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# 3 Rootstock Breeding: Current Practices and Future Technologies

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## 3.1 Introduction

The aim of plant breeding is to 'maximize the probability of creating, and identifying, superior genotypes which will make successful new cultivars. In other words, they will contain all the desirable characteristics/traits necessary for use in a production system' (Brown and Caligari, 2011). Prior to commercial release of a new cultivar, the breeding process requires: (i) identification of variable germplasm; (ii) hybridization to combine genetic materials from different sources into a single entity; (iii) selection of superior genotypes with a favourable combination of characteristics; and (iv) multiplication of stable cultivars.

The available germplasm resources for vegetable rootstock breeding are described in detail in Chapter 2 (this volume). Here, we will consider how to combine different sources of genetic variation, for example by overcoming species barriers, and how to select useful rootstocks. The many traits that can be associated with the root system or conferred by a rootstock to the scion are outlined in Plate 6, and are the topic of more detailed discussion elsewhere in this book. First, we will consider the impact of the practice of grafting on breeding strategies.

## 3.2 Stacking Traits: Meiosis or Grafting or Both?

Combining characteristics from different sources into a single seed line, known as 'trait stacking' or 'pyramiding', is challenging. The first reason is genetic linkage:

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positive and negative alleles may occur at closely linked loci, and recombining them in the most favourable way may require screening of huge numbers of individual plants to find rare meiotic recombination events, a process that becomes increasingly difficult as the number of genetic loci involved increases. Secondly, there may be negative consequences from pleiotropic effects of a single allele on many traits; for example, a gene that increases root growth may also be expressed in a fruit and have a negative effect on fruit quality, but the two effects cannot be separated in a genetically homogenous non-grafted plant. Thirdly, there may be complex epistatic interactions whereby the effect of an allele at one locus depends on the presence of a specific allele at another locus; such interactions can be described, but their complexity makes breeding more difficult. Finally, we must recognize the difference between qualitative and quantitative traits; the former are determined by a single locus that can produce a large effect on the trait, for example a disease resistance gene, which is relatively easy to track. In contrast, quantitative traits are controlled by a large number of quantitative trait loci (QTLs), each of which has a small effect on the trait, leading to a continuous distribution of trait values. Selecting for improvement of a quantitative trait clearly is far more challenging because of the need to combine alleles at a large number of genetic loci.

Historically, the primary goals of most breeders of graftable vegetable crops were enhanced yield, fruit quality and disease resistance. This has led to the release of commercial elite scion cultivars that are high yielding and have fruit characteristics suitable for postharvest handling, and that are attractive to consumers, usually combined with a range of key qualitative disease resistance loci. Adding further rootzone-expressed traits to such cultivars requires crossing and reselection of all favourable traits, a long and expensive process that can take up to 10 years. In addition, desirable rootzone-expressed traits tend to be highly dependent on the environment due to local soil conditions and the associated abiotic and biotic pressures, and therefore differ among growing systems. Trait stacking by grafting has many advantages: the breeding goals for the rootstock and scion are different, and they can be bred independently, breaking the breeding scheme into two more easily addressed challenges. The rootstock can also include a much larger proportion of wild-species DNA than the scion without impacting on domestication traits, such as fruit size and quality. The impact of the traits from each are then combined through the graft, and scion and rootstock combinations can be chosen by the grower or plant nursery to address specific market or environmental requirements. In comparison, a non-grafted system requires a larger number of whole-plant cultivars to provide the same degree of flexibility and choice to the grower, and the deployment of wild-species germplasm is severely restricted due to impacts on marketable yield.

For growers, the only disadvantage of the grafting strategy is the additional expense of purchasing grafted transplants, but it is clear from the rapid increase in the industry take-up of grafting that the economic advantages outweigh the extra costs. For commercial seed companies, the situation is more complex: grafted crops tend to have lower planting densities, often with two main stems grown from a single rootstock, and therefore seed sale volumes are lower in grafted crops, even if supplying both rootstock and scion seeds; rootstock × scion compatibility is an additional complication to the selection criterion when breeding rootstocks,

and selection protocols must discover successful rootstock × scion × environment combinations (Cohen *et al.*, 2007). Seed companies must also pay great attention to rootstock seed quality to provide the very high germination uniformity and disease-free status demanded by grafting nurseries. Finally, if not policed, the possibility of illegal propagation of commercial $F_1$ hybrids by grafting side shoots on to rootstocks becomes a threat to seed company sales when grafting is widely practised. However, seed companies have been reacting strongly to the increasing trend towards grafting, with most vegetable seed companies actively involved in rootstock breeding, and there are significant commercial opportunities in some markets to develop effective rootstocks where the benefits of grafting are still marginal and uptake is low (e.g. in pepper), or to increase market share where a few well known rootstock cultivars dominate (e.g. in tomato). Vegetable seed companies, including those that breed rootstocks (Table 3.1) have been undergoing considerable consolidation, resulting in a handful of large multinational corporations (Howard, 2009), a process that has accelerated in recent years, and inevitably this reduces the tendency to breed for specific local environments. As a result, there is also an important role for breeding of vegetable rootstocks for 'public good', especially where there can be rapid gains in crop production by overcoming biotic and abiotic constraints in developing countries (Palada and Wu, 2008; Keatinge *et al.*, 2014).

Perhaps the ultimate goal of the vegetable breeder is to combine all the rootstock and scion traits into one non-grafted cultivar, a challenge that could be met eventually if a greater understanding of the genetic loci is achieved. However, some alleles have positive and negative effects in the root and shoot, respectively, which might give a neutral or negative overall effect on yield in a non-grafted

**Table 3.1.** Major companies that breed and supply vegetable rootstock seeds. This is a non-exhaustive list in alphabetical order. All websites accessed 16 April 2016.

| Company/group | Website |
|---|---|
| Asahi Industries | https://www.asahi-kg.co.jp/en/products/agriculture/seed/index.html |
| Bejo Zaden | http://www.bejo.com/ |
| BHN Seed | http://www.bhnseed.com/ |
| Capgen Seeds | http://www.capgenseeds.com/en/ |
| De Ruiter/Monsanto | http://www.monsanto.com/products/pages/deruiter-seeds.aspx |
| DP Seeds | http://www.dpseeds.com/rootstock |
| Enza Zaden | http://www.enzazaden.us/products/fruitvegets/ |
| Gautier Semences | http://www.gautiersemences.com/ |
| Hazera/Limagrain | http://hazerainc.com/ |
| Vilmorin/Limagrain | http://www.vilmorin.com/ |
| Nunhams/Bayer | http://www.nunhems.com/ |
| Origene | http://www.origeneseeds.com/ |
| Rijk Zwaan | http://www.rijkzwaan.com/ |
| Sakata | http://www.sakata.com/ |
| Seminis/Monsanto | http://www.monsanto.com/products/pages/seminis.aspx |
| Syngenta | http://www.syngenta.com/global/corporate/en/Pages/home.aspx |
| Takii Seed | http://www.takiiseed.com/ |
| Zeraim Gedera/Syngenta | http://www.zeraim.com/ |

plant but a positive effect when deployed only in the rootstock of a grafted crop, as reported for IL8-3 in tomato (Gur *et al.*, 2011). In addition, grafting will always provide the most rapid strategy to deploy new traits, such as a new soilborne disease resistance allele in the rootstock or a new fruit-quality gene in the scion, without the need to recombine and reselect all traits into a single non-grafted cultivar: breaking the complex breeding task into two simpler parts, later joined by grafting, speeds up the process.

## 3.3 Developing Stable Core Collections of Germplasm for Breeding

Breeding programmes, including for rootstocks, rely on the genetic diversity present in germplasm collections; in Chapter 2 (this volume), the wealth of plant genetic resources for the Solanaceae and Cucurbitaceae was presented. Typically, germplasm collections include landraces (local varieties), locally and internationally bred cultivars, wild species and wild relatives, each of which is registered in a database and conserved by seed multiplication. Core collections are established after morphological and genetic diversity analysis (Yetişir *et al.*, 2008) to efficiently represent the full range of allele diversity and wealth, without unnecessary duplication. Where possible, the open-pollinated members of the core collection are often converted to inbred lines by five or more rounds of self-fertilization and selection to gradually fix segregating loci to homozygosity; this allows the collection to be easily conserved and multiplied by avoiding further genetic segregation, but the process can take several years (Fig. 3.1).

Alternatively, homozygous lines can be generated by forming doubled haploid (DH) plants. In this process, haploid plants are produced from gametophytic cells by anther, microspore or ovary culture. The resulting haploid plants are sterile, but their chromosome numbers can be doubled through natural processes or chemically (using colchicine) to produce fertile DH plants in a much shorter time than classical self-pollinating methods (Germanà, 2011). However, the success of this process varies among species.

In pepper (Irikova *et al.*, 2011; Ochoa-Alejo, 2012; Cheng *et al.*, 2013; Kim *et al.*, 2013) and aubergine (Başay *et al.*, 2011; Salas *et al.*, 2011; Başay and Ellialtioğlu, 2013), anther culture is used effectively to produce DH lines by seed companies, but this does not work in tomato (Bal and Abak, 2007; Seguí-Simarro *et al.*, 2011; Moreno *et al.*, 2012). Anther culture has also been used for the production of dihaploids by halving the chromosome number from the tetraploids that arise from somatic hybridization in aubergine (Rizza *et al.*, 2002).

Regenerating haploid plants through ovule and ovary culture in the Cucurbitaceae family is possible although often challenging (Li *et al.*, 2013). However, haploid embryos may be induced by pollination with irradiated pollen or with pollen of a different species (Sari *et al.*, 1994; Kiełkowska *et al.*, 2014), and this has been investigated in melon (Abak *et al.*, 1996), watermelon (Gursoz *et al.*, 1991; Sari *et al.*, 1994), cucumber (Caglar and Abak, 1999) and pumpkins (Kurtar, 2009; Kurtar *et al.*, 2002); these techniques are now used routinely by breeders and researchers.

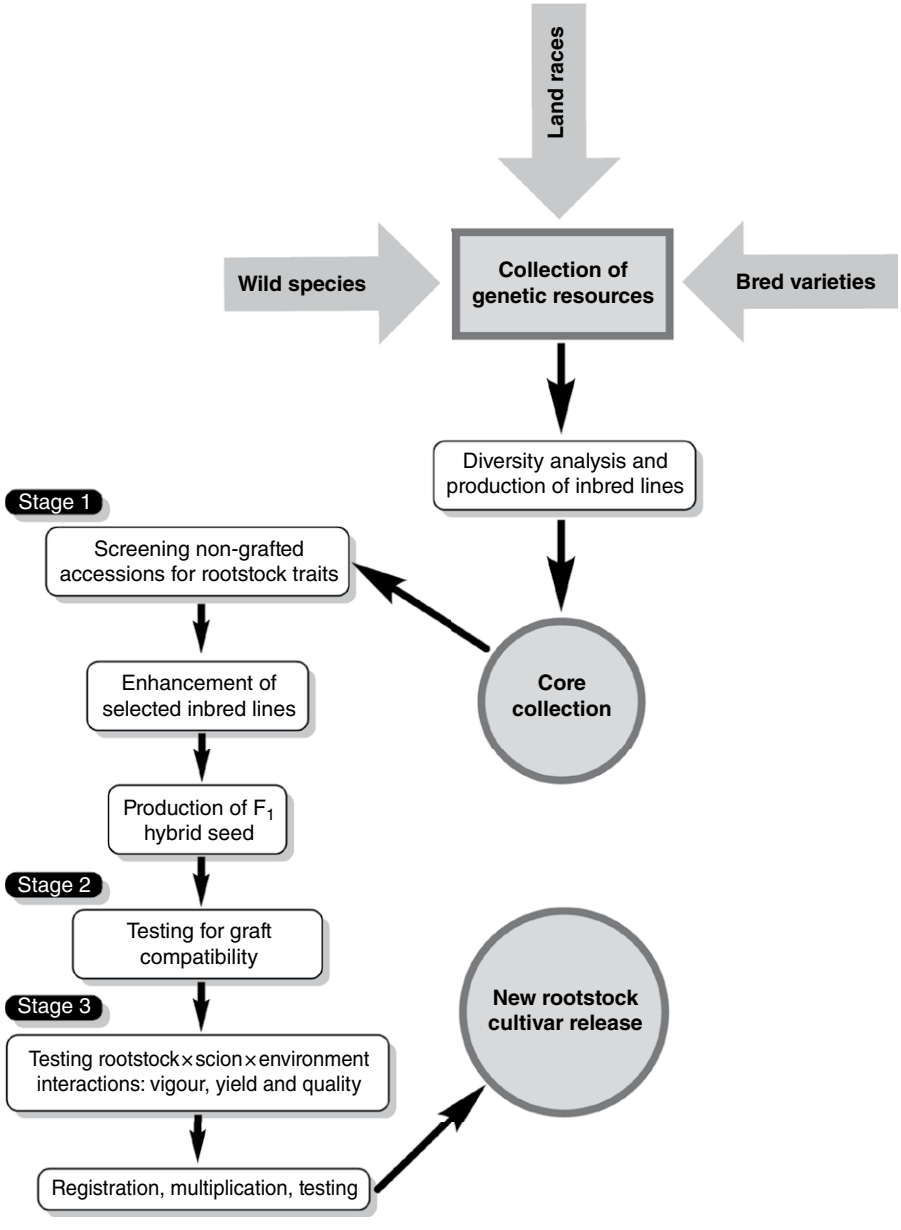


**Fig. 3.1.** Example of the process of breeding a new rootstock cultivar. The different stages are described in more detail in the main text.

## 3.4 Deploying Genetic Diversity for Rootstocks

### 3.4.1 General principles

Useful traits, particularly disease resistances, exist in wild relatives of the key grafted crops tomato, aubergine, pepper, cucumber, melon and watermelon, but

these wild species often have poor germination, low vigour or poor graft compatibility. For example, germination rate and uniformity can be a problem with the wild species *Solanum torvum* Sw. (Hernández-Verdugo *et al.*, 2010). Development and selection of heterotic $F_1$ hybrids has been a successful approach for creating rootstock cultivars that can overcome some of the issues associated with using pure wild-species rootstocks while retaining useful traits. These hybrid rootstocks are most often grafted to $F_1$ hybrid scions to create a grafted transplant with four interacting genomes; hence, there are many opportunities for interactions and compatibilities controlled by the molecular factors that influence heterosis (Ryder *et al.*, 2014), including the bidirectional movement of small RNAs and mRNAs across graft junctions (Haroldsen *et al.*, 2012; Tsaballa *et al.*, 2013; Goldschmidt, 2014; Avramidou *et al.*, 2015; Yang *et al.*, 2015; Lewsey *et al.*, 2016). However, it must be recognized that the breeding of commercial hybrids is done in secrecy; the origins of the most successful hybrids are trade secrets, and rootstock cultivars are protected by plant breeders' rights. Equally, whether there has been a scientific basis to the selection of the most successful hybrids, or whether it has been through the 'art' of skilled and experienced breeders, is not publically known; and the degree to which marketing versus crop performance influences how growers and nurseries choose commercial elite rootstock cultivars is unclear.

### 3.4.2 Use of *Cucurbita* $F_1$ hybrids

Historically, *Cucurbita* rootstock breeding has been dominated by work in Korea, China and Japan, and as a result of this breeding effort, commercial cucurbit scions, including cucumber, watermelon and melon, are most often grafted on to interspecific *Cucurbita maxima* Duch. (pumpkin) × *Cucurbita moschata* Duch. (squash) $F_1$ hybrid rootstocks. This interspecific hybrid has become the accepted paradigm for vigorous rootstocks that improve yield and disease resistance in cucurbits; this paradigm appears to have arisen largely from the worldwide historical popularity of one such hybrid rootstock, 'Tetsakabuto', developed by Japanese breeders, which is believed to be exceptionally vigorous due to heterosis. In addition to being used as a rootstock, 'Tetsukabuto' is marketed as a winter squash cultivar and is a *C. maxima* 'Delicious' × *C. moschata* 'Kurokawa No. 2' interspecific cross; the apparently good seed production and combining ability are probably the factors that led to its popularity (Robinson, 1999) and to the widespread use of interspecific hybrid rootstocks in general. *C. maxima* × *C. moschata* crosses generally give rather poor fruit set and seed yield, and usually produce a sterile hybrid plant; a recent study in Turkey tested many combinations, and, although some were more favourable than others, further breeding work is required to reach acceptable seed yields (Karaagˆaç and Balkaya, 2013). The latter report underlines the remarkable commercial success of the Asian breeding programmes that created 'Tetsukabuto'; however, there is a lack of published scientific evidence to show that this particular hybrid type is superior to other hybrid or non-hybrid lines. Indeed, a recent study has shown that the performance of *C. maxima* × *C. moschata* $F_1$ hybrid rootstocks is not conclusively better than the parental lines, depending on the specific cross or the key traits examined (M. Edelstein *et al.*, unpublished

data), and, as discussed in Chapter 2 (this volume), there is a huge amount of genetic diversity still not used or tested in hybrid rootstocks; for example, highly diverse *C. moschata* inbreds are available (Kong *et al.*, 2014) and, although *Lagenaria* are used extensively in the Far East as rootstocks, landraces in Europe and Turkey have untapped potential (Yetişir *et al.*, 2008; Karaca *et al.*, 2012).

### 3.4.3 Use of *Solanum* $F_1$ hybrids

In the Solanaceae, utilizing a hybrid with at least one parent that retains a large complement of wild-species DNA is typical, and *Solanum lycopersicum* L. (tomato) × *Solanum habrochaites* S. Knapp & D.M. Spooner (wild species) is the most widely used commercial hybrid rootstock for tomato. However, as with *Cucurbita*, there is a lack of published evidence for the superiority of *S. lycopersicum* × *S. habrochaites* $F_1$ hybrid rootstocks in comparison with the many other hybrid combinations or non-hybrids that are possible; further public research to explore wider genetic diversity, to understand the role of heterosis and to link phenotypes to genetic loci is required.

Despite the understandable lack of public information about the origins and reasons for the predominance of specific commercial rootstocks, there is a considerable scientific literature, described in the following sections, that considers *how* to deploy genetic diversity, that is, from studies on the sexual compatibility between different wild species and the methodologies available for breaking species barriers, including embryo rescue and somatic hybridization. These are the techniques that enable alleles to be moved between species in breeding materials, and that underpin the first stages of rootstock breeding.

### 3.4.4 Interspecific hybrids and hybridization barriers

Chapter 2 (this volume) details the sexual compatibility within the various groups of Solanaceae and Cucurbitaceae. In breeding programmes, the cultivated Solanaceae parent is usually used as the female and the wild species is the pollen donor (Bletsos *et al.*, 1998, 2004; Premabati Devi *et al.*, 2015), and the success of the cross is determined by the percentage of fruit set, number of seeds per fruit and percentage germination of the $F_1$ seed. The hybridization barriers can act before fertilization, for example by inhibition of pollen-tube growth, as for *Capsicum annuum* L. × *Capsicum pubescens* Ruiz & Pav. (da Silva Monteiro *et al.*, 2011), and this can be overcome by somatic hybridization. Where barriers occur after fertilization, embryo rescue has been used extensively to produce viable $F_1$ hybrids. The success of producing interspecific hybrids is easily tested using molecular markers (Reddy *et al.*, 2015).

In somatic hybridization, also called protoplast fusion, cells from two different species of plants are fused together to create a new plant with the characteristics of both species. Protoplasts are first isolated through mechanical or enzymatic procedures, and osmotic agents or electricity are then used to cause fusion. One of the earliest examples was the pomato, the potato–tomato fusion product (Melchers *et al.*, 1978). In aubergine, the first interspecific somatic hybrid was

obtained between *Solanum melongena* L. and *Solanum sisymbriifolium* Lam. to provide new sources of disease resistance (Collonnier *et al*., 2003; Daunay, 2008).

The cultivated and wild relatives of aubergine are an important source of tolerance to drought (*Solanum macrocarpon* L.), salinity (*Solanum linnaeanum* Hepper & P.-M.L. Jaeger) and frost (*Solanum grandiflorum* Ruiz & Pav., *Solanum mammosum* L. and *Solanum khasianum* C.B. Clarke) (Rotino *et al*., 2014), and efforts have been made to develop somatic hybrids between *S. melongena* and wild or cultivated relatives for the improvement of aubergine rootstocks. Somatic hybrids have been developed for *S. melongena* × *Solanum integrifolium*, *S. melongena* × *Solanum sanitwongsei* W.G. Craib (Asao *et al*., 2001) and *S. integrifolium* × *S. sanitwongsei*, producing fertile hybrids with increased resistance to bacterial wilt (Iwamoto *et al*., 2007), and for *S. melongena* × *Solanum marginatum* L.f. (an arborescent species) (Borgato *et al*., 2007).

Embryo rescue was one of the earliest successful applications of *in vitro* culture used to assist the development of plant embryos that otherwise would be non-viable because of incompatibilities between genomes in a particular hybrid (Cisneros and Tel-Zur, 2010). Embryos are removed from the ovary after fertilization but before abortion, and cultured aseptically on suitable medium to generate viable plants. This has been applied, for example, to interspecific hybrids: *S. melongena* × *S. torvum* (Kumchai *et al*., 2013), *S. melongena* × *S. khasianum* (Rattan *et al*., 2015) and *Capsicum annuum* × *Capsicum baccatum* (Jae *et al*. 2006; Eggink *et al*., 2014), although further development of embryo-rescue techniques in *Capsicum* peppers is needed (Manzur *et al*., 2013). In the case of *S. lycopersicum* × *Solanum peruvianum* L., the development of lines carrying resistance to tomato yellow leaf curl virus, tomato spotted wilt virus and tomato chlorosis virus was possible (Picó *et al*., 2002; Encina *et al*., 2012; Julián *et al*., 2013). Embryo rescue has been reported to be successful from the following crosses in the cucurbits: *Cucurbita ficifolia* Bonché × *Cucurbita pepo* L. and *Cucurbita martinezii* L.H. Bailey × *C. pepo* (Rakha *et al*., 2012); *C. maxima* × *C. pepo*, *C. pepo* × *C. moschata*, *C. ficifolia* × *C. maxima*, (*C. maxima* × *C. moschata*) × *C. pepo* and *Cucurbita argyrosperma* C. Huber × *C. moschata* (de Oliveira *et al*., 2003; Šiško *et al*., 2003).

The capture of wild-species DNA in a somatic or embryo-rescued hybrid necessarily requires substantial further germplasm enhancement before this novel germplasm can be deployed as true breeding parental lines for the creation of $F_1$ hybrid rootstock cultivars. Further crossing and then inbreeding, or production of DH lines to produce stable parental lines, must be completed and then many $F_1$ hybrids must be tested phenotypically for rootstock traits (Fig. 3.1).

## 3.5 Grafting as a Tool for Genetic Hybridization and Chimera Production

The agronomic practice of vegetable grafting demands that breeders continually generate and select new rootstock genotypes, which is the main subject of this chapter. However, the formation of the graft union itself also opens up new opportunities, described below, for: (i) combining genetic material within cells (genetic hybridization); and (ii) creating novel cellular chimeras. Grafting can therefore be regarded as an extra tool for the researcher and breeder to address fundamental questions or pursue genetic improvement in graftable crops.

### 3.5.1 Genetic hybridization: transfer of nuclear and organellar DNA between cells of the graft union

Grafting of two genetically different plants together to form a graft union brings cells of distinct genotypes into intimate proximity. This proximity offers the opportunity for the exchange of genetic material, either through cell fusion, in a process akin to protoplast fusion, or by movement of nuclei, chromatin or organelles through widened plasmodesmata (Fuentes *et al.*, 2014). This genetic exchange could occur naturally when plants fuse together, as is commonly observed in appressed stems of woody perennial species (Warschefsky *et al.*, 2016); if the resulting cells then underwent shoot regeneration, for example through the wound-induced formation of adventitious shoots, this could lead to individual plants with unique combinations of genetic material that could be inherited in subsequent generations. This mechanism for genetic exchange can be exploited by researchers and plant breeders to generate novel genetic resources. Grafting can therefore be considered a potential tool for genetic hybridization that can overcome sexual compatibility barriers.

Early evidence of the transfer of plastid genomes came from experiments where two transgenic tobacco lines were grafted together, one with a nuclear genome marker and one with a plastid marker. Cells that contained both markers were detected at the graft unions at high frequency (Stegemann and Bock, 2009). Later, transfer of whole plastid genomes in the absence of nuclear genome introgression (Stegemann *et al.*, 2012) was observed to occur across graft junctions, and this was proposed as a mechanism for the natural horizontal transfer of DNA between sexually incompatible species that had been predicted previously from phylogenetic analysis. Similarly, cell-to-cell movement of mitochondria was observed in tobacco graft junctions: restoration of *Nicotiana tabacum* L. fertile flower anatomy was observed in plants regenerated from an *N. tabacum*/*Nicotiana sylvestris* Speg. & S. Comes graft due to replacement of a defective mitochondrial genome (Gurdon *et al.*, 2016). More dramatically, the transfer of the entire nuclear genome was observed in the junction of a *N. tabacum*/*Nicotiana glauca* Graham graft; in this case, the *N. tabacum* parent (2n = 4x = 48 chromosomes) carried a hygromycin resistance gene, and the *N. glauca* parent (2n = 2x = 24 chromosomes) carried a kanamycin resistance gene. The graft junction was excised and cultured in the presence of kanamycin and hygromycin to produce callus and then shoots; some of the regenerated plants were stable and fertile and carried a full complement of parental chromosomes (2n = 6x = 72) that were successfully transmitted to the subsequent generations on meiosis by disomic inheritance segregating as an amphidiploid (Fuentes *et al.*, 2014). These plants therefore represented a new allopolyploid plant species (named *Nicotiana tabauca*) created by a grafting-induced asexual process.

Allopolyploidization is highly advantageous and common in crop species, and is believed to have occurred through sexual hybridization and chromosome doubling; grafting-induced allopolyploidization provides a new method for crop improvement that can combine genomes without the need for sexual compatibility between parents, and may be a mechanism that has played its part in genome evolution and crop domestication. Although grafting-induced genetic hybridization is conceptually similar to protoplast fusion in its outcome, it may be technically

easier to achieve and could give a viable alternative in graft-compatible species where protoplast fusion has failed.

### 3.5.2 Use of grafting to generate chimeras

While genetic hybridization allows new combinations of DNA to occur in a single cell, chimeric plants are defined as those that contain cells arising from more than one genetic origin that are propagated by cell division within an individual plant. Interchange of DNA between cells is not a requirement for chimeric plant formation, and chimerism cannot therefore be inherited through the gametes. Here, chimeras are of interest because they can arise from the graft union, and their potential utility is discussed.

According to the Tunica-Corpus model (Reeve, 1948), shoot apical meristems of dicotyledonous plants contain three cell layer classes: L1, L2 and L3. Of these, L1 is the outermost epidermal layer, L2 is the next inner mesophyll/palisade layer and L3 comprises the remaining internal cells including vascular cell types. As a meristem develops, the genetic lineage of cells in each layer is preserved in the developing shoot because the cells of L1 and L2 divide only in the anticlinal plane, while the innermost cells divide in both anticlinal and periclinal directions. If all the cells of one layer arise from a single genotype that differs from another layer, then a periclinal chimera (i.e. differing between layers) can be produced that is stable when vegetatively propagated (Filippis *et al.*, 2013); this is the basis of many variegated ornamental plants. In the case of adventitious shoot formation from a graft union, a meristem can be generated from a group of cells, and if this group contains cells from the rootstock and scion, it can develop into a chimera, and sometimes will form a stable periclinal chimera if one cell layer becomes homogeneous (Zhu *et al.*, 2007). Although *in vitro* protoplast fusion and protoplast co-culture can also be used to create chimeras, these techniques are technically challenging, and many researchers prefer to use grafting followed by adventitious shoot formation to generate chimeras for studying multicellular development and its molecular and genetic control; typically, parental lines with visible markers such as colour and surface traits are chosen to allow visible detection of chimeras. Some examples are described below.

Splice grafts made between *N. glauca* and *N. tabacum* were decapitated just above the graft union and treated with auxin/lanolin pastes to stimulate callus formation. Of 209 adventitious shoots that were formed from such graft unions, three were found to be interspecific mericlinal chimeras, and these could later be stabilized as periclinal chimeras (Marcotrigiano and Gouin, 1984). In this study, no chimeras could be produced from alternative *in vitro* methodologies such as mixed callus cultures, so grafting was essential to recover chimeric shoots in this case.

Approach grafting between red and green cabbage varieties, followed by sectioning and culturing of the graft junction, produced up to 53% of resulting adventitious shoots showing a visible red/green chimeric structure, and microscopic examination of the presence of anthocyanins arising from the red cabbage cells showed chimeric structures classified as 'complex sectorial-peripheral' (Noguchi *et al.*, 1992). These authors considered that symmetrical vertical heterografting

through the centre of the shoot apex should give the optimal production of chimeric plants. In another *Brassica* study, the shoot apical meristems of *Brassica juncea* L. Czern. (tuber mustard) and *Brassica oleracea* L. (cabbage, red cultivar) seedlings were grafted vertically in the presence of different concentrations of auxin and cytokinin; after graft formation, the fused shoot tips were cut and cultured *in vitro* for adventitious shoot formation, and the most successful treatment resulted in 6% of adventitious shoots being visibly chimeric (Chen *et al.*, 2006).

In *Solanum*, stable periclinal chimeras were produced between tomato (*S. lycopersicum*) and the nightshade *Solanum nigrum* from graft unions (Lindsay *et al.*, 1995), and in a more recent study in the post-genomic era, tomato 'Heinz 1706' was grafted to *Solanum pennellii* Correll LA716, and one periclinal chimera was observed in the adventitious shoots derived from 50 grafts (Filippis *et al.*, 2013). This chimera had an L1 layer derived from *S. pennellii*, while the L2 and L3 cell layers were from the cultivar. Due to the known sequence polymorphisms between the two species, both of which now have reference genomes (Bolger *et al.*, 2014), the chimera could be used to study the gene expression differences between L1 and L2/L3 using RNA sequencing.

The above examples show that chimeras are readily generated, but are they useful? In clonally propagated fruit trees, the production of stable chimeric plants via grafting could be used to combine different traits that are controlled by a specific cell layer for agronomic advantage (Zhou *et al.*, 2002), but, as commercial grafted vegetable crops are invariably raised from seedlings, and stable propagation of chimeric plants occurs only through vegetative (clonal) means, chimeric individuals generated in vegetable crop species are unlikely to have direct commercial applications. However, formation of chimeric plants may create greater opportunities for DNA transfer (i.e. between genetically distinct cell layers of the whole shoot in contrast to the rather limited graft union), and could allow further possibilities for generating novel stable genetic combinations.

## 3.6 Selection of Improved Rootstocks

### 3.6.1 Phenotypic selection

Before defining breeding aims, information on the performance of the currently used commercially available rootstocks in different locally conducted experiments should be gathered, where it exists (Kubota *et al.*, 2008). Breeding aims can then be shaped by the market needs, and suitable selection schemes developed.

Each breeder will have their own unique views and strategies for breeding an improved rootstock, but a convenient framework for discussion divides the process into three stages (Fig. 3.1). The first stage is the development and selection of promising inbred lines in the non-grafted state, and the use of these as parental lines to make $F_1$ hybrid seed. Generally, non-grafted accessions and breeding lines are selected for resistance to soilborne pests and diseases, abiotic stresses, root vigour, germination and seedling uniformity. The second stage is to select lines with the desired grafting affinity and scion compatibility, and the third stage is to evaluate the effects of the selected rootstock × scion combinations on crop performance,

focusing on fruit yield and quality under targeted agroclimatic conditions and specific production systems.

### *Stage 1: screening of non-grafted breeding lines*

Inbred lines from core collections of germplasm can be evaluated for key traits before moving to more complex evaluations. Seed traits are one such area for early selection: seed evaluation can be based on seed yield, seed mass, germination rate and uniformity of emergence. These seed traits tend to have high heritability, that is, a high influence of genetic factors relative to environmental factors, and germination of rootstock seeds takes place in protected grafting nursery conditions where the environment is controlled and has less impact on the outcomes for seed traits (Huarachi Morejon, 2013; Premabati Devi *et al.*, 2015).

The non-grafted genetic material can easily be screened phenotypically for soilborne diseases at this stage in glasshouse pot experiments if molecular markers are not available for a particular disease; for example, aubergine and pepper cultivars and breeding lines were pre-screened for root rot resistance to *Phytophthora capsici* isolates (Foster *et al.*, 2013), tomato germplasm was screened for nematode resistance (Cervantes-Moreno *et al.*, 2014) and exotic watermelon accessions were screened for multiple pests and diseases (Cohen *et al.*, 2014). Particularly for disease resistance in the Solanaceae, there are a range of molecular markers available, so phenotypic screening is only necessary if resistances are overcome or new diseases appear. However, for resistance to abiotic stresses, to the best of our knowledge, there are no molecular markers that are currently in commercial use, so phenotypic screening is the only option. For example, screens for resistance to low temperature or high salinity are used by many breeding companies, some of whom have a special emphasis on breeding for such root traits (Rootility, 2016).

The ability to maintain growth at lower rootzone temperatures can be taken as a general indicator of vigour under optimum conditions, although there is little literature on this type of germplasm screening because it is usually carried out as part of proprietary breeding programmes and therefore the scientific basis is unknown. Although rapid growth of non-grafted germplasm can be taken as an indication of vigour, this is likely to be a highly complex trait, in which heterosis and graft compatibility play major roles, so it is not clear if the screening of non-grafted plants for vigour is a good indicator of later performance of grafted $F_1$ hybrids. However, it is common practice in rootstock breeding to select for accessions with large, well developed root systems after excavating or pulling up soil-grown plants, an approach that can be quantified for QTL studies, at least in the non-grafted crop maize, by 'shovelomics' (Colombi *et al.*, 2015). There is considerable current research to discover QTLs and gene alleles that can improve abiotic stress traits and vigour in rootstocks so that molecular markers can be developed to replace some aspects of phenotypic selection (*see* Marker-assisted selection, below). A greater understanding of the genetic loci that control heterosis, where only a few examples have been described so far (Krieger *et al.*, 2010), is also needed to improve the selection of vigour.

Typically, selected inbred accessions would go through further rounds of genetic enhancement to combine traits. This is achieved by backcrossing and recurrent selection to create parental lines suitable for the production of $F_1$ hybrid seeds.

*Stage 2: testing for graft compatibility*

$F_1$ hybrid rootstocks are next tested for their ability to form graft junctions and to support the growth and development of the grafted plant. This 'graftability' depends on having suitable hypocotyl diameters to match the scion, and the developmental cellular capacity for making vascular connections. Graft compatibility refers to the success of specific rootstock × scion combinations in terms of both graft formation and the subsequent growth and development of the successfully grafted plant. Compatibility screens can only be achieved by grafting each rootstock genotype to a range of scion genotypes and then evaluating the formation of the graft junction and the continued development of the grafted transplant. Such trials are expensive, and therefore the skill of the breeder is to bring forward a relatively small number of promising hybrids to this second step.

Knowledge of the genetic basis of grafting compatibility is limited, but information on molecular and cellular aspects of graft formation, including heterografts, is beginning to emerge (Milien *et al.*, 2012; Cookson *et al.*, 2014; Moreno *et al.*, 2014; Melnyk *et al.*, 2015) (*see* Chapter 5, this volume). There are no known molecular genetic markers for rootstock × scion compatibility, but this could be addressed by using suitable grafted mapping populations to find QTLs.

*Stage 3: evaluation of the performance of rootstock × scion combinations*

Breeding for rootstocks is a complicated process because it involves not only the rootstock genotype but also its effects on the scion, which can vary in different environments and cultivating systems (King *et al.*, 2010). Rootstock cultivars that will cover large market areas are desirable, and thus the compatible rootstock × scion combinations should be tested across many locations and over multiple years to evaluate the genotype (rootstock) × genotype (scion) × environment interactions against existing elite rootstocks and self-grafted scions in the presence of the most important biotic and abiotic stresses. Typical variables for assessment are: (i) vegetative versus generative crop development; (ii) marketable fruit yield in the early and late season; (ii) fruit quality; and (iv) resistance to pest and diseases.

Alongside the activities of commercial breeders, scientific researchers often collect existing commercial rootstock cultivars to evaluate their effectiveness at improving scion performance under different environmental conditions, and to understand the physiological mechanisms involved (Leonardi and Giuffrida, 2006; Rivard and Louws, 2008; Rivard *et al.*, 2010; Zhang *et al.*, 2016). Such scientific understanding could lead to the development of phenotypic screens that have greater predictive power for selecting improved rootstocks.

### 3.6.2 Marker-assisted Selection

In breeding populations, seedlings with a specific complement of chromosome segments of known origin can be selected by marker-assisted selection (MAS) using DNA-based genetic markers. If a causal relationship is already established between a genetic locus and a phenotype, then expensive phenotypic selection of plants at more advanced stages of development, such as late-season fruit yield due to rootstock genotype, can be greatly reduced. MAS is applied routinely in breeding

companies using high-throughput genotyping platforms; some of these have the capacity to run more than 200,000 polymerase chain reaction (PCR)-based genotyping assays per day (Douglas, 2016). Technologies for genotyping have developed rapidly over the last decade, with single-nucleotide polymorphisms (SNPs) now dominating; SNPs are currently assayed by technologies such as the PCR-based KASP™ (He *et al.*, 2014), and genotype-by-sequencing using next-generation sequencing is emerging as an alternative approach (Kim *et al.*, 2016). Molecular markers for well characterized pest and disease resistance alleles are commonly used commercially and in publicly funded breeding programmes (Hanson *et al.*, 2016) but are more available in the Solanaceae than in the Cucurbitaceae. The challenge remains to find molecular markers for large-effect, robust QTLs for selection for resistance to abiotic stress, vigour, yield and fruit quality.

### *Identification of genetic markers for rootstock traits*

Classical QTL approaches can be used to identify chromosomal locations that control rootstock traits, with the eventual aim of developing markers for MAS in rootstock breeding, and there are a few examples reported in the scientific literature.

Field trials with an introgression line (IL) mapping population, where single introgressions of *S. pennellii* LA716 were present in the background of processing tomato 'M82', were conducted using the ILs as rootstocks; this showed that IL8-3 contained a recessive QTL that conferred approximately 20–75% greater yield on the scion compared with non-grafted plants over three seasons (Gur *et al.*, 2011). The detection of an effect in IL8-3 but not in IL8-3-1 or IL8-2, implied that the rootstock effect was within a 1.4 Mb region of chromosome 8 containing approximately 180 genes (Chitwood *et al.*, 2013).

Using two recombinant inbred line populations from crosses between *S. lycopersicum* var. *cerasiforme* E9 and *Solanum pimpinellifolium* L. L5 or *Solanum cheesmaniae* (Riley) Fosberg L2, in which each member of the population was grafted to a common scion, several QTLs were discovered that affected scion traits under salinity stress. The most robust QTL with the highest log of odds (LOD) scores from this work, *gTW3.1* (total fruit weight per plant, LOD = 4.29) and *gFN3.1* (fruit number per plant, LOD = 4.59), were located on chromosome 3 at approximately 50 cM; the two QTLs at this locus had additive effects of 2.3 fruits per plant and 130 g fruit per plant, compared with mean trait values of around 37 fruits per plant and 1750 g fruit per plant, respectively, in the self-grafted controls (Estañ *et al.*, 2009). However, when the same population was grown at a different level of salinity, different QTLs for grams of fruit per plant were found and their LOD scores were marginal (Asins *et al.*, 2015). This illustrates the difficulty of defining complex quantitative traits controlled by many small-effect QTLs with environmental interactions. In the same study, a robust QTL for the effect of rootstock genotype on scion $Na^+$ concentration was found when plants were grown under moderate salinity, but this did not co-locate to QTLs for fruit yield, so its utility for breeding is unclear. QTLs for mineral content were also described (boron, potassium, magnesium and molybdenum), and these could be useful to breed rootstocks that enhance dietary nutritional value of fruit, or that mitigate plant mineral deficiencies (Asins *et al.*, 2015). To the best of our knowledge, there are no other published studies on vegetable rootstock QTLs, but the literature on perennial rootstock

QTLs, such as apple, pear and citrus, is more expansive, despite the problems with perennial genetics; for example, two rootstock loci, *Dw1* and *Dw2*, have been well characterized for their ability to confer dwarfing, vigour and precocity (Rusholme Pilcher *et al.*, 2008; Fazio *et al.*, 2014; Knäbel *et al.*, 2015), and are beginning to be used in MAS (Bassett *et al.*, 2015).

### *Genomic resources for vegetable rootstock breeding*

QTL studies are underpinned by the genomic resources now available in all grafted vegetable crops. Next-generation sequencing technologies have facilitated the development of reference genomes for all of the major grafted crops: cucumber (Huang *et al.*, 2009), tomato (Tomato Genome Consortium, 2012), melon (Garcia-Mas *et al.*, 2012), watermelon (Xu *et al.*, 2013), hot pepper (Kim *et al.*, 2014) and aubergine (Hirakawa *et al.*, 2014). We are now in the post-genomic era, with large numbers of accessions being resequenced to show their DNA sequence differences (polymorphisms) compared with reference sequences. For example, in tomato, more than 500 accessions have been resequenced and the data are publically available (Aflitos *et al.*, 2014; Lin *et al.*, 2014); in addition, wild species related to tomato are being sequenced by *de novo* assembly to create species-specific reference genomes, with the *S. pennellii* LA716 genome completed to a high standard (Bolger *et al.*, 2014). The parents of a tomato population that has been used extensively to study rootstock QTLs (Estañ *et al.*, 2009; Asins *et al.*, 2010, 2015) and physiological mechanisms (Albacete *et al.*, 2015a,b) have also been resequenced (Kevei *et al.*, 2015). These genomic sequences, and genotyping platforms such as Illumina BeadChip (Asins *et al.*, 2015), greatly accelerate the process of identifying genetic markers associated with traits, and also in finding the causative polymorphisms; these huge advances in the description and assay of genetic variation leave phenotyping as the bottleneck in the discovery of useful markers for rootstock MAS.

## 3.7 Transgenic Rootstocks

Transgenic rootstocks have been created and tested as potential means to enhance scion performance or to understand signalling mechanisms. For example, overexpression of the cytokinin biosynthesis gene *IPT* in tomato rootstocks led to increased cytokinin in xylem sap and a 30% increase in yield under a moderate salinity stress of 75 mM NaCl (Ghanem *et al.*, 2011a,b); overexpression of *S*-adenosyl-1-methionine led to the accumulation of polyamines in the root system, and as a rootstock this genotype increased yield under alkali stress (Gong *et al.*, 2014); and overexpression of a $H^+$-pyrophosphatase in *Lagenaria* rootstocks increased root system size and the salinity tolerance of watermelon scions to which they were grafted (Han *et al.*, 2015). Engineering virus resistance in rootstocks has also been attempted in watermelon by overexpression of coat proteins from the cucumber green mottle mosaic virus (Park *et al.*, 2005). Numerous further ideas can be tested for their ability to transgenically enhance rootstocks based on current knowledge, either to alter root system vigour and architecture or to generate hormonal or RNA-based signals, and this can be done using genome

editing, which may or may not be considered a gene-modification technology from a regulatory perspective (Cyranoski, 2015).

It has been suggested that transgenic rootstocks could be used to produce non-genetically modified fruit from a non-transgenic scion, to avoid some regulatory issues, but recent descriptions of the movement of nucleic acids across the graft junction (Zhang *et al.*, 2008; Haroldsen *et al.*, 2012; Tsaballa *et al.*, 2013; Avramidou *et al.*, 2015), and the broad nature of the regulatory framework currently make this an unworkable proposition.

## 3.8 Rootstock Registration and Commercialization

The International Union for the Protection of New Varieties of Plants (UPOV) has established test guidelines for tomato rootstocks, including hybrids of *S. lycopersicum* with *S. habrochaites*, *S. peruvianum* or *S. cheesmaniae*, which were adopted in March 2014 (CPVO, 2014). UPOV has also established test guidelines for a *C. maxima* × *C. moschata* interspecific hybrid (UPOV, 2014b) and for bottle gourd (UPOV, 2014a). A database of commercial rootstocks for cucurbits, aubergine, pepper and tomato is updated annually (USDA, 2016). A database of National Listings of plant varieties is also available (CPVO, 2016). A list of major companies that breed and supply vegetable rootstock seeds is given in Table 3.1.

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# Chapter 3: Rootstock breeding: current practices and future technologies

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