BIOTECHNOLOGICAL ASPECTS OF ONOIN BREEDING

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Abstract

A review is presented of two research areas in *Allium* breeding, in which biotechnological techniques play a major role, namely molecular genetics and in vitro culture.

1. Introduction

Over the past two decades significant progress has been made in integrating biotechnology into *Allium* breeding research. Cell biological research has primarily focused on *in vitro* propagation. Efficient plant regeneration protocols have been developed for explant, meristem, embryo and callus cultures (Novak, 1990). Genetic manipulation techniques such as protoplast fusion, particle gun bombardment and *Agrobacterium*-mediated gene transfer are still in an experimental phase (Klein *et al.*, 1987; Dommisse *et al.*, 1990). The application of molecular biological techniques in *Allium* has been primarily directed towards the introduction of a widely usable marker to aid the selection process in breeding programmes as well as to study genome organization. The aim of this paper is not to review all the biotechnological research carried out in *Allium* species but to discuss the current developments in two major areas of biotechnological research in *Allium*, namely, molecular genetics and *in vitro* culture.

2. Indirect selection by means of markers in *Allium* breeding

Knowledge about the genetics of *Allium* species is very scant (Pike, 1986). This is mainly due to the long generation time of most *Allium* species and the strong inbreeding depression (Jones and Davis, 1944). As a result, the breeding of *Allium* species is completely based on phenotypic selection (e.g. mass selection and family selection). It goes without saying that phenotypic selection methods are not particularly efficient, especially in those cases where a trait is difficult to assess, or is inherited recessively. With the advent of genotypic markers, which can be used for indirect selection, opportunities for increasing selection efficiency in *Allium* breeding programmes are created. In this section the usage of these new markers with special reference to molecular markers will be discussed.

2.1. Phenotypic versus genotypic markers

Markers can either be based on the phenotype (morphological markers) or on the genotype (biochemical, cytogenetical and molecular). A comparison between both categories shows that phenotypic markers are less effective for purposes of mapping and indirect selection than genotypic markers (Tanksley *et al.*, 1989). The main reasons being a. the undesirable effects morphological markers (may) cause on the phenotype, in contrast to genotypic markers which are acting in a phenotypic neutral manner and b. the shortage of phenotypic markers as compared to genotypic markers.

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2.2. Genotypic markers: biochemical and cytogonetical markers

Extensive research has taken place describing isozyme variation in *Allium*. Most studies were focussed on *A. cepa*, *A. fistulosum*, their interspecific hybrid and backcross populations with *A, cepa* (Peffley and Currah, 1988; Van der Valk *et al.*, 1991). Furthermore isozyme studies have been carried out in *A. tuberosum* to estimate the degree of apomixis (Kojima *et al.*, 1991) and in *A. fistulosum* to determine the amount of variability in relation to cultivar identification (Haisima *et al.*, 1993). The picture emerging from these studies is that isozymes are useful markers in *Allium*, but their number is quite low: twelve isozyme systems have been found to show polymorphism. This means that only a small part of the *Allium* genome is covered by this type of marker.

The number of polymorphisms detected by cytogenetical markers such as Giemsa-C and Giemsa-G bands proved to be either too low (De Vries and Jongerius, 1988) or unusably high (Peffley and De Vries, 1993). *In situ* hybridization using biotin - of fluorescein-labelled probes may perhaps change this situation (Ricroch *et al.*, 1992).

A preliminary conclusion with respect to the usage of both biochemical and cytogenetical markers in *Allium* research therefore is that both markers are valuable but their use is restricted to specific areas of fundamental research (e.g. karyotyping, biosystematics).

2.3. Genotypic markers: molecular markers

In recent years a number of DNA marker systems has been developed. The marker developed first, the RFLP marker, has had an enormous impact on fundamental (De Vicente and Tanksley, 1993) as well as on practical plant breeding research (Nienhuis *et al.*, 1987).

2.4. Restriction Fragment Length Polymorphism (RFLP) marker

The *Allium* genome belongs to the group of large plant genomes: onion has a genome size of 15500 Megabasepairs (Mbp) per 1C nucleus and leek one of 24400 Mbp per 1C nucleus. Most other plant genomes are much smaller, e.g. cauliflower and tomato are 650 Mbp per 1C nucleus and 1000 Mbp per 1C nucleus respectively (Arumuganathan and Earle, 1991). The consequence of having a large genome for RFLP analysis is that after the (process of Southern hybridization almost no hybridization signals are observed, because too few copies of the genome are present on the blot. For example, given the same amount of DNA, fifteen times fewer copies of the onion genome are present compared to the number of copies present of a tomato genome. Therefore, the sensitivity of the Southern hybridization technique would have to be increased approximately fifteen times in *Allium*, to yield the same result as for tomato. This technical problem has proven to be a major obstacle to the application of the RFLP marker in *Allium*.

In contrast to the nuclear genome, organellar genomes can easily be analyzed by means of Southern hybridization because the number of mitochondrial (mt) and chloroplast (cp) genomes per plant cell is in the order of several hundreds of genome copies, whereas the nuclear genome is present in the diploid plant cell in only two copies. Analyses of the mt genome of onion have shown that the N and T cytoplasms resemble each other to a large extent, while the S cytoplasm deviates significantly (Havey, 1993). For the chloroplast genome of onion a physical map is available (Katayama *et al.*, 1991). Phylogenetic research of the genus by means of cpDNA restriction enzyme analyses has been carried out by Havey (1991, 1992).

In conclusion: the RFLP marker is potentially useful to study the nuclear genome of *Allium* provided that technical problems with respect to Southern blotting can be solved. The use of the RFLP marker in organellar genomes has already proven to be very valuable in biosystematic studies.

2.5. Polymerase Chain Reaction (PCR) - based markers

Based on PCR technology (Sakai *et al.*, 1985) a new category of molecular markers has become available for *Allium*. In this context, the Randomly Amplified Polymorphic DNA (RAPD) marker and the Sequence Tagged Site (STS) marker will be discussed.

The RAPD marker is based on the amplification of DNA fragments by using synthetic oligonucleotides (usually 10-mers) as primers for the PCR reaction. By means of PCR, parts of the genome are being amplified to a large extent. This completely overcomes the problem of having too few copies of the genome, as is experienced with the RFLP technique. Wilkie *et al.*, (1993) observed that RAPD polymorphisms are detected at a high frequency both between *Allium* species and also within *Allium* species: seven out of twenty primers showed polymorphisms between onion cultivars. De Vries *et al.* (1992) showed that the Bulked Segregant Analysis (Michelmore *et al.*, 1991), using RAPD markers, was a very powerful technique to tag an agronomically important trait. They found three RAPDs closely linked to the locus conferring resistance to downy mildew (*Peronospora destructor*) which originated from *Allium roylei* (Kofoet *et al.*, 1990). We have now cloned the RAPD which was most closely linked to the resistance locus (2.7 cM; unpubl. results) and subsequently sequenced and developed 20-mer primers. This presently enables marker-aided selection for downy mildew resistance in commercial plant breeding programmes. The major advantage of marker-aided selection in this specific case is that the downy mildew fungus is not needed anymore for the resistance screening, so problems of getting a proper infection and subsequent problems of interpreting the results due to escapes are eliminated. Furthermore, a quick and reliable screening test can already be performed at the seedling stage.

For the construction of large molecular marker maps of cross-fertilizing species, RAPDs are less useful because they are population-specific, have a dominant nature (a heterozygote cannot be distinguished from a homozygote) and are not always reproducible (Waugh & Powell, 1992). The current opinion about the usage of RAPD markers is therefore, that they are very useful in quickly identifying the genes determining agronomically important traits. However, for further usage in plant breeding programmes, the primers of a linked RAPD marker need to be replaced by specific and enlarged primers (as in the downy mildew example) or the RAPD marker needs to be mapped on an existing RFLP map (not available for *Allium*) to enable the subsequent use of neighbouring RFLP markers. This means that for *Allium* there is still a need for a codominant, easy to handle, marker. The sequence tagged site (STS) marker might be such a marker.

The STS marker, or to be more specific the STMS (sequence tagged microsatellite marker), is based on the ubiquitous presence of so-called microsatellite sequences in the genome (Hearne *et al.*, 1992; Bruford and Wayne, 1993). Microsatellites are repetitive sequences of generally less than four nucleotides and with an overall length usually less than 100 bp (Thomas *et al.*, 1993). In the few plant species analyzed so far A_n/ A_{Tn}/ CA_n/ GT_n are the microsatellite sequences which occur most frequently (Bell and Ecker, 1994). The frequency of microsatellites in the nuclear genome has not been yet fully established for plant species. However, it is estimated that they occur at a maximum frequency of once in 200 kB (Wu and Tanksley, 1993). Especially the high degree of polymorphism and its codominant nature (Weissenbach *et al.*, 1992) renders this marker potentially interesting for *Allium* species. However, several problems have yet to be solved before the STS marker can be successfully applied in plants. The frequency of microsatellite sequences in plant genomes seems to be lower than in animal genomes. Consequently, efficient enrichment methods for microsatellite sequences in genomic libraries would be valuable (cf. Bell and Ecker, 1994). Furthermore genotyping needs to be improved, because of the occurrence of shadow bands, possibly resulting from mispairing of the template and the newly-synthesized strand or terminal transferase activity of the Taq polymerase (Hearne *et al.*, 1992).

3. *In vitro* culture

Although, substantial progress has been made regarding tissue culture techniques for *Allium* species, there are only a few reports on the isolation and culture of protoplasts. Most of these reports deal with enucleation or the development of fusion procedures (Opatmy and Havránek, 1977; Bradley, 1978; Bracha and Sher, 1981; Kim *et al.*, 1986J. For the isolation and culture of protoplasts of *Allium* species different types of starting material have been used. In several studies conducted with onion and garlic, protoplasts isolated from leaf material were cultured. In these experiments, only cell wall formation or an initial cell division was observed (Opatmy and Havránek, 1977; Bradley, 1978; Bracha and Sher, 1981; Kim *et al.*, 1986, Novak *et al.*, 1986). There is one report of successful plant regeneration from mesophyll protoplasts of *A. cepa* (Wang *et al.*, 1986). Fellner (1993) mentioned the formation of small cell colonies from callus protoplasts of *A. longicuspis*, a wild ancestor of garlic. The primary calli were derived from leaf bases and protoplasts from these calli showed a higher viability and divided more easily, as compared to protoplasts isolated from leaves. Microcalli have also been obtained from callus protoplasts of *A. cepa* and *A. fistulosum* (Balakrishnamurthy *et al.*, 1990). However, none of these authors mentioned sustained cell division from callus-derived protoplasts.

3.1. Protoplast culture in leek (*Allium ampeloprasum*)

In leek, F_1 hybrid breeding for improving uniformity is of great economical importance. Unfortunately, hybrid breeding based on cytoplasmic male sterility (CMS) is hampered because there is no source of CMS available in leek and introduction of this trait through interspecific hybridization has not yet been successful. Somatic hybridization may therefore become an important tool to overcome these problems. However, this technique requires the use of protoplasts, and consequently we have set up a research programme in order to develop a reliable regeneration procedure from leek protoplasts.

Protoplasts were isolated from embryogenic suspension cultures. In monocotyledonous species, embryogenic suspension cultures have proven to be a valuable source of protoplasts. In most cases, when regenerable suspension cultures were available, plants were also obtained from the protoplasts (Vasil, 1987; Vasil, 1988; Shillito *et al.*, 1989; Vasil *et al.*, 1990). For the establishment of fast-growing and finely dispersed suspension cultures embryogenic callus cultures are often used, which are compact and white in appearance (Vasil and Vasil, 1984; Redway *et al.*, 1990). Immature embryos seem to be the most suitable explant for the establishment of such embryogenic cultures (Armstrong and Green, 1985; Vasil and Vasil, 1986). In leek, we have used mature embryos as explant, on which exclusively compact, embryogenic callus was initiated (Buiteveld *et al.*, 1993). Attempts to initiate cell suspensions from this compact and embryogenic callus type have, up until now, not succeeded. In maize, two embryogenic callus types, a compact and friable type, can be distinguished, but only the friable type has proven to be a good source for the production of embryogenic cell suspensions (Vasil and Vasil, 1986). For this reason, our research was aimed at the initiation of such a friable type of callus. This callus could be successfully initiated from immature embryos of leek (Buiteveld *et al.*., 1994) and could easily be distinguished from the compact callus type. The friable callus was characterized by having a soft, white friable appearance, while consisting of numerous globular structures or somatic embryos embedded in a mucilage. These callus cultures showed a very high regeneration capacity. Upon transfer to regeneration medium, the calli disintegrated into numerous somatic embryos, which germinated easily. Friable callus cultures with the highest regeneration capacity were then selected for the initiation of suspension cultures. The suspension cultures exhibited pronounced differences with respect to growth and degree of dispersion.

We found that these differences were genotype-dependent and therefore, genotypes were screened for their amenability to culture in liquid medium. Only well-dispersed embryogenic suspension cultures with a moderate to good growth rate were selected. Protoplasts isolated from these cultures were richly cytoplasmic and formed microcalli when cultured in a modified K8p-medium. Culturing the protoplasts in alginate had a positive effect on the plating efficiency. Further growth of the microcallli obtained led to the formation of embryogenic calli from which well-rooted plants could be regenerated at high frequency (Buiteveld and Creemers-Molenaar, 1994)

Summarizing, it can be said that, for leek, the choice of starting material was an important step towards developing a successful procedure for the isolation and culture of regenerable protoplasts. When working with other *Allium* species, results may prove even better when the same type of explant and starting material is used.

4. Perspectives

With the introduction of biotechnological techniques into *Allium* breeding programmes new possibilities become apparent. For example, the development of a broadly applicable molecular marker in *Allium* can lead to a significant increase in the selection efficiency. This minimizes the amount of labour required to carry out a breeding programme and shortens the breeding cycle. Cell biological techniques, such as protoplast culture, may prove to be very beneficial for the transfer of agronomically important traits between species which do not normally intercross.

Therefore, it is expected that progress in both areas of research will eventually lead to the use of a wide range of *Allium* germplasm for plant breeding purposes and will allow a more analytical plant breeding than hitherto was possible.

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