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Overcoming Interspecific Barriers in Ornamental Plant Breeding

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ABSTRACT

In ornamentals, interspecific breeding is considered to be the most important source of genetic variation. The introgression of genes may be part of a breeding program towards disease resistance, better growth vigor, winter hardiness or morphological alteration in general. Both sexual and asexual hybridization, by pollination and protoplast fusion respectively, are fit to induce the desired introgression into the acceptor species. However, the number of possible barriers is very high. Obviously, apart from the actual "fusion barriers" also regeneration barriers are met; moreover, a thorough screening procedure of possible hybrids is needed. Whereas the hybrid status can be confirmed by molecular markers, visualization of introgression needs to be performed by cytogenetic techniques. In sexual crosses, both prezygotic and postzygotic barriers can inhibit the creation of a fertile interspecific hybrid; in asexual crosses regeneration of hybrid cells is often a bottleneck. For this reason *in vitro* techniques are indispensable for interspecific breeding in a large number of crops. Polyploidization protocols, whether driven by unreduced gametes or by mitosis arresting chemicals, can ease the fertilization or restore fertility of F_1-hybrids. In the latter case, unreduced gametes arisen after FDR (first division restitution), SDR (second division restitution) and IMR (indeterminate meiotic restitution) still offer the possibility of direct gene introgression in the next generation, even if the hybrid produces no normal (haploid) gametes.

1. INTRODUCTION

Breeders of ornamentals are in a constant search for new technologies that may offer a valuable aid in cost reduction, product quality enhancement and assortment enlargement. Plant tissue culture comprises a set of *in vitro* techniques, methods and strategies. It has been exploited to create genetic variability to improve crop plants and their health status and to preserve germplasms (Taji *et al.* 2002). *In vitro* techniques for the culture of protoplasts, anthers, microspores, ovules and embryos have been used to create new genetic variation in breeding lines (Brown and Thorpe 1995). Apart from micropropagation, the most important *in vitro* applications are induction of somaclonal and gametoclonal variation, pathogen eradication, germplasm conservation, synthetic seed production through somatic embryogenesis, haploid breeding, polyploid breeding and interspecific hybridization. Interspecific hybridization is a means of transferring genes within related species; as such, it offers an alternative for genetic modification. Van Tuyl and De Jeu (1997) considered interspecific hybridization to be the most important source of genetic variation in ornamentals. The introgression of genes may be part of a breeding program towards disease resistance or morphology alteration (Uhlinger 1982).

The difficulty of creating interspecific hybrids increases along with the phylogenetic distance between the parents (Sharma 1995). Different barriers have been met from the first attempts on (Stebbins 1958). Growth and development of "alien" pollen tubes can be impeded in the female style (prezygotic barriers); after fertilization embryo development can be arrested by malformation of nurse tissue, mostly endosperm (postzygotic barriers). *In vitro* methods allow developing an integrated procedure for overcoming fertilization barriers, since environmental conditions can be controlled and optimized (Eeckhaut 2003). As Hermsen (1984) quotes "the statement, that two species are not crossable, is controversial unless a broad genetic variation of the parental species has been used and the cross combinations have been carried out on a large scale under a wide range of environmental conditions". This quotation implies that cross ability is determined both by genetic and environmental factors.

2. INCONGRUITY OR INCOMPATIBILITY?

As suggested by Hogenboom (1973 1975) breeding incompatibility between plants may be controlled by either of two major mechanisms, incompatibility or incongruity. Incompatibility has developed numerous times during the evolution of flowering plants (Franklin-Tong and Franklin 2000). It is typically involved in crosses between closely related genotypes that possess at least some elements of an S-gene controlled self- or

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Abbreviations: BC, back-cross; **FDR**, first division restitution; **IMR**, indeterminate meiotic restitution; **SDR**, second division restitution inter-incompatibility system. Incompatibility is a natural defense mechanism against inbreeding, as only the growth of "self" pollen is arrested, which may occur at various stages between pollination and fertilization (Wheeler *et al*. 2001) or even afterwards (Williams *et al*. 1984). The exact stage is determined by the nature of the incompatibility system (Newbegin *et al.* 1993) that may be dependent on the pollen genotype (gametophytic) or on the pollen parent genotype (sporophytic). Different flower types within a single species may cause heteromorphic incompatibility. Although it is mostly controlled by one single gene, systems governed by two or more loci have been described, e.g. in Poaceae (Gaude and McCormick 1999).

Incongruity is typically seen in crosses where independent evolution of the partners (being different species) has led to a breakdown in control of pollen tube behavior in the pistil. It may therefore be considered as an isolating mechanism developed as a by-product of evolutionary divergence (Hogenboom 1975). The development of the hybrid genome may also be hampered or even arrested by multiple barriers in a later stage (**Table 1**). An alternative model proposed by de Nettancourt (1977 1997) contrasts with incongruity since it suggests that cross-hybridization is prevented by an active process that inhibits what would otherwise be a compatible pollination. Although these kind of responses have been observed within several families (Wheeler *et al.* 2001), their extent remains unclear so far.

Incongruity can be caused by both pre- and postfertilization barriers; incompatibility mainly occurs before gamete fusion (Raghavan 1997). Whereas incompatibility promotes outbreeding, incongruity limits the possibilities for species hybridization (McCubbin and Kao 1996). Neither incompatibility nor incongruity is caused by sterility (non-viability of the gametes, Knox *et al.* 1986). A connection between the underlying mechanisms behind incongruity and self-incompatibility has been suggested by the so-called SI x SC rule, stipulating that pollen from self-compatible species is often rejected by pistils of self-incompatible species, whilst the reciprocal crosses are viable. However, there are exceptions to this rule (Wheeler *et al.* 2001). Up to now, several techniques have been used to overcome fertilization barriers: the application of exogenous growth substances and immunosuppressants, embryo rescue, intraovarian pollination *in vivo* and *in vitro*, *in vitro* fertilization of ovules, and somatic cell hybridization (Zenkteler 1990). Six main hybridization barriers may be distinguished (**Table 1**) in incongruous crosses.

2.1. Prezygotic incongruity

The exchange of signals between females and males mediates successful fertilization and is one of the central themes in biology. Gametophytes of angiosperms (producing egg cells and sperm cells) play critical roles in the fertilization. Pollination can be divided into characteristic stages that include: (a) pollen hydration and germination; (b) growth of the pollen tube through the stigma, style and ovary; (c) pollen tube guidance to the ovule micropyle; and (d) delivery of the sperm to the embryo sac (Wilhelmi and Preuss 1997). The time required for this series of events varies considerably depending on the species, from a few hours to several months. The development of pollen tubes from the stigma towards the ovules can be easily monitored by staining with aniline blue (Cuevas *et al.* 1994).

The influence of phytohormonal compounds on signaling has not been unraveled, though interesting data are already available (Baker *et al.* 1997, Van Creij *et al.* 1999, Katsukawa *et al.* 2000). Phytohormones produced in the fertilized ovules also affect fruit set, seed set and abscission (Pickersgill *et al.* 1993, Van Creij *et al.* 1997). ABA-concentrations increase fast in incompatibly pollinated flowers and slowly in unpollinated ones, while decreasing after compatible pollinations. Auxin concentrations increased after compatible pollination. Ethylene levels remain relatively unaffected after compatible pollinations but increase after incompatible pollinations or non-pollination. ABA and ethylene cause abscission of incompatible or non-pollinated flowers. A practical application might be the use of ethylene antagonists (CO_2, Ag) for the realization of incompatible/incongruous crosses (if at least the increase of ethylene levels is one of the causes of incongruity – and not one of the consequences). On the other hand, in *Nicotiana* there appears to be a correlation between ethylene production after pollination of mature pistils and pollen growth. The production of ethylene differs depending on the pollen used; in some cases ethephon can minimize the arrest of incongruous pollen tubes inside the style (Sanchez and Mariani 2002). Ethylene may inhibit both incompatibility (by decreasing the synthesis of S-products) and incongruity (by stimulating pollen growth in senescent flowers; Williams and Webb 1987). From this data it appears that ethylene might have a dual effect: a promotion of the growth of incongruous pollen, but also a triggering of abscission after incongruous pollination, thus limiting the possibility for the sperm nuclei to reach the ovules.

Generative and vegetative nuclei of pollen exhibit different sensitivity towards irradiation. As the division capacity of the generative nucleus is easily disturbed at certain (plant specific) doses, the vegetative nucleus remains unaffected. This so-called mentor pollen is therefore sterile, but still able to penetrate the style and has a stimulatory function (Stettler 1968). It is often applied in interspecific pollinations, upon mixing with incongruous pollen (Villar and Gaget-Faurobert 1997, Wenslaff and Lyrene 2000). An increasing flower age (Ascher and Peloquin 1966) as well as pollination at high temperatures (Van Tuyl *et al.* 1982) may affect fertilization efficiencies in a positive way. Also specific lipids are able to promote the growth and penetration of the pollen tube into the pistil (Wolters-Arts *et al.* 1998).

Cut-style pollination is often suitable for hybridization between parents with different pistil lengths since pollen tube growth and development are often impeded in (too) short or long pistils (Williams and Rouse, 1990); it was applied successfully in different interspecific pollination in ornamental genera, like *Vriesea* (Vervaecke 2001), *Tulipa* (Van Creij *et al.* 1997), *Fritillaria* (Wietsma *et al.* 1994) and *Lilium* (Van Tuyl *et al.* 1991, Janson 1993, Fukai *et al.* 2005). The style can be completely removed in order to pollinate the ovules directly (placental pollination). Another method is style grafting where a compatible style is grafted on an incompatible ovary (Van Tuyl *et al.* 1991). The technique is referred to as 'ovary grafting' when next to the donor style (a part of) the donor ovary is grafted on the ovary of the seed parent. In lilies, hormone treatment of the style was more efficient than ovary grafting, placental pollination and mentor pollination to circumvent the inhibition of ovule penetration (Van Creij *et al.* 1997). Also a positive effect of high temperature in overcoming incongruity has been detected and applied in breeding programs of lily by pollinating at high temperatures (Van Tuyl *et al.* 1982, Okazaki and Murakani 1992). Also in *Rhododendron*, prezygotic barriers are clearly present in many parent combinations (Preil and Ebbinghaus 1985, Okonkwo and Campbell 1990, Rouse 1993) and pollen tube growth is significantly influenced by temperature treatment (Kho and Baer 1973). Probably heat sensitive inhibitors of pollen tube growth are inactivated.

A rather marginal technique is hexane treatment of the stigmatic surface (Whitecross and Willing 1975). *In vitro* fertilization and protoplast fusion do not require *in vivo* pollen tube growth but mostly confront the breeder with complicated technical problems or poor regeneration of the fusion products (Brown and Thorpe 1995).

2.2. Postzygotic incongruity

2.2.1. Absence of seed production or germination

Ploidy differences between parent plants might be one of the main incongruity barriers according to Badger (1988) by causing a 'triploid block' resulting in the malformation of endosperm and the inhibition of germination. Endosperm absence or retarded development is often the cause of spontaneous abortion of hybrid embryos after interspecific pollinations (Van Tuyl *et al.* 1991, Pickersgill *et al.* 1993, Sharma 1995). Johnston *et al.* (1980) proposed the endosperm balance number theory to explain the basis for normal seed production but currently there is no consensus on the requirements for the formation of hybrid endosperm. Fertilization-independent endosperm and fertilization-independent seed *Arabidopsis* mutants have been isolated in which an endosperm-like tissue arises from the central cell in the absence of fertilization, confirming the degree of autonomy of the central cell at this stage (Ohad *et al.* 1996, Chaudhury *et al.* 1997). These mutants suggest that endosperm development is probably triggered by a combination of maternal factors, which are interpreted differently by the central cell and the egg cell (Slocombe *et al.* 1999). Starch is deposited in modified plastids, the amyloplasts; loci responsible for the major enzymes involved in starch biosynthesis have been identified (Gallusci *et al.* 1996).

Using *in vitro* media, which replace the endosperm and allow maturation of the hybrid embryo, can overcome the lack of endosperm. This would not only allow to rescue seedlings from more crosses, but also to rescue more seedlings per cross. Next to rescuing hybrid (or haploid) embryos the main applications of embryo culture are solving the problems of low seed set, seed dormancy, slow germination, germination of obligatory parasites, shortening the breeding cycle and vegetative propagation (Zenkteler 1990, Pierik 1999). The main factors affecting its success are the genotype and growth conditions of the mother plant, the developmental stage of the embryo at isolation, the composition of the nutrient media and the culture environmental conditions (oxygen, light and temperature; Pierik 1999). The term "embryo rescue" is restricted to only those cases where the embryos, if not rescued are endangered and would not form seedlings. There are heterotrophic and autotrophic stages in embryo development. In the heterotrophic stage, the embryo (called proembryo) is smaller and usually requires growth regulators (Raghavan 1980). During this latter phase, up to the heart stage, embryos fully depend on the endosperm and other maternal tissues of the ovules. Hybrid embryos which start to degenerate at the heterotrophic phase, after isolation and culture in artificial media, die soon after transfer (Zenkteler 1990).

'Embryo rescue' therefore offers a promising tool for interspecific breeding as an aid in hybridization by sexual means (Zenkteler 1990, Pickersgill *et al.* 1993). Hybridization through this technique has proven possible on *Capsella* (Monnier 1988), *Tulipa* (Custers *et al*. 1995, Van Creij *et al.* 1997), *Lilium* (Asano and Myodo 1977, Van Tuyl and Van Holsteyn 1996, Arzate-Fernandez *et al.* 1998, Obata *et al.* 2000, Chi 2002), *Arachis* (Feng *et al.* 1996), *Alstroemeria* (De Jeu and Jacobsen 1995, Burchi *et al.* 1998), *Allium* (Nomura and Oosawa 1990), *Camellia* (Hwang *et al.* 1992), *Nicotiana* (Reed and Collins 1978, Nikova and Zagorska 1990), *Phaseolus* (Mok *et al.* 1978), *Cajanus* (Mallikarjuna and Moss 1995), *Zantedeschia* (Yao and Cohen 1996), *Delphinium* (Honda and Tsutsui 1997, Honda *et al.* 1999 2003), *Hydrangea* (Reed 2000, Reed *et al.* 2001), *Clethra* (Reed *et al.* 2002), *Rhododendron* (Eeckhaut *et al.* 2003), *Hibiscus* (Eeckhaut *et al.* 2004) and within the Brassicaceae (Inomata 1978, Quazi 1988, Gundimeda *et al.* 1992, Thierfelder *et al.* 1992, Lelivelt 1993, Li *et al.* 1995, Momotaz *et al.* 1998); reviews have been published by Sharma (1995), Sharma *et al*. (1996) and De Jeu (2000). 'Embryo rescue' can be performed in one step (embryo culture) or two steps (*in ovulo* culture + embryo culture). The most applied culture methods are ovary slice culture, ovule with placenta culture, single ovule culture, and embryo sac culture (Van Tuyl and De Jeu 1997, Chi 2002). Monnier (1988) has shown that the success of an embryo rescue medium partly depends on sugar, Fe and Ca-concentration. Immature embryos are known to require higher osmotic strength (lower potential) of the medium compared to the relatively mature ones. High osmotic strength of the medium prevents precocious germination of young embryos and supports normal embryonic growth. Therefore sugar concentration is at least as important for the osmolarity of the medium as for providing nutrition, at least when culturing immature embryos (Sharma *et al.* 1996). Though embryo rescue is not always required to obtain hybrids it might improve crossing efficiency significantly compared to natural crosses (Faure *et al.* 2002).

2.2.2. Hybrid albinism

Incompatibility between the plastome and nuclear genome inhibits chloroplast development and chlorophyll formation. Heavy chlorosis may very well cause inviability of the hybrid seedlings (Przywara *et al.* 1989 1996, Badami *et al.* 1997, Ha *et al.* 1998) and occurs after interspecific hybridization in many genera (Yao and Cohen 2000). Affected plants are either white (total albinism), either having extended white sectors (variegated) or either becoming green with ageing (virescent). *Zantedeschia* is a model crop for researching plastid DNA-inheritance after interspecific crosses. Yao *et al*. (1994 1995) found that the use of bridge plants (plants that can be successfully crossed with both partners of an incongruous cross) may help in obtaining viable hybrids between 2 sections of this genus. *In vitro* embryo rescue experiments (Yamaguchi 1986) on *Rhododendron* reveal that, although more seedlings were obtained, the relative number of albinos increased.

All plastids develop from proplastids according to the requirements of each differentiated cell, and which type is present is determined in large part by the nuclear genome (Gruissem 1989). Proplastids are present in meristematic cells and can develop into specialized plastid types such as chloroplasts, amyloplasts or chromoplasts. Chloroplast development is associated with significant morphogenic and molecular changes, the most visible being the accumulation of chlorophyll and the assembly of the complex thylakoid membrane system. In darkness, proplastids normally develop into etioplasts, which are characterized by the presence of one or several prolamellar bodies (Ryberg *et al.* 1993). Following the transfer of dark-grown leaves to light, the regular structure of the prolamellar bodies gradually disappears, and the prothothylakoids of the etioplastids are transformed into thylakoids and the membranes start to overlap, forming typical grana.

According to Yao and Cohen (2000) hybrid *Zantedeschia* albinos do not develop prolamellar bodies in their etioplasts in darkness and do not form grana in light, which indicates that the block to chloroplast development occurs before or during the development of etioplasts. They propose a multiple gene control of plastome-genome incompatibility and plastid DNA-inheritance, based upon backcrosses that show a higher compatibility as the number of genes from the parent contributing the plastids increases.

During interspecific *Rhododendron* breeding, albinism occurs rather frequently, even after intrasubgeneric pollinations (Michishita *et al.* 2002). In Japan, evergreen *R. indicum* hybrids could be crossed with deciduous azaleas thanks to embryo rescue techniques provided the evergreen azalea was the seed parent (Ureshino *et al.* 1998 1999, Miyajima *et al.* 2000). It appeared that after a three-way cross more seedlings inherited plastid-DNA from the pollen donor than after a control cross. The reason for this phenomenon remains unclear; however the inheritance of plastid-DNA from pollen donors has been described before (Hagemann 1992, Yao *et al.* 1994). Using a rapid screening method through DAPI fluorescence, Corriveau and Coleman (1988) detected putative plastid-DNA in the generative and/or sperm cells of pollen from 43 species (out of 235 species tested). One of these 43 species was *R. maximum* Linn. (the only Ericaceae species in the test). Biparental influences on plastid development had already been proposed by Noguchi (1932). The occurrence of albinism is often linked to the inheritance of plastid-DNA and therefore to application of either parent as pollen donor or receptor. According to Krebs (1997) the inhibition of synapsis is mainly a problem in intersubgeneric crosses.

2.2.3. Lack of growth vigor

Lack of growth vigor is often correlated with low chloroplast content. However, also growth of green seedlings may get arrested due to the incompatibility between both parental genomes and the formation of non-functional (consisting of hybrid subunits) proteins. Lack of growth vigor can not be resolved and therefore needs to be prevented to the largest possible extent by other parent plant combinations or a larger quantity of pollinations. Hybrid weakness, dwarfism and inviability have been reported in many plant genera: according to Levin (1978) these effects are caused by genome disharmony and incompatible development cues, the deleterious, complementary action of one or a few genes, and/or cytoplasmic effects.

2.2.4. Hybrid breakdown

Pollination with pollen of related species can induce embryo development but later on elimination of the chromosomes of the wild species can occur, causing the development of a haploid plantlet. This technique is widely used in barley (*Hordeum vulgare* x *Hordeum bulbosum*) (Kasha and Kao 1970), wheat (Ahmad and Comeau 1990), oat (Riera-Lizarazu *et al.* 1996) and potato (*Solanum tuberosum* x *Solanum phureja*) (Rowe 1974). DNA-sequence elimination is one of the major responses of the wheat genome to wide hybridization (Shaked *et al.* 2001). Also pollen of a triploid cultivar was able to induce parthenogenesis in *Citrus* (Germana and Chiancone 2001).

3. HYBRID STERILITY

Errors in chromosome segregation at meiosis may cause F_1 sterility (Heslop-Harrison 1999). The occurrence of mismatches is prevented by so-called "mismatch repair" genes which prevent non-homologous chromosomes to cross-over (Hunter *et al.* 1996). This failure leads to an unbalanced chromosome number in the gametes which results in sterility. Even when chromosome pairing is able to proceed, low F_1 fertility may be controlled by sterility genes (Ha *et al.* 1998). One of the most striking consequences of a disturbed synapsis is the abortion of the malformed embryo sac in many intersubgeneric hybrids, causing female sterility. Microspores resulting from a disturbed meiosis usually are non-viable (Van Tuyl and De Jeu 1997, Shamina *et al.* 1999).

Embryo rescue may need to be combined with allopolyploidization to result in fertile offspring (Chen *et al.* 2002), which can be used as pre-breeding material. Two strategies for allopolyploidization may be implemented: mitotic chromosome doubling by chemical agents or meiotic chromosome doubling by the application of unreduced (2n) gametes. After allopolyploidization, the presence of homologous genome pairs will allow normal meiosis to proceed.

A myriad of genetic and epigenetic reactions may be induced after allopolyploid formation (Soltis and Soltis 1993 2000, Chen and Pikaard 1997, Hieter and Griffiths 1999, Matzke *et al.* 1999, Comai 2000, Comai *et al.* 2000, Liu and Wendel 2000 2002, Otto and Whitton 2000, Wendel 2000, Baumel *et al.* 2002, Kashkush *et al.* 2002, Osborn *et al.* 2003, Madlung and Comai 2004, Adams and Wendel 2005, Han *et al.* 2005). Next to 'normal' genome additivity or gene introgression, karyotypic stabilization owing to rapid chromosomal repatterning, translocations, sequence elimination, transposon activity and epigenetic effects may largely affect phenotypic behavior of the hybrids obtained.

3.1. Mitotic chromosome doubling

Colchicine, an allelopathic compound produced by *Colchicum autumnale*, is known to inhibit the formation of spindle fibers and effectively arrest mitosis at the anaphase stage (Hancock 1997). Since chromosomes have already multiplied but cell division is arrested, polyploid cells are created. However, colchicine is a carcinogenic compound and generally less efficient than modern alternatives. During the seventies, nitrous oxide was the first applied alternative (Taylor *et al.* 1976); nowadays, there is a tendency towards the use of herbicides with a similar mode of action, such as the dinitroanilines oryzalin and trifluralin (Morejohn *et al*. 1987, Verhoeven *et al.* 1990, Vaughn and Lehnen 1991, Van Tuyl *et al.* 1992, Rao and Suprasana 1996, Hansen and Andersen 1996), the phosphoric amides such as amiprophos methyl (Hansen *et al.* 2000) and the carbamate herbicides like chlorpropham and propham. Other widely used mitotic disrupter herbicides include dithiopyr and pronamide. All of these compounds block mitosis in a similar way, by preventing the formation of the microtubules of the mitotic spindle along which chromosomes are separated (Morejohn *et al.* 1987).

The more detailed mode of action of the aforementioned herbicides is described by Vaughn (2000). Antimitotic herbicides are mostly used in lower doses than colchicine, are more efficient and less toxic for humans (Vaughn and Vaughan 1988). *In vivo* material appears to be less sensitive than *in vitro* material; surfactants, wetting agents and other carriers like DMSO sometimes enhance efficiency (Kehr 1996).

It should be kept in mind that mitotic allopolyploids produce homogenous gametes, due to preferential pairing between homologous chromosomes, which increases the predictability of the F_2, but on the other hand limits the expression of recessive genes that are only present in the chromosomes of one of both parental species. Only meiotic allopolyploidization (after the fusion of 2n gametes) may result in direct introgression of desired genes.

3.2. Meiotic chromosome doubling

Evidently, 2n gametes would provide a better tool for allopolyploidization since FDR (first division restitution), SDR (second division restitution) and IMR (indeterminate meiotic restitution; **Fig. 1**) allow homoeologous recombination (Mendiburu and Peloquin 1977, Karlov *et al.* 1999).

Unreduced gametes occur in most plant species (Harlan and De Wet 1975, Veilleux 1985, Lamote *et al.* 2002) and also in doubled haploids (Crespel *et al.* 2002). In *Lilium*, the formation of 2n gametes is dramatically increased upon hybrid formation (Van Tuyl *et al.* 1989), a phenomenon that is also observed in other crops (Van Tuyl and De Jeu 1997, Ramanna *et al.* 2003, Lim *et al.* 2003a 2004). Therefore 2n gamete formation would provide a very convenient tool for efficient introgression of desired genes in F_2 interspecific hybrids. Unreduced gametes have indeed been applied in a number of breeding programs (Lim *et al.* 2003b, Lyrene *et al.* 2003, Barba-Gonzalez *et al.* 2004).

According to several authors different mechanisms of nuclear restitution are determined by single genes (Mok and Peloquin 1975, Bretagnolle and Thompson 1995). If only one of the parents in a cross contributes a 2n gamete it is called unilateral sexual polyploidization, as opposed to bilateral sexual polyploidization (both parents contributing an unreduced gamete; Mendiburu and Peloquin 1977).

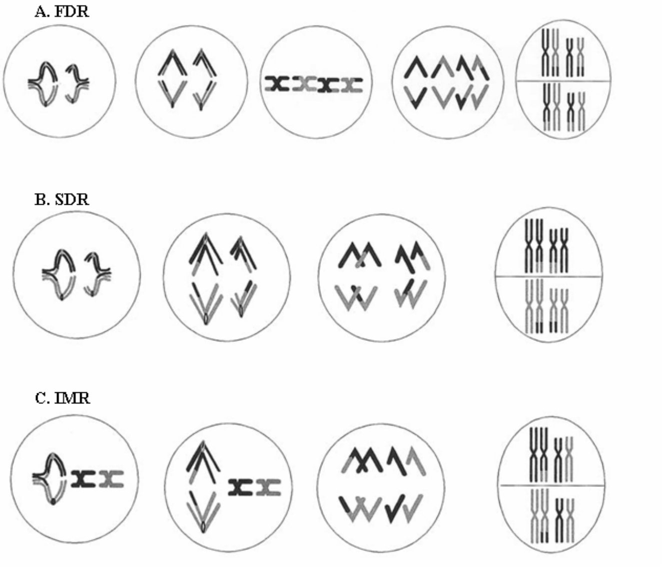

Fig. 1 Consequences of FDR, SDR and IMR (after Ramanna and Jacobsen, 2003). (**A**) **FDR.** Both pairs of homoeologous chromosomes have paired. During the second meiotic division all chromosomes divide as single unit by forming one equatorial plate (fused spindle). Parental chromosomes are preserved in the restitution gametes. (**B**) **SDR.** Homoeologous chromosomes pair normally but after normal disjunctional separation, the nuclei at both poles restitute; centromeres divide but the chromatids do not separate. The parental chromosome constitution is disrupted. (**C**) **IMR.** Both bivalent and univalents are formed in the meiocyte. The bivalent disjoins normally whereas the univalents divide equationally and the nuclei restitute. As in SDR, the parental chromosome constitution is not preserved.

The types of intergenomic recombination that occur during the origin of 2n-gametes depend on the meiotic abnormalities that give rise to 2n-spores (Mok and Peloquin 1975, Mendiburu and Peloquin 1977, Veilleux 1985, Lim *et al.* 2001a, Ramanna and Jacobsen 2003). If only univalents are formed at metaphase I the entire chromosome complement may be oriented on the equatorial plate, and all of them divide equationally. This is similar to mitosis and gives rise to 2 identical nuclei; after cell wall formation and without the second division a dyad with two 2n-spores is formed. This is typical for FDR without homoeologous recombination. FDR can also occur when both univalents and bivalents are formed during metaphase I; bivalents fall apart as half-bivalents which divide equationally (like the univalents). Recombinant chromatids are present in the 2n-gametes and therefore they form FDR gametes with recombination.

SDR occurs when all the homoeologous chromosomes pair at metaphase I and disjoin normally at anaphase I, leading to random assortment of homoeologous chromosomes. Centromeres then divide but the sister chromatids of each half-bivalent are present in the same nucleus. As a consequence, not all parental chromosomes are present in the 2n-gamete and every chromosome is present in pairs, as opposed to FDR 2n gametes in which all hybrid chromosomes are represented once. Genetic recombination due to crossing-over as well as homoeologous chromosome assortment occurs.

A novel restitution type has been discovered in interspecific *Lilium* hybrids (Lim *et al.* 2001). IMR is an in-between system compared to FDR and SDR. Chromosome associations can equally be univalent or bivalent. The unique feature of the system is that all bivalents and univalents are oriented on the equatorial plate in the microspore mother cell and all of them divide simultaneously at the modified anaphase I. The bivalents disjoin as in normal anaphase I so that the half-bivalents move to the opposite poles (like in SDR). At the same time all univalents divide equationally (like in FDR) and the chromatides move to the opposite poles. As a result, the exact sporophytic chromosome number is restored but some of the chromosomes will be represented as single copies (like in FDR) whereas the others will be present in pairs (like in SDR).

4. PROTOPLAST FUSION

Somatic hybridization by the fusion of protoplasts allows circumventing sexual crossing barriers between different species. With the discovery of an enzymatic method for protoplast isolation (Cocking 1960) isolation of large amounts of protoplasts became possible. For fusion, the two most powerful systems to date are the PEG-high pH/Ca2+ method and electrofusion (Pelletier 1993) which together account for virtually all the fusions currently conducted. The latter offers the advantage of a better control of the fusion conditions, and is more suitable for fragile protoplast types (Waara and Glimelius 1995). According to Trigiano and Gray (2000) the fusion product yield is increased 20-fold by using electrofusion.

Nurse culture, plating technique and feeder layer, and culture in a microchamber are the three basic techniques used for protoplast culture (Eigel and Koop 1989). The plant regeneration capacity is affected by many factors, of which genotype, donor tissue, culture medium and protoplast manipulation are the most decisive (Skarzhinskaya *et al.* 1996). 'Traditional' somatic fusion has yielded interspecific plants in several genera (Al-Atabee and Power 1990, Nakano and Mii 1993, Reichert and Liu 1996, Kim *et al.* 2003).

Theoretically, fusion is possible between cells of completely different origin. However, the regeneration ability decreases as the phylogenetic difference between fusion partners increases. In symmetrical fusions the genetic contribution of both partners is equal, which often decreases the viability, regeneration capacity and fertility of the fusion products. In these cases, the fused cell stops dividing, shoot meristems fail to develop, albinism appears, shoots root insufficiently or the acclimatized plantlets are sterile. This is the result of a 'gene conflict' caused by unbalanced genomes, as certain chromosomes repel one another, so cell division creates aneuploid cells that fail to develop any further. Also the different developmental programs of both partners contribute to the gene conflict.

A solution is provided by asymmetric protoplast fusion. In this approach, the chromosomes of the donor protoplast are fragmented by γ-, X- or UV-irradiation (Hall *et al.* 1992) or ultracentrifugation; these fragments are to be integrated into the acceptor genome (Forsberg *et al.* 1998). Only in the case of UV (254 nm)-irradiation, increasing doses always result in a more efficient fragmentation and integration in the recipient genome (Forsberg *et al.* 1998, Yamagishi *et al.* 2002, Xiang *et al.* 2003, Xu *et al.* 2003, Cheng *et al.* 2004). Moreover, UV-radiation causes relatively many single strand cleavages and is responsible for pyrimidin dimerisation, cytosine hydratation and crosslinking between different strands or between DNA and proteins (Setlow 1972, Cieminis *et al.* 1987). Asymmetric hybrids tend to root better, to regenerate easier and to be more fertile owing to the smaller 'gene conflict'. The technology allows to transfer polygenous traits between different genera, even if the genes involved have not been characterized. Especially Solanaceae and Brassicaceae species are suitable for regeneration, although some regeneration experiments within Poaceae, Fabaceae and various woody plants have also been successful (Parokonny *et al.* 1994, Rasmussen *et al.* 1997, Rutgers *et al.* 1997, Forsberg *et al.* 1998, Ilcheva *et al.* 2001, Zhou *et al.* 2001, Yamagashi *et al.* 2002, Xiang *et al.* 2003, Xu *et al.* 2003, Li *et al.* 2004). Within the ornamental section results were achieved within the Liliaceae (Saito and Nakano 2002, Horita *et al.* 2003). Using embryogenic callus or somatic embryos as a source for acceptor protoplasts is responsible for the establishment of regeneration in various genera. (Matsumoto *et al.* 2002). A supplementary advantage of asymmetric fusion is that the required number of backcross and selection cycli is strongly reduced (Waara and Glimelius 1995, Kinoshita and Mori 2001).

Microprotoplast mediated chromosome transfer (MMCT) allows to transfer a single chromosome from donor to acceptor. After treatment with mitosis inhibitors cells or microspores produce micronuclei, from which protoplasts are isolated. By ultracentrifugation, these disintegrate into small microprotoplasts that contain only 1 or 2 chromosomes. In other words, the technique is very suitable for the creation of addition lines. For the liliaceous monocot *Hemerocallis hybrida*, Saito and Nakano (2002) have described a protocol starting from suspension cultures initiated from creamy-white calli. The cultures, consisting of fine clumps of 20-50 cells, are subcultured on Murashige-Skoog based medium and then treated with DNA synthesis inhibitor (8 µM propyzamide) to induce micronucleation. In dicot plants, other mitosis inhibitors are usually more efficient. After 78h of the initiation of micronucleation treatment, suspension cells were incubated for 6h in a cell-wall digesting enzyme solution. Protoplast suspensions are then filtered through a 50 µm nylon sieve. The suspension is then layered on top of a continuous iso-osmotic PERCOLL gradient, that is prepared by the addition of 0.5M sorbitol to PERCOLL followed by ultracentrifugation at 200000g for 30 min at 4°C. After layering, a second ultracentrifugation cycle of 1.5h is performed. All bands are gathered, diluted with 0.5M sorbitol and filtered through nylon sieves with decreasing pore sizes. This results in a population containing predominantly smaller protoplasts (containing 1 or 2 chromosomes). The actual transfer is performed by PEG-mediated fusion.

Altogether, it is a proper alternative for UV-irradiation, although application is limited to a distinct number of genera (Rutgers *et al.* 1997, Binsfeld *et al.* 2000, Saito and Nakano 2002). Theoretically, UV-irradiation and MMCT may be combined to produce small fragments of a single chromosome.

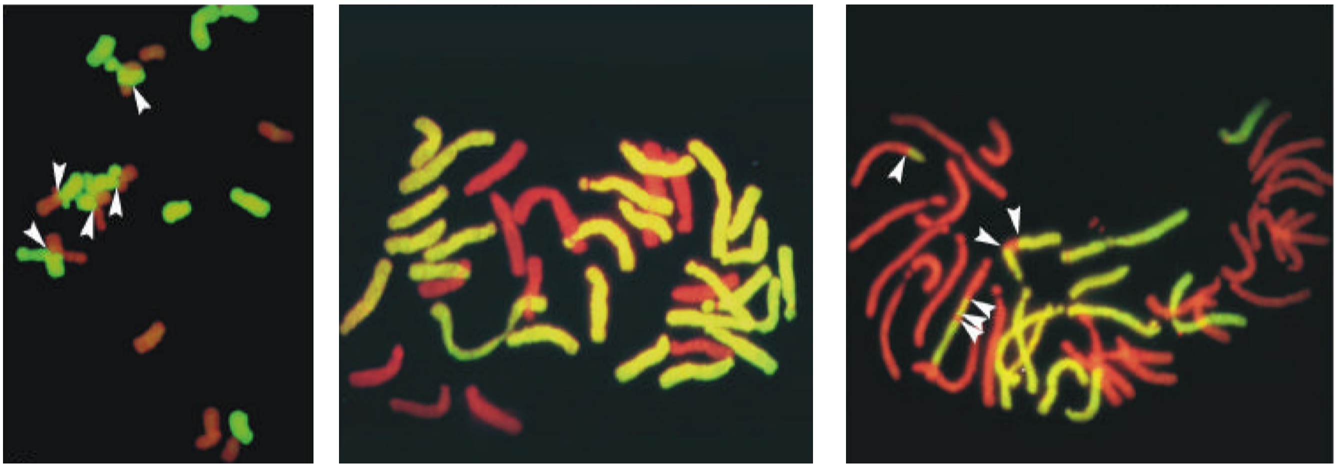

Fig. 2 GISH applications in interspecific *Lilium* hybrids (after Lim *et al.* 2001b). Left: Chromosome differentiation due to GISH in metaphase I of an interspecific hybrid, showing 5 bivalents (arrowheads) and 14 univalents. Middle: Chromosome constitution of a triploid interspecific hybrid, showing 24 (yellow) and 12 (red) chromosomes of either parent. Homoeologous recombination is totally absent. Right: Chromosome constitution of a triploid interspecific hybrid, showing 24 (red) and 12 (yellow) chromosomes of either parent. Arrowheads indicate recombinant segments.

5. MOLECULAR AND CYTOGENETIC CHARACTERIZATION OF HYBRIDS

One of the important requirements for introgression breeding is the availability of techniques for monitoring the presence or absence of alien chromosomes or the recombinant segments in the back-cross (BC) progenies. By the development of DNA in situ hybridisation methods during the last two decades, that identification of alien genetic material in the hybrids and BC is possible with unprecedented accuracy. One of these methods is genomic *in situ* hybridisation (GISH), a technique that involves the direct hybridisation of labelled, single stranded DNA of one species to denatured metaphase chromosomes of mitotic or meiotic stages that are spread out on a microscopic slide (**Fig. 2**). Either total genomic DNA of one of the parents or cloned DNA sequences of other origin are used. The chromosome preparations are then evaluated under a fluorescent microscope. Several fluorochromes that enable the simultaneous detection of chromosomes from 2 or more sources in a hybrid can be used (Gill and Friebe 1998, Raina and Rani 2001).

Cytogenetic studies after interspecific pollination have been performed in the genera *Crocus* (Orgaard *et al.* 1995), *Alstroemeria* (Kuipers *et al*. 1997, Kamstra *et al.* 1999, Ishikawa and Ishizaka 2002), *Allium* (Keller *et al.* 1996, Friesen *et al.* 1997, Khrustaleva and Kik 1998), *Lilium*

(Karlov *et al*. 1999, Lim *et al*. 2000) and *Helianthus* (Atlagic *et al.* 2005). Besides being suitable for detection of alien genetic material in hybrids, the GISH technique is of great value for elucidating certain basic questions on the phylogeny (Leitch and Bennett 1997), meiotic nuclear restitution mechanisms and genetic recombination (Kamstra *et al*. 1999, Lim *et al*. 2001).

RFLP and PCR-based DNA-marker techniques such as RAPD, AFLP and microsatellites have been found efficient for the control of effectiveness of a cross in several ornamental genera, such as *Allium* (Friesen *et al.* 1997), *Alstroemeria* (Han *et al.* 1999), *Rhododendron* (Kron 1993 1998, Kobayashi *et al.* 1996, De Riek *et al.* 1999 2000, Kita *et al.* 2005). For this purpose, co-dominant marker techniques such as microsatellite markers that enable to visualise the parental alleles of both crossing partners are much preferred. However, if such markers are not available, a (dominant) multilocus fingerprinting technique (like AFLP) can be successfully applied. In such case, primer pairs that amplify the best unique markers, present only in the crossing partner to be introgressed, have to be selected. Especially in remote crosses using e.g. unrelated wild species in *Solanum* (Grumeza *et al*. 2004), quite a high number of AFLP primer combinations have to be tested to pinpoint the relatively small fragments that are introgressed.

A combination of *in situ* hybridisation and molecular markers techniques is not only useful for monitoring the hybrids and back cross progenies for introgression, but also for elucidating the modes of origin of 2n-gametes, the extent of genetic recombination and the phylogeny of the species and hybrid cultivars.

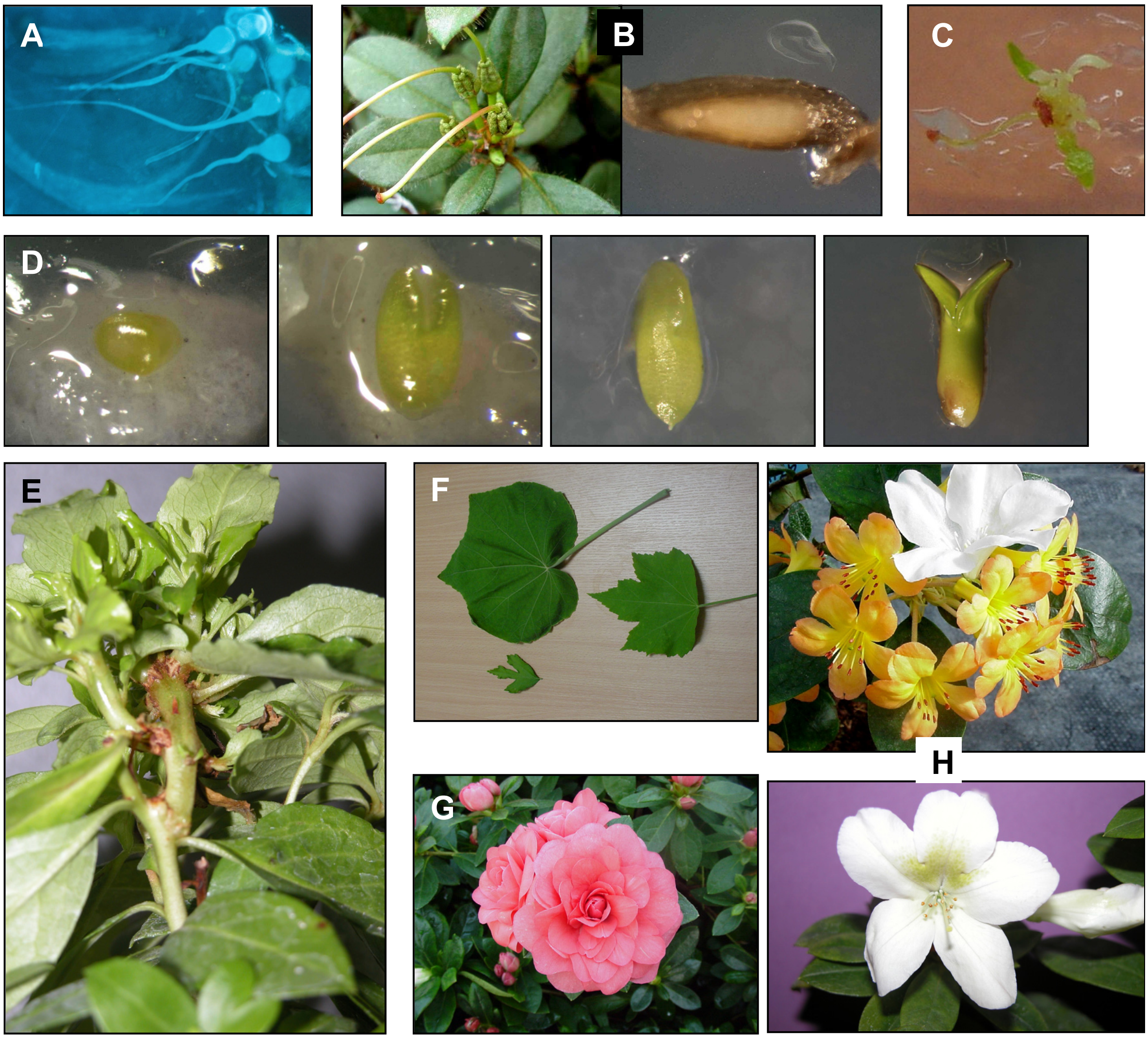

Fig. 3 Different interspecific crossing barriers and techniques used to characterize and overcome them. (**A**) Aniline blue staining of pollen tube growth (*Calathea*). (**B**) Poor fruit and seed development (*Rhododendron*). (**C**) Virescent albinism (*Rhododendron*). (**D**) Isolation of globular, heart-shaped, torpedo-shaped and cotyledonary *Hibiscus* embryos *in vitro*. (**E**) Growth aberration in *Rhododendron* interspecies hybrids. (**F**) Leaf of an interspecific *Hibiscus* seedling (right) compared to both parents (left). (**G**) Introduction of new flower shapes in Belgian pot azalea after interspecific pollination. (**H**) Flowering *Rhododendron* hybrid (lower picture) with intermediary flower shape compared to its parents (upper picture).

6. CONCLUSION

The creation of interspecific hybrids, along with chromosome doubling technology, offers extended opportunities for ornamental breeders (Van Tuyl and Kim 2003), as demonstrated in many genera (Eikelboom and Van Eijk 1990, Sparnaaij and Koehorst van Putten 1990, Lu and Bridgen 1997, Kato and Mii 2000, Nomura *et al.* 2002, Eeckhaut 2003, Sujatha and Prabakaran 2003, Kobayashi *et al.* 2004, Van Huylenbroeck *et al.* 2004).

In interspecific breeding programs, the frequent occurrence of multiple barriers necessitates an integrated approach by the use of various techniques, aiming for sexual or asexual fusion, regeneration and screening (**Figs. 3, 4**). Moreover, incongruity is determined by both genetic and environmental factors, and the variation within genotypes and environmental conditions applied in experiments should therefore be substantial. Somatic fusions can be an alternative for sexual breeding, and especially asymmetric protoplast fusion offers extended possibilities. Whatever the fusion technique applied, *in vitro* techniques can play an important part in improving the overall efficiency in many crops. Molecular and cytogenetic screening procedures are to be included to determine the genomic composition of all alleged hybrids.

Polyploidization, whether mitotically or meiotically induced, is closely linked to interspecific breeding. Crossability between parents may be improved in the process of plant breeding by equalizing their functional ploidy level. Breeding at polyploid levels is widely used in interspecific hybridization programs of many ornamental crops such as *Alstroemeria*, *Dendranthema*, *Freesia*, *Gladiolus* and *Lilium* (Van Tuyl and De Jeu 1997). F_1-sterility of interspecific hybrids is very common and may, among others, be the consequence of reduced chromosome pairing during meiosis. Often, the only viable gametes produced have the same chromosome number as the somatic cells of the hybrid. However, these unreduced gametes are to be preferred over mitosis inhibiting chemicals for the induction of polyploids, as they allow introgression. A better understanding of allopolyploid/interspecific hybrid associated phenomena would undoubtedly lead to substantial innovation in the commercial assortment of many ornamentals.

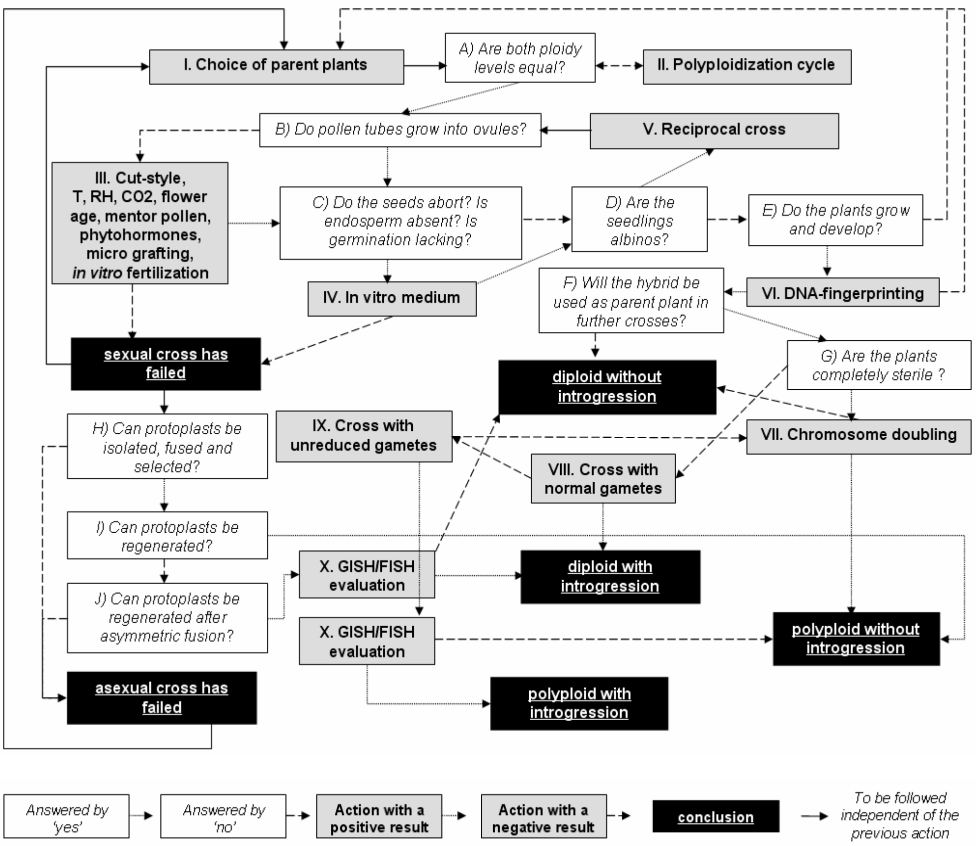

Fig. 4 Integrated approach to circumvent interspecific incompatibility in ornamental plant breeding. After performing technical steps and actions (grey boxes, labeled by Roman figures) different barriers may be encountered (white boxes, labeled by letters). Depending on technical feasibility of the actions, the presence of barriers and the efficiency of various techniques that were applied, new barriers can be met and new actions will be undertaken.

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