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Somatic versus sexual hybridization: features, facts and future

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CONTENTS

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INTRODUCTION

The main goal of plant breeding is the construction of new genotypes. This can be achieved by the introduction (sexual and somatic hybridization and genetic transformation) and manipulation (recombination and selection) of genetic variation. The introduction of foreign genes through the generative cycle can be successfully achieved, for example in cereals, by means of chromosome addition and radiation-induced chromosome engineering. Reference is made to reviews of Sybenga (1983) and Brar & Khush (1986). The principle of hybridization has been extended to the somatic cycle by protoplast fusion with hybrids and cybrids being produced at different levels of

ploidy and taxonomic distance (Harms 1983a,b; Gleba & Sytnik 1984; Glimelius 1986; Hinnisdaels *et al.* 1988).

The objective of this paper is to present, in parallel, the respective achievements, merits, and limitations of sexual and somatic hybridization. Exploitation of the generative cycle has resulted in important contributions to plant breeding, but too often such discoveries had empirical bases. It was considered that plant tissue culture in general, and somatic hybridization in particular, have the intrinsic potential to fill many of the gaps in plant biology. More specifically, it was expected that somatic hybridization would allow one to bypass most of the incompatibility barriers built by nature to preserve genomic identity. However, this is not yet the case, though the evolution in techniques on sexual and somatic hybridization is revealing interesting convergence and complementarity. The discussion on the practical implications and applications of the hybridization–recombination–segregation process will be limited (they are extensively covered by most cited works); instead, emphasis will be put on analytical aspects, so important at a time when plant biotechnology is looking for more rational, less empirical principles that govern plant metabolism and development.

SEXUAL HYBRIDIZATION—BACKGROUND, PROBLEMS AND OBJECTIVES

Sexual hybridization produces hybrid combinations within specific taxonomic distances. The practice of plant breeding was developed to widen the range of combinations over taxonomic distances that varied with the group of species used and the level of ploidy. Segregation of gene combinations after recombination in meiosis represent, together with mutation breeding (Broertjes & van Harten 1988), the main sources of variability and the basis of genetic manipulation in plants. Aberrant traits resulting from the process of hybrid disgenesis, such as sterility, segregation and sex ratio distortions, high frequency mutations, chromosome structural changes, rearrangement and non-disjunction, split corolla phenotype and variegation, have been observed in specific hybrid combinations and are conditioned by the direction of the cross in both plants (e.g. *Oenothera, Petunia*) and *Drosophila* (D'Amato 1977; Schnabelraum *et al.* 1985, and references therein).

Thus hybrids are usually sterile, due to disorders in the progress of meiosis (of which reciprocal translocations are known to disturb regular disjunction) (Wilby & Parker 1988): such chromosome rearrangements can be inherited in a Mendelian or a non-Mendelian manner. Sometimes fertility can be restored by chromosome doubling (synthesis of amphiploids, cf. Brar & Khush 1986). With the exception of *Triticale*, none of the several amphiploids created so far has become a new crop.

In the success of wide crosses via classical breeding methods, however, cytogenetics has played a major role in explaining some of the mechanisms which today allow breeders to establish original genomic associations, to obtain restructured chromosomes which normally do not pair, cytogenetic tester sets and new karyotypes (McKey 1981, Cauderon 1986; Hageberg 1986).

Barriers to wide hybridization, techniques to overcome crossability problems, and approaches to gene introgression via sexual hybridization have been described in Harms (1983a), Brar & Khush (1986), Sybenga (1983), Zenkteler & Slusarkiewicz-Jarzina (1986). They will be dealt with more specifically on page 257.

SOMATIC HYBRIDIZATION—BACKGROUND, PROBLEMS AND OBJECTIVES

Protoplasts can be isolated from most plant species (Roest & Gilissen 1989) and efficient methods of protoplast fusion have been worked out (Negrutiu *et al.* 1986 and references therein). Provided that protoplasts of one of the two fusion partners have the ability to undergo divisions in culture, heterokaryons have been observed to undergo a variable number of division cycles in most of the combinations tested (Gleba & Sytnik 1984). Their analysis has allowed one to establish that the incompatibility reactions are absent or highly attenuated mainly at fusion or during the early stages of somatic hybridization, and made it possible to assess (a) the type of incompatibility mechanisms that act in somatic hybrid cells maintained in culture and that undergo regeneration induction processes, and (b) the phylogenetic range within which somatic hybridization can operate to produce material ranging from viable fusion products to fertile hybrid plants.

With the increasing number of wide somatic hybrids produced, it became more and more clear that somatic incompatibility reactions at various levels (Harms 1983a) do operate in somatic hybrids and therefore limit the somatic approach to wide hybridization as well (see page 260). These limitations parallel those encountered with sexual hybridization. Somatic hybrid clones were produced between species which apparently could not be crossed sexually, from interfamily to interspecies combinations (Evans *et al.* 1980; Schenck & Röbbelen 1982; Schieder *et al.* 1985; Ehlenfeld & Helgeson 1987). In other words, a comparative evaluation of somatic versus sexual hybridization is possible (see page 257).

Spontaneous asymmetrization of fusion products. Fusion between phylogenetically remote species frequently results in an asymmetric combination of the two genomes, with parts of one or both genomes being lost during the *in vitro* passage (reviewed by Gleba & Sytnik 1984; Imamura *et al.* 1987 and references therein). The extent to which the two genomes undergo polyploidization, chromosome elimination and rearrangements is largely unpredictable (Harms 1983a).

There is evidence that a certain asymmetrization has to occur before differentiation processes can be initiated (Potrykus *et al.* 1984; Schieder *et al.* 1985). This could be particularly important not only in understanding the basis of wide hybridization conditions together with the role of somatic incompatibility reactions, but also in terms of applied breeding objectives. The fact that the asymmetrization is a gradual process, where morphogenesis resumes after prolonged callus proliferation, makes spontaneous asymmetrization a less attractive condition, as there is little, if any, control on this process. The best documented cases are *Arabidobrassica* (Hoffmann & Adachi 1981), *Datura+Atropa* (Schieder *et al.* 1985) and *Nicotiana tabacum+Hyoscyamus muticus* (Jia *et al.* 1983). These examples were analysed in more detail in a previous discussion (Negrutiu *et al.* 1988).

Induced asymmetrization of fusion products. From the above discussion it appears desirable to control and direct the process of chromosome elimination in particular, and the asymmetrization process in general, in order to ensure the production of highly asymmetric, fertile hybrid plants. Two methods for transferring only part of a donor genome have been established, namely irradiation of donor protoplasts with sublethal or lethal doses and the occurrence of premature chromatin condensation (PCC) in aphasic fusion; these will be discussed later.

EXPLOITATION OF GENETIC MARKERS IN SOMATIC HYBRIDIZATION EXPERIMENTS

The production of symmetric and asymmetric (both nuclear or cytoplasmic) somatic hybrids has been facilitated by an appropriate choice of selectable marker genes (Lazar 1983; Harms 1983b; Gleba & Sytnik 1984; Flick 1983; Negrutiu *et al.* 1988). Useful mutant cell lines include nitrate reductase deficiency, amino acid- and vitamin-requiring mutants and mutants resistant to 5-methyltryptophan (5MT), s-aminoethylcysteine (AEC), streptomycin, kanamycin, lincomycin, picloram, hydroxyurea, and methothrexate. Such mutants have been used to demonstrate three main aspects of plant biology (reviewed in Negrutiu *et al.* 1988). These are, specifically:

(i) partial genome transfer by protoplast fusion between species that have not been crossed so far by classical breeding techniques;
(ii) a more frequent event than previously thought, paternal transmission of organelles at low rates; and
(iii) the rare occurrence at fusion and subsequent *in vitro* culture of recombination events at the level of chloroplast DNA and the high recombination frequency amongst mitochondrial DNA molecules.

Further progress in this area will need more refined tools in order to demonstrate systematically at the DNA and chromosome levels the fate of the donor traits. This implies the use of cloned genes that upon transfer into the recipient cells express a selectable phenotype, and can be identified either as a DNA sequence or at the protein level.

Among the cloned genes already available, one can list antibiotic- and herbicide-resistance genes, as well as genes involved in the hormonal and opine metabolism of *Agrobacteria.* Thus, the neomycin phosphotransferase, an enzyme detoxifying amino-glycoside compounds such as kanamycin, G418 and promomycin by phosphorylation (Potrykus *et al.* 1985; Klee *et al.* 1987b), or alternatively genes conferring resistance to hygromycin (Van den Elzen *et al.* 1985), bleomycin (Hille *et al.* 1986), methothrexate (Eichholz *et al.* 1987) and streptomycin (Mazodier *et al.* 1986), have been used to produce an important range of phenotypes with well-defined characteristics.

Examples of cloned genes that confer herbicide resistance are: acetolactate synthase (ALS), the target enzyme for sulphonylurea and imidazolinone herbicides (Schloss *et al.* 1988), 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), the primary target of the herbicide glyphosate (Della-Cioppa & Kishore 1988), and the *bar* gene, an acetyltransferase that acts upon phosphinothricin (PPT), an inhibitor of glutamine synthase, one component of the tripeptide bialaphos, an antibiotic and non-selective herbicide (Thompson *et al.* 1987 and references therein).

Other interesting reporter genes are GUS (β-glucuronidase, cf. Jefferson 1987) and luciferase (Ow *et al.* 1986). Finally, cloned genes derived from the T-DNA and responsible for the synthesis of plant hormones or opines in transformed plant cells have been already used as markers in fusion experiments (Gleba *et al.* 1986) and are discussed in Klee *et al.* (1987a,b).

Selectable markers of this sort represent valuable tools for an early and unequivocal identification of transgenomes, and one can expect to see them used extensively in both somatic and sexual hybridizations.

PARAMETERS INVOLVED IN THE HYBRIDIZATION/ INTROGRESSION PROCESS

This section analyses several parameters that are characteristic of the hybrid condition and participate in gene-introgression phenomena.

Unidirectional elimination of chromosomes. **Sexual hybrids.** One of the best studied cases of spontaneous genome/chromosome elimination at cell division is that of *Hordeum* hybrids, which is under genetic control, resulting in haploid nuclei in embryos and diploid nuclei in endosperm (Finch, 1983 and references therein), and may occur at several developmental stages. Similar results are available in wheat × maize and wheat × *Sorghum* (Laurie & Bennett, 1987). An interesting case of uniparental elimination has been reported in barley, involving different parental genomes in different tissues (endosperm versus embryo) of the same cross. The eliminated chromosomes differed from the ones being retained in having smaller centromeres and tending before, during, and after elimination to occupy more peripheral regions of the mitotic spindle (also see page 259). Another important factor involved in the elimination or maintenance of parts of the donor genome may be the control in time of chromatin condensation between or within the partner genomes: early condensing elements can be maintained preferentially as compared to late condensing structures. **Somatic hybrids.** Culture conditions more suitable for protoplasts of one species as compared to those of the other, sources of protoplasts (plant tissues versus cultured cells), the relative stages in the mitotic cycle at which the protoplasts were isolated before fusion and the relative length of the cell cycle in the parental species have all been shown to influence the direction and/or extent of chromosome elimination in proliferative hybrid cells (Ashmore & Gould 1982; Harms 1983a; Gleba & Sytnik 1984; Gould & Daines 1986).

Thus PCC has the potential to provoke chromosome elimination and fragmentation in fused cells, the phenomenon having been observed as early as 3 h after fusion (Szabados & Dudits 1980). G_0/G_1 and mainly S-stages appear to be the most exposed to damage in aphasic fusions. PCC could account for several reported asymmetric hybrids (Dudits *et al.* 1979; Schieder *et al.* 1985).

Similarly, studies on interspecific hybrids in animal cells have shown that in the particular case of nascent hamster–human hybrids, the segregating human chromosomes assumed an aberrant central position within a ring of hamster chromosomes (Graves & Zelesco, 1988).

Irradiation effects and gene expression in hybrids. **Sexual hybrids.** In plants, the irradiation system was mainly employed at fertilization to create ovule-derived haploids (Raquin 1985), to generate maternal phenotypes in one sexual generation (matromorphs) (Pandey 1983 and references therein), or to provoke introgression of specific traits in wide-cross combinations by translocations of chromosome segments (e.g. transfer of resistance genes from *Aegilops umbellulata* to the 6B or from *Agropyron elongatum* to the 6A chromosome of wheat) (Brar & Khush 1986).

The cytological effects of radiation include a large array of nuclear abnormalities observed at meiosis or mitosis (Gaul 1977 and references therein; Nicoll *et al.* 1987) including polyploid restitution nuclei, nuclear bridges, disrupted mitotic synchrony, pseudo-isochromosomes, bivalents and the appearance of chromosome fragments. Such

phenomena seem to occur early after treatment, suggesting rapid sorting of aberrant or unbalanced nuclear constituents. In diploid organisms, the reciprocal translocations were by far the most common amongst surviving induced chromosome rearrangements, while in polyploids very often rather dramatically unbalanced induced structural changes survived mitosis or meiosis and passed over to later generations (Hageberg 1986). **Somatic hybrids.** The association of irradiation with fusion, *in vitro* culture, selection, regeneration, and passage through meiosis is expected to represent a more versatile experimental tool of gene introgression compared to the available breeding methods. It is well established that irradiation favours a rapid and unidirectional elimination of donor chromosomes generating asymmetric, nuclear or cytoplasmic, hybrid plants (Dudits *et al.* 1980, 1987; Gupta *et al.* 1982, 1984; Somers *et al.* 1986; Galun & Aviv 1986; Imamura *et al.* 1987; Müller-Gensert & Schieder 1987; Gleba *et al.* 1988) (Fig. 2). The treatment determined the direction of elimination but not the extent of elimination of the irradiated genomes (Gleba *et al.* 1988; Famelaer *et al.* 1989), which suggests that the number of induced breaks (single or double) is relatively small, and that the recipient cells have the ability to rescue efficiently the damaged donor nucleus by cross-acting repair mechanisms.

Experiments to compare irradiated with non-irradiated combinations (for example Gupta *et al.* 1984; Gleba *et al.* 1988; see also Dudits *et al.* 1987) have demonstrated the role of the irradiation-induced asymmetrization in stabilizing the clones that arise from fused protoplasts and in promoting plant regeneration.

Deleted or minichromosomes have been observed in combinations such as *N. plumbaginifolia* + *A. belladonna* or *N. sylvestris* (Fig. 2a–d and f). By analysing sufficient clones (up to 100) a few individuals were identified to contain low numbers of donor chromosomes (Gleba *et al.* 1988; Famelaer *et al.* 1989). It was estimated that 9–50% of the donor haploid genome was present in clones from the *N. plumbaginifolia* + *A. belladonna* combination. Furthermore, in the absence of specific selection pressures it is probably impossible, at present, to direct the maintenance or elimination of specific chromosomes. Chromosome fragment introgression has not been clearly documented and thus remains a rare and unpredictable event (see page 265). Donor marker genes selected for infusion products appeared to be expressed in a stable way under selection conditions, while non-selectable genes, such as isozymes or opine genes, exhibited a highly variable level of expression (Gleba & Sytnik 1984; Famelaer *et al.* 1989).

Although the maintenance of non-selectable markers from the few studied so far seems to be a random event, there is not yet evidence for preferential retention/elimination of certain chromosomes; the mechanical–physical constraints in the spatial arrangement of chromosomes in hybrid nuclei (cf. page 259) should play a role in making chromosome elimination non-random (also see page 260).

Other facts should also be taken into consideration.

(i) The physical presence of alien genes may not necessarily result in appropriate expression, both because one of the adverse side-effects of irradiation is inactivation of non-selected genes by mutation, and because correct expression of these genes may be altered in the recipient genomic context. Arguments to support this can be found in Mujeeb-Kazi *et al.* (1987); Zelesco & Graves (1987); Gleba *et al.* (1988); Famelaer *et al.* (1989).

(ii) Currently it is almost impossible to make any predictions on the fate of polygenic traits located at different positions in the donor genome. However, all these factors considered, there is a need to devise methods that allow recombinogenic events to occur as early as possible after fusion.

It is interesting that in many symmetric or asymmetric hybrids under investigation, changes in the copy number of rDNA from both parents were shown to occur independently of other (isozymes, RFLP) markers (Miesfeld & Arnheim 1984; Pijnacker *et al.* 1987; Gleba *et al.* 1988; Moore & Sink 1988).

To conclude, highly asymmetric hybrids may represent cases where large portions of the maintained donor chromosomes became 'silent', not only because of fragmentation and physical loss, but also because of radiation damage and/or 'under-expression' in a new genomic environment.

Spatial organization of chromosomes following sexual and somatic hybridization. **Sexual hybrids.** Chromosomes are assumed to have specific organizational relationships with the nuclear membrane and between each other. Studies on DNA interactions with the nuclear matrix have provided information on the spatial organization of replication and transcription (Razin 1987). There are several types of DNA–matrix associations: (a) permanent attachment sites, such as those involving the origins of replication and others detected in both transcriptionally active and inactive nuclei; (b) attachment sites in transcriptionally active nuclei associated with the matrix-expressed genes; and (c) sites that maintain the fixed positions of individual chromosomes in interphase nuclei.

There is evidence that chromosomes assume specific arrangements in nuclei and on the mitotic spindle and these arrangements have important implications for chromosome behaviour.

(i) In *Ornithogalum virens*, chromosomes in the haploid complement exhibit end-to-end order to provide a model that shows how the arrangement of two such haploid arrays in diploid nuclei could facilitate meiotic pairing (Ashley & Pocock 1981).

(ii) In grasses, the two haploid chromosome sets occupy separate domains on the mitotic spindle (Bennett 1982). In interspecies and intergeneric hybrids, one parental chromosome set is nearer the outside of the spindle, concentric with the second parent's chromosomes. Such hybrids tend to loose chromosomes of one parent non-randomly. The model has been challenged by Callow (1985).

(iii) The position of chromosomes within gametic nuclei is also determined by telomere-to-telomere attachment of non-homologous chromosomes in a specific sequence (chromosome chain) and anchoring of telomeres to the nuclear membrane (Ashley & Pocock 1981).

Somatic hybrids. What is still not yet understood is how this structural organization of chromosomes and the nuclear matrix operates in heterokaryons. Gleba *et al.* (1987) studied the spatial arrangement of chromosomes at first division as well as after prolonged culture in metaphase plates of interspecific (*N. sylvestris* + *N. plumbaginifolia*) and intertribal (*N. chinensis* + *A. belladonna*) hybrids obtained by protoplast fusion. At the first mitotic division, the chromosomes of the two fusion partners were spatially separated and followed a 'segmented' pattern, while after long-term culture, the topology of genome separation in both callus cells and root tips showed changes from 'segmental' to 'radial'.

In this last case, the small *Atropa* chromosomes occupied the metaphase centre, while the large chromosomes of *Nicotiana* were scattered about the periphery of the plate. In the other combinations, teleocentric chromosomes of *N. plumbaginifolia* were positioned predominantly at the periphery of the metaphase plates whereas *N. sylvestris* chromosomes occupied the centre. One can speculate that the spatial separation of genomes in fusion combinations reflects a probable chimeric structure of

non-chromosomal nuclear constituents. It remains to be established whether this is also the case with highly asymmetric fusion products.

The in vitro *culture passage.* The role of the *in vitro* culture period following fusion is envisaged as a situation in which attenuation of incompatibility reactions occurs. The integrated pattern of differentiation within a species is fixed as a tight genetically controlled programme. Hybridization brings new genetic material which alters the system either by producing, in addition to gene infiltration, a variety of unusual, novel or transgressive morphogenesic responses (Smith 1974) (see page 254), or by creating a brutal imbalance and irreversible damage (hybrid inviability). Hybridization products benefit from the *in vitro* passage in two ways.

(i) Explants of wide hybrids can be taken through tissue culture cycles in an attempt to enhance the frequency of genetic exchanges between alien and cultivated genomes (Lapitan *et al.* 1984), as shown in wheat × rye hybrids (Fig. 1c,d).
(ii) Somatic hybrids evolve for many division cycles under *in vitro* culture conditions, during which time genetic exchange can occur prior to morphogenesic processes.

In culture, cells evolve in a permissive and destabilizing environment (Bayliss 1975), where the tight regulatory controls acting upon cells within an organized plant structure cease operation, or at least operate under non-specific, dedifferentiation conditions (in a way similar to those known to act in certain plant–pathogen interactions, e.g. the crown-gall induction). The result is the early occurrence of gross (Fig. 2e) and/or cryptic chromosome rearrangements, transposon activation, gene amplification, somatic crossing over and sister chromatid exchange (Peerbolte *et al.* 1987; Benzion & Phillips 1988; Evans & Sharp 1988).

The ability to regenerate fusion products can be considered as a further selection pressure to reveal or accentuate the (spontaneous or induced) asymmetrization process (Sidorov *et al.* 1987; Gleba *et al.* 1988; Famelaer *et al.* 1989).

Regeneration of recipient-type plants may operate to select specifically against or in favour of certain chromosomes of the donor species. In fact, genes involved in cell proliferation have been identified and assigned to corresponding chromosomes in *Crepis capillaris* (Rueda *et al.* 1988). An improved regeneration response was observed at higher levels of asymmetrization, and this was true irrespective of the species combination tested (Negrutiu *et al.* 1988).

Screening for recipient-type phenotypes implies identification of individuals that undergo important unidirectional elimination of donor material. As centromere retention is the major event with respect to both intra- and intergenomic recombinations in heterokaryons produced by fusion, retention of asynthenic gene combinations must also be poor.

Incompatibility reactions and the phylogenetic range: somatic versus sexual hybridization. Sexual hybridization has a long and well-established history and practice. Sufficient information has been collected during the last decade on somatic hybridization for parallels to be drawn between the two approaches.

The fertility constraints are such in plant breeding and seed production that the unavoidable limitation in sexual hybrids is the fidelity of genetic transmission. As somatic hybrids are usually produced between diploid cells, an increased and/or earlier meiotic balance has been expected to be achieved compared with the situation in sexual hybrids (Sundberg *et al.* 1987).

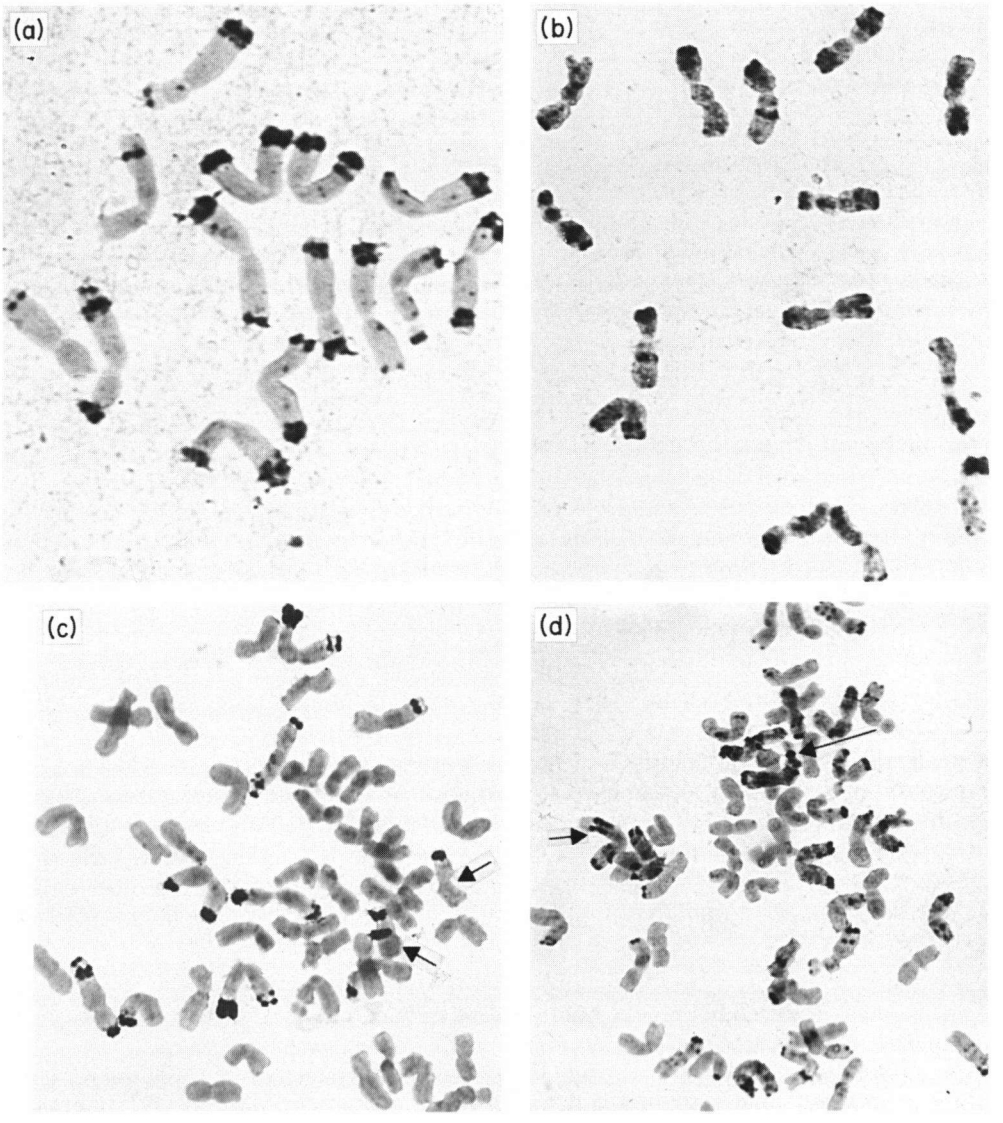

Fig. 1. (a) Chromosomes of diploid rye hybridized *in situ* with a biotin-labelled probe pSC74 (480 bp repeat). The tandem by repeated DNA sequence is a major component of terminal and sub-terminal heterochromatic knobs in rye. The sequence is specific to the rye genome among the annual species of the Triticinae. (b) Chromosomes of diploid rye hybridized *in situ* with a biotin-labelled probe pSC119 (a complex repeat). This is a dispersed-type repeated DNA sequence and marks the entire rye genome. This sequence is widespread among the species in the *Triticinae*. However, the organization of the sequence is different in different species of the *Triticinae*. (c) Chromosomes of octoploid triticale (tissue culture regenerated) *in situ* hybridized with a biotin–labelled probe pSC74. Several kinds of chromosomal changes can be observed. There are only 11 rye chromosomes, three rye chromosomes have been lost. Some rye chromosomes have undergone translocations or deletions because of their altered size or arm ratio (arrows, see (a) for the normal rye chromosome set). Also note the amplified hybridization site on a pair of chromosomes (at six o'clock and 12 o'clock). The wheat chromosomes were unlabelled. (d) Chromosomes of octoploid triticale [the same plant was used in (c)] hybridized *in situ* with a biotin-labelled probe pSC119. This probe labels rye chromosomes all-over [see (b)] and also labels 10 pairs of wheat chromosomes at one or a few localized sites. One pair of rye chromosomes showed an unlabelled segment at the tip indicating deletion of this sequence (arrows), or translocation with an unlabelled wheat chromosome segment. As the same segment is heavily labelled with the pSC74 sequence [see (c)], it indicates deletion of the pSC119 sequence followed by amplification of the pSC74 sequence.

Observations at nuclear level. **Sexual hybrids.** Cytogenetic instability and lack of fertility have been documented extensively in interspecific and intersection hybrids of tobacco (more than 300 sexual hybrids have been reported, cf. Smith 1968), or in chromosome addition lines of sugarbeet (De Jong *et al.* 1985), to mention just two classic cases. Intergeneric hybrids, as shown in cereals (Blanco *et al.* 1986), set the ordinary limits of sexual hybridization with classical means.

Embryo rescue alone or in combination with *in vivo* treatments or *in vitro* fertilization has been useful in extending the frontiers of the hybridization process (Blanco *et al.* 1986; Zenkteler 1986; Gill & Raup 1987; Altman 1988; Shintaku *et al.* 1988): in remote crosses, embryogenic development was observed but stopped after a few cell divisions both in dicots (Zenkteler & Melchers 1978) and cereals (Brar & Khush 1986).

These results allow conclusions to be drawn. Embryo structures can be produced in wide crosses by means of *in vitro* culture techniques, but the developmental stage observed is a function of the phylogenetic distance between the partners. Zygotic incompatibility reactions exhibit a phylogenetic gradient while developmental constraints are likely to have strong genetic bases, and seem to operate mainly beyond the globular stage of embryo development. **Somatic hybrids (symmetric combinations).** Fusion products from wide crosses usually proliferate actively at the callus stage, which suggests that somatic incompatibility may be bypassed mainly because of intrinsic characteristics of *in vitro* cultured cells (and an association of radiation effects and *in vitro* culture passage in the case of fusions with an irradiated donor partner). This erases and suppresses (more or less reversibly) certain 'domains' involved in somatic incompatibility processes. Such a loss of genomic 'identity' is most likely to be a gradual phenomenon which could be associated, in time, with the loss of morphogenetic potential in established cultured cells. As a matter of fact, in some short-lived abortive intergeneric syncaryons, mainly produced by the fusion of protoplasts derived from differentiated tissues (Binding 1976), the somatic incompatibility reactions apparently were still acting within cells that had not been exposed previously to the cell culture environment. On the other hand, proliferative wide-cross hybrid cells are usually highly polyploid, genetically unstable or unbalanced.

Zenkteler & Melchers (1978) demonstrated that hybrid plants could be regenerated from calluses produced by somatic hybridization only in those cases where hybrids arose through sexual hybridization as in *Nicotiana, Petunia* and *Daucus* species.

Somatic interspecific hybrids show, as their sexual counterparts do, low or no self-fertility (Hamill *et al.* 1985; Schnabelraum *et al.* 1985; O'Connell & Hanson 1987; Sundberg *et al.* 1987; Sidorov *et al.* 1987; Wright *et al.* 1987). By recurrent selection of the most fertile individuals over two generations it was possible to increase the level of self-fertility in *Nicotiana* hybrids (Hamill *et al.* 1985). In *Petunia* (Schnabelraum *et al.* 1985), the sexual cross of *P. parodii* × *P. inflata* was successful only with *P. parodii* as the female partner (prezygotic incompatibility), while in the somatic hybrids dysgenic mechanisms were considered to operate.

In two other cases, somatic hybridization is the preferred choice, as demonstrated with somatic hybrids between *N. rustica* and *N. tabacum* (Hamill *et al.* 1985) and in the intergeneric combination of the sexually incompatible orange species *Citrus sinensis* and *Severinia disticha* (Grosser *et al.* 1988).

All the interfamilial hybrids reported so far are genetically unstable (show a tendency to rearrange and loss of chromosomes of one of the parents) and unable to undergo morphogenesis (Kao 1977; Binding and Nehls 1978; Wetter & Kao 1980; Chien *et al.*

1982; Niizeki *et al.* 1985; Sala *et al.* 1985), while intergeneric and intertribal nuclear hybrids, although genetically more stable, resulted in highly abnormal or sterile plants (Gleba & Hoffman 1979; Krumbiegel & Schieder 1979; Dudits *et al.* 1980; Gleba *et al.* 1982, 1983; Potrykus *et al.* 1984). A few classical examples are *Arabidobrassica*, soybean + tobacco or barley, *Datura* + *Atropa* and tomato + potato.

Somatic hybridization may produce hybrids that reach genomic balance, and subsequently meiotic stability, more rapidly than their sexual equivalents. Improvement of methods of *in vitro* fertilization, embryo rescue, etc., may turn out to be as efficient as protoplast fusion in producing the desired range of hybrids between wild type and cultivated plant species. **Somatic hybrids (asymmetric combinations).** As far as the obtention of highly asymmetric hybrids between phylogenetically unrelated species is concerned, the protoplast technology may represent the method of choice. Highly asymmetric hybrid plants with a recipient-type morphology have been obtained following irradiation of the donor material in combinations such as *N. tabacum* + *D. carota* (Dudits *et al.* 1987), *N. plumbaginifolia* + *N. sylvestris* (Famelaer *et al.* 1989), *N plumbaginifolia* + *A belladonna* (Gleba *et al.* 1988), *N. plumbaginifolia* + *P. hybrida*, and *N. plumbaginifolia* + *L. esculentum* (S. Hinnisdaels, unpublished data). A relatively rapid asymmetrization has to take place in order to maintain fusion products between phylogenetically remote species capable of proliferation and able to regenerate in culture. As in the case of sexual hybrids (Brar & Khush 1986), wide somatic hybrids, symmetric or asymmetric, are almost impossible to obtain at the diploid level: all hybrid combinations containing the true diploid *N. plumbaginifolia* as a recipient exhibited a tetraploid chromosome complement (Gleba *et al.* 1988 and Fig. 2b,c).

Recurrent backcrossing with the diploid wild-type parent was performed in order to reduce the ploidy level and to produce a further and significant elimination of the retained donor genomic complement in the *N. plumbaginifolia* + *A. belladonna* combination (Gleba *et al.* 1988 and Fig. 2c,d). Thus, a combination of genetic manipulation at the somatic level with regeneration and generative cycling enabled the production of single chromosome addition lines in intergeneric hybrids within two sexual generations (Fig. 2d,f). In another study on asymmetric hybrids (cybrids) in Solanaceae, Glimelius *et al.* (1986) demonstrated that the nuclear incompatibility 'border' between the five tribes of the Solanaceae was set between the subfamilies Solanoideae and Cestroideae.

As the extent of asymmetrization in fusion products seems to increase with the phylogenetic distance (compare the results from Sidorov *et al.* 1987; Dudits *et al.* 1987; Gleba *et al.* 1988; Famelaer *et al.* 1989 and Fig. 2b,c), the transfer of limited amounts of genetic information from one species to the other may be more easily and rapidly achieved in wider cross combinations. A similar picture is documented in animal cell hybrids (Campbell & Worton 1981 and references therein).

Observations at the level of cytoplasmic genetic determinants. Uniparental (maternal) inheritance of cytoplasmic organelles is recognized as a widespread phenomenon in plants. Protoplast fusion has demonstrated the ability to create a variety of nuclear and cytoplasmic combinations (Gleba & Sytnik 1984). This situation may actually be more complex than desired, because there is bulk transmission of all cytoplasmic determinants, followed by random sorting out and eventually recombination events (mainly in the case of mitochondria, cf. Rothenberg & Hanson 1987; Morgan & Maliga 1987). Nevertheless, the production of hybrids allows the transfer, in one somatic cycle, of chloroplasts from any of the parental species as shown in *Brassica* (Pelletier *et al.* 1986; Sundberg *et al.* 1987).

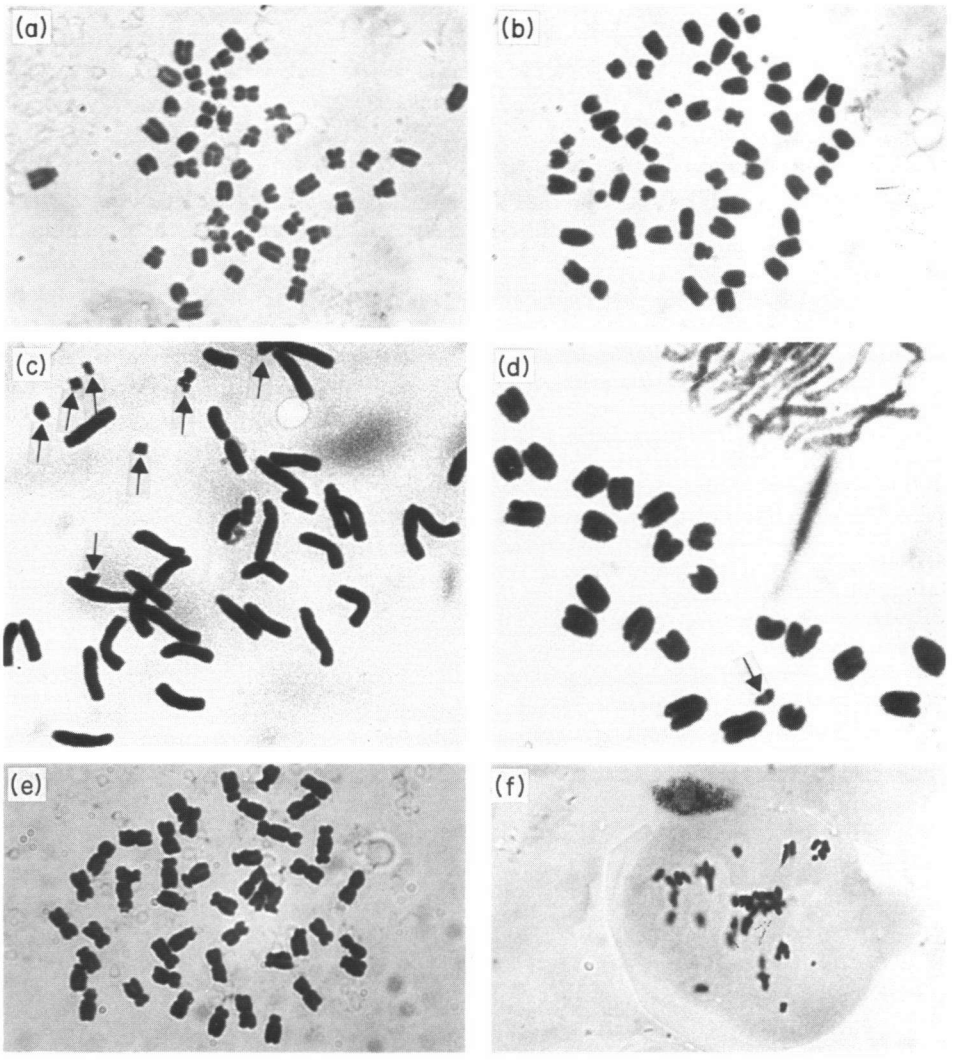

Fig. 2. (a) Metaphase plate of a symmetric somatic hybrid of *N. plumbaginifolia* ($2n = 20$) + *N. sylvestris* ($2n = 24$). Telocentric chromosomes are from *N. plumbaginifolia*, metacentrics are typical of *N. sylvestris*. (b) Metaphase plate from an asymmetric somatic hybrid R_0 plant of *N. plumbaginifolia* + *N. sylvestris* following irradiation of *N. sylvestris* protoplasts (1000 Gy). There are 39 telocentrics and 17 donor (from mini to deleted) chromosomes. (c) Metaphase plate from an asymmetric somatic hybrid R_0 plant of *N. plumbaginifolia* ($2n = 20$) + *A. belladonna* ($2n = 72$) following irradiation of *Atropa* protoplasts (300 Gy). There are 34 *N. plumbaginifolia* and seven *Atropa* chromosomes. (d) The plant from (c) was backcrossed twice and chromosomes analysed in BC-2 progeny: the metaphase plate shows 23 *N. plumbaginifolia* chromosomes and one *Atropa* chromosome. (e) Metaphase plate from freshly induced callus in *N. sylvestris* leaf disks: a dicentric chromosome was produced, followed by ploidization of the chromosome set. (f) A meiotic spread in a R_0 asymmetric somatic hybrid of *N. plumbaginifolia* + *N. sylvestris* [as in (b)] showing transmission of several strongly deleted chromosomes of *N. sylvestris*.

Interestingly there are two examples from sexual hybridization in *Nicotiana* (Medgyesy *et al.* 1986) and *Hordeum* × *Secale* (Soliman *et al.* 1987), which show that paternal transmission of cytoplasmic determinants can also be achieved, which indicates that strict maternal inheritance of organelles may not always occur.

The asymmetrization of the cytoplasmic organelles remains as unpredictable as that of the nuclear genomes (Pelletier *et al.* 1986). In general, a rapid sorting out of parental chloroplasts has been observed (Sheppard *et al.* 1983), especially under defined selection pressures (Fluhr *et al.* 1983; Flick *et al.* 1985; Pental *et al.* 1986). To date there are two well-documented cases of intergeneric cybridization, one containing the nucleus of *Petunia* and chloroplasts of tobacco (Pental *et al.* 1986), the other containing the nucleus of tobacco and the chloroplasts of *Atropa* (Kushnir *et al.* 1987). Tools are now available to study nuclear–cytoplasmic interactions in the building of zygotic or postzygote incompatibility barriers that act in asymmetrization of fusion combinations following irradiation of the donor partner (Thanh *et al.* 1988).

Recombination events and gene introgression. **Sexual hybrids.** Gene transfer from alien species by sexual hybridization and recombination in meiosis requires chromosome pairing between the two species. The DNA 'block' containing the gene of interest from the recurrent parent becomes incorporated by chromosomal exchange into the recipient parent. In species that are sufficiently remote phylogenetically, there is partial or complete failure of chromosome pairing, with a resultant lack of homologous recombination. However, gene introgression can be achieved eventually by illegitimate recombination (Sybenga 1983). In any of these situations, repeated backcrossing and selfing, followed by selection or screening operations, are required in order to identify what appears to be a rare recombination event.

The extent of recombination between two species varies for different chromosomes or chromosome segments, depending on their degree of homology (Brar & Khush 1986). In wheat and rye, although sufficiently close genetically, very limited recombination has been observed even in the presence of *Ph* mutation (Koebner & Shepherd 1987; also see Riley *et al.* 1981; Lapitan *et al.* 1986). In *Nicotiana* species, five categories of pairing were distinguished based on the amount of conjugation at the first meiotic metaphase (Smith 1968).

The extent of knowledge on meiotic recombination can be summarized as follows: meiosis is conditioned by a large number of genes; many of them have been identified through mutations that affect the premeiotic, meiotic, or postmeiotic course of events (reviewed by Kaul & Murthy 1985). A large proportion of the mutations are involved in male meiosis, causing male sterility (*ms* genes), or inhibiting synapsis and chiasma formation, the result is univalent formation. Genes that regulate synaptonemal complex (SC) formation and that simultaneously reduce or increase recombination are found in barley (Enns & Larter 1962), maize (Miller 1963; Nel 1969), and tomato (Moens 1969). There is also evidence to indicate distinct genetic control over different chromosomes which results in chromosome-specific chiasma variations, associated with a compensatory mechanism of overall chiasma frequency per genome (Parker 1975; Tease & Jones 1976).

Breeding has developed useful tools to increase recombination, mainly by breaking gene blocks in which there is negligible crossing over. Chiasma formation is localized and large segments of chromosomes (frequently those close to the centromere) show decreased or lack of recombination (Hageberg *et al.* 1977). Among the tools in use to increase recombination are: (a) translocation stocks (with relatively distally located break points) covering all chromosomes; (b) trisomics; (c) mutations affecting the control of meiotic chromosome pairing and manipulation of the chromosome 5B or *Ph* gene (i.e. induced homolgous pairing); (d) radiation treatments, known to induce translocation and to enhance crossing over in the proximal region adjacent to the centromere, and

(e) misdivision of univalents which can produce translocations with breaks at the centromere or telocentric fusions; an opportunity to transfer parts of chromosomes (Lukaszewsky & Gustafson 1983). However, raising the overall level of recombination is possible only to a limited extent by external agents or special genotypes, and can have adverse consequences in addition to the desired effects (Sybenga 1983). **Somatic hybrids.** The occurrence of intragenomic chromosome breakage and union (Fig. 2e) has been identified in the initial stages of callus induction from leaf disks of *N. sylvestris*, while Pijnacker & Ferwerda (1987) identified megachromosomes as well as fused chromosomes in suspension cultures of potato. On the other hand, direct gene transfer experiments with truncated antibiotic-resistant genes has demonstrated that homologous recombination occurs at very low rates in cultured plant cells (Paszkowski *et al.* 1988). In animal cells, somatic hybrids in culture exhibit high frequency segregation of recessive drug-resistant phenotypes, which was shown to result from gene inactivation and chromosome non-disjunction rather than from mitotic recombination (Campbell & Worton 1981).

So far there is mainly circumstantial evidence to suggest that protoplast fusion can result in introgression, via illegitimate or legitimate recombination, of limited amounts of genetic information from one fusion partner into the other, in both symmetric and radiation-induced asymmetric hybrids (Dudits *et al.* 1979, 1980 and 1987; Jia *et al.* 1983; Schieder *et al.* 1985 and Bates *et al.* 1987). The tobacco + barley combination (Somers *et al.* 1986) deserves a few comments here. It gives electrophoretic and immunological evidence for transfer, via fusion with an irradiated donor partner, of the barley nitrate reductase (*NR*) gene into nitrate reductase-deficient tobacco protoplasts, and succeeds in obtaining fertile NR^+ regenerants at high rates. Not only were NR^+ clones produced in control mixtures of parental protoplasts (no fusion treatment), but the frequency of NR^+-selected clones did not seem to be significantly different among reversion tests and fusion samples. Moreover, all reported segregations were allelic to the wild type locus implying systematic correction via 'targeted' homologous recombination. In one report (De Vries *et al.* 1987), intra- and intergeneric translocation fragments and deletion were demonstrated to occur in regenerants from polyploid bilateral asymmetric hybrid calli of *N. plumbaginifolia* + *Solanum tuberosum* after 24 months in culture.

Gene introgression appears therefore to be mainly the unpredictable consequence of the *in vitro* passage (see page 260) of fusion products. As a constant feature of the hybrid condition is the pattern-specific physical separation within a common nucleus of the chromosomes of the fusion partners, the opportunities for intergenomic exchanges remain rather limited.

In the case of fusions with an irradiated donor partner, the radiation *per se* provides free ends within the fragmented donor chromosomes, but the rapid loss of fragmented acentric chromosomes during the first division(s) seriously reduces the chances of introgression.

DNA regions are known where DNA is late replicating; such regions correspond to fragile sites and exhibit constrictions, gaps or breaks, which are more recombinogenic, and which are also important in evolutionary terms (Laird *et al.* 1987). Treatments known to disrupt replication patterns (such as those used to synchronize cells) have been shown to result in over-replication of DNA, which in turn can generate a wide variety of chromosome aberrations and rearrangements as a consequence of recombination in the over-replicating strands (Schimke *et al.* 1986).

Experimental conditions and treatments need to be devised that enhance the opportunities for recombination at the very first division of a fusion product. Treatments with colchicine (Gleba *et al.* 1987), or other milder reversible mitogenic or DNA replication

blockers that alter the pattern of spatial separation of chromosomes in the hybrid cells, can be envisaged, as well as weak ultraviolet or gamma irradiation of fusion products, in order to provide breaks in the recipient genome. Alternatively, one must screen for highly asymmetric hybrid fertile plants and count (as in classical breeding schemes) on the role of meiosis to provide opportunities for segment exchange.

CONCLUSIONS

(i) The methods concerning sexual hybridization and the exploitation of the reproductive cycle aimed at both distant hybridization and associated gene introgression are time consuming [e.g. in the breeding of the amphiploid *Triticale*, in intergeneric combinations in Triticeae among 18 genera (Cauderon, 1986 and references therein), and in Solanaceae, Caryophyllaceae and *Zea* species]. The main obstacle at present is bypassing the globular stage of rescued distant hybrid embryos (Glimelius 1985; Zenkteler *et al.* 1986).

(ii) Meiotic techniques of exchange (homolgous, homoeologous, and restricted non-homologous) remain the main means of manipulation and are indispensable for the transfer of large gene blocks (Sybenga 1983). Manipulation of recombination is best documented in wheat and oats, but each species may have particular mechanisms. Methods are needed to induce or enhance homologous recombination, as well as to induce somatic crossing over (directed non-homologous) in hybrids that lack chromosome pairing and meiotic recombination.

(iii) Data accumulated in sexual and somatic hybrids from both plants and animals share important similarities in a number of essential processes, such as chromosome elimination, pairing and recombination. Functional, sexual or somatic, plant hybrids are restricted to interspecific and, eventually, some intergeneric combinations, with some evidence that somatic hybrids can reach a more rapid meiotic balance or cover a wider range of species combinations than their sexual counterparts.

(iv) Somatic cell genetics has recently been shown to be highly efficient due to the development of selectable and/or detectable nuclear and cytoplasmic markers that allow one to perform refined experiments at the cellular level. In addition, protoplast fusion has extended the range of species combinations beyond most of the phylogenetic constraints that nature has built. The most common feature of induced asymmetric hybrids is donor chromosome transfer. Passages through meiosis appear to be necessary to reduce the level of ploidy and further eliminate donor chromosomes.

(v) To increase the speed of introgression, at present, one has access to androgenetic techniques (haploid production), a series of *in vitro* culture techniques, as well as a series of refined biochemical and molecular tools such as isozyme analysis, and, more recently, restriction fragment length polymorphism (RFLP) and *in situ* hybridization (Tanksley & Rick 1980; Moore & Sink 1988; Rayburn & Gill 1985, 1987; Mouras *et al.* 1989 and Fig. 1a, b). Such analyses should allow monitoring of the occurrence of recombination events much earlier than before.

(vi) Somatic hybridization incorporates a range of versatile experimental tools including an almost free choice of fusion partners, application of radiation and/or antimitogenic drugs at defined points in the cell cycle or proliferation stage, controlled conditions of callus growth and plant regeneration, specific and tight selection pressures, and the availability of DNA and immunological probes.

(vii) In terms of applied objectives, the identification of desirable, well-defined traits within donor parents is a prerequisite for breeding and commercial achievements. At present, somatic and sexual hybridization are sharing an increasing number of common as well as complementary perspectives, which are evolving on a basis of convergent concepts and experimental tools.

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