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REVIEW

Progress in plant protoplast research

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Abstract In this review we focus on recent progress in protoplast regeneration, symmetric and asymmetric hybridization and novel technology developments. Regeneration of new species and improved culture techniques opened new horizons for practical breeding in a number of crops. The importance of protoplast sources and embedding systems is discussed. The study of reactive oxygen species effects and DNA (de)condensation, along with thorough phytohormone monitoring, are in our opinion the most promising research topics in the further strive for rationalization of protoplast regeneration. Following, fusion and fragmentation progress is summarized. Genomic, transcriptomic and proteomic studies have led to better insights in fundamental processes such as cell wall formation, cell development and chromosome rearrangements in fusion products, whether or not obtained after irradiation. Advanced molecular screening methods of both genome and cytoplasmome facilitate efficient screening of both symmetric and asymmetric fusion products. We expect that emerging technologies as GISH, high resolution melting and next generation sequencing will pay major contributions to our insights of genome creation and stabilization, mainly after asymmetric hybridization. Finally, we demonstrate agricultural valorization of somatic hybridization through enumerating recent introgression of diverse traits in a number of commercial crops.

Keywords Genome fragmentation · Hybrid screening · Interspecific hybridization · Protoplast regeneration · Somatic fusion

Abbreviations

CMS	Cytoplasmic male sterility
MPP	Microprotoplast
ROS	Reactive oxygen species

Introduction

Plant cells from which the cell wall has been enzymatically or mechanically removed are called protoplasts. Theoretically, protoplasts are totipotent, meaning that they have the capability to dedifferentiate, re-enter the cell cycle, go through repeated mitotic divisions and then proliferate or regenerate into various organs. Fusion of protoplasts from different species, therefore, supplies a practical breeding tool (Johnson and Veilleux 2001) and circumvents sexual hybridization related prezygotic or postzygotic barriers. It can create different homokaryon or heterokaryon types, as well as alloplasmic hybrids (cybrids) (Xia 2009).

Regeneration is often the bottleneck in somatic hybridization breeding programs, which has forced researchers to come up with more innovative approaches, such as electrical stimulation, non-ionic surfactants and artificial gas carriers (Davey et al. 2005). Nonetheless, protoplast fusion became a common technique for the introduction of novelties in commercial crops. During the last decade, interest in protoplast research was renewed, partly due to public antagonism toward genetically modified organisms.

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Recently, Davey et al. (2010) and Grosser et al. (2010) published practical manuals with numerous protocols for isolation, culture, fragmentation and fusion.

In this review we highlight the technological breakthroughs that were made during the last decade in spermatophytes protoplast related research. This survey does not aim to cite all protoplast related research, but to provide an overview of the most innovative developments and insights. We critically discuss recent achievements and speculate on new multidisciplinary approaches that can enhance further implementation.

Rationalization of regeneration

Table 1 summarizes the main plant species for which progress in protoplast culture has been documented over the last 10 years. The most suitable culture method, including optimal protoplast density, and the most efficient protoplast source have been listed. Donor material type has often been decisive for successful regeneration, demonstrating protoplast variability within a single genotype depending on the exact protoplast source. For instance, suspension cells contain more mitochondria than mesophyll cells, and for monocotyledonous species often represent the most suitable donor material (Chabane et al. 2007). Within a single genotype, protoplast variability can arise due to somaclonal variation, but also to different antioxidant and phytochemical concentrations that may be source inherent and can affect regeneration capacity (Pan et al. 2005). In our opinion, explant variability, whether genetically or physiologically, should be further exploited to enable protoplast regeneration in recalcitrant crops. Indeed, relatively few efforts have been done to increase variability within the explants. Pretreatments usually are narrowed down to phytohormonal treatment, whereas ploidy manipulation, antioxidant treatments and metabolism enhancers could possibly prove more efficient in a number of species.

Recent results have led to a better understanding of the importance of culture systems. The relatively low colony formation in liquid medium is assumed to be caused by a shortage of aeration and light (Azad et al. 2006) or a release of toxic components (Duquenne et al. 2007). Semi-permeable membranes (Niedz 2006) for improving oxygen supply or microfluidic polydimethylsiloxane channels with microtubes for continuous medium supply (Ko et al. 2006) can significantly enhance regeneration efficiency. A general finding was the better regeneration of alginate or agarose-embedded protoplasts. In *Cichorium*, a universal regeneration system could be accomplished by agarose bead culture (Deryckere et al. 2012). For other crops, as well beads, discs, layers, thin layers or extra thin films are used (Pati et al. 2005; Rakosy-Tican et al. 2007; Prange et al. 2010a; Grzebelus et al. 2012a; Kielkowska and Adamus 2012). A major advantage of embedding systems is the easy refreshment of the cultures. This minimizes possible negative effects toward microcolony development and microbial contamination. When discs are used, protoplasts divide at a higher rate at the edge (Rakosy-Tican et al. 2007). The thinner the matrix, the higher were the plating efficiencies (Pati et al. 2005). Rotation of the alginate/protoplast suspension during application and before polymerization minimizes layer thickness (Grzebelus et al. 2012a). Also the embedding agent type affects the final outcome, possibly by interacting with genotype, osmolarity, temperature, culture system or aeration (Prange et al. 2010a; Kielkowska and Adamus 2012). This is in accordance with earlier postulations on the positive effect of embedding by membrane stabilization through lipid peroxidase inhibition, the prevention of leakage of cell wall precursors or other metabolites, and lower ethylene levels (Bajaj 1989). Moreover, protoplast aggregation leading up to poor oxygen supply and browning is avoided (Pati et al. 2008; Lian et al. 2011). Also, the osmotic pressure changes steadily instead of stepwise (Kanwar et al. 2009). Another advantage of protoplast embedding may be an improved signaling cascade before the first cytokinesis. Before dedifferentiated plant cells enter cell division, the vacuole develops a complex architecture. This enhances division through better nuclear positioning (Sheahan et al. 2007). In the model proposed by Zaban et al. (2013), the cytoskeleton interacts with this nuclear positioning and thus controls activation and release of molecules involved in cell wall synthesis. Subsequently, dynamic actin filaments are indispensable for the induction of a new cell pole, required for elongation. Briere et al. (2004) suggested that in agarose-embedded protoplasts the actin microfilament network is involved in this signaling process, leading to polarity acquisition and embryoid determination. The aforementioned techniques designed for culturing protoplasts in very narrow matrices hold potential for many previously recalcitrant crops. In addition to further technical improvements, a better biochemical characterization of oxygen deficiency in protoplast culture could provide useful data for further breakthroughs.

Original media supplementations have recently contributed to regeneration of some afore recalcitrant species or materials. In *Beta vulgaris*, the plating efficiency of mesophyll cells drastically increases after adding 100 nM phytosulfokine, a peptide growth factor, which has antioxidant properties but possibly also generates a nurse cell effect (Grzebelus et al. 2012b). The supply of exogenous arabinogalactan protein-rich extracts significantly improved organogenesis from protoplast derived callus (Wisniewska and Majewska-Sawka 2007). Galactoglucomannan-derived oligosaccharides are signaling molecules in plant cell elongation and differentiation. They positively influenced protoplast viability and regeneration (Kakoniova et al. 2010).

Table 1 Progress on protoplast regeneration in different plant species in the period 2004–2013

Plant species	Protoplast source[a]	Culture method	Protoplast density ($\times 10^4$/ml)	Result[b]	References
Alstroemeria spp.	C	Liquid	10	P	Kim et al. (2005)
Anthurium scherzerianum	SE	Agarose beads	10	MCO	Duquenne et al. (2007)
Beta vulgaris	M	Thin alginate layer	40	MC	Grzebelus et al. (2012b)
Brassica oleracea	H	Agarose embedded, coculture	10	P	Chen et al. (2004)
Brassica oleracea	H	Agarose embedded, coculture	10	P	Sheng et al. (2011)
Brassica oleracea	H	Alginate layer	40	P	Kielkowska and Adamus (2012)
Calibrachoa spp.	M	Liquid or alginate embedded	15	S	Meyer et al. (2009)
Chrysanthemum indicum	M	Liquid	10	C	Eeckhaut and Van Huylenbroeck (2011)
Cichorium intybus	M	Agarose embedded	5	P	Deryckere et al. (2012)
Citrus sinensis	C	Alginate beads	25	E	Niedz (2006)
Cyclamen coum	SC	Agarose or alginate embedded	15	P	Prange et al. (2010b)
Cyclamen persicum	SC	Alginate films	15	P	Winkelmann et al. (2006)
Cyclamen spp.	SC	Agarose or alginate embedded	15	P	Prange et al. (2010a)
Daucus carota	H	Thin alginate layer	40	P	Grzebelus et al. (2012a)
Dianthus acicularis	M, SC	Solid (Gelrite)	10	P	Shiba and Mii (2005)
Echinacea purpurea	M	Alginate block/liquid	10	P	Pan et al. (2004)
Gentiana kurroo	SC (CO)	Agarose bead cultures	20	P	Fiuk and Rybczynski (2007)
Gossypium davidsonii	SC	Liquid over solid	20–100	P	Yang et al. (2007)
Gossypium hirsutum	SE, SC	Liquid	20–100	P	Sun et al. (2005b)
Gossypium hirsutum	SC	Liquid	20	P	Wang et al. (2008a)
Gossypium klotzschianum	SE, SC	Liquid	20–100	P	Sun et al. (2005a)
Helianthus annuus	H	Alginate discs	80	P	Rakosy-Tican et al. (2007)
Hypericum perforatum	HC	Alginate blocks	20	P	Pan et al. (2005)
Ipomoea cairica	M	Liquid	1–2	P	Guo et al. (2006)
Iris fulva	SC	Agarose block	10	P	Inoue et al. (2004)
Kalanchoë blossfeldiana	M	Liquid	10	P	Castelblanque et al. (2010)
Lilium japonicum	SC	Agarose embedded, nurse cells	10	P	Komai et al. (2006)
Lotus corniculatus	CO	Extra thin alginate film	20	P	Pati et al. (2005)
Morus indica	M	Liquid	10	P	Umate et al. (2005)
Musa acuminata	SC	Liquid, feeder layer	100	P	Xiao et al. (2007)
Musa paradisiacal	SC	Liquid, feeder layer	100	P	Dai et al. (2010)
Nicotiana tabacum	M	Extra thin alginate film	10	P	Pati et al. (2005)
Petunia spp.	M	Liquid	15	S	Meyer et al. (2009)
Phalaenopsis sp.	SC	Solid, gellan gum	10	P	Shrestha et al. (2007)
Phellodendron amurense	M	Solid, gellan gum	40	P	Azad et al. (2006)
Phoenix dactylifera	C	Liquid, feeder layer	100	C	Chabane et al. (2007)
Robinia pseudoacacia	C	Liquid	20–40	P	Kanwar et al. (2009)
Spathiphyllum wallisii	SE	Agarose beads	10	MCO	Duquenne et al. (2007)
Ulmus minor	M	Agarose droplets	20	MC	Conde and Santos (2006)
Zea mays	SC	Solid/feeder/liquid	20–40	P	He et al. (2006)
Zingiber officinale	SC	Liquid	10–50	P	Guo et al. (2007b)

[a] *C* callus, *CO* cotyledon, *H* in vitro hypocotyls, *HC* hypocotyls derived callus, *M* mesophyll cells from in vitro leaves, *SC* suspension cells, *SE* somatic embryos

[b] *C* callus, *E* embryos, *MC* microcalli, *MCO* microcolonies, *P* plants, *S* shoots

Polyamines are involved in a variety of growth and developmental processes in higher plants, and in stress responses. Isolation increases putrescine levels, especially in non-totipotent protoplasts (Papadakis et al. 2005). The intracellular polyamine levels and metabolism are possibly related to totipotency expression of plant protoplasts. Rakosy-Tican et al. (2007) propose spermidine to stimulate mitosis and to reduce stress impacts. Profiling multiple internal hormone levels during different regeneration phases with chromatographic tools (LC-HRMS and LC-MS/MS) will allow to compare recalcitrant and regenerative genotypes, to administer exogenous hormones at the proper time and to monitor their metabolism closely. Furthermore, nowadays significant efforts are made to establish high throughput bioassays to discover novel molecules with potent cytokinin activities, as exemplified by Motte et al. (2013) for phenyl adenine. These could contribute to organogenesis in crops where traditional cytokinins have not induced shoot formation.

Oxidative stress evoked during protoplast isolation and culture may contribute to protoplast recalcitrance (Cassells and Curry 2001). Inclusion of ascorbate in the protoplast isolation medium of *Arabidopsis* leaves prevented protoplast damage (Riazunnisa et al. 2007). On the other hand, oxidative stress and auxins may act complementary to enhance growth cycle activity or differentiation (Pasternak et al. 2005). In *Nicotiana tabacum* protoplasts, glutathione induced cell dedifferentiation, similar to high auxin concentrations, whereas dehydroascorbate counteracted auxin mediated leaf protoplast development by its internal reduction to ascorbate; consequently, cell division was inhibited and cell expansion stimulated (Potters et al. 2010). Changes in the production of reactive oxygen species (ROS) and reactive nitrogen species were studied in more detail in cucumber protoplasts (Petrivalsky et al. 2012). The crucial role of controlled reactive oxygen and nitrogen species production in both regeneration and cellular growth and regeneration was confirmed. The different levels of as well ROS and antioxidant enzymes and scavengers in *Citrus* callus and mesophyll are suggested to play a key role in defining the regeneration potential of protoplasts of both cell types (Xu et al. 2013). Regeneration research would greatly benefit from more detailed studies on the effects of isolation and early culture on model plants antioxidant mechanisms. Further research could, therefore, be directed toward in vivo localization of diverse ROS and enzymes involved in either their production or detoxification. Specific consequences of ROS accumulations and enzyme activity could be monitored in both responsive and recalcitrant genotypes.

As mentioned before, protoplast isolation generates significant levels of stress and influences polyamine and ROS biosynthesis, both of which may negatively interfere with subsequent regeneration (Cassells and Curry 2001; Papadakis et al. 2005). Xylanase and pectin lyase are components of commercial cellulase and pectinase that contribute to ROS formation (Ishii 1987); purified enzymes indeed generate less ROS (Papadakis and Roubelakis-Angelakis 1999). However, also non purified enzyme mixtures are efficient scavengers of extracellular peroxide (Yasuda et al. 2007); this indicates that mainly intracellular ROS are responsible for protoplast recalcitrance. To limit cell damage during isolation to the best possible extent, Wu et al. (2009) have developed the 'Tape *Arabidopsis* Sandwich' method that allows an easy removal of the lower epidermis and a more efficient exposure of mesophyll cells to the enzyme mixture. During protoplast isolation, endophytic bacteria can be released and subsequently cocultured (Klocke et al. 2012); their interaction with plant cells is probably quite complex, and we can speculate that their effect on protoplast regeneration can be either stimulative or inhibitory depending on the exact developmental phase, culture techniques and other circumstances.

Recent tools, e.g., suppression subtractive hybridization (Yang et al. 2008) have shown potential for genome studies, but also the proteome of developing protoplasts has received more attention (Kwon et al. 2005; De Jong et al. 2007). Holme et al. (2004) identified two quantitative trait loci that contribute to the regeneration ability of protoplast derived microcalli. This can be an initial step toward marker assisted selection of regeneration ability and/or gene introgression into recalcitrant species. In coming years, more fundamental knowledge on early divisions and microcolony formation will become available. To this end, transcriptome and proteome analyses should be implemented further.

Exploration of the link between osmolarity decrease and chromatin over condensation as hinted by Ondřej et al. (2009) may also provide significant contributions toward understanding the complex interaction between genetic background and environmental conditions in early regeneration. Indications for the importance of antioxidants have yet been published in this matter (Ondřej et al. 2010). On the other hand, a connection between ROS and cytosine hydroxymethylation that decreases chromatin recondensation after protoplast isolation, has not been demonstrated (Moricová et al. 2013).

Figure 1 shows a general model of the different steps of protoplast regeneration, compiling the most promising research areas for further research in the near future.

Advances in protoplast fusion and fragmentation

In recent years, chemical fusion and electrofusion were equally used, depending on the plant family. Olivares-Fuster et al. (2005) developed the electrochemical protoplast

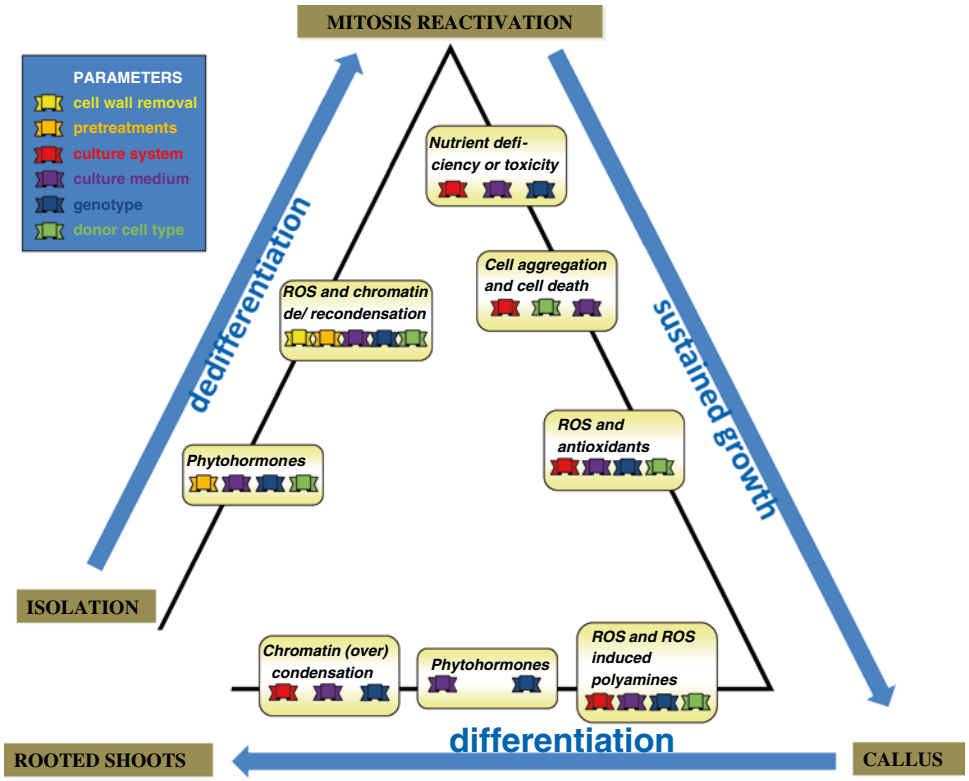

Fig. 1 Proposed general model of protoplast regeneration, representing parameter driven dedifferentiation, growth or differentiation inducers and inhibitors

fusion method that combines the advantages of the two methods. It is based on chemically induced protoplast aggregation and direct current pulse promoted membrane fusion. According to the authors, high yields were obtained, but the system is not regularly implemented outside Rutaceae and requires the purchase of an electropulser.

Fusion events can be monitored with fluorescent markers, or by observing cell organelles. Pati et al. (2008) isolated heterokaryons by an innovative colony tracking technique in *Rosa*, based on differential fluorescent staining, and Borgato et al. (2007) monitored the efficiency of magnetic activated cell sorting. GFP transgenic lines have been used as tools for fusion monitoring (Table 2). The theoretical potential of flow cytometry for cell sorting is extended, but practical obstacles such as the preservation of a stable osmotic potential and the efficient recuperation of sorted protoplast populations are omnipresent and have so far inhibited flow cytometrical hybrid selection on a wide scale. Some novel technologies may offer an alternative sorting system for (fused) protoplasts or even cell nuclei. The Zeiss CombiSystem combines MicroTweezers for optical trapping and particle positioning with laser beam microdissection and subsequent laser directed transport in a collection vessel. Another potential innovative tool for cell sorting is DEPArray™ (Silicon Biosystems), a new platform based on moving dielectrophoresis cages. In the latter device, each suspended cell is trapped in a single cage and sorted after multiplexed fluorescent and morphological characterization. Cells can be selected individually, and friction is avoided. To our knowledge, these systems have not yet been used for selection of somatic fusion products.

Several techniques can be used for genome fragmentation, such as UV irradiation (Hall et al. 1992) or microprotoplasts (MPPs) (Yemets and Blume 2009). Genome fragmentation of the donor partner stimulates the elimination of much of its redundant genetic material in the somatic hybrid. Moreover, most karyotype instability causing donor genes are eliminated during the first post-fusion mitoses, as opposed to symmetrical fusions after which eliminations can occur up to the first sexually derived generation (Cui et al. 2009). In some cases, asymmetric fusions were realized without fragmentation treatment (Li et al. 2004). Asymmetric hybrids were obtained after protoplast fusion of UV treated *Bupleurum scorzonerifolium* and wheat. However, instead of *B. scorzonerifolium* chromosome fragments integrated in wheat, the reverse was found. This study can contribute to physical wheat genome maps (Zhou and Xia 2005). The same was observed when untreated *Arabidopsis thaliana* protoplasts were fused with UV treated *Bupleurum* protoplasts (Wang et al. 2005). It is suggested that the high amount of secondary metabolites is quenching ROS (Wang et al. 2011c).

A general problem is the quantification of DNA damage after an irradiation treatment. Abas et al. (2007) presented Comet assay single cell gel electrophoresis as a reliable tool to observe single and double strand breaks, and Xu

Table 2 Innovative approaches for interspecific somatic hybrid selection or characterization in the period 2004–2013

Tools	References[a]
Screening after fusion with GFP transformed fusion partner	Guo and Grosser (2005); Cai et al. (2006); Ovcharenko et al. (2011)*
Heterofusion labeling of mitochondria	Sheahan et al. (2005)
Genomic in situ hybridization	Fu et al. (2004); Li et al. (2004); Xiang et al. (2004)*; Wang et al. (2005)*, (2011a)*; Zhou and Xia (2005)*; Feng et al. (2006); Iovene et al. (2007); Tu et al. (2008); Cui et al. (2009); Yang et al. (2009); Lian et al. (2011); Patel et al. (2011); Jiang et al. (2012)*
InterRetroelement and retroelement microsatellite amplified polymorphism	Patel et al. (2011)
Microsatellite-anchored fragment length polymorphism	Thieme et al. (2010)
5.8S gene based species specific DNA marker	Prange et al. (2012)
Chloroplast DNA cleaved amplified polymorphic sequence	Fu et al. (2004); Takami et al. (2005); Trabelsi et al. (2005); Xu et al. (2005), (2007)*; Cai et al. (2006); Bidani et al. (2007); Iovene et al. (2007); Patel et al. (2011); Sarkar et al. (2011)
Mitochondrial DNA cleaved amplified polymorphic sequence	Fu et al. (2004); Takami et al. (2005); Xu et al. (2005); Cai et al. (2006); Guo et al. (2007a); Iovene et al. (2007); Wang et al. (2010); Ovcharenko et al. (2011)*; Patel et al. (2011); Sarkar et al. (2011)
Real-time PCR	Ondřej et al. (2010); Liu et al. (2012); Wang et al. (2013b)
High resolution melting analysis	Deryckere et al. (2013)
Microarray transcriptome analysis	Liu et al. (2012); Wang et al. (2013b)
Reverse transcription PCR	Wang et al. (2011a*; Liu et al. (2012)
Real-time reverse transcription PCR	Yu et al. (2012)

[a] Publications on asymmetric fusions are labeled with *

et al. (2007) revealed extensive DNA fragmentation with the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling assay.

We expect that alternative tissues, such as immature pollen, will be relatively more applied in the future for MPP production, because of the lack of synchronization requirement, as demonstrated for *Lilium* and *Spathiphyllum* (Saito and Nakano 2002; Lakshmanan et al. 2013). Moreover, MPPs could be selected based on filtration properties, which would result in different genome types, enabling researchers to attribute plant traits to particular chromosomes and further utilize MPPs accordingly. For subsequent characterization of MPPs, confocal laser scanning is a promising tool (Famelaer et al. 2007). Combining multiple techniques, such as irradiation of MPPs, or the creation of MPPs from unreduced gametes formed by interspecific hybrids offer a palet of innovative research in genome fragmentation and the transfer of recombined chromosomes.

Characterization of somatic hybrids

Chromosome constitution and stability

Both fertile and sterile hybrids are obtained after symmetric and asymmetric protoplast fusions. Frequent chromosomal abnormalities are a probable cause for male sterility in somatic hybrids (Iovene et al. 2012), and their occurrence has, therefore, been better studied of late. Various abnormal meiotic behaviors can occur in somatic hybrids: univalents, multivalents, lagging chromosomes, triads, polyads and chromosome bridges. The appearance of different numbers of univalents suggests the lack of homology of different chromosomes. The formation of multivalents may enable fusion partners to exchange genes and may suggest intergenome homology or another effect on hybrid fertility. Lagging chromosomes could contribute to the formation of small pollen grains that are linked to sterility (Guo et al. 2010). Apart from meiotic abnormalities, chromosome fragment deletion and rearrangements also occur in sterile somatic hybrids (Iovene et al. 2012).

For fertility, chromosome addition (meaning that the number of chromosomes in the hybrid equals the sum of the chromosomes in the fusion partners) is not absolutely required. Somatic hybrids that have the complete nuclear complements of both parents are generally rare (Xia 2009), and this fact has drawn more attention to the evaluation of chromosome number and structure stability following fusion. Fu et al. (2004) described an additive *Citrus* fusion in which chromosome translocations were observed. After various fusions, additive products were found alongside hybrids with reduced chromosome numbers (Wang et al. 2008b; Szczerbakowa et al. 2010; Lian et al. 2012), and in some cases the hybrid 2C level is lower than the 2C sum of both parent species (Sheng et al. 2008). Furthermore, regenerated fused genotypes are not always ploidy

stable (Prange et al. 2010b; Sheng et al. 2011). Chromosome rejection as a consequence of the distant relationship between both partners is very likely (Guo et al. 2006). Whole chromosome block elimination and fragment loss upon genomic rearrangements are possible consequences (Guo et al. 2010). SSR markers are suitable tools to prove recombination through the occurrence of new bands and the disappearance of others (Guo et al. 2008). Regeneration ability is by itself the best possible tool to select hybrids with favorable genome complementation (Xiang et al. 2004).

The possible establishment of these complementations is evidently affected by the application of irradiation, which acts as a chromosome fragmenting or even eliminating agent on its own and thus interferes with the regeneration process (Jiang et al. 2012). Irradiation can speed up chromosome elimination, incompatibility alleviation, and differentiation. In the research of Wang et al. (2011b), irradiation driven ROS and flavonoid production were demonstrated; in our opinion, this can be of major importance toward the interference of ROS with regeneration in other crops. More profound research efforts could be directed toward the exact function of antioxidants during early divisions and genome stabilization. Possibly flavonoids are linked to the enigmatic preferential elimination of chromosomes of the non irradiated fusion partner after asymmetric hybridization described by Wang et al. (2005).

Chromosomal reduction due to transposon activity occurred in two somatic *Oryza* hybrids and suggested a potential to generate breeding lines with novel chromosomal constitutions. Shan et al. (2009) attributed the elevated transposon activity to the one-step introgression of multiple donor (*Zizania latifolia*) chromatin segments. Substantial loss of its original copies accompanied the activation of the element; this was not reported previously. This indicates that wide hybridization and subsequent introgression may activate transposable elements that are otherwise quiescent, and also suggests that, under certain conditions, along with mobilization of a transposon its original copy numbers can be dramatically reduced.

Genome stability and chromosome elimination require close monitoring. Causes for genome instability are extended: different cell cycle of genetically remote parents, smaller centromeres of eliminated chromosomes, DNA methylation of centromere function involved genes and cytoplasm containing secondary metabolites (Wang et al. 2008b). However, protoplast fusion based models may provide an excellent model to unravel the acquirement of karyotypic stability. For instance, somatic fusions have confirmed the probable harm of higher ploidy levels in this respect (Szczerbakowa et al. 2011). Cytogenetic techniques are indispensable for a thorough characterization of hybrid genome evolution upon fusion.

GISH has shown its potential for chromosome analysis, but requires well trained skills. The preparation of well-dispersed chromosomes is the most important factor. Yet in Escalante et al. 1998, used it for somatic hybrid screening, and over the last years it developed into a standard screening tool (Table 2). We expect that the contribution of GISH to genome characterization studies after fusion will further increase, and researchers will exploit its complementarity with other techniques. For instance, a combination of FISH and GISH precisely identified *Avena sativa* chromosome segments introgressed in an asymmetric hybrid with *Triticum aestivum* (Xiang et al. 2010). When needed in other crops, the sensitivity of this combined technique could be increased by the application of tyramid FISH that can visualize chromosomal targets as small as 500 bp on metaphase spreads (De Jong 2003). GISH acted complementary with SSR in demonstrating the genetic background of hybrids arisen after somatic fusion of 2 *Triticum* genotypes and *Psathyrostachys* (Li et al. 2004), and thus enabled the compilation of an asymmetric fusion protocol without the need for genome fragmentation in this particular combination. GISH could also be used for monitoring genomic stabilization. If chromosome elimination occurs preferentially rather than randomly, e.g., in *Solanum* (Trabelsi et al. 2005), it is a convenient tool to evaluate the effects of fusion and regeneration related parameters, and thus to optimize these parameters in an effort to stimulate or impede regeneration of particular genome types. A particular possible application of cytogenetic tools is the study of meiosis after somatic fusion to define chromosome affinity and phylogenetic relationship between the original fusion partners. On the other hand, the complementarity of two specific parents can be prescreened with GISH preceding fusion. As the probability toward successful hybridization increases along with parent complementarity, prescreening can be used to select partner combinations with relatively higher chances of success.

Genome, cytoplasmome, transcriptome and proteome screening

Molecular tools based on DNA analysis have known significant progress over the last years and are now routineously used for characterization of somatic hybrids. SSR, tandemly repeated in eukaryotic genomes, was used in SSR-PCR and in ISSR-PCR for somatic hybrid characterization. ISSR analysis showed that the level of intergenomic recombination can be increased by reducing ploidy level of *Solanum* hybrids, through androgenesis, by tetrasomic inheritance (Toppino et al. 2008). SSR markers are codominant and results are highly reproducible, whereas RAPD provides a useful screening tool when little is known about the DNA sequence of the test plants. Generally, the former

marker is recommended over the latter, except in particular *Solanum* fusions (Sarkar et al. 2011). Alternative nuclear DNA markers are occasionally used for somatic hybrid screening (Table 2).

Recent tools such as microarray analysis or reverse transcription PCR have enabled hybrid transcriptome study (Table 2). Gancle et al. (2006) suggested proteomics as a good approach to better understand inheritance and regulation rules in somatic hybridization. In proteome analysis of *Citrus* somatic hybrids, among the differentially expressed spots proteins that can be linked are involved in photosynthesis, metabolism, and stress response, particularly to antioxidative stress. Cytosolic ascorbate peroxidase is upregulated, whereas it is downregulated in the chloroplasts; due to the peroxidase link with the ascorbate/glutathione cycle an effect on ROS scavenging may be expected. The antioxidative system is thus clearly affected by the hybrid status. Further proteome analysis indicates a better adaptation of the cybrid to cold or drought stress and an upregulated Rubisco activity (Wang et al. 2010). The complicated regulation mechanism between gene and protein can interfere with downstream hybrid monitoring; Liu et al. (2012) demonstrated introgression on the RNA level, but found little correlation between transcriptome and proteome.

The high demand for low-cost sequencing has stimulated high throughput (next generation) sequencing technologies development. These parallelize the sequencing process and thus simultaneously produce thousands or millions of sequences (Hall 2007). 500,000 Sequencing by synthesis operations may be run in parallel in ultra high throughput sequencing. Next generation sequencing enables fairly cheap high quality nucleic acid sequence data obtention of complete genomes in a short period of time, and will therefore definitely contribute to somatic hybrid genome screening and stability studies in the near future.

Somatic fusion can yield a combination of cytoplasms from different sources, unlike sexual cross hybridization that leads to maternal inheritance of cytoplasmic genomes (Xu et al. 2005). Before cytokinesis, unbiased chromosome partitioning is ensured by highly ordered nuclear inheritance. Likewise, the endoplasmatic reticulum, chloroplasts and mitochondria display distinctive partitioning strategies that guarantee unbiased inheritance before dedifferentiating cells have completed mitosis (Sheahan et al. 2004). For mitochondrial interaction after protoplast fusion at the subcellular level, Sheahan et al. (2005) fused protoplasts that contained either green fluorescent protein or MitoTracker-labeled mitochondria. This allowed them to report the phenomenon of massive mitochondrial fusion that within 24 h led to a near-complete mixing of the mitochondrial population. It occurs in *Medicago* and *Arabidopsis* mesophyll protoplasts but not in protoplasts from already dedifferentiated cells like tobacco BY-2 or callus cultures. These results allow to more clearly interprete novel mitochondrial genotype development upon cell fusion. Sytnik et al. (2005) demonstrated that also chloroplasts can be transferred to remote species by protoplast fusion.

PCR–RFLP and CAPS analysis using mitochondrial or chloroplast universal primer pairs have been efficient and reliable tools for cytoplasmic genome characterization (Table 2). It is a relatively new somatic hybrid characterization tool (Cheng et al. 2003). Compared to RFLP with labeled probes, CAPS is more rapid, less expensive, and simpler (Guo et al. 2004). Chloroplast SSR is even more convenient and efficient, because enzyme cutting following PCR reaction is not required (Cheng et al. 2005). Also sequencing of common bands and searching for restriction endonuclease sites could be cheaper and more convenient than actual CAPS analysis, though after sequencing CAPS could be used to confirm the results.

Compared to nuclear DNA, inheritance of cpDNA and mtDNA is relatively complex. For *Citrus* fusion, the general consensus is that cpDNA is randomly transmitted; as for mtDNA, nearly all hybrids get theirs from the suspension parents (Fu et al. 2004; Guo et al. 2004; Takami et al. 2005). Guo et al. (2007a, b) described cpDNA coexistence in *Citrus* fusions. Whether this was persistent or just temporary due to incomplete elimination of cpDNA from 1 fusion partner, is still unclear. As the plant grew less vigorously and had fewer leaves, it is less competitive than the other fusion products, which may account for a selection toward non cpDNA coexistent types. In *Solanum* hybrids, coexistence of mtDNA was recorded (Sarkar et al. 2011).

Like nuclear genomes, cytoplasmic genomes are not always stable upon fusion. Intergenomic chloroplast recombination occurs rarely in higher plants, as opposed to the high level of mitochondrial recombination (Trabelsi et al. 2005). The latter occurs after various fusions (Xiang et al. 2004; Iovene et al. 2007; Yamagishi et al. 2008). In *Triticum aestivum* + *Setaria italica* hybrids, cpDNA coexistence as well as recombination occur (Xiang et al. 2004). It was also observed in *Solanum tuberosum* + *verneï* (Trabelsi et al. 2005), *Solanum berthaultii* + *tuberosum* (Bidani et al. 2007) and *Bupleurum* + *Swertia* (Jiang et al. 2012).

High resolution melting analysis, a screening technique based upon insertions, deletions or SNPs induced altered dissociation behavior of double stranded DNA, has become a highly sensitive method for genotyping (Wu et al. 2008). Deryckere et al. (2013) applied it to unravel mitochondria and chloroplast constitution in *Cichorium* somatic hybrids. High resolution melting can become a standard for mtDNA and cpDNA screening, as, through combination with a PCR reaction, it can outcompete laborious and costly sequencing analysis. Promising as it

may be, it has its shortcomings in establishing recombination events and should for that aim be combined with sequencing.

Agricultural valorization

Genomic variation is of major interest in agricultural or industrial crops for plant quality and yield improvement. Salt tolerance, quality improvement, cytoplasmic male sterility (CMS) transfer, disease resistance, seedless triploids and rootstock improvement are the most common breeding goals for somatic hybridization in cash crops (Wang et al. 2013a). Most practical results were recently achieved in 'model families' Rutaceae, Brassicaceae and Solanaceae.

Somatic hybridizations and cybridizations in *Citrus* resulted in rootstocks resistant to biotic and abiotic constraints and in increased yield and fruit quality (Dambier et al. 2011), as well as in brown spot resistant scions (Soriano et al. 2012). New seedless triploid *Citrus* cultivars are produced via haploid + diploid fusion and symmetric fusions of elite diploid cultivars can lead to superior allotetraploid breeding parents (Grosser and Gmitter 2005). The endosperm balance number complicates sexual crosses in *Solanum* (Johnston et al. 1980). The most important objective in *Solanum tuberosum* somatic breeding is the introduction of resistance against the PVY virus, Colorado beetle and late blight (*Phytophtora*). Most hybrids are fertile and indeed contain some partial resistance against these parasites. Multiple resistances were also found, along with high morphological and agronomic variation (Thieme et al. 2010). Jiang et al. (2009) obtained *Brassica napus* + *Camelina sativa* hybrids with increased linolenic acid content compared to the *B. napus* partner. Scholze et al. (2010) produced the first resistant raphano-brassica symmetric and asymmetric hybrids. These showed new resistance types along with multiple resistances, including turnip mosaic virus. Other agriculturally relevant properties modified by somatic hybridization included chilling tolerance in *Actinidia* (Xiao et al. 2004), photoperiodical response in *Gossypium* (Sun et al. 2005b) and storage root formation in *Ipomoea* (Yang et al. 2009).

An important practical application of new genome/cytoplasmome combinations is the introduction of CMS (Cai et al. 2006). Fitter et al. (2005) demonstrated the possibility of introgressing CMS carried by mtDNA from a wild species into the cultivated crop. Yamagishi et al. (2008) proposed mitochondrial recombination as a tool for CMS introduction in cabbage. Lian et al. (2011) introduced CMS after *Brassica juncea* + *B. oleracea* fusion. Most hybrids were male sterile, although stamina appeared normal. Likewise, CMS and normal stamina simultaneously appeared after *Arabidopsis* + *Bupleurum* fusions (Wang et al. 2008b).

Conclusions

Somatic hybridization is one of many breeding tools available to create various new genomic combinations, and is essentially different from other techniques in many respects. When comparing somatic hybridization to transgenic approaches, the former enables to broaden the germplasm base, allows to transfer uncloned multiple genes and generates products that are not bound to the same legal regulations as transgene plants (Grosser and Gmitter 2005). Also, it transfers both mono- and polygenic traits (Thieme et al. 2004). Over the last years, it was frequently used as an alternative for incompatible sexual crossing, although apart from polyploidization other genomic effects, like chromosome rearrangements, are more typically observed in somatic hybrids than in their sexual counterparts (Chevre et al. 1994). Like sexual crosses, somatic fusions are confronted with their own particular troubleshooting and opportunities. Future studies will not only enlighten us on the particular differences in establishment of karyotypic stability through either method or their phenotypical consequences, but generate tools to overcome some of the drawbacks inherent to protoplast mediated hybridization, and increase the potential of the technique to create novelties with agriculturally improved traits. To this end, a further rationalization of protoplast regeneration is indispensable, and we expect that regeneration efforts will continue to steadily drop out of trial and error based experimentations. Especially, the further development of asymmetric hybrids can tackle problems that appear after somatic fusion, as it may limit genome conflicts. The fast evolution in marker development will allow more profound studies on genome stability. We will gain better insights on genetic backgrounds that are responsible for hybrid selection during the entire regeneration process.

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