Chapter 1
Apples

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Abstract The overall objectives of modern apple breeding programs are to increase the marketability of fruit and reduce production costs. Developing well adapted cultivars with resistance to major pests is also a focus of all breeding programs. The apple is generally grown as a composite tree with a rootstock and a fruiting scion, making rootstock breeding as important as the development of scion cultivars. Genetic resistance has been found for a number of the major pests of apple. Engineering resistance to apple scab and fire blight has been the focus of many of laboratories. Most of the traits associated with adaptation and productivity have been shown to be quantitatively controlled, including chilling requirement, cold hardiness, plant vigor, season of flowering and duration of the juvenile period. Many of the traits associated with fruit quality are also quantitatively inherited including flavor, skin color, shape, size and texture. Several cDNA libraries have been developed to identify genes associated with pollination and apple fruit development. A number of apple linkage maps have been published using several different sets of parents and molecular markers have been linked to a number of monogenic traits. Mining of existing apple EST information promises to expand our knowledge of many genes important in the genetic improvement of apple.

1.1 Introduction

Apples are cultivated all across the temperate world. Their adaptive range extends from the extreme cold of places such as Siberia and North China to the much warmer environs of Columbia and Indonesia. More than 60 countries produce over 1000 or more metric tons of apples, with China, U.S.A., Turkey, Iran, France, Italy, Poland and Russia being the leading producers. World production now exceeds 57,000 million metric tons (FAOSTAT, 2004).

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Apples are an extremely versatile crop. They can be eaten directly from the tree or stored for up to a year in controlled atmospheres. They can be processed into juice, sauce and slices, and are a favorite ingredient in cakes, pies and pastries. The juice can be consumed fresh or fermented into cider, wine or vinegar. The ornamental crab apples are also known for their floral display and attractive foliage.

There are over 6,000 regionally important cultivars and land races across the world, but a few major cultivars now dominate world fruit production (O'Rourke 2003). 'Delicious' is the most important cultivar grown, followed by 'Golden Delicious', 'Granny Smith', 'Fuji' and 'Gala'. These varieties represent over 60% of the world's production. Emerging varieties include 'Cripps Pink' (often sold under the trademark Pink Lady®), 'Honeycrisp' (sold in Europe as Honeycrunch®) (Fig. 1.1), 'Scifresh' (fruit marketed under the trademark Jazz®), 'Delblush' (fruit sold as Tentation®), 'Civni' (fruit marketed as Rubens®), 'Corail' (fruit marketed as Pinova® or Pinata®) and 'Ariane'.

The genetic base of the cultivated apple has greatly eroded over time as regional cultivars have been replaced. This has been compounded by the loss of many public apple breeding projects and their associated apple cultivar collections (Brooks and Vest 1985). Forsline and his group at the USDA Germplasm Repository at Cornell University has worked hard to counter this trend by actively collecting and cataloging native apple germplasm and making it available to apple breeders (Forsline et al. 1994, Hokanson et al. 1997).

Fig. 1.1 Fruit of the Honeycrisp apple cultivar (photographed at the University of Minnesota Horticultural Research Center, Excelsior, Minnesota, U.S.A.)

1.2 Evolutionary Biology and Germplasm Resources

The genus of apples, *Malus*, belongs to the subfamily Pomoideae of the Rosaceae family. Another important fruit tree, pear (*Pyrus*), belongs to the same subfamily. There are over 30 primary species of apple and most can be readily hybridized (Korban 1986, Way et al. 1991). The cultivated apple is likely the result of initial domestication followed by inter-specific hybridization (Harris et al. 2002). Its primary wild ancestor is *M. sieversii* whose range is centered at the border between western China and the former Soviet Union. Apples are the main forest tree there and display the full range of colors, forms and tastes found in domesticated apples across the world (Forsline et al. 1994, Hokanson et al. 1997). The domesticated apple has been referred to with the epithet *Malus* × *domestica* (Korban and Skirvin 1984), although recently Mabberley et al. (2001) proposed that *Malus pumila* should properly refer to the domesticated apple and its presumed wild relative *M. sieversii*. Other species of *Malus* which contributed to the genetic background of the apple likely include: *M. orientalis* of Caucasia, *M. sylvestris* from Europe, *M. baccata* from Siberia, *M. mandshurica* from Manchuria, and *M. prunifolia* from China. It is likely that these species hybridized with domesticated apples as they were spread by humans (Harris et al. 2002).

The bulk of the apple species are $2n = 2x = 34$ (Table 1.1), although higher somatic numbers of 51, 68 and 85 exist; several of the cultivated types are triploid (Chyi and Weeden 1984). It is possible that the high chromosome number of apple represents an ancient genomic duplication, since there are several other Rosaceous fruit species with lower haploid chromosome numbers of $n = 8$ and 9. Based on cytology and analysis of morphological characters, the *Maloideae* likely have a polyploid origin (Phillips et al. 1991). Isozyme studies in *Malus* support an allopolyploid origin based on the presence of duplicated gene systems, allele segregation and fixed heterozygosities (Chevreau et al. 1985, Weeden and Lamb 1987, Dickson et al. 1991). An allotetraploid origin involving ancestral *Spiroideae* (mostly $x = 9$) and *Amygdaloideae* ($x = 7$) was proposed by Sax (1931) and is supported by flavonoid chemistry (Challice 1974, Challice and Kovanda 1981) and morphological traits (Phillips et al. 1991). Apples are largely self-incompatible and some are apomictic. They are propagated vegetatively, usually as composites with a separate rootstock and scion.

Apples were certainly one of the earliest fruits to be gathered by people, and their domestication was probably preceded by a long period of unintentional planting via garbage disposal. It is difficult to determine exactly when the apple was first domesticated, but the Greeks and Romans were growing apples at least 2,500 years ago. They actively selected superior seedlings and were budding and grafting 2,000 years ago (Janick et al. 1996). People in Central Asia where *M. sieversii* is native still save desirable trees when the forest is cleared for agriculture (Ponomarenko 1983) and commonly graft and plant desirable *M. sieversii* from the forest into their gardens. Planting desirable trees from root suckers may also have been a common practice prior to grafting, as *M. sieversii* trees sucker freely. Conversely, people may have cloned and moved some of their horticulturally desirable trees to areas where they

Table 1.1 Distribution of selected apple species in subsection Pumilae and their chromosome numbers

Species	Chromosome number ($2n$)	Distribution
M. asiatica Nakai	34	N. & N.E. China, Korea
M. baccata (L.) Borkh.	34, 68	N. & N.E. China
M. × *domestica*	34, 51, 68	Worldwide
M. floribunda (Siebold) ex. Van Houtte	34	Japan
M. halliana Koehne	34	Japan
M. hupehensis (Pamp.) Rehder	51	Central China
M. mandshurica (Maxim.) Kom. ex Skvortsov	34	Manschuria
M. micromalus Makino	34	S.E. China, Korea
M. orientalis Uglitzk.	?	Caucasia
M. prunifolia (Willd.) Borkh.	34	N. & N.E. China, Korea
M. pumila Mill.	34	Europe
M. sieversii (Ledeb.) M. Roem.	?	N.W. China
M. spectabilis (Aiton) Borkh.	34, 68	China
M. sikkimensis (Wenz.) Koehne ex C. K. Schneid.	51	Himalaya
M. sylvestris (L.) Mill.	34	Europe

Adapted from Way et al. 1991

seasonally grazed their animals. These trees or their open pollinated descendants may be among the horticulturally elite specimens observed in some of the forests today.

The most likely beginning of cultivation was in the region between the Caspian and Black seas (Vavilov 1949–1950); apple cultivation had reached the Near East by 3,000 B. P. (Zohary and Hopf 1993). The Romans spread the apple across Europe during their invasions and it was dispersed to the New World by European settlers during the sixteenth century.

The passage of trade routes from China to the Middle East and Europe through Central Asia probably facilitated repeated short and long distance dispersal to the east and west, either intentionally or unintentionally, of *M. sieversii* and its hybrid derivatives. The *M.* × *domestica* Borkh complex may have arisen through hybridization with species native to China including *M. prunifolia* (Willd.) Borkh.,

M. baccata (L.) Borkh., *M. mandshurica* (Maxim.) Kom. ex Skvortsov, and *M. sieboldii* (Regel) Rehder. To the west, hybridization with the local species *M. sylvestris* (L) Mill. and *M. orientalis* Uglitzk. is conjectured (Ponomarenko 1983, Morgan and Richards 1993, Hokanson et al. 1997, Juniper et al. 1999).

During the late nineteenth and twentieth centuries, *M.× domestica* cultivars found or bred in Europe, Russia, North America, New Zealand, Japan, and Australia were introduced throughout the world and form the basis for most current commercial apple production (Way et al. 1991, Janick et al. 1996). Several species are known to have contributed to the *M.× domestica* complex in modern breeding programs including *M. floribunda* Siebold ex Van Houtte, *M.× micromalus* Makino, *M.× atrosanguinea* (hort ex Späth) C.K. Schneid., *M. baccata* (L.) Borkh., *M. zumi* (Matsum.) Rehder, and *M. sargentii* Rehder (Ponomarenko 1983, Way et al. 1991, Janick et al. 1996).

In southern and eastern Asia, Nai, or the Chinese soft apple, *M.× asiatica* Nakai, was the primary cultivated apple for over 2000 years until *M.× domestica* was introduced in the late nineteenth and early twentieth centuries (Morgan and Richards 1993, Zhang et al. 1993, Watkins 1995, Zhou-Zhi 1999). *Malus ×asiatica* is likely a hybrid complex derived primarily from *M. sieversii* with *M. prunifolia* and perhaps other species.

Prehistoric remains and historical records, reviewed by Morgan and Richards (1993), provide evidence of the cultivation, dispersal, and human use of the apple in the Asia and Europe over the last several thousand years. Archaeological remains of apple that dated to about 6500 BC were found in Anatolia, though it is impossible to know the source of this fruit or whether it was cultivated. Historical evidence referring to apple cultivation dates to the second millennium BC from Anatolia, and northern Mesopotamia. By 500 BC, the apple likely was cultivated widely throughout the Persian Empire as fruit orchards are prominently featured in writings from the period. When Alexander the Great conquered the Persians around 300 BC, the cultivation of fruits was dispersed throughout the Greek world. By this time, the Greek philosopher, Theophrastus, distinguished the sweet cultivated apple from astringent wild forms.

The ascendance of the Roman Empire spread cultivation of the domesticated apple north and west through Europe where it supplanted and likely hybridized with, the native crab apple, *M. sylvestris*. Multiple varieties were recorded by the Roman writer, Pliny, and they attained an important place in Roman cuisine, medicine, and aesthetics by the first century AD. The Roman goddess Pomona was revered as the deity associated with apple and other fruits. With the rise and spread of Christianity and Islam over the next several centuries, apples were carefully maintained, even through wars and difficult times, in the abbey gardens throughout Europe and the orchards of Iberia. These apparently replaced the native crab apples that had a place in the diet of early Celts, Gauls, Franks, Scandinavians and other peoples of northern Europe in fermented, dried, or cooked forms. Maintenance of fruit gardens was encouraged as a basic monastic skill and many abbeys developed large orchards with many *M.× domestica* cultivars. Likewise in the Muslim world of the eastern

Mediterranean and Iberia, fruit growing was revered in keeping with Koranic teachings and skills of grafting, training and pruning became highly developed.

Today, the largest collection of apple germplasm is held at the Plant Genetics Resource Unit at Cornell University, Ithaca New York, where there are almost 4,000 accessions being maintained (http://www.ars.usda.gov/main/site_main.htm?modecode=19100500). Many of these genotypes were collected from the apples center of diversity in Central Asia (Hokanson et al. 1997, Forsline 2003).

1.3 History of Improvement

From the thirteenth century, apples became more and more widely planted throughout Europe in gardens of royalty and commoners. Raw apples were occasionally consumed, but they were more greatly prized when cooked and sometimes blended with spices and sugar or honey. Fermented juice, or cider, like beer, was preferred to the sometimes questionable local water supply. By the seventeenth century there were at least 120 cultivars described in western Europe. The rise and spread of Protestantism, which saw the apple as the special fruit of God, is credited with expanding apple cultivation across northern and eastern Europe after beginning in Germany in the early seventeenth century. By the end of the eighteenth century, many hundreds of cultivars were recognized throughout Europe. The Royal Horticultural Society of England acknowledged at least 1200 in 1826. The eighteenth and nineteenth centuries saw apple cultivars recognized and classified based on their suitability for their end uses. Aromatic dessert apples were more widely appreciated by this time, while good cooking types were still appreciated for puddings and pastries. Flavorful varieties with moderate acid and tannin levels were prized for cider production. The late nineteenth and early twentieth centuries represented the maximum of diversity in apple cultivation in Europe with hundreds of locally popular varieties being grown in thousands of small orchards. In the twentieth century, the rise of imported fruit from the Americas, New Zealand, Australia, and South Africa forced European orchards to increase in size and decrease in number and, to a large extent, to adopt the very same cultivars that were developed in, and imported from the New World.

Apples were established in the 1650s near Cape Town in South Africa to sustain settlers and to supply the ships of the Dutch East India Company. The commercial orchard district in the Western Cape apple was started by Cecil Rhodes and his associates in the late nineteenth and early twentieth century to replace a faltering wine industry.

Apples were introduced to Australia, on the island of Tasmania and at the present site of Sydney, in 1788. Orchards were established by settlers in Tasmania and New South Wales by the early 1800s. Significant production areas were eventually developed in Tasmania and the southeastern mainland. In 1814, English missionaries brought apples from Australia to New Zealand where two large apple production districts became established in the districts of Hawkes Bay and Nelson during the nineteenth and twentieth centuries.

Beginning in the sixteenth and seventeenth centuries, European colonists brought apples to the Americas. Spanish priests introduced them to their missions in Chile and California. Spanish and Portuguese settlers introduced apples to their settlements in suitable temperate climate zones of South America. European settlers brought apple seeds to establish orchards in the eastern United States and Canada. Apples grew well from northern Georgia through eastern Canada and, as in Europe, were soon highly prized for food and drink, and as a source of sugar and alcohol. The first orchards in New England were recorded in the 1620s and 1630s and became important components of the New England farmstead. Likewise, they became important on the large plantations of the mid-Atlantic colonies by the mid-1700s, including those of the early United States presidents, George Washington and Thomas Jefferson. Jefferson, an astute horticulturist, acquired and carefully trialed dozens of cultivars for his Monticello gardens in Virginia.

In Canada, French colonists established orchards in the seventeenth century along the St. Lawrence Valley. Settlers also established orchards around Lake Ontario, and in the milder valleys of Nova Scotia and New Brunswick.

As settlers moved westward in the United States, apple orchards were a requirement of homesteading throughout the territories of the Ohio River Valley. Jonathan Chapman, known as Johnny Appleseed, devoted his later life, from 1806 to 1847, to helping settlers establish thousands of apple trees on their new farms in the Ohio River drainage. The Great Lakes region of the United States, especially the states of New York, Michigan, and Ohio, continues to be a major apple production area.

In 1847, as settlers moved in to the productive valleys of western Oregon, Washington and northern California, Henderson Llewelling brought 700 trees with his family on the Oregon Trail and eventually established the first fruit nursery in the Pacific Northwest. As irrigation schemes were eventually developed in the Pacific Northwest, especially in the basin of the Columbia River and its tributaries west of the Cascade Mountains and extending to the Okanagon River valley in British Columbia, this region became one of the preeminent apple production areas of the world.

By the early twentieth century, the United States and Canada were the two largest apple producing nations. Later in the century, the Soviet Union also became important. At the beginning of the twenty-first century, China become the largest apple producer, with a large proportion of the crop being exported as concentrated juice. Major southern hemisphere production, much of it for export to northern hemisphere countries during their spring and summer, occurs in South Africa, Chile, Argentina, New Zealand, and Australia. As previously mentioned, production is currently dominated by strains of just a few cultivars: ‘Delicious’, ‘Golden Delicious’, ‘McIntosh’, and ‘Jonagold’ from North America; ‘Braeburn’ and ‘Gala’ from New Zealand; ‘Granny Smith’ from Australia; and ‘Fuji’ from Japan. Though many other cultivars remain locally important, these dominate current production and are also widely used in breeding programs around the world.

From its origins among the millions of wild *M. sieversii* trees in the mountains of central Asia (Fig. 1.2), and from the early development of thousands of local

Fig. 1.2 Photo showing the diversity of fruit collected from wild *Malus sieversii* apple trees in the Tarbagatai mountains of eastern Kazakhstan

cultivars in Europe and America, the domesticated apple, as cultivated in twenty-first century, has shrunk drastically in diversity.

1.4 Current Breeding Efforts

The overall objectives of modern breeding programs are to increase the marketability of fruit and reduce production costs. Apples are sold fresh, juiced and processed in numerous ways, but the largest overall market involves fresh fruit. Numerous apple species are also important as ornamentals (Fiala 1994).

Dessert apples are sold primarily based on appearance (size, color, shape and freedom from blemishes) and quality (taste and texture) (Janick et al. 1996; Laurens 1999; Brown and Maloney 2003). There is considerable regional variation in taste preferences, from a desire for tartness in Europe and the U.S. Midwest, to a preference for sweetness and low acidity in Asia. Low allergenic apples have become a priority in Europe. Favored colors range widely from solid green, yellow to red and bicolors of many combinations. In general, apples are expected to be blemish free, large (>70 mm in diameter), and ovate or conic shaped. Storage life is also a critical parameter, as most apples are stored for long periods of time. Resistance to apple scab and powdery mildew are common breeding goals. Niche markets are also arising for improved nutritional aspects such as higher antioxidants.

The attributes needed for processed fruit depends on their final market. Some of the most important markets are for cider, sauce and slices (Crassweller and

Green 2003). Less browning is a particularly critical parameter in the fresh cut and slices market.

Production costs are greatly reduced by maximizing yields, increasing picking efficiency and incorporating disease and pest resistance. Adaptation is a key parameter associated with yield, particularly in marginal climates with extreme winters or low chilling hours. Pest and disease resistance is critical to productivity in areas where other means of control are not available or undesirable. This applies particularly to growers interested in producing 'organic' apples.

The apple is generally grown as a composite tree with a rootstock and a fruiting scion, making rootstock breeding as important as the development of scion cultivars. There are a number of important attributes of rootstocks including; ease of propagation, clean upright stems, easy to bud or graft, well anchored root systems, no suckering, and good stock-scion compatibility (Janick et al. 1996). Rootstocks should also offer a range of tree size control from dwarfing to vigorous, induce early, heavy cropping, tolerance to cold and wet or dry soils, and be resistant the prevailing pests and diseases (Webster and Wertheim 2003).

Brown and Maloney (2003) reviewed current breeding programs and activities throughout the world. In the US, a new program was started in 1994 at Washington State University. Other programs in the US include the PRI (Purdue University, Rutgers University and the University of Illinois) cooperative that has concentrated on developing scab resistant cultivars, the University of Minnesota (of 'Honeycrisp' fame), and Cornell University, best known for 'Empire' and 'Jonagold'. The New Zealand program has been an innovator in the licensing and restricted availability of selections from their program. Increasingly, programs are partnering with private industry, examples include the collaboration between breeders, nurseries and packers in France (Laurens and Pitiot 2003).

1.5 Genetics of Economically Important Traits

1.5.1 Pest and Disease Resistance

Genetic resistance has been found for a number of the major pests and diseases of apple including: Fire blight (*Erwinia amylovora*), Alternaria blotch (*Alternaria mali*), apple blotch (*Phyllosticta solitaria*), apple canker (*Nectria galligena*), apple scab (*Venturia inaequalis*), cedar apple rust (*Gymnosporangium juniperi-virginianae*), crown rot (*Phytophthora cactorum*), powdery mildew (*Podosphaera leucotricha*), wooly apple aphid (*Eriosoma lanigerum*), rosy apple aphid (*Dysaphis plantaginea*) and rosy curling aphid (*D. devecta*) (Table 1.2).

Resistance to Alternaria blotch, crown rot, wooly apple aphid, rosy apple aphid and rosy curling aphid are regulated by a single dominant gene. RFLP markers have been found for resistance to rosy leaf curling aphid (Roche et al. 1997). Apple blotch and apple rust resistance are regulated by two dominant genes, and a

Table 1.2 Genetics of pest and disease resistance in apple

Pest or disease	Observations and source
Bacterial	
Fire blight *Erwinia amylovora*	Immunity is present in some *Malus* species (Janick et al. 1996); QTLs for resistance reported by Khan et al. (2006)
Fungi	
Alternaria blotch *Alternaria mali*	Resistance is controlled by a single dominant gene (R^{alt}) which is epistatic to a dominant gene (Alt) controlling susceptibility (Saito and Niizeki 1988)
Apple blotch *Phyllosticta solitaria*	Susceptibility is regulated by two dominant genes (Ps_1 and Ps_2) with duplicate recessive epistatic interaction between gene pairs (Mowry and Dayton 1964)
Apple canker *Nectria galligena*	Highly resistant cider apples and rootstocks have been identified (Moore 1960)
Apple scab *Venturia inaequalis*	Both quantitative and qualitative resistance exists; major source is a dominant gene, V_f; multiple resistance genes at the V_f locus have been found in several species; the resistance of V_f is enhanced by polygenes (Dayton and Williams 1968, Crosby et al. 1992, Bus et al. 2002); numerous QTL and markers identified (Tartarini and Sansavini 2003, Durel et al. 2004, Bus et al. 2005a,b, Hemmat et al. 2004)
Cedar apple rust *Gymnosporangium juniperi-virginianae*	Regulated by two, dominant genes (Gy-a and Gy-b) and perhaps other modifying genes; resistance mechanisms vary (Mowry 1964, Aldwinckle et al. 1977, Chen and Korban 1987)
Crown rot *Phytophthora cactorum*	Regulated by a single dominant gene (P_c), but polygenes are important (Alston 1970, Watkins and Werts 1971)
Powdery mildew *Podosphaera leucotricha*	Regulated by several dominant genes (Pl_1 and Pl_2) and polygenes; resistance may be enhanced by polygenes (Alston 1977, Korban and Dayton 1983, Gallott et al. 1985); quantitative resistance will be needed for durable resistance (Caffier and Parisi 2007); markers identified for the dominant alleles Pl1, Pl2, Pl-d, Pl-w, Pl-m (Markussen et al. 1995, Durel et al. 2002, Evans and James 2003)
Insects	
Wooly apple aphid *Eriosoma lanigerum*	Regulated by a single dominant gene (*Er*); 'Northern Spy' has high level of resistance, along with several other cultivars; resistance gene is closely linked to incompatibility gene (Knight et al. 1962, Knight 1962, Cummins et al. 1981); markers identified for *Er1* and *Er3* (Sandanayaka et al. 2003)
Rosy apple aphid *Dysaphis plantaginea*	Regulated by a single dominant gene (Sm_h); resistance found in open pollinated selection of *M. robusta* (Alston and Briggs 1970)
Rosy curling aphid *D. devecta*	Regulated by a single dominant gene; four different resistance genes have been identified in 'McIntosh' (SD_{pr}) 'Cox's Orange Pippin' (Sd_1), 'Northern Spy' (Sd_2) and *Malus robusta* (Sd_3); a precursor gene must be present for effective resistance (Alston and Briggs 1977); markers identified to *Sd1* (Roche et al. 1997, Cevik and King 2000)

number of polygenes. Genes for resistance to powdery mildew have been identified and markers have been developed: Pl_1 (Markussen et al. 1995), Pl_2 (Dunemann et al. 1999), *Pl-d* (James et al. 2004), *Pl-w* (Evans and James 2003). A marker has yet to be developed for *Pmis* from Mildew immune seedling (MIS). Resistant *Malus* genotypes have also been identified for fire blight and apple canker, although the genetics have not been elucidated. 'Delicious' has fairly good resistance to fire blight, but immunity is only found outside of the cultivated species of apple (Janick et al. 1996).

At least 10 different resistance genes have been identified for apple scab (Bus et al. 2002), and one of them, *Vf* from the ornamental crabapple *M. floribunda 821*, has been used all over the world to create new scab resistant cultivars (Laurens 1999). This gene has been cloned and shown to confer scab resistance to a transgenic cultivated variety 'Gala' (Belfanti et al. 2004). Scar markers have been identified for *Vbj* from *Malus baccata jackii* (Gygax et al. 2004) and *Vm* from *M. atrosanguinea* 804 (Cheng et al. 1998). Patocchi et al. (2005) used genome scanning to identify a microsatellite tightly linked to *Vm*. Numerous other QTL have been identified for scab resistance, which will be described in the section on genetic mapping of apple. Gessler et al. (2006) review scab resistance in apple.

In breeding for multiple disease or pest resistance a balance of resistance and commercial fruit quality may be difficult to achieve. Adequate levels of resistance to the major fungal pathogens (scab and powdery mildew) coupled with resistance to fire blight requires large populations and great attention to other secondary problems such as leaf spot, moldy core and summer fruit pathogens.

Attempts have been made to associate specific enzyme activities with resistance to superficial storage scald. The total activities of guanacol-dependent peroxidase (POX), superoxidismutase (SOD) and catalase (CAT) were not found to be significantly associated with susceptibility to scald in a segregating population of 'White Angel' × 'Rome Beauty'; however, there were associations with the presence and absence of individual isozymes (Kochhar et al. 2003).

Several genes have been isolated that are related to disease resistance. A cDNA has been cloned from fruit of 'Fuji' that encodes a pathogenesis-related 5/thaumatin-like protein (PR5/TL) that was named Mdt 1 (*Malus domestica* thaumatin-like protein) (Oh et al. 2000). A salicylate-inducible PR-10 gene (designated as *APa*) was found to be expressed during infection of a compatible vs. a non-compatible race of *V. inaequalis* (Poupard et al. 2003). Eighteen genes were identified as having higher expression levels during infection of 'Golden Delicious' by *Penicillium expansum* (Sáchez-Torres and González-Candelas 2003). Two of these genes likely encoded *SS*-glucosidase and phosphatase 2C.

A number of apple sequences have been identified that are similar to the R (resistance) genes of other plants that contain a nucleotide binding site (NBS). NBS-containing genes are the most common class of resistance genes found in plants. Over 20 families of NBS-containing genes have been identified in apple that include the two major groups described in dicot plants, one lacking a toll-interleukin element and one containing it (Baldi et al. 2004, Calenge et al. 2005). A cluster of receptor-like genes has been identified in bacterial artificial clones derived from the

Vf scab resistance locus that are similar to the *Cladosporium fulvum* (Cf) resistance gene family of tomato (Vinatzer et al. 2001).

1.5.2 Morphological and Physiological Traits

Most of the traits associated with adaptation and productivity have been shown to be quantitatively controlled, including chilling requirement, cold hardiness, plant vigor, season of flowering and duration of the juvenile period (Table 1.3).

Several aspects of plant habit have been shown to be regulated by single genes in inheritance studies (Alston et al. 2000). A dominant gene regulates the columnar habit in the 'Wijcik' clone of 'McIntosh' (Lapins and Watkins 1973, Lapins 1974). A series of recessive alleles have been identified that regulate dwarfing (Decourtye 1967, Alston 1976). Recessive genes have been associated with the spur-habit in sports of 'Golden Delicious', 'Redspur' and 'Starkrimson' (Decourtye and Lantin 1969, Alston and Watkins 1973). RAPD markers have been identified for terminal bearing, initial bud break, root sucker formation (Weeden et al. 1994) and columnar tree habit (Hemmat et al. 1997).

Several cDNA libraries have been developed to identify genes associated with pollination and apple fruit development (Dong et al. 1997, 1998b, Sung et al. 1998; Yamada et al. 1999). *Mdh3* encoding a Phalaenopsis O39-like homeodomain protein was found to be expressed in apple ovules and may initiate the program of ovule development (Dong et al. 1999). A homologue of mammal *DAD1* (defender against cell death 1) was cloned that is expressed after flower pollination and during senescence of leaves, petals and fruit (Dong et al. 1998c). A gene encoding polygalacturonase-inhibiting protein (PGIP) was isolated that has two peaks of expression during apple maturity and is activated by wounding and fungal infection (Yao et al. 1999).

A number of MADS-box genes have been cloned and characterized from apple (Table 1.4). These genes produce transcription factors which play an important regulatory role in the development of floral meristems in all plants. To date, one of the most interesting MADS-box genes that has been cloned is a mutation of *MdP1* caused by a retrotransposon insertion, which abolishes gene expression and leads to parthenocarpic fruit development (Yao et al. 2001).

Homeobox genes have also been identified in apple that encode homeodomain proteins which are transcription factors that regulate a number of developmental processes. Watillon et al. (1997) identified three KNOTTED1 (*kn1*)-like homeobox genes, *KNAP1–3*. Transcripts from *KNAP3* accumulated in a wide rang of vegetative and reproductive organs, while mRNAs from *KNAP1* and *KNAP2* were present primarily in elongated parts of stems. Sakamoto et al. (1998) isolated two additional (*kn1*) -like homeobox genes, *APHB1* and *APHB2*. *APHB1* are expressed in shoot apical tissues, stems and flowers but not mature leaves and fruit. *APHB2* is expressed in all organs involving mature leaves and developing fruit. Another homeobox gene, *MDH1*, was isolated from developing fruit, flowers and leaves of apple that has a homeodomain similar to BEL1 which is involved in ovule development in *Arabidopsis* (Dong et al. 2000).

Table 1.3 Genetics of adaptation, productivity, plant habit and fruit quality in apple

Attribute	Observations and source
Adaptation	
Chilling requirement	Generally quantitatively inherited, although the low chill requirement of 'Anna' is thought to be controlled by one major gene and a number of minor ones (Hauagge and Cummins 1991, Labuschagne et al. 2002); bud break number is highly heritable (Labuschagne et al. 2003)
Cold hardiness	Quantitatively inherited, largely additive (Watkins and Spangelo 1970)
Growth rate	Quantitatively inherited; QTL identified (Conner et al. 1998)
Vigor	Quantitatively inherited, largely additive (Watkins and Spangelo 1970, Durel et al. 1998)
Season of flowering	Quantitatively inherited, largely additive (Janick et al. 1996); QTL identified (Liebhard et al. 2003a)
Harvest date	Quantitatively inherited; QTL identified (Liebhard et al. 2003a)
Productivity	
Flower number	Quantitatively inherited; QTL identified (Liebhard et al. 2003a)
Fruit number	Quantitatively inherited; QTL identified (Liebhard et al. 2003a)
Juvenile phase length	Quantitatively inherited; QTL identified (Liebhard et al. 2003a)
Incompatibility	Numerous S-alleles exist, are semi-compatible combinations (Broothaerts et al. 2004a,b)
Plant habit	
Compact	Single dominant gene (*Co*) identified for compact columnar habit in 'Wijcik' clone of 'McIntosh'; genetics more complex in other sources of the compact branching habit (Lapins and Watkins 1973, Lapins 1974); markers identified (Conner et al. 1997, Kim et al. 2003a,b, Tian et al. 2005)
Dwarfing	Several sources of dwarfing exist that are regulated by recessive alleles (d_1–d_4) (Decourtye 1967, Alston 1976)
Internode length and N°	Quantitatively inherited; QTL identified (Conner et al. 1998)
Spur bearing habit	Generally quantitatively inherited, but spur-habit in sports of 'Golden Delicious', 'Redspur' and 'Starkrimson' result from a single recessive gene (Decourtye and Lantin 1969, Alston and Watkins 1973)
Russett	Single dominant gene identified with many modifying polygenes (Alston and Watkins 1973, Durel et al. 1998); some russeted clones do not transmit russet to offspring
Fruit quality	
Acidity	Quantitatively inherited; QTL identified for malic acid gene, *Ma* (Maliepaard et al. 1998, Liebhard et al. 2003a)
Bioactive compounds	High variability among cultivars in ascorbic acid and phenolic compounds (Schmitz-Eiberger et al. 2003); QTL identified by Davey et al. (2006)
Firmness	Quantitatively inherited; QTL identified (Seymour et al. 2002, Liebhard et al. 2003a)

Table 1.3 (continued)

Attribute	Observations and source
Skin color	Quantitative but may be regulated by a few major genes; one possibility is that three dominant, major genes produce color (Lespinasse et al. 1988, Brown 1992); marker identified for a fruit color gene (*Rf*) regulating the red/yellow dimorphism (Cheng et al. 1996); EST research indicated that myb transcription factors are important (Espley et al. 2007, Takos et al. 2006)
Shape	Quantitatively inherited with a low genotype by environment interaction (Currie et al. 2000)
Size/weight	Quantitatively inherited (Durel et al. 1998); highly heritable (Volz et al. 2001); QTL identified (Liebhard et al. 2003a)
Storage disorders	High heritability for soft scald and superficial scald; moderate heritability for water core; low heritability for external pit, internal pit, brown heart, breakdown and chilling injury (Volz et al. 2001)
Sugar content	Quantitatively inherited; QTL identified (Liebhard et al. 2003a)
Texture	Quantitatively inherited (Durel et al. 1998); QTL identified (Seymour et al. 2002); *Md-ACS1* found to be closely associated with fruit softening (Costa et al. 2005)

1.5.3 Fruit Quality

Many of the traits associated with fruit quality are quantitatively inherited including flavor, shape, size and texture (Table 1.3). Skin color is also quantitatively inherited, but the number of major genes regulating it may be limited. Anthocyanin stripes are regulated by a single dominant gene in 'Cox's Orange Pippin' (Klein 1958). It has been proposed that three, dominant major genes regulate color (A, B and C) (Lespinasse et al. 1988). Yellow is produced by one dominant allele, red if more than two dominant alleles are present. The yellow cream flesh color of 'Cox's Orange Pippin' is dominant (Alston 1981). Russett is regulated by a single dominant gene, with numerous interacting polygenes (Alston and Watkins 1973), yet some russetted clones must only have the mutation in the L1 as they do not transmit this trait to their offspring. A RAPD marker has been identified for fruit skin color (Cheng et al. 1996). Recently the myB transcription factor has been suggested to regulate apple red fruit color (Takos et al. 2006, Espley et al. 2007).

Genes have been identified and cloned that influence fruit quality. An allele of the 1-methycyclopropene softening slower gene (*Md-ACS1*) was found that was significantly associated with softening (Oraguzie et al. 2004 and 2007).

Genes associated with anthocyanin biosynthesis were cloned from apple fruit skin, and cDNAs were identified that encode flavanone 3-hydroxylase (F3H), dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT). Each gene was found to be a member of a multigene family. The mRNAs of these genes were detected preferentially in the skin tissue and were light induced. The transcripts were more abundant in the skins

Table 1.4 MADS-box genes cloned and characterized in apple

Gene	Observations and source
AFL1	Expressed only in the floral bud during the transition from vegetative to reproductive growth. Orthologue of *Arabidopsis FL* (Wada et al. 2002)
AFL2	Expressed in vegetative shoot apex, floral buds, floral organs and root. Orthologue of *Arabidopsis FL* (Wada et al. 2002)
MdMADS1	Expressed in all floral organs and young fruits, but not in leaves. Expression highest in early stages of flower and fruit development. Shows significant sequence homology with *Arabidopsis* AGL2 (Sung and An 1997)
MdMADS2	Transcribed in all four floral organs and at all stages of flower development. Member of SQUA subfamily of snapdragon. Transgenic tobacco expressing it flowered early and had shorter bolts, but showed no homeotic changes in the floral organs (Sung et al. 1999)
MdMADS3	Expressed in the inner three whorls of the floral primordium, but not in fruit. Showed high sequence homology with *Arabidopsis* AGL2 and AGL4 (Sung et al. 2000)
MdMADS4	Expressed ubiquitously in inflorescence meristem, floral meristem, fruit and seeds. Showed high sequence homology with *Arabidopsis* AGL2 and AGL4 (Sung et al. 2000)
MdMADS5	More strongly expressed in fruit than flower buds; not expressed in leaves. Highest expression in young fruit. Most strongly expressed in cortex and skin, little expression in core. Showed high sequence homology with *Arabidopsis AP1* (Yao et al. 1999b). Expressed specifically in sepals during flower bud formation (Kotoda et al. 2000). Transgenic *Arabidopsis* with this gene flowered early (Kotoda et al. 2002)
MdMADS6	More strongly expressed in fruit than flower buds. Highest expression in young fruit. Most strongly expressed in cortex and skin, but significant expression in core. Showed high sequence homology with *PrMADS1* and *MdMADS7* (Yao et al. 1999)
MdMADS7	More strongly expressed in fruit than flower buds. Highest expression in older fruit. Most strongly expressed in cortex and skin, but significant expression in core. Showed high sequence homology with *PrMADS1* (Yao et al. 1999)
MdMADS8	More strongly expressed in fruit than flower buds. Highest expression in young fruit. Most strongly expressed in core and cortex; weak expression in skin. Showed high sequence homology with *AGL2*, *AGL4*, *MdMADS1* and *MdMADS9* (Yao et al. 1999)
MdMADS9	More strongly expressed in fruit than flower buds. Highest expression in younger fruit. Most strongly expressed in core and cortex; weak expression in skin. Showed high sequence homology with *Arabidopsis AGL2* and *AGL4* (Yao et al. 1999)
MdMADS10	More strongly expressed in fruit than flower buds. Highest expression in young fruit. Only expressed in core. Showed high sequence homology with *Arabidopsis AGL11* (Yao et al. 1999)
MdMADS11	High expression in both fruit and flower buds. Preferentially expressed in fruit after pollination. Highest expression in young fruit. Evenly expressed in all three fruit tissues. Showed high sequence homology with *Arabidopsis AGL6* (Yao et al. 1999)

Table 1.4 (continued)

Gene	Observations and source
MdMADS12	Isolated from leaf tissue but has significant homology with the *Arabidopsis* floral identity gene *AP1*. Expressed at similar levels in leaves, vegetative shoots and floral tissues. May play a role in the transition from juvenile to adult stage (van der Linden et al. 2002)
MdMADS13	Isolated from leaf tissue but has significant homology with the *Arabidopsis* floral identity gene *AP3*. Mainly expressed in petals and stamens (van der Linden et al. 2002, Kitahara et al. 2004)
MdMADS14	Isolated from leaf tissue but has significant homology with the *Arabidopsis* floral identity gene *AGAMOUS*. Preferentially expressed in carpels. Possible orthologue of *SHATTERPROOF* (van der Linden et al. 2002)
MdMADS15	Isolated from leaf tissue but has significant homology with the *Arabidopsis* floral identity gene *AGAMOUS*. Highly expressed in stamens and carpels (van der Linden et al. 2002)
MdPI	Shows high amino acid sequence identity with *Arabidopsis PI*. A retrotransposon insertion was identified that abolished expression of the gene and probably led to parthenogenic fruit development (Yao et al. 2001)

of cultivars with red skin than non-red, indicating that these genes have major roles in determination of apple skin color.

The expression of six genes (*PAL, CHS, CHI, F3H, DFR* and *ANS*) involved in anthocyanin production was also studied during flower development (Dong et al. 1998a). Maximum accumulation of all 6 RNAs was highest during early flower development and dropped drastically after petal expansion. Blocking of UV or natural light greatly reduced expression of these six genes and inhibited anthocyanin production, and after re-exposure to light, white flowers were not able to resynthesise anthocyanins.

In a study of the genetics of commonly found storage disorders, Volz et al. (2001) found high heritability for soft scald and superficial scald, moderate heritability for water core, and low heritability for external pit, internal pit, brown heart, breakdown and chilling injury. Two of the genes that are likely associated with superficial scald have been cloned from apple, *hmg1* and *hmg2* (Rupasinghe et al. 2001, Pechous and Whitaker 2002). These genes encode 3-hydoxy-3-methylglutaryl coenzyme A reductase (HMGR) which catalyses the synthesis of mevalonate from HMG-CoA. Superficial scab is thought to be caused by the oxidation of α-farnesene in apple skin and α-Farnesene is produced in the mevlonate pathway. A gene encoding (E,E)-α-farnesene synthase gene (*AFS1*) has also been cloned that uses farnesyl diphosphate as a substrate. (Pechous and Whitaker, 2004).

Boss et al. (1995) identified the gene for a full length polyphenol oxidase (*pAPO5*) from a 'Granny Smith' fruit peel cDNA library whose expression was induced by wounding and was elevated in peel with superficial scald. Polyphenoloxidase (PPO) is thought to play an important role in browning after the wounding of apples. Kim et al. (2001) cloned *pPPO5* from 'Fuji' and identified another full length PPO gene, *pMD-PPO2*, which shared about 55% identity. *MD-PPO2*

was expressed in all stages of flower development, while the *APO5* transcript was detectable only in late anthesis. Both genes were expressed during early fruit ripening; however, only *APO5* was significantly induced by wounding.

Overall, flavor is a quantitative trait, but some of its individual components have been found to be regulated by single genes. The distinct aroma of 'Cox's Orange Pippin' is the result of a single dominant allele (Alston and Watkins 1973). A dominant allele also determines moderate to high acidity, with the specific levels being inherited quantitatively (Nybom 1959, Brown and Harvey 1971). Resistance to bitter pit was reported to be regulated by two dominant alleles (Korban and Skirvin 1984). Souleyre et al. (2005) have isolated an alcohol acyltransferase that produces esters involved in flavor.

A number of recent studies have shown that the antioxidant capacity of apples can have wide ranging health effects including the inhibition of colon- and liver-cancer cells (Eberhardt et al. 2000, Schirrmacher and Schempp 2003). Schmitz-Eiberger et al. (2003) found high variability among cultivars in ascorbic acid and phenolic compounds. 'Topaz', 'Berlepsch', 'AW 93', 'Golden Delicious', 'Rubinette', 'Braeburn' and 'Honeycrisp' had among the highest levels of ascorbic acid, while the highest levels of phenolics were found in 'Scesterimuher', 'Bortlinger', 'Bohnapfel' and 'Dulmener Rosenapfel'. Lee et al. (2003) found 'Rhode Island Greening' to have unusually high levels of antioxidants, while Lata (2007) documented the effect of cultivar and seasonal variation. Davey et al. (2006) identified QTL affecting vitamin C in apple using the mapping population of 'Braeburn' × 'Telamon'.

Two types of genes associated in hormone biosynthesis have been cloned and isolated. Kusaba et al. (2001) isolated a cDNA encoding gibberellin (GA) 20-oxidase that was mainly expressed in immature seeds. Wegrzyn et al. (2000) cloned an α-amylase gene from apple fruit that was transiently upregulated during low temperature exposure. Stanley et al. (2002) also isolated several α-amylase genes from apple and *Arabidopsis* that they suggested might be targeted to different compartments within the cell (cytosol, secretory pathway and plastid).

Genes for ACC-synthase, ACC-oxidase and polygalacturonadase (PG) have been cloned and characterized from apple (Dong et al. 1991 and 1992, Castiglione et al. 1998) and the promoter sequences of the genes for ACC-oxidase and PG have been characterized (Atkinson et al. 2005). Castiglione et al. (1998) examined restriction products across 12 *Malus* species and found two allelic forms of a gene for ACC-oxidase but very little variability in a gene for ACC-synthase. They suggested that the two allelic forms of ACC-oxidase might control the rate of ethylene synthesis and could be used in marker assisted selection. Harada et al. (2000) found a specific allele of ACC-synthase (*Md-ACS1-2*) that was associated with low levels of ethylene production in a screen of 35 cultivars. Atkinson et al. (1998) examined the expression of PG and ACC-oxidase mRNAs and detected them earlier in 'Royal Gala' apples relative to internal ethylene concentration than 'Braeburn' and 'Granny Smith'.

Tao et al. (1995) identified a cDNA encoding NADP-dependent sorbitol-6-phosphate dehydrogenase (S6PDH) from apple and found that protein levels were

highest in mature fruit. Sorbitol is the end product of photosynthesis in apple. Tao et al. (1995) incorporated the gene into tobacco and found that sorbitol levels were positively correlated with S6PDH activity levels. Yamada et al. (1998) identified a full-length cDNA for NAD-dependent sorbitol dehydrogenase (NAD-SDH) and showed that the mRNA is expressed in mature apple fruit.

A number of plant-derived allergens have been identified and placed into specific groups, including pathogenesis-related proteins (PR), seed storage proteins and structural proteins (Hoffmann-Sommergruber 2002). Representatives of three families of PR genes have been cloned and characterized in apples including: (1) *Mal d 2*, producing a thaumatin-like protein (Krebitz et al. 2003), (2) *Ypr10*, producing a intercellular protein with unknown enzymatic action (Puhringer et al. 2000), and (3) *Mal d 3*, producing a lipid transfer protein (LTP) (Diaz-Perales et al. 2002). The promoter of *Ypr10* is both stress- and pathogen-inducible, and the product of *Mal d 2* has anti-fungal properties. Gao et al. (2005a,b) cloned and mapped *Mal d 1* and also mapped *Mal d 2* and *4*. The location of allergens was studied by Marzban et al. (2005). *Mal d 1* and *2* were distributed in peel and flesh, while *Mal d 3* is restricted to the peel.

Breeding programs are assessing low allergenicity as a breeding objective. Gao et al. (2005a) cloned and mapped the major apple allergen *Mal d 1*, and then studied *Mal d 3* (2005b). Carnes et al. (2006) demonstrated differences in antigenic and allergenic profiles for 10 different apple varieties and found significant variation in content of *Mal d 3*.

1.6 Crossing and Evaluation Techniques

1.6.1 Breeding Systems

Most apples require cross-pollination; in the orchard, pollination is carried out primarily by bees. Self-fertility is limited in apple by gametophytic self-incompatibility, where the growth of self pollen tubes is prevented by cytotoxic proteins that are produced in the stigmatic tissue. Attack is avoided if specific inhibitors of these proteins are expressed. The style-encoded toxic proteins are RNases which are produced by the *S-gene.* The pollen-expressed inhibitors have not been identified. Allele-specific PCR primers have been developed to selectively amplify and identify individual S-alleles, and 28 S-alleles have been cloned and identified in 150 diploid and triploid European, American and Japanese cultivars (Broothaerts et al. 2004a). Three S-alleles (S_2, S_3 and S_9) are very common and seven are very rare (S_4, S_6, S_8, S_{16}, S_{22}, S_{23} and S_{26}). The allelic composition of the most widely grown cultivars are: Delicious (S_9S_{28}), Golden Delicious (S_2S_3), Granny Smith (S_3S_{23}), Fuji (S_1S_9) and Gala (S_2S_5). S-RNase analysis has been used to identify the parents of Japanese cultivars (Kitahara et al. 2005, Matsumoto et al. 2006). Broothaerts (2003) suggests that some S-alleles be renumbered based on new research.

1.6.2 Pollination and Seedling Culture

The blossom is typically composed of five petals, five sepals, about 20 stamens and a pistil with five styles. The flowers are borne in cymose clusters on short pedicels. Each ovary generally has five carpels with two ovules each, resulting in a total of 10 seeds (although some varieties can have up to 18 seeds).

For pollen collection, flowers are gathered at the balloon stage before petal expansion. Blossoms can be collected from rooted plants in the field or greenhouse, or flowers can be forced in the greenhouse by cutting flowering shoots and holding them in water. Anthers are first removed from flowers by passing them over a screen and then allowed to dehisce overnight in containers such as Petri plates or 'paper boats'. The dry pollen is ready to be used directly in crosses and remains viable for several days at room temperature. It can remain viable for several weeks if refrigerated under low relative humidity. For long term storage, pollen can be held for at least a year at -15°C in loosely stoppered vials in a desiccators with calcium chloride.

To emasculate flowers, fingernails or scissors are generally used to remove sepals, petals and stamens at the balloon stage. Pollinations are generally made soon after emasculation, although flowers are sometimes re-pollinated one day later if conditions are thought to be too cool for normal pollen germination and tube growth. Flowers can be successfully fertilized over a period of several days. Generally, two flowers per cluster are emasculated and the rest are removed. There is no need to cover the emasculated flowers after pollination, as insects do not visit flowers without stamens and petals (Visser 1951). Some breeders do not emasculate at all and rather rely on the self incompatibility system to prevent self fertilization. In this case, flowers are bagged to prevent contamination. Keulemans et al. (1994) discussed the effect of number of flowers pollinated on fruit set in crosses.

Pollen is often placed on the stigmas after dipping a small brush into vials or Petri plates of pollen. Pencil erasers or fingertips are also sometimes used to transfer pollen. After each cross, the pollination vehicle is washed or dipped into 95% alcohol and allowed to dry to prevent cross contamination.

Several other techniques are sometimes employed to eliminate the emasculation step. Small trees can be enclosed in a bee-proof structure with a bouquet of open flowers of the desired parent and honeybees. Bouquets of potential parents with dominant marker genes can also be placed adjacent to several recipients in a field, and the desired hybrids can be identified in the seedling blocks. Seed can also be collected from orchards that contain two cultivars of interest.

Most breeders attempt to get at least 200–300 seeds per cross, although thousands of seeds are sometimes generated of crosses that are thought to have great commercial potential or when seedling screening is planned using inoculation or markers. About 50–100 pollinations are typically required to generate a few hundred seeds, but if flowers are emasculated, the general rule is that on average one flower produces one seed due to damage from emasculation. The number of crosses made varies greatly depending on program objectives, available resources and philosophy of the breeder, but can range from 5 to 50. If both parents are heterozygous for pale

green lethal (Way et al. 1976), dwarfing genes (Alston 1976) or sub-lethals linked to the *Vf* gene (Gao and van de Weg 2006) then greater numbers are needed, due to the expected 25% loss due to lethals and dwarfs.

Apple seeds must be stratified in the cold for successful germination. Seeds can be left in the fruit at slightly above freezing temperatures to naturally after-ripen, but when this is done, molds often become a problem. More commonly, seeds are harvested just before the fruit reach maturity, but late enough for the seed coats to have become dark-brown. The seeds are then held in the cold at 3–5°C for 60–80 days in plastic bags containing moist filter paper or peat moss. Length of stratification may vary depending on the genetic background. Thomsen and Eriksen (2006) found that two *Malus* species (*Malus sargentii* and *M. sieboldii*) differed in their response to pretreatments and stratification temperatures. When their radicals begin to emerge, the seeds are transplanted into pots or trays at 1–2 cm depth. They are maintained under greenhouse conditions for about 60 days until they become 30–45 cm tall, and then they are planted in a nursery or moved outside in larger pots. Plants are also sometimes pre-selected before field planting for plant vigor, large mature-phase leaf type and growth habit/architecture. Often seedlings are inoculated with scab in the greenhouse, particularly if at least one parent is known to be resistant; this technique can reduce the progeny population by 50–80% (Janick et al. 1996).

1.6.3 Evaluation Techniques

The juvenile period without fruit varies from 3 to 10 years, depending on genotype and growth environment. A number of techniques have been used to shorten the duration of the juvenile period, including shoot pruning, root pruning and bark ringing (Janick et al. 1996); however, most of these are tedious and difficult to utilize with large populations. Probably the most helpful approach is to maintain active growth in the greenhouse before planting and throughout the entire evaluation period. Many programs will graft seedlings onto dwarfing rootstocks (M9 most common, also Bud 9 and EMLA 27) either in the first or second year. Dwarfing rootstock promotes more precocious flowering and saves space, but the use of rootstocks adds to the cost of the program. The rootstocks must be from virus-indexed stock to avoid infecting scions.

Field selection is generally performed in three stages: *Phase 1* – Genotypes are replicated only once whether grafted or not. If they are on their on own roots, spacing is usually 1.5–2.0 m in row and 5.0–7.0 m between rows. If they are on dwarfing rootstocks, they are usually spaced 0.6–1.0 m in the row and 4.0–5.0 m between rows. *Phase 2* – the most promising seedlings are cloned by grafting on dwarfing rootstock (M9 most common); usually 4–6 trees are produced and are planted as a single unit or split into two replications. The replications are usually evaluated at only one location but sometimes they are planted at two sites. *Phase 3* – Pre-commercial testing is conducted with multiple trees (10–50 is common) placed at many locations across the world (10–20 would not be uncommon). Virus indexing (and thermotherapy if needed) is usually performed at this stage on the most promising selections.

More breeding programs are starting to evaluate what quality traits are most important to its consumers. Fruit quality, aroma, consistent and high soluble solids, a range of acidity, high juiciness, crispness and non-browning flesh are desirable as are methods to quantify these traits. Sensory testing is becoming a part of many programs, as are studies of quality components (Harker et al. 2006).

1.7 Biotechnological Approaches to Genetic Improvement

1.7.1 Genetic Mapping and QTL Analysis

Molecular markers have been linked to a number of monogenic traits in apple (Tartarini and Sansavini 2003). The most work has been done on the *Vf* gene for scab resistance, where over 40 markers have been identified. Markers for the other scab resistance genes have also been developed by many groups and include *Vh* from Russian seedling R12740-7A of *M. sieversii* (Hemmat et al. 2002, Bus et al. 2005a,b, Boudichevskaia et al. 2006), *Vm* (Cheng et al. 1998, Patocchi et al. 2005), *Va* and *Vb* (Hemmat et al. 2004, Erdin et al. 2006), *Vd* (Tartarini et al. 2004), *Vbj* (Gygax et al. 2004) and *Vg* (Durel et al. 2000, Calenge et al. 2005). Gessler et al. (2006) reviewed the literature in this area from type of resistance through gene pyramiding.

Markers have also been linked to the pest resistance genes *Sd1* for *Dysaphis devecta*, and *Er1* and *Er2* for *E. lanigerum* (Table 1.2). A few markers have also been linked to genes regulating morphological traits including the columnar habit (*Co*), fruit color (*Rf*) and fruit acidity (*Ma*) (Table 1.3). Recently a cDNA/AFLP approach was used to identify a gene that contributes to lowering of fruit acidity (Yao et al. 2007).

A number of apple linkage maps have been published using several different sets of parents: 'White Angel' × 'Rome Beauty' (Hemmat et al. 1994), 'Wijcik McIntosh' × NY 75441-58 (Conner et al. 1997), 'Prima' × 'Fiesta' (Maliepaard et al. 1998), 'Iduna' × A679-2 (Gianfranceschi et al. 1998), 'Fiesta' × 'Discovery' (Liebhard et al. 2003b; Silfverberg-Dilworth et al. 2006) and 'Telamon' (a columnar genotype) × 'Braeburn' (Kenis and Keulemans 2005). The one with the greatest genome coverage and marker density is that of Liebhard et al. (2003b), with 475 AFLPs, 235 RAPDs, 129 SSRs and 1 SCAR marker. Two parental maps were constructed that spanned 1,140 and 1,450 cM, respectively. While their map was composed primarily with normally segregating markers, several linkage groups were found to carry groups of markers with the same distorted ratios. The highly transferable SSR frame of this map will make it a useful starting point for future *Malus* mapping projects.

In the first QTL study in apple, randomly amplified polymorphic DNAs (RAPDs) were used to locate genes associated with juvenile tree growth and development in the cross between the columnar mutant 'Wijcik McIntosh' and a standard form, disease resistant selection NY 75441-58 (Conner et al. 1998). One to eight QTL were identified for a number of traits including height increment, internode number,

internode length, base diameter, branch number and leaf break. The amount of variation explained by regression on individual loci ranged from 3.9 to 24.3, with an average of 7%. Most QTL were significantly associated with a trait in only one or two years.

Several groups in Europe have been especially busy mapping the QTL associated with resistance to apple scab into various linkage groups (LGs). The cross of 'Prima' × 'Fiesta' and other related F_1 progenies have been used to identify major genes associated with resistance in the D.A.R.E. project (Durable Apple Resistance in Europe) (Durel et al. 2002, 2003). The major genes for scab resistance *Vg* were found on LG 12. Several different NBS-type resistance gene analogues were clustered at bottom of LG 5 and at the top of LG 10. Numerous QTL for partial scab resistance were identified that mapped to four genomic regions. Most of these QTL were race specific with a few exceptions that included a QTL on LG 2 for resistance to races 6 and 7, and a QTL on LG 17 for resistance to races 1 and 6. A major non-race-specific QTL was identified near an NBS-analog cluster on linkage group LG 10. Three major genes for powdery-mildew resistance were also identified by bulked segregant approaches, and one of them on LG 2 was located in the same region as scab resistance.

Vinatzer et al. (2004) used the inverse polymerase chain reaction and simple sequence repeats to identify BAC clones containing the apple scab resistance gene *Vf* and found the gene in scab-resistant accessions of *Malus micromalus* and 'Golden Gem' of *M. prunifolia*., which were previously not known to carry this gene. They also found a mistake in the published pedigree of the *Vf* cultivar 'Florina' by comparing SSR patterns of its presumed progenitors to characterized ones.

Five apple progenies were used in the D.A.R.E. project to identify QTL with broad spectrum of resistance towards a wide range of strains of the fungus (Durel et al. 2004). It was verified that four major genomic regions exist that carry resistance to multiple strains of the fungus, with a QTL region on LG 17 carrying the widest spectrum of resistance. Several other linkage groups carry QTL or major resistance genes to specific isolates.

Resistance to apple scab was also mapped in the cross 'Fiesta' × 'Discovery' (Liebhard et al. 2003a and c). Eight genomic regions were identified in this study, with six conferring resistance to leaf scab and two to fruit scab. The amount of variation attributed to the various genes ranged from 4% to 23%, with all but one of the QTL being present across multiple years and locations. Two of the scab resistance QTL reported by Durel et al. (2002) were located on the same linkage groups (LG 10 and 17) and two were not. While 'Discovery' showed more resistance to scab in the field, the most QTL were identified in the more susceptible parent 'Fiesta'. This may indicate that the resistance genes in 'Discovery' are largely homozygous and can not be detected because they do not segregate.

Resistance gene homologues have been mapped in two segregating populations, 'Fiesta' × 'Discovery' (Baldi et al. 2004) and 'Discovery' × TN10-8 (Calenge et al. 2005). The gene homologues are widely distributed across the genome, but often reside in clusters. A high number of the markers mapped close to major genes

or QTL for resistance to scab and mildew. Research on nucleotide binding site (NBS)-encoding resistance gene homologs (RGHs) among the Rosaceae revealed synteny of a genomic region that encompasses powdery mildew resistance locus among *Malus*, *Prunus* and *Rosa* (Xu et al. 2007).

Progeny from the cross of 'Prima' × 'Fiesta' were used to detect QTL associated with physical and sensory descriptors related to fruit flesh firmness (King et al. 2000, 2001). Significant QTL were identified on nine linkage groups that were associated with firmness, stiffness, slow breakdown, crispness, granularity, hardness, juiciness, sponginess and overall liking. Considerable variability was noted across years and sites for penetrometer and acoustic resonance readings, and the presence of the QTLs associated with these traits was also highly variable. A highly significant QTL was detected on LG 16 for firmness, crispness, juiciness, sponginess and overall liking. QTL for penetrometer or acoustic resonance measures were not detected in this region, although it did map with *Ma*, the malic acid gene (Maliepaard et al. 1998). Several significant QTL associated with firmness and juiciness on linkage group LG 1 are found in proximity to the locus *Vf*, originating from the scab resistant crab apple *Malus floribunda*.

1.7.2 Regeneration and Transformation

The first apple transformation was done by James et al. (1989) using *Agrobacterium tumefaciens*. Since this seminal study, much work has been conducted to improve the efficiency of gene transfer and regeneration and a wide array of cultivars have been transformed and regenerated (Hammerschlag and Liu 2000, Brown and Maloney 2003). Particle bombardment of apple leaf explants has received much less emphasis, although Gercheva et al. (1994) developed protocols for 'Royal Gala'.

Several genes have been inserted into apple to provide resistance to fungal diseases, although their efficacy in generating pathogen resistance has not yet been published. The stilbene synthase gene from grapes and polygalacturonase-inhibiting protein from kiwi were transferred to 'Holsteiner Cox' and 'Elstar' (Szankowski et al. 2003). The antimicrobial peptide gene *A1-AMP* was incorporated into 'Jonagold' (Broothaerts et al. 2000a). Transgenic lines of 'Orin' and 'JM 7' have been selected with genes encoding chitinase, glucanase and sarcotoxin (Soejima et al. 2000).

Engineering resistance to apple scab has been the focus of several laboratories. Belfanti et al. (2004) isolated the *HcrVf2* gene for apple scab resistance from wild *Malus floribunda* and found it confers resistance in transgenic 'Gala'. Bolar et al. (2001) found that expression of endochitinase from the biocontrol fungus *Trichoderma atroviride* increased resistance to apple scab in transgenic 'Marshall McIntosh'. In other work, this group inserted genes for both endochitinase and exochitinase from *T. atroviride*, and found they had a synergistic activity against the pathogenic fungi *V. inaequalis* (Bolar et al. 2000). Chevreau et al. (2001) inserted the

gene for puroindoline-b from wheat in both susceptible and resistant apple cultivars, but did not report on whether resistance was achieved.

A considerable amount of work has been undertaken to develop fire blight resistant apples through genetic engineering (Aldwinckle et al. 2003, Norelli et al. 2003). Initial efforts focused around transferring genes for anti-microbial proteins to apple, including attacin E, avian lysozyme and the cecropin analogs, SB-37 and Shiva-1. The highest levels of resistance were found in the attacin-transgenics, but the cecropin- and avian lysozyme analogues were also effective. In long term field trials, fruit from transgenic lines of 'Royal Gala' and 'Galaxy' containing the antimicrobial genes was indistinguishable from the fruit of non-transformed trees.

In the most recent attempts to engineer resistance to fire blight, antimicrobials proteins have been introduced into apple that act directly against the pathogen, *E. amylovora* (Norelli et al. 2003). Two genes are being investigated: (1) the harpin gene (*hrpN*) from *E. amylovora*, which produces an effector molecule that induces resistance when applied to apple flowers, and (2) genes for DspE-interacting kinases, which interfere with the DspE pathogenesis factor from *E. amylovora*. The gene for NPR1 protein (*MpNPR1*) has also been studied, whose homologue in *Arabidopsis* is thought to be a key regulator in the induction of disease resistance.

In other work on pest resistance, Viss et al. (2003) developed transgenic 'Jonagold' that was resistant to crown gall disease, by inserting genes designed to express double-stranded RNA from the *iaaM* and *ipt* sequences of the ocogenes of *Agrobacterium tumefaciens.* These genes are responsible for the excessive hormone production that leads to gall formation. Markwick et al. (2003) found that apple plants of 'Royal Gala' expressing biotin-binding proteins were resistant to the lightbrown apple moth. Yao et al. (1995) produced 'Royal Gala' plants resistant to the herbicide Glean™ by transforming them with pKILI110, a mutant of the *Arabidopsis* acetolactate synthase gene.

Several transgenes have been shown to have significant effects on apple growth and development. Early flowering was induced in transgenic apple when BpMADS4 from silver birch (*Betula pendula* Roth.) was overexpressed (Flachowsky et al. 2007). Holefors et al. (2000) found the *Arabidopsis* phytochrome B gene to reduce shoot, root and plant dry weights in transformed M26 rootstock. Bulley et al. (2005) isolated an apple GA 20-oxidase gene and inserted it into 'Greensleeves' in the sense and antisense orientations and produced dwarf lines with both constructs. Application of GA_3 restored the internode length and number of these transgenic lines, and the scion remained dwarfed after grafting to normal rootstocks. Atkinson et al. (2002) found overexpression of polygalaturonase in transgenic 'Royal Gala' led to a range of novel phenotypes including silvery colored leaves and premature leaf shedding. Mature leaves also had malformed stomata that effected water relations and lead to brittle leaves.

The role A, B, C genes from *Agrobacterium rhizogenes* are known to influence hormone metabolism and root development during infection, and as such have been tested as a means of influencing apple growth and development. The integration of the rolA gene into the genome of apple rootstock A2 reduced plant

height and shortened internodes (Zhu et al. 2001a). The transformation of apple rootstock M.9/29 with the rolB gene reduced node number and stem length, but not relative growth rate (Zhu et al. 2001b). Root percentage and root number was increased in shoots of Jork 9 rootstock and the apple scion 'Florina' through the insertion of *rolB* (Sedira et al. 2001, Radchuk and Korkhovoy 2005). Introduction of *rolC* into the apple rootstock 'Marubakaidou' produced four different phenotypes in transformants: a group with reduced height and shortened intervals, a group with reduced height but normal internode lengths, a group with normal height with shortened intervals and a group that was phenotypically similar to control plants (Igarashi et al. 2002).

To reduce browning, Murata et al. (2000) produced transgenic 'Orin' apples carrying the antisense of polyphenol oxidase (PPO). The approach worked, as some of the transgenics had significantly lower levels of PPO than non-transgenic shoots and less browning. Broothaerts et al. (2000b) developed a spectrophotometric assay to rapidly screen PPO activity in apple.

Transgenic apple trees have been produced that possess extra copies of the endogenous S-gene controlling self-incompatibility in apple to induce self-fertility (Broothaerts et al. 2004a,b). In controlled self and outcrosses over a 3-year-period, the transgenic lines had normal levels of fruit and seeds after selfing, while the control plants had significant reductions. The self-fertile transgenic type was associated with an absence of pistil S-RNase proteins.

Gilissen et al. (2005) silenced the major allergen *Mal d* 1 using the RNA interference approach. Allergen levels were reduced but not eliminated.

Transgenic approaches are adding to our knowledge of flavor and ethylene responses. Dandekar et al. (2004) examined the effect of down-regulation of ethylene biosynthesis on fruit flavor complex in apple fruit. In related studies, the relationship of ethylene biosynthesis to volatile production, related enzymes, and precursor availability in apple peel and flesh tissues was studied by Defilippi et al. (2005a,b) who found that alcohol acyltransferase, a rate limiting step for ester biosynthesis important in aroma, is regulated by ethylene.

James et al. (2001) and his group (Gittins et al. 2000, 2001, 2003) have studied the ability of a number of heterologous and homologous promoters to drive expression of β-glucuronidase in tissues of apple. They found the ribulose-1,5-bisphosphate carboxylase/oxygenease small-subunit promoter (*RBCS3C*) from tomato and *SRS1P* from soybean to primarily drive activity in vegetative tissues of apple that had chloroplasts. The *SRS1P* promoter was regulated by light, while *RBCS3C* was not. They also found the *extA* promoter from rape to be very active in all apple tissues, even though its activity is root-specific in its own species. The vascular tissue promoters, *rolC* from *Arabidopsis rhizogenes* and *COYMV* from the *Commelina* yellow mottle virus were found to have localized expression in structural tissues. The group has also been active in identifying ethylene inducible promoters from apple.

Cisgenic approaches, using genes from apple in transformation, is discussed by Jacobsen and Schouten (2007).

1.7.3 Genomic Resources

Mining of existing apple EST information, such as the studies of Newcomb et al. (2006) and Park et al. (2006), and the use of microarrays (Lee et al. 2007, Pichler et al. 2007) promises to expand our knowledge of many genes important in the genetic improvement of apple. The development of public databases such as the GDR (*G*enome *D*atabase for the *R*osaceea; Jung et al. 2004) and the European HIDRAS AppleBreed (Antofie et al. 2007) also offer excellent prospects for enhanced collaboration amongst breeders, bioinformatics researchers and those involved in molecular biology. In the GDR database alone, over 50,000 ESTs are available from several species, tissues and developmental stages.

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