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# Apple Breeding – From the Origin to Genetic Engineering

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## ABSTRACT

Apple is the most important temperate fruit crop and ranks fourth in world production of fruits after citrus, grapes and bananas. Although more than 10,000 cultivars are documented, only a few dozen are grown on a commercial scale worldwide. Despite the abundant number of cultivars there is a demand for new cultivars better adapted to climatic conditions/changes and sustainable production. Yet, the challenge for apple breeding is the establishment of improved, multiple disease resistant cultivars with high and regularly yield suited for modern production systems. Due to the fast development of molecular techniques an increasing knowledge on the genome of apple is available, e.g. the whole genome sequence of 'Golden Delicious' (Velasco *et al*. 2010). Molecular markers for a lot of major traits, mostly resistance genes, and QTLs facilitate marker assisted selection, especially the pyramiding of resistance genes to achieve more durable resistance. Mainly the breakdown of the *Rvi6* (*Vf)* scab resistance enhanced the breeding for pyramided resistance genes. But nevertheless, until now there is a gap between the existing molecular knowledge and its application in apple breeding. This paper will focus on the origin and domestication of apple, breeding objectives and classical as well as molecular approaches to achieve breeding aims. Besides this an update on molecular knowledge and the current state and progresses in genetic engineering will be presented.

**Keywords:** classical breeding, molecular breeding, genetic engineering, *Malus*, MAS, molecular markers, resistance
**Abbreviations:** **ACW**, Agroscope Changins Wadenswil; **AFLP**, amplified fragment length polymorphism; **BAC**, bacterial artificial chromosome; **cDNA**, complemantary DNA; **cfu**, colony forming unit; **DArT**, diversity array technology; **db**, data base; **dCAPS**, derived cleaved amplified polymorphic sequence; **DUS**, distinct, uniform and stable; **ECPGR**, European Cooperative Programme for Plant Genetic Resources Networks; **EST**, expressed sequence tag; **EUFRIN**, European Fruit Research Institutes Network; **GDR**, genome database for Rosaceae; **GRIN**, Germplasm Resources Information Network; **HiDRAS**, High Quality Disease-Resistant Apples for a Sustainable Agriculture; **HRM**, high resolution melting; **JKI**, Julius Kühn-Institute; **LG**, linkage group; **MAS**, marker assisted selection; **MMT**, million metric tons; **PCR**, polymerase chain reaction; **QTL**, quantitative trait loci; **RAPD**, random amplified polymorphic DNA; **RFID**, radio frequency identification; **RFLP**, restriction fragment length polymorphism; **S-SAP**, sequence-specific amplified polymorphism; **SCAR**, sequence characterized amplified region; **SNP**, single nucleotide polymorphism; **SSR**, simple sequence repeat; **t**, tons; **UPOV**, Union pour la Protection des Obtentions Végétales, **USDA-ARS**, United States Department of Agriculture – Agricultural Research Station; **USPP**, United States Plant Patent; **ZIN**, Züchtungsinitiative Niederelbe

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## ORIGIN AND HISTORY OF THE CROP

The cultivated apple is probably the result of interspecific hybridization and at present, the binominal *Malus* × *domestica* Borkh. has generally been accepted as the appropriate scientific name, replacing the previous common usage of *M. pumila* (Korban and Skirvin 1984). *Malus sieversii* Lebed., a wild apple species native to Central Asia, is now thought to be the primary progenitor of the cultivated apple. Fruits of *M. sieversii* show an extensive diversity from almost inedible 'crabs' to fruits not very dissimilar to some modern cultivars. In ancient times, apple seeds and trees were probably dispersed from Central Asia east to China and west to Europe via trade routes popularly referred to as the 'Silk Road' (Juniper and Mabberley 2006). Selected cultivars, deriving from apparently random hybridisation and playing a significant role in Old World horticulture, appeared following the introduction of grafting technologies.

Apple was well-known to the ancient Greeks and they were familiar with the art of grafting. In the 3rd century B.C., Theophrastus wrote in '*Historica Plantarum*' about the maintenance of apple using this technique and referred to various cultivars. Roman horticulturalists knew all about budding, grafting and rootstocks. The Elder Pliny noted about 20 varieties of apple (Watkins 1995). The Romans brought the whole package to Western Europe and, for the

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last 2,000 years, the domesticated apple has diversified and flourished worldwide (Harris *et al.* 2002). Apple cultivars, deriving originally from the Old Silk Road, were now cut off from all their parental origins, except possibly from a small degree of cross-pollination with the European Crab-apple *Malus sylvestris L.* Mill. Archaeological evidence for the collection of apples from the wild in Europe can be found in Neolithic (11,200 years ago) and Bronze Age (*ca*. 4,500 years ago) sites throughout Europe (Schweingruber 1979). But it is unlikely that the native crab *M. sylvestris* has produced hybrids of any commercial value. Any possible influence of *M. sylvestris* is likely to have been mainly on cider apples (Juniper *et al.* 1999). The alien apple cultivars found, when they spread over the whole Europe, an agreeable climate. They hybridised, principally amongst themselves is surmised, mutated to a degree, and proliferated to the point.

After the migration period it was Charlemagne (A.D. 742-814) who wrote in his '*Capitulare de villis et cortis imperialibus*' a list of some fruits grown in his kingdom. During the Middle Ages, especially the new founded monasteries promoted horticulture and the apple cultivars at that time were connected with history of monasteries. By the 17th and 18th century fruit cultivation was already well established and a general written guidance was available as summarized by Juniper and Mabberley (2006). With the development of industrial centres and cities in the beginning of the 19th century, the production of apples had to be improved including new practical methods of growing. The number of cultivars escalated whereby every town and village in West/Central Europe could claim a local apple cultivar.

A European style of horticulture was also established by the early colonists on the eastern seacoast of North America. From the end of the 16th century European crops were pouring into the new colonies. Apples were soon established in the Tidewater region and elsewhere, grafting was rarely practised in the 17th and 18th centuries. English cultivars were not so successful under the American climate, but they often set seed, where they were fertile and could find compatible partners. As a result, the genetic diversity accumulated in North America orchards soon became considerably greater than that in Europe (Watkins 1995), and the potential for hybridization gave rise for screening new American cultivars, e.g. 'Jonathan', 'Wagener' and 'Golden Deliciuos' (Juniper *et al.* 1999). 'Golden Delicious' was found as a seedling of unknown parentage in a hedgerow in West Virginia in about 1890. A secondary centre of diversification was established in the United States in the 18th and 19th centuries (Janick 2005). 'Granny Smith' arose as a chance seedling near Sydney, Australia, in the 1850s (Votteler 1998), 'Braeburn' as a chance seedling from 'Lady Hamilton' in the 1950s in Nelson in New Zealand. The chance seedlings 'Braeburn', 'Golden Delicious' and 'Granny Smith' still have large economic importance. Today the production of apple represents also a strong economical factor on the South hemisphere. The industrialisation of horticulture in the beginning of the 20th century, especially after the Second World War, led to a devastating erosion of the genetic diversity of apple. Nowadays apple production focuses on a very limited number of cultivars which results in the loss of the other cultivars grown on a commercial scale at one time (Way *et al.* 1990; Höfer 2006).

## BOTANICAL DESCRIPTION AND GENETIC RESOURCES

The apple, along with many of important temperate fruit crops, belongs to the *Rosaceae* or rose family. The genus *Malus* Mill. comprises 25 to 47 species, depending upon the rank given to several taxa and the acceptance of putative hybrids. *Malus* classifications differ primarily in the taxonomic level at which infrageneric groupings of species are recognised. Rehder (1920, 1927, 1949) created his own system and most authors have now adopted Rehder's sytem. Newer reports divide the genus *Malus* in six sections (Forsline *et al.* 2003) or even in seven sections (Qian *et al.* 2006). In China, as the centre of origin of the genus *Malus*, about 80% of all species are native – among them eight newly described species were found (Zhou 1999). Most of the species intercross and, since self-incompatibility is common; seeds obtained from a botanic garden are mostly interspecific or intercultivar hybrids. Some taxon formerly listed as species are now classified as cultivated species because there is no record of having wild origin (Forsline *et al.* 2003). The *Malus* species are very polymorphic by its nature and represent a complicated system of ecotypes, forms and varieties (Li 1996).

First apple populations appeared already in the Cretaceous period in South-East Asia about 70 million years ago. However, a large-scale development and differentiation of apples took place in the Palaeoneogene, i.e. 50 to 20 million years ago (Langenfelds 1991). Today only a few of them have survived and greatly differentiated relict apple species appeared (sect. *Eriolobus* and sect. *Docyniopsis*). The docynio-like apples (sect. *Docyniopsis*) flourished in South-West China, then differentiated and gave rise to sorbo-like apples (sect. *Sorbomalus*) which spread in Central and East Asia. The green-fruited apples (sect. *Chloromeles*) evolved from a common initial ancestor when they migrated to North America during the time when the Asian and North American continents were not separated yet. The differentiation of the original apples in the Himalayas produced two most highly developed branches of the genus *Malus*, **berry–like apples** adapted to harsh conditions and evolved along the ornithophilous line (tiny, berry-like fruit adapted to seed dispersal by means of birds) and **true fruit apples** (sect. *Malus*) spread in Euroasia. Younger species, particularly true apples, are characterized by great diversity of forms of different origin, polymorphism, and tending to mutual hybridization.

The origin of the basic haploid number of *Malus* and other *Pomoideae* (n = 17) is attributed to hybridization between two remote ancestral types (Darlington and Moffet 1930). A second, more recent hypothesis is based on modern molecular techniques (Morgan *et al.* 1994): early diploid *Spiraeeae* (x = 9) became autopolyploid (x = 18), followed by a reduction in chromosome number (x = 17). This hypothesis is supported by the study of Tang *et al.* (2008) who developed a model system explaining preferential gene retention or loss after genome duplication. Evans and Campbell (2002) clarified this hypothesis by DNA sequence data from duplicated GBSSI genes. Most species are diploid and cross-pollinated, but Way *et al.* (1990) list *M. coronaria* from the American section *Chloromeles* as apomictic and either triploid or tetraploid. They also note deviations from the normal type in section *Malus*: *M. sikkimensis* (Wenzig) Koehne, *M. hupehensis* (Pampan.) Rehd. and *M. toringoides* (Rehd.) Hughes (apomictic and triploid), *M. sargentii* Rehd. (apomictic and tetraploid), and *M. spectabilis* (Ait.) Borkh. and *M. baccata* (L.) Borkh. (non-apomictic and either diploid or tetraploid). The domesticated apples, with few exception – a small number of triploids, and a mere handful of commercially insignificant tetraploids – retain the original diploid chromosome complement of 2n=34 (Juniper and Mabberley 2006).

Especially since the early 1990s when the Convention on Biological Diversity was concluded, the political arena has undergone significant changes and many 'National Programs for Genetic Resources of Plants' were designed to provide long-term preservation, utilization, research and development for these species and large, crop specific germplasm collections were established. Apple was chosen to be on the Annex 1 list of the International Treaty on Plant Genetic Resources for Food and Agriculture. Because clonal integrity is the key to a successful conservation program for apple, maintenance of genetic resources presents special problems and opportunities not found in seed-propagated crops.

The National Germplasm Repository for Apple in

**Fig. 1 Accession of *Malus hupehensis* in the Fruit Genebank Collection at Dresden-Pillnitz.**

**Fig. 2 *Malus sylvestris* scion successfully grafted on rootstock M9 after six months of cryoconservation according to the dormant bud method.** (Forsline *et al*. 1998)

Geneva, USA is part of the National Plant Germplasm System and maintains as the largest collection of apple germplasm nearly 4,000 accessions. Many of these genotypes were collected from the apple centre of diversity in Central Asia (Forsline *et al.* 2003). As a preventive measure against field disasters, large portions of the national Canadian and USDA *Malus* collections are also backed-up in liquid nitrogen vapour at the USDA-ARS National Center for Genetic Resources Preservation (NCGRP) in Fort Collins, Colorado (Volk *et al.* 2008). Cryopreservation methods for *Malus* were originally reported by Saki (1960). The NCGRP routinely cryopreserves *Malus* dormant buds by first desiccating single bud sections to 25-30% moisture content, slow-cooling tubes of buds sections at 1 to -30°C, holding the samples at -30°C for 24 h, and then quickly placing tubes into liquid nitrogen vapour for long term storage (Forsline *et al.* 1998). While the active collection focuses on germplasm maintenance, characterization, documentation and distribution to the user community, the base collection provides long term preservation and is distributed only when needed for reproduction at the active site. Cryopreservation of dormant vegetative buds provides several advantages as a method of conserving fruit crop genetic resources:

- Clonal integrity is maintained.
- The technique is simple and space-efficient.
- Low-maintenance requirements consist of liquid nitrogen as a coolant in storage vessels.
- Very long storage is theoretically possible.
- Recovery of flowering plants for crossing is possible within about two years after grafting (Stushnoff 1991).

In Europe, the gene bank curators cooperate under the frame of the European Cooperative Programme for Plant Genetic Resources (EC/PGR), a collaborative program among most European countries aimed at facilitating the long-term conservation on a cooperative basis and the increased utilization of plant genetic resources in Europe. The program operates through networks in which activities are carried out either in the framework of Working Groups or as *ad hoc* actions. The genetic resources of apple are included into the Fruit Network, Working Group *Malus*/*Pyrus*. Following objectives are included in the common work:

- conservation of *ex situ* collections,
- definition and selection of descriptors,
- characterization of the genetic diversity using molecular markers and elaboration of a European database http://www.nationalfruitcollection.org.uk/ecpgr.php.

At a collaborative workshop the EC/PGR *Malus*/*Pyrus* Working Group agreed upon a standard set of eight reference accessions and 12 SSR markers for molecular fingerprinting of apple (van Teuren *et al.* 2010). Using the standard sets for fingerprinting reliable comparisons can be made between laboratories and datasets, and collections can be screened cost-effectively. Regarding the conservation activities, National Networks for preservation of fruit genetic resources were and will be created in Europe. National inventories and decentralized network activities are the base.

In Germany, the 'National Program for Genetic Resources of Agricultural and Horticultural Plants' was designed to provide long-term preservation, utilization, research and development for these species. For fruit species the establishment of a nationwide German Fruit Genebank and, especially for apple, the establishment of cryopreservation as a method for conservation were incorporated as main objectives. The German Fruit Genebank has been recently established to minimize the risk of losing fruit genetic resources. The German Fruit Genebank is a decentral network, aimed at the coordination of different germplasm collections in Germany. The work will be organized in species specific networks and preservation guidelines for each fruit species will be developed. The German Apple Genebank preserves 950 cultivars, and seven partners which maintain the collections are cooperating. The Genebank of the Julius Kühn-Institut (JKI) situated at the Institute for Breeding Research on Horticultural and Fruit Crops in Dresden, Germany (**Fig. 1**) is the main partner of the German Apple Genebank, and

maintains 775 cultivars as well as 530 *Malus* wild species accessions representing 46 primary and hybrid species currently maintained as two trees in the field. In addition to the clonal collection, approximately 1,000 seedlings of wild *Malus* spp. from centres of origin are preserved at JKI originating from collection expeditions to China. A main part of the *Malus* wild species collection is represented by accessions of *Malus sieversii* that have been collected in Central Asia and were analysed by a collaborative evaluation for disease resistance and horticultural and molecular characterization (Luby *et al.* 2001; Wiedow 2006; Höfer 2007). On the base of a comprehensive evaluation, a core collection of *Malus sieversii* was established which contains maximum diversity for the species. For building a backup cryopreservation, the method using winter vegetative buds was successfully applied to the first *Malus* accessions maintained at the JKI genebank (Höfer 2007; **Fig. 2**). Further specifications of the cryopreservation protocol are necessary to adapt the method to mild winter conditions in Europe. The work will be supported in the frame of COST Action 871, CRYOPLANET (European Cooperation in the field of Scientific and Technical Research, www.cost.esf.org/index.php?id=181&action_number=871).

## ECONOMIC IMPORTANCE, USES AND AREAS OF PRODUCTION

Apple is the most important temperate fruit crop and has been cultivated in Asia and Europe from antiquity (Janick *et al.* 1996). The main advantages of apples are the availability throughout the year, due to growing in different regions and the possibility of long term storage, and the adaptability to different growing regions. The temperate zone between 35 and 50° latitudes is the optimum for apple production, although apple is adaptable to wider regions (Kellerhals 2009a). Due to the relatively good winter hardiness and late blooming apples are more suited to the northern hemisphere than many other fruit trees. Worldwide apple production ranks fourth within the fruit crops behind citrus, grapes, and bananas (FAOSTAT 2008). More than 50 MMT are produced worldwide annually (FAOSTAT 2006) with a slightly increase in the last years. The value of the apple production in 2005 worldwide, about 55 MMT, was estimated at about $ 10 billion. The World Apple Report 2006 predicted a significant increase in production and consumption. The estimated world apple production in 2015 will be around 73 MMT. Whereas the production in Europe and North America will be more or less stable, an increase from 28 MMT in 2005 to 33 MMT in 2015 is expected for Asia. This predominantly is due to the development of better cultivation techniques and progress in economics in China, the major apple producer of the world. In 2004, China produced around one third of the whole world production and the proportion is increasing. **Table 1** lists the ten countries leading in apple production 2008. Germany ranks 13 with a production of 1.046.995 t.

Processing of apple to juice has a huge economic importance. In the market year 2003/2004 about 1.2 MMT were produced world wide (FAS/USDA report) with China being the top producer followed by Poland, Germany and the United States of America. Often the production of juice is made of culled fruit from fresh apple lines and only few apples are specifically grown for juice production. However, in several areas of Europe such as Switzerland, Germany, France, Austria and others juice and cider production from traditional standard trees of local cultivars is still common and appreciated also for landscaping and environmental view.

Besides fresh consumption of apples and juicing, apples can be processed to products like puree, jam, cider, and others, and they are well suited for the production of baby food. Fresh consumption of apples often has been associated with beneficial health effects: "An apple a day keeps the doctor away", and it was shown that it can reduce risk of some cancers, cardiovascular diseases, asthma, and diabetes, thereby explaining the popularity of apple throughout the world besides the taste.

**Table 1** Apple production in 2008 of the ten leading countries in apple production (source: www.fao.faostat.org)

| Country | Apple production in tons in 2008 |
|---|---|
| China | 29,851,163 |
| United States of America | 4,431,280 |
| Poland | 2,830,870 |
| Turkey | 2,504,490 |
| Islamic Republic of Iran | 2,660,000 |
| Italy | 2,208,227 |
| India | 2,001,400 |
| France | 1,940,200 |
| Russian Federation | 1,467,000 |
| Chile | 1,370,000 |

An increasing market is the organic production of apples. The whole amount of marketed organic apples either for fresh market or processing in Europe in 2005 was estimated to about 97,000 t (Kelderer 2007) with an increasing tendency.

## BREEDING OBJECTIVES

Laurens (1999) reported about an inquiry performed in 1996 among 42 apple breeders from 29 countries representing four continents. The main objectives and partners included in the breeding programs were reviewed. The main common objective was to combine in new cultivars high fruit quality with disease and pest resistance. Adaptation to climatic conditions is also of prime interest for the countries located in marginal areas. Many apple breeders are also involved in tree habit studies in order to obtain productive and regular cropping trees. Storage ability and harvesting period are other objectives pursued in some programs. Presently, due to economic pressure on fruit production, high and regular yielding cultivars that easy produce fruit with a high pack out of commercial quality are required.

Genetic diversity provides the raw material for breeding and plant improvement. Some breeding programs, e.g. in New Zealand, are considering new features such as red-fleshed apples and novel flavours. Red-fleshed apple accessions came into the HortResearch gene pool from the remarkable wild apple forests of Kazakhstan and Kyrgyzstan where the seeds were collected by Dr. Dominique Noiton in the 1990's (Anonymous 2006). Fruit quality is supposed to further increase in importance in the future. Consumers are becoming increasingly aware of quality. New processing procedures and new products will require fruits with specific quality attributes. Cultivars specific for certain uses will be demanded. One aspect of quality that will require much more attention in future is food value: higher vitamin content; better sugar: acid ratios; fruit which are high in particular minerals (e.g. calcium) or non-allergenic. As food additives become less available for use by processors, natural colour, flavour and firmness will also become more important (Muggleston 1995a, 1995b).

Another challenge for breeders will be the proposed climatic change. The environmental conditions for growing will change; biotic and abiotic stresses will appear or disappear, respectively enhance or decrease. The breeding for cultivars adapted to specific environments could be become more important but a reliable prediction of prospective climatic conditions and their aftermath regarding the growing of apple is required to define new necessary breeding aims. Selection methods according to new breeding aims have to be developed.

The topic of resistance breeding, especially the pyramiding of resistance genes to gain durable resistance, will be discussed below.

## CLASSICAL APPLE BREEDING AND GENERAL CONSIDERATIONS

Breeding is a major innovation factor in apple growing worldwide. As in other products and commodities, consumers require products with exciting, new and up to date features. However, the breeding process is long term and so is the commercial introduction phase for a new cultivar. It is therefore a great challenge for every breeder to head for new cultivars that might be on the market only 30 to 40 years after designing the crosses. Compared with annual crops, breeding perennial crops is complex, long lasting and time consuming due to their long juvenile phase and long economic lifespan (Antofie *et al.* 2007). There is a continuous search among breeders to speed up the breeding process and to streamline the selection process. Breeding for high quality apples combined with excellent agronomic features and durable disease resistance is a highly relevant approach for sustainable production systems.

During the last 20 years the developments in molecular genetics lead to new and more precise breeding approaches. A major step forward in breeding efficiency is being achieved by combining classical phenotypic selection methods with new molecular techniques. Apple breeding is requiring good foresight of future trends on the market on one side and good knowledge on the breeding material. The breeder's experience and the breeder's eye are still important features. However, it is possible that respective databases and computer-aided decision systems will give support and help to integrate a huge amount of information. Despite of these new tools, apple breeding will remain a multitasking and interdisciplinary approach requiring highly sophisticated long term management. Historical aspects of breeding, breeding objectives and methods as well as cultivar protection and marketing will be outlined.

### From chance seedlings to controlled apple breeding

Until the latter half of the twentieth century most of the world's apple cultivars were chance seedlings selected by fruit growers (Janick *et al.* 1996). Presumably the first apple breeder was Thomas Andrew Knight who lived from 1759 to 1838 in Britain. He started to make intentional crosses between selected parents in 1800. However, only about 100 years later with the rediscovery of Mendel's laws fruit breeding became more relevant. The University of Minnesota fruit breeding program in the USA started in 1888. In Germany, the association of pomologists defined the objectives of a German apple breeding program in 1912 (Götz and Silbereisen 1989). However it took up to 1929 that a formal German apple breeding program started in Müncheberg in Easter Germany with the objective to improve yield and fruit quality (Fischer and Fischer 1996, 2004, 2008). In 1930, research started on apple scab and frost resistance within this program.

In the first half of the 20th century the only important US cultivars derived from controlled breeding were 'Cortland' ('McIntosh' x 'Ben Davies') and 'Idared' ('Jonathan' x 'Wagener') (Janick *et al.* 1996). However after that time a whole range of new varieties developed from controlled crosses were successfully released such as 'Elstar', 'Gala', 'Jonagold', 'Mutsu', 'Maigold' (all seedlings from 'Golden Delicious'), and 'Empire' and 'Fuji' (seedlings from 'Red Delicious').

In Switzerland, shortly after the foundation of the Research Station at Wädenswil in 1890, apple breeding activities have been started. The main interest was focused at that time to apple cultivars suitable for processing to juice and cider. Only after 1920 also table quality started to be considered. However, only in 1955 the first significant and successful cultivar was released under the name 'Schweizer Orangenapfel'. It was derived from a cross performed in 1935 between 'Cox Orange' and 'Ontario'. Even more successful at least in Switzerland was the cultivar 'Maigold' relased in 1964 (Kellerhals 1991).

A tremendous impact on the 20th century apple breeding till today came from the PRI program in the USA (Janick 2006, see also box "The PRI Apple Breeding Program") as it developed a range of disease resistant selections and varieties which were subsequently used as parents in many breeding programmes worldwide.

### Scab resistance

Scab is in many areas of apple growing considered as being the most harmful disease. Therefore, many breeding programs worldwide are focused on durable disease resistance towards apple scab (*Venturia inaequalis*) and often combined with resistance to powdery mildew (*Podosphaera leucotricha*) and fire blight (*Erwinia amylovora*). The first attempts to breed scab resistant apple cultivars were undertaken in Germany (Kellerhals 1989). The aim was to combine resistance with good fruit size and quality. It was already at that time suggested that a mass screening method for selection of resistant seedlings should be developed and that resistant selections should be tested a second time with different races or sources of the fungus. Schmidt (1938) describes a mass infection method for one-year-old apple seedlings in outdoor frames. The floor of the frames was covered with infected leaves which were watered several times to release ascospores. During the growing season seedlings were inoculated every two weeks with a water suspension of conidia. By these means an intensive infection pressure was created. However, the prospects of combining the high scab resistance found in several *Malus* species with good fruit size and quality did not appear promising. Therefore the work was continued with large-fruiting sources carrying polygenic scab resistance such as 'Antonovka' and 'Ernst Bosch' (Schmidt 1938). After World War II the Coop-Programme started in the USA involving the following Universities:

- Purdue University (E.B. Williams, F.H. Emerson and J.R. Shay),
- Rutgers University (L.F. Hough, Catherine Bailey),
- University of Illinois (D.F. Dayton and J.B. Mowry).

It was also called PRI-programme (Purdue, Rutgers, Illinois). The gene for scab resistance used in this program was identified by L.F. Hough from a collection of *Malus* species and hybrids assembled by Prof. C.S. Crandall at the University of Illinois in the early 1900's. Crandall made studies on the inheritance of fruit size in apple and used small-fruited *Malus* species for that purpose (Crandall 1926). In 1914, he made a cross between 'Rome Beauty' and '*Malus floribunda* 821', and in 1926 a full-sib cross between two F1 selections. Basically all *Rvi6* (old nomination: *Vf*) resistant modern apple cultivars go back to this cross (see **Box 1** "The PRI Apple Breeding Program").

While breeding for scab resistance, phenotypic early selection for scab resistance in the greenhouse is nowadays often applied using the scale of Chevalier *et al.* (1991). Depending on the progenies varying proportions can be discarded as susceptible to scab in the glasshouse test. In the case of one parent carrying a major scab resistance gene in a heterozygous state crossed with a susceptible one, a 1:1 segregation can be expected. However, when pyramiding different scab resistance genes, the proportion of resistant progeny plants will be higher, in the case of two genes such as *Rvi6* and *Rvi2*, 75% of the progeny plants are expected to be resistant.

A reduction of the risk of resistance breakdown is being achieved by combining several functionally different resistances in a cultivar. Molecular selection for genotypes with such pyramided genetic resistance against scab and powdery mildew is current practice for example in the Swiss apple breeding program at Agroscope Changins-Wädenswil. Marker assisted selection (MAS) promises great potential for improving the efficiency in apple breeding. Genetic markers can reduce the number of plants, and the time required for evaluation, thus new cultivars become

**Box 1: The PRI Apple Breeding Program**

A remarkable contribution to world wide efforts to develop disease resistant apple varieties was the PRI Apple Breeding Program in the USA which was reviewed by Janick (2006). The PRI cooperative scab-resistant apple breeding program between Purdue University, Rutgers, the State University of New Jersey and the University of Illinois had a long and interesting history. The original program dates to a formal 1945 collaboration between J.R. Shay, pathologist at Purdue University and L.F. Hough, horticulturist at the University of Illinois to develop scab-resistant apples. The germplasm exploited by Shay and Hough can be traced back to Crandall at the University of Illinois who maintained a large collection of *Malus* species. Crandall attempted to determine scab inheritance patterns in *Malus*. A selection he labelled '*Malus floribunda 821*' is the original source of the *Rvi6* (*Vf*) gene. Spring 1943 was exceptionally cool and wet resulting in severe epidemic of scab. Hough noted that a F2 of '*Malus floribunda* 821' x 'Rome Beauty' segregated in a ratio of 1 resistant to 1 susceptible, suggesting the involvement of a single gene. Selection F2 26829 had unusually good quality and was already about 5 cm in fruit diameter. Techniques for controlled greenhouse screening of hybrid progenies were developed by Shay (Shay and Hough 1952). According to Janick (2006) up to 2006 more than 1,500 selections have been made of which 44 have been released for advanced testing as Coop-Numbers. Sixteen of these have been named as cultivars, such as 'Priscilla', 'Crimson Crisp', 'Gold Rush', 'Enterprise', 'Juliet'. Nowadays the PRI program and its output are integrated in many apple breeding activities worldwide and there is also a shift to more fundamental research on genomics. Many breeders and research groups in the world could profit from the achievements of this program.

commercially available sooner. The availability and application of MAS will be discussed below.

## Powdery mildew resistance

Knight and Alston (1968) gave a good overview on sources of field immunity to mildew (*Podospaera leucotricha*) in apple. By considering the literature and by their own work at that time, they concluded that the cultivated apple provides a complete range of reaction from full susceptibility to what is often described as "immunity". Major gene sources of mildew immunity have been sought as part of the apple breeding program at East Malling (UK). However, examination of some 2,000 cultivars at East Malling and the National Fruit Trials near Faversham, England suggests that it is improbable that true immunity exists in the cultivated apple (Alston 1969). Brown (1959) suggested that mildew resistance in the cultivated apple is invariably under polygenic control. Genetic studies at East Malling showed that the high resistance found in open-pollinated seedlings of *M. robusta* and *M. zumi*, respectively, is determined by dominant genes (Knight and Alston 1968). First it was expected that these open pollinated seedlings carry single dominant genes named *Pl1* and *Pl2*, respectively. However, later this hypothesis was revised to account for two different genes plus modifiers. In the meantime, the genetics of these mildew resistances have been further elucidated by molecular approaches. The position of the *Pl1* locus together with the AT20-SCAR and the RGA15G11 markers at the bottom part of apple LG 12 indicate that *Pl1* might be a further gene of an important resistance gene cluster located in this region of the apple genome (Dunemann *et al.* 2007)

In greenhouse tests for mildew resistance, Dayton (1977) identified a single plant grown from open-pollinated seeds of 'Starking Delicious' as highly resistant. One parent appears to have been a *Malus* species. It was named 'Mildew immune selection' (MIS). Progeny tests indicated a single gene from MIS conferring immunity to *P. leucotricha* in both the greenhouse and the field and therefore distinct from those reported by Knight and Alston (1968). Lespinasse (1983) reported a virulent biotype of mildew which seemed to overcome the resistance of MIS in Dayton's plantation. Other sources for mildew resistance were found in the Netherlands (D-Series, in *Malus hupehensis* and the ornamental cultivar 'White Angel', in 'David', *Malus robusta* 'Robusta 5', *M. robusta* 'Korea' and *M. robusta* '24-7-7,8') (Gallot *et al.* 1985).

## Fire blight resistance

Fire blight caused by the Gram-negative bacterium *Erwinia amylovora (Burrill) Winslow et al.* is a devastating disease in apple production. The disease spread from North America in the early 1950 first to the British Isles and subsequently to continental Europe (Peil *et al.* 2007). A series of crab apples and selections have consistently been found to be resistant to fire blight.

There is also scope for breeding fire blight resistant apple cultivars by exploiting genetic variation in the germplasm and by using molecular markers associated to QTLs. For fire blight no major resistance genes have yet been found. However, Peil *et al.* (2007) assumed a major resistance gene for fire blight on linkage group 3 of *Malus x robusta* 5. This strong QTL explained 67%-83% of the phenotypic variation and in each family tested about 20-25% of the seedlings remained disease-free (Peil *et al.* 2008b). Another strong QTL explaining 50-70% of phenotypic variation was identified in 'Evereste' (Durel *et al.* 2009). The QTLs from 'Robusta 5' and 'Evereste' in contrast to the QTL from 'Fiesta' confer (near) immunity but have been found in small-fruited crab apples with low fruit quality. Therefore, they would require several generations of backcrossing before a new commercial cultivar carrying this resistance could be obtained.

At the ACW Research Station at Wädenswil the screening of promising advanced selections for relative fire blight tolerance is conducted in a quarantine glasshouse. Scion material is grafted onto M9 rootstock. Trees are planted in early spring in plastic tubes grown in the glasshouse for several weeks prior to inoculation. For each cultivar, usually 10 replicate trees are inoculated by puncturing the distal tip of shoots 15-30 cm long with a syringe containing an *E. amylovora* solution of $10^9$ cfu/ml of defined strains. Spreading of disease symptoms is evaluated in weekly intervals during three to four weeks by measuring the expansion of the necrotic lesion from the shoot tip in relation to the total shoot length. Recently within a European collaborative action, the different methods for phenotyping fire blight resistance applied by different institutes were discussed and comparisons were performed. There is still scope for further harmonization and coordination.

Glasshouse screening of advanced selections with a shoot inoculation test for fire blight resistance highlighted considerable differences among selections (Kellerhals, pers. communication). Genotypes such as FAW 14995 displayed low susceptibility to fire blight. They are interesting as parents and as cultivars. However, further testing under field conditions is required to confirm their low susceptibility to fire blight. As concerns their potential interest as commercial cultivars a whole range of other selection criteria need to be fulfilled as well.

One of the topics of the Dresden-Pillnitz breeding program for resistance to fire blight is the introgression of resistances from wild species like *M. baccata*, *M. fusca* or *M. robusta*, and the combination of different resistance mechanisms to obtain stable and durable resistance. Besides wild species, which are used to establish pre-breeding material, fire blight resistant cultivars, e.g. 'Rewena' and 'Enterprise', and advanced selections are used as resistance donors for cultivar breeding. Screening for resistance to fire blight is similar to the screening at ACW, but inoculation is per-

formed by cutting the tips of the two youngest leaves with scissors dipped in bacterial suspension instead of using a syringe. In fire blight screenings the selection Pi-AS 50,74 was identified as valuable donor for resistance to fire blight in cultivar breeding.

## Other diseases and pests

There are several other diseases of importance in apple growing such as apple canker (*Nectria galligena*), crown rot (*Phytophtora cactorum*), storage diseases, apple rust (*Gymnosporangium juniperi-virginiane*) as well as sooty blotch and fly speck.

Apple is attacked by both fruit-damaging and leaf-damaging lepidopteran pest insects, which require regular control such as the carpophagous codling moth, *Cydia pomonella*, or frequent control such as the phyllophagous apple leaf miner, *Lyonetia clerkella* (Stoeckli *et al.* 2009a). It is known that insect resistance is partly based on morphological plant traits such as pubescence, fruit colour or fruit shape and surface waxes (Smith 2005). Alston and Briggs (1970) reported on a hypersensitive response of '*Malus robusta* 5' to rosy apple aphis (*Dysaphis plantaginea*).

As many environmentally friendly pest control tactics are only effective at low levels of infestation, host plant resistance is a promising component of integrated pest management systems. Stoeckli *et al.* (2009a) examined the number of *C. pomonella*-infested fruits and the number of *L. clerkella* mines as measures of apple resistance or susceptibility. Herbivore surveys on 160 apple genotypes, representing a segregating F1 cross of the apple cultivars 'Fiesta' and 'Discovery', were carried out during two consecutive years and at two sites in Switzerland. Broad-sense heritability was 29.9% (*C. pomonella*), 18.2% (*L. clerkella*), and 21.9% (fruit number) and 16.6% (fruit diameter). A subsequent analysis identified a quantitative trait locus (QTL) associated to *C. pomonella* susceptibility on the 'Discovery' linkage group 10. No significant QTL was identified for resistance to *L. clerkella*.

## Tree architecture

The consideration of tree architecture and shoot morphology traits might be a promising manner to obtain trees that are adapted to training systems while reducing intrans and improving the control of vegetative development and yield regularity (Lespinasse 1992; Laurens *et al.* 2000). Liebhard *et al.* (2003b) estimated genetic and environmental variances and highlighted QTL's for growth (tree height and basis diameter) and phenological traits in an apple progeny. Tobutt (1985) has described breeding of apples inheriting the remarkable columnar growth habit of 'McIntosh Wijcik', a natural sport of 'McIntosh'. Tobutt (1994) reported on crosses from 'Wijcik' with 'Wellington Bloomless' and 'Spencer Seedless' which have apetalous flowers and bear parthenocarpic seedless fruit if not pollinated.

## Fruit quality

Fruit quality is the principal breeding objective. Important fruit quality aspects include colour, size, shape, appearance, flesh texture, firmness, juiciness, sugar, and acidity. Flavor is important but difficult to assess and consumer requirements may differ in different areas of the world.

The success of newly developed disease resistant apple varieties is largely dependent on their fruit quality (Kellerhals and Eigenmann 2006). Fruit quality is composed of many, mostly quantitative, traits.

According to Alston (1987) quality can be considered under four main categories:

- Appearance which concerns size and shape as well as colour and skin finish,
- Eating quality which covers all aspects of flavour, flesh colour and texture,
- Storage which depends on a number of factors including resistance to bitter pit, core flush, low temperature breakdown, senescent breakdown, scald and diseases such as *Gloeosporium* and *Phytophthora* fruit rots,
- Processing quality which includes canning and cooking performance, ease of peeling and juice quality.

First fruits in apple seedlings can usually be expected at earliest in the fourth year after crossing. However, most often the first fruit selection steps are performed from the fifth to the seventh year after crossing using phenotypic characterisation. For advanced selections, the methods and tests gradually become more sophisticated. Expert panels, instrumental and sensory analysis at different stages of storage are performed and finally consumer tests should give answer as to how the market chances can be rated. Currently research is carried out to develop marker-assisted selection tools for fruit quality (Gianfranceschi and Soglio 2004). This would considerably increase selection efficiency, as fruit quality traits can only be selected relatively late in the selection process.

Several other apple traits determining fruit quality in a broad sense are catching the attention of consumers and are being considered by breeders. This awareness is about to find expression in joint scientific efforts of pomologists, diet experts, clinical pharmacologists, and biochemists (Sansavini *et al.* 2004).

Apple fruit ripening is accompanied by volatile aroma production. This is important for the eating quality. Zini *et al.* (2009) applied Proton Transfer Reaction-Mass Spectrometry analysis in order to identify genomic regions related to volatile organic compounds. Today, researchers and consumers have great interest in phenolic compounds (or polyphenols) because of their strong antioxidant properties, great abundance in the human diet, and their probable role in the prevention of various diseases associated with oxidative stress such as cancers, cardiovascular diseases, and inflammation (Scalbert and Williamson 2000). Phenolics are regarded as one of the functional food components in fruits and are thought to contribute to the health effects of fruits (Hamauzu 2006). Phenolic compounds relate to sensory qualities of fruits such as colour, astringency, bitterness, and aroma (Macheix *et al.* 1990), and to different quality aspects of fruits including health benefits. As a result, it is important to know how the evolution and accumulation of phenolics in fruits tissues are regulated and how their content or composition can be modified. Breeding for processing characteristics is also of interest, e.g. non-browning apples for fresh cut products (Khanizadeh *et al.* 2009).

Fruit quality is evaluated in most apple breeding programs with increasing intensity during the selection steps increasing precision towards higher stages. Whereas in step 1 usually only one person evaluates the genotypes, during following stages several evaluators are included and additional instrumental analyses are performed. Gradually expert panels and also finally consumers are included. Based on the developments within projects such as the HIDRAS project (Gianfranceschi and Soglio 2004), markers flanking QTL's for fruit firmness and acidity could be integrated in a marker-assisted selection process for fruit quality.

Apple breeding is a complex management and planning task with interactions to many disciplines such as phytopathology, entomology, genetics, informatics, social sciences, and marketing. Some breeding programs are oriented towards the development of world apple cultivars; other activities are more oriented towards locally adapted specialities or cultivars for specific niches such as organic production, baby food, or juices etc. In the Midwest of the USA there is an apple breeding program, organized by the Midwest Apple Improvement Association, specifically designed for this growing area as current apple breeding programs are unlikely to produce cultivars that will be economically viable for the lower Midwest. A similar approach is followed in northern Germany by the breeding initiative Lower Elbe (Züchtungsinitiative Niederelbe, ZIN) (Dierend and Schacht 2009). In 2002, the ZIN was founded with the aim to establish a private financed apple breeding program

in North Germany. Members of the breeding initiative are growers, fruit co-operatives, and some fruit retailers. Breeding is performed in close cooperation with a Belgian nursery and the Institute of Fruit Growing and Nursery Production of the University of Applied Sciences Osnabrück, Germany. Scientific research projects are flanking breeding.

Climatic, economic and market changes and evolutions will require a continuous cultivar innovation. However, there is also to consider that markets and consumers tend to like tradition and well-known traditional cultivars. In breeding, the range of possible breeding objectives that might be considered is huge and it is therefore a great challenge for breeders to focus on the most important and promising criteria.

## Breeding efficiency

Selection efficiency is a key issue in a breeding program. Breeding consists of identifying as quickly and as precisely as possible the most promising progeny plants (Kellerhals *et al*. 2009b). It is a challenge to efficiently find the outstanding genotypes prone to become a successful commercial cultivar out of a large quantity of progeny plants. Selection efficiency in apple breeding starts with the appropriate choice of parents. To breed for disease resistant varieties, early phenotypic screening tests of progenies, e.g. for scab resistance, are performed. However, for other diseases such as powdery mildew and fire blight selection is usually made only at a later stage. Marker-assisted selection (MAS) allows selecting for tree and fruit characters more efficiently. It is applied in the ACW and Dresden-Pillnitz breeding programs to select for scab, mildew, fire blight resistance, and primarily to pyramid resistances.

Besides selecting for disease resistance at an early breeding stage, juvenility is an important issue. Juvenility is expressed in young apple seedlings by spines, horizontal side branches, small and narrow leaves and generally wildness (Visser and Schaap 1967). Juvenility is not only hampering the change to the generative phase but according to Visser (1965) it is also related to low precocity. Breeders should therefore select against juvenile symptoms. The long juvenile phase hampers the development of new cultivars; therefore breeders try to speed up the generation cycle. In Dresden-Pillnitz seedlings are grown in a greenhouse, in the first four month with 16 h additional lightening, to accelerate the switch from the juvenile to the adult phase. Within one year, seedlings usually grow up to more than 2 m length and will reach the adult phase (**Fig. 3**). One year after sowing seedlings are grafted onto a rootstock/interstem combination (M9/'Hibernal') already planted in the field, resulting in fruiting usually three years after sowing (Fischer 1994). On average 2,000 seedlings are grafted on the rootstock/interstem combination in the orchard every year and around 20 to 40 seedlings are selected every year for the second selection step. **Table 2** shows the efficiency of the Dresden-Pillnitz scab resistance breeding program from 1972 to 1992. From around 50,000 seedlings sown out, 26,058 were selected as resistant in scab inoculation screenings and planted in different plots in the orchard. Only three out of 26,058 seedlings evaluated in field trials were estimated as good enough to get breeders rights, 'Rebella', 'Recolor', and 'Rekarda'.

**Fig. 3 Apple seedlings in the greenhouse at Dresden-Pillnitz 18 weeks after sowing.**

Volz *et al.* (2009) developed a fast-breeding approach by growing up elite seedlings rapidly under controlled environment conditions with the objective to get them to flower within 12 to 15 months from germination. A reduction to only a few months from germination to flowering was achieved by a transgenic fast-breeding approach developed

**Table 2** Selection efficiency in scab resistance breeding in Dresden-Pillnitz from 1972 to 1998.

| Plot | Number of seedlings | 2nd selection step | | 3rd selection step | | Released cultivars | |
|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % |
| D1 | 1,155 | 48 | 4.16 | 37 | 3.20 | 1 | 0.09 |
| C9 | 575 | 43 | 7.48 | 4 | 0.69 | 0 | 0 |
| B11 | 10,404 | 159 | 1.53 | 17 | 0.16 | 0 | 0 |
| C4 | 6,599 | 215 | 3.26 | 8 | 0.12 | 2 | 0.03 |
| E6 | 2,992 | 37 | 1.24 | 14 | 0.47 | 0 | 0 |
| E8 | 2,814 | 27 | 0.96 | 12 | 0.43 | 0 | 0 |
| E10 | 1,519 | 25 | 1.65 | | | | |
| Total | 26,058 | 554 | 2.12 | 92 | 0.37 | 3 | 0.01 |

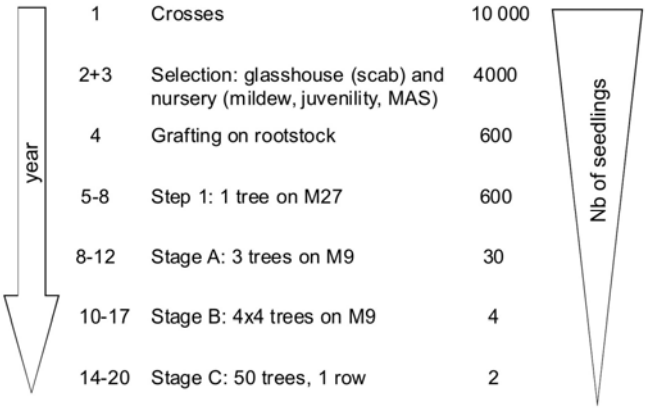


**Fig. 4 Selection scheme used at the Research Station ACW Wadenswil in apple breeding.** (from Kellerhals *et al.* 2008)

in Dresden-Pillnitz (Flachowsky *et al.* 2007, 2009; see below).

The selection process in the ACW apple breeding program is shown in **Fig. 4** following the way of an annual population of about 10,000 seedlings to two outstanding selections that are candidates to become a new cultivar. On average we expect one commercial cultivar out of about 30,000 original seedlings. While designing the crosses, the most advanced and promising breeding material and commercial cultivars are considered. Prior to finalizing the crossing program the envisaged parents are subjected to molecular analysis to confirm the presence of the desired genes by the amplification of the expected marker alleles. The traits involved mainly relate to disease resistance but recently also fruit quality.

Seedlings scored as being resistant in the glasshouse scab screening are planted in an outdoor nursery field. In the second year they are selected in the nursery for mildew resistance, vigour and lack of juvenile symptoms. Good measures for juvenility in young apple seedlings on their own roots are trunk diameter and leaf area (Visser 1965; Murawski 1957). Currently at this first and second year seedling stage, MAS is applied for progenies from crosses where resistance genes have been pyramided. At the nursery stage, juvenility and growth habit as well as powdery mildew incidence are important selection features. Our additional aim is to speed up the process by selecting the promising seedlings already in the first year of growth by allowing better growing conditions. Moreover the graftwood taken from the seedlings will be grafted on rootstock with an interstem in order to allow for early development, flowering and fruiting of the grafted trees.

The total number of seedlings is reduced from originally about 10,000 to 600 at the age of two years by means of phenotypic selection and in specific crosses with molecular selection. Those selected seedlings are grafted on the dwarfing rootstock M 27 to evaluate tree and fruit performance. At ACW MAS currently is mainly applied with specific and pre-selected seedling progenies in their second leaf.

## Data and information management

To successfully conduct an apple breeding program a sophisticated data and information management is essential as the breeding cycle is very long. There is a continuous shift from intuitive breeders experience and intuition towards through data and information management.

Currently, there are various public databases for perennial crops that are related to different aspects of genetics and breeding (Antofie *et al.* 2007). The USDA-ARS Germplasm Resources Information Network (GRIN 2011) is a database which stores information about clonal germplasm in the USDA system, including various tree species as apples, pears stone fruits, grapes, etc. The Genome Database for Rosaceae (GDR 2011) is a curated and integrated web-based relational database. GDR contains data on physical and linkage maps, annotated EST sequences and all publicly available *Rosaceae* sequences. Although this database started as a database for *Prunus*, it is now extending to other families of the *Rosaceae*. Various databases for the management of genetic resources were created by the European Cooperative Programme for Plant Genetic Resources Networks (ECPGR). These databases are crop specific and include apple (Maggioni *et al.* 2002; ECGPR 2011), pear (http://pyrus.cra.wallonie.be/) and various stone fruits (http://www.bordeaux.inra.fr/urefv/base/). The HiDRAS SSRdb (http://www.hidras.unimi.it/) contains detailed information on more than 300 SSR markers that have been mapped in apple. The *AppleBreed DB* uploaded the HiDRAS data, most of which are likely to become public. All these databases are relational, curated and web based. They are continuously extending in content and functionality and it can be foreseen that future generations of breeders will much more relay on such databases and bioinformatics in general.

To ease selection work and to minimize exchanges in the breeding process, the ZIN attempts to acquire and process data more efficiently. Therefore, they developed a data base for selection purposes and the data are collected on a laptop in the field recognising each tree by RFID-technique (Dierend and Schacht 2009).

## Cultivar innovation and marketing

Weber (2008) gave a good overview on apple cultivar innovation and marketing. Apple orchards are usually established for 15 to 20 years and need considerable investment in tree material and other resources. Nowadays new cultivars are usually protected and royalties have to be paid to the owner. A careful choice on which cultivar or which cultivars should be planted is essential. Today mostly there are not the fruit growers and nurseries which primarily decide on which cultivar should be promoted but rather the fruit traders. This trend was mainly reinforced by so called club-cultivars. The introduction of such cultivars includes the whole chain from breeding to cultivar testing, extension service, fruit-growing, quality control and sale to the shelf in the supermarket. Rules are defined for all partners and included in the promotional concept. The successful introduction of new cultivars became more and more difficult in the last 20 years. Prices were dropping, competition has become stronger and some new cultivars turned out to be unsuccessful in the marketplace. The build-up of cultivar clubs is aimed to oppose there trends and to establish a win-win situation for all partners involved. Ideally the grower is no more left alone with the decision about a new cultivar and the risk related to its introduction. He is being integrated in a whole club organization. The cultivar club should give security and confidence to the whole chain up to the consumer. The new apple and the related trademark should become a highly desirable profile of a modern commodity. The aim of many clubs is to control also the quantity of production in order to avoid overproduction with related price drop.

A main reason for the build-up of cultivar clubs was also the increasing private investment in former publically financed apple breeding programs. In many countries the government resigned partially or fully from apple breeding. New models for funding were necessary and cultivar clubs allow to create income through tree and fruit licences which at least partially are financing breeding.

Successful launch of promising selections resulting from breeding programs is a key procedure for their success. Competition among breeding institutes and companies and representatives of cultivars has increased in recent years. This trend was reinforced by the introduction of new cultivars in a club system including exclusivities for growers, marketers and sellers. In parallel with this trend, new cul-

tivars are getting more and more introduced not only by using the cultivar name but also by the attachment of a brand name (Weber 2008). Main objectives of this approach are to protect the whole innovative process of introduction, to get the brand established at business or consumer levels and to receive sustainable returns for the up-front investments. Most of the brands need considerable budgets for promotion and sales campaigns. Therefore, cultivar managers and their companies are using royalties on trees and fruit to finance the introduction of the new cultivar and to co-finance the respective breeding program.

The product life cycle of an apple orchard spans in general about 15 years. Therefore and because of the high investment to establish the orchard, careful evaluation whether to invest into a new apple cultivar or not needs to be carried out. In comparison to cultivars being produced without any royalty system, the new business model of a managed cultivar implicates four parameters which need to be considered by the grower prior investing in a new cultivar and brand. Weber (2008) pointed out the following parameters and targets to create a sustainable integrated system:

- Return on investment by sustainable management,
- Profile of intellectual property orientation and responsibility,
- Supply and value chain by quality of relationships and communication,
- Market response by dedication to a production system (organic, integrated), fruit quality and ethics.

Within such a scheme, all technical questions about yield, fruit size, regular crop, disease susceptibility etc. shall be answered under the aspect "return on investment". Innovative new apple cultivars shall add value in order to stay competitive in the market and to pay back the investments. The success story of today's world leading varieties such as 'Gala', 'Braeburn' and 'Fuji' demonstrated that significant attributes were different from formerly existing varieties. All those varieties became fully accepted only after a growing period of 15 to 20 years within their area of origin and that significant market penetration and consumer awareness can be achieved through promotion and branding (e.g. Pink Lady®). Variety managers and their companies take one part of the risk involved when launching a new cultivar.

### Cultivar protection

Cultivar protection and trade marks have become an important issue in the successful introduction of new apple cultivars within and outside of cultivar clubs. Gary Langford (2007) established a comprehensive overview on the current situation related to cultivar protection and trade marks which is subsequently summarised.

Usually plant material of a new cultivar or information about it is made with a confidentiality agreement such as an experimental agreement (e.g., EUFRIN-agreement). Such an agreement allows early and controlled testing of promising advanced breeding material under diverse growing conditions in order to examine the performance. Breeders should be aware that publication of specific information about a selection or cultivar can trigger the time frame for protection.

Certification of the plant material is an important component of its development. Viruses, such as apple mosaic, apple chlorotic leaf spot, apple stem grooving and apple stem pitting viruses, have a significant impact on the performance of the tree in the nursery and the orchard. Growers need high quality planting material of the specific cultivar to allow for economic success. Certification systems in the USA and Europe will not accept propagating material infected with these viruses. In Europe, nursery trees of the cultivar being entered for PBR trials will not be accepted unless they are tested negative for a set of defined viruses. Virus testing should be completed prior to the introduction of a new cultivar. Plant protection is coordinated by an international convention established by the Union pour la Protection des Obtentions Végétales (UPOV) with its headquarters at Geneva, Switzerland. The UPOV conventions are regularly updated and concern a large number of cultivated plants. The technical guidelines detail the descriptors that are used globally to describe apple cultivars. A new cultivar has to be distinct, uniform and stable (DUS) when compared to the most similar cultivars of common knowledge. To establish the DUS characteristics, a comparative trial at a specifically designated and accredited institute is required. In the case of apple cultivars these trials take around four to six years to be completed. About 40 countries have laws that comply with the UPOV convention. In the European Union it is possible to apply for community cultivar protection. The USA has quite unique plant protection laws via a plant patent (USPP). This is particularly important with regard to the timing of applications. There is a time limit of one year only from the first overseas application or the publication of information about the cultivar. In other countries that are signatories to UPOV there is a period of six years from the first PBR application date to submit applications in these countries.

Therefore the timing of the first commercial sale and release of information is critical in the completion of PBR and a USPP. PBR protection if granted for a period of 25 years. Unlike PBR, trademarks, once granted, last as long the trademark is in use and the renewal fee is paid. Trademarks are used in association with products of a specific description and in the case of apples describe the fruit. Trademarks are generally used in conjunction with PBR. Trees protected by PBR become authorised trees for the production of a product that can use the trademark. A trademark cannot be registered if it can be proven that it is an existing cultivar name. The trademark Re-cultivars® was protected for a group of cultivars, the Dresden-Pillnitz scab resistant cultivars.

Good control of the cultivar protection is important as the commercial development of a new cultivar causes significant costs.

## MOLECULAR TECHNOLOGIES, EFFORTS AND APPLICATION

Breeding is aimed on the creation of new, improved cultivars which will be competitive on the market. Since controlled crosses became a tool in apple breeding, breeders in general created genetic variability by crossing well by well. They subsequently selected for the best progenies to test their suitability for becoming a cultivar. Before molecular techniques were explored, decisions to select progenies were exclusively based on phenotypic data. The development of the first molecular tools, e.g. RFLPs (restriction fragment length polymorphisms) (Grodzicker *et al.* 1974), offered the possibility to determine the genotype and to understand the genetics of traits. The association of a distinct RFLP-marker to a specific phenotypic trait allowed the estimation of the phenotype before it is expressed by applying markers. Since the detection of a thermostable DNA polymerase (Chien *et al*. 1976) and the development of PCR (Saiki *et al*. 1988) the tools available for the genotypic detection of traits, i.e. the estimation of phenotype, increased rapidly. Since that time different PCR based marker systems, like RAPDs (random amplified polymorphic DNAs; Williams *et al*. 1990; Koller *et al*. 1993); dCAPS (derived cleaved amplified polymorphic sequences; Neff *et al.* 1998), AFLPs (amplified fragment length polymorphisms; Vos *et al.* 1995; Tignon *et al*. 2000), SSRs (simple sequence repeats; Guilford *et al*. 1997; Gianfranceschi *et al*. 1998), SCARs (sequence characterized amplified regions; Evans and James 2003) and SNPs (single nucleotide polymorphisms; Schmid *et al*. 2003; Chagne *et al*. 2008) as well as other technologies, like DArT (diversity array technology; Kilian *et al.* 2005), micro array systems (Lee *et. al.* 2007; Schaffer *et al*. 2007) and MassARRAY (a technology particularly well suited to distinguishing gene paralogues; Irwin 2008) were developed. Accordingly to the develop-

ment of marker systems statistical methods for mapping of major genes and QTLs (quantitative trait loci) and computer software (Lincoln *et al.* 1993; Stam 1993; Utz and Melchinger 1996; van Oijen 1996) for implementing these procedures have been created (Bernado 2008).

## ESTs, BACs, microarrays and genome sequencing

The possibilities and application of functional genetics increased with the development of cDNA libraries, the sequencing of ESTs (expressed sequence tags), the identification of RGAs (resistance gene analogues) by genome walking approaches in BAC (bacterial artificial chromosomes) or the use of degenerated primers, and will dramatically increase when the whole apple genome sequence will be publically available.

The large number of 250,000 to over than 300,000 ESTs from apple derived from different tissues, conditions, developmental time points, and genotypes for apple have been reported (Newcomb *et al*. 2006; Park *et al*. 2006) forming around 30,000 non-redundant clusters of sequence (Gardiner *et al*. 2007). The number of sequences and singletons from *Malus* cDNA libraries publically available have been listed by Allan *et al*. (2009). The libraries were screened, e.g. to identify genes associated with flavor and aroma components (Park *et al.* 2006) or to study gene expression in response to abiotic stress (Wisniewski *et al.* 2008). By the application of functional genetics several candidate genes for various traits could be discovered, e.g. disease resistance (Baldi *et al.* 2004; Calenge *et al.* 2005b), ethylene pathway (Oraguzie *et al.* 2004; Costa *et al.* 2005), fruit texture (Costa *et al.* 2005, 2008) or fruit flesh color (Chagné *et al.* 2007).

The EST sequencing programs led to the next step in molecular analysis: the simultaneous analysis of gene expression by microarray technology. Two approaches have been published so far. A 3,484 feature cDNA array was used to identify 192 apple cDNAs involved in early fruit development (Lee *et. al*. 2007) and a 15,726 feature oligonucleotide array was screened for genes differentially expressed during fruit ripening (Schaffer *et al.* 2007). Apple arrays are also valuable for the study of heterologous hybridization. Allan *et al*. (2009) describe the use of apple arrays in hybridization experiments with pear (*Pyrus communis*) and peach (*Prunus persica*).

Several BAC libraries have been constructed which led to successful map based cloning of the scab resistance gene *Rvi6* (Vinatzer *et al.* 1998, 2001; Xu *et al*. 2001; Xu and Korban 2002) and the identification of a single BAC clone containing *Rvi15* (Galli *et al.* 2010). A scaffold physical map using BAC fingerprinting and BAC end sequencing was established recently (Han *et al.* 2007; Han and Korban 2008).

The whole genome sequencing project of apple, done by an international consortium led by the Edmund Mach Foundation (FEM), Italy, recently finished the genome sequencing of the diploid cultivar 'Golden Delicious' (Velasco 2009; Velasco *et al.* 2010). Around 80% of the genome, including more than 90% of the genes was assembled in scaffolds and 70% could be anchored to the apple linkage maps using 1,700 molecular markers. The abundant number of approximately 2,000,000 SNPs detected in the 'Golden Delicious' genome indicates the highly heterozygous status of apple.

## Markers, population size and cost efficiency

Whereas the primary interest of a breeder is the development of improved cultivars and the application of markers can support this aim, the main interest of geneticists is to understand the inheritance and variation of traits (Bernado 2008). The cooperation between breeders and geneticists can promote both tasks,

- the creation of genetic variation to analyse traits and
- the identification of molecular markers for major genes and QTLs precisely predicting the loci.

According with the development of tools for genotyping the number, of genetic linkage maps for apple and their saturation with molecular markers, respectively (Hemmat *et al.* 1994; Maliepaard *et al.* 1998; Liebhard *et al.* 2003a; Kenis and Keulemanns 2005; Silverberg-Dilworth *et al.* 2006; Fernández-Fernández *et al.* 2008; Igarashi *et al.* 2008; N'Diaye *et al.* 2008 presented a consensus map), and of markers linked to traits in apple, major genes and QTLs increased rapidly (Oraguzie and Bell 2008, reviewed in Gardiner *et al*. 2007). Patocchi *et al*. (2009a) presented a set of 21 multiplex SSRs spanning most of the apple genome for the rapid detection of markers linked to specific traits.

The overall advantage of molecular markers is the possibility to predict the phenotype of a mature plant at a very early stage, i.e. in apple when the first leaves of a seedling are developed allowing DNA extraction. But the application of a molecular marker in a breeding program depends mainly on the cost efficiency of marker assisted selection (MAS). Byrne (2007) stated that less than 50% of breeders from over 100 fruit and ornamental breeding programs of perennial crops from around the world apply molecular markers due to the high costs. The cost-benefit relationship in fruit trees seems to be more favourable than for annual crops due to their long juvenility and large size (Luby and Shaw 2001). The application of MAS for the prediction of fruit traits, which can be evaluated only after the long juvenility, of traits that require difficult and expensive phenotyping, and for pyramiding of genes could be cost-efficient (Mehlenbacher 1995). The development of new marker technologies (e.g. SNPs and DArT-markers) have increased the number of markers and decreased the cost of marker analyses in several crop species. Especially the development of high-throughput technologies for SNP genotyping (Jenkins and Gibson 2002; Syvänen 2005) increased the number of data points 40-fold together with a 6-fold reduction of costs per datapoint in a commercial maize breeding programme (Eathington *et al.* 2007). But even if the cost for one datapoint decreases, the total amount of money necessary for genotyping will not decrease necessarily. For example, the cost of SNP genotyping ranges from only 3 to 15 Eurocents per datapoint (i.e. one plant sample genotyped for one locus) depending on the number of SNPs multiplexed and analysed in a single reaction – from 256 to 1,536 SNPs (Schaeffer 2006; Ha *et al.* 2007; Hyten *et al.* 2008). But due to the large scale required for the reduction of per sample costs for genotyping, the total costs of genotyping will remain high (Bernado 2008). Additionally, the presence of non-informative markers on a standard SNP chip, since not all SNP markers will be polymorphic in each population, will increase the costs per datapoint (Hyten *et al.* 2008).

Nevertheless, the development of high-throughput techniques and the technique required for their application led to the funding of companies offering DNA isolation and marker application services. Breeders can use these services to apply MAS without the need to invest any money in the implementation of the technique needed. Even in labs of breeders multiplexing markers (Frey *et al.* 2004) or applying techniques like HRM (high resolution melting; Liew *et al*. 2004) the costs reduction and the time needed for genotyping can make MAS profitable compared to phenotyping. Although the development of large scale genotyping together with lower costs per datapoint seem to be attractive for application in breeding, a question about the practical usefulness of these techniques has to be answered: What population size that has to be considered for an efficient use of molecular markers? Two examples:

- If a combination of eight independently inherited resistance genes is aimed to be achieved, only one out of 256 progeny individuals will contain all resistance genes together. The breeder has to create a progeny of 76,800 plants to get 300 plants with all desired resistance genes for further selection steps. The number of progeny plants required increases if the main objective

is the combination of oligo-/polygenic controlled traits.

- An example was delivered by Janick *et al.* (1996). For the establishment of one desirable seedling possessing a combination of five polygenic controlled traits, and the estimation of percentage of desirable seedlings for the five traits are 40, 20, 20, 10 and 10%, at an acceptable level of expression, 6,250 seedlings have to be produced. The creation of 300 plants possessing the desired traits for further selection steps would require the production of 1,875,000 seedlings.

Regarding only the space needed for sowing in a greenhouse a number of around 80,000 seedlings cannot be handled in normal breeding programs independently of the costs for genotyping. A strategy to overcome this problem is the production of plants homozygous for some desired traits (Kellerhals *pers. communication*) or the indexing in multi-trait breeding programs (Bernado 1991) by weighting of traits followed by the selection of a distinct proportion of the seedlings with the highest indices.

Effective MAS of major traits depends mainly on the distance between marker and gene. The prediction value can be increased if two markers closely surrounding the gene are used to avoid selection of recombinants. MAS of QTLs is not that confidential, because genotype-environment interaction as well as epistatic, additive and dominance effects are mostly not well understood (Ribaut and Ragot 2007). Both, the markers for major genes and the marker for QTL are often limited to the progeny in which they were determined but transferability to other progenies is often necessary. Most economically important traits in fruit crops are considered to be QTLs, i.e. they are controlled oligo-/polygenic and inherited quantitatively (Luby and Shaw 2001). QTL mapping studies revealed an average of three to five QTLs for each trait and the reported QTLs accounted in general for 40 to 60% of the phenotypic variation for the trait (Kearsey and Farquhar 1998; Bernado 2002).

## Application of markers, marker-assisted selection

In cases where phenotyping is impossible on seedlings, for example if no phenotyping procedure is available, or phenotyping does not produce distinct phenotypes for identification of resistance genes or phenotyping requires bigger or mature trees, MAS offers the opportunity for early selection. The number of false positive of false negatives individuals depends on the recombination rate between marker and resistance gene as was shown for pyramiding of scab resistance genes (Bus *et al.* 2009a). The reliability of MAS can be proved by using two molecular markers enclosing the gene of interest. Another opportunity is the introduction of traits not present in the geographical location of the breeding program (Collard *et al.* 2005).

As many resistance genes introduced into the apple germplasm came from wild species (and wild species can be regarded as reservoir for the detection of new resistance genes), the introduction of resistance genes requires several pseudo-backcrosses to remove unwanted genome proportions and to minimize genetic drag. A double recombination is necessary to remove linkage drag down- and upstream of the resistance gene but double recombination events are very low within a distance of 15 to 20 cM (Kearsey 1997). Nevertheless, the introduced resistance gene from a wild species will be accompanied by linkage drag. MAS can also be used for the detection of progeny with reduced linkage drag as was shown by King *et al.* (1999) for the introduction of the scab resistance gene *Rvi6* (old denomination:*Vf*) from '*M. floribunda 821*'. But also the function of the gene can depend on linkage drag if it contains resistance enhancing factors (Durel *et al.* 2003), accordingly resistance has to be phenotyped in the respective genetic background to prove the functionality of the introduced gene.

The presence of marker alleles in susceptible plants or the expression of the same allele size for two different genes (e.g. SCAR marker OPL19 produces a 430-bp band for both scab resistance genes *Rvi2* and *Rvi8*, Patocchi *et al.* 2009b) could raise problems in MAS.

Besides marker assisted selection molecular markers are also valuable for the exploitation of germplasm, identification of cultivars and verification of pedigrees, as was shown in the verification of the parents of the Dresden-Pillnitz cultivars (Reim *et al.* 2009).

The application of MAS in breeding to shorten and enhance breeding efficiency is called SMART breeding.

## Marker-assisted selection in resistance breeding

Marker-assisted selection in apple is mainly applied for validation and applications concerning resistance (Bus *et al.* 2009a) and in particular for pyramiding of scab resistance genes (Peil *et al.* 2007; Bus *et al.* 2009a; Kellerhals *et al.* 2009c). This may be due to the fact that:

- most markers were developed for resistance to scab (Gardiner *et al.* 2007) and
- the break-down of the scab resistance inherited from '*Malus floribunda* 821' (Parisi *et al.* 1993; Bénaouf *et al*. 1997).

Pyramiding of (major) resistance genes is regarded as a tool to achieve more durable resistance to a pathogen in apple (MacHardy *et al* 2001; Gessler *et al.* 2006; Kellerhals 2009c; Patocchi *et al.* 2009b). The presence of natural pyramids in apple consisting of major genes has shown to be durable, e.g. scab resistance of the 'Russian seedling' (Bus 2006). On the other hand, examples in other plant-pathosystems have shown that pyramiding of major genes can be risky and not durable (Lebeda 1992). Scab studies in apple demonstrated that at least race 6 is able to overcome the pyramided genes *Rvi1*/*Rvi6* (Parisi and Lespinasse 1996; Laurens *et al.* 2004). Bus *et al.* (2009b) stated that *V. inaequalis* isolates can be expected to overcome several resistance genes and QTLs as an increasing number of them are identified and used in apple breeding. Nevertheless, an improvement in breeding for durability of resistance could be the combination of major genes and polygenic quantitative resistance (Fischer and Fischer 1999; Soufflet-Fresslon *et al.* 2008) since polygenic quantitative resistance is considered to be more durable (Parlevliet 1993; Lindhout 2002; Parlevliet 2002).

Pyramiding of resistance genes requires the application of MAS since the detection by phenotyping alone cannot prove the presence of pyramided resistance genes, but phenotyping can be performed to reduce the number of progenies directed to MAS. MAS gene pyramiding (for inbred lines) has been reviewed by Ye and Smith (2008a, 2008b).

If resistance genes are inherited independently, breeding of pyramided resistance using MAS can be done straight forward. More attention has to be paid if pyramiding of genes in coupling or repulsion on a linkage group will be done, because sometimes a molecular marker linked to two resistance genes cannot distinguish between both (Bus *et al.* 2005b). Multiallelic SSR markers could solve these problems (Gardiner *et al.* 2006). MAS for genes in repulsion can be done with the respective markers for each gene. Since sometimes apple genotypes with pyramided resistance genes in repulsion are not suited to become a cultivar but will be used as resistance donor, a recombination event is required for the combination of these genes in coupling to be inherited. According to the linkage of these genes, molecular markers surrounding each gene closely have to be applied to prove for real recombinants. Depending on the linkage, resistance genes in coupling will be inherited together and promoting resistance breeding. The respective markers are needed for MAS.

As polygenic resistances are controlled by several loci and these QTLs are inherited in a Mendelian manner (Hospital and Charcosset 1997), MAS for QTLs follows the same rules as for major genes (Bus *et al.* 2009a) with the difference that the inheritance of the whole QTL has to proved with flanking markers which was mentioned above for resistance traits with low linkage to the markers. But

nevertheless, a quantitative inherited trait consists of several loci and to achieve a high effect most QTLs involved need to be inherited. This hampers the successful inheritance of QTLs.

### *1. Scab*

The scab resistance genes mentioned in this paper will be named according to the new nomenclature proposed by Bus *et al.* (2009b). Until now a lot of major scab resistance genes and QTL for resistance to scab have been discovered (reviewed in Gessler *et al.* 2006; Patocchi *et al.* 2009b). But due to new sources and increasing knowledge more genes will be detected. Recently, a new scab resistance gene *Vd3* from the founder D3 has been mapped in close vicinity to *Rvi6* (the old denomination was *Vf*) but on the homologous chromosome (Soriano *et al.* 2009), and Bus *et al*. (2010) mapped *Rvi16* on LG3. Although several major genes have been reported conferring resistance to scab, only few besides *Rvi6* have been introduced to commercial cultivars, i.e. *Rvi4*, *Rvi5* and *Rvi13* (Patocchi *et al.* 2009b).

Two clusters for scab resistance genes on LG1 (Soriano *et al.* 2009) and LG2, comprising four major genes as well as several QTLs (Bus *et al*. 2004), could be detected. The organisation of resistance genes in coupling on one LG allows the inheritance of pyramided genes. The cultivar 'Regia' bred in Dresden-Pillnitz (Fischer and Fischer 2008), known to carry the *Rvi4* scab resistance gene (Peil *et al*. 2008a; Patocchi *et al*. 2009b), is assumed to carry indeed two major scab resistance genes. 'Regia' is a decendend from the 'Russian Seedling', the donor of *Rvi2*, *Rvi4* and *Rvi15*. Analysis with molecular markers for *Rvi2* and *Rvi4*, both located on LG2 with a distance of about 40 cM (Bus *et al.* 2005a, 2005b), support the hypothesis that 'Regia' carries both, *Rvi2* and *Rvi4*, resistance genes (Kellerhals and Peil, unpublished data). The hypothesis that 'Regia' carries also *Rvi2* is promoted by the occurrence of stellate necrosis on progeny of a 'Regia' by 'Idared' cross after inoculation with scab (**Fig. 5**). Inoculation experiments with scab strains 2 and 4 to prove if 'Regia' really carries *Rvi2* and *Rvi4* have been performed in 2010 and will be continued in 2011.

Most advances in MAS selection were made in pyramiding of scab resistance genes (Kellerhals *et al.* 2008; Peil *et al.* 2008a; Kellerhals *et al.* 2009c; Bus *et al.* 2009b). Whereas the combination of two major scab resistance genes were reported by Peil *et al.* (2008a) (*Rvi4/Rvi6*) and Bus *et al.* (2009a) (*Rvi2/Rvi6* and *Rvi2/Rvi4*), Kellerhals *et al.* (2008, 2009c) reported the pyramiding of up to three major scab resistance genes (*Rvi2/Rvi4/Rvi6*). In the Dresden-Pillnitz breeding program the cultivar 'Recolor' was developed which decendend from the cross ('Regine' (*Rvi6*) x 'Reglindis' (carrying a polygenic scab resistance inherited from an 'Antonvka' source). It is supposed that 'Recolor' carries *Rvi6* and a QTL of the polgenic resistance, but until now no molecular data support this hypothesis. Kellerhals and his group (pers. comm.) started a breeding program to generate progeny homozygous for pyramided resistance genes. Application of MAS could identify progeny homozygous for *Rvi2/Rvi4/Rvi6.*

Regrettably up to now none of the progeny with pyramided scab resistance genes developed in Dresden-Pillnitz and Wadenswil showed a satisfied fruit quality to become a cultivar (Kellerhals, Peil pers. comm.).

### *2. Powdery mildew*

The phenotypic evaluation of powdery mildew on young seedlings in the glasshouse for selection of resistant progeny using natural inoculum is difficult because it is highly influenced by plant age (seedlings vs. adult plants) and environmental conditions (Korban and Tartarini 2009). In contrast to scab where the application of MAS for the detection of only one major resistance gene is not necessary due to the fact that precise prediction of resistance based on phenotyping of seedlings is possible, MAS for a single


**Fig. 5 Progeny of a 'Regia' by 'Idared' cross after inoculation with scab.** (**A**) Compatible reaction – V*enturia inaequalis* is sporulating. (**B**) Incompatible reaction – stellate necrosis indicating *Rvi2*.

major powdery mildew gene makes sense. Korban and Tartarini (2009) described the present state of powdery mildew research and listed the major genes for resistance detected so far (*Pl1*, *Pl2*, *Pld*, *Plm*, *Plw*) and the available markers linked to these genes. Two more genes were mentioned in an overview of Gardiner *et al.* (2007), *Pln* and *Pla*. *Pln* derived from 'Novosibirsky Sweet' and was detected applying the *Pl1*-marker AT20 to a X3191 x 'Novosibirsky Sweet' population (Dunemann *et al.* 2004). An AU-CAPs and a SNP marker is linked to *Pln*, which might be a new gene or allelic to *Pl1* (Dunemann *et al.* 2004). The rootstock 'Aotea 1' is the donor of *Pla*. Marker analysis indicated a positional relationship of *Pla* to *Pl2* and *Plm*. A new powdery mildew resistance gene *Plbj*, originating from the donor *Malus baccata jackii*, recently was located on LG10 between the two SSR markers Ch02a10 and Ch02c11, and an AFLP derived SCAR marker, PLBJ-SCAR, was developed by Dunemann und Schuster (2009). Genes *Pl1* from *M.* x *robusta* and *Pld* from D12 seem to be located on a resistance cluster on LG12 (James *et al.* 2004; Dunemann *et al.* 2007), and *Pl2* and *Plm* map close together on LG11 (Bus *et al.* 2010), both genes are linked to the N18-SCAR (Gardiner *et al.* 2003). But it is still unclear if the regions of *Plm* and *Pl2* are homologous or homeologous (Gardiner *et al.* 2007).

Besides major genes several QTLs for powdery mildew resistance and markers linked to the QTLs have been reported (Kellerhals *et al.* 2000; Stankiewicz-Kosyl *et al.* 2005; Calenge and Durel 2006).

At Dresden-Pillnitz three powdery mildew resistance genes (*Pl1*, *Pl2*, *Plm*) were pyramided, and the pyramid *Pl1/Pl2* was successfully combined with the pyramid *Rvi4/Rvi6* (Peil *et al.* 2008a). Because fruit quality cannot be expected from the cross *Pl1Pl2* x *Plm*, progeny with all three powdery mildew genes have to be crossed with a high

quality cultivar. If the genomic regions of *Plm* and *Pl2* are homolog, recombination is required to inherit all three genes. Application of molecular markers to a progeny *Pl1Pl2Plm* x fruit quality will help to determine the position of *Plm*.

### *3. Fireblight*

All QTLs determined for resistance to fire blight so far were discussed in a review regarding the improvement of fire blight resistance in apple and pear (Peil *et al.* 2009). For the breeders challenge to develop a new fire blight tolerant cultivar, the QTL identified on LG7 of 'Fiesta' (Calenge *et al.* 2005a; Khan *et al.* 2007, 2009) seems to be most appropriate. However it explains only up to 46% of phenotypic variation. A QTL identified on LG3 of '*Malus* x *robusta* 5' (Mr5) explains up to 80% of the phenotypic variation (Peil *et al.* 2007, 2008b). The most important reason is that 'Fiesta' is already a cultivar and Mr5 is a wild species with abundant genetic drag only useful for establishing prebreeding material. The application of the two markers, AE10-375 and GE8019, bracketing the QTL on 'Fiesta' LG7, FBF7, proofed the stability of the QTL effect in different backgrounds and showed that plants carrying both markers were significantly more resistant than those that did not amplify both markers (Khan *et al.* 2009). Kellerhals *et al.* (2009b, 2009c) already used these markers for selection in a 'Milwa' x 'Enterprise' population. But nevertheless breeders have to keep in mind that a plant/progeny/cultivar carrying the QTL region identified by both markers is not inevitably resistant or more resistant than another not carrying both markers. Only a preselection is possible and reasonable. The resistance to fire blight has to be confirmed by artificial inoculation.

### *4. Pests caused by insects*

Major genes for resistance to leaf curling aphid (*Dysaphis* cf. *devecta*) and woolly apple aphid (*Eriosoma lanigerum*) and available markers have been summarized by Gardiner *et al.* (2007). Recently Bus *et al.* (2008, 2010) mapped the four woolly apple aphid resistance genes *Er1*, *Er2*, *Er3*, and *Er4* to LGs 8, 17, 8, and 7, respectively, and developed markers for all four genes and proved their applicability in MAS. Bus *et al.* (2009a) pyramided *Er1* and *Er3* by crossing 'Northern Spy' (*Er1*) x S26R01T053 (*Er3*) and identified 38 progenies homozygous for the marker allele of NZsn_O05, linked close to both genes. In this first generation all progenies with both resistance genes will carry them in repulsion phase and recombination during the next cross is required to put them in coupling.

Both major resistance genes to leaf curling aphid (*Sd1* and *Sd2*) are located on LG7 (Maliepaard *et al.* 1998; Cevik and King 2000, 2002). Stoeckli *et al.* 2008b identified a QTL for *D.* cf. *devecta* resistance in 'Fiesta' on LG7 explaining up to 20% of the phenotypic variance at the same position like *Sd1* and mentioned that the resistance is therefore due to *Sd1*.

Stoeckli *et al.* (2008b, 2009a, 2009b) also mapped a putative QTL for resistance to *D. plantaginea* explaining only up to 8.5% on 'Fiesta' LG17, a QTL for resistance to codling moth (*Cydia pomonella*) explaining up to 8.2% of the phenotypic evaluation and two significant QTLs for rust mite (*Aculus schlechtendali*) resistance both on 'Fiesta' LG7 explaining up to 12.3 and 16.6% of phenotypic variance.

Due to the difficulties in reliably phenotyping insect pests, MAS for insect resistance will offer a good opportunity for preselection. However, whether MAS for the so far located QTLs will be valuable for breeding is still quite unclear.

## Fruit quality and marker-assisted selection

Fruit quality is the main topic for a breeder and for the decision to release a new cultivar. Since apple has a very long juvenile period, which can last up to seven to eight years (Hanke *et al.* 2007) and during this time the formation of fruits is impossible; MAS for a reliable prediction of fruit quality would be of greatest benefit for breeders. Despite two major genes for fruit color, *Rs* and *Rf* (Weeden *et al.* 1994; Maliepaard *et al.* 1998) and a major genetic determinant for red colour in the core of the apple fruit (Chagné *et al.* 2008), most traits determining fruit quality analysed genetically so far are controlled quantitatively. A number of QTLs were detected for sensorial and physical measurements of fruit quality parameters like fruit flesh firmness, stiffness, crispness, juiciness, sponginess, acidity, brix-content and others (Maliepaard *et al.* 1998; King *et al.* 2000, 2001; Liebhard *et al.* 2003b; Kenis *et al.* 2008). However, most of those QTLs are largely dependent on the environmental conditions and only a few were stable over several years. The confirmation of these QTLs in other genetic background, in different environments and over several years is a presumption for the development of reliable markers and the application of MAS to improve fruit quality.

Recently, two papers concerning aroma compounds of apple have been published (Zini *et al.* 2005; Dunemann *et al.* 2009) and an abundant number of QTLs were identified. Dunemann *et al.* (2009) detected 50 putative QTLs for only 27 different apple fruit volatiles. Assuming that the real number of volatiles is much higher and that a specific combination of numerous volatiles is required to give a good aroma, breeding for aroma using molecular markers still seems to be far away.

Additionally, numerous loci for the four allergenic groups of apple, respective candidate genes and identification of alleles most likely involved in allergenicity (Gao *et al.* 2005a, 2005b, 2005c, 2008), QTLs for vitamin C content of fruit flesh and skin (Davey *et al.* 2006), genes involved in the ripening process/ethylene production (Costa *et al.* 2005; Wiersma *et al.* 2007; Costa *et al.* 2008) and three malic acid related genes (Yao *et al.* 2009) have been detected or isolated.

Numerous QTLs for fruit harvest date, diameter, height, size and weight have been reported (King *et al.* 2000, 2001; Liebhard *et al.* 2003b; Kenis *et al.* 2008).

The number of QTLs and genes involved in the building of fruit ingredients, determining physical and sensorial fruit traits is abundant. Most of the QTLs discovered are highly dependent from environmental conditions. If these genes or QTLs will be applied in MAS has to be examined critically by the expected gain in relation to additional costs.

## Growth traits and marker-assisted selection

Markers for growth traits might be valuable if tree architecture and shoot morphology suited for specific production systems, regular bearing, productivity and/or self thinning could be predicted reliably, thus saving costs for growing and screening seedlings in an orchard. But since growth traits are controlled by QTLs and underlying ontogentic factors and environmental conditions as well as pruning systems, the predictability of these traits until now is low.

Tree topology and geometry on a one-year-old apple progeny of 'Starkrimson' x 'Granny Smith' was phenotyped for the detection of QTLs involved in architectural traits (Segura *et al.* 2007). QTLs for tree geometry, i.e. tree form, internode length and long sylleptic axillary shoots were identified on LGs 2, 3 7, 8, 10 and 16. The QTL for basis angle of long sylleptic axillary shoots on LG10 could explain up to 29% of the phenotypic variation, the highest value discovered for a single QTL observed in this study. Regarding tree topology Segura *et al.* (2007) identified QTLs for the number of axillary spurs on LGs 1, 9, 13 and 16, QTLs for the number of long shoots on LGs 3 and 13, and QTLs for the number of spurs on LGs 1 and 16. The

global model explained up to 64% of the phenotypic variation for the number of axillary shoots followed by 38 and 27% for the number of long shoots and number of spurs, respectivly. Furthermore, two QTLs on LGs 6 and 8 were detected for day of budbreak with a broad sense heritability of 0.58. Kenis and Keulemans (2007) studied tree architecture in apple on a 'Telamon' x 'Braeburn' population and analysed the influence of the *Co* gene, inducing columnar growth, on growth traits. The *Co* gene was mapped on LG15 of 'Telamon' but with a distance of 23.6 cM to the col marker, i.e. a significantly higher recombination frequency as observed by Hemmat *et al.* (1997) in two 'Wijcik McIntosh' populations. Kim *et al.* (2003) reported on a RAPD marker located close to the *Co*-gene, i.e. 1.8 cM. The location of the *Co*-gene is contradictory to the mapping on LG10 in a 'Fiesta' x 'Prima' population by Maliepaard *et al.* (1998), but maybe LG15 of 'Telamon' is homologous to LG10 of 'Fiesta' x 'Prima'. QTLs for several growth traits clustered close to the *Co*-gene. Conner *et al*. (1998) determined QTLs for internode number, internode length, branch number and branch diameter were close to *Co.* These QTLs, except the QTL for internode number, were confirmed by Kenis and Keulemans (2007).

QTLs for tree habit in seedlings and mature trees were mapped by Liebhard *et al.* (2003b). Kenis and Keulemans (2007) reported that QTLs for tree architecture were instable over years and for different root systems and that the genetic control changes during maturation and is influenced by environmental conditions denying the possibility of successful MAS for growth traits at the current stage of knowledge. Recently Segura *et al.* (2009) dissected tree architecture into genetic, ontogenetic and environmental effects, thus enhancing the possibility for the application of MAS.

A major QTL for dwarfing, *Dw*, was located on LG5 in a M9 by '*M.* x *robusta* 5' population (Rushholme-Pilcher *et al.* 2008).

### Cultivar identification

Molecular markers can also be applied to distinguish cultivars and clonal selections to assign synonyms and to fingerprint collections. Molecular identification/ discrimination of cultivars was performed with RAPDs (Koller *et al.* 1993), AFLPs (Tignon *et al.* 2000) or SSRs (Guilford *et al.* 1997; Galli *et al*. 2005). SSRs were also applied for the genotyping of genetic resources to improve collection management and cultivar identification in the Netherlands (van Treuren *et al.* 2010) and in Switzerland (Szalatnay *et al.* 2009). Moreover, genetic relationships in apple were analysed and synonyms were detected (Bassil *et al.* 2009). The discrimination of sports from mother cultivars could facilitate the process of getting breeders rights and would be helpful in litigations. Whereas AFLP markers failed to distinguish mutant cultivars like 'Golden Reinders', 'Queen Cox' or a fruit color mutant of 'Jonagold' from the mother cultivars, a thermoclone of 'Jonagold' produced different AFLP patterns on three distinct apple trees (Tignon *et al*. 2000), indicating a certain level of genetic instability within this cultivar. The usefulness of AFLPs for this purpose is still quite unclear since Donini *et al.* (1997) revealed AFLP polymorphisms between template DNA extracted from different plant organs. Pancaldi *et al*. (1998) used a RAPD strategy, template mixing, to discriminate mutants without success. But nevertheless by applying RAPDs they could distinguish 'Starking VF' from two other 'Red Delicious' mutants 'Starkrimson' and 'Red Chief'. Successful discrimination of sports from the mother cultivar was reported for pear. A retrotransposon based marker technique, sequence-specific amplified polymorphism (S-SAP), was successfully applied to apple and pear. Venturi *et al*. (2006) could discriminate 'Gala' from six skin color mutants ('Ruby Gala', IG31, 'Gala Must', 'Galaxy', 'Royal Gala', and 'Mondial Gala'), all bud sport mutants (Dickinson and White 1986), and between the mutants, and 'Hillwell' from the mother cultivar 'Braeburn', indicating that the sports are due to retrotransposon insertion. The S-SAP approach was successful in pear, too, 'Max Red Bartlett', 'Rosired' and 'Sensation' could be distinguished from 'Bartlett' (Venturi *et al*. 2009). This tool will be a valuable tool for discrimination of sports in apple and could help in the result of disputes regarding PBR.

Furthermore, molecular markers are valuable in determining of pedigrees. Reim *et al.* (2009) analysed the parents of the cultivars bred in Dresden-Pillnitz and detected that the assumed pedigree of some of the cultivars was wrong.

A set of 12 SSR markers was assigned by the ECPGR *Malus/Pyrus* working group for the fingerprinting of cultivars, clones and wild species. The application of a common set of markers allows the comparison of fingerprints from different collections. To standardize the fragment size a set of eight cultivars/wild species was chosen as reference (van Treuren *et al.* 2010).

## *IN VITRO* CULTURE AND GENETIC ENGINEERING

*In vitro* culture and the role of cytokinins in the regeneration process in apple was recently reviewed by Dobránszki and Teixeira da Silva (2010) and Magyar-Tábori *et al.* (2010), respectively, and lies beyond the scope of this review. Genetic transformation in apple was recently summarized by Dolgov and Hanke (2006), Aldwinckle and Malnoy (2009) and Hanke and Flachowsky (2010). Many transformation efforts have been made since the publication of the first report on apple transformation in 1989 (James *et al.* 1989). Since that time many protocols for transformation and regeneration of apple scion cultivars, rootstock cultivars and apple wild species were described, but the most effective method has remained the *A. tumefaciens*-mediated leaf disc transformation and regeneration of genetically modified plants through adventitious shoot formation (Hanke and Flachowsky 2010). Even though a number of selectable marker genes like the *bar* gene of *Streptomyces hygroscopicus* (de Bondt *et al.* 1996; Lebedev *et al.* 2002; Szankowski *et al.* 2003; Dolgov and Skryabin 2004), the *manA* gene of *Escherichia coli* (Flachowsky *et al.* 2004; Zhu *et al.* 2004; Degenhardt *et al.* 2006, 2007), the *Vr-ERE* gene of *Vigna radiata* (Chevreau *et al.* 2007) or the *Dao1* gene of *Rhodotorula gracilis* (Hättasch *et al.* 2009) have been tested on apple, the selection of transgenic apple plants is still performed by using the *nptII* selectable marker gene and kanamycin as a selective agent.

Beside the establishment of the transformation technology work was focused on the improvement of agronomically important traits such as resistance to biotic and abiotic stress and herbicides. Other studies have been engaged with self fertility, dwarfing, rooting ability or precocity. Furthermore, much work was dedicated to improve traits, which are related to the fruit (for review see Aldwinckle and Malnoy 2009; Hanke and Flachowsky 2010). Most of these studies have been performed with foreign genes originating from non-crossable, genetically distant species such as viruses, bacteria, fungi or other plant species like pea, rice, wheat, barley and silver birch (reviewed in Aldwinckle and Malnoy 2009).

However, as tempting as it seems to have a construction kit system with different selectable marker genes and transgenes for each trait whichever needs to be improved, the ultimate aim is a marker-free transgenic plant (Gessler and Patocchi 2007) containing only native DNA or at least DNA from crossable species. Marker-free GM apple plants can be produced by using clean vector technologies or by transformation without the use of any marker gene. A first clean vector system was recently tested on apple (Krens *et al.* 2004) and transformation without the use of marker genes has been described several times (Flachowsky *et al.* 2004; Malnoy *et al.* 2007, 2010). Both technologies resulted in the production of more or less marker-free plants. Using such a technology and a native resistant gene like the *HcrVf2* scab resistance gene of '*Malus floribunda* 821', the

development of so-called "cisgenic" plants as suggested by Schouten *et al.* (2006a, 2006b) and Joshi *et al.* (2009) should be a realistic aim. Another promising strategy was recently published by Flachowsky *et al.* (2009). The authors suggested the use of transgenic early flowering apple plants in a classical crossbred breeding program to reduce the amount of time which is needed to produce a new apple cultivar. Using the transgenic line T1190 (cv. 'Pinova') containing the *BpMADS4* gene of silver birch (Flachowsky *et al.* 2007) as crossbred parent, one generation per year is feasible. The system can now be used to introgress resistance genes from apple wild species into the cultivated apple by natural crossing, to realize several backcross generations and to produce a new and highly resistant apple cultivar, free from any foreign DNA sequences, within a few years.

A total of 57 field test applications (notifications and release permits) were found for the U.S. within the Environmental Releases Database (2011). To date only eleven full release permits have been listed. The majority of the GM apples which were intended to be released were modified in a way that fruit quality parameters (e.g. fruit ripening, low browning, storability etc.) or the resistance to insects or bacterial and fungal diseases were improved. In Europe, releases of GM plants have to be notified according to Directive 2001/18/EC. Since 1991 a total of ten summary notifications can be found for GM apples, but only two field trials have really been performed until now. The others were refused by the national authorities or by the regional minister or they could not start because of the lack of consent given by the competent authority. One field trial has been performed in The Netherlands. This field trial was performed with GM apple plants over-expressing the *hth* gene of barley, which encodes for an alpha-hordothionine. These plants were tested in the field on their resistance to apple scab, powdery mildew and fire blight. The second field trial has been carried out in Sweden. The released GM apple rootstocks were transformed with the *rolB* gene of *Agrobacterium rhizogenes* to improve the rooting ability. No GM apple cultivars are in the market to date, but different international activities are going on. The Okanagan Specialty Fruits Inc. in Summerland (British Columbia, Canada) for example is working on the development a low browning GM apple for processing and apple chip production. The commercialization of the first low browning apple cultivar is expected in the near future (http://www.okspecialtyfruits.com). Research groups in Switzerland and in The Netherlands are working on the development of cisgenic apple plants containing the *HcrVf2* (*Rvi6*) scab resistance gene from the crab-apple '*Malus floribunda* 821'. The projects are promising, but the commercialization of the first cisgenic apple cultivar in Europe is not expected before 2013.

## FUTURE WORK AND PERSPECTIVES

There is a good scope in apple breeding to develop high quality and multiple resistant cultivars with pyramided resistance genes, to achieve durable resistance, suited for sustainable production with minimal applications of pesticides. However, the desired reduced plant protection by growing multiple resistant cultivars may lead to an increase in other diseases or new diseases will appear challenging future breeding. Anyway, breeding is laying the genetic basis for the development and adapted sustainable growing and protection strategies. The increasing knowledge on marker trait associations and the availability of molecular markers offers the opportunity for early selection. Approaches such as the development of plants homozygous for several resistance loci will improve breeding by inheriting the homozygous traits surely. The development of high throughput techniques for DNA isolation or marker application facilitates the simultaneous screening of a high number of loci and/or distinct alleles. But nevertheless, there is a gap between application of markers in apple breeding and available knowledge and techniques. This gap has to be filled for example by the education of a new breeder generation in molecular techniques to promote molecular breeding. Classical and molecular breeding approaches need to be combined for successful and economic breeding.

The publication of the entire sequence of the 'Golden Delicious' genome will promote the development of markers, identification of genes and enables powerful tools for functional genomics. An abundant amount of knowledge will be generated in a relatively short time and this knowledge should be implemented in breeding tools. According to the abundant amount of data that can be generated, e.g. by SNP-chips, software tools are needed for the analysis of the data. If multiplexing techniques, simultaneously analysing thousand of data points, will be really valuable for practical breeding in the future depends on population size and the number of traits to select for. The larger the number of traits to be analysed the larger the population size has to be, but the number of manageable seedlings is limited. The breeders' interest will concentrate on extensive genotyping of putative parents to combine as much as as possible positive alleles. If sequencing costs will decrease as fast as in the last years the complete sequencing of individual genotypes seems to be possible.

Alternatively to classical breeding it will also be possible to improve already existing cultivars. Methods are available to produce cisgenic apple plants containing only DNA of *Malus*. The genome sequencing project of apple will deliver huge information and facilitate the detection and isolation of new resistance genes for transformation. Cisgenic cultivars carrying several introduced traits will be developed allowing sustainable production. The advantage of cisgenic cultivars is that cultivars already accepted by growers, markets and consumers can be improved by adding genes of *Malus*. The precondition for growing cisgenic cultivars in production orchards is the acceptance of the product, the apples by the consumer. The introduction of specific traits by transformation can be useful to improve breeding cultivars with outstanding quality.

Both cisgenic approaches and classical breeding implementing molecular tools will complement each other in the development of new cultivars.

The number of cultivars getting breeders rights has dramatically increased in recent years but the market for new cultivars is limited. The marketing of cultivars in so called "cultivar clubs", the cooperation of breeders and nurseries or trading companies enforces the need of reimbursement of costs. This tendency enlarges the competition of breeders and decreases the volition and/or the possibility to exchange breeding material while the cooperation on scientific issues is increasing. There is a challenge that breeders will cooperate in the future to build and process very large populations with modern breeding tools.

Whereas the development of breeding and the cooperation in breeding in the future is quite unclear, SMART breeding, i.e. the use of molecular markers to shorten and improve the selection process, is already applied by some breeders and will be the future for more and more breeders and absolutely necessary for the successful development of better cultivars with pyramided resistances.

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