the emergence of the condition (N. Jennings pers comm.). In addition, environmental factors appear to play a role as variation in the extent of crumbliness is apparent from year to year (A. Dolan pers comm). A study on the 'Latham' × 'Glen Moy' population examined the occurrence of crumbly fruit over a six-year period, in both open field and under polytunnel. This highlighted that seasonal, environmental and genetic factors all influence the condition. Two QTLs that are important for the genetic control of the condition were located on linkage groups one and three. Contrary to the suggestion by Jennings (1967a) that the crumbly fruit syndrome was related to the gene H region, no genetic association with this region on LG 2 could be identified. However, the longer the fruit takes to set fruit and reach green fruit stage, the more likely it is to be crumbly. This may explain the association hypothesised by Jennings, as the *Hh* genotype of gene H is associated with a slowing down of ripening across all stages from open flowers to the green/red stage compared to the *hh* genotype (Graham et al. 2009b).

Poor fruit set that affects raspberry fruit crumbliness has been associated with over frequent bee visits that impair fruit or seed production and/or quality by damaging flowers during visitation. Pollination and drupelet set in 16 raspberry fields was assessed along a gradient of bee abundance (Saez et al. 2014). Using pollen supplementation, they found pollen loads on stigmas increased with visit frequency in a subset of six fields. Drupelet set was not therefore pollen limited, but decreased with the proportion of damaged styles. In fields with the highest bee frequency equivalent to 300 visits per flower per day, 80% of styles were damaged and these developed into fruits with up to 30% fewer drupelets compared to flowers in fields with the lowest bee visitation rates of four visits per flower per day. Extreme bee visitation, particularly by *Bombus terrestris*, damaged the styles of raspberry flowers, precluding ovule fertilisation by deposited pollen and limiting crop production by reducing drupelet set (Saez et al. 2014).

14.12 Systems Biology Approaches to Studying Fruit Development

The development, improvement and integration of large-scale sequencing technologies and gas/liquid mass spectrometry techniques into horticultural breeding are changing our understanding of fruit developmental physiology, metabolic pathways and leading us to new important genes to important traits. Genomes of important *Rosaceae* species have been sequenced https://www.rosaceae.org/ and are providing the physical genetic maps that allow comparative differences to be discovered within these economically important species. Most recently, a 243 Mb whole genome assembly was established for black raspberry (*Rubus occidentalis)*, a

relative of red raspberry (VanBuren et al. 2016). The assembly consists of 2226 scaffolds spanning over an estimated 83% of the genome, which is largely collinear with the strawberry genome. In comparison with genetic maps, 87% of the selected red raspberry genetic markers match the position of the black raspberry markers on the physical map (VanBuren et al. 2016). The assembly of a red raspberry genome sequence will allow comparison between these closely related species and cultivar re-sequencing will further establish cultivar-specific genes significant to different traits.

A red raspberry 'fruit transcriptome' comprising of a comprehensive database of 56,000 unigenes has recently been established by combining sequences from Roche 454 transcripts, Illumina GAII assembled transcripts, Sanger expressed sequence tags and BAC coding sequences (unpublished). Probes were designed for the generation of a custom Agilent microarray and used to screen Glen Moy and Latham across five stages of fruit development. Developmental and genotype-specific gene expression patterns were found with expected roles in cell wall hydrolysis, water movement, fruit ripening and cell wall flexibility (unpublished). QTL mapping analysis for a selection of 20 candidate genes showed a distribution throughout all seven *Rubus* linkage groups, with the majority located on LG 3, 5 and 7, and several, for example, Pectinmethylesterase (PME) and β-1,4 xylan hydrolase (XL), were significantly associated with softening QTLs. Expression analysis of selected genes across the same fruit stages found that aquaporin and pectinmethylesterase show a steady rate of decline during ripening while polygalacturonase and β-1,4 xylan hydrolase significantly showed highest transcriptional levels in ripe fruit (Simpson et al. 2016). The evidence from microarray analysis indicates a co-ordinated expression between many genes during fruit development and ripening.

The analysis of a whole range of fruit phytochemicals associated with quality or health beneficial traits has now become relatively facile using chromatographic methods linked to mass spectrophotometry to aid the identification and facilitate quantification of compounds. It is well established that during the transition from an organ that functions to protect developing seeds to one that functions to act as an attractant to seed-dispersing organisms, fruit undergoes huge changes in their phytochemical profile. For example, in tomato fruit a range of primary metabolites including sugars, organic acids and amino acids see a tenfold or greater changes in concentration between 25 and 55 days after pollination (Osorio et al. 2011). Secondary metabolites see even greater changes; for example, levels of delphinidin the most abundant anthocyanin in blueberry change more than 80-fold over the course of development (Zifkin et al. 2012). These changes in phytochemical profile are accompanied by vast changes in transcript abundance with over 4000 transcripts over-expressed at least threefold or higher at specific stages of grape development, while over 1300 transcripts were expressed at least tenfold or higher and almost 350 50-fold or higher (Sweetman et al. 2012). Such vast changes in both phytochemical and transcript profiles can be effectively utilised to associate specific genes with the accumulation of specific compounds, and work illustrating such an approach is presented elsewhere in the current volume.

Integration of data from genomics, transcriptomics, proteomics and metabolomics (and other 'omic' technologies) from the same developmental fruit series, creates comparable reference datasets. For example, transcriptional results from the *Rubus* microarray allow the correlation of gene expression patterns with the accumulation of specific phytochemicals in developing fruit samples. With knowledge of biochemical pathways, an association of specific genes with important raspberry fruit components can be constructed and integrated network pathways developed. Using genomic information, the identification of gene transcripts has the potential to identify and validate gene markers by associating allelic variation within specific chromosomal regions with genes shown to correlate with phytochemical accumulation. Alternatively, such methods can be used to identify specific genes that are likely to influence the level of a specific

phytochemical or class of phytochemicals within fruit, thereby providing specific targets for the identification of allelic variation and molecular markers. These system biology approaches expand the reliability and predictability of raspberry breeding strategies to improve fruit yield and quality. These approaches will further offer the basis for the development of genetic manipulation and novel breeding strategies that improve and produce new varieties of this economically important and health beneficial crop.

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