Somatic Cell Genetics and Its Application in Potato Breeding

13

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Abstract

This chapter presents a summary of published work on the development, achievements and interconnections of research on potato somatic cell genetics. To maintain genetic stability the main topics include the establishment and maintenance of in vitro cultures, micropropagation, shoot and meristem culture, somatic embryogenesis, production of micro- and mini-tubers and conservation of germplasm. In the second section, the methods presented are based on the induction and utilization of genetic variability (diversity): production of haploids, somatic hybridization via protoplast fusion, somaclonal variation and gene transfer. Another significant aspect of this review is the presentation of numerous methods used in clonal propagation, the production of healthy plants, germplasm conservation for medium-term and long-term storage, potato breeding and utilization of germplasm for the production of advanced breeding clones and potato cultivars with improved resistance to pathogens, pests and abiotic stress, and of high quality and with other specific traits for other purposes. Finally, new methods of breeding, including molecular marker development and genome editing, are briefly described to indicate the potential of somatic cell genetics for the future improvement of potato.

13.1 Introduction

Each plant cell contains one complete set of chromosomes with the genetic information for the development of an individual plant, which is the basis of the ability to regenerate a plant from cells in culture. Starting with the first report of the cultivation of potato plants in vitro (Stewart and Caplin 1951) diverse biotechnological techniques have been successfully used for more than

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65 years. These techniques are generally referred to as somatic cell genetics, which both increase the supply of genetic diversity and make selection more efficient. Potato can be cultivated in vitro and is amenable to biotechnological improvement (Barrell et al. 2013). Somatic cell genetics has developed since the demonstration of potato cell totipotency in vitro, i.e. plant regeneration from isolated protoplasts (Shepard and Totten 1977). The definition of plant somatic cell genetics includes all in vitro genetic techniques that can be used to culture organs, tissues, cells and isolated protoplasts and obtain insights into the genetics of plant somatic cells (Terzi et al. 1985). Some of them are routinely used for many practical applications in potato breeding, maintenance and production. Micropropagation using two node explant culture and development of micro-tubers is today commonly used in all tissue culture laboratories for the propagation and medium-term preservation of potato germplasm. Cryopreservation techniques are used for the long-term conservation of potatoes and wild species of *Solanum* (Li et al. 2016). In vitro selection of somaclones and protoclones of potato has been successfully used as well as genetic manipulation through gene transfer or protoplast fusion to bypass sexual incompatibility and introgress many of the resistance genes of wild species to improve potato (Rokka 2015). New biotechnological techniques such as CRISPR-Cas9 can be used to genetically manipulate potato, which opens up new horizons for potato improvement (Wang et al. 2015). Relevant biotechnological methods and approaches for the development of potato based on somatic cell genetics are summarized in Fig. 13.1.

In this chapter the use of potato somatic cell genetics is discussed and brought up to date regarding the latest achievements and introduction of new techniques such as iRNA and genome editing and the prospects of potato somatic cell genetic studies for potato crop improvement. With the broadening of the genetic knowledge and approaches, like genomic selection, gene editing, transformation and hybrid breeding, gene identification and diagnostic molecular marker techniques, it will be possible to manipulate and successfully control and change the patterns of development of tissues to suit our interests and needs. In particular, DNA markers for the precise characterization of germplasm, the construction of saturated linkage maps, defined molecular markers for marker-assisted gene pyramiding and alien gene introgression should improve the breeding of potatoes. Cell and tissue culture techniques are invaluable in achieving these goals.

13.2 Methods of Maintaining Genetic Stability

13.2.1 Micropropagation: Shoot, Meristem Tip Culture, Somatic Embryogenesis, Micro- and Mini-tubers and Their Use in Potato Breeding

13.2.1.1 In Vitro Multiplication and Shoot Culture

Methods of plant tissue culture include the growing of plant cells, tissues or organs isolated from a plant on artificial media under axenic conditions in a suitable environment. One practical objective is the rapid clonal propagation of potato. Micropropagation is a much faster and more efficient way of asexually propagating in vitro plantlets of single shoot cuttings on artificial media than the traditional propagation by cuttings in soil in a glasshouse. The shoots are cut into single node explants, each containing an axillary bud and cultivated individually in glass tubes or vessels of different sizes. After transfer to a fresh medium, which supports shoot elongation and rooting, the axillary buds of these explants rapidly develop into rooted plantlets consisting of several internodes within 3–4 weeks. The culture of shoots is the basic technique for establishing in vitro cultures using shoot tips, or apexes, and material for use in other techniques such as cell, tissue and organ culture, protoplast culture, somatic embryogenesis and transformation. Other methods of

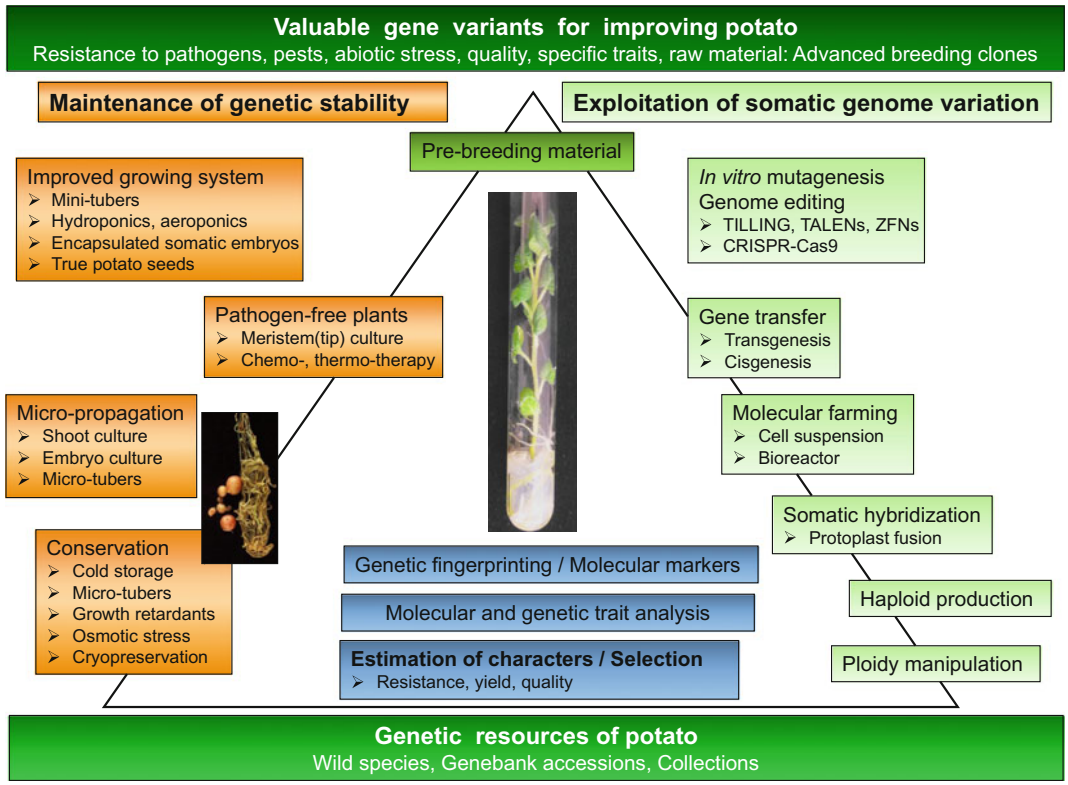

Fig. 13.1 Approaches and manipulation steps for improvement of potato based on somatic cell genetics

propagation, like multiple shoot production by activating axillary buds, were favoured in the 1970s (Westcott et al. 1977; Roca et al. 1978) and used for transferring genetic resources into potato (Roca et al. 1979).

A lot of research has been undertaken to determine the main factors for ideal propagation, including media for the production and maintenance of potato shoot cultures (details in Vinterhalter et al. 2008). The MS medium, as described by Murashige and Skoog (1962), contains carbohydrates (sucrose), macro and microelements, vitamins, but no plant growth regulators, like phytohormones, and is still the best and most widely used medium for potato propagation. Mainly during the 1970s and up to the beginning of 2000, the effect of medium supplements and various cultural conditions that affect the growth of in vitro plants were being intensively investigated. The use of liquid, agar or gelrite-solidified media, mineral nutrition and specific substances and inoculation density (overview in Vinterhalter et al. 2008) was investigated. Different types of closures for tubes and vessels were used for analysing growth and morphology of potato shoots in vitro (Chanemougasoundharam et al. 2004; Genound-Gourichon et al. 1993). Based on these studies and our experience we recommend cotton wool plugs, which in terms of avoiding morphological abnormalities, are the best type of closure.

Seabrook (2005) reviews the studies on the effects of irradiance, photoperiod and spectral composition of light on potato growing in vitro. Recently, trials were carried out on the use of LED light for growing potato plants and tissue in vitro to minimize the energy costs of climate rooms for propagation and storage (Jao and Fang 2004; Luz et al. 2016; Da Rocha et al. 2015).

An efficient method for mass propagation of single-leaf cuttings is published by Haapala (2005). Other details were published, like using

food containers as a cheaper alternative to the traditional culture vessels, which can also be effectively sterilized using NaOCl solution, which significantly reduces the costs of micropropagation (Weber et al. 2015). Temperature pre-treatment of transplants from in vitro plantlets influences their growth and yield in the field (Tadesse et al. 2001). Attempts were made to automate micropropagation using robots monitored by cameras and computer programs (Aitken-Christie et al. 1995). This interesting approach was not generally accepted because of problems involved in arriving at accurate decisions about how to manipulate plant material.

Recently the main use for shoot cultures is clonal propagation but it is also the basic technique of other biotechnological methods. This method of shoot culture guarantees, when development of callus tissue is avoided, high multiplication rates and the production of genetic identical, healthy and stable potato plants. Therefore the term 'rapid or mass propagation' is generally used.

13.2.1.2 Meristem Tip Culture

The essence of meristem-tip culture is the excision of an organized shoot apex, 0.3–1.0 mm in length from a donor plant for subsequent culture in vitro. An apical meristem includes the apical dome and a limited number, mostly two to four of the youngest leaf primordia and no differentiated provascular or vascular tissues. Meristem tips are removed by sterile dissection under a microscope and cultured in a liquid medium with filter paper bridge supports or on an agar-solidified medium containing low concentrations of plant growth regulators. There are a lot of studies on meristem tip culture but only a few will be mentioned in this chapter. An advantage is a genetic stability inherent in this technique, since plantlet development is from an already undifferentiated apical meristem and the development of shoots directly from the meristem avoids callus tissue formation and adventitious organogenesis. A major advantage of working with such small explants is the potential this has for excluding pathogenic organisms that may have been present in the donor plants. Therefore, this technique is used to eradicate harmful viruses, based on the observation that only a few virus particles are present in meristem cells. Using very small explants, the chance of producing a virus-free plant is high, but the survival rate is directly proportional to the size of the explant. The efficiency of virus eradication depends on the type of virus, potato variety or genotype. To increase the probability of successfully producing virus-free material, thermotherapy can be used separately or in combination with meristem tip culture as the second step in the procedure (Stace Smith and Mellor 1968; Šip 1972; Faccioli 2001). For thermotherapy, ex vitro or in vivo plants or tubers are kept at a high temperature of 32–36 °C. Another method of virus eradication is chemotherapy: media are supplemented with viricidal substances, like ribavirin (Klein and Livingstone 1982; Faccioli and Colalongo 2002) or jasmonic acid (Ravnikar and Gogala 1989). All these methods could be used for the mass production of virus-free potato plants, which could be used as seed for the routine establishment of potato crops in the field.

Based on demand, meristem tip culture is used by breeding companies in combination with thermo- and chemotherapy to eliminate virus diseases to produce virus-free (disease-free) plants.

13.2.1.3 Somatic Embryogenesis

Somatic embryogenesis is the development of a bipolar structure consisting of both a root and a shoot, from any sporophyte cell via the same key stages of embryo development as zygotic embryogenesis via globular, hart and or torpedo stages. The cells first de-differentiate and then re-differentiate towards the embryogenic pathway. Somatic embryos are produced using different media and explants like: cotyledons/hypocotyls or shoot/leaf explants (Pret'ová and Dedicova 1992; De García and Martínez 1995; JayaSree et al. 2001) or suspension cultures (Vargas et al. 2005). An efficient system for inducing somatic embryogenesis in potato is reported by Seabrook and Douglass (2001) and Sharma and Millam (2004), with the potential for mass clonal propagation. The internodal

segments are subjected to a three-stage culturing regime of shoot multiplication, the induction of somatic embryogenesis and the regeneration of somatic embryos using specific culture media. After transferring explants from an initial incubation on a medium containing auxin to an auxin-free medium, embryos develop within three weeks, which is confirmed by histological studies. It is reported that this unicellular mode of origin can successfully be used to produce such embryos (Sharma and Millam 2004; Sharma et al. 2007a). After transferring to a plant growth regulator-free medium, the resulting plantlets develop into potato plants, which produce tubers of good quality when grown in a glasshouse. The use of somatic embryogenesis by regenerating from single cells is an interesting tool for producing seed material and propagation of transgenic potato plants. Producing synthetic seeds by encapsulating somatic embryos could have advantages for handling, storage and transportation (Sharma et al. 2007b). Furthermore, it is a novel biological system for studies on gene expression and regulation. The use of somatic embryogenesis for potato improvement is summarized by Nassar et al. (2015).

13.2.1.4 Micro-tubers

Induction and Propagation

Micro-tubers are produced in vitro by culturing shoots. Their size is between 4 and 15 mm depending on cultural conditions and potato genotype. The fresh weight varies from 100 to 400 mg. Several different in vitro culture systems are used in tuberization studies (Ewing 1987). The most common method uses shoot cultures involving at least one subculturing to develop tubers. For practical purposes it is necessary to understand each of the different phases in the production of micro-tubers: initial explants, tuber induction, and dormancy response, development of new plants and abiotic factors and conditions. Hussay and Stacey (1984) studied tuberization in single node cuttings of several potato cultivars. Their results were confirmed by later studies and, therefore, are described here in more detail. On a medium containing 2.0 mg/l BA and 6% sucrose, micro-tubers develop after 6–8 weeks. The upright leafy shoots develop on horizontally growing stolons. Photoperiod also affects tuberization as under long-day conditions stolons form tubers in the medium and under short-day conditions most tubers develop above the solidified agar medium. All tuberization-inducing factors are inhibitors of gibberellin biosynthesis. The presence of GAs inhibits tuberization and promotes the elongation of stolons (Kumar and Wareing 1972; Vreugdenhill and Struik 1989; Xu et al. 1998).

Using this single-node tuberization system has revealed that in vitro tuberization is stimulated by increasing the sucrose concentration to 5–8% compared to glucose, fructose, maltose (Khuri and Moorby 1995; Fufa and Diro 2013) or mannitol (Lo et al. 1972) and by the addition of 2–10 mg/l cytokinins (Wang and Hu 1982; Abbott and Belcher 1986; Estrada et al. 1986; Gopal et al. 2004) and supplementary nutrients (Dhital and Lim 2011). Fluctuating temperature also affects the in vitro production of micro-tubers (Otroshi et al. 2009).

By adding various compounds, such as the plant growth inhibitor CCC (Hussay and Stacey 1984; Estrada et al. 1986; Lentini and Earle 1991), auxins (Ewing 1987; Dragićević et al. 2008), coumarin (Stallknecht 1972), jasmonic acid (JA, Pelacho and Mingo-Castel 1991), activated charcoal (AC, Bizarii et al. 1995) or hydrogen peroxide (López-Delgado et al. 2012) to a medium, it is also possible to stimulate the induction and development of micro-tubers.

Dormancy and Mass Propagation of Micro-tubers

Dormancy of micro-tubers is strongly dependent on genotype (Leclerc et al. 1995; Pruski et al. 2003) and tuber size. Small micro-tubers manifest a greater tendency to become dormant than large tubers (Ranalli et al. 1994; Leclerc et al. 1995). Lê (1999) presents data that indicates that a period in cold storage decreases the tendency to become dormant. Micro-tubers produced in cultures exposed to light had a short dormancy and sprouted prematurely (Gopal et al. 1997). Short-day treatments reduce the duration of

dormancy compared to tubers developed in darkness (Coleman and Coleman 2000). Micro-tubers produced under long-day conditions tend to sprout more readily than those kept under short-day conditions (Vecchio et al. 2000). These results demonstrate that the dormancy of micro-tubers ranges from strong to completely absent (Leclerc et al. 1995; Coleman et al. 2001). Therefore, they are clearly not similar to field-grown plants (Coleman et al. 2001). Summarizing, dormancy is determined by the method of production. Before selecting a procedure for producing micro-tubers, it is important to consider the reason for producing them. Other traits of micro-tubers are essential for the propagation of healthy material for transfer into glasshouses or grown on in the field. Liquid MS medium in fermenters has been used for the large-scale production of micro-tubers (Akita and Takayama 1988). This involves a two-step method starting with the cultivation of single node cuttings in 2 litres of liquid medium containing 3% sucrose contained in jars exposed to weak light until they produced shoots. In the second step, when the shoots were 20 mm long, the medium is replaced by one containing 9% sucrose and cultivated in darkness. Within two weeks this results in the production of 223 tubers per fermenter. The production of micro-tubers using different types of bioreactors is reviewed by Piao et al. (2002).

13.2.1.5 Mini-tubers

Mini-tubers are mostly 5–30 mm in diameter and weigh 0.5–5 g, and are larger than micro- tubers, but smaller than seed tubers, which weigh about 50–70 g. Mini-tuber production is based on the rapid in vitro propagation of a virus-free stock of micro-plants and their subsequent culturing hydroponically or other similar derived technologies (Ahloowalia 1999). It is used as the starting point for a field multiplication system. After acclimation, in vitro propagated plants or micro-tubers develop their own system of stolons and tubers after they are transferred to glasshouses or net-houses. The production of pathogen-free mini-tubers is possible within 70–90 days of growing them in soil under protected and controlled conditions. Some commercial companies quote rates of up to 1000 mini-tubers per square metre following non-destructive harvesting every 40–50 days from a crop derived from a single micro-plant under optimal glasshouse conditions (http:www.quantumtubers.com/techinfo.htm).

The larger the mini-tubers, the easier they are to handle and select because the characters of parental cultivars expressed in the tubers, like shape, skin colour and texture, are more easily visible. The effectiveness of using these tubers for selecting for agronomic characters is demonstrated by Gopal et al. (2002). The age of transplants from in vitro derived potato plantlets affects crop growth and seed tuber production in the field (Milinkovic et al. 2012; Lommen 2015). Healthy mini-tubers are the basis of seed multiplication programmes, as this reduces the number of multiplications and hence the risk of contamination of diseases and pests in the field. For the large-scale production of mini-tubers, growing them hydroponically in a nutrient solution is an efficient technique (Lommen 2007). The roots of the plants are enclosed in a water-filled container and the liquid nutrient solution is directly taken up by the roots. The shoots develop well under controlled temperature conditions. The mini-tubers repeatedly can be harvested as they can be removed from the plants once they have grown to a minimum size. This leads to the initiation of new extra tubers (Lommen 2007). Muro et al. (1997) compared two contrasting culture systems for propagating first generation potatoes: a system using peat or sand mixed with mineral fertilizers and a hydroponic culture method using perlite as a matrix and a nutrient solution. The total production and number of tubers were significantly higher in the hydroponic cultures. Compared to this, an aeroponic system, in which nutrients are applied as mist to the root system is more efficient for producing mini-tubers, but they have a lower average weight (Ritter et al. 2001). Hydroponic or aeroponic systems for producing disease-free mini-tubers for pre-basic seed production are used in countries where the climatic conditions are very unfavourable, such as high temperatures and humidity during the vegetation period, as in

Latin America (Mateus-Rodriguez et al. 2013), Africa (Vanderhofstadt 1999; Mbiyu et al. 2012; Prossy et al. 2014) and South Korea (Chang et al. 2011). Because the roots of the plants are cooled by the culture medium, the plants develop well and quickly. Several cycles of potato production per year is possible, resulting in a highly productive system.

Potato breeding companies commonly use in vitro cultures of plants, micro-tubers and mini-tubers to rapidly multiply their varieties and to maintain a collection of disease-free, and true breeding material. The mini-tubers can be classified as Elite Seed and used for the production of certified seed.

13.2.1.6 Long-Term Storage for Conservation of Potato Germplasm and Plant Genetic Resources, (Living Collection, Gene Bank)

Maintenance of Cultures of in Vitro Plants

The standard duration of the subculture of potato in a MS medium is 4–6 weeks at 20 °C and with a photoperiod of 16 h. This has been the standard procedure for the clonal propagation of potato plants since it was used successfully in the 1970s (Westcott et al. 1977). For a large collection of valuable genotypes, this method is expensive in terms of time and labour. The growth of the plants is determined by the number in a cultural vessel (Sarkar et al. 1994), but there are more efficient methods of prolonging the period for which cultures of shoots can be stored.

In vitro techniques for the medium- to long-term storage of potato tissue must satisfy the following requirements (Thieme 1992):

- extended storage life of the material must not be associated with reduced viability;
- low material, energy and labour inputs;
- can be used for a wide range of genotypes;
- no greater risk to the genetic stability of the stored material than growing in the field.

Investigations on how to fulfil these criteria resulted in the development of protocols, which require subculturing once per year or more and guarantee the genetic identity and a high percentage survival of explants.

Micro-tuber Induction and Storage

Nodal parts of in vitro plants are cut and transplanted into an MS-medium enriched with 8–10% sucrose but without phytohormones and cultivated under long-day conditions at 20 °C. After two to three weeks, culturing continues under tuber-inducing conditions at 9 °C under short-day conditions of 8/16 h light/dark cycle. Two to four months later during tuber formation the stems slowly die. The micro tubers left in the tubes are stored in the dark at 4 °C. Tubers are examined after 16 months (of total culturing time) and their germination and preservation status are assessed. Propagation involves cutting and transferring germinated stem parts from the old tuber to a fresh medium (Thieme 1992). At each stage in the storage cycle, first, the young stems and later the sprouting tubers can be harvested and used as the first step in their rapid propagation.

To produce a stock or living collection, micro-tuberization of tuber-bearing cultivars and genotypes is widely used (Donnelly et al. 2003; Pett and Thieme 1982; Kwiatkowski et al. 1988; Lizarraga et al. 1989).

Plant Growth Retardants

A simple, efficient and cheap method for reducing growth is to use plant growth retardants, which are routinely employed for small collections of germplasm (Dodds et al. 1991). Substances such as abscisic acid (ABA, Westcott 1981b), chlorcholine chloride (CCC, Miller et al. 1985) and acetylsalicylic acid (ASA, López-Delgado et al. 1998) can extend subculture duration by up to 12 months.

Reduction of Nutrition and Manipulation of Osmotic Stress

Reduced carbohydrate and mineral nutrition induces a slower growth of shoots in vitro. Sugar

alcohols, like sorbitol or mannitol are used instead of sucrose, which increases the osmotic value of the medium (Westcott et al. 1977). The best result is 18 months storage without sub-culturing and a 58% survival of potato micro-plants, which was achieved by growing them in an MS medium supplemented with 20 g/l of sucrose and 40 g/l sorbitol at a low temperature (Gopal and Chauhan 2010). Shoot tips encapsulated in calcium alginate beads (Nyende et al. 2003) can be stored at 10 and 4 °C for 180 and 270 days, respectively.

Cold Storage and Cryopreservation

A reduction in the temperature from 22 to 6–12 °C can extend the subculture duration from 4 weeks up to 12 months (Westcott 1981a). This method is used for the medium-term storage of a living collection.

The best option for the long-term maintenance of vegetative propagated plants is cryopreservation, using storing explants in or above liquid nitrogen, which has been intensively studied. There are numerous reviews and articles on the theoretical and methodological aspects of cold storage and cryopreservation (Harding 2004; Halmagyi et al. 2005; Benson et al. 2006; Benson 2008a, b; Harding et al. 2009; Sakai and Engelmann 2007; Benson and Keith 2012; Panta et al. 2015) and the details of the techniques used for potato are cited by Bajaj (1977, 1995), Grout and Henshaw (1978), Towill (1984), Keller et al. (2008), and Wang et al. (2008), which focus on currently used potato cryopreservation protocols. Kaczmarczyk et al. (2011) indicate the historically important, currently used and most recent advances in potato tip cryopreservation of various species and varieties of potato.

Basically this approach includes the main steps and modifications in the techniques (mentioned below) for the propagation and preparation of donor plants, isolation of explants (tuber sprouts, axillary buds and apical shoot tips), pre-culture, dehydration, cooling, storage, rewarming, regeneration (of explants) and propagation.

Different techniques have been successfully used for the cryopreservation of a wide range of species (Kaczmarczyk et al. 2011):

- two-step cooling
- ultra-rapid cooling
- droplet freezing
- vitrification
- droplet vitrification
- encapsulation/dehydration
- encapsulation/vitrification.

The advantages and disadvantages of these methods based on a lot of single observations are discussed. The parameters that affect cryopreservation, such as the physiological state of the donor plants and shoot tips and their pre-culture, and specific cryogenic factors, type of cryoprotectants, the cooling and rewarming process or media and light regime for further cultivation of plants after recovery are summarized by Kaczmarczyk et al. (2011).

Based on studies on genomic DNA stability, no genetic changes occur in plants after cryopreservation (Benson et al. 1996; Harding and Benson 2000). Harding and Benson (2001) demonstrate that stable somatic inheritance of genomic regions occurs by means of microsatellite profiles, which are identical in the regenerated material, the parental plants and their progeny. The successful conservation of characteristics of cultivars is confirmed, by estimating the ploidy level or by AFLP, RAPD or inter-simple sequence repeat (ISSR) markers (Zarghami et al. 2008; Hirai and Sakai 1999; Li et al. 2016).

There are cryopreserved collections of potato cultivars and accessions of wild species of potato in different countries: the Czech Republic (Zámečnik et al. 2007), Germany (Keller and Dreiling 2003; Kaczmarczyk et al. 2009), Peru (Panta et al. 2006; Gonzalez-Arnao et al. 2008), South Korea (Kim et al. 2006), Spain (Barandalla et al. 2003), the UK (http://www.scri.ac.uk) and USA (http://www.ars.usda.gov; http://www.ars-grin.gov/nr6), preserved using different

cryopreservation methods. The regeneration capacities of cryopreserved genotypes, which is the key factor in this approach, vary widely from 0 to 93%. The DMSO droplet method, improved by the use of alternating temperatures during preculture and a solid medium for regeneration is currently successfully used for storing 1119 accessions at IPK Gatersleben, for which the mean regeneration capacity is 46% (Kaczmarczyk et al. 2011).

A problem with this technique still remains the genotype-dependent ability to regenerate after cryopreservation. But one should bear in mind that none of the conservation strategies, like cryopreservation, cell and tissue culture and field culture, are completely safe (Kaczmarczyk et al. 2011). A selection of conservation techniques should be recommended based on the kind and number of potato genotypes, the length of time for which they are to be stored, the existing technical equipment and staff and other specific aspects of the research.

Cryopreservation can also be used to eradicate viruses, because many viruses are unable to survive or multiply under freezing conditions (Benson 2008b; Wang and Valkonen 2009). Potato leaf roll virus (PLRV), Potato virus Y (PVY), Potato virus M (PVM) and Potato virus S (PVS) are eliminated by the cryotherapy of virus-infected potato shoot tips (Wang et al. 2006) and in combination with ribavirin treatment (Kushnarenko et al. 2015), respectively. Ukhatova et al. (2016) used cryotherapy and complex chemo- and thermotherapies to eradicate PLRV in Chilean samples of *Solanum tuberosum.*

Potential applications of cryogenic technologies for plant genetic improvement and pathogen eradication are summarized by Wang et al. (2014).

13.3 Methods of Inducing and Utilizing Genetic Variability (Diversity)

13.3.1 Organ Culture

13.3.1.1 Production of Haploids

To obtain haploid cells and plants, gametophytes are cultured in vitro. In androgenesis it is the young anthers, pollen or microspores from flower buds that are cultured, in gynogenesis it is the ovules. In nutritional media containing phytohormones mitotic activity is induced in the haploid nucleus of gamethophytic cells. The resulting cells, tissues, embryos and plants can be haploid, but also diploid or polyploid. Haploid plants are weaker than diploid plants and are also sterile. The treatment of their meristems with the alkaloid colchicine induces endomitosis and the production of diploid fertile shoots, which are homozygotes. The results of the detailed studies carried out in the 1970s are summarized by Bajaj and Sopory (1986). The androgenic regenerants are very variable and 'androgenic competence' reduces the success of this method. Androgenesis protocols in terms of media and method were significantly improved by Uhrig (1985). A positive androgenic response was obtained by adding cytokinins and auxins alone or in combination. More recent results in potato androgenesis are reviewed by Pret'ová and Dedicova (2006).

Gynogenesis can be obtained by cross-pollination using *S. phureja* as a pollen donor. Seed is produced parthenogenetically by the mother plants. Using this method 500 monohaploid plants ($2n = x = 12$) from 2 million seeds were identified based on the colour of the spots at the base of the leaves (Uijtewaal et al. 1987) and stored in vitro. Androgenic monoploids are superior in

terms of most agronomic traits, including leaf size and tuber yield (Lough et al. 2001).

Anther culture is an alternative to selfing for the production of inbred lines of potato. Androgenesis does not require completely functional gametes to generate monoploid plants. The doubled monoploids could be used as female plants in hybrid schemes but male fertility is lacking (Paz and Veilleux 1999). Although this approach is limited, anther culture remains a tool for germplasm development in the conversion of potato into diploid crops (Jansky et al. 2016). Diploid inbred line breeding of potato was started and proved by Lindhout et al. (2011).

13.3.1.2 Embryo Rescue and Seed Culture

Embryo rescue techniques are based on the isolation of embryos from seeds and their cultivation on artificial media in vitro. It is used in potato after specific crosses to save embryos from ovules, which are fertilized but do not develop into viable seed (Singsit and Hanneman 1991). The fruit is removed over a period of ca. 20 days and embryo rescue conducted after the berries are surface-sterilized with ethanol. Using a scalpel and dissecting needles, seeds and embryos are isolated and placed in glass tubes containing MS media. Depending on the size and stage of development of the embryo (Thieme 1991), a plant develops after culturing for eight weeks the root system of which is robust enough for the plants to be grown on in a glasshouse (Ramon and Hanneman 2002). To overcome hybridization barriers, potato embryo rescue alone or in combination with other methods, such as mentor pollination, hormone treatment and reciprocal crosses can be used (Jansky 2006). Successful crossing between non-tuber-bearing and tuber-bearing species of *Solanum* is also possible (Watanabe et al. 1995) and is used to produce novel inter-series hybrids of *Solanum* (Dinu et al. 2005) and for the introgression of late blight resistance of 1EBN wild species *Solanum pinnatisectum* into *S. tuberosum* (Ramon and Hanneman 2002).

To obtain important offspring from crosses between partners that are 'difficult' to cross, immature seeds from recently harvested berries or dried stored seeds can be isolated, sterilized and cultivated in vitro, and can result in two to six weeks in the development of plants.

In addition to somatic hybridization, embryo rescue and seed culture have been successfully used to acquire interspecific and intergeneric hybrids for use as pre-breeding material in potato breeding programmes.

13.3.2 Somaclonal and Epigenetic Variation

13.3.2.1 General Aspects: Definition, Origin and Causes, Mechanisms and Molecular Basis

Somaclonal variation is defined as genetic and phenotypic variation among clonally propagated plants from a single donor clone resulting from the use of tissue culture. This phenomenon is recorded for many crop plants (Larkin and Scowcroft 1981, 1983; Ahloowalia 1986; Kaeppler et al. 1998, 2000; Veilleux and Johnson 1998). It is manifested as cytological abnormalities, frequent qualitative and quantitative phenotypic variations, DNA sequence changes, gene activation and silencing (Kaeppler et al. 2000). Somaclonal variation mimics induced mutations. Only a few of these mutations are expressed as phenotypic and cytogenetic changes in the regenerated plants (Jain et al. 1998).

There are discussions in the literature about the different mechanisms that result in somaclonal variation including point mutations induced by exogenous factors such as radiation and chemical mutagens, changes in chromosome number and structure, changes in organelle DNA, somatic crossing-over and sister chromatid exchange, chromosome breakage and rearrangement, somatic gene rearrangements, DNA amplification, DNA methylation, epigenetic variation, that may result from micro-environmental conditions in tissue culture, histone modification and RNA interference (iRNA), segregation of pre-existing chimeric tissues and insertion or excision of transposable elements or

non-specific interaction inducing changes in gene expression (Jain et al. 1998; Kaeppler et al. 1998, 2000; Krishna et al. 2016). Transposable elements can be activated by tissue culture. Insertions of these elements and retrotransposons can function as insertional mutagens of plant genomes, which may also cause chromosomal rearrangements (Tanurdzic et al. 2008). Li (2016) points out that the role of de-differentiation and re-differentiation during cell culture can contribute to the detected ploidy variation, given that different culture methods often induce different frequencies of somaclonal variation. The expression of genes that are responsible for centromere and ploidy stability are expected to change during de-differentiation and re-differentiation and may therefore result in a variation in the number of chromosomes in some cultured cells. The epigenetic changes in gene expression may last for many mitotic generations, may even be heritable over a certain number of reproductive generations, and may consequently still cause genome instability in the original and the immediately following generations of regenerated plants (Li 2016).

Recently, epigenetic variation in in vitro cultures of potato cells has attracted interest. Demarly and Sibi (1989) coined the term 'epigenetic variation' for this somaclonal variation, the inheritance of which is neither Mendelian nor cytoplasmic. Epigenetic control of gene expression is defined as a somatically or meiotically heritable alteration in gene expression that is potentially reversible and is not due to a DNA sequence modification. It involves gene silencing or gene activation that is not due to chromosomal aberrations or sequence changes, which might be unstable or reversible somatically or through meiosis (Kaeppler et al. 2000). Authors point out that these epigenetic changes could be manifested in the activation of quiescent loci or as an epimutation of loci sensitive to chromatin-level control of expression. They suggest that somaclonal variation is manifested as quantitative and qualitative trait mutations, karyotype changes and sequence modification. More aspects of the epigenetics of somaclonal variation in plants are discussed by Kaeppler et al. (2000). Epigenetic events defined as structural adaptations of chromosomal regions that register, signal, or perpetuate altered states of activity have also to be considered (Bird 2007).

The analysis of DNA methylation is a well-described epigenetic mechanism for detecting and evaluating epigenetic variation in in vitro cultures of plant cells (Miguel and Marum 2011). For potato, amplified fragment-length polymorphism (AFLP) and methylation-sensitive amplified polymorphism (MSAP) are used to study variation in micro-plant morphology (Siobhan and Cassells 2002), somatic embryos (Sharma et al. 2007b) and cryopreserved shoot tips (Kaczmarczyk et al. 2010). Furthermore modifications of histones and small RNAs are reported occurring in cell suspension cultures of potato (Law and Suttle 2005). In general, advances that have uncovered highly dynamic mechanisms of chromatin remodelling occurring during cell de-differentiation and differentiation processes on which the in vitro adventitious plant regeneration are based, are presented in Miguel and Marum (2011).

Li (2016) introduces an interesting concept of genome network to describe different types of variations as natural attributes of somatic genomes in crops and horticultural plants and reviews the agricultural implications of these variations. He proposes the term 'somatic genome variation' which covers the variation in an organism and the generation of new genotypes through somatic means from a sexually produced individual. He assumes that it displays many more attributes than genetic mutation and is important for agriculture.

13.3.2.2 Callus, Cell Suspension and Protoplast Culture

Somaclonal variation occurs in plants obtained by using tissue culture (Larkin and Scowcroft 1981). Plants regenerated from various cells and tissues, such as cultures of protoplasts (protoclones, Shepard 1980), apical meristems (mericlones), anthers or microspores (gametoclones), callus (calluclones) and leaf and stem tissue (somaclones) vary. Callus is defined as an unorganized mass of tissue growing on a solid

substrate. In liquid media, callus quickly dissolves into small aggregates of cells called a cell suspension (Bajaj and Dionne 1967; Lam 1977). Cell suspensions are used as starting material for protoplast culture (Opatrny et al. 1980) and the production of somatic embryos (Vargas et al. 2005).

During the 1960 to the 1980s research focused on methodologies to produce somaclones and factors that influence their variability or stability. The external application of plant growth regulators to a callus induces it to differentiate organs in vitro like shoots, leaves, roots or other organs. Induction of callus was first reported by Stewart and Caplin (1951) and further studied by Bajaj and Dionne (1967), Skirvin et al. (1975) and Roest and Bokelmann (1976). The effect of indolyle-3-acetic acid (IAA), indole-3-butyric acid (IBA), naphthyl acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D) and kinetin was investigated by Okazawa et al. (1967). Calluses on potato tuber explants usually form after about one to two weeks of culturing on media with the auxins, 2,4-D and NAA. The conditions and balance of plant growth regulators in cultures were measured in terms of successful direct shoot regeneration from tuber discs or leaf explants of a number of cultivars (Okazawa et al. 1967; Lam 1975; Skirvin et al. 1975; Jarret et al. 1980a, b; Mix and Sixin 1983; Kikuta and Okazawa 1984; Esna-Ashari and Villiers 1998; Wheeler et al. 1985). Shoot regeneration is a two-stage procedure (Webb et al. 1983) involving different plant growth regulators. In the first stage the media are supplemented with NAA, BA and GA3, and in the second stage, with GA3. Thieme and Griess (2005) used leaf and stem explants from 17 potato cultivars and breeding clones for callus induction on MS medium with 0.2 mg/l NAA, 2 mg/l zeatin and 5 mg/l GA3. After two weeks, the explants were transferred into a shoot induction medium (Webb et al. 1983; Wheeler et al. 1985). Then the successful shoot regeneration of leaf and petiole explants in combination with a wide range of plant growth regulators can be analysed (Park et al. 1995; Hansen et al. 1999; Yee et al. 2001).

In the 1990s, somaclonal variation was studied in terms of estimating the changes in traits after growing them in the field and its application in potato breeding. This revealed that tissue culture per se appears to be an unexpectedly rich and novel source of genetic variability generated during the tissue culture cycle (Larkin and Scowcroft 1981). This tissue culture cycle starts with the establishment of a de-differentiated cell or tissue culture, the proliferation of cells for a number of cell generations and the subsequent regeneration of plants. The expectation was that the somaclonal variation recorded for many crops, such as potato, may result in genetically stable and useful genotypes with novel or changed traits useful for breeding programmes. Investigations indicate that the source of the explant (Sree Ramulu et al. 1986), the culture medium, the age of the donor plants, the duration of the culture and the genotype itself are important factors affecting the extent and frequency of somaclonal variation.

The chromosome stability of somaclonal variants has been investigated. Polyploidy, aneuploidy and structural changes including chromosomal deletions, inversions and translocations occur in plants regenerated from callus culture (Ahloowalia 1986). Sree Ramulu et al. (1986) indicate that the initial ploidy of the donor plants influences the degree of polyploidization that occurs during protoplast isolation and culture. All protoclones derived from diplopid donor clones become tetraploid or aneuploid. A high frequency of protoclones of cv. Bintje retain tetraploidy but are morphologically abnormal. Munir et al. (2011) demonstrate somaclonal variation in cv. Désirée by using random amplification of polymorphic DNA (RAPD) markers.

Callus induction, maintenance and shoot regeneration were the basis of protocols for *Agrobacterium*-mediated transformation of potato at the end of the 1980s. The practical application of callus culture, however, is uncertain because high genomic instability of the regenerated shoots resulted mostly in aberrant plants unsuitable for breeding purposes and clonal propagation. Somaclonal variation is

undesirable for large-scale mass propagation of clones, germplasm preservation and production of transgenic plants. For this, genetic uniformity of the plants at an early stage is essential. The genetic fidelity of plants can be revealed using morpho-physiological, biochemical, cytological and DNA-based, molecular markers. Krishna et al. (2016) review the strengths and weaknesses of the different marker systems including morphological traits, cytological, isoenzyme and DNA markers. Next-generation sequencing technology is used to realize the whole-genome sequencing of individual plants (Miyao et al. 2012). New technologies will help in arriving at a better understanding of somaclonal variation and its potential use in crop improvement.

13.3.2.3 Success in Inducing Somaclonal Variation for Potato Breeding

In this review the terms protoclones are used for clones derived from protoplasts and somaclones for those derived from any other tissues.

There are relatively few experiments on the use of somaclonal variation for the improvement of potato cultivars and breeding lines for breeding purposes (Table 13.1). The objective of these studies is to analyse somaclones of cultivars or breeding clones derived from protoplasts, explants and callus culture, including mutagenic treatment and in vitro selection, in the field in terms of their suitability to improve agronomic traits of potato. Shepard et al. (1980) found clones with different types of morphology in a population of 10,000 protoplast-derived clones of the cv. Russet Burbank. Protoclones with improved characteristics had deficiencies in other agronomic traits, with some of them being more resistant to diseases than their parents. Sebastiani et al. (1994) report the potential of somaclonal variation in producing potato clones resistant to *Verticillium dahlia* and Cassells et al. (1991) discuss the resistance in the field of somaclones of potato to late blight in potato associated with instability and pleiotropic effects. Secor and Shepard (1981) document differences in 22 of 35 traits of protoclones, which are associated with variation in the starch content of the protoclones. Extensive morphological variation occurs in protoclones of cv. Maris Bard (Thomas et al. 1982). Of 33 protoclones from cv. Crystal, none have a higher tuber yield, but are better in terms of tuber bruising, resistance to tuber soft rot and chip colour. Rietveld et al. (1991, 1993) used somaclones of three potato cultivars derived from tuber discs explants. A multistage selection procedure used to characterize these somaclones in field plots over five generations at three locations revealed less variation in tuber shape than in other traits, but they produce longer tubers, as previously reported by Pavek and Corsini (1982) and Cassels et al. (1986). A higher mean tuber number for somaclones compared to controls is recorded (Rietveld et al. 1991; Thieme and Griess 1996, 2005). The latter authors studied 13,000 somaclones of 17 potato donor cultivars or breeding clones, transferred the in vitro plants to a glasshouse, followed by several generations grown in a field and a multistage selection procedure commonly used in potato breeding. Over a period of five years and three field generations, yield, tuber characters, haulm growth, earliness, starch content, starch yield and tuber appearance of somaclones were assessed and compared with that of the controls. This revealed that these traits varied depending on donor genotype. The haulm growth, yield and tuber quality of the majority of the somaclones were poorer than in control. Earliness varied in one maturity group. There was no variation in the skin and flesh colour of tubers. In the second field generation, the frequencies of negative variants for individual donor genotypes ranged between 0.7 and 22%, of invariants between 71 and 98% and strong positive variants between 0 and 9%. Summarizing all results depending on trait, the average percentage for all donor genotypes ranged between 0.1 and 1.4% for positive variants (Thieme and Griess 2005). These results led to the conclusion that somaclonal variation can be used to modify one or few traits in a commercial cultivar while preserving other important traits. Therefore, this variation should be exploited in potato breeding as an additional tool to improve specific agronomic traits of specific cultivars. For example, a cultivar that has desirable agronomic traits could

Table 13.1 Utilization of somaclonal variation to improve commercial varieties/breeding clones of potato, including somatic cell selection

Source of explants/culture, mutagenic treatment	Potato cultivar or clone	Character of somaclones	References
Leaf/culture filtrate, callus, regeneration	6 dihaploid clones	Improved resistance to *Phytophthora infestans*, *Fusarium* species	Behnke (1979, 1980)
Mesophyll protoplasts/callus, regeneration	Russet Burbank	Increased tuber number, starch content, lower proportion of 'crull'	Secor and Shepard (1981)
Leaf, rachis, stem/callus, regeneration	Désirée	High yield, increased resistance to *Streptomyces scabies*, higher dry matter content	Evans et al. (1986)
Protoplasts/callus, regeneration	Feltwell, Maris Piper, Foxton	Improved yield, tuber appearance, resistance to common scab, PVY, PLRV	Thomson et al. (1986)
Protoplasts/pathogen filtrate/callus, regeneration	Dihaploid clones	Non-significant improvement in resistance to *Fusarium* species (*F. sulphureum*, *F. coeruleum*), *Phytophthora infestans*	Wenzel and Foroughi-Wehr (1990)
Internodes/adventitious regeneration	Bintje	Improved stable resistance to *Phytophthora infestans* in the field (but mutants often appear at maturation)	Cassells et al. (1991)
Tuber discs/callus, adventitious regeneration	Superior	Stable improved yield, tuber number and shape, and enhanced vigour	Rietveld et al. (1991)
Tuber discs/callus, adventitious regeneration	Kennebec, Russet Burbank, Superior	High total tuber number and weight, earlier maturing and a more elongated tuber shape	Rietveld et al. (1993)
Mesophyll protoplasts/callus, regeneration	Crystal	Improved resistance to tuber bruising, bacterial soft rot, enhanced chip colour and processing quality	Taylor et al. (1993)
Mesophyll protoplasts/callus, regeneration	Irish Cobbler	Non-browning cv. White Baron	Arihara et al. (1995)
Stem/callus, regeneration	17 cvs. and breeding clones	Improved tuber number and size, starch content and yield, early maturing	Thieme and Griess (1996, 2005)
Leaf/callus, regeneration	Kennebec	Salt tolerant plants	Ochatt et al. (1998)
Stem segments/irradiation, adventitious regeneration	Golden Wonder	Improved yield and resistance to *Phytophthora infestans* (foliage blight)	Kowalski and Cassells (1999)
Stem/mutagenesis by irradiation	Kufri Jyoti, Kufri Chandramukhi	Improved heat tolerance	Das et al. (2000)
Stem, tuber, protoplasts/callus, regeneration	Bintje	Alterations in general appearance, leaf morphology, tuber characteristics, unstable	Jelenić et al. (2001)
Callus culture/*in vitro* mutagenesis	Désirée	Improved resistance to *Alternaria solani* and *Streptomyces scabies*	Veitia-Rodriguez et al. (2002)

(continued)

Table 13.1 (continued)

Source of explants/culture, mutagenic treatment	Potato cultivar or clone	Character of somaclones	References
Leaves, irradiated callus, culture filtrate/regeneration	Désirée	Improved resistance to *Alternaria solani*	Rodríguez et al. (2007)
Cells/pathotoxins, callus, regeneration, selection	Iwa, Russet Burbank	Improved resistance to *Streptomyces* spec., high tuber weight	Wilson et al. (2009, 2010a, b)
Meristem tissue/regeneration after thermotherapy	Reet	Positive differences in yield, tuber number and weight, and resistance to late blight	Rosenberg et al. (2010)
Tuber tissue/somatic embryogenesis	Russet Burbank	Superior processing qualities	Nassar et al. (2011)
Tuber sprouts/spontaneous chimeras	Red Nordland	Improved skin colour, resistance to *Strepto-myces scabies*, *Spongospora subterranea*	Waterer et al. (2011)
Cells/thaxtomin A, callus culture, regeneration	Russet Burbank	Improved resistance to common scab and powdery scab	Tegg et al. (2013)
Tuber sprouts/mutagenesis by chemicals	Cardinal, Diamant, Asterix	High yielding	Hoque and Morshad (2014)
Tuber tissue/somatic embryogenesis	Russet Burbank	High content of phytonutrients and antioxidants	Nassar et al. (2014)

be improved by increasing tuber number per plant, starch content or earliness.

Mixoploidy and chimeric structures in nine somaclones of cv. Bintje are associated with alterations in its appearance, leaf morphology and tuber characteristics. This phenotypic instability is correlated with aneuploidy or polyploidy, which can be detected at high frequencies in the chromosome counts of root tips of these somaclones (Jelenić et al. 2001).

After eradicating viruses, using thermotherapy, meristem culture regenerated plants of the variety Reet differ in yield, number and weight of tubers and resistance to late blight, and meristem clones also deviate in otherwise invariable morphological characteristics (Rosenberg et al. 2010). A high-yielding genotype obtained by using chemical mutagens (Hoque and Morshad 2014) and the non-browning cv. White Baron was developed by using somaclonal variants of cv. Irish Cobbler (Arihara et al. 1995).

Of 800 somaclones of cv. Russet Burbank produced using somatic embryogenesis, 25 lines were selected on the basis of their yield and processing quality, which indicates that somaclonal selection offers clear benefits for phytonutrient improvement and in improving the processing quality of potato (Nassar et al. 2011, 2014). Three somaclones derived from cv. Désirée are more resistant to *Alternaria solani* and *Streptomyces scabies* (Veitia-Rodriguez et al. 2002). The best cultivar, with the smallest somaclonal variation, for producing synthetic seed was selected based on the results of a RAPD analysis (Bordallo et al. 2004).

If naturally occurring mutations in potato are stable and beneficial, they can be used in breeding programmes. In field trials, over 30 lines derived from chimeric tubers of the cultivar Red Norland were studied, and new lines developed from plants exhibiting spontaneous mutations that caused chimeras in terms of tuber

skin colour with the dark red coloration stable over several generations of vegetative propagation and were higher yielding than the original cultivar (Waterer et al. 2011). In vegetatively propagated potato plants, some traits resulting from somaclonal variation, such as chip colour quality, are quite stable over at least several generations of vegetative propagation (Nassar et al. 2011). Gamma-irradiation can be used during in vitro propagation of plants to induce heat tolerance mutants in two commercial potato cultivars (Das et al. 2000). There have been attempts to select salt-tolerant potato cell lines and plants (Ochatt et al. 1998; Queiros et al. 2007). Potter and Jones (1991) confirm that plants of cv. Désirée produced by multiplication of organized meristems or serial subculture of stem nodes using morphological and RFLP analysis are genetically stable. Plants derived from regeneration after a short leaf callus phase vary in banding patterns and morphology. Significant differences between clones derived from meristem tips of four potato cultivars after field experiments at different locations persist for several years (Nielsen et al. 2007). This variation was in the number of plants per plot, maturity, skin and flesh colour, tuber form, time of emergence, flowering, number of stems and tubers per plant.

The genetic and phenotypic stability of potato plants of the cv. Désirée obtained using four different propagation methods have been compared (Sharma et al. 2011). Plants from synthetic seed (somatic embryos), axillary buds, micro-tubers and true potato seed have been analysed phenotypically, cytologically and using AFLP markers. Compared to clonally propagated plants that do not vary phenotypically, plants from true potato seed show phenotypic segregation. None of these plants varied in genome constitution, assessed using flow cytometry. In plants regenerated by means of axillary bud proliferation, the AFLP-marker profile was identical but there were some differences among the somatic embryo and micro-tuber-derived plants (Sharma et al. 2011). To discriminate intra-clonal variants of cv. Russet Norkotah, there are AFLP and microsatellite markers, which are suitable for detecting epigenetic differences (Hale et al. 2005).

Based on published results (summary in Table 13.1), somaclones of potato can be used as a source of new variation (Karp 1995). There are suitable tools for detecting, evaluating, identifying and improving traits in order to realize the benefits of these variations. But the former very optimistic appreciation of their practical utilization (Bottino 1975; Larkin and Scowcroft 1981) has not been confirmed. There is a need for further attempts to improve potato in terms of agronomic traits and resistance to biotic and abiotic stresses.

It is recognized that the recovery of somaclones exhibiting beneficial traits without any negative side effects is rare. For many applications somaclonal variation is something to be avoided (Barrell et al. 2013; Dann and Wilson 2011). Methods aimed at producing uniform plants from cell and tissue culture, such as for the large-scale clonal propagation and multiplication after virus/pathogen elimination (Rosenberg et al. 2010), long-term storage (Dann and Wilson 2011), cell screening and polyploidization (Chauvin et al. 2003), cell fusion (Kumar 1994) or gene transformation (Dale and McPartlan 1992; Heeres et al. 2002) are examples of when somaclonal variation is undesirable. There are no ways to avoid the production of somaclonal variants in transgenic potato lines (Meiyalaghan et al. 2011; Barrell and Conner 2011). Therefore, the exploitation of somaclonal variation is currently not widely used in potato breeding programmes.

Plant tissue culture has resulted in the development of many novel tools, which have recently been used by potato breeders. Nevertheless, a combination of biotechnological methods such as cell and tissue culture, genetic engineering, marker- and genome-assisted technologies have a high potential to improve potato crops.

Advances in the use of new techniques, like DNA microarrays, RNA transcriptomic, metabolomic and proteomic approaches and the identification of genes will help in resolving the challenge of providing enough food in the future for the ever-growing world population.

13.3.3 Somatic Hybridization via Protoplast Fusion

13.3.3.1 Protoplast Isolation and Culture

Plant protoplasts are cells from which the cell wall has been removed by dissection or enzymatic digestion (Davey et al. 2005). Mechanical procedures involving slicing of plasmolyzed tissues are today rarely used for protoplast isolation. Plant protoplasts isolated from somatic cells are still totipotent and can produce, in suitable culture conditions, a new cell wall, colonies of cells, micocalluses, calluses and finally new plants. Lacking a cell wall, protoplasts are very good systems for gene transfer, induced fusion (also called somatic hybridization), targeted mutagenesis and somatic cell genetic research (Davey et al. 2005). Potato was one of the first plants to be used in protoplast culture and somatic hybridization. After the discovery of the utility of enzymes like cellulases and pectinases for plant protoplast isolation (Cocking 1960) and their use for tobacco protoplast isolation and plant regeneration (Carlson et al. 1972), potato was one of the next species that proved amenable to protoplast isolation and culture (Shepard and Totten 1977; Zuba and Binding 1989). This opened the way for using isolated potato protoplasts in somatic hybridization and gene transfer. Protoplasts are versatile cell systems that can be used to manipulate the genome of the somatic cells of potato (*Solanum tuberosum* L. $2n = 4x = 48$) including its monoploids ($2n = 1x = 12$), (di) haploids ($2n = 2x = 24$) and related wild diploid species ($2n = 2x = 24$) of *Solanum* (Wenzel 2006). Since the 1980s, many laboratories have optimized the methods for protoplast isolation and culture of crop potatoes and many of its wild relatives (Zuba and Binding 1989). Nowadays these methods are well refined and routinely used for culturing many wild species and crop potatoes (Thieme et al. 1997; Sharma et al. 2011; Rokka 2015).

After many years of research on different tissues, like leaves of glasshouse-grown plants, mesophyll tissue of in vitro shoots, single cell suspensions (Jones et al. 1989a, b), in vitro-induced micro-tubers (Jones et al. 1989a) and true potato seedlings derived from hypocotyl tissues (Dai and Sun 1994), the tissue of choice is leaf mesophyll harvested from three-week-old shoots of in vitro plants (Thieme et al. 1997). Protoplast yield and viability are greater for potato and wild *Solanum* shoots cultured in jars than in test tubes. This may be due to the greater volume of the jars and the resultant lower levels of ethylene. When STS (silver thiosulphate), an inhibitor of ethylene biosynthesis, is added to the culture media, it stimulates leaf area growth in *Solanum chacoense* (Rakosy-Tican et al. 2011). Mesophyll tissue can yield approximately 10^6 pp ml^{-1} g^{-1} fresh weight. The key factors for good protoplast yield are: the source and age of the donor tissue, the growth conditions (vigorous plants with well-developed leaves), tissue slicing and enzyme solution. For the digestion of leaf tissue, proper enzyme solutions have to be developed for each species or genotype. Digesting solution contains mainly two enzymes: Cellulase R-10 (1%) and Macerozyme R-10 (0.5%), but adding slightly lower concentrations of different and very active digestive enzymes like Pectolyase Y-23 or Driselase may improve cell wall removal. In order to maintain protoplast integrity, mannitol, sorbitol or sucrose at iso-osmolar concentration need to be added to the enzyme solution. Macroelements or sometimes microelements might also improve protoplast viability after isolation, at least the presence of Ca ions is essential for membrane stability (Davey et al. 2005). The incubation in the enzyme solution is also a critical step, incubation at room temperature overnight (16 h) being the most convenient. After incubation, protoplast release from mesophyll tissue can be improved by shaking at a high temperature and low rotation for at least 30 min and up to 1–2 h. Protoplast isolation has to be checked using an inverted microscope and can be further improved by squeezing the tissue. After removing undigested tissue by filtration and cellular debris by two to three centrifugation steps in an iso-osmolar solution, depending on the protocol (Rokka 2015), the protoplasts can be counted by using a haemocytometer and cell viability can be

evaluated by using FDA (fluorescence diacetate assay) (Rakosy-Tican et al. 1988 and references herein). The viable protoplasts are then mixed for further use in somatic fusion experiments, gene transfer, somatic cell genetics or other basic studies.

If protoplasts are to be cultured to regenerate plants or to induce protoclonal variation, different cultural steps have to be followed. At each step different media and plant growth regulators (PGRs) are used. There are many reports on potato protoplast culture in the literature, starting with the first successful plant regeneration from mesophyll protoplasts (Shepard and Totten 1977), followed by many improvements made by many groups as presented in a previous review (Vinterhalter et al. 2008). Today, there are protocols for plant regeneration from mesophyll protoplasts, as described by Thieme et al. (1997), which involve mainly four steps:

1. Cultivation up to visible cell colonies from isolated protoplasts in the dark at 25 °C on liquid VKM-media.
2. Transfer of cell colonies to solid CUL-media kept under fluorescent light, at a photoperiod of 16 h and 25 °C, until a macro-callus develops.
3. Cultivation of the callus on JKM-media for initiation of shoot regeneration.
4. Transfer of shoots to propagation media (MS modified by reducing the NH_4NO_3 content to 1.2 g/l).

This method for protoplast regeneration is widely used for many combinations of somatic fusion and is a reliable and useful way of regenerating a large number of somatic hybrids (Thieme et al. 2008, 2010; Rakosy-Tican et al. 2015).

Two main issues are encountered in protoplast culture: protoclonal variation caused by callus genetic instability and genotype-dependent response to protoplast culture (see Sect. 13.3.2). When maximum genetic variation is required, somaclonal variation provides a useful tool for the more technologically demanding approaches like somatic hybridization and transformation. The attraction of protoclonal variation is that it requires no knowledge of the genetic basis of a specific trait, it needs no recombinant DNA, it does not require mutagenesis, specialized equipment or containment measures and can be exploited by using standard in vitro culture procedures. In contrast, when the production of true-to-type plants is the goal, clonal propagation from protoplasts assures the cloning is of single cells.

Although the genotype effect in protoplast regeneration occurs in potato and its wild relatives, the optimization of culture media made it possible to use similar media to regenerate cell colonies, calluses and shoots from protoplasts for several species of *Solanum*. These standard culture conditions (media and physical factors) are useful for isolated protoplasts and somatic hybrids or fusion products and intra as well as interspecific combinations (Thieme et al. 1997, 2008, 2010), but the efficiency of regeneration varies for each particular fusion combination (Rakosy-Tican et al. 2015).

13.3.3.2 Protoplast Fusion and Somatic Hybridization

Plant protoplasts might fuse spontaneously during protoplast isolation due to plasmodesmata enlargement between adjacent cells, but this spontaneous homo-specific fusion occurs at a low frequency. There are chemical, physical and a few biological tools used to induce protoplasts to fuse (Davey et al. 2005). These techniques were developed to induce protoplasts with negative charges, the so-called zeta potential, to attract each other. The fusion can be achieved only when protoplasts are first forced to agglutinate and further factors will result in the disorganization of protoplast membranes leading to fusion or merging of two or more agglutinated cells. Fused cells can belong to the same (homo-specific) or different (hetero-specific) species. Protoplast fusion can be induced in any combination of intra-, interspecific, inter-generic or even cells of organisms belonging to different kingdoms. But, the fusion products can only express totipotency when phylogenetic relationships are close. Closely related species generate

fusion products that can de-differentiate and finally regenerate new plants. Although many different fusion methods are used in laboratories, only two are widely used, i.e. electrofusion and PEG (polyethylene glycol) induced fusion (Davey et al. 2005). Electrofusion is by far the preferred method since its discovery in 1979 (Senda et al. 1979). It consists of protoplast agglutination induced by the use of an alternating current (AC) field, the so-called dielectrophoresis or pearl chain formation driven by the mutual attraction of protoplasts based on electrical charges and their movement towards each other and to the electrodes (Zimmermann and Scheurich 1981). In the second phase of electrofusion, the agglutinated aligned protoplasts are induced to fuse by using direct current (DC) square wave pulses with a high intensity (2000 V cm^{-1}) and very short duration (10–100 μs) (Rakosy-Tican et al. 1998). Electrofused plant protoplasts are also influenced by these electric fields in a stimulatory way, although the so-called electrostimulation effect is not well understood, with the responses expressed in the first and a few subsequent generations of plants (Goldsworthy 1996; Davey et al. 1996). Electrostimulation attracted interest during the 1990s but these methods for stimulating the growth of protoplasts and other plant tissues have received less attention in the past few decades. This is an area, which deserves more investigation in the future, mainly in relation to plant regeneration from recalcitrant protoplasts, but also from a basic point of view. Understanding the cellular and molecular mechanisms involved in plant cells or other responses of cells to electromagnetic fields is, in our opinion, worth investigating for future use in such fields for stimulating cell development. The culture media used to stimulate protoplast response and regeneration of protoplasts from other species contain different additives (Davey et al. 2005). The division of potato protoplasts, isolated from cell suspensions, is enhanced by the addition of Erythrogen™, an oxygen carrier, when the protoplasts are embedded in agarose semi-solid droplets (Power et al. 2003).

Moreover, the electrofusion of preselected pairs of protoplasts of tobacco (Rakosy-Tican et al. 2001) is a more refined technique, in which the two protoplasts to be fused are selected using micromanipulation and the electrofusion is induced in a controlled manner. The electrofusion of preselected pairs of potato protoplasts is not used as the mass fusion is preferred to scale up somatic hybridization experiments in the case of this important tuberous crop. PEG-induced fusion generally has a similar efficiency as electrofusion in inducing double fusion of protoplasts, especially when washed with calcium solution (Davey et al. 2005). The value of the fusion efficiency is around 45%, but higher values are reported for electrofusion, which depends on species, fusion chamber, number of DC pulses and protoplast lysis dependent on electrofusion parameters, as shown for cereal mesophyll protoplasts (Rakosy-Tican et al. 1988, 1998).

After fusion, fusion products have to be selected or regenerated plants have to be analysed for hybridity using molecular and cytogenetic techniques. Over the last decade selection of potato somatic hybrid cells was mainly based on the presumption of vigorous growth, which is revealed by using green fluorescent protein (*gfp*) reporter genes when potato is electrofused with transgenic *Solanum chacoense* expressing *gfp* (Rakosy-Tican and Aurori 2015). For the characterization of somatic hybrid plants, there are many PCR-based molecular tools, such as Random Amplification of Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), Interspaced Simple Sequence Repeats (I-SSR), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP) or Microsatellite-anchored fragment length polymorphism (MAFLP) (Baird et al. 1992; Thieme et al. 2008; Iovene et al. 2012). Due to their stability and universality SSR markers are widely used (see Tables 13.2 and 13.3) (Eeckhaut et al. 2013; Thieme et al. 2008, 2010). Recently the application of Diversity Array Technology (DaRT) has made it possible to characterize completely the composition of the genome of somatic hybrids between potato and

Table 13.2 Inter- and intra-specific somatic hybrids of potato, *Solanum tuberosum* (*S. tbr*), the techniques used for their characterization and/or selection, traits of interest and references, in the interval 2003–2017

Combination	Tools for characterization and/or selection	Traits of interest—resistance or other traits	References
Interspecific somatic hybrids			
S. tbr (+) *S. acaule*	Glycoalkaloid aglicones	*Clavibacter*	Rokka et al. (2005)
S. tbr (+) *S. berthaultii*	ISSR, cytoplasmic DNA, FC	Salt tolerance, tuber yield	Bidani et al. (2007)
S. berthaultii (+) *S. etuberosum*	NS	*Phytophthora erythroseptica, Pythium ultimum*	Thomson et al. (2007)
S. tbr (+) *S. brevidens* (*S. palustrae*)	RFLP, GISH, FISH	Tuber soft rot, early blight	Tek et al. (2004)
	RAPD, GISH, FISH	Addition and substitution lines	Dong et al. (2005)
	Laboratory and field resistance tests	*Streptomyces*	Ahn and Park (2013)
S. tbr (+) *S. bulbocastanum*	RAPD chromosome specific	Loss of one specific chromosome	Bołtowicz et al. (2005)
	Laboratory and field resistance tests	*Meloidogyne chitwoodi* (*Mc*)	Brown et al. (2006)
	MAS for RB gene (*Rpi-blb1*) GISH, cytoplasmic DNA	MAS applied to *Pi* resistance Genetic characterization	Colton et al. (2006), Iovene et al. (2007)
	MAS $R_{Mc1(blb)}$	Selection of breeding lines resistant to *Mc*	Zhang et al. 2007
	Anther culture of 4× somatic hybrids	Haploidization for breeding	Yermishin et al. (2008)
	ISSR, microsporogenesis, anther culture	Haploidization for breeding	Iovene et al. (2012)
	SSR, cytogenetics, *Rpi-blb1*; *Rpi-blb3* gene	*Pi*	Rakosy-Tican et al. (2015) (genes and resistance, under publication)
S. tbr (+) *S. cardiophyllum*	Morphology, chromosome number, RAPD	Characterization	Shi et al. (2006)
	RAPD	CPB, *Pi*	Chen et al. (2007)
	SSR, AFLP, MFLP, ploidy	PVY, CPB, *Pi*	Thieme et al. (2010)
	RAPD, SSR, ISSR, AFLP, cyto-plasmic type molecular markers, FC	*Pi*	Chandel et al. (2015)
S. tbr (+) *S. circaeifolium*	Morphology, RAPD, chromosomes	*Pi*	Espejo et al. (2008)
S. tbr (+) *S. chacoense*	RAPD, morphology	*Pi*	Chen et al. (2007)
	SSR, cytoplasm type, MAS, BC_1 characterization	Bacterial wilt	Chen et al. (2013, 2016)
	MMR deficiency, SSR, RAPD marker for leptines	CPB (antibiosis and antixenosis)	Molnár et al. (2016)
S. tbr (+) *S. commersonii*	Southern analysis of organelles	*Verticillium* wilt	Kim-Lee et al. (2005)

(continued)

Table 13.2 (continued)

Combination	Tools for characterization and/or selection	Traits of interest—resistance or other traits	References
S. tbr (+) *S. etuberosum*	RAPD, SSR, GISH, cytoplasm type	PVY	Gavrilenko et al. (2003)
	Characterization of BC populations	PRLV	Novy et al. (2007)
	Cytoplasm type, FC, RAPD, SSR	PVY	Tiwari et al. (2010)
S. tbr x S. *berthaultii* (+) *S. etuberosum*	NS	PVX, PVY, PRLV, green peach and potato aphids, CPB, wireworm	Novy et al. (2004, 2006)
S. tbr (+) *S. x michoacanum*	Ploidy, RAPD	*Pi*	Szczerbakowa et al. (2010)
	DaRT	*Pi*	Smyda et al. (2013), Smyda-Dajmund et al. (2016)
S. tbr (+) *S. nigrum*	Morphology, ploidy, RAPD	*Pi*	Szczerbakowa et al. (2003)
S. tbr (+) *S. pinnatisectum*	RAPD, cytological analysis, *Pi* resistance analysis	No resistance	Szczerbakowa et al. (2005)
	RAPD, morphology	*Pi,* CPB	Chen et al. (2007)
	Ploidy, cytoplasm type	*Pi*	Polzerova et al. (2011)
	RAPD, SSR, cytoplasm type, FC	*Pi*	Sarkar et al. (2011), Tiwari et al. (2013)
S. tbr (+) *S. stenotonum*	Isoenzymes, SSR, PEPC/RUBISCO ratio	*Ralstonia solanacearum*	Fock et al. (2007)
S. tbr (+) *S. tarnii*	SSR, AFLP	PVY, *Pi*	Thieme et al. (2008)
S. tbr (+) *S. vernei*	Isozymes, RAPD, I-SSR,	Recombinant plastome	Trabelsi et al. (2005)
S. tbr (+) *S. verrucosum*	RAPD	*Pi*	Greplova (2010)
S. tbr (+) *S. villosum*	RAPD, GISH, ROS	*Pi*	Tarwacka et al. (2013)
Intraspecific somatic hybrids			
S. tbr cvs. Aminca (+) Cardinal Cardinal (+) Nicola	Isoenzymes, SSR, ISSR	PVY, *Pythium aphanidermatum*	Nouri-Ellouz et al. (2006)

NS not specified; *Pi Phytophthora infestans* (late blight); CPB Colorado potato beetle; PRLV potato leaf roll virus; PVX potato virus X; PVY potato virus Y; PEPC phosphoenolpyruvate carboxylase, RUBISCO ribulose-1,5-bisphosphate carboxylase oxygenase, FC flow cytometry, SSR Simple Sequence Repeats (microsatellites), I-SSR Inter SSR, MAS marker assisted selection

S. x michoacanum (Smyda-Dajmund et al. 2016), which demonstrates that all the chromosomes of both species are present in the hybrids but many markers are still missing.

Cytogenetic characterization of potato somatic hybrids depends on indirect and direct methods of assessing the ploidy level. Molecular cytogenetic methods are used to determine the composition of genomes. An example of an indirect method is the flow cytometry determination of DNA, which in combination with the quantity of parental DNA can be used to obtain a good

Table 13.3 Molecular approaches for the selection and characterization of somatic hybrids during 2004–2017 (after Chao and Park 2004, up-dated)

Approaches	References
Isozyme analysis	Trabelsi et al. (2005), Nouri-Ellouz et al. (2006)
Flow cytometry analysis	Cai et al. (2004), Tek et al. (2004), Trabelsi et al. (2005), Bidani et al. (2007), Greplová et al. (2008), Thieme et al. (2008, 2010), Tiwari et al. (2010), Polzerova et al. (2011), Sarkar et al. (2011), Ahn and Park (2013), Yu et al. (2013), Rakosy-Tican et al. (2015)
Indirect cytogenetics tools/number of chloroplasts per guard cell	Sarkar et al. (2011), Denes (2015), Molnár (2017)
Chromosome counting	Tek et al. (2004), Boltowicz et al. (2005), Nouri-Ellouz et al. (2006), Shi et al. (2006), Przetakiewicz et al. (2007), Chen et al. (2008b), Espejo et al. (2008), Szczerbakowa et al. (2010), Ahn and Park (2013), Tarwacka et al. (2013), Yu et al. (2013), Rakosy-Tican et al. (2015)
SSR markers	Cai et al. (2004), Tek et al. (2004), Trabelsi et al. (2005), Nouri-Ellouz et al. (2006), Bidani et al. (2007), Lightbourn and Veilleux (2007), Thieme et al. (2008, 2010), Tiwari et al. (2010), Polzerova et al. (2011), Sarkar et al. (2011), Iovene et al. (2012), Ahn and Park (2013), Chen et al. (2013), Smyda et al. (2013), Yu et al. (2013), Rakosy-Tican et al. (2015), Molnár et al. (2016)
AFLP/MAFLP markers	Tek et al. (2004), Thieme et al. (2010), Ahn and Park (2013)
RAPD markers	Cai et al. (2004), Boltowicz et al. (2005), Rokka et al. (2005), Trabelsi et al. (2005), Shi et al. (2006), Przetakiewicz et al. (2007), Chen et al. (2008b), Espejo et al. (2008), Greplova et al. (2008), Szczerbakowa et al. (2010), Tiwari et al. (2010), Polzerova et al. (2011), Sarkar et al. (2011), Ahn and Park (2013), Smyda et al. (2013), Tarwacka et al. (2013), Molnár et al. (2016)
RFLP markers	Tek et al. (2004), Przetakiewicz et al. (2007)
CAPS/SCAR markers	Nouri-Ellouz et al. (2006), Sarkar et al. (2011), Smyda et al. (2013), Yu et al. (2013)
DNA sequence analysis	Bidani et al. (2007)
Fluorescence in situ hybridization (FISH)	Tek et al. (2004)
Genomic in situ hybridization (GISH)	Tek et al. (2004), Iovene et al. (2012), Tarwacka et al. (2013), Yu et al. (2013), Denes (2015), Molnár (2017)
Diversity array technology (DArT)	Smyda-Dajmund et al. (2016)
Cytoplasmic DNA markers	Smyda-Dajmund et al. (2016)

estimate of the ploidy level of potato somatic hybrids (Thieme et al. 2008, 2010; Rakosy-Tican et al. 2015). Flow cytometry has proved very useful for selecting hexaploid or near hexaploid shoots after the electrofusion of tetraploid potato cultivars with diploid *Solanum* wild species in many combinations (Thieme et al. 2004, 2008, 2010; Rakosy-Tican et al. 2015). An example of an indirect method is the correlation between chloroplast counts in guard cells or the number of guard cells per area of abaxial epidermis, and somatic hybrid ploidy (Sharma et al. 2011). Direct estimation of ploidy relies on chromosome counts in root meristems after staining with DAPI (Rakosy-Tican et al. 2015), or other non-fluorescent stains used in classical cytogenetic studies (aceto-carmine or orceine) (Przetakiewicz et al. 2007). The most widely used, simple and reliable method for the rapid estimation of ploidy is the number of chloroplasts per guard cell. Recently it was shown that counts of chloroplasts in guard cells obtained using a

fluorescence microscope (Molnár 2017) correlate with chromosome counts in root meristems (Molnár 2017, Sharma et al. 2011). Flow cytometry can be used on the first shoots produced by protoplast-derived calluses (Thieme et al. 2008; Rakosy-Tican et al. 2015). This technique is useful for selecting the hexaploid shoots after the fusion of tetraploid potato cultivars with different diploid wild species. Although the selection reduces the number of shoots transferred and maintained in vitro, the ploidy level might change after a long time in in vitro micro-propagation and micro-tuber storage, as is the case of the somatic hybrids between potato tetraploid cultivars and *S. bulbocastanum* (Rakosy-Tican et al. 2015). This genome instability and chromosome loss after long-term culture and repeated back-crosses might make it possible to eliminate the inheritance of non-desired traits from the wild parent (Rakosy-Tican et al. submitted). It is now possible to increase the homeologous recombination by inducing mismatch DNA repair (MMR) deficiency using *AtMSH*2 antisense or a dominant negative gene. The *Agrobacterium*-mediated transfer of these genes into *S. chacoense* (Rakosy-Tican et al. 2004), followed by somatic hybridization through electrofusion, reveals that Colorado potato beetle resistance traits can be introgressed into somatic hybrids (Molnár et al. 2016). There are few studies on the composition of the genome of potato somatic hybrids using in situ hybridization techniques: genome in situ hybridization (GISH), or fluorescence in situ hybridization (FISH). Potato and its related wild species of *Solanum* have very small somatic chromosomes of 1.0–3.5 μm in length (Dong et al. 2000) and show slight differences in their morphology, so classical cytogenetic methods, are not very useful for the genome analysis of potato somatic hybrids (Gavrilenko 2007). Consequently, normal cytogenetic techniques like C banding cannot be used to determine the composition of the genome of somatic hybrids of potato. Genome in situ hybridization (GISH) has been used to distinguish the genomes of the two species in some somatic hybrid combinations, such as potato (+): *S. brevidens* (Dong et al. 1999; Gavrilenko et al. 2002) *S. bulbocastanum* (Iovene et al. 2007; Denes 2015), *S. etuberosum* (Gavrilenko et al. 2003) and *S. nigrum* (Horsman et al. 2001).

GISH was first used to distinguish chromosomes and fragments of chromosomes in potato by Schwarzacher et al. (1989) and its use in analysing the composition of genomes in somatic hybrids depends mainly on genome sequence complementarity and stringency conditions (Gavrilenko 2007). The standard GISH protocol differentiates chromosomes when genome complementarity is 80–85% or less, but more similar genomes are difficult to distinguish, as in the case of the somatic hybrid *Solanum tuberosum* (+) *S. chacoense*, which is partially identified by using multicolour (mc) GISH and high stringency conditions (Molnár 2017). GISH can be successfully used to determine the genome composition of somatic hybrid clones and their descendants (back-crosses), and also to discriminate between intra- and/or inter-genomic pairing in wide hybridizations, in order to study genome interactions such as chromosome specific eliminations and inter-genomic translocations (Gavrilenko 2007 and references).

Fluorescence in situ hybridization (FISH) has also been used for identification and physical gene positional mapping in potato and its somatic hybrids (Gavrilenko 2007). FISH helped to clarify, for instance, the genome composition of the somatic hybrids with *S. brevidens*, by using the clone pST3 that signals only the telomeric regions of *S. brevidens* chromosomes (Rokka et al. 1998). FISH with tandemly repeated species-specific DNA sequences has also been used for comparative karyotyping and studying introgressions in the genome of potato. The use of FISH with genome DNA inserted into large vectors such as bacterial artificial chromosomes (BACs), a technique also called BAC-FISH, has been used successfully to map small sections (only a few kilobases long) of physical chromosomes (Jiang et al. 1995). Subsequently, Jiang and colleagues were able to use RFLP-marker specific BAC clones as FISH probes to identify each potato chromosome in a haploid complement (Dong et al. 2000). This made it possible

using other specific probes and multiple in situ hybridization cycles to identify the chromosomes of a species in hybrids using FISH (Dong et al. 2000). Although there are fewer cytogenetic studies using modern molecular tools on meiotic chromosomes, the development of FISH and more recently the so-called Fiber-FISH has enabled the comparative analysis of single chromosomes (Lou et al. 2010).

There are other genomic techniques that have been less used to investigate potato somatic hybrids, although they have yielded very interesting results in studies of other species of plants (Eeckhaut et al. 2013). Transcriptomic studies using micro- and macro-arrays or RT-qPCR are likely to provide a better understanding of the genetics of somatic cells and the complex interaction between the fused protoplasts of two species. Moreover, next-generation sequencing or high resolution melting analysis are currently the most likely to provide advances in somatic hybrid characterization and practical exploitation in breeding.

Intraspecific and Interspecific Hybridization

The production of somatic hybrids from protoplasts, which circumvents pre- and post-zygotic crossing barriers, can be used to insert resistance to stress into vegetative propagated crops (Lössl et al. 1999) and might be widely accepted by breeders (Hofferbert 1996). It has a greater potential for self-generating biodiversity in numerous nuclear and cytoplasmic genome combinations than sexual hybridization (Kumar and Cocking 1987). It also provides an opportunity for initiating recombination events between parental genomes. Moreover, homeologous recombinations can also be increased by inducing a DNA repair deficiency, for instance, mismatch repair deficiency (MMR, Rakosy-Tican et al. 2004, 2016; Molnár et al. 2016). Potato is a good example of the availability of a great genetic diversity in related wild species, more than 200 of which occur in the area from which potato originated (Bradshaw et al. 2006). This diversity of resistance genes cannot be exploited by crossing the species sexually because of many barriers, including the endosperm balance number (see Rokka 2015). Somatic hybridization can contribute to overcoming these barriers in potato-wide hybridization.

The first intergeneric somatic hybrid was produced between potato and tomato (Melchers et al. 1978), called 'pomato or topato', but the regenerated plants produced fibrous-like tubers and were sterile or set only parthenocarpic fruit. Although from a practical point of view these hybrids are a great disappointment, they indicate that although complex somatic incompatibility prevents the somatic hybridization of distantly related species, it might be more successful in hybridizing more closely related species. Moreover, many subsequent studies on inter-generic hybrids provide a better understanding of somatic cell genetics and cytoplasmic inheritance in somatic hybrids (Guri et al. 1991). The next somatic hybrid of potato was *S. chacoense* Bitt. (+) *S. tuberosum* (Butenko and Kuchko 1979) and *S. nigrum* L. (+) *S. tuberosum* (Binding et al. 1982). Potato breeders were more interested in both of these hybrids because of their resistance to diseases and the possibility of using them to produce breeding clones. Since the 1980s, different wild *Solanum* species have been hybridized with potato using protoplast fusion, and many of them express various traits, including resistance to viruses (Thach et al. 1993; Pehu et al. 1990), bacteria (Austin et al. 1988), fungi (Mattheij et al. 1992) or insect pests (Cooper-Bland et al. 1994; Molnár et al. 2016). Recent data are presented in Table 13.2. A previous review presented an extensive list of potato somatic hybrids (Orczyk et al. 2003), but after 14 years this information needs to be up-dated. In Table 13.2 there are many examples of the transfer of resistance traits and multiple resistance genes conferring resistance to the most important potato pathogens and pests, like late blight caused by *Phytophthora infestans* (*Pi*), viruses (PVY, PVX, PRLV, etc.) or the most voracious pest of potato, Colorado potato beetle (CPB). Furthermore, multiple resistance can be transferred from wild relatives into the potato gene pool (Thieme et al. 2010) and even more somatic hybrids of species can be produced, as in

the case of the tri-species somatic hybrids (Novy et al. 2006). Pathogens and pests are considered to be responsible for at least a 22% loss of yield in potato worldwide (Aversano et al. 2007). Indeed, some potato pathogens and pests can completely destroy the plants, especially the voracious and adaptable CPB, which is notorious for its resistance to almost all of the pesticides currently used (approximately 53 insecticides based on different active components, Alyokhin et al. 2008). One of the first very successful examples of how somatic hybridization might be used for potato improvement and in studies of somatic cell genetics are the somatic hybrids between the incompatible species *S. bulbocastanum* and cultivated tetraploid potato (Helgeson et al. 1998), which were first assayed for late blight resistance caused by *Phytophthora infestans* in the laboratory and then in a field under intense disease pressure. These somatic hybrids were back-crossed with potato cultivars and shown to carry durable resistance to this disease. Subsequently, a gene involved in durable resistance, was characterized, isolated, sequenced and located on chromosome VIII (Song et al. 2003). Transgenic plants with this gene, first known as RB, were regenerated after *Agrobacterium*–mediated transfer and durable resistance was maintained in transgenic plants (Lozoya-Saldana et al. 2005). Since these first results with this wild species that demonstrate its value as a resource of durable resistance genes against late blight, there has been an increasing interest in transferring these resistance traits to cultivated potato (Naess et al. 2001; Iovene et al. 2007). RB gene was the first durable resistance gene described for late blight but soon many other genes were discovered both in *Solanum bulbocastanum* and other wild species. In *S. bulbocastanum* to date there are four characterized resistance genes: *Rpi-blb*1 (formerly RB), *Rpi-blb*2, *Rpi-blb*3 and *Rpi-bt*1 (van der Vossen et al. 2003; Song et al. 2003; Oosumi et al. 2009; Lokossou et al. 2009; Orbegozo et al. 2016). In addition, late blight resistance from other sources was also accessed by means of interspecific somatic hybrids with the wild species *S. pinnatisectum* (Sarkar et al. 2011), *S. tarnii* (Thieme et al. 2008), *S. cardiophyllum* (Thieme et al. 2010) and more recently *S. x microachanum*, a wild diploid derived from a spontaneous cross between *S. bulbocastanum* and *S. pinnatisectum* (Smyda et al. 2013). All these new somatic hybrids were tested in the field and shown to be resistant after two or three years of assessement, hence they are suitable for breeding. Somatic hybrid lines originating from fusion between potato and *S. bertaultii* are more tolerant of salt stress (Bidani et al. 2007). As a source of resistance to bacterial wilt caused by *Ralstonia solanacearum*, another wild species, *S. stenotomum,* was used (Fock et al. 2001). All the somatic hybrids tested were as resistant as the wild species (Fock et al. 2001). Similarly, *S. chacoense* was explored for molecular markers associated with bacterial wilt resistance, and for introgressing resistance into the potato gene pool (Chen et al. 2013) (see Table 13.2). A very successful approach involving the transgenesic induction of MMR deficiency in a high leptine-producing accession of *S. chacoense*, followed by somatic hybridization, generated many plants exhibiting both antixenosis and antibiosis against Colorado potato beetle (Molnár et al. 2016).

In any scheme of introgressive hybridization, restoration of agronomically acceptable cultivars often requires one or more back-crosses of the somatic hybrid with cultivars, along with selection for a trait of interest and against undesirable traits and inappropriate 'wild' to 'cultivar' genome or gene interactions (Thieme et al. 2008, 2010). With increasing restrictions on the use of pesticides to control potato diseases and pests, deployment of resistance genes from wild species will likely assume greater importance in the future. While it is clear that resistance genes can be introgressed from wild species into potato by somatic hybridization, the processes of introgression and related mechanisms and their interactions are not completely understood (Rieseberg and Wendel 1993). Studies on hybridization followed by gene introgression indicate that these processes may have played a significant role in the evolution of many plant taxa (Heiser 1973). Moreover, as suggested by

other authors, there is currently an increase in the interest for genomic and functional genomic analysis of the somatic hybrids of different crop plants (Eeckhaut et al. 2013), analyses that have yet not been used in studies on potato.

Starting in the 1990s, somatic hybridization was used to study different dihaploid lines of potato generated by sexual crossing with *S. phureja* (Rokka 2009) or pollen and anther in vitro culture. The results of the protoplast fusion of two dihaploid potato lines were at first not very promising, but the restoration of tetraploids from two dihaploid lines with valuable yield and resistance traits soon proved to be a valuable approach to potato breeding (Table 13.2). Resistance to nematodes, viruses (PVY) and *Phytium* bacterial diseases was combined by intra-specific protoplast fusion (Cooper-Bland et al. 1994; Nouri-Ellouz et al. 2006).

Symmetric and Asymmetric Somatic Hybrids: Basic and Practical Achievements

Fusion of two different species results in symmetric hybrids with the combined genomes from both species. Incorporation of the genomes of both parents, especially their nuclear genomes, in a hybrid has two obvious disadvantages: (1) transfer of too much exotic, wild species, genetic material along with the gene(s) of the desirable trait; and (2) genetic imbalance leading to somatic incompatibility. These limitations result either in abnormal growth and development of the somatic hybrids, or regeneration of infertile plants. In the case of potato there are many reports of symmetric somatic interspecific somatic hybridization between diploid wild species and potato dihaploid lines (Rokka 2015). Although genetically more stable, many of these hybrids are infertile and hence it is not possible to introgress resistance genes from a wild parent. For this reason symmetric somatic hybridization between tetraploid potato cultivars and diploid wild species became more popular (Helgeson and Haberlach 1999). Many such $4x$ (+) $2x$ somatic hybrids, in addition to being hexaploid, were also aneuploid or mixoploid (Rakosy-Tican et al. 2015). Genetically, such hybrids may be unstable and eliminate wild species chromosomes during the next stages of tissue culture, as occurs in potato and *S. bulbocastanum* hybrids. But, after two back-crosses with cultivated potato, many of them re-stabilize at a tetraploid level (Rakosy-Tican et al. 2015; under publication). Theoretically hexaploid or near hexaploid somatic hybrids of potato will tend to eliminate, after two back-crosses with potato tetraploid cultivars, wild species chromosomes and maintain very few alien chromosomes or introgress some genes from the wild parent (Fig. 13.2). Chromosome elimination in some interspecific somatic hybrids of potato largely depends on the phylogenetic relationship, type of genome: A, B, C, D and P (Gavrilenko 2007), cell cycle synchronization after fusion and two species chromosome interaction during mitosis, to name but a few of the mechanisms responsible for the instability of the fusion products (Orczyk et al. 2003). The elimination of chromosomes by somatic hybrids of many crop plants has stimulated interest in directing and possibly controlling this process. Therefore, efforts were made to reduce the proportion of the wild relative's nuclear genome in the hybrid.

Asymmetric fusion allows the transfer of part of the nuclear genome of one species into another. Somatic asymmetric hybrids can result after symmetric fusion or can be induced by fragmenting the donor species DNA by using the donor-recipient method (Lakshmanan et al. 2013). In most protocols (Fig. 13.2), both donor and recipient species are treated to reduce a genome's participation in the fusion product, but it is also possible to treat the donor protoplasts in order to direct their elimination of the genome (Grosser and Gmitter 2011). Usually, the donor protoplasts are treated with sub-lethal doses of ionizing irradiation, such as gamma or X rays (Dudits et al. 1987; Oberwalder et al. 1998) or UV irradiation (Hall et al. 1992a), in order to induce double-stranded breaks and hence partial genome elimination (Gleba et al. 1988). It was initially thought that there is a direct correlation between irradiation dose and the amount of DNA fragmentation and elimination, but this is only the case for up to approximately 65% of nuclear

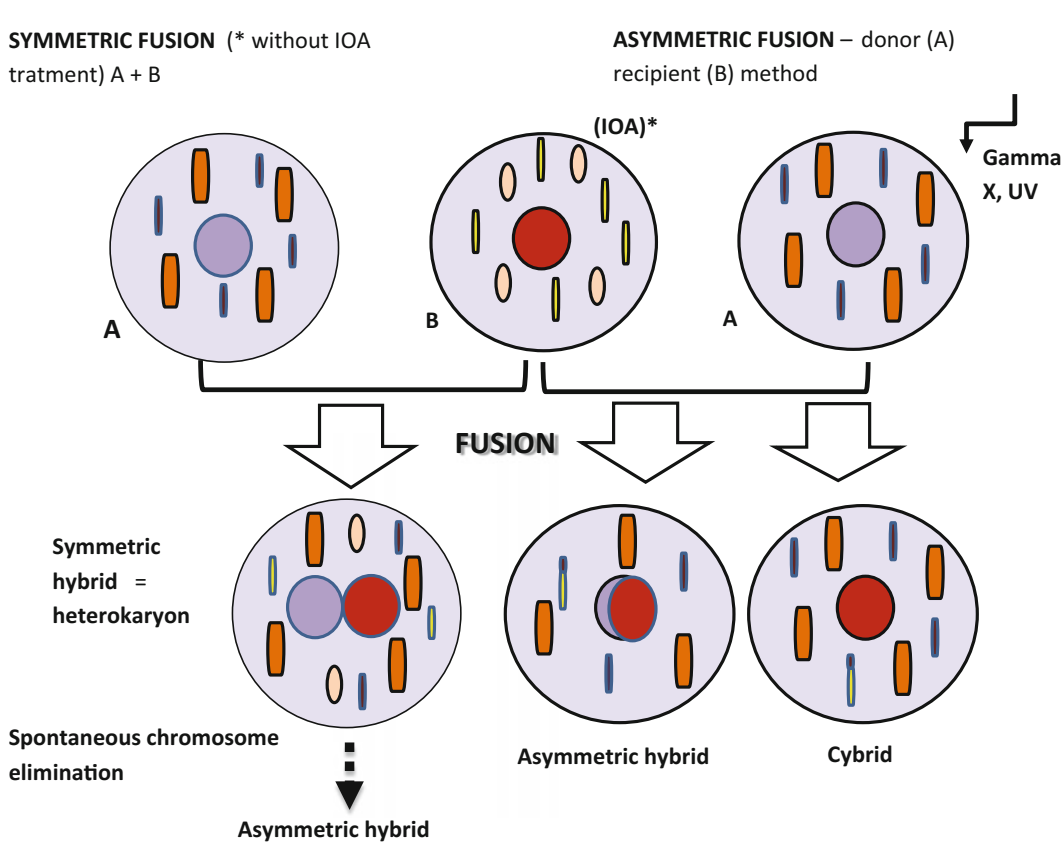

Fig. 13.2 Comparision between symmetric and asymmetric protoplast fusion and resultant hybrid cells from the perspective of nuclear and cytoplasmic genome fate

DNA elimination (Hall et al. 1992a, b). Further increase in irradiation dose did not increase the sorting out of donor DNA. In addition to irradiation, chemical agents can be used to induce chromosome elimination, such as restriction endonucleases, spindle toxin or chromosome condensation agents (Ramulu et al. 1994). Using these methods, asymmetric potato hybrids with some wild *Solanum* species (Valkonen et al. 1994) and intergeneric somatic hybrids can be produced (Wolters et al. 1993; Ali et al. 2000). When the genome of the recipient species, potato, is eliminated, this treatment targets the cytoplasmic genome and iodoacetic acid (IA), iodoacetamide (IOA) and actinomycin D can be used (Liu et al. 2005). If both treatments are used, cybrids will be regenerated. Potato cybrids produced by using the donor-recipient method have a nuclear genetic constitution from one parent in combination with cytoplasmic genomes of the other parent (Perl et al. 1990). Cybrid plants are used to produce new genetic diversity and understanding the interrelations between nuclear genes and cytoplasmic DNA and for the transfer of cytoplasmic inherited traits such as male sterility (Melchers et al. 1992; Liu et al. 2005).

Characterization of Cytoplasmic DNA

In comparison to other techniques of chromosomal and gene engineering, somatic hybridization is unique in its potential to simultaneously transfer both nuclear and cytoplasmic genes. Therefore, it is relevant to analyse the new genetic configuration of hybrid DNA in order to confirm not only the hybrid status, but also to follow the segregation of organelles after merging the protoplasts of two species. In potato interspecific somatic hybrids, the fate of

organelles after fusion is assessed by using different molecular markers of chloroplast and mitochondrial DNA (Lössl et al. 1999). As a general rule, the organelles in somatic hybrids segregate independently, chloroplasts sorting out but the mitochondria of both parents often combining (Sheahan et al. 2005). Such common features are frequently reported, and also occur in potato somatic hybrids (Lössl et al. 1994; Iovene et al. 2007). There are a few exceptions to the general interaction and segregation of organelles, for example, in somatic hybrids between cultivated potato and a wild species of potato, *Solanum verneï,* where recombination between the chloroplast genomes of both parents occurs (Trabelsi et al. 2005). Similarly, in somatic hybrids between potato and *S. berthaultii*, both co-existence and recombination of chloroplast DNA occur (Bidani et al. 2007). Co-existence of mitochondrial DNA is also recorded (Sarkar et al. 2011). Scotti et al. (2007) identified a molecular mitochondrial region, *rpl*5-*rps*14, as a hotspot for mitochondrial DNA rearrangements in potato somatic hybrids. Moreover, in the somatic hybrids between five potato tetraploid cultivars and one cloned accession of *S. bulbocastanum*, in addition to the elimination of the wild species chromosomes depending on recipient cultivar, the type of chloroplast DNA in the two parents plays an important role in the regeneration capacity and genetic stability of the resulting somatic hybrids (Rakosy-Tican et al. 2015). Haplotype w of chloroplasts in potato cvs. Delikat and Rasant, as in *S. bulbocastanum,* increases the incidence of plant regeneration in these fusion combinations (Rakosy-Tican et al. 2015). Reduction in the survival of somatic hybrids when nucleo-cytoplasmatic incompatibility is present is also reported for other fusion combinations (Leon et al. 1998; Orczyk et al. 2003). In future, more detailed studies on several fusion combinations and their contribution to nuclear and cytoplasmic DNA should shed some more light on the complex mechanisms involved in the six different genome interactions after two protoplast fusions. Different haplotypes of chloroplast (ct), mitochondrial (mt) and nuclear (n) DNA, analyzed using RFLP and/or SSR markers are extensively used in phylogenetic and co-evolutionary studies on cultivated potato accessions and their wild relatives (Hosaka 2002; Hosaka and Sanetomo 2009). A 241 bp deletion in ctDNA as well as a shorter deletion of 41 bp (Ames et al. 2007), indicate that some populations of the diploid *S. tarijense* are the maternal parent of cultivated potato. In addition, phylogenetic studies reveal the co-evolution of chloroplasts and mitochondrial genomes and that the correlation between nDNA and ctDNA is even closer. Recently Sanetomo and Gebhardt (2015) analyzed different types of cytoplasmic DNA in European potatoes and correlated them with some agronomic traits such as tuber starch content and late blight resistance. Such basic studies are a good starting point for breeding better potatoes both by classical and biotechnological means.

Future Application in Potato Breeding

After the intensive efforts during the last century to further increase the yield of potato cultivars failed (Douches et al. 1996), the main objectives of the potato breeding switched to improving processing attributes and resistance to diseases and pests, while maintaining or even improving such traits as tuber colour, shape, quality and/or yield. Over the past fifty years these objectives have mainly been achieved by using wild species of *Solanum* as resources of resistance and other new traits via classical breeding. The number of wild species that could be integrated into potato breeding was and is quite limited because of sexual incompatibility, although there are techniques other than sexual crosses, such as manipulations of ploidy levels (Jansky 2009), breeding 2*n* gametes or using bridging species to integrate genes from 25 wild *Solanum* species into modern cultivars (Ross 1986). The main source of resistance genes is still *S. demissum*, with more than half of the modern cultivars with introgressions from this species (Ross 1986). The main limitations to the classical breeding of potato are tetraploidy and heterozygosity, which make breeding very complex (Muthoni et al. 2015). Millions of progeny have to be screened to detect one line with the potential for a new cultivar and this may

take more than 11 years (Plaisted et al. 1984; Barrell et al. 2013). Moreover, when genes from an incompatible wild species have to be exploited, as is the case of *S. bulbocastanum,* which is a source of genes for durable resistance to late blight, the use of a bridging species to produce new cultivars took 49 years and then only one gene was integrated into the potato gene pool, i.e. *Rpi-blb*2, producing two new cvs. Bionica and Toluca (Haverkort et al. 2009).

Over the last six decades plant biotechnology has contributed many new less time-consuming opportunities for potato improvement and has provided valuable solutions to conventional breeding difficulties (Barrell et al. 2013; Luthra et al. 2016).

Somatic hybridization has also resulted in the production of many resistant somatic hybrids, integrating multiple genes and traits or even multiple species hybrids as detailed in this section. The limitation of somatic hybridization is that it can result in the production of somatic hybrids that are resistant but sometimes have misshapen tubers or the initial somatic hybrids have poor fertility. Solutions to these disadvantages have been proposed, such as haploidization and the use of intra-specific hybridization of dihaploid potato lines (Rokka 2009), or the use of somatic fusion in which tetraploid potato cultivars are fused with sexually incompatible diploid wild species. The resulting hexaploids are often fertile and crossable with other tetraploid cultivars (Thieme et al. 2008, 2010; Rakosy-Tican et al. 2015).

A new concept for exploiting all new and old technologies to improve potato in a concerted way is combinatorial biotechnology (Rakosy-Tican 2012) and schemes for its application are proposed (Rakosy-Tican et al. 2016). A general scheme for the application of combinatorial biotechnology to improve potato is presented in Fig. 13.3. The main goal of such schemes is to transfer several genes and traits from wild relatives of potato into potato cultivars by first using the somatic hybridization of the wild donor with potato tetraploid cultivars and then integrating other in vitro techniques like transgenesis, embryo rescue, in vitro or marker-assisted selection, etc. and different analytical, biochemical, biophysical and genomic and phenome analyses. There is scope in the future for improving such schemes by using new omic approaches and genomic technologies like next generation sequencing, micro and macro-arrays or directed mutagenesis (Eeckhaut et al. 2013).

A successful use of somatic hybridization in potato breeding is the release of a new cultivar 'Jeseo' which was produced in Korea (Jeju

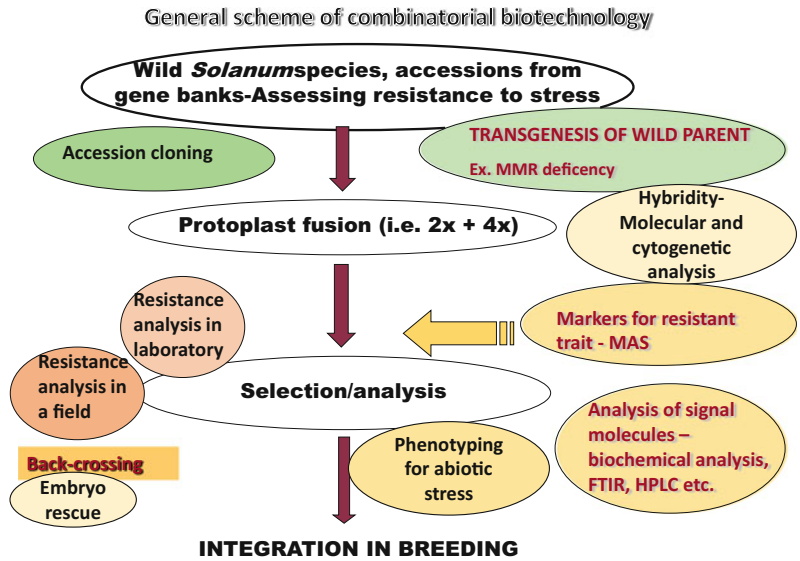

Fig. 13.3 Schematic representation of a general combinatorial biotechnology approach involving somatic hybridization and additional in vitro techniques as well as analytic biochemistry methods and phenotyping aiming to transfer resistance traits into potato crop

Special Self-governing Province Agricultural Research & Extension Services). This cultivar was obtained after two back-crosses of a somatic hybrid clone with cv. Dejima. The new cultivar is highly resistant to potato common scab (*Streptomyces scabies*, *S. turgidiscabie* and *S. acidiscabie*), soft rot and potato leaf roll virus (PLRV). However, it is susceptible to potato virus Y (PVY) and late blight (*Phytophthora infestans*). The tubers of this cultivar are round, with shallow eyes, yellow skin and a short dormant period and the yield, although lower than that of the cultivated parent, reaches 38.8 t/ha (Kim et al. 2013).

13.3.4 Transfer of Genes into Crop Potatoes

The potato was one of the first crops transformed successfully using the *Agrobacterium*-mediated transformation of many potato cultivars (An et al. 1986; Sheerman and Bevan 1988; Stiekema et al. 1988). There are many examples of attempts to transfer and integrate economically important genes into crop potatoes and some of the previous reviews have presented the state of the art for this tuberous crop (Kumar 1995; Solomon-Blackburn and Barker 2001; Christou et al. 2006; Mullins et al. 2006; Millam 2007; Rakosy-Tican 2013). *Agrobacterium tumefaciens*-mediated transformation works well with many cultivars of potato and a few wild species of the genus *Solanum* (Rakosy-Tican et al. 2004, 2007). The efficiency of this method of transferring genes varies depending on the genotype, with cv. Désirée the model variety (Stiekema et al. 1988; Sheerman and Bevan 1988; Rakosy-Tican et al. 2007). Transformation efficiency was improved by using particular marker genes, the most frequently used being the *npt*II gene (bacterial neomycin phosphotransferase gene). Later on reporter genes were also transferred into the potato. The most commonly used reporter gene is *gus* (glucuronidase gene), but in the last few years green fluorescent protein (*gfp*) was also frequently used to transform different species of plants including potato and some of its wild relatives (Rakosy-Tican et al. 2007; Rakosy-Tican 2013). Both *gfp* and *npt*II combined in a binary vector to improve the transgenesis of potato cultivars and dihaploid lines as it makes it easier to identify chimeras and escapes, which are quite common when the selection is only based on the use of the resistance to antibiotics, such as kanamycin (Rakosy-Tican et al. 2007). This strategy enabled us to achieve a high efficiency in *Agrobacterium*-mediated gene transfer into potato cultivars and one dihaploid line. These cultivars were then used to transform a marker-free hairpin construct containing two antisense coat protein (CP) genes separated by an intron and then generate hairpin structures and posttranscriptional gene silencing, which resulted in cultivars resistant to PVY (Rakosy-Tican et al. 2010).

Worldwide, transgenic plants with a number of different traits are being developed: (1) resistance to herbicides; (2) pollination control mechanisms —CMS (cytoplasmic male sterility); (3) insect resistance (genes from bacteria and plants); (4) virus resistance, including reverse genetics; (5) resistance to fungi (antifungal proteins or R genes); (6) nutritional improvement—Golden potato; (7) senescence retardation; (8) tolerance of abiotic stresses; and (9) production of valuable pharmaceuticals and secondary metabolites (use of plants as bioreactors). The application of gene transfer and the results obtained using crop plants were recently reviewed by Davey et al. (2010) and Rashid and Lateef (2016) and for only potato by Rakosy-Tican (2013), and in this section, the results obtained in the last few years are highlighted and presented in Tables 13.4 and 13.5. The disadvantages of transgenesis are the constraints on transferring genes between species, the possibility that only a limited number of cloned genes can be transferred, and the concern of consumers over their introduction as human food have all increased the interest in developing new strategies like cisgenesis and transfer of genes between the plants of the same species (see Jacobsen and Schouten 2008; Haverkort et al. 2008). Unfortunately, scientists were not able to convince the European Commission on the non-GMO status of plants generated by

transferring genes from the same species or a related inter-crossable species of plants (http://www.efsa.europa.eu/en/efsajournal/pub/2561.htm). In the frame of the DuRPh Project in the Netherlands, Zhu et al. (2012) stacked three late blight-resistance genes: *Rpi-sto1* (*S. stoloniferum*) homologue of *Rpi-blb1*, *Rpi-vnt1.1* (*S. venturii*) and *Rpi-blb3* (*S. bulbocastanum*), and put them into a single binary vector pBINPLUS. The susceptible cv. Désirée was transformed and that the stacked genes functioned was revealed by using a detached leaf assay (DLA) and field assays over a period of two years (Zhu et al. 2012; Haesaert et al. 2015). Thus cisgenesis might prove very useful if exempted from GMO rules in Europe. Such a strategy could be used to stack dominant genes in a variety that improves its resistance to late blight and other diseases. For all quantitative traits, which depend on multiple genes, somatic hybridization and combinatorial biotechnology may be a better way of improving potato.

13.4 New Breeding Technologies Used for Improving Potato

In recent years new biotechnological techniques have been adopted for plant breeding which make use of RNAi (RNA interference) or miRNA (micro RNA) and which allow for precise gene editing via directed mutagenesis. In potato, hundreds of miRNAs have been identified (Zhang et al. 2013; Kim et al. 2011). Methods like targeting induced local lesions in genomes (TILLING), mega nucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the bacterial clustered regularly interspaced short palindromic repeats associated with protein 9 nuclease (CRISPR-Cas9) have lately been applied to different crops. These technologies achieve specific and precise silencing or knockout of a given gene or its activation and carry a huge potential for understanding gene function and regulatory processes in different organisms including plants. Precise genome engineering like TALENs or CRISPR-Cas9 makes use of isolated protoplasts and bacterial systems to induce directed mutagenesis. Compared to earlier technologies like ZFNs or TALENs, CRISPR-Cas9 proves to be easier and more efficient and hence has been widely used in recent years (Gaj et al. 2013). The Cas9 endonuclease is driven by a 20-base pair (bp) sequence at the end of the single-guide RNA (sgRNA), which acts as a guide to a specific site of the genome. Once the genome is targeted, the nuclease Cas9 is able to cleave double-stranded DNA, leading to deletion, substitution or insertion at the target site (Sander and Joung 2014). Genome editing tools provide a potential alternative to traditional *Agrobacterium*-mediated introduction of a gene of interest (Halterman et al. 2016).

Since 2013, CRISPR/Cas9 has been applied either in transient expression and/or stable transgenesis in several plant species, such as *Arabidopsis thaliana* and *Nicotiana benthamiana*, as well as in several crops like rice, wheat, maize, and tomato (Brooks et al. 2014; Jiang et al. 2013; Li et al. 2013; Miao et al. 2013; Nekrasov et al. 2013; Shan et al. 2013). It has been also shown that mutations generated in the primary transgenic plants by the CRISPR/Cas9 system can be stably transmitted to the next generation (Brooks et al. 2014; Feng et al. 2014). Thus, the CRISPR/Cas9 system is becoming a powerful tool for genome editing in plants, whereas the reports of the usage and efficiency of the CRISPR/Cas9 system-mediated plant genome engineering are still limited.

In potato, reverse genetics was applied to induce virus resistance by transgenesis (Missiou et al. 2004), also in combination with marker-free gene transfer (Bukovinszki et al. 2007; Rakosy-Tican et al. 2010). Elias et al. (2009) showed the utility of enzymatic mismatch cleavage for TILLING and ECOTILLING in three varieties of potato. The three mutant cultivars exhibit salinity tolerance after treatments with gamma irradiation. This method allowed a rapid germplasm characterization. For identification of novel starch variants in potato dihaploid, seeds were treated with ethylmethanesulphonate (EMS) for 16 h. By using a granule-bound starch synthase I gene

Table 13.4 Transgenesis and cisgenesis used to improve potato tubers or their use as bioreactors: a synthesis of the more recent results

Goal	Specific trait	Genes transferred	Results	References
Tuber quality	Glycoalkaloid content Reduction of α-solanine/increase α-chaconine	*Sgt1*	Reduced tuber toxicity	McCue et al. (2005)
		GmSTM1	Study of sterol biosynthesis	Arnqvist et al. (2003)
	Protein content	Amaranth albumin gene-*AmA1*	Increase in protein and essential amino acids	Chakraborty et al. (2000)
	Cysteine and glutathione content	SAT-coding *cysE* gene	Increase in essential amino acids-healthier tubers	Stiller et al. (2007)
	Increase in 14-3-3 isoforms	Proteins 14-3-3; *CHS, CHI, DFR*	Increase in antioxidants	Łukaszewicz et al. (2002, 2004)
	Increase in flavonols and anthocyanins	*DFR, UGT*	Better tuber content with same yield and starch content	Aksamit-Stachurska et al. (2008)
	Carotene and lutein—Golden potato	*crtB, crtl, crty*	Golden potato rich in A vitamin	Diretto et al. (2007, 2010)
	Biofortification in vitamin E	*At-HPPD*, *At-HPT*	Increase in vitamin E	Crowell et al. (2008)
	Waxy starch	Not the case	Low amylose	McPherson and Jane (1999)
	High amylopectin starch	*GBSS*	Change in starch composition	Visser et al. (1991)
	Biofortification in inulin	*1-FFT, 1-SST*	Inulin synthesis—healthier tubers	Hellwege et al. (2000)
	Tuber storage qualities	LbPFK with tuber-specific promoter	Reduction in low temperature sweetening	Navrátil et al. (2007)
		RNAi	Reduction in low temperature sweetening	Chen et al. (2008a)
	Processing low acrylamide[a]	Two asparagine synthetase genes	Reduced acryl-amide in chips and French fries	Rommens et al. (2008)
Tuber yield	Tuber number	*OsSUT5Z* and *OsSUT2M*	Increase in yield	Sun et al. (2011)
	Tuber development	*AtPAP2*	Carbon metabolism and yield	Zhang et al. (2014)

(continued)

Table 13.4 (continued)

Goal	Specific trait	Genes transferred	Results	References
Potato plants as bio-reactor	Surface antigen for hepatitis B	*HBsAg*	Vaccines	Guan et al. (2010), Thanavala and Lugade (2010a, b)
	Producing salmon interferon	Interferon gene	Interferon biosynthesis	Fukuzawa et al. (2010)
	Vaccines	Rotavirus CP VP6	Trials on mice	Yu and Langridge (2003)
		Papilloma virus genes	Vaccines for papilloma virus	Biemelt et al. (2003)
	Human serum albumin	*HSA*	Human serum production	Farran et al. (2002)
	Human tumour necrosis factor	TNF-α	Cancer therapy	Ohya et al. (2002)
	Antibodies	IgGs and Fab fragments of genes	Production of antibodies	DeWilde et al. (2002)
	Production of diagnostic reagent	SimpliREDTM	HIV diagnosis	Schunmann et al. (2002)
	Staphylokinase overexpression	*SAK*	Plasminogen activator—treatment of poor blood circulation	Gerszberg et al. (2012)

[a]All-native DNA transformation

(*waxy*), a series of point mutations were identified that affect gene expression for enzyme function. It was possible to establish elite breeding lineages lacking granule-bound starch synthase (GBSS) I protein activity and producing high amylopectin-starch (Muth et al. 2008).

TALENs was used to improve cold storage and processing traits in potato (Clasen et al. 2015). The CRISPR/Cas9 system was established in potato recently (Wang et al. 2015). Altered starch quality with full knockout of GBSS gene function in potato was achieved using CRISPR-Cas9 through transient transfection and regeneration from isolated protoplasts (Andersson et al. 2017). The authors have demonstrated that this system is an effective tool in potato, and can promote functional studies of hitherto uncharacterized genes.

Aside from these novel technologies, some other aspects and approaches have to be taken into consideration if the breeding system of potato is to be improved (Jansky et al. 2016):

- management of the nearly 100 crop wild relatives mostly sexually compatible with cultivated potato at diploid level;
- production of inbred lines by selfing for systematically combining genes or alleles of interest, as well as for exploiting heterosis;
- production of near-isogenic or other introgression lines;
- hybrid production supported by a cytoplasmic male sterility system;
- successful TPS (true potato seed)-based cultivars with improved heterosis, uniformity, cytoplasm male sterility, combining ability, disease resistance, or seedling vigour;
- stacking of new genes into well-established inbred lines;
- cybrid production by protoplast fusion between male sterile cytoplasic sources and male fertile cultivars to change male fertile potato cultivars into male sterile ones without altering the nuclear genome as a step in developing TPS parents (Perl et al. 1990);

Table 13.5 Examples of transgenesis and cisgenesis results in improving biotic and abiotic stress in potato during 2003–2017

Goal	Trait	Genes used	Results	References
Biotic stress resistance	Insect resistance	*cry1Ac9*	Resistance to tuber moth	Davidson et al. (2004)
		Hybrid Bt endotoxin	Resistance to both coleopteran and lepidopteran pests	Naimov et al. (2003)
		Cysteine *Pls*	Resistance to Western flower thrips	Outchkourov et al. (2004)
	Resistance to bacteria	5-*UGT*	Tuber yield, starch and anthocyanin increase, resistance to *Erwinia carotovora*	Lorenc-Kukuła et al. (2005)
		*ScSN*1	Resistance to *Erwinia carotovora* and *Rhizoctonia solani*	Almasia et al. (2008)
	Resistance to late blight	*Rpi-vnt1.1*	Increased yield and *Pi* resistance in field trials	Jones et al. (2014)
		*Rpi-vnt*1.1 and *Rpi-sto1*	Cisgenic marker-free *Pi* resistant cvs.	Jo et al. (2014)
		RB (*Rpi-blb1*)	Tolerance to *Pi* and gene stability	Listanto et al. (2015)
		Rpi-vnt1.1, *Rpi-sto1*, *Rpi-blb3*	Stacking three cisgenes—durable resistance *Pi*	Zhu et al. (2012)
	Resistance to diseases	*MsrA2*	Broad-spectrum fungal and bacterial resistance	Osusky et al. (2005)
		MsrA3 with tissue-specific promoter	Mitigates biotic and abiotic responses	Goyal et al. (2013)
	Nematode resistance	Peptide-disrupting chemoreception of nematodes	*Globodera pallida* resistance—no side effects on non-targets	Green et al. (2012)
	Virus resistances	dsRNA PVY coat protein (CP)	RNAi-induced resistance to PVY	Missiou et al. (2004)
		shRNA with *ipt* gene	Resistance to PVY^{NTN} in a marker-free system	Bukovinszki et al. (2007)
		CP gene	Resistance to PVY in the field	Dusi et al. (2009)
		shRNA with I	Resistance to PVY^{NTN} in a marker-free system	Rakosy-Tican et al. (2010)
		shRNA	Resistance to PVY	Tabassum et al. (2016)

(continued)

Table 13.5 (continued)

Goal	Trait	Genes used	Results	References
Abiotic stress tolerance	Heat tolerance	*CaPF*1	Tolerance to high temperature	Youm et al. (2008)
		*AtCBF*3	Heat tolerance	Dou et al. (2015)
	Freezing tolerance	*Atrd*29*A::DREB1A*	Tolerance to freezing	Behnam et al. (2007)
	Drought tolerance	*ScTPS*	Studies on water content and photosynthesis	Stiller et al. (2008)
		ggpPS	Increased glucosyl-glycerol in tubers/drought, salt tolerance	Sievers et al. (2013)
		PaSOD	Increased photosynthesis under drought	Pal et al. (2013)
		TPS1	Trehalose increase and tolerance to drought	Kondrak et al. (2012)
	Two stress factors	*BADH*	Drought and salt tolerance	Zhang et al. (2011)
		*StEREBP*1	Cold and salt tolerance	Lee et al. 2007
		SOD, APX	Tolerance to oxidative stress and high temperature	Tang et al. (2006)
		*At DREB*1*B*	Drought and freezing tolerance	Movahedi et al. (2012)
		*StDREB*1 or *StDREB*2	Salt or drought tolerance	Bouaziz et al. (2012, 2013)
		GB	Salt and cold tolerance	Ahmad et al. (2014)
	Multiple stresses	*StnsLTP*1	Multiple tolerance to heat, salt and drought	Gangadhar et al. (2016)
		CodA/chloroplast	Tolerance to oxidative, salt, and drought stresses	Ahmad et al. (2008)
		SOD, APX, CodA/ chloroplast	Tolerance to oxidative, salt, and drought stresses	Ahmad et al. (2010)

At DREB1B dehydration response element B 1B; *CodA* choline oxidase; GB glycinebetaine; *BADH* betaine aldehyde dehydrogenase; *MsrA*2 gene for frog antimicrobial peptide; *SOD* superoxide dismutase; *APX* ascorbate peroxidase; *Pi Phytophthora infestans*; Pls protease inhibitors; *ScTPS Saccharomyces cerevisiae* trehalose-6-phosphate synthase gene; *ScSN*1 *Snakin*-1, a cysteine-rich peptide from *Solanum chacoense*; *StEREBP*1 *Solanum tuberosum* ethylene responsive element binding protein 1; *TPS*1 yeast trehalose-6-phosphate synthase 1; 5-*UGT* anthocyanin 5-*O*-Glucosyltransferase

- mapping and sequencing male-fertility genes in diploids, using CRISPR-Cas9 to create male sterile plants for use as female parents in hybrid production (Belhaj et al. 2015);
- the dominant self-incompatibility inhibitor (*Sli*) gene, identified in the sexually compatible wild species *S. chacoense* should be used to produce inbred lines (Hosaka and Hanneman 1998);
- use of back-cross breeding to introgress small chromosome regions from wild species into a cultivated background.

When comparing the main classical techniques for potato improvement with the modern ones based on biotechnology and genome editing (Fig. 13.4), one has to weigh up the advantages and drawbacks in applying them in practice.

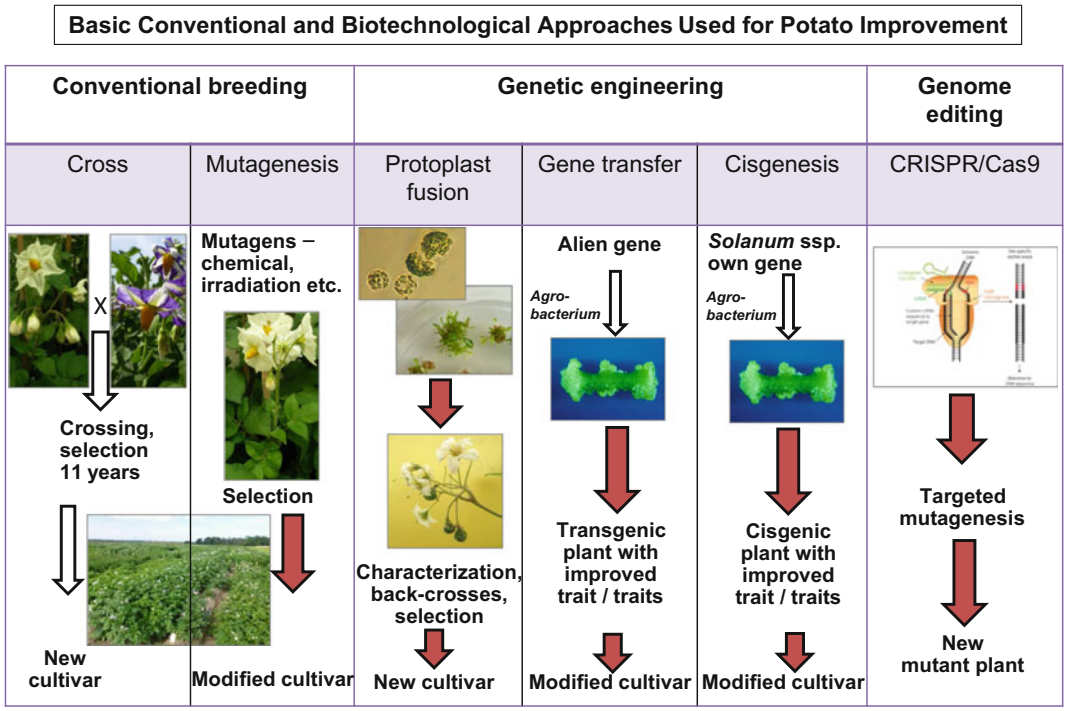

Fig. 13.4 Valuable conventional and biotechnological techniques for potato improvement

Classical breeding still is a time-consuming process, which involves many years of selecting a huge number of clones. Classical mutagenesis is based on chemical or physical treatment acting randomly and at multiple sites in the genome. Biotechnological tools were developed to bypass these drawbacks. In this chapter we tried to show the advantages and state of the art in using in vitro techniques in potato improvement and somatic cell genetic studies. The main approaches to increase genetic variability and select improved varieties as for productivity and resistance to biotic and abiotic stress are presented in Fig. 13.4. Somatic hybridization through protoplast fusion allows sexual incompatibilities to be bypassed and the transfer of both multiple genes and traits from wild relatives into the potato crop genome. It still needs back-crossing for at least two or more generations and selection for the desired traits. Gene transfer from distant or related species needs a good knowledge of dominant genes and their transfer into well-characterized potato varieties. Stacking of transgenes or cisgenes has proven its utility in potato crop but it is still not well accepted by the consumer in Europe. One better way to achieve the goals of improving the crop resistance traits is combinatorial biotechnology already discussed in this chapter as a complex combination of different biotechnological and analytic tools in accordance with the classical and newest genome studies. The latest technologies of reverse genetics and targeted mutagenesis have already proved to be very precise and have apparently no drawbacks but are still in the beginning and will most probably contribute to new achievements at the basic research level and applied potato improvement in the future.

From a practical point of view and to achieve the goals of our actual agriculture challenged by climate change and the exponential increase of world population, we have to bear in mind that all possible modern and classical tools are needed to improve crops and assure food and resources for the next generation.

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