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METHODS FOR OVERCOMING INTERSPECIFIC CROSSING BARRIERS

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SUMMARY

Crossing barriers occur frequently when intra- or interspecific crosses are attempted. These barriers are the result of incompatibility and incongruity. Sexual barriers preventing interspecific hybridization have been distinguished into pre- and post-fertilization barriers. The nature of the barrier determines the method to be used to overcome the specific barrier. A range of techniques such as bud pollination, stump pollination, use of mentor pollen and grafting of the style have been applied successfully to overcome pre-fertilization barriers. *In vitro* methods in the form of ovary, ovary slice, ovule and embryo culture are being used to overcome post-fertilization blocks which cause endosperm failure and embryo abortion. An integrated method of *in vitro* pollination and fertilization followed by embryo rescue has been applied in many crosses. Vital hybrid plants may display lack of flowering or male and female sterility resulting in failure of sexual reproduction. If sterility is caused due to lack of chromosome pairing during meiosis, fertility may be restored by polyploidization, enabling pairing of homologous chromosomes in the allopolyploid hybrid. Integration of these techniques to the breeding program would enable the breeder to introgress genes across the species barriers.

INTRODUCTION

The phenomena underlying crossing barriers are incompatibility and incongruity. Incompatibility operates in intraspecific crosses and is the result of the activity of S-alleles. Incongruity occurs in interspecific crosses as a result of a lack of genetic information in one partner to complete pre- and post-pollination processes in the other (Hogenboom 1973). This chapter focuses on different methods used to overcome incongruity.

Interspecific and intergeneric crosses are made to introduce new genetic variation into cultivated plants. In breeding ornamental crops, interspecific hybridization is the most important source of genetic variation. Many of the cultivars have originated from complex species crosses which have given rise to a broad range of shapes and colours to plants and flowers (Ohri and Khoshoo 1983; Van Eijk *et al.* 1991; Ramanna 1992; Van Creij *et al.* 1993). Many examples in this chapter are chosen from research on ornamental plants especially *Lilium*, because lily is a model plant for style manipulations and *in vitro* culture methods. For an overview of the entire field of reproductive barriers the reader may refer to reviews by Frankel and Galun (1977), Raghavan and

Srivastava (1982), Shivanna (1982), Sastri (1985), Williams *et al*. (1987), Khush and Brar (1992), Liedl and Anderson (1993) and Pickersgill (1993).

The sexual barriers hampering interspecific hybridization have been distinguished into pre- and post-fertilization barriers (Stebbins 1958). Many studies deal with methods for overcoming pre-fertilization barriers. Once fertilization has occurred, hybrid embryo growth may be restricted by post-fertilization barriers. Both embryo and endosperm have to develop an equilibrium for sharing nutrients in an undisturbed developmental process. In general the first division of the zygote is delayed to favour the first division cycles of the endosperm cells. When the equilibrium in the development of the zygote and endosperm is disturbed an abortion of the young embryo or disintegration of endosperm follows. This abortion can take place in various stages of development of the young seed. Depending on the stage of embryo abortion various *in vitro* techniques can be applied to rescue the abortive embryo.

Crossability barriers imposed by temporal and spacial isolation of the parents (see Chapter ...) can be effectively overcome through pollen storage. As pollen storage is discussed in detail in Chapter .., it is not covered here.

TECHNIQUES FOR OVERCOMING STIGMATAL AND STYLAR BARRIERS

Genetic variation in interspecific crossability

'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 of Hermsen (1984a) implies that crossability is determined both by genetic and environmental factors. It is therefore necessary to test different accessions of both the parents for hybridization programs.

Unilateral incongruity is the phenomenon that a cross is successful only in one direction, whereas the reciprocal cross fails. In lily, crossing barriers can be overcome using cut-style pollination, but mostly in one cross direction only (Van Creij *et al*. 1993). In interspecific crosses between *Sorghum* and maize Laurie and Bennett (1989) found the variation of pollen tube inhibition to be due to one single gene.

Use of mixed and mentor pollen

The use of mixed pollen i.e. mixture of compatible and incompatible pollen (Brown and Adiwilaga 1991) and mentor pollen, i.e. compatible pollen genetically inactivated by irradiation but still capable to germination, is reported to overcome inhibition in the style in many plant species, when used together with incongruous pollen (see chapter 14 of M. Villar on the mentor pollen technique). For lily, mentor pollen was effective in overcoming self incompatibility but not in interspecific crosses (Van Tuyl *et al*. 1982).

Influence of environmental conditions

The presence of the optimal level of receptivity of the stigma can vary from several hours (in mango) to more than one week (in lily). It determines the optimal time of pollination.

A positive effect of high temperature in overcoming self incompatibility and incongruity has been detected and applied in breeding programmes of lily by heating the style (Ascher and Peloquin 1968) or by pollinating at high temperatures (Van Tuyl *et al*. 1982; Okazaki and Murakami 1992). Probably heat-sensitive inhibitors of pollen tube growth are inactivated. Comparable effects of floral aging on pollen tube growth are reported by Ascher and Peloquin (1966).

Style and ovary manipulations

As it was firstly demonstrated more than 60 years ago in *Datura* (Blakeslee 1945), pollen

tube growth inhibition in the style can be overcome using different pollination techniques in which style and ovary are manipulated (*Fritillaria*: Wietsma *et al*. 1994; *Lilium*: Myodo 1963; Van Tuyl *et al*. 1988, 1991; Janson *et al*. 1993; *Lathyrus*: Davies 1957; Herrick *et al*. 1993; *Nicotiana*: Swaminathan and Murti 1957). One of these manipulations involves removal of the stigma and a part or whole of the style and pollinating the cut end. This is referred as stump pollination or 'cut-style', 'intrastylar' or 'amputated-style' pollination. In lily two types of incongruity are distinguished based on the arrest of pollen tube growth at different levels of the style. In the first type the site of arrest of pollen tube growth is just below the stigma. This 'upper-inhibition' occurs 12 to 24 h after pollination and results in short pollen tubes. In the other type, pollen tube growth ceases halfway in the style 3 to 4 days after pollination. This 'lower-inhibition' results in medium sized pollen tubes (Asano 1980b 1985). In a comparison of several pollination methods, it was shown that pre-fertilization barriers in lily can be circumvented by using the cut-style technique (Van Tuyl *et al*. 1991; Janson *et al*. 1993). Following stump pollination, many pollen tubes of e.g. lily, *Lathyrus* and *Fritillaria* grow normally into the ovary. In this way pollen circumvents stylar and stigmatal barriers which can inhibit pollen tube growth. However, a complication associated with this method in lily is the low seed set, probably caused by the premature arrival of pollen tubes in the ovary (Janson *et al*. 1993). A majority of the pollen tubes either grow past the inner integument or did grow along but not into the micropyle after cut-style pollination. Activation of the ovary by a pre-pollination preceding intrastylar or placental pollination, did not result in an increase of the percentage of ovule penetration. The percentage of ovule penetration after cut-style pollination did increase when a longer part of the style was retained on the ovary (Fig. 1). Despite the low seed set, a large number of unique interspecific lily hybrids was obtained using this method (Asano and Myodo 1977; Asano 1980a; Okazaki *et al*. 1992; Van Creij *et al*. 1993). Also, in crosses between *Fritillaria imperialis* and *F. raddeana* Wietsma *et al*. (1994) were able to obtain interspecific hybrids using the cut-style technique.

The stylar graft technique was applied successfully in order to improve the cut-style technique. (Van Tuyl *et al*. 1991). In this method, pollen grains are deposited on a compatible stigma. After one day, the style of the pollen donor is cut 1-2 mm above the ovary and grafted on the ovary of another plant. Style and stigma are joined *in vivo* using a piece of a straw filled with *L. longiflorum* stigmatic exudate or are stuck together with only the exudate. *In vitro* a piece of 'water agar' was placed on the style (Fig. 2ab). Table 1 shows the effect of different pollination methods on average seed set. It can be observed that cut-style pollinations result in a low seed set, whereas the grafted-style method gives a better seed set, but was not successful in a high percentage of the pollinated ovaries.

Chemical treatments

Application of growth regulators, such as auxins, cytokinins and gibberellins to the pedicel or the ovary at the time of or soon after pollination may improve fruit and seed set after interspecific pollination (Emsweller and Stuart 1948; Dionne 1958; Al Yasiri and Coyne 1964; Pittarelli and Stavely 1975). Application of growth regulators to delay abscission of the style and show positive effects on the development of young fruits (Kruse 1974; Larter and Chaubey 1965; Islam et al. 1975; Fedak 1978; Alonso and Kimber 1980; Mujeeb-Kazi 1981). In many crosses, application of growth substances promote post-pollination development up to a stage when hybrid embryos can be excised and cultured (Islam 1980; Subrahmanyam 1979; Sastri et al. 1981, 1983). In interspecific crosses of *Populus*, treatment of the stigma with organic solvents such as hexane and ethyl acetate before pollination has been reported to be effective in overcoming pre-fertilization barriers (Willing and Pryor 1976). Also immunosuppressors such as amino-n-caproic acid, salicylic acid and acriflavin have been used to produce wide hybrids in many cereals (Baker et al. 1975; Tiara and Larter 1977; Bates et al. 1979; Mujeeb-Kazi and Rodriguez 1980; Mujeeb-Kazi 1981) and legumes (Baker et al. 1975; Chen et al. 1978) The female parent is treated with immunosuppressors before and/or after pollination for many days and the resulting embryo is cultured on a suitable medium. It has been suggested (Bates and Deyoe 1973) that crossability barriers between distantly related taxa are mediated through a specific inhibition reaction analogous to immunochemical mechanism of animals; treatment with immunosuppressors inactivates these immunochemical reactions. However, there is so far no evidence to indicate the involvement of immunochemical reactions in interspecific crosses.

TECHNIQUES FOR OVERCOMING POST-FERTILIZATION BARRIERS

A range of *in vitro* methods have been developed to overcome post-fertilization barriers in a number of plant species. When abortion occurs in a very young stage and maternal tissue has no negative influence on the development of seeds, ovary culture can be applied: young fruits can be grown *in vitro* to a stage at which dissection of embryos is possible. In some crops, the ovary is large and slicing the ovary in small parts and influencing them, is a better option for rescuing the young seedlings *in vitro*. This technique is referred to as 'ovary slice culture'. When the mismatch between embryo and endosperm development starts very early and ovary culture and/or ovary slice culture fails, ovules can be dissected out of the ovaries and be cultured *in vitro*. If young fruits

can stay for a longer time on the mother plant, embryo rescue can be applied by different methods: *in ovulo* rescue and embryo rescue. Since Hännig (1904) employed embryo culture for the first time 90 years ago, these techniques are applied in numerous crops (Williams *et al*. 1987).

Ovary culture and ovary-slice culture

Ovary culture has been applied in many species: *Brassica* (Inomata 1980; Gundimeda et al. 1992; Kerlan *et al*. 1992; Sarla and Raut 1988; Takeshita *et al*. 1980), *Eruca-Brassica* hybrids (Agnihotri *et al*. 1990), *Lilium*, *Nerine* and *Tulipa*, (Van Tuyl *et al*. 1993) and *Phaseolus* (Sabja *et al*. 1990). Ovary-slice culture was applied by Kanoh *et al*.(1988), Straathof *et al*. (1987) and Van Tuyl *et al*. (1991) for the production of interspecific *Lilium* hybrids. Ovaries were harvested 7-40 days after intrastylar pollination and, after surface sterilising, sliced into 2 mm thick disks. Seed germination occurred 30-150 DAP. By this method plantlets were obtained from very small embryos.

Ovule culture

In those crops in which the fruit is aborted before embryo culture can be applied, ovule culture is an easy and fast method. This technique is applied in *Alstroemeria* (Bridgen *et al*. 1989), *Cyclamen* (Ishizaka and Uematsu 1992), *Lycopersicon* (Neal and Topoleski 1985), *Nicotiana* (Iwai *et al*. 1986) and *Vitis* (Gray *et al*. 1990). The production of interspecific hybrids in *Alstroemeria* will be discussed as an example. In *Alstroemeria* fertilization occurs 24 hours after pollination (De Jeu *et al*. 1992). Ovaries are harvested 2 DAP, the ovules are dissected and placed on an MS medium containing 9 % sucrose. Six weeks later the ovules are transferred to an MS medium with 4-5 % sucrose. Germination of the ovules starts 1-2 weeks later (De Jeu *et al*. 1992). Histological studies of the *in vitro* cultured ovules revealed, that the ovules within the first two weeks of culture enlarged to twice their size due to proliferation of the inner and outer integuments. No development of cellular endosperm *in vitro* was found, whereas the embryo cells divided almost normally (Fig. 3A). By 42 DAP, the first plantlets arose from the cultured ovules (Figs. 3BC) whereas normal seed development in *Alstroemeria* takes 2½-3 months (De Jeu 1992). Depending on the genotypic combination of the interspecific crossing, the percentage of seedlings obtained from ovule culture varied from 0.5-22.5%, whereas in the *in vivo* situation on the plant no seeds could be harvested.

Monnier and Lagriffol (1985) compared the growth of *Capsella* embryos when cultivated either *in vitro* within ovules, or individually on a medium or *in vivo*. They concluded that an embryo grown *in vivo* was more differentiated than that arised from *in vitro* culture. Embryos from *in ovulo*

culture (ovule culture) had a better growth and survival than the individually cultivated embryos.

Interspecific hybrids between *Nicotiana tabacum x Nicotiana acuminata* were obtained through ovule culture in a liquid Nitsch H medium (Nitsch and Nitsch 1969) after incubating the ovules 2-10 DAP (Iwai *et al*. 1986). Ovules from 4-6 DAP onwards gave a good germination rate, younger ovules died. In *Zea mays* Campenot *et al*. (1992) isolated from 1 DAP ovules of isolated embryo sacs that were partially surrounded by nucellar tissue and contained a zygote and a few endosperm nuclei, and cultured them *in vitro*. They achieved germination of the embryos from 7 DAP onwards using basic media. Modification of the basic medium by the addition of the growth regulator BAP resulted in germination of 1 DAP zygotes. Through this method a zygote resulting from an *in vitro* fertilization as described by Kranz (Chapter 14) could be developed into a mature plant (Campenot *et al*. 1992).

Embryo culture

Embryo culture can be applied successfully in crosses in which pollinated flowers can stay on the plant for a notable time, before natural abscission occurs. This method has been applied in a large number of crops (see Raghavan 1986 for details). Some recent examples are: *Allium* (Nomura and Oosawa 1990), *Alstroemeria* (Buitendijk *et al*. 1992), *Freesia* (Reiser and Ziessler 1989), *Howea* (Moura and Carneiro 1992), *Lilium* (Van Tuyl *et al*. 1991), *Lycopersicon* (Imanishi 1988) and *Solanum* (Singsit and Hanneman 1991).

To overcome the problems involved in isolating the young embryos from ovules and providing suitable conditions for their growth, embryo culture has been modified in some systems (Harberd 1969; Buitendijk et al. 1992). The ovule is cut in half and the cut halves, or only the halves containing the embryo, are cultured in a liquid medium. Out of these half ovules germinating embryos emerged, which could be raised to plantlets on a solid medium.

A large number of interspecific and intergeneric hybrids have been produced in *Brassica* through sequential culture of ovary, ovule and often embryo (Nanda Kumar et al. 1988; Agnihotri et al. 1990; Gundimeda et al. 1992). In sequential culture, ovaries are initially cultured for 6-10 days. Enlarged ovules are excised from cultured ovaries and recultured on a fresh medium. Hybrids are realized either directly from cultured ovules or after excising and culturing the embryos. Przywara et al. (1989) followed sequential culturing of ovules and embryos to raise hybrids between *Trifolium repens* and *T. hybridum*.

In interspecific hybridization of *Phaseolus vulgaris x P. acutifolius* (Sabja *et al*. 1990) pods were collected from 10 days after pollination (DAP) and cultured upright in a modified liquid Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supported by glasswool. In the

in vivo situation, the hybrid embryos stopped growth by 21 DAP and soon thereafter the pods abscise. The ovary culture resulted in the development of small and weak embryos; 90 % of them survived when they were excised and cultured. Only the ovaries from 14 DAP and older gave viable hybrid plants (Sabja *et al*. 1990). In this example a combination of ovary culture and embryo culture is used. In most cases ovary culture is applied during the growth of small undifferentiated embryo inside the ovule, and the differentiated embryo is taken out and cultured.

In many of the distant crosses, the number of hybrids realized are rather limited. However, hybrids can be multiplied through in vitro culture techniques (Agnihotri et al. 1990; Nanda Kumar and Shivanna 1991; Chen and Adachi 1992). In some hybrids embryonal callus and subsequent differentiation of hybrid plantlets has been achieved. In others, culture of single node segments or shoot tips have been used to propagate the hybrid. The use of embryo callus for multiplication of hybrid is also of importance for induction of variations in the hybrid and for experiments on genetic transformation.

INTEGRATED TECHNIQUES FOR OVERCOMING PRE- AND POST-FERTILIZATION BARRIERS

In many interspecific and intergeneric crosses, integrated techniques to manipulate both pre- and post-fertilization barriers have been applied. In vitro pollination and fertilization is one such technique. Unlike the other techniques, which retain the zone of inhibition (stigma and style) and manipulate pollen germination and pollen tube growth to overcome pre-fertilization barriers, in vitro pollination brings pollen grains in direct contact with the ovules, and is, therefore, considered more effective (see Rangaswamy 1977).

Kameya and Hinata (1970) obtained hybrids between *Brassica chinensis* and *B. pikenensis* through pollination of excised, cultured ovules. This approach has not been successful in other systems, but a modified technique of in vitro pollination, termed placental pollination, has been successful. Placental pollination involves removal of the stigma, style and ovary wall, pollination of the ovules intact on the placenta, and culturing of the ovule mass on a suitable medium (Rangaswamy and Shivanna 1967). In this method ovules do not come in contact with the medium as only the pedicel is inserted into the medium. Keeping the pollen and ovules free from the moisture is critical for achieving success in many systems. Using this technique, hybrids have been produced between *Melandrium album* and *M. rubrum*, *M. album* and *Silene schafta*, and *M. album* and *Viscaria vulgaris* (Zenkteler 1990), *Nicotiana tabacum* x *N. oesoebila* (Reed and Collins 1978), *Petunia parodii* x *P. inflata*, *Zea mays* x *Z. mexicana* (Dhaliwal and King 1978). In some of

the crosses between species of *Melandrium* and *Datura*, although fertilization was achieved through placental pollination, proembryos aborted.

In *Brassica* and related taxa, a part of the ovary wall was removed to expose the ovules and pollen grains were deposited on the surface of the ovules; the pollinated ovary was implanted through the pedicel in the culture medium. Developing embryos were subsequently dissected from the enlarged ovules (15-21 DAP) and cultured. Through this integrated techniques of in vitro pollination and embryo rescue many hybrids were produced: *B. napus* x *B. campestris* (Zenkteler et al. 1987), *B. napus* x *Diplotaxis tenuifolia*, *B. napus* x *Moricandia arvensis*, *B. oleracea* x *D. tenuifolia* and *D. tenuifolia* x *B. napus* (Zenkteler 1990). By combining *in vitro* pollination, placenta culture and *in ovulo* embryo culture, incongruity barriers between *Nicotiana* species could be bypassed (DeVerna *et al*. 1987).

In *Lilium* various combinations of *in vitro* pollination (cut-style and grafted-style method) and embryo rescue (ovary, ovule and embryo culture, placental pollination, Fig. 4AB) were applied in order to control the whole fertilization process (Janson 1993; Van Tuyl *et al*. 1991). This resulted into a range of new interspecific hybrids (Van Creij *et al*. 1993). In crosses between *L. longiflorum* cultivars, ovaries with complete pistil were cultured three days before anthesis and were pollinated with aseptic pollen when the stigma was receptive (Fig. 4C). The ovaries were sliced 30-45 DAP and either the embryos were dissected from the ovules for embryo culture or mature seeds were harvested out of these *in vitro* pollinated cultured ovaries 60-90 DAP. In other combinations between *Lilium* species, an ovary-slice culture in combination with ovule culture was applied. Ovary slice culture started 5-8 DAP and the swollen ovules were excised 42 DAP and cultured on a medium until germination started (Fig. 4DE). The emerged plantlets were transferred to an embryo culture medium. Using this method, plantlets were obtained 52-54 DAP, whereas in the normal *in vivo* situation seedlings were not produced before 121 DAP (Table 2). Fig. 5 shows a diagram of alternative routes for application of *in vitro* culture methods for *Lilium* (Van Tuyl *et al*. 1991). Similar results were obtained from interspecific crosses in *Tulipa* and intergeneric crosses between *Nerine* and *Amaryllis* (Van Tuyl *et al*. 1990 1993). Recently in vitro fertilization has been achieved using isolated sperms and eggs; this aspect is discussed in Chapter...

TECHNIQUES FOR OVERCOMING F1-STERILITY

Barriers occurring after a successful embryo rescue are hybrid breakdown and F1-sterility. Hybrid breakdown results in the loss of the hybrid before flowering and is a result of the

unbalanced new gene combinations. F1-sterility of interspecific hybrids is very common and among others may be the consequence of reduced chromosome pairing during meiosis. Breeding at polyploid levels is widely used in interspecific hybridization programmes of many ornamental crops such as *Alstroemeria*, *Chrysanthemum*, *Freesia*, *Gladiolus*, *Lilium*.

Chromosome doubling

Interspecific F1-hybrids may display sterility owing to lack of chromosome pairing during meiosis. This sterility hampers further breeding. Somatic (mitotic) chromosome doubling may induce homologous pairing of chromosomes and restores fertility (Hermsen 1984a,b). Colchicine was used successfully to produce fertile allotetraploids in many crops such as *Anigozanthos* (Griesbach 1990), *Arachis* (Singh 1985), *Lilium* (Asano 1982; Van Tuyl 1993), *Phaseolus* (Weilenmann *et al*. 1986), *Tagetes* (Bolz 1961). As an alternative for colchicine, oryzalin, a herbicide with anti-mitotic activity, was recently used successfully (Van Tuyl *et al*. 1992). Crossability between parents may be improved in the process of plant breeding by equalizing their functional ploidy level. Allopolyploids may function as fertile bridges for gene introgression into the cultivar assortment (Hermsen 1984a,b; Nanda Kumar and Shivanna 1993).

Application of 2n-gametes

Application of meiotic polyploidization in interspecific breeding programmes may be of great importance for the introgression of characters from diploids to tetraploids (Hermsen 1984a,b; Veilleux 1985; Ramanna 1992). In many species, polyploidization is the result of functional 2n-gametes in one or both the parents. Such gametes may result from mechanisms of meiotic restitution. Normally, the frequency of 2n-gametes is low. Wide interspecific lily hybrids are usually completely male and female sterile. In rare cases, however, some fertile pollen is detected. In a group of more than 50 hybrids from the cross *L. longiflorum* x *L. candidum*, raised through embryo culture, only one hybrid showed pollen fertility of 25%. Meiosis in this hybrid was highly irregular and all pollen contained 2n-gametes (Van Tuyl 1989 1993). Comparable cases were found in lily by backcrossing oriental hybrids with 'Shikayama' x *L. henryi* and *L. auratum* x *L. henryi* (Asano 1982) resulting in triploid progenies. Backcrossing these triploids with *L. auratum* x *L. henryi* resulted in aneuploids with chromosome numbers intermediate between 36 and 48 (Van Tuyl 1989 1993). In contrast to the wide interspecific crosses, the cross between the Asiatic hybrid 'Enchantment' and the related *Lilium pumilum* produced fertile pollen. Meiotic studies of several of these hybrids showed, the formation of not only haploid pollen was formed but also relatively high percentages of 2n-pollen (Van Tuyl *et al*. 1989).

In *Alstroemeria* interspecific hybrids produce 2n-pollen of up to 60-70% in some of the genotypes of crosses between Chilean and Brazilian species (Ramanna, pers. comm.). In this crop sexual polyploidization appears to be a convenient system for breeders to produce triploid and tetraploid interspecific hybrids. For example, if a trispecific triploid generates 2n-gametes, in one step of unilateral sexual polyploidization, a tetraploid with four different genomes can be produced (Ramanna 1992).

CONCLUDING REMARKS

For many crops, especially for ornamentals, interspecific hybridization is a very effective method for the introduction of desired characters into breeding material and achieving crop improvement (Uhlinger 1982). The possibilities for interspecific crosses are nevertheless limited due to various crossing barriers. Many diverse techniques have been developed to overcome these barriers. A relatively underexposed aspect in this field of research is the use of genetic variation to overcome these barriers.

The application of *in vitro* pollination and other pollination methods combined with embryo rescue is a very powerful breeding approach. A more controlled environmental conditions during the processes of pollination, fertilization and embryo development would result in repeatability of experiments almost independent of the season. Conditions for each process can be optimized, and crossing barriers can be studied more systematically. *In vitro* methods make it possible to develop an integrated procedure for overcoming pre- and post-fertilization barriers.

In future tissue specific promotors along with RNAse genes may enable breeders to eliminate the recognition barriers in the stigma and style by preventing the formation of these organs. In this way Goldman *et al*. (1994) have developed a female sterile tobacco plant by eliminating the stigmatic tissue. This stigma does not permit pollen germination, but when pollen together with the stigmatic exudate is used for pollination, pollen germination occurs and normal fertilization and seed set take place. By this method, the whole pollen tube pathway and thus the recognition and barrier functions could be eliminated which opens a way for new applications. These techniques provide a very powerful tool to the plant breeders to transfer genes across species barriers.

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Tables and table captions

Table 1. Effect of pollination technique on the number of seeds. A comparison between three types of pollinations: GSM (grafted-style method), CSM (cut-style method) and N (normal pollination) in a compatible cross between *L. longiflorum* 'Gelria' and *L. longiflorum* 'Albivetta'.

Pollination technique	Number ovaries pollinated	Number of fruits developed	Total number of seeds	Seeds per fruit
GSM exudate	26	1	15	15.0
GSM straw exudate	26	7	96	13.7
GSM straw medium	26	5	36	7.2
CSM	25	19	85	4.5
N	25	19	451	23.7

Table 2. Comparison of the number of plantlets obtained per ovary from the incongruous combinations *L. longiflorum* x *L. henryi* and *L. longiflorum x L. dauricum* using three pollination methods (GSM= grafted-style method, CSM=cut-style method and N=normal pollination). As a control, the intraspecific cross *L. longiflorum* cv 'Gelria' x cv 'White American' was studied both in the climate room (cl) and in the greenhouse (gr). Interspecific pollinations were carried out in the climate room; 8 DAP, ovaries were cultured; 42 DAP, ovules were excised and the swelling score was determined (on a scale from 1-9 in which 1=no swelling, 9=very strong swelling). Subsequently, swollen ovules were cultured; plantlet development was scored from 10 to more than 40 weeks after start of ovary culture. (nd=not determined).

L. longiflorum hybrids	Pollination method	Number ovaries cultured	Swelling score of ovules	Number Plantlets per ovary cultured
Intraspecific	N (gr)	41	nd	nd	143
Intraspecific	N (cl)	24	nd	nd	19
x *L. henryi*	N (cl)	10	4.8	2206	0
x *L. henryi*	CSM (cl)	10	5.6	2622	2.1
x *L. henryi*	GSM (cl)	10	3.2	1195	0.1
x *L. dauricum*	N (cl)	13	3.9	3475	0
x *L. dauricum*	CSM (cl)	14	5.1	4568	3.5
x *L. dauricum*	GSM (cl)	14	2.7	2446	4.8

Figure captions

Fig. 1. The percentage of ovules with a pollen tube in the micropyle (•, experiment with 69 flowers, solid lines represent the standard deviation, $ó_{n-1}$) and the percentage of seeds with an embryo (⅄, experiment with 30 flowers, dotted lines represent $ó_{n-1}$) achieved after cut-style pollination in *Lilium longiflorum* carried out with compatible pollen and at different style lengths: stigma present only at 100% (non-cut).

Fig. 2a. GSM (grafted style method of pollination), (A) Style of Oriental hybrid 'Star Gazer' compatibly pollinated with pollen of another Oriental hybrid and attached to an ovary of the Asiatic hybrid 'Esther' and, (B) Style of 'Esther', compatibly pollinated with pollen of 'Connecticut King' and attached to an ovary of the Oriental hybrid 'Star Gazer' Bar= 1 cm.

Fig. 2b. Detail of graft end from situation (A) in Fig. 2a. Both style ends are slightly separated to show the bundle of pollen tubes growing through the graft. Bar= 0.1 cm.

Fig. 3A. Ovule culture in *Alstroemeria* 35 DAP. Transverse section showing embryo development (EM) without the development of cellular endosperm in an incongruous cross between a tetraploid cultivar and *A. aurea*. Bar= 400 ìm.

Fig. 3B. Ovule culture in *Alstroemeria* 42 DAP. The embryo emerges out of the ovule; histological investigation in an incongruous cross between a tetraploid cultivar and *A. aurea*. Bar= 200 ìm.

Fig. 3C. Ovule culture in *Alstroemeria* 42 DAP. Germination *in vitro* of an ovule in an incongruous cross between *A. pelegrina x A. aurea*.

Fig. 4A. *In vitro* pollinated ovaries from *L. longiflorum* 'Gelria' 10 days after cut-style (left) and stigmatal pollination (right) with an Asiatic hybrid lily.

Fig. 4B. Placental pollination of *L. longiflorum* 'Gelria' with the oriental hybrid 'Star Gazer': pollen directly pollinated on placentas, some swollen ovules can be observed.

Fig. 4C. Asiatic hybrid lily 'Enchantment' ovary slices, 4 weeks after compatible pollination, on MS medium supplemented with 9% sucrose and 1 mg/l NAA, in continuous presence of anthers. Note

the swollen ovules inside the ovary slices. Bar= 1 cm.

Fig. 4D. Seedling of a directly germinated ovule of the Asiatic hybrid lily 'Enchantment', 12 weeks after compatible pollination, on MS medium supplemented with 5% sucrose and 0.1 mg/l NAA. Bar= 0.5 cm.

Fig. 4E. Hybrid plantlet with bulb formation, from an ovule obtained after the interspecific cross *L. longiflorum x L. dauricum*, 40 weeks after pollination, ready to be transferred to soil. Bar= 1 cm.

Fig. 5. Diagram of alternative routes for application of *in vitro* culture methods. Numbers represent minimal number of days in a compatible intraspecific situation (crosses between different *L. longiflorum* cultivars). Note that in the normal *in vivo* situation, seedlings are produced after 121 DAP, whereas in the embryo rescue and ovary-ovule route, plantlets emerge from 52-54 DAP.

Figures 1-2

Figures 3-4

Figure 5.