1.
New Technology

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DEVELOPMENTS IN THE CULTURE OF PLANT PROTOPLASTS AND CELLS AND THEIR REGENERATION TO PLANTS

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ABSTRACT

Steady progress is being made on culture and regeneration of plants from protoplasts and cells. Callus culture in itself can cause variation in regenerated plants, and this aspect is outlined. Developments in protoplast fusion methodology using electric fields are also discussed. The need for efficient culture systems for useful genotypes of crop plants is emphasised as a pre-requisite for other aspects of genetic manipulation.

INTRODUCTION

There are two distinct areas in which plant tissue culture is an important technique. The first includes situations where the maintenance of genetic stability is a pre-requisite. This encompasses such approaches as micropropagation (the clonal multiplication of plants in vitro), germplasm storage, virus elimination (the eradication of latent virus infection by meristem culture), embryo rescue (the recovery of hybrids, usually from wide sexual crosses when endosperm development is disturbed), and production of haploids via anther or ovule culture (Jones and Karp, 1985). The second involves situations where isolated cells or tissues are first induced to form a disorganised cell mass (callus), from which shoots and new plants are subsequently regenerated. Under these circumstances, culture-induced changes may occur spontaneously ('somaclonal variation'). This second area is discussed in more detail, because most of the developing techniques of genetic manipulation of plants involve a callus phase at some stage of the procedure. In this article, the present state of the art of plant regeneration from cells and tissues, and some applications, are outlined, with emphasis on more recent developments. Some of the examples are taken from work at Rothamsted which has centred on cereals (wheat, barley) and dicotyledonous crop plants (potato, oilseed rape, sugar beet).

Regeneration from explants

Surface-sterilized plant tissues (e.g. leaf, petiole, stem etc.), placed on a culture medium containing salts, organic components, sucrose as a carbon source, plant growth regulators, and solidified with agar, can be induced to form callus tissues. With suitable combinations of growth regulators (auxins and cytokinins), shoots may be induced. The shoots may be excised, rooted, transferred to soil and grown to form intact plants.

The range of plants that can be regenerated in this way is considerable, and now extends to many crop species such as potato, tomato, brassicas, sugar beet, pea, forage legumes and cereals (Flick et al. 1983; Jones and Karp, 1980; Bright and Jones, 1985). For example, at Rothamsted, starting from immature embryos, plants have been regenerated from somatic tissues of every one of over 40 spring and winter wheat cultivars examined (Maddock et al. 1983) (Fig. 1).

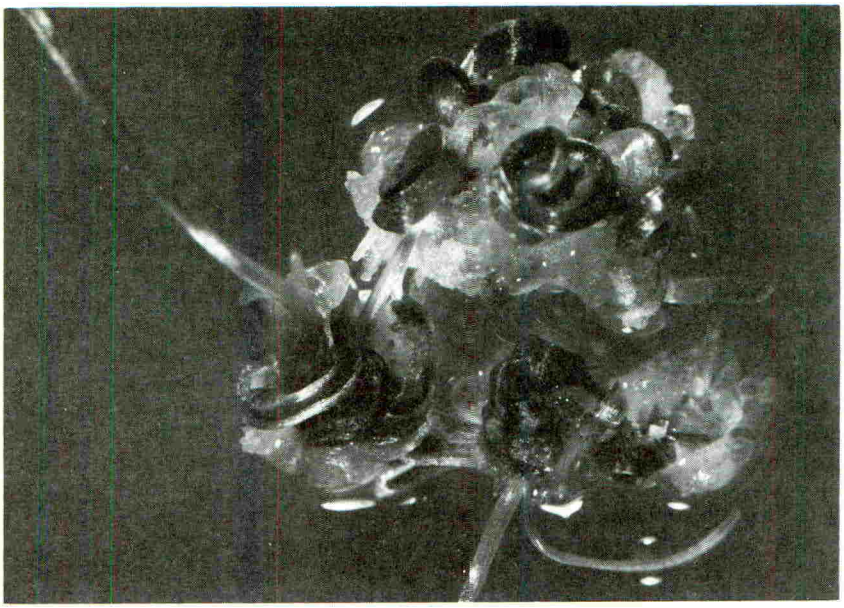

Fig. 1. Shoots developing on cultured tissues of immature wheat embryo. (Courtesy Dr. S.E. Maddock).

Regeneration from protoplasts

Protoplasts are single plant cells, isolated following enzymatic removal of cell walls, which are initially spherical (Fig. 2). They must be maintained in an osmoticum (e.g. 9% mannitol) that will counteract the tendency for the protoplasts to take up water osmotically and burst. The range of enzymes available for protoplast isolation, and their purity, has improved recently. In particular, the enzyme Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd. Japan), with pectin endo-lyase and endo-polygalacturonase activity, has proved particularly effective

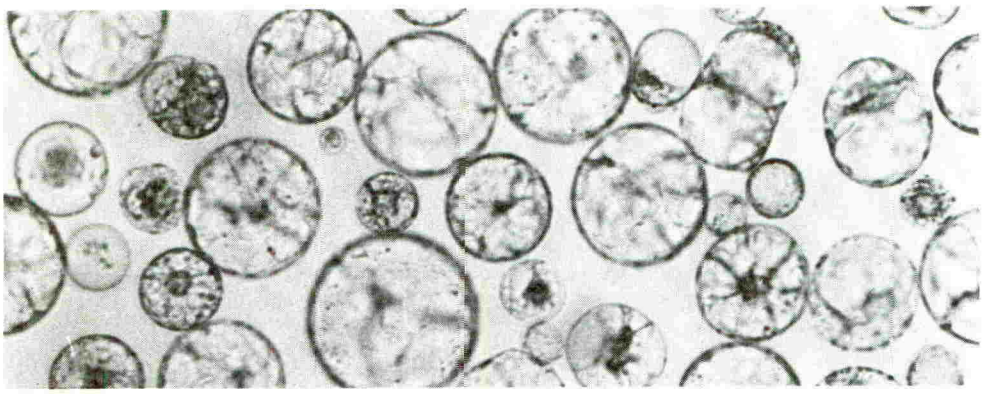

Fig. 2. Protoplasts isolated from hypocotyl calls of oilseed rape (*B. napus*).

(Nagata & Ishii, 1979). Most procedures for isolation involve washing the protoplasts in fresh medium, perhaps with a step including spinning onto a more dense sucrose cushion, to remove debris and wall degrading enzymes. The protoplast density is adjusted, and then they are plated out in appropriate culture medium. A method that has proved successful, particularly for selection of transformed colonies, is the embedment of protoplasts in low-gelling temperature agarose ('agar-bead' technique, Shillito et al. 1983). This allows media to be changed easily without disturbing the cells, and agarose also provides mechanical support for protoplasts. On culture, protoplasts form new cell walls, divide, and form colonies, during which time the osmotic pressure of the medium is gradually lowered. One problem with plant protoplasts, that they usually need to be plated out at fairly high densities, has been solved at least for tobacco by the technique of Caboche (1980). Rather than try to add many components to the medium, (Kao and Machayluk, 1975), transfer from medium containing high auxin to low auxin after 4 days culture, allows protoplasts at the density of 1-10 ml^{-1} to form colonies.

Once protoplast-derived colonies have been obtained, the next step is to induce shoot formation by transfer to media usually containing higher cytokinins and lower auxin levels (Fig. 3). This may either be by

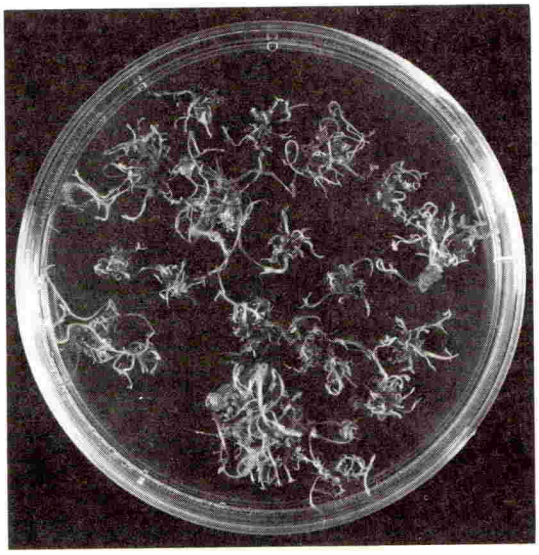

Fig. 3. Shoots regenerating from protoplast-derived colonies of potato.

'organogenesis' - when shoots and roots develop separately, or by 'embryogenesis' - when structures resembling zygotic embryos (embryoids) develop and germinate to give shoots and roots. The ideal regeneration sequence from protoplasts would be direct development of embryoids within as short a time as possible, followed by their germination to give plants. This scheme has been attained for haploid oilseed rape protoplasts (Li and Kohlenbach, 1982).

As far as crop plants are concerned, there has been a steady increase in the number of species and genotypes that can be regenerated from protoplasts to plants (Jones and Karp, 1985). The fact that culture-ability is often restricted to specific genotypes or cultivars of a crop plant is a drawback that needs further study. The initial approach is

often to screen a range of genotypes to find one that appears responsive, and work with that one. However, with careful attention to detail, it should be possible to modify conditions such that any genotype or advanced breeding line may be cultured, as shown by the successful culture of apparently unresponsive cultivars of potato (Foulger and Jones, 1986). However, it is notable that grain legumes, sugar beet and cereals have proved to be most difficult to regenerate. Some advances have been made both for the legumes (e.g. soybean), and sub-tropical cereals. In the latter case, plants have been reported to be regenerated from protoplasts of Pennisetum americanum, Panicum spp and sugar cane (Vasil and Vasil, 1980; Lu et al. 1981; Vasil et al. 1983). However, the major cereals such as wheat, maize and rice, still cannot be regenerated from protoplasts, although protoplasts obtain from liquid cell suspension cultures that divide to form callus are available for most of these graminaceous species. The reasons why this important group of plants are unresponsive is unknown. Clearly the developmental pattern differs from dicots, this is reflected in the general maxim that it is possible to maintain division in dividing cereal cells, but not to induce division in mature, differentiated cells. In contrast, ' mature' cells (e.g. in the leaves) of dicots can readily be induced to divide. For cereals this situation in unfortunate because, as is described in more detail (Potrykus, this volume) it is now possible to genetically engineer graminaceous protoplasts, but so far such methods can yield only engineered callus, not intact plants (Jones, 1985a,b).

Nature of plants regenerated from culture

It was originally envisaged that regeneration of plants from protoplasts or explants would produce clones identical to the parental plants. However, it has become increasingly obvious that this is usually not the case. This culture-induced variation has been called 'somaclonal variation', and has been documented for a wide range of plants regenerated from culture (Fig. 4) (Larkin and Scowcroft, 1981; Karp and Bright, 1985; Jones and Karp 1985). This variation may manifest itself in changes of such characters as height, general morphology, disease response, yield; altered tuber, grain or flower colour etc. Research is moving from the phase of describing and cataloguing this variation, towards trying to understand its molecular basis. One factor is obvious changes in cytology -regenerants may be aneuploid - this has been particularly well documented for potato (Karp et al. 1982; Creissen and Karp, 1985) and wheat (Karp and Maddock, 1984) and appears most common in polyploid species. Other cytological changes can include deletions or interchanges of parts of chromosomes (Creissen and Karp, 1985; Fish and Karp, 1986). However, variant plants are still found in the absence of identifiable cytological changes. The latter variants are of most interest, since variation can occur in useful agronomic characters (Karp and Bright 1985, Evans et al. 1986). Research is in progress to describe the underlying basis of this variation - whether and how it differs from classical mutants, whether it can be controlled, reduced or usefully directed. Breeders are having to decide whether or how much effort they should put into such approaches, if any. For crops such as potato, there is a real possibility that established cultivars may be upgraded by identification of superior variants. On the other hand, from the viewpoint of techniques aimed at modifying plants genetically (e.g. protoplast fusion, transformation), somaclonal variation is an unwanted complication that it would he useful to

eliminate if possible. This is the case for genetic manipulation by protoplast fusion, which is discussed next.

Fig. 4. Variation in hight of Triticale plants regenerated from the same immature embryo.

Protoplast fusion

The isolation of plant protoplasts offers possibilities for genetic manipulation that by-pass the normal limitations to sexual combination of characters. Thus, in principle, it should be possible to combine any two protoplasts and obtain hybrid plants. In practice, with more knowledge of protoplast culture, it has been found that the production of hybrid plants in this way has not been as straight-forward as predicted, and some of the earlier claims have turned out to be rather exaggerated. Nevertheless, there has been steady progress, and useful work in the following areas is emerging: (1) combination of complete genomes (2) partial genome transfer, either by spontaneous loss of genetic material or induced by irradiation of the 'donor' protoplasts population (3) transfer of cytoplasmically-(chloroplast, mitochondrion) encoded traits. Even if a sexual cross is possible, for example to introduce genes from a wild species into a cultivated species, fusion may extend the range of genotypes that can be hybridized, and also leads to a greater variety of hybrids. This is because in sexual crosses maternal inheritance of cytoplasmic traits is the rule, whereas after fusion, in general, there is recombination of mitochondria from both parental types, but segregation of chloroplasts such that hybrids may contain chloroplasts from one or other parent (Gleba and Evans, 1983).

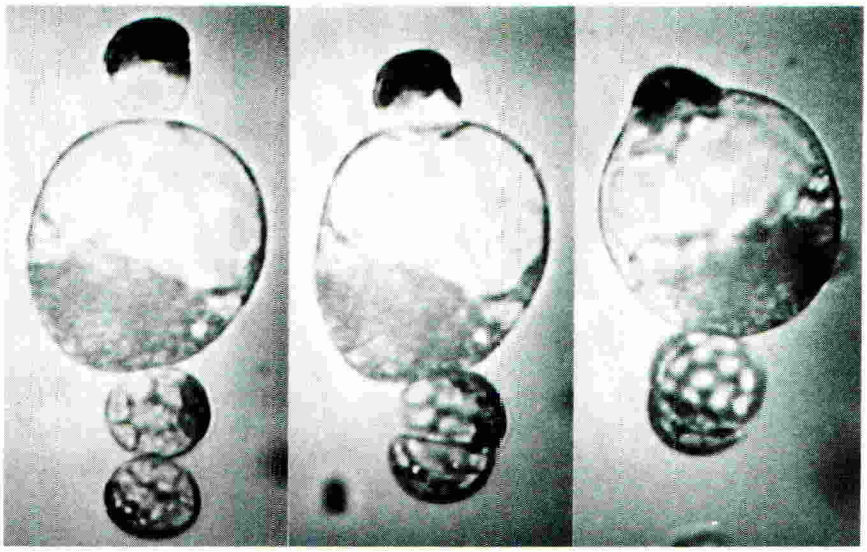

Fig. 5. Fusion of large hypocotyl protoplast of B. napus with small leaf protoplast of S. brevidens, and self fusion of S. brevidens leaf protoplasts, following electrofusion. (From Tempelaar and Jones, 1985a).

Fusion methodology

Fusion of plant protoplasts has been accomplished over the past decade by variations on chemical methods involving adhesion of protoplasts by polyethylene glycol with membrane fusion induced by treatment with solutions containing high calcium concentrations at high pH (e.g. pH 10.5) (e.g. Evans, 1983). More recently, the physical method of 'electrofusion' has emerged (Zimmermann and Scheurich, 1981; Zimmermann 1982). When protoplasts are suspended in a medium of low conductivity (e.g. 9% mannitol), application of a high frequency alternating field (e.g. 1 MHz) polarizes protoplasts, so they act as dipoles, and are attracted towards one another by 'dielectrophoresis' to form chains. Once in contact, fusion can be induced by application of one or a series of short DC pulses (15-300 usec, 1-3 $KVcm^{-1}$) that cause transient membrane breakdown and pore formation, and leads to fusion of contacting membranes (Fig. 5). In initial work, extrapolated from the animal cell field, an electrode separation of 1-200 um was used. However, for plant work, wide electrode separations are more useful (1-5 mm). The measurement of pulse duration-fusion response curves (Fig. 6) has indicated that leaf protoplasts are more fusogenic than suspension culture protoplasts, and this in turn means that by controlling pulse parameters, fusion can be directed between different protoplast populations (Tempelaar and Jones, 1985a,b). It is also possible to fuse cells on a bulk scale, and still apply these principles to obtain a good proportion of 1:1 fusion products even when the chains of protoplasts are fairly long (e.g. 60% 1:1 products at an overall fusion frequency of 50%). Electrofused cells can divide, form colonies and regenerated shoots in the same way as chemically fused cells.

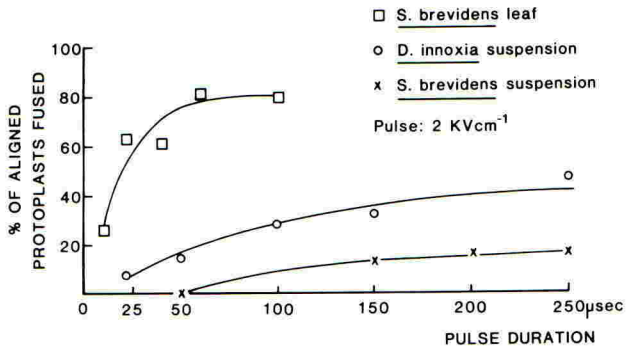

Fig. 6. Pulse duration-fusion response curves of mesophyll and suspension protoplast after electrofusion. The differences in response can be used to direct fusion of different protoplast types. (Modified from Tempelaar and Jones, 1985a).

Applications of fusion

The application of protoplast fusion technology is described in more detail by Cocking (this volume). Here, one crop, potato, is taken as an example. Hybrid plants have been produced by fusion of potato protoplasts with various different solanaceous species (Jones and Karp 1985; Jones, 1986), incuding tomato, wild potatoes S. chacoense, S. brevidens, S. nigrum, and N. sylvestris. In the earlier work, aneuploid hybrid plants were produced in the main. More recently, Austin et al. (1985a) succeeded in fusing dihaploid potato and diploid S. brevidens protoplasts to produce sexually fertile tetraploid hybrids - thus introducing the potentially useful germplasm of S. brevidens, reported to exhibit resistance to potato leaf roll virus and frost tolerance, for introgression into potato. Similarly, the synthesis of novel tetraploids from selected diploid lines has been achieved (Austin et al. 1985b).

The background of cytological changes nevertheless will still prove a problem in future work on protoplast fusion, and will necessitate production of many hybrid plants from which useful genetic combinations can be selected.

Direct gene transfer

The need for efficient regeneration systems from protoplast to plant, and the need to control genetic stability during this process, has been highlighted by the recent successful direct introduction of genetic information into protoplasts. This aspect is described in more detail by Potrykus (this volume). The approach opens up exciting new areas of methodology for modification of plant genomes, but exploitation will be limited at present to those systems where efficient plant regeneration from protoplasts can be achieved.

CONCLUSION

There has been steady progress in broadening the number of species that can be regenerated from protoplasts, and the efficiency of plant regeneration in such systems. Nevertheless, continued careful work on protoplast and tissue culture of important crop plants is required for full exploitation of cellular and molecular techniques that can be applied to their genetic manipulation, and for future biotechnological applications.

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THE USE OF RECOMBINANT DNA TECHNIQUES IN THE PRODUCTION OF VIRUS RESISTANT PLANTS

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ABSTRACT

The use of recombinant DNA technology may be useful in the production of virus resistant plants in two ways. One of these uses cloned fragments of viral genomes as a probe in a diagnostic assay for viral infections in crude plant extracts. The improved efficiency of this method over older symptom based detection methods has been used to benefit potato breeding programmes. The second application of recombinant DNA will permit the transfer of virus resistance genes to the genome of susceptible plants. The vector systems for this transfer and the likely source and mechanism of action of resistance genes are described.

INTRODUCTION

The techniques of recombinant DNA work have developed over the last few years such that it is now possible to isolate and amplify pure genes in large amounts. Subsequently the genes can be manipulated, modified or moved from genome to genome in spectacular ways. This has lead to the expectation by some that plant breeding, which is a discipline of gene manipulation and transfer, will benefit as a result. However, Bingham (1984) reminds us that many of the current plant breeding requirements can be met by breeding methods currently in use and implies that the genetic engineer should appraise his goals carefully. Repetition of the achievements of conventional breeding will benefit no-one and in the market place, results rather than novelty carry the premium. This is undoubtedly wise advice, but should not necessarily induce pessimism about the prospects that molecular genetic engineering will contribute towards the production of better crop plants. In this presentation I shall outline two methods where recombinant DNA work has, or may, contribute towards the breeding of virus resistant plants. One of these is in the area of diagnostics where the new technology is used to improve the efficiency and effectiveness of conventional breeding.

The second area involves the possibility of introduction of virus resistance genes into the genome of susceptible plants. The vector systems which have been developed for this will be described and various sources of the actual resistance genes compared.

VIRUS DIAGNOSIS USING CLONED DNA PROBES

The screening of progeny for virus resistance in a breeding programme involves measurement of virus production following a test infection. In the potato breeding programme at the Plant Breeding Institute this is normally carried out in the fourth year of a breeding cycle when several thousand different plant clones are to be tested. This measurement of virus accumulation thus needs to be rapid and accurate. For potato virus Y and potato leafroll virus, symptom production cannot be detected easily in the primary infection so that tubers of infected plants need to be grown for a second year when symptoms appear. The development of a rapid hybridisation assay which is effective on extracts of young tuber sprouts was therefore a useful advance. It became possible to detect virus susceptibility before

the second year so that the second year of growth can be eliminated (Baulcombe et al., 1984a).

The method of sample preparation in this procedure is quite simple (Baulcombe et al., 1984b). Crude sap samples are simply spotted onto nitrocellulose filters and dried. The filters are subsequently hybridised with radiolabelled DNA probes which are copies of the viral genome. After hybridisation and washing to remove the non-hybridised probe the presence of virus is detected by autoradiography. Because of the straightforward nature of this procedure hundreds of samples may be processed simultaneously. In extensive tests we have found that the procedure is as reliable as immunological methods. It has therefore been accepted readily by plant breeders and is now in routine use at this Institute. The use of cloned DNA copies of the viral RNA in this assay has several advantages. Firstly the probe is absolutely pure and free of sequence which might react with sap of non-infected plants. The cloning step is of course an amplification as well as a purification. It becomes possible therefore to prepare large quantities of probe, without repeated purification of the virus. The cloned probes also enable defined regions of the viral genome to be used. This may be advantageous when it is necessary to diagnose different strains of virus. For example, in the potexvirus group it has been shown that different regions of the genome hybridise to different extents between type II and type III strains. This is a flexibility not given by immunological probes (which do not discriminate readily between type II and type III strains of potato virus X) (Baulcombe et al., 1984b).

This nucleic acid hybridisation assay is therefore one of the first examples where recombinant DNA techniques are used to advantage by plant breeders. The many reports of different probes from different laboratories suggest that in time this will become a major contribution of molecular biologists to plant breeding. It is less spectactular perhaps than the hopes for molecular genetic engineering of plants, but a useful contribution nevertheless. Diagnostic applications of recombinant DNA are likely to serve other areas too. Cloned DNA probes may be useful as markers for nuclear genetic loci or particular cytoplasmic genes (Flavell et al., 1983) and possibly also in the diagnosis of fungal and bacterial disease.

GENETIC ENGINEERING OF VIRUS RESISTANCE IN PLANTS

The application of molecular techniques to the engineering of virus resistance in plants requires two elements. Firstly one needs a vector system for the delivery of the resistance gene into the plant genome. The second requirement is a means of identifying and isolating genes, as fragments of DNA, which are likely to confer resistance to the recipient plant.

Vector systems

Until now, two categories of vector system have been used to transfer foreign DNA into the genomes of higher plants. The more widely used of these is based on the Ti plasmid of Agrobacterium tumefaciens and the other system is direct DNA uptake, which will be described by Potrykus in a separate contribution to this meeting.

The use of Agrobacterium as a vector followed from the demonstration that following infection of plant cells with this bacterium, part of the Ti plasmid DNA, known as the T-DNA, became integrated into the genome of the infected plant (Chilton et al., 1977). The natural Ti plasmid was not immediately suitable as a vector system, however, for several reasons.

The T-DNA region of natural Agrobacterium contains several genes which are responsible for the production of auxins and cytokinins. As a result the infected transformed cells are tumourogenic and cannot be regenerated into sexually mature plants. Therefore these genes and other non-essential regions of the T-DNA region were deleted (De Greve et al., 1982).

The Ti plasmid also presented difficulties because of its large size. The presence of multiple sites for restriction enzymes and the difficulties of isolating intact DNA lead to several strategies which facilitated the transfer of novel DNA into T-DNA and eventually into the plant genome. Perhaps the most novel of these followed from the discovery that transfer of T-DNA required the action of the virulence genes, located elsewhere on the Ti plasmid, but that these genes could act in trans (De Framond et al., 1983). It was therefore possible to create a small plasmid containing T-DNA which could be maintained and selected in E. coli. This plasmid could be transferred by mating into an Agrobacterium strain which contains a plasmid with the vir genes but not a T-DNA region (Bevan, 1984). These vir genes subsequently catalyse the T-DNA transfer from the small plasmid into the plant genome (Bevan, 1984).

Other elements of the T-DNA vector are a dominant marker which allows the selection of transformed plant cells and an expression cassette. In the vector currently used in this laboratory, both of these components contain two elements in common (Bevan et al., 1985). They both contain the same promoter from cauliflower mosaic virus responsible for transcription of the 35S RNA. The transcriptional terminator is also common, being derived from the nopaline synthase gene of T-DNA. The expression cassette, however, contains a unique Bam HI site for the insertion of novel genes at a position where the marker contains the gene for neomycin phosphotransferase. The promoter is of moderate strength and active in different tissue types, so that this serves as a general purpose vector. It is likely that in the future, other promoters will be used to drive high level expression of genes in a tissue specific manner. Probably one of the first promoters to be used in this way will be from the chlorophyll a/b binding protein gene which has been shown to drive the expression of a bacterial chloramphenicol transacetylase gene in transformed plants (Herrera-Estrella et al., 1984).

The actual use of Agrobacterium to infect and transform plant cells is now very straightforward. Leaf discs are incubated with the Agrobacterium for a few hours and then cultured on a layer of feeder cells, initially, and then the drug selective medium with hormones to induce shoots (Horsch et al., 1985). Shoots are obtained routinely by 21 days and these are then excised and cultured on root-inducing medium. With tobacco and petunia it is possible to obtain mature transformed plants within a few months of the initial transformation. The procedure, with modifications may be applicable to potato, tomato, Arabidopsis and legumes. Unfortunately, the tissue culture of monocotyledonous plants is not yet developed to the stage where mature plants can be regenerated from somatic leaf tissue.

The second method of DNA transfer into plants by direct transformation is also applicable in a biotechnological context to a range of plant species where protoplasts can be regenerated into plants. Unfortunately this also excludes monocotyledonous plants. The features of this method of DNA transfer are described in an accompanying presentation by Potrykus.

Although further development work needs to be done on vector systems, they are currently useful on some important crop plants. It should be

possible therefore, provided that virus resistance genes can be isolated, to transfer these into the genomes of susceptible plants.

Isolation of virus resistance genes

In principle there are two sources of virus resistance genes as DNA fragments. It may be possible to isolate naturally occurring resistance genes from resistant plants. Alternatively expression of gene sequences from other diverse sources may interfere with the viral infection. Which of these will be the most effective is as yet unresolved. However, in the view of this author the latter is likely to prove the most accessible source. The reason for this is likely to be the difficulty of identifying DNA fragments from resistant plants containing the resistance genes.

Normally either of two strategies may be used to identify recombinant DNA clones containing a particular gene. If the protein product of the gene is known it is possible to determine at least a partial sequence or to raise antibodies against the protein. Antibodies may be used to screen recombinant DNA clones either directly or indirectly. Peptide sequences may indicate short gene sequences and so oligonucleotide probes prepared which may be used to screen clones. These are now routine and highly effective procedures (Szostak and Rothstein, 1984).

The second procedure, involving transposon tagging, will be discussed by Weinand in an accompanying presentation and is a powerful method when a gene can be identified easily by its gross phenotype.

However, neither of these two methods is readily applicable to the isolation of virus resistance genes. In no case, to my knowledge, has the protein product of the viral resistance genes been identified, thus ruling out the first approach. The transposon tagging method can be used easily only in plants where moveable DNA has been characterised at the molecular level and where movement of the transposon can be controlled genetically. In 1985 this restricts the list to Zea mays and Antirrhinum majus, although evidence for transposon-like DNA has been obtained in wheat (Martienssen and Baulcombe, unpublished) and soybean (Goldberg et al., 1983). In addition the genetical transfer of the transposon into the resistance gene may involve periods of several years and large scale resources to screen for this event.

It is likely that, given time and effort, these difficulties in isolation of virus resistance genes from plants could be overcome. However, in doing this the ostensible benefits of molecular genetic engineering of plants, namely speed and directness, will have been negated. For this reason, in this laboratory, we have chosen to approach virus resistance using genes which are readily accessible. Two examples are given below, one of which is the basis of our work.

Virus resistance through the production of neutralising antibodies

The development of monoclonal antibody-producing cell lines has now opened up the possibility of isolating the immunoglobulin genes which are specific for defined antigens. This has been achieved for the genes of antibodies which react against a (4-hydroxy-3-nitrophenyl)acetyl hapten (Bothwell et al., 1981). It should be quite possible, therefore, to isolate the genes for the immunoglobulin which is reactive against viral proteins. Expression of this gene in transformed plant cells might be reasonably expected to neutralise the activity of the viral protein. Whether this strategy will succeed is still to be determined. However, there are no steps which should provide unusual technical difficulties. Viral genes can

be cloned and easily identified and the gene products produced by expression of the genes in E. coli. The recombinant produced protein may be used then as antigen to stimulate antibody production and the antibody gene cloned and identified using a probe to the immunoglobulin constant region. One can expect, therefore, that this strategy will be tested in the near future.

Virus resistance through the mimicking of interviral cross protection

If a plant is infected with a virus (the inducer), that plant becomes resistant to infection by a related virus (the challenger). This phenomenon, known as interviral cross protection, is now well established for many viruses and has been used as a form of crop protection, when the inducing strain produced mild infection (Fletcher and Rave, 1975). Use of a complete virus as inducer does, however, have disadvantages. For example, the virus may serve as a reservoir of infection in other plants or produce significant yield losses even as a mild infection. It has been suggested therefore by several workers that viral genes or RNA could be produced from incomplete viruses which are maintained in the plant by expression from viral sequences integrated into the genome with Ti plasmid vectors.

This approach is technically simpler even than the antibody approach, as the sources of potential resistance genes are the actual viruses. These can be purified and the genes cloned with ease. It is only necessary to characterise the actual gene regions before creating the constructions in the T-DNA expression cassette. Precisely how well the transformed plants resist infection depends on the mechanism of cross protection.

One model suggests that production of coat protein by the inducer inhibits unpacking of the challenge strain (de Zoeten and Fulton, 1975). However, this model cannot explain all cross protections as inducing strains which lack coat protein or which produce defective coat protein are effective (Zaitlin, 1976; Cadman and Harrison, 1959). By expression of the viral coat protein in transformed plants it will be possible to test this model. The coat protein produced from the transcribed nuclear gene should inhibit a challenge infection by viruses which are similar or identical to the source of coat protein gene. Failure to observe this would suggest that the model is wrong.

In a second model of viral cross protection it is proposed that the target molecule of the challenge virus inhibition is the negative strand replicative intermediate RNA molecule (Palukaitis and Zaitlin, 1984). The suggestion is that the large amount of viral strand sequence produced by the inducer anneals to the replicative intermediate of the challenger. This, it is proposed, would inhibit the transcription of challenge virus viral strand RNA. In this case, the expression of the region complementary to the promoter on the replicative intermediate (i.e. the 5' of the viral strand) would affect cross protection in the transformed plant. This model would not, however, explain cross protection between DNA viruses. It is also difficult to reconcile with the fact that during single infections there may be a large excess of viral strand over replicative intermediate. This does not apparently inhibit production of further viral strand RNA.

A final suggestion for the mechanism of cross protection assumes that essential viral functions involve an interaction between the virus or viral RNA with a plant component and a viral gene product. This is undoubtedly the case for the replicase which comprises both plant and viral components (Hall et al., 1982) and seems almost inevitable for viral transport. This process, at least in TMV, involves the virus and viral RNA, a viral

transport protein (Ohno et al., 1983) and, it is assumed, plant components.

It is proposed that when the three-way interaction involves homologous viral components it functions more effectively than if heterologous components are involved. Thus a challenge virus or its RNA will enter almost always into interactions with the highly abundant products of the inducer. As a result the essential functions of the challenger will proceed more slowly than those of the inducer so that the inducer prevails. If this model is correct, then expression at a high level of any essential viral gene product will protect against a challenge infection if the challenge infection differs from the source of viral gene. If the challenge infection is the same as the source of the transcribed viral gene in the nuclear genome then the interactions with the product of this gene will not be recognised as different and the three-way interactions will proceed normally.

CONCLUSIONS

Vector systems and methods are now well-developed for the transfer of DNA into the genome of some species of transformed plants and promoters have been isolated which can drive the expression of this DNA. The way is now open, therefore, for attempts to engineer agronomically useful traits including virus resistance into crop plants using these methods. The source of genes encoding these useful traits may well not be from plants but viruses, mammals or other organisms.

While these approaches are speculative, a definite contribution of recombinant DNA work to plant improvement will be in the area of diagnostics.

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TECHNIQUES FOR TRANSFERRING GENES INTO PLANTS

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Recent progress in cell and molecular biology has made available a great variety of techniques for the transfer of genes to plants, which exeed those possibilities which are based on the natural process of pollination and fertilisation. These novel techniques include combinations of complete genomes via somatic hybridisation (Gleba & Sytnik 1984), transfer of partial genomes via subprotoplast fusion (Maliga et al. 1982), irradiation prior to fusion (Aviv et al. 1980), and the transfer of isolated nuclei (King & Saxena 1985, Saxena & King 1985) and metaphase chromosomes (De Laat & Blaas 1985). The following review will not discuss all these techniques but rather focus only on those which allow for the transfer of isolated, single genes.

1. Gene transfer via bacterial vectors

The discovery that the soil bacterium Agrobacterium tumefaciens uses a natural gene transfer system for tumor transformation of plants has opened up the area of genetic engineering of plants. This phenomenon has been discussed at so many occasions that it is probably common knowledge and does not require, therefore, more than a brief reminder (for a recent review see Gheysen et al. 1985).

Agrobacterium tumefaciens carries an extrachromosomal circular DNA, the Ti-plasmid ('tumor inducing') with a group of genes (the 'vir' genes from 'virulence') which play an essential role in the transfer process of a stretch of the plasmid (the T-DNA) into competent plant cells. Competence is, probably, a specific physiological state of wound-adjacent cells which is the consequence of wounding in plants, which are within the hostrange of Agrobacterium. Although the mechanism of the DNA transfer from the bacterium into the plant cell is not yet understood, it can be routinely used for gene transfer. The T-DNA is flanked by 'border' sequences which have a function in the transfer process. The T-DNA carries, amongst others, at least three genes which interfere with the cellular auxin and cytokinin regulation, and thus with the controlled proliferation of transformed cells, causing tumorous growth. Inactivation of either or both groups of genes led to the construction of 'disarmed' Ti-plasmids which still function in gene transfer but no longer interfere with plant regeneration. Foreign genes engineered between the border sequences of the T-DNA are cotransferred into the host genome by this natural gene transfer process. They are integrated into the genome and inherited to sexual offspring. The very large Ti-plasmid (150 kb) has been made more easy to manipulate by the development of a 'binary vector' system, where the T-DNA is separated from the 'vir genes' on independent and small plasmids. Foreign genes inserted into the T-DNA of such 'mini'-Ti-plasmids are transferred into the plant as from the normal Ti-plasmid. Efficient 'shuttle vectors' for gene exchange between E.coli, Agrobacterium and plants are available which contain cassetes of a collection of convenient restriction sites for the facile insertion of foreign genes. Infection leading to gene transfer is achieved either by simple injection of bacteria into a tissue, e.g. stem, by infection of cut wound surfaces e.g. decapitated shoots or leaf discs, or by co-incubation of protoplast-derived cells with Agrobacterium. Gene transfer through Agrobacterium is, to date, a routine and efficient method and in use in numerous laboratories all over the world.

The only other bacterial vector known, so far, is Agrobacterium rhizogenes (Chilton et al. 1982) which transfers part of its very similar Ri-plasmid, and which, therefore, can be used in a very similar way for gene transfer. The specific advantages of this system may be related to the fact that the roosts produced in response to transformation are of clonal origin and may facilitate plant regeneration from transformed cells, where this is not always easily achieved from A. tumefaciens transformed tissue.

2. Gene transfer via viral vectors

DNA viruses, entering plants via a natural infection process and spreading in the plant systemically, would offer an alternative vector system, if it were possible to introduce foreign genes into their genome without interfering with its biological function. This has been achieved, so far, for one case(Brisson et al. 1984, Brisson & Hohn 1985): A part of the Cauliflower Mosaic Virus genome has been deleted to provide space for the integration of foreign DNA and a selectable marker gene has been inserted in such a way that the hybrid viral genome could continue to replicate and spread systemically. Following inocculation and an incubation time which allowed the distribution of the engineered virus throughout the plant, biological and molecular analysis clearly demonstrated that indeed the foreign gene had been introduced into, and systemically spread throughout, the plant, and that it was expressed. Further experiments studying a) the space which can be made available for passenger DNA, b) the size of the passenger genes which can be introduced, c) the stability of the foreign genes in the viral genomes, and d) possibilities for an extension of the host range (group T.Hohn) and binary vector systems (group B.Hohn) are in progress in our institute. The advantages associated with such viral vector systems, compared to the A. tumefaciens system are systemic spread throughout the entire plant and replication to multiple copies (thousands) of the foreign gene per plant cell. Disadvantages which can be seen at the present state of knowledge may concern the relatively narrow hostrange, the limited space available for foreign genes, the genomic instability of the insert and the exclusion of the virus from transmission to the next sexual generation.

There is considerable interest to date to explore the possibilities for gene transfer to graminaceous monocots, especially cereals, via gemini viruses (e.g. Mullineaux et al. 1984, Goodman 1985). We are, however, not aware of any case where a foreign gene had been transferred by this route. If this were possible, advantages and disadvantages would be, probably, similar to those mentioned for CaMV.

An interesting combination of viral and bacterial vectors (agroinfection), in which viral functions are introduced into the nuclear DNA of plant by Agrobacterium is under study in the group of B.Hohn in our institute. Following infection of Brassica plants the virus was shown to recombine from the T-DNA and give rise to systemic infection (Grimsley et al. 1986). This approach may allow the development of meiotically transmissible multi-copy vectors, which would also allow more foreign DNA to be incorporated than presently possible in viral vectors.

3. Vectorless systems

The use of biological vectors for gene transfer was essential at the onset of genetic engineering of plants and has been developed to a convenient and efficient technique with A. tumefaciens. There is, however, one problem which led to attempts to develop vectorless transformation systems: Biological vectors will always be limited by a hostrange. The severe limitation which is, so far, visible with the otherwise perfect A. tumefaciens

system, is its inability to transfer genes to graminaceous monocots, e.g. the cereals.

a) Direct gene transfer

From work with isolated protoplasts it was known that naked plant cells take up foreign DNA (Lurquin & Kado 1977). As it is also well documented that plants can be regenerated from protoplasts (Potrykus & Shillito 1985, Davey 1983, Dale 1983), the obvious approach to vectorless gene transfer was via protoplasts. Davey et al. (1980), Draper et al. (1982) and Krens et al. (1982) had shown that the isolated Ti-plasmid of Agrobacterium can be integrated into protoplast-derived clones. On this basis we aimed at developing a routine and efficient protocol for a hostrange independent gene transfer to plants (Paszkowski et al. 1984).

1) Protocol and efficiency: Our partially optimized protocol includes incubation of freshly isolated protoplasts with an isolated gene under plant expression signals, followed by a heatshock treatment, polyethyleneglycol and electroporation, and yields stable integrative transformants at 1-2% of unselected clones recovered (Shillito et al. 1985).

2) Hostrange: We did not find any limitation of this procedure with regard to plant species. Transformation was possible with protoplasts from every plant species attempted, and this included also graminaceous monocots, e.g. Lolium multiflorum (Potrykus et al. 1985a) and Triticum monococcum (Lörz et al. 1985) and Zea mays (Fromm et al. 1985). The remaining problem in graminaceous monocots is plant regeneration from protoplasts (Potrykus et al. 1979).

3) Inheritance and stability: We have analysed, so far, ca. 150 primary transformants of N. tabacum, several of those have been followed into the sixth sexual generation. In the great majority of cases (>95%) the inheritance of the foreign gene follows Mendelian rules. In most cases we observed inheritance of a single (dominant) Mendelian factor (Potrykus et al. 1985b). We also found, however, integration of two, three, and several independent loci, as well as integration of multiple copies of one gene at the same locus. The foreign gene can be maintained absolutely stably (no loss or inactivation through six sexual cycles and over two years without any selective pressure) and it can be unstable (loss at a specific frequency or a variable frequency from a homozygous situation) (Potrykus et al. 1985c). The cause for stability and instability is, so far, not understood.

4) Proof for transformation is based on a strict correlation of treatment, expected phenotype, physical presence of the foreign gene, its activity, and inheritance (Paszkowski et al. 1984, Potrykus et al. 1985b).

5) Localization and integration: The foreign gene integrates (randomly) at different loci of different chromosomes by a molecular mechanism which is also, so far, not understood. We have developed a method to visualize transformed genes by in-situ hybridization on metaphase chromosomes (Mouras et al. 1986). Localization via linkage to marker genes is in progress with a complex heterozygote Petunia hybrida line.

6) Co-transformation: Recovery of clones having integrated a foreign gene not producing a phenotype for which selectable conditions can be established at the cell culture level is possible a) via screening of a few hundred clones (1-2% transformants) (Paszkowski et al. 1986) and, more efficient, b) by using co-transformation: a selectable marker gene is mixed (physically unlinked) with the non-selectable gene, transforme clones selected, and then screened for the presence of the non-selectable gene. We found up to 88% co-transformants (Schocher et al. 1986).

7) Gene transfer from total genomic DNA: Reconstruction experiments, where a selectable marker gene was mixed with total genomic plant DNA yielded transformants down to a ratio of 10 copies of the gene per haploid tobacco genome. Treatment of protoplasts with partially digested tobacco DNA isolated from a plant containing probably one copy of a selectable gene per haploid genome produced transformants at the predicted frequency of one in ca. 2 million clones. This result indicates that even the direct transfer of genes from total genomes without prior isolation of the gene might be feasible, if tight selection for this gene is possible (Müller et al., 1986).

8) Reproducibility: Direct gene transfer has been working routinely in our laboratory in, so far, more than 200 independent experiments. It is also in use in several other laboratories including the Max-Planck-Institut for Plant Breeding, Cologne, Stanford University, and the Boyce Thompson Institute at Cornell. The high transformation frequency mentioned (1-2%) is routine with leaf protoplasts of N. tabacum 'Petit Havana' SR1; other species (Petunia hybrida and N. plumbaginifolia) have been optimized to routine value of 0.1% so far.

9) Advantages and disadvantages: The main disadvantage of this method is that it requires protoplasts, and plant regeneration from protoplasts is more difficult than from complex explants, or still not possible in as important crop plants as cereals and soybean. Advantages are seen in the independency of any hostrange, and the possibility of co-transformation and gene transfer directly from total genomic DNA.

b) Liposome fusion

Liposomes are artificial lipid vesicles. They can be used to encapsulate DNA. Fusion of liposomes with protoplasts transfers the DNA into the cell. This approach has been used to convincingly show uptake, integration, and inheritance of a foreign gene in tobacco (Caboche & Deshayes 1984). The method requires, as direct gene transfer, protoplasts as acceptor cells. The efficiency is not as high as with direct gene transfer, and DNA uptake into the protoplast does not appear to be the limiting step in this technique in any case. There might be an advantage for gene transfer into organelles; there are however, so far, no data to support this idea.

c) Spheroplast fusion

Spheroplasts are in analogy to protoplasts, naked bacteria. They can be fused with protoplasts. If they contain plasmids carrying a gene of interest, this gene can be transferred into the plant genome after release from the spheroplast. This has been achieved in one case (Hain et al. 1984). Advantages and disadvantages are as for liposome fusion.

d) Microinjection

Mechanical injection of DNA into nuclei of protoplasts via microscopic injection needles has been developed very recently to an efficient method (Steinbiss et al. 1985, Miki 1985, Reich et al. 1985, Goodman 1985). Microinjection requires such an effort in instrumentation and manual skill that it will, probably, not be possible to compete with other techniques as far as straightforward gene transfer into protoplasts is concerned. It will, however, become a very important technique for gene transfer as soon as it will work with cells or meristems. Microinjection into embryogenic microspores may then open the route for genes into cereals.

4. Other approaches

There have been numerous attempts since the early seventies to use germinating pollen as vehicles for gene transfer into the zygote (e.g. Hess 1975, de Wet et al. 1985). Despite the fact that numerous interesting phenotypes have been recovered from such experiments, in not a single case could definite proof be established that the novel phenotype was based on the physical presence and activity of a foreign gene. During the last two years numerous laboratories around the world have performed rigorous tests with molecular defined genes, to find out whether indeed gene transfer via the pollen route is real. As far as we are aware, there was not a single clear positive case. These results do not disprove the possibility of pollen transformation. They demonstrate, however, that this will not be an easy route, and tight proof will have to be provided, before this method can be accepted. If it were to work, however, it might outdate all other techniques for applied purposes.

There are numerous biological facts which may make gene transfer via germinating pollen very hazardous for the foreign gene, for instance extracellular nucleases, pollentube wall, intracellular nucleases, cell walls of the male pollentube cells, extreme heterochromatization of the male nucleus, intracellular environment in the synergists etc., most of which may not apply to an alternative system: in many plants 'embryogenic microspores' are available from which haploid plants can be regenerated (Heberle-Bors 1985). These microspores also represent the only single cell system in cereals for which experimental proof for totipotency is available. We have, therefore initiated experiments on gene transfer into embryogenic microspores - so far with negative results only.

What has been discussed with regard to pollen transformation also applies for the many experiments where DNA has been fed to plants or where seedlings have been incubated in DNA (e.g. Hess 1969, Ledoux et al. 1975). It might, however, still be worthwhile to challenge these ideas with clearly defined and selectable marker genes for which molecular probes are available which will allow one to rigorously analyse phenotypes for the presence and activity of the foreign gene.

SUMMARY

There is a collection of routine and efficient techniques for transferring genes into plants at our disposal, and foreign genes, once introduced, can be inherited like original plant genes. The limitations, which still exist for cereals and other important crop plants, may be overcome by progress in protoplast culture or extension of the hostrange of biological vectors. Progress in the application of recombinant DNA technology to crop improvement and crop protection is, therefore, more limited by lack of basic knowledge on agronomically important genes as well as by the lack of methods for the isolation of single copy genes or groups of genes from plant genomes. We hope that the method of direct gene transfer will contribute to a solution of the latter problem.

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Transposon Mutagenesis And Its Role In Gene Isolation

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ABSTRACT

Mutations induced by the integration of transposable elements into plant genes have genetically been studied in great detail in Zea mays and Antirrhinum majus. Several elements from Z.mays (Ac, En (Spm), Mu) and A.majus (Tam1, Tam2, Tam3) recently have been characterized molecularly and could be used as specific probes for the isolation of genes from mutants induced by the integration of transposable elements.

RESULTS

Unstable mutations, possibly induced by transposable elements are known from many plants and have been extensively studied in Zea mays by Barbara McClintock and Peter A.Peterson and in Antirrhinum majus by Hans Kuckuck. These elements have been discovered during studies of unstable mutations, induced in genes which are easily visible, like such influencing kernel color and shape in Zea mays or flower color in Antirrhinum majus.

Such elements, if integrated into one of the about fifteen genes involved in the anthocyanin pathway, influence the degree of anthocyanin biosynthesis by either reducing or blocking gene activity. This then leads to a pale or colorless flower or kernel. However, due to the instability of these elements they can leave the site of integration thereby restoring gene activity. This process can happen very frequently during plant development and leads to a variegated phenotype. Excision of an element usually is followed by the reintegration in another position in the genome, a process which is called transposition.

The genetical analysis of transposable elements in Z.mays showed that many of them are so-called two element systems, composed of a receptor element, receiving transacting signals from the fully active regulator component also called autonomous element (for review: Fedoroff 1984) These two parts of a system specifically act

together in the way that a regulator element interacts with only its own receptor component. The best known of these systems are the Ac/Ds (Activator/Dissociation; McClintock 1965) and the En/I (Enhancer/Inhibitor, Peterson 1970) or Spm (Supressor mutator; McClintock 1965) elements.

Some transposable elements have been cloned molecularly, like the Ac (Fedoroff et al. 1983), the Enl (Pereira et al. 1985) and Mul (Bennetzen et al. 1984, Barker et al. 1984) element in Z.mays and the Tam3 (Sommer et al. 1985) element in A.majus. This was possible by the isolation of mutants induced by the integration of the element into a gene already characterized at the molecular level. The tagging of genes by transposable elements is done by crossing of a line homozygous dominant for the gene to be tagged (and in addition carrying the functional transposable element) to a line carrying the same gene in a homozygous recessive allele. Variegated kernels (or plants with variegated flowers) in the progeny of this cross are putative candidates for the integration of the transposable element into the specific gene and have to be analyzed in further crosses for linkage analysis (see for example: Pereira et al. 1985). The frequency of such an event in maize is about $5x10^{-5}$ - $2x10^{-6}$ and seems to vary between different loci (Peterson 1983, Robertson 1985).The genes were the Ac, Enl, Mul and Tam elements have been originally isolated from, are the waxy gene (Ac isolated from wx-m9;Fedoroff et al. 1983; En/I isolated from wx-844, Pereira et al. 1985), and the alcohol dehydrogenase gene of Z.mays (Mul element, Freeling et al. 1982) and the chalcone synthase gene in A.majus (Bonas et al. 1984).

The genetical analysis of Z.mays mutants induced by functional elements (like Ac and En (Spm)) have shown that these elements are unique structures in the genome of those mutants. If this would be true for the molecular structure as well, these elements would provide a very specific sequence to be used in gene isolation from mutants, where the element is integrated into the gene of interest.

The molecular cloning of the transposable elements Ac (Fedoroff et al. 1983) and Enl (Pereira et al. 1985) from Z.mays indeed showed that these elements are unique structures in the genome in terms of size, restriction pattern and sequence and could be used to clone a number of genes from the anthocyanin pathway from Z.mays. The Ac element for example was used to identify the bz gene from the mutant Acbz-m2 (Fedoroff et al. 1984). The Enl element was useful as molecular probe to isolate the al (O'Reilly et al. 1985), the c2 and c gene (Wienand pers.commun.) from the En (Spm) induced mutant al-m(papu)(Peterson 1970), c2-ml (McClintock 1967) and c-m668655 (Reddy and Peterson 1983).

The method described can become complicated however, if the element used for gene tagging is repetitive in the genome. This is the case if a receptor part of an element is present at the specific gene of interest. These structures (called Ds elements in the Ac/Ds and inhibitor in the En/I system) are present in high copy numbers in the Z.mays genome. They are deletion derivatives of the autonomous elements and vary manyfold in length compared to the autonomous elements (Döring et al. 1984, Fedoroff et al. 1983, Schwarz-Sommer et al. 1984). Cloning of a gene from such mutants is very laborious, because the molecular structure of such a deletion derivative cannot be predicted. However, using mutants of the same gene induced by different independent tags can solve the problem by cross screening element containing clones from each mutant line. Clones containing homologous sequences flanking the elements should cross hybridize and in addition to the element contain at least part of the gene. Thus the al gene from Z.mays could be cloned by O'Reilly et al. (1985) from mutable al alleles tagged by the Mul and the En element.

The procedure of gene isolation using controlling element induced mutations can become a very powerful method for gene isolation, since it does not require information on the gene product. Therefore in principle any gene of interest can be tagged by transposable elements.

This in respect of crop improvemant is of particular interest in the isolation of genes desired for gene transfer, like for instance a resistance gene.

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THE CONTRIBUTION OF MICROBIAL GENETICS TO THE STUDY OF PLANT-MICROBE INTERACTIONS

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INTRODUCTION

All manner of plants play host to a wide variety of microorganisms that have many representatives amongst viruses, bacteria and fungi. These interactions vary in their degree of intimacy and complexity and also in the effects they have on the plant; some do it good, some do it harm and yet others appear to have no consequence.

Many of these relationships are of economic importance in agriculture and have long attracted the attention of microbiologists, pathologists, plant breeders and physiologists who, by one means or another, have tried to augment the beneficial ones and deter the deterimental ones. It should be emphasized, though, that these interactions are of inherent interest, over and above their practical importance; the fact that a given microbe can elicit a precise and sometimes very complex response in the plant (e.g. a legume root nodule) offers an opportunity of investigating plant growth and differentiation by novel means. In a sense, the microbe can be viewed as a 'growth regulator' and if the molecular bases of the microbes' abilities to interact with plants can be determined, the potential exists to identify a new set of signals that may dramatically affect plant differentiation and development.

As in virtually all fields of biological research, the topic of plant-microbe interactions is now being subjected to analyses using the tools of molecular biology. Although the molecular biology of Tobacco Moasic Virus has a history of some thirty years, the discipline as a whole is really in its infancy but has already given novel insights into the basic mechanisms of several interactions. More than that, genes have been put into and taken out of a selection of microbes that naturally associate with plants in attempts to construct engineered strains that possess features that may be of agronomic advantage.

In this paper I will review some of the ways in which microbial genetics has been used to study particular plant-microbe interactions. The emphasis will be on the interactions between bacteria and plants; this is more a reflection of the author's competence rather than an indication of the relative importance of viruses and fungi.

NOVEL TOOLS FOR GENETIC ANALYSIS OF BACTERIA

There are two fundamental requirements for any genetic analysis in any organism; first, a system of gene transfer and secondly, a mechanism for generating novel variants or mutants. Studies on the molecular genetics of Gram negative bacteria, including several that interact with plants, have been greatly facilitated by the use of two novel tools plus a neat trick that combines the powers of both.

Wide host-range cloning vectors

Although DNA from any source can be cloned and analysed physically in *E. coli*, it is clearly advantageous to be able to test its function in the bacterium from which it was derived. Since many *E. coli* cloning vectors,

such as pBR322 or bacteriophage lambda, fail to replicate in non-enteric bacteria (e.g. Agrobacterium, Rhizobium, Xanthomonas and Pseudomonas) it has been necessary to develop new vectors that are derived from wide host-range plasmids. To date these have, for the most part, been derived from P1 and Q-group plasmids; these occur naturally in Pseuodmonas strains but are transmissible to a wide range of Gram negative genera including those named above.

A widely used vector for the construction of clone banks is pLAFR1, a P1-group cosmid that can accommodate approximately 30 kilobases of DNA (Friedman et al. 1982) and such recombinant plasmids can be efficiently mobilized into a range of Gram negatives. Bagdasarian et al. (1981) constructed a variety of plasmids based on the Q group plasmid RSF1010; these too have had applications in the functional analysis of cloned DNA (see for example Rossen et al., 1984).

Transposon mutagenesis

Many of the bacterial genes that are of interest in terms of their role in the interaction with plants, such as those concerned with virulence in pathogens or nodulation in Rhizobium, specify no obvious phenotype on Petri dishes, but are expressed only when the microbe encounters the host plant. Therefore the genetic and physical location of (for example) a chemically induced mutation that abolishes pathogenesis in Xanthomonas would be a time-consuming and arduous job.

The power of transposons as mutagens is that they 'tag' the mutated gene both physically and with an easily selectable marker. Transposons are discrete elements of DNA that have the ability to transpose or jump from one position in the genome to another and in doing so interrupt, i.e. mutate any gene into which they transpose. Since the most frequently used transposons specify antibiotic resistance, it follows that, in order to locate the mutant gene, all that is required is to map the position of the antibiotic resistance (see for example Scott et al. 1982). Physical analysis is also greatly facilitated as illustrated by the following example.

Consider a non-pathogenic mutant of Xanthomonas isolated by mutagenesis with the transposon Tn5. This transposon specifies kanamycin resistance and has no EcoR1 sites. To isolate the gene in which the transposon is inserted the following straightforward steps are required.

(a) Digest the total genomic DNA of the mutant with EcoR1.

(b) Ligate the genomic DNA fragments to an appropriate vector cut with EcoR1.

(c) Transform E. coli selecting kanamycin resistance.

These transformants will thus contain the Tn5 and, more importantly, the Xanthomonas DNA that flanks the transposon. This cloned DNA can then be used as a probe to fish out the coresponding wild-type copy of the gene from a clone bank. Having got the interesting DNA in cloned form, it is obvious that it can be subjected to detailed physical (e.g. DNA sequencing, site-directed mutagenesis, transcriptional mapping) as well as genetic analyses.

The principle used for the initial introduction of transposons into bacteria such as Rhizobium, Pseudomonas or Xanthomonas involves the use of so-called 'suicide' plasmids. These are plasmids which are stable in E. coli and can be mobilized into these other genera but which fail to replicate in

them. Therefore, if such a plasmid contains a transposon (e.g. Tn5), random insertions of the transposon into the genome of the recipient can be obtained by transferring the plasmid by conjugation from E. coli to (e.g.) Pseudomonas selecting for the kanamycin resistance specified by Tn5. Since the plasmid containing the transposon is "doomed" in Pseudomonas the only way to retain Tn5 in the strain is if the Tn5 jumps into a location in the genome of the Pseudomonas. Several such suicide vectors have been developed (see for example Beringer et al. 1978, Simon et al. 1983, Morales & Sequeira 1985).

Marker exchange mutagenesis

An elegant way of harnessing the tools of transposon mutagenesis and wide host-range cloning vehicles was devised by Ruvkun & Ausubel (1981). In essence the procedure involves the insertion of a transposon into DNA cloned in a wide host-range plasmid, the transfer of the mutant plasmid into the species from which the cloned DNA was derived and finally, through recombination between the cloned DNA and the corresponding region of the genome, the transposon is transferred by marker exchange into the corresponding position in the genomic DNA. Then the phenotype of the resultant mutant can be examined. This means that a large number of mutations can readily be isolated and mapped in a defined region of DNA and their phenotype easily ascertained. This approach has been of real value in, for example, the analysis of symbiotic genes of Rhizobium (e.g. Ruvkun et al. 1982) and pathogenesis genes in Xanthomonas (e.g. Turner et al. 1984).

The use of these tools, together with other techniques of molecular biology have provided the ability to identify, isolate and, to greater or less degrees, to assign functions to genes that specify the ability of individual bacteria to interact with plants in their own specific fashion.

In such studies on the bacterial genes involved in particular plant-microbe interactions, two rather different approaches have been taken. One is to isolate directly mutations or genes identified primarily on the basis that they are concerned with the interaction (e.g. isolation of non-tumerogenic mutants of Agrobacterium) followed by attempts to identify the biochemical nature of the relevant genes. The other philosophy involves the deliberate isolation of mutants or genes that affect a defined biochemical function and then to test the mutations in the relevant gene with regard to their phenotype effects on the interaction (e.g. isolation of mutants of Erwinia defective in pectinase).

In what follows, space permits only a listing of some of the highlights of genetic research in some of the bacteria that interact with plants.

Agrobacterium

A. tumefaciens and A. rhizogenes respectively induce tumours and proliferation of roots on dicotyledonous plants. Their ability to do this is determined by a large plasmid (the Ti plasmid in A. tumefaciens) that contains a specific piece of DNA (T-DNA) which is transferred to the host when Agrobacterium occupies a wound. This DNA can integrate in single or multiple copies into the DNA of the host (either nuclear or more rarely, the chloroplast) and because the integrated DNA contains 'eukaryotic' genes that specify the plant growth hormones cytokinin and auxin, cell proliferation is induced and a tumour forms. Recent work has shown that the T-DNA is excised as a circle from the Ti plasmid (Koukolikova-Nicola et al. 1985) and that this requires the presence of other virulence (vir) genes that are also on the plasmid. Interestingly, these vir genes are expressed only in the presence of a chemical present in plants (Stachel et al. 1985). It is not clear yet how Agrobacterium 'mates' with its host nor how the transferred DNA

gets into the cells and integrates. Nor has the basis of host-range been determined; although it has recently been established that, contrary to previous belief, certain monocotyledenous plants such as asparagus can be infected by A. tumefaciens though tumour-inducing ability is greatly reduced compared to that found when dicots are infected.

Over and above the inherent interest of this interaction, Agrobacterium has achieved fame as nature's genetic engineer. As will be seen elsewhere in this volume the fact that this bacterium can transfer DNA into plants has been exploited by inserting foreign genes into the T-DNA in order to expedite their incorporation into plants.

Xanthomonas and Pseudomonas

These two genera contain many bacterial pathogens ranging from blackrot on brassicas (caused by X. campestris) to fireblight on tobacco (induced by Pseudomonas tabaci). In several species, non-pathogenic mutants have been isolated following random mutagenesis and screening on plants and the corresponding DNA has been cloned and subjected to physical analyses but, as yet, no information is available concerning the biochemical basis of these defects (Daniels et al. 1984; Boucher et al. 1985).

The concept of race specificity and avirulence in phytopathogens has been well established by conventional genetic methodology. Briefly, it is thought that a particular race of pathogen is avirulent on a specific host, not because it lacks something but because it contains genes that cause the resistant host to mount a defence response. In Pseudomonas syringae p.v. glycinea a gene bank (in the vector pLAFR1) was made from a race that was avirulent on a particular race of soybean (Staskawicz et al. 1984). A plasmid was identified which, when transferred to another race of Pseudomonas that was normally virulent on the same host, caused it to lose its virulence confirming that strains that are avirulent on a particular variety of host plant contain gene(s) that specify products which in some way elicit a defence response in the host. Again, the molecular basis of this is unknown but presumably involves a component on the bacterial surface which, if recognised by the host as being 'foreign' causes the hypersensitive defence to be mounted.

An interesting and unusual case of pathogenicity concerns the ice nucleation ability of certain epiphytic strains of Pseudomonas, which, when they form colonies on leaves cause ice to form in the vicinity of the colony at relatively high temperature ($0^{\circ}C$) and this is a major component of (ice nucleation) frost damage to plants. Mutants which fail to nucleate have been isolated and the gene responsible identified. To the consternation of certain environmentalists it has been proposed that the nucleation negative mutants could be released in large numbers and, by competing with the resident population, reduce the risk of frost damage. Whether this proves to be feasible (or allowable) remains to be seen. The greater potential commercial application of these bacteria may in fact lie in the lollipop or snow-making industries where they could be used to raise the freezing temperature of water!

Erwinia

These enteric bacteria cause a series of soft rot diseases and it had been predicted that pectic lyase which is made and secreted by these bacteria was an important determinant of the pathogenicity. Recently the pectinase genes of Erwinia have been cloned, transferred to Escherichia coli and it was found that the E. coli transformants were capable of inducing soft rot though the symptoms were not so severe as with Erwinia itself (Collmer et al. 1985).

Analogous studies to those that have been conducted on plants that do harm bacteria have also been done on beneficial partners.

Rhizobium

Bacteria of this genus have the ability to induce nitrogen-fixing nodules on legume plants which allows many crop plants to be grown without the need for nitrogenous fertilizer and the genetic basis of nodulation and nitrogen-fixation though by no means understood, has been studied in considerable detail. In the various Rhizobium species that nodulate temperate legumes, the genes that determine nitrogen fixation (nif) are on large symbiotic plasmids (e.g. Banfalvi et al. 1982) and these are linked to the cluster of nod genes (Downie et al. 1983) that specify the ability to nodulate and which determine the particular host-range of a given species. Fortunately, the nif genes are very similar to the corresponding genes of Klebsiella which have been studied in great detail (see Ausubel et al. 1985). Thus, by comparing nif gene sequences in the two genera it is possible to deduce if a given nif gene in Rhizobium specifies the synthesis of the enzyme nitrogenase, the production of co-factors or are involved in nif gene regulation. The nodulation genes have also been studied and although they have been sequenced in some species, their precise function remains unknown. It is clear though that, similar to the vir genes of Agrobacterium, the nod genes are only activated in the presence of a chemical present in the root exudate of legumes (Mulligan & Jong 1985, Rossen et al. 1985). In addition to the direct identification of the nod genes, it has been shown that some mutants defective in exopolysaccharide synthesis are also defective in nodulation or nitrogen fixation, implicating a role for this polymer in the symbiotic interaction (Finan et al. 1984). Also, mutants that fail to transport dicarboxylic acids fail to fix nitrogen in the nodule suggesting that these are the sources of energy to support nitrogen fixation (Ronson & Atwood, 1985).

Frankia

This genus is an Actinomycete and can induce nitrogen-fixing nodules on a range of non-leguminous woody shrubs and trees such as Alder and Cassurina. Although the bacterium is difficult to grow, plasmids have been isolated from it for use as potential vectors (Normand et al. 1985). Given the sophistication of the genetic analyses of other Actinomycetes it may indeed be feasible to develop vector systems that might be used to investigate the location and function of symbiotic genes in this genus.

Plant Growth Promoting Bacteria

Certain strains of Pseudomonas actively colonize roots and it appears that some of these promote the growth of seedlings of certain crops. It is thought that they do this by suppressing sub-clinical infections by pathogens by synthesizing a siderophere that has a high affinity for iron and starves potentially pathogenic fungi of the metal. Genetic evidence supports this notion; the genes that are responsible for the synthesis of the siderophere have been identified (Moores et al. 1984) and mutants defective in its synthesis have been isolated and shown to have lost their ability to enhance growth rates of the plant. There is interest in exploiting the native ability of certain bacterial species to colonize roots to introduce foreign genes into them. For example, the gene that specifies the Bacillus thuringensis toxin has been cloned into a strain of rhizospheric Pseudomonas in the hope that inoculation of roots by this engineered strain would confer resistance to attack by insect larvae.

CONCLUSION

This listing of the different genera of bacteria that interact with plants which have begun to be analysed by genetic techniques is not comprehensive but may give an indication of the range of organism and of the properties they possess. Clearly much needs to be done to translate the increasingly detailed information on genes involved in pathogenesis or symbioses from the language of DNA to that of biochemical function. Although it is becoming increasingly simple to identify, clone and sequence genes of interest whether they be in a virus or a mouse there really is a stumbling block in extending the knowledge of gene structure to defined gene function. Even with plant viruses, where the entire genomic sequences of many are known, the viral polypeptides purified and the transcriptional organization determined, there is no real understanding of the distinction between a virus that causes spots on tomatoes from one that induces wilt on maize or indeed, an unpleasant disease in man.

If it remains a challenge to define the precise methods by which a microbe 'signals' a plant, the difficulties to be encountered in analysing the precise response of the host may be an order of magnitude more complex. For example, in a legume root nodule some five hundred genes may specifically be expressed in response to inoculation by Rhizobium; what signals are involved in their induction, what do they do - at this stage the answers are still unknown.

I do not wish to close on a note of pessimism, just one of reality. The progress made in the last five years in the science of plant molecular biology including the study of plant-microbe interactions has been impressive and the subject is really only in an early exoponential phase. There are real problems, the basis of which are only now beginning to be addressed; based on earlier performance, there are some exciting times ahead.

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2.
Prospects for Crop Plant Improvement

Chairman and Session Organiser:
R. B. AUSTIN

A CASE STUDY OF THE APPLICATION OF PROTOPLAST FUSION TO TOMATO IMPROVEMENT

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ABSTRACT

This case study of the consequences of fusion of protoplasts of the cultivated tomato (Lycopersicon esculentum) and a wild tomato species (Lycopersicon peruvianum) demonstrates that it is now possible to obtain fertile somatic hybrid plants using such cell fusion methodology coupled with in vitro culture. Wild tomato species which possess a vast store of potentially useful genetic variation are usually isolated sexually from the cultivated tomato; the production of such fertile hybrids may lead to breeding improvements in the cultivated tomato.

INTRODUCTION

Species of the genus Lycopersicon can be divided into two major groups on the basis of their sexual crossing relationships; a group of species that can be hybridised with the cultivated tomato with relative ease (the 'esculentum complex'), and another group separated from the cultivated species by severe sexual barriers (the 'peruvianum complex'). The species of the 'peruvianum complex' represent a vast store of potentially useful genetic variation which tomato breeders are frequently deterred from utilising because of the daunting sexual barriers which have to be overcome. (Taylor & Al-Kummar 1982.) These workers have emphasised the need for a simple and generally applicable method of transferring genes between members of the 'peruvianum complex' and the cultivated tomato. As will be discussed further later, even when sexual hybrids have been produced these have frequently lacked fertility.

Gene transfer by protoplast fusion is an attractive additional procedure for evaluation for its applicability in transferring genes between members of the 'peruvianum complex' and the cultivated tomato, and generally for the better exploitation of wild species of Lycopersicon. Protoplasts of L. esculentum can be readily isolated, and in a wide range of varieties can be induced to undergo sustained division to form callus. However, reproducible regeneration of plants from such protoplast-derived callus is, in our experience in our Plant Genetic Manipulation Group here at Nottingham, restricted to a few varieties (Morgan & Cocking 1982), even though it has been claimed that this is now possible in fourteen cultivated tomato cultivars (Shahin 1985). The wild species, L. peruvianum is readily capable of regeneration into plants from protoplast-derived callus (Zapata et al. 1977). Most investigators have assumed that somatic hybridisation requires an ability to regenerate plants from protoplasts of the particular variety of cultivated tomato being utilised in the hybridisation assessment. It has for instance even been suggested that regeneration capability should be inbred sexually from L. peruvianum into a range of cultivated tomato varieties to achieve this objective (Thomas & Pratt, 1981). However, our experience from an extensive investigation of somatic hybridisation in Petunia is that regeneration capability is a dominant characteristic from the wild species which is expressed in somatic hybrids (Frearson et al. 1977); and that it should be possible to utilise the good plant regeneration capability of L. peruvianum in fusions with L.

esculentum.

This illustrative case study of the consequences of fusion of protoplasts of these two Lycopersicon species demonstrates the usefulness of somatic hybridisation for gene transfer in this genus. This investigation of the production of somatic hybrid plants of Lycopersicon esculentum Mill. and Lycopersicon peruvianum Mill. is fully described elsewhere (Kinsara, Patnaik, Cocking & Power 1986). Here are highlighted the key aspects which have enabled this ability to begin to utilise somatic hybridisation for crop improvement.

Before describing these aspects it will help to obtain a perspective by describing briefly the work of others that has already been undertaken to facilitate introgression of desirable genes from wild tomato species both sexually, and by protoplast fusion procedures. When Lycopersicon esculentum (the cultivated tomato) is used as the male parent in sexual crosses with L. peruvianum, pollen tube elongation is normally stopped before fertilization is effected. Ovule penetration by any pollen tubes that happen to elongate sufficiently is also inhibited. The result is that no fruits are set. When L. peruvianum is the male parent the cross is prevented by embryo abortion. Fruits are set, but no viable seeds are formed (Hogenboom 1972a). As discussed by Thomas and Pratt (1981) each of the reciprocal sexual crosses can succeed under certain circumstances. Interspecific hybrids have been produced with L. esculentum as the male parent using specially selected rare strains of L. peruvianum in which unilateral incompatibility barriers have been partially broken (Hogenboom 1972b). In the interspecific cross when L. peruvianum was the male parent plants were successfully produced by embryo culture (Smith 1944). Sexual fertility of such F1 hybrids is vital to bring about suitable introgression of L. peruvianum alien genes into the cultivated tomato. Sterility of such sexual hybrids is unfortunately common. Embryo culture can sometimes be used successfuly for the first backcross of the hybrid to L. esculentum (Alexander 1963). More recently the formation of complex hybrids between L. esculentum & L. peruvianum to promote interspecific gene transfer has been described which may ultimately form the basis for an efficient sexual genetic bridge between the cultivated tomato and L. peruvianum. (Taylor & Al-Kumar 1982).

Somatic hybridization by the fusion of protoplasts has unique features which clearly suggest its use when such difficulties are being encountered, since it can bring together genomes of sexually incompatible species; and because it can be used to synthesise allopolyploids in which heterozygosity and resulting hybrid vigour have been fixed in one step. Also usually in sexual reproduction cytoplasmic organelles are inherited maternally (as in tomato), whereas somatic cell fusion creates a novel situation by making a cytoplasmic mix of the two parents. (Pental & Cocking 1985). Understandably, therefore, investigators have explored the possibilities in this respect within a range of genera. Even, however, when adequate selection, coupled with suitable plant regeneration procedures has been employed, the resultant somatic hybrid plants have often lacked fertility and have not been able to be incorporated in an ongoing breeding programme. This has been the situation in the hybrids synthesised between the sexually incompatible species Petunia parodii and Petunia parviflora (Power et al. 1980) which are infertile on selfing and crossing. Various approaches have been suggested to overcome this difficulty. Clearly, basic studies are required such as those recently undertaken by White & Rees (1985) on the chromosome cytology of the somatic

hybrid between P. parodii and P. parviflora, which have established the extent and manner the two complements respond differently and specifically to control exercised upon their behaviour at meiosis by the novel hybrid genotype. Recently Hamill et al. 1986 have analysed the fertility in somatic hybrids of Nicotiana rustica and Nicotiana tabacum and progeny over two sexual generations. Fertility was non-uniform in the population; and even though only partially fertile somatic hybrids were recovered, it was possible to increase the level of fertility by recurrent selection of the most fertile off-spring over two generations. Also recently, tetraploid somatic hybrids were produced by protoplast fusion between Solanum brevidens and the cultivated potato (Solanum tuberosum). S. brevidens has resistance to potato leaf roll and frost, but is difficult to cross sexually with the cultivated potato. Hybrids produced fertile pollen but there are, as yet, no reports on the incorporation of this potentially useful hybrid in a breeding programme. (Austin et al. 1985) It has been suggested that somatic hybridization with limited gene transfer, and associated fertility, might be achieved by X - or γ - irradiation of protoplasts of the alien parent before fusions with crop plant protoplasts, and also that the generation of interspecific triploids by protoplast fusions might achieve a similar result. (Pental & Cocking 1985). Encouragingly, evidence has been obtained for the transfer of the barley nitrate reductase structural gene to Nicotiana tabacum by protoplast fusion (Somers et al. 1986); and triploid gametosomatic hybrid plants with high pollen viability and a high capacity for seed set have been regenerated following the fusion of Nicotiana tabacum leaf protoplasts with N. glutinosa pollen tetrad protoplasts (Pirrie & Power 1986). Attempts have been made to generate somatic hybrid plants between L. esculentum and L. pennellii (which belongs to 'the esculentum group'), but only calli were selected and characterised (O'Connnell & Hanson 1985). Somatic hybrid plants have, however, been obtained between L. peruvianum & L. pennellii, but these hybrids were sterile (Adams & Quiros 1985).

Our studies here in the Plant Genetic Manipulation Group at Nottingham have centred on investigating the consequences of fusion of protoplasts from leaves of L. esculentum with protoplasts from cell suspension cultures of L. peruvianum. Our assessment of work so far on somatic hybridization has been that, in most instances, insufficient numbers of heterkaryons have been selected and regenerated into whole plants for hybrid evaluations. We therefore undertook a large number of experiments (twenty one) in each of which there was a satisfactory fusion frequency. In eighteen of the twenty one fusion experiments regenerated shoots and plantlets were similar in terms of morphology to shoots of L. peruvianum arising in the appropriate control. However, in three separate experiments some of the regenerated shoots were atypical morphologically, and it was amongst this population that somatic hybrids were detected and characterised morphologically, chromosomally and by Fraction 1 protein analysis.

MATERIALS AND METHODS

Protoplast isolation

Protoplasts were isolated from fully expanded leaves of cultivated tomato plants (L. esculentum c.v. Ailsa Craig) as previously described (Zapata et al. 1977), and from cell suspension cultures of L. peruvianum (accession S37/68 Botanic Gardens, University of Birmingham, U.K.).

Fusion of protoplasts

The fusion medium employed was 30% (w/v) polyethlylene glycol, M.W. 6000 (Koch-Light), 2.36% (w/v) $Ca(NO_3)_2.4H_2O$ and 9% (w/v) mannitol, pH 5.8.

Culture of protoplasts

The cultural procedures have recently been described in detail (Kinsara et al. 1986).

Analysis of regenerated plants

A detailed comparison of morphology was carried out for L. esculentum, L. peruvianum and the putative somatic hybrids. Characteristics distinguishing the two parental species were specifically stem, leaf, inflorescence, fully expanded flower, sepals, stigma (inserted or exerted) and fruit morphology. Biochemical characterisation utilised Fraction 1 protein profiles following the procedure of Cammaerts & Jacobs (1980) with some modifications which enabled each of the two parental species and the somatic hybrids to be distinguished in relation to the small (nuclear-coded) subunit of Fraction 1 (Kinsara et al. 1986). Chromosomal analysis using standard procedures was used to determine chromosome number and meiotic pairing behaviour.

RESULTS

Following fusion, heterokaryon frequency was 5-7%. Confirmation of hybridity of regenerated plants was based on morphological comparisons and unambiguous characterisation by Fraction 1 protein analysis. The selected somatic hybrid plants were hexaploid (2n = 72), fertile, and set seed upon self pollination. For a detailed presentation of these results see Kinsara et al. 1986.

DISCUSSION

This is an instance where protoplast fusion has resulted in the production of fertile somatic hybrid plants, when sexual crosses (albeit produced with difficulty) in this important crop usually do not produce fertile plants. Moreover, since the variety of cultivated tomato utilised is a non-regenerating variety from protoplasts (Morgan & Cocking 1982), the presently described procedures will probably have wide applicability to a range of other non-regenerating cultivated tomato varieties, and regenerating wild type Lycopersicon species.

It is now more than forty years since Smith (1944) produced sexual crosses between L. esculentum (as the female parent) and L peruvianum using embryo culture. Now somatic hybridisation may enable better utilisation of the disease resistance, variation and adaptability in L. peruvianum, enabling breeding improvements in the cultivated tomato. Initially these improvements (as suggested by Rick (1983) for sexual hybridisation) may be restricted to the "prebreeding" stage in which the genes from the vast germplasm reserves of a range of tomato exotics are transferred into the milieux of cultivated tomato varieties without carrying the projects to the ultimate goal of 'polished' new cultivated tomato cultivars. A central requirement is that such somatic hybrids should have adequate fertility, and it is encouraging in this respect that the presently described somatic hexaploid (L. esculentum (+) L. peruvianum) can be selfed and backcrossed to L. esculentum. The high ploidy may, however, be a disadvantage in on-going breeding development. If so, it may be useful to explore the breeding behaviour of any gametosomatic hybrids (L. esculentum (x) L. peruvianum) produced by fusions between somatic cultivated tomato protoplasts and pollen tetrad protoplasts of L. peruvianum. Recently gametosomatic hybrids have been produced between N. tabacum and N. glutinosa which can be selfed and backcrossed to N. tabacum (Pirrie & Power, 1986). High ploidy levels in the hybrids are thereby avoided and

this may be an asset in on-going breeding programmes aimed at improvements in cultivated tomato.

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LIGHT ACTIVATED GENES: PROSPECTS FOR MODIFYING THEM TO INCREASE CROP PRODUCTIVITY

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ABSTRACT

By the application of molecular biological techniques considerable advances have been made in understanding the photoregulation of gene expression. These techniques provide the potential to manipulate plant growth and development for commercial exploitation. There still remain, however, many fundamental cellular mechanisms that require further characterisation before this potential can be fully realised. In this review we will discuss the perception of light by the plant, the photoregulation of genes and also identify specific areas where manipulation of light-regulated genes may be utilised commercially.

INTRODUCTION

The major environmental determinant of plant growth and development is light. Light acts not only as the source of energy for photosynthesis, but it also has a major influence upon the morphology and pattern of differentiation throughout plant growth. Thus light acts upon processes as diverse as seed germination, stem elongation, chloroplast development and the conversion from vegetative to reproductive growth. However, only recently, with the advent of molecular biological techniques, has direct evidence been obtained that light regulates the expression of genes.

It is not our intention in this short review to list the many examples that indicate the effect of light at the transcription level on protein synthesis (for comprehensive reviews, see Tobin & Silverthorne 1985, Harpster & Apel 1985, Thompson et al. 1985). Instead, specific examples have been selected that demonstrate the application of molecular biology to investigate light activated genes, while discussing the areas of knowledge that remain uncertain and that require further study. In addition, we will assess characteristics of the light environment that make it potentially attractive to exploit modern technological opportunities and identify particular proteins or metabolic pathways to which it is applicable.

PHOTOPERCEPTION

The laws of photochemistry hold that only absorbed photons can elicit a response. Light-activated genes do not themselves absorb light but rather require the mediation of a light-absorbing or photoreceptor molecule, such as phytochrome, one or more blue light receptors, protochlorophyllide and a photoreceptor for ultraviolet (UV) radiation. It is through these pigments that the plant response to light is mediated. Phytochrome, however, is believed to play the dominant role and remains the most extensively studied.

The major route through which light is absorbed by the plant is the photosynthetic system. Although it is believed that the photosynthetic apparatus is capable of self-adaptation to different lighting conditions,

it is not known at present whether activation of genes results from light absorption by photosynthetic pigments.

In addition an indirect mechanism of photoperception operates in plants in the form of the photoperiodic system. It is known that phytochrome is involved in this process, but the timing of the light treatments is the decisive feature. Under natural conditions, daylength is the signal which causes the most dramatic changes in the pattern of development in non-etiolated plants. The molecular events that take place in response to this signal have not been investigated rigorously and consequently the biochemical mechanism remains unknown.

The function of a photoreceptor is to transduce light energy to a biochemical signal to which the cell is preprogrammed to respond and gene activation is only one of a number of possible consequences of photoreceptor excitation. There is no reason to suppose that the stimulation of light-reponsive genes will involve mechanisms which differ, in principle, from those involved in the activation of genes by other stimuli such as hormones. The difference lies in the way in which the original stimulus is perceived and transduced. Specificity of response is introduced in the transmission of the signal from the photoperception system to the genetic material and in the responsivity of the genes themselves.

GENE ACTIVATION

It is well established that light plays a major role in chloroplast development (Lichtenthaler & Meier, 1984). As a consequence, the major research effort on light-regulated genes has been focussed upon events taking place during the transition of the etioplast of dark-grown seedlings into chloroplasts of fully green seedlings. The changes that take place involve the complete rearrangement of the etioplast ultrastructure with a co-ordinated synthesis of components required for the development of the mature chloroplast. The synthesis of proteins essential for the functional activity of the chloroplast is determined by both the nuclear and chloroplast genetic systems (Ellis, 1981). Light, moreover, is known to act upon gene expression of both chloroplast and nuclear DNA.

Nuclear encoded chloroplast proteins

The majority of chloroplast proteins are nuclear encoded, although frequently a component of the holoprotein is coded for within the chloroplast. The products of the cytoplasmic mRNA are usually larger molecular weight precursors which are then capable of entering the chloroplast by recognition of the additional peptide.

The most abundant chloroplast protein, ribulose 1,5-bisphosphate (RuBP) carboxylase, is composed of equivalent numbers of two subunits. The smaller subunits (SSU) are nuclear encoded and after transport and processing to its mature size they are assembled into the holoprotein by combination with the chloroplast encoded larger subunits (LSU). There have been many reports of photoregulation of this enzyme, but the first direct evidence for the regulation being mediated via changes at the mRNA level were obtained using in vitro translation of poly(A) RNA extracted from Lemna (Tobin, 1978). This experimental protocol, however, does not distinguish between increased transcription and changes at the translational level such as activation of nascent mRNA.

Evidence that the 'steady state' transcript levels were increased by light was obtained using a SSU cDNA probe hybridised to complementary RNA

(Stiekema et al. 1983). This increase was rapid (within 2 hours of light treatment) and shown to be red/far-red reversible; typical of a phytochrome response. A similar response is found in soya bean (Tobin & Silverthorne, 1985), although for pea a lag of 24 hours is found before a response to light is obtained (Thompson et al. 1983, Jenkins et al. 1983). Using nuclei isolated from pea, Gallagher & Ellis (1982) fed labelled UTP to study light regulation of *in vitro* transcription. They found that SSU transcription was 18-fold greater for nuclei isolated from light-grown plants as compared to dark-grown ones.

In both *Lemna* and soya bean there is a decrease in transcripts for the SSU within 2 hours of light-grown plants being placed in the dark and this decline continues for 48 hours. If a 15 minute far-red light treatment is given prior to the dark period the decline is more rapid, reaching the level of dark-grown plants within 2 hours. Either phytochrome in the far-red absorbing form is required to maintain the light levels of transcription for the SSU, or the response has a long escape time. Using isolated nuclei from pea to study these responses by *in vitro* transcription, more rapid responses to the light/dark transition have been obtained (Gallagher et al. 1985).

An innovative application of modern technologies to the study of light-regulated sequences has been carried out by Broglie et al. (1984b) and Herrera-Estrella et al. (1984). They have studied the structure and properties of the isolated gene that contains light inducible sequences. Broglie et al. (1984b) used the (Ti) plasmid of *Agrobacterium tumefaciens* as a transformation vector to insert a pea SSU gene and its surrounding sequences into calli of *Petunia*. The inserted gene was light-regulated in the *Petunia* tissue and the fidelity of expression was retained in this heterologous system. Light increased the transcript level in the *Petunia* tissue by an equivalent amount to that found in pea and the processing/assembly into the holoenzyme was as predicted on the basis of previous studies. The first report of a functional analysis of the control sequences of a light regulated plant gene was carried out by Morelli et al. (1985) using the same system. They investigated the light-regulation of transcription in 5' deletion mutants. It was considered that a 33 bp sequence (-35 to -2) around the TATA box was required for the light-sensitivity of this particular SSU gene. Control of maximum transcription resided in the -1052 to -352 region as mutants without this region gave only 15-20% of the wild type activity while retaining their photoresponse. To determine the action of light on post-transcriptional events, these authors combined the SSU coding sequence with a light-insensitive 35s promoter of cauliflower mosaic virus. Significantly, the level of SSU transcripts produced were identical in the light or dark, and clearly demonstrated that light has no effect upon post-transcriptional modification or stability of the transcripts. Using a similar approach, Herrera-Estrella et al. (1984) constructed a chimaeric gene by linking the 5'-flanking region of pea SSU to the coding region of bacterial chloramphenicol acetyltransferase gene (CAT). This construction was then inserted into *Nicotiana tabacum* via an *A. tumefaciens* (Ti) plasmid vector system and the expression of both CAT activity and its mRNA were found to be light-regulated. In agreement with Broglie et al. (1984b), it was confirmed that the light response was controlled primarily at the level of transcription. Furthermore, the construction of these chimaeric genes involving pea promoter regions transformed into other species suggests that the mechanism by which the light stimuli is transmitted to the promoter region is strongly conserved.

Another important nuclear encoded chloroplast component is the chlorophyll a/b binding protein (CAB). This protein is associated with the chlorophyll and carotenoids of the light-harvesting antennae of photosystem II. A wheat genomic fragment of 6.5 kb containing CAB sequences has also been introduced into tobacco protoplasts using A. tumefaciens and plants regenerated from the calli (Lamppa et al. 1985). As no routine transformation system is available for monocotyledonous plants, this transfer has allowed the regulatory sequences of the wheat gene to be examined. The CAB gene was found to be both light-regulated and organ-specific. The increased transcription in the light was largely confined to the leaves (though low levels of transcripts were found in stem tissue) and the most abundant expression followed a dark to light transition.

The steady state CAB transcript level has been shown to be light-regulated via phytochrome, but the kinetics and precise extent are dependent on both species and developmental stage (Tobin & Silverthorne, 1985). It has been found that the fluence rate of red light required to increase the steady state mRNA level for CAB genes in pea is 10^4 less than that required for SSU. This result indicates a differential response of nuclear coded genes to the quantity of red light and may relate to a physiological requirement to rapidly assemble the thylakoid membrane as the seedling emerges (Kaufman et al. 1984). These experiments have been extended to investigate differential expression for a number of mRNA transcripts (Kaufman et al. 1985a). Furthermore, those genes that show a very low fluence response to red light (i.e. CAB) show no response to blue light. The presence of a blue light response appeared to correlate with the phytochrome responsivity (Kaufman et al. 1985b).

Chloroplast encoded proteins

Chloroplast DNA is a relatively small molecule ranging in size from 120-150 kb and its size greatly improves the potential for genetic manipulation using recombinant DNA technology (for a detailed review of chloroplast DNA see Whitfield & Bottomley, 1983). The number of identified proteins coded for still remains small, although a number of light-regulated polypeptides are known including LSU, the 32kD herbicide-binding polypeptide (Link, 1982), subunits of the chloroplast coupling factor (Rodermel & Bogorad, 1985) and polypeptides of the P700 chlorophyll a-protein complex of photosystem I (Fish et al. 1985). In maize, positively photoregulated areas of the plastid genome account for c. 19% of the total (Rodermel & Bogorad, 1985). The light-regulated genes are distributed around the plastid DNA and are transcribed either individually or as polycistronic mRNA. The photogenes differ in their response to light, reaching maximum increases at different times and in most cases then falling back to pre-illumination levels. The time course for transcription of known polypeptides suggest that it may relate to the order in which the photosynthetic components are assembled.

Most maize photoregulated genes give rise to a number of different size transcripts whose response to light is not uniform. For example, CF1 a-subunit gene gives 9 transcripts and CFo III gives 11, all increasing with illumination, but not to the same extent (Rodermel & Bogorad, 1985). These results could occur as a result of different transcription initiation sites or by modification of larger common transcripts by processing enzymes. However, the precise role of light remains to be elucidated in these complex polycistronic genes, particularly as the stoichiometry of individual subunits within multi-subunit protein complexes are often different and yet may be coded for in the same polycistronic region eg. CF1

stoichiometry is 3:1 for b:e subunits. Furthermore, C4 plants such as maize that contain two distinct cell types in their leaves are known to show differential gene expression for chloroplast encoded proteins (Broglie et al. 1984a). It would be interesting to know what role light plays in the expression of the plastid genomes in these specialised cells. The nature of the photoreceptor associated with light-regulated gene expression in chloroplasts has not been extensively studied and the exact role and identity of the photoreceptors concerned requires further investigation. For instance, in pea tissue both the 32kD herbicide-binding protein and LSU transcripts show red/far-red reversible characteristics in their light response, indicative of phytochrome (Thompson et al. 1983). The timing of these responses, however, suggests that general developmental events may be occurring. In mustard the 32kD protein may be phytochrome regulated (Link, 1982), but in Spirodela a blue light receptor has been implicated (Gressel, 1978).

UV Regulation of phenyl-propanoid pathway

In addition to visible light, plants are able to perceive UV irradiation. Plants respond to this irradiation by the activation of the phenylpropanoid biosynthetic pathway (300 nm wavelength being most effective). This is a typical plant defense response to environmental stress and produces flavonoid glycosides. These pigments are strong UV absorbing pigments and therefore afford the plant cell protection. Fungal elicitors also activate phenylpropanoid metabolism, but the products are furanocoumarins which have fungicidal properties. Both flavonoid glycosides and furanocoumarins have the same enzymes in the initial stages of their biosynthesis. By the application of cDNA hybridisation probes corresponding to enzymes of the general phenylpropanoid pathway, (phenylalanine-ammonia lyase and 4-coumarate:CoA ligase) or the flavonoid pathway (chalcone synthase), the steady state mRNA levels were found to correspond to changes in the induction pattern of these enzymes in resonse to UV irradiation or fungal elicitor. Furthermore, similar results were obtained when ^{32}P-UTP was used to determine the in vitro transcription of these genes in isolated nuclei (Chappell & Hahlbrock, 1984 and reference therein). From these experiments, it was concluded that flavonoid glycoside accumulation in response to UV irradiation is determined predominantly at the level of gene transcription. These results are particularly important because Hahlbrock's group have attempted to correlate the actual transcription rate to the level of the final product. Thus the radiolabelling of RNA for PAL and chalcone synthase was similar to the response obtained with the isolated nuclei. In addition, although the fungal elicitor and UV irradiation do not give precisely the same pattern of activation, they may well function by a common, as yet unidentified mechanism or intermediate. It is this mechanism by which light perception is translated into activity at the gene level that is of fundamental importance to the application of light as a controllable, non-invasive methodology for regulation of plant growth and development.

Photo-inactivation of genes

In addition to those genes that are increased in their expression in response to light, there are a number of important examples including phytochrome and protochlorophyllide reductase where light causes a reduction in transcription. The amount of phytochrome declines rapidly in response to its photoconversion by red light to the presumed active form Pfr. Studies using in vitro translation revealed that the translatable mRNA for phytochrome falls rapidly upon red-irradiation (Gottmann & Schafer, 1982; Colbert et al. 1983). Furthermore, cDNA hybridisation has

confirmed that the steady state levels of sequences for phytochrome decrease within 15 minutes of red light treatment (Hershey et al. 1984). Such responses become all the more important when one considers the evidence that phytochrome may exist as immunologically distinct populations and consequently reflect different gene products in green and etiolated tissue (Thomas et al. 1984), in association with membranes (Jordan et al. 1984), and in seeds (Hilton & Thomas, 1985). For reviews and discussion see Thomas et al. 1986; Jordan et al. 1986).

Photoperiodic gene regulation

One of the major morphological changes in plants is the switch from a vegetative to reproductive mode of growth, which is of major significance in horticulture and crop production. The morphological change may be photoperiodically induced, although the interactions of daylength with other factors such as temperature (e.g. vernalization) may be complex (Thomas & Vince-Prue, 1984). Grafting experiments have shown that the light stimulus is perceived in the leaves and transmitted by an unknown mechanism to the shoot apex (Schwabe, 1984). Changes in the RNA of apical meristems are an early event in floral induction, but recent evidence suggests that specific m-RNA is transcribed in the leaves of Hyoscyamus niger in response to inductive cycles (Warm, 1984). Although the gene products of these light-regulated genes have not yet been identified, genes determining response to photoperiod have been localised on the chromosomes of wheat (Law & Scarth, 1984).

EXPLOITATION OF LIGHT-REGULATED GENES

Many of the identified photogenes have important roles in plant development and could in theory be manipulated for improved crop productivity. The practicalities of this manipulation are likely to prove obdurate problems and the benefits may be less tangible in the short term. RuBP carboxylase has been selected as a target for genetic manipulation because of its key position as the common enzyme of both photosynthesis and photorespiration. A reduction in photorespiratory activity would be of agricultural significance, because it would decrease the loss of fixed CO_2 (Ellis & Gatenby, 1984). Increasing the amount of RuBP by photoactivation of multigene families may be only limited benefit, as the enzyme is already extremely abundant. Possibly photo-inactivation (cf. phytochrome and protochlorophyllide reductase) of other photorespiratory enzymes is a plausible approach, although the loss of the enzymes which process the two carbon product of photorespiration is likely to have detrimental effects upon cell function (Somerville & Ogren, 1982).

Another photogene that is central to the electron transport capabilities of the thylakoid membrane and may be part of the PS II reaction centre (Deisenhofer et al. 1985), is the chloroplast encoded 32 kD protein of PS II. Not only is the protein rapidly synthesised in response to light, but the rate of degradation is dependent on the rate of photosynthetic electron transport and light intensity (Bennett, 1984). A better understanding of the complex role of light in the properties of this protein may provide opportunities for its manipulation, especially as this protein also confers herbicide resistance. Furthermore, as the components of the thylakoid membrane are in general reduced under low light (i.e. CF_1, cytochrome b_6f, reaction centre polypeptides, etc.) and an increase may stimulate photosynthetic activity, their manipulation by light may also offer potential for commercial exploitation.

By the construction of chimaeric genes, it is now possible to place

non-photoregulated genes under the control of light and target them to certain organelles. Schreier et al. (1985) have demonstrated this by fusing a neomycin phosphotransferase gene with a sequence from the SSU gene from pea including the promoter sequence and coding sequence for the transit peptide and 22 further amino acids. Using A. tumefaciens as a vector the chimaeric gene was transferred to tobacco and the gene shown to be integrated into nuclear DNA. The fusion protein was expressed in a light-dependent manner, was transported to, and expressed in, the plastid. This capability should have far-reaching potential for agricultural and biotechnological exploitation. The development of effective strategies for the application of this technique is critically dependent on our understanding of the physiological and biochemical principles involved in the photoregu lation of plant development. With the exception of a few parasitic species, light is an essential requirement for plant growth under natural conditions. Genes which are directly light-stimulated will therefore normally always be expressed in the aerial portions of plants, although their expression may be modulated according to the light microenvironment pertaining to a particular plant or part of a plant. Light-activation would under these circumstances enable co-ordination of the degree of expression of an alien gene with that of the native light-regulated genes. Differential expression of alien genes within a single plant could possibly be exploited to manipulate patterns of assimilate partitioning within a plant in response to self shading of older leaves. Another approach may be to use light-activation to enable the expression of genes at stages of plant development during which they would normally be inactive e.g. during senescence.

Flowering is probably the best characterised example of photoperiodic control although many other aspects of plant development may be strongly affected by daylength. Flowering is of considerable economic significance, and its control by daylength is used commercially, illustrating the benefits of this type of approach. In chrysanthemum plants manipulation of daylength by using artificial light in winter and blackouts in the summer enables the grower to control the duration of the vegetative phase to give the most desirable stem lengths, and the transition to flowering, so that flowers are produced at the same time for each member of the crop and at the time required by the grower. Photoperiodic regulation of genes in field grown material would enable them to be expressed at a particular time, possibly in co-ordination with events such as flowering or seed formation (Miflin, 1984).

The unique feature of light-activated genes which confers them with special potential is that they may be remotely activated with a non-invasive stimulus. It is precisely for this reason that light-activated genes have been the experimental subjects of choice for those studying mechanisms of transcriptional control in plants. However, commercial exploitation of this feature requires that the light environment of the plant material in question be controlled. This is not easily achieved under field conditions and this approach will probably be more effective for plants grown with artificial illumination. These would include those in bioreactors, in vitro cultured plant material and possibly glasshouse grown material. In vitro material may be grown heterotrophically in the absence of light and gene activation could be totally controlled under these conditions. Another approach is to use photoperiodically regulated genes, as daylength is particularly easy to control for plants growing in vitro under artificial illumination. One further possibility is to exploit the selective nature of light absorption by photoreceptors as compared to the photosynthetic system. For example, many species will grow adequately

in red light which provides an effective energy input for photosynthesis and photomorphogenetic input for phytochrome activated genes. An alien or native gene which is blue light dependent would only be expressed when supplementary blue light is supplied and hence be completely controllable.

PHOTOREGULATION IN PERSPECTIVE

The photoregulation of the genetic system demonstrates many of the advances that have been made in understanding the mediation of light in fundamental developmental processes. Moreover, technology is becoming available that can allow one to predict the application of light regulation to modify plant growth, either by direct manipulation of photogenes or by the insertion into plant tissue of chimaeric constructions containing light regulated sequences. However, many of the apparent transcription responses to light are clearly integrated with a general responsivity to light. Light can induce a general increase in transcription during greening by increasing the total DNA content, or by stimulation of RNA polymerase activity, yet it is also able to cause differential gene expression. Genes that are clearly light-regulated in some tissues, i.e. SSU in pea, are present in the dark in others i.e. cucumber and castor bean (Tobin & Silverthorne, 1985). Thompson et al. (1983) suggested that the potential for light regulation may depend on the state at which development is arrested in the dark. Furthermore, the light regulation of genes must be sufficiently subtle to bring about differential expression of genes that are members of multigene families, regulate polycistronic sequences, to obtain tissue and organ specificity and perhaps even regulate expression within different specialised cell types as found in C4 species. The regulatory system may also have to account for endogenous rhythms in transcription, as has been suggested for pea CAB and SSU (Kloppstech, 1985).

Most of the evidence for light-regulated genes has been obtained in greening systems or seedlings. It is most important to remember that fully mature leaves respond both to light quality and light quantity e.g., by changes in the composition, ultrastructure and functional activity of the chloroplast (Bjorkman, 1981; Lichtenthaler & Meier, 1984; Davies et al. 1986a,b) or by switching from vegetative to reproductive growth. Is the regulation at the transcriptional level important under these conditions and which photoreceptors are involved?

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ALTERATION OF THE ACTIVITY OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE THROUGH MANIPULATION OF ITS STRUCTURE AND REGULATION

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ABSTRACT

The principal carbon-fixing enzyme of plants and bacteria, ribulose-1,5-bisphosphate (RuBP) carboxylase, is inefficient because it also catalyses the oxygenation of the RuBP substrate. Variation between the enzymes from different organisms in the relative specificity for CO_2 and O_2 suggests that this varies according to the structure of the protein. Genes for several forms of the enzyme have been isolated and expressed in bacteria. These genes are now being manipulated *in vitro* in attempts to alter the relative rates of the carboxylase and oxygenase activities. Work has also been carried out on the factors which may control the enzyme activity within the plant chloroplast.

INTRODUCTION

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is the most important enzyme in plants, catalysing the first step in photosynthetic carbon metabolism. The enzyme catalyses the reaction between atmospheric CO_2 and ribulose-1,5-bisphosphate (RuBP), a 5-carbon sugar, yielding two molecules of 3-phosphoglycerate. However, the enzyme also catalyses a second reaction between molecular O_2 and the RuBP substrate in the initial step of photorespiration. The products of this reaction are one molecule of 3-phosphoglycerate and one of 2-phosphoglycollate (see Figure 1). This oxygenase activity may be regarded as wasteful, since not only does this reduce the amount of RuBP available for reaction with CO_2, but also the recycling of the 2-phosphoglycollate to 3-phosphoglycerate results in the evolution of CO_2. The relative proportion of oxygenase activity has been shown to vary between different organisms, achieved through either modification of the enzyme structure or morphological changes to increase the CO_2 concentration at the active site. Such changes lead to significant increases in carbon assimilation, and hence higher growth rates. Other factors that may limit the efficiency of the enzyme are associated with its activation in the chloroplast. Magnesium ions and CO_2 have long been known to be essential for the activation process, but it is now clear that other more subtle mechanisms may also operate.

INEVITABILITY OF THE OXYGENASE REACTION

RuBisCO from higher plants is an oligomer of eight large and eight small subunits. The carboxylase and oxygenase reactions are catalysed at the same active site within the larger subunit. Both reactions proceed via an enediol intermediate. The enzyme first binds the RuBP substrate

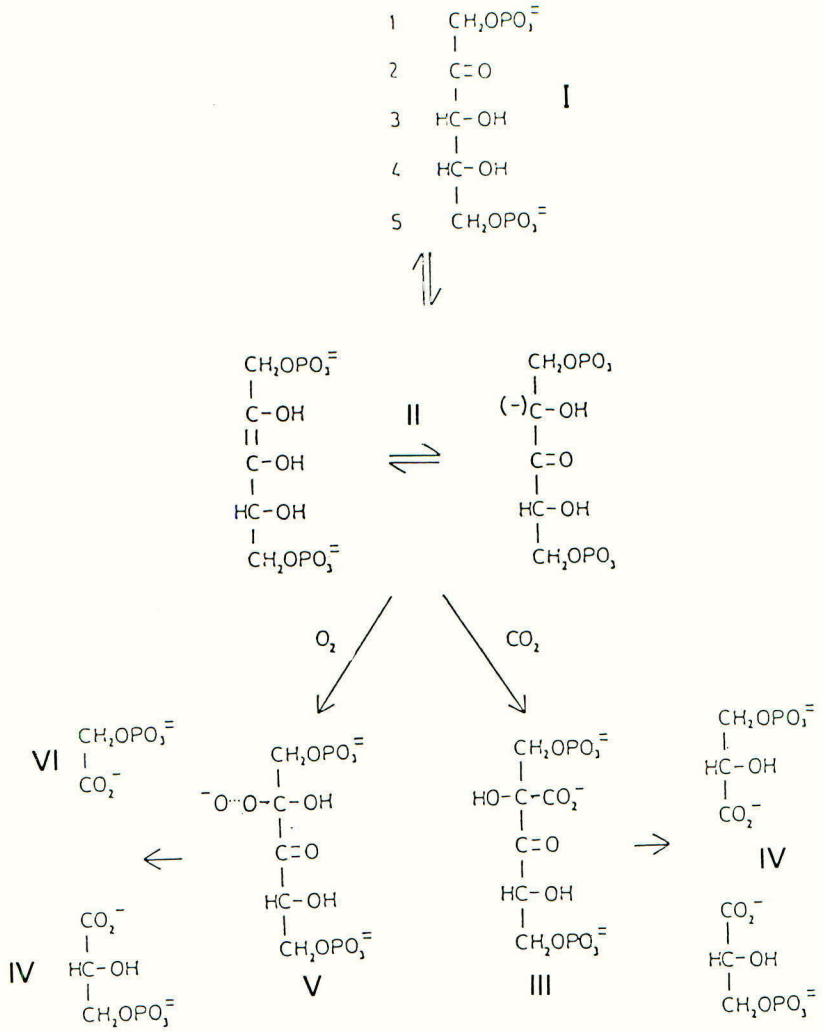

Fig. 1. The reactions of carboxylation and oxygenation of RuBP (I) catalysed by RuBisCO. Apart from the product molecules 3-phosphoglycerate (IV) and 2-phosphoglycollate (VI), the enediol intermediates (II) and 3-keto-2-carboxy derivative (III) are also known to be implicated in the reactions. The 3-keto-2-peroxy derivative of oxygenation (V) has not yet been identified directly.

and then catalyses the abstraction of the C_3 proton to generate the enediolate anion (Gutteridge *et al.* 1984a). In the presence of either CO_2 or O_2 the enediolate is rapidly processed to the reaction products (Figure 1). The partitioning between the carboxylase and oxygenase reactions is therefore, presumably, due to competition between the two gaseous substrates. This has led to the proposal that the oxygenase reaction is an inevitable consequence of the carboxylase mechanism (Andrews & Lorimer 1978). Clearly this cannot be the case since considerable variation in partitioning coefficient or specificity factor exists for RuBisCO isolated from different species. The lowest specificity factor (highest proportion of oxygenase) reported is for the enzyme isolated from *Rhodospirillum rubrum*, a dimeric protein of identical subunits which are homologous to the larger subunit of the plant enzyme. Even within the hexadecameric higher plant enzymes there is considerable variation in specificity factor (see Table 1). This must mean that there is a structural basis for the discrimination between the gaseous substrates and provides encouragement for an attempt to modify the specificity factor through genetic engineering.

TABLE 1

Specificity factor for RuBisCO isolated from several organisms (M. A. J. Parry & S. Gutteridge, unpublished). Specificity factor is defined as VcKo/VoKc where Vc and Vo are the maximal rates of carboxylation and oxygenation, and Kc and Ko are the Michaelis constants for CO_2 and O_2. A high specificity factor indicates a high specificity for CO_2.

Organism	Specificity factor
Rhodospirillum rubrium	9
Anacystis nidulans	52
Tobacco (*Nicotiana tabacum*)	91
Wheat	107

REGULATION OF RUBISCO ACTIVITY

If we are to attempt to change the kinetic parameters of crop plant RuBisCO, it is essential to understand how the activity is controlled *in vivo*. One particular paradox exhibited by RuBisCO concerns the conditions necessary to generate full activity. RuBisCO assayed *in vitro* is inactive unless it is incubated with CO_2, usually supplied as HCO_3^- and Mg^{2+}. However, the concentrations of these needed to obtain maximum catalytic activity are an order of magnitude greater than those available within the chloroplast. The inclusion of certain effectors reduces the concentration of cofactor needed for full activation to those expected in the stroma (Gutteridge *et al.* 1982), presumably by altering the equilibrium between enzyme, CO_2 and Mg^{2+}. However, the pools of most effectors reported to influence activity *in vitro* are not large enough to do this and are unlikely to be

physiologically relevant (McCurry et al. 1981).

Inorganic phosphate (Pi) is also a potent stimulator of RuBisCO activity. When RuBisCO is purified in the absence of phosphate or effectors of activation, subsequent activation in the presence of Pi yields maximum activity at relatively low concentrations of CO_2 and Mg^{2+}. This activity is about double that obtained in the absence of Pi with saturating CO_2 and Mg^{2+}. Analysis of the number of activated (i.e. with CO_2 and Mg^{2+} bound) sites shows no difference between presence and absence of Pi. Thus Pi does more than simply stimulate the activation process, it must stimulate individual sites to become more active. 6-Phosphogluconate is the only other effector that acts in a similar fashion to phosphate, but stimulation is only achieved if the concentration is at least equal to the concentration of active sites. Higher than stoichiometric amounts leads to increased binding of the activating cofactors, the results expected if 6-phosphogluconate were favouring a ternary enzyme complex.

Recently two further mechanisms for the regulation of activity in vitro have been reported. Salvucci et al. (1985) have identified a soluble chloroplast protein that is required for the activation of RuBisCO in a reconstituted light-activation system. Although an Arabidopsis mutant defective in activation appears to lack this protein, a direct effect has yet to be proven, since activation may result from an indirect effect on phosphate concentration or pH. Servaites (1985) and Seemann et al. (1985) have discovered a light-modulated inhibitor that can regulate activity by binding at the active site, but the structure of the inhibitor has yet to be determined. Although the concentration of this inhibitor in leaves could account for observed changes in RuBisCO activity, the inhibitor does not appear to be present in all species. Plants possessing the inhibitor can regulate RuBisCO activity by apparently maintaining the enzyme in an activated but catalytically inactive form in the dark and at low light intensities. Since RuBisCO activity changes in parallel with photosynthesis, manipulating the control of enzyme activity may provide a route for increasing photosynthesis.

MANIPULATION OF THE L_2 RUBISCO

An essential prerequisite for genetic manipulation of RuBisCO is the isolation of the structural genes and their expression in a suitable host, to produce sufficient quantities of enzyme such that the properties of mutant forms of the enzyme can be studied. The simplest form of RuBisCO is found in a photosynthetic bacterium, Rhodospirillum rubrum. This enzyme is composed of two identical subunits, denoted as L_2. The gene coding for the protein was obtained from a clone library and inserted into plasmid pBR322, with part of the lacZ gene of the bacteriophage M13mp7 included to increase expression (Somerville & Somerville 1984). The resulting recombinant plasmid, pRR2119, was introduced into the bacterium Escherichia coli, where it produced relatively high levels of active enzyme. This plasmid has been used as the basis for site-specific mutagenesis to modify the structure of the carboxylase, in order that the contribution of specific amino acids residues to the kinetic parameters can be studied.

Since the three-dimensional structure of the active site of RuBisCO is, at present, unknown, the choice of which amino acid residue to alter must be restricted to those identified with affinity labels as being close to the active site. For example, the only amino acid residue with a known function is a Lysine at position 191 in the R. rubrum enzyme, which is carbamylated during activation of the enzyme by CO_2 and magnesium ions. Of the ten amino acids surrounding this site, an Aspartate at position 188 is conserved in all the RuBisCOs known, and this was chosen for mutation in an attempt to perturb the metal ion binding site (Gutteridge et al. 1984b). Site-directed mutagenesis was carried out at this site by isolation of the double-stranded DNA plasmid containing the gene and the exposure of a short region of single stranded DNA. To this was annealed a 20-base oligonucleotide, exactly complementary to the region to be mutagenised except for a single nucleotide change of C to A, which would result in a change of Aspartate to Glutamate at position 188. The plasmid was then repaired with DNA polymerase and introduced back into E. coli. Bacteria carrying the mutated gene were identified by hybridisation with radio-labelled oligomer, and the carboxylase enzyme was isolated from a large-scale liquid culture. Analysis of the mutant enzyme revealed that the rate constants of both carboxylase and oxygenase activities had decreased by 30%, but that the specificity factor was unchanged. pRR2119 has now been used as the basis for several mutagenesis experiments, and these are outlined in Table 2.

TABLE 2

Site-directed mutagenesis of Rhodospirillum rubrum RuBisCO in pRR2119. Kc and Ko are as defined in Table 1.

Amino Acid	Location	Effect	Reference
glu - asp	188	activity x 0.7 Kc x 2	Gutteridge et al. 1984b
lys - glu	191	inactive	Estelle et al. 1985
met - leu	330	Kc x 2 Ko x 36 activity x 0.2	Laing et al. 1985

MANIPULATION OF THE L_8S_8

RuBisCO from blue-green algae and higher plants combines two types of subunit, large (L) and small (S), arranged in a hexadecameric L_8S_8 complex. The L subunit is homologous to the single subunit of R. rubrum, and affinity probe binding shows that it contains the active site. The S subunit is essential for catalysis, but its actual function is unclear. In higher plants, the genes for these two subunits are located separately: the gene for the L subunit is in the chloroplast DNA (Coen et al. 1977, Bottomley & Whitfeld 1979), whereas the S subunit is encoded in the nucleus (Coruzzi et al. 1983, Broglie et al. 1983, Dunsmuir et al 1983) and is synthesised in the cytoplasm as a large precursor (Highfield

& Ellis 1978) which is processed to the correct size on transport into the chloroplast. The first constructs of the genes coding for this type of RuBisCO were the L subunit from maize and wheat. The maize L subunit gene was cloned in the thermoinducible plasmid pHUB4 under control of the strong transcriptional promotor P_L (Gatenby & Castleton 1982). This resulted in high levels of expression in *E. coli*, approximately 2% of total cell protein. Several groups are currently working to produce a construction containing both L and S subunits of higher plant RuBisCO which will produce active enzyme, but agglutination and precipitation of the plant L subunit has proved to be an as yet insurmountable problem.

The RuBisCO from cyanobacteria is also of the form L_8S_8, and is unusual in that the subunits can be reversibly dissociated from the holoenzyme (Andrews & Abel 1981). The genes for the two subunits of the *Anacystis nidulans* RuBisCO are located close together on the chromosome, with a small intervening region, and are transcribed together as a dicistronic message (Shinozaki *et al.* 1983, Shinozaki & Sugiura 1983, 1985). The restriction endonuclease fragment containing these genes has been cloned into a plasmid under control of the P_L promotor (Gatenby *et al.* 1985). When introduced into *E. coli* cells this construct produced only low levels of enzyme activity, due in part to an under-production of S subunits such that the enzyme was of the form L_8S_3. Introducing the same restriction fragment into plasmid pUC18 however (A. L. Phillips, C. A. Kettleborough, M. A. Parry, S. Gutteridge, unpublished) under control of the *lac* promotor, yielded a construct which produced active RuBisCO, probably with an L_8S_8 stoichiometry, to a level of the order of 10% of the host bacterial protein.

Until such time as the expression in bacteria of an a catalytically active crop plant RuBisCO is achieved, the construction of a plasmid which produces acceptable amounts of the very similar cyanobacterial L_8S_8 RuBisCO promises to be very useful. This will be used for both mutagenic experiments to investigate the reaction mechanism and to change the kinetic parameters of the enzyme, and also for the purification of large amounts of the enzyme for crystallographic studies, which may lead to detailed information about the active site.

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VARIETAL IMPROVEMENT IN THE BREAD-MAKING QUALITY OF WHEAT: CONTRIBUTIONS FROM BIOCHEMISTRY AND GENETICS, AND FUTURE PROSPECTS FROM MOLECULAR BIOLOGY

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ABSTRACT

In the wheat breeding programme at the Plant Breeding Institute high priority is given to the development of new, high yielding varieties with improved bread-making properties. This should lead to a further reduction in the importation of expensive, high quality wheat from Canada for use in UK bread grists. Current home-grown varieties do not have optimal mixing properties, causing bread doughs to be too weak. This has been identified at the molecular level to a deficiency in the elastic properties of the glutenin protein complex. The subunits which make up glutenin fall into two groups: low-molecular-weight (LMW) subunits amounting to 80% of the total, and HMW subunits. Each group is coded by genes at three different loci and all show extensive allelic variation. Variation in HMW glutenin subunits has a major influence on dough strength. By analysing random lines of many crosses from the breeding programme these subunits have been partially ranked for their effects on quality. The wheat breeders are being advised which varieties to cross to achieve optimal compositions of HMW glutenin subunits and progeny from single-seed descent are being selected from electrophoresis of half grains. In other work, bread-wheat landraces from ancient agriculture are being screened for novel HMW glutenin subunits. The genes from some of them are being transferred to commercial wheats by repetitive backcrossing so that their effects on quality can be assessed. The molecular biology of the glutenin subunit genes is being studied extensively. This work will probably make an important impact on wheat breeding in the future; indirectly by indicating to geneticists which novel HMW glutenin subunits, from landraces and diploid species related to wheat, are likely to have a structure which will impart strong elasticity to glutenin, and directly by the insertion of cloned subunit genes into the wheat genome.

INTRODUCTION

The maritime climate of the United Kingdom favours the production of high yielding wheat crops with a low grain protein content. This has led primarily to the development of feed wheat and soft milling, low protein wheats for biscuit and cake making. At The Plant Breeding Institute (PBI), Cambridge, bread-quality wheat varieties, whose grain must be hard milling and have a much higher protein content (10-13%), have also been bred since about 1910, but until recently they have never been grown extensively in British agriculture because they have been relatively low yielding. Traditionally, the United Kingdom has imported wheats for bread-making from Canada, the U.S.A. and Australia, whose continental climates favour the production of strong, high-protein wheats which are highly suited for bread-making.

Wheat production and trade has changed dramatically in the U.K. over the last few years. For the first time in its history the U.K. has become a net exporter of wheat and is likely to remain in surplus for the forseeable future. This has been accomplished by the release of varieties which give much greater yields, by improving agricultural practice and by increasing the acreage planted with wheat. Since the UK joined the European Economic Community in 1973, North American wheats have become much more expensive to buy, both through the introduction of import tarifs and the rise in their price on world markets. These changes have lead, in the growing season 1982-83 for instance, to 2.19 million tons of the UK crop of 10.3 million tons being exported cheaply and 1.43 million tons of high quality wheat being imported at much greater expense (HGCA, 1984).

Clearly there is a very strong incentive to reduce the level of these imports by increasing the percentage of home-grown wheat in UK bread grists. This is now being achieved, partly by the development of new bread-making procedures and by gluten supplementation and partly by the introduction of more suitable varieties. Whereas in 1967, only about 20% of the flour used to make a typical white British Loaf was home-grown, this has increased in 1985 to 80-90%. To raise this percentage even higher and to raise the contribution of home-grown wheat in wholemeal bread, which contains a much higher proportion of imported wheat, new varieties are required which approach the quality of their North American counterparts. To achieve this the biochemical basis for differences in bread-making quality between varieties needs to be understood in genetic terms so that strategies for varietal improvement can be implemented by the wheat breeder.

There are several characters which contribute to the overall bread-making quality of a variety but it is generally recognised that two of the most important are protein content and protein quality. The protein content of a grain is primarily governed by agricultural practice and climate and genetic variation for this character is both complex and small. It is very difficult to breed for increased protein content without reducing grain yield. In contrast, variation in protein quality for bread-making is primarily genetically determined but until recently it has hardly been understood in biochemical and genetic terms. The object of my research group at the PBI is to identify proteins which confer good bread-making quality to a dough, and, with the aid of the wheat breeders, to bring them together in breeding programmes. Our research progress over the last six years will be reviewed and future prospects, with inputs from molecular biology, will be discussed.

RESULTS

Gliadin and glutenin

The endosperm of a single variety of bread wheat contains many different proteins and they are resolved into about 60 major and 100 minor components by two-dimensional polyacrylamide-gel electrophoresis. (Payne *et al*, 1984a). Most of the major proteins are constituents of gluten, a visco-elastic complex which is formed by kneading dough in a stream of running water. Modern biochemical work has shown that the great majority of proteins in gluten are storage proteins, synthesised on the endoplasmic reticulum and deposited in protein bodies during grain development. Two different protein groups form the gluten complex, gliadin and glutenin.

Gliadin molecules are small (molecular weight approximately 35,000), and spherical and they give extensibility or viscous flow to dough. They have no recognisable subunit structure. By contrast glutenin molecules consist of long, linear molecules of variable length with a molecular weight range of 200,000 to several million. They are built up of disulphide-linked subunits and they impart viscoelasticity to a dough. The subunits belong to two groups, the low-molecular-weight (LMW) subunits, which are in the majority, and the high-molecular-weight (HMW) subunits.

A dough which is optimally suited for bread-making will have a balance of strong elasticity and high extensibility. The bread-making qualities of many English wheats are limited by their elasticity, causing them to have weak mixing properties. Variation in protein quality for bread-making is therefore likely to be mainly determined by glutenin structure. An important finding by Huebner & Wall (1976), later confirmed by Bottomley et al., (1982), was that the longer the glutenin molecules were the greater was their elasticity. Although glutenin chain length may not be the only reason for differences in elasticity it is clear that the biophysical properties of glutenin must be determined by the composition of its subunits. A detailed study of the genetics of glutenin subunits was therefore made.

Genetical analysis of glutenin subunits

Bread wheat is a hexaploid with 42 chromosomes; the diploid chromosome number of its ancesters, which contributed the A, B and D genomes, being 14. Spontaneous, partial or whole chromosome deletions are thus tolerated and this has been exploited by cytogeneticists in the development of a large collection of aneuploid lines for several varieties. To locate the genes controlling a particular protein to a chromosome the biochemist has simply to determine by electrophoresis which of the aneuploids lack the protein. By this approach, the genes coding for HMW subunits of glutenin were shown to occur on the long arms of chromosomes 1A, 1B and 1D (Bietz et al., 1975). Subsequent linkage analysis and recombination mapping showed that the genes occur at locus Glu-1, close to the centromere (Payne et al., 1982). In equivalent studies it was shown that genes coding for LMW glutenin subunits occur close to the ends of the short arms of the same group 1 chromosomes (Jackson et al., 1983; Payne et al., 1984a). For any one chromosome, linkage between these genes and those at Glu-1 is very weak so that recombination between HMW and LMW glutenin subunits will be very great in breeding programmes.

The genes encoding LMW glutenin subunits occur at a large locus on each group 1 chromosome called Gli-1 which also contains families of genes coding for ω- and γ-gliadins (Payne et al, 1984b). Recent nucleotide and amino-acid sequencing studies of these protein groups (see Shewry & Miflin, 1985) strongly suggest that they are closely related and their genes probably arose from a single, ancestral gene by gene replication and divergence. The rate of mutation of these genes has been great so that today there are many complex alleles for each of the Gli-1 loci. Sozinov & Poperelya (1980) have made an extensive study of this variation by comparing the mobilities of ω- and γ-gliadins by starch-gel electrophoresis. More recently the same research group changed their analytical procedure to polyacrylamide-gel electrophoresis and, using predominantly varieties from the USSR, described 15 complex alleles for Gli-1 on chromosome 1A, 18 for Gli-1 on 1B and 8 on chromosome 1D

(Metakovsky *et al*, 1984). The total number of permutations of protein blocks arising from the three *Gli-1* loci, and that are possible for a single variety of wheat, is therefore 15 x 18 x 8 = 2160. Although Sozinov's group did not study variation in LMW subunits of glutenin, whose genes also occur at the *Gli-1* loci, it is known from preliminary studies that these too display extensive allelic variation (Jackson, 1984).

The *Glu-1* loci on the long arms of chromosomes 1A, 1B and 1D, which code for HMW glutenin subunits, contain many fewer genes than the *Gli-1* loci (Thompson *et al*., 1983) and each locus produces just one or two major subunits (Payne *et al*., 1981b). However, there is much allelic variation although it is not as extensive as that for the *Gli-1* proteins. Payne and Lawrence (1983) described three alleles for *Glu-1* on chromosome 1A, 11 on 1B and 6 for 1D in a sample of 300 wheat varieties. The possible number of permutations of HMW glutenin subunits is large at 198, and the combinations of HMW and LMW subunits vast, perhaps up to 400,000.

It is our hypothesis that the variation in the composition of HMW and LMW subunits of glutenin is primarily responsible for the genetic component of differences in the bread-making qualities of British varieties. The primary objective of our research programme is to identify which allelic group from each locus confers the strongest elasticity to glutenin and to help the breeder bring the élite alleles together by conventional plant breeding.

Relating individual glutenin subunits to bread-making quality

This has been determined primarily by analysing random progeny of various crosses taken directly from the PBI wheat breeding programmes. The crosses chosen were between parents with contrasting HMW glutenin subunits and contrasting bread-making qualities (Payne *et al*., 1979 and 1981a). For each cross at least 70 progeny were analysed, for bread-making quality by the SDS-sedimentation test (Axford *et al*., 1979) and for HMW subunits of glutenin, by SDS-PAGE. The latter technique additionally enabled segregation at the *Gli-1* locus on chromosome 1B to be followed because the 1B-encoded ω-gliadins are well resolved by this technique. The progeny of some crosses were additionally analysed by gliadin electrophoresis to detect allelic variation at all the *Gli-1* loci and at one or two of the three *Gli-2* loci, the latter occuring on the homoeologous group 6 chromosomes and coding for α- and β-gliadins.

By the random-progeny method, significant associations were demonstrated between the presence of certain storage protein alleles and good quality as assessed by the SDS sedimentation test. Quality associations were particularly marked for the HMW subunits of glutenin (Payne *et al*, 1981a) and from the analysis of many different crosses a partial ranking order of alleles at each *Glu-1* locus with respect to SDS-sedimentation volume was constructed (Payne *et al*., 1984a and Fig. 1). Thus for chromosome 1D, glutenin subunits 5 and 10 were associated with good quality for all crosses and were always superior to subunits 2 and 12. The latter subunit pair was not significantly different to subunits 3 and 12 but progeny containing either of these two allelic pairs gave, on average, greater SDS-sedimentation volumes than those with 4 and 12. The ranking order is thus 5+10 > 2+12 = 3+12 > 4+12. Some highly significant

associations with quality were found with certain Gli-1 alleles. Thus, the allele on chromosome 1D coding for ω-gliadins 12.5, 15.5 and 17.5 (using the nomenclature of Zillman and Bushuk, 1979) was associated with better quality than the allele coding for ω-gliadins 16 and 18.5. Similarly those progeny containing the 1BL/1RS translocation chromosome, in which the rye locus Gli-R1 replaces the gliadin locus on chromosome 1B, are always associated with poor quality.

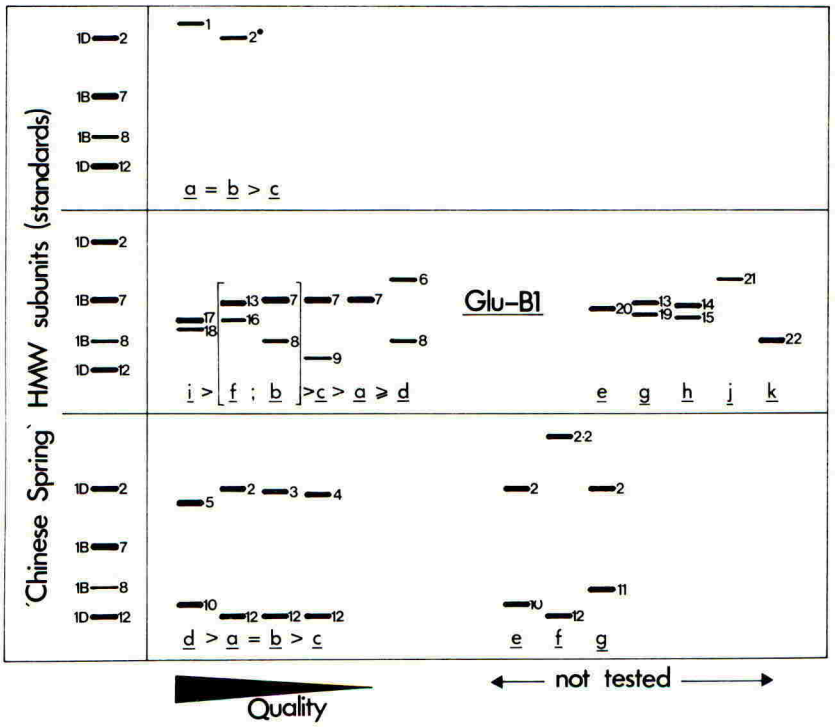

Fig.1. Allelic variation in HMW subunits of glutenin and its relationship to bread-making quality. Subunits were fractionated by SDS-PAGE with the direction of migration from top to the bottom of the Figure. On the left-hand side the subunit pattern of the control, Chinese Spring, has been triplicated to show the relative mobilities of: Top, chromosome 1A-encoded variants; middle, 1B- encoded variants; bottom, 1D-encoded variants. The lower case letters refer to the allele designations of Payne and Lawrence (1983).

The great complexity of the gliadin proteins and their extensive allelic variation makes it an extremely difficult and prolonged task to rank the major alleles at all three Gli-1 loci with respect to quality. However, after several decades of work by Sozinov and colleagues in the USSR, using the same general approach described above for Glu-1 alleles, quality ranking orders for the major alleles were elucidated (Sozinov & Poperelya, 1980). Our very limited findings, including the two examples given above, are consistent with the ranking orders of these workers.

There are several disadvantages to the random-line approach in relating

individual proteins to bread-making quality. The major one is that crossing two parents generates considerable genetic variation. To reduce the affects of this in the above studies, many progeny of a cross need to be analysed. This means effectively that only simple, and probably less exacting quality tests such as the SDS-sedimentation test can be performed. The random line method has been adopted to reduce the severity of background genetic variation by using Chinese Spring (CS) as a parent and crossing it with several intervarietal chromosome substitution lines of CS. Thus, as an example, CS was crossed with CS (Hope 1A) and the progeny were brought through the generations by single-seed descent to F_5. Since the parents only differ from each other with respect to chromosome 1A, one coming from CS and the other from the variety Hope, the progeny will be genetically identical apart from having unique combinations of chromosome 1A. The two parents were chosen because they have contrasting alleles of Glu-1 and Gli-1 on this chromosome. The experiment was in two parts and the results are described below.

In the first part all the progeny were tested for protein composition by SDS-PAGE and for quality by the SDS-sedimentation test (Fig 2). Approximately half of the progeny contained the HMW glutenin subunit, called subunit 1 (Payne et al., 1979) which was inherited from Hope, and these on average gave much larger sedimentation volumes (indicating superior quality) than the remaining progeny which lacked a 1A-encoded HMW subunit as they contained the null allele at Glu-1 from CS. This result is consistent with previous findings (Payne et al., 1979) but is far more clear cut because most of the other proteins which influence quality are not varying. However a new finding was that the Gli-1 allele from CS was associated with good quality in the progeny compared to the corresponding allele from Hope (Fig. 2). These effects at the two loci were additive for the progeny which contained both the good quality alleles, i.e. subunit 1 and the CS Gli-1 allele, had an even higher mean sedimentation volume whereas those which contained the two alleles associated with poor quality, had the smallest volumes.

In the second part of the experiment, all the progeny at F_5 were screened by SDS-PAGE and 5 plants for each of the 4 possible permutations of storage-protein composition were grown in the glasshouse for bulking up seed. A subsequent field trial enabled sufficient white flour to be produced for bread-making and for rheological tests. Variation in loaf volume between protein genotypes reflected exactly the variation in SDS-sedimentation volume. However the lines with the two "good-quality" alleles produced loaves which had a much finer crumb structure than the other genotypes, which were all similar to each other in this respect. Further experiments with the Simon Research Extensometer showed that the line which produced superior loaves made doughs which had stronger elasticity.

The large differences in quality associated with the two alleles at Gli-1 are consistent with the ranking order of alleles by Sozinov & Poperelya (1980) and this has encouraged us to use other intervarietal chromosome substitution lines as parents in further work.

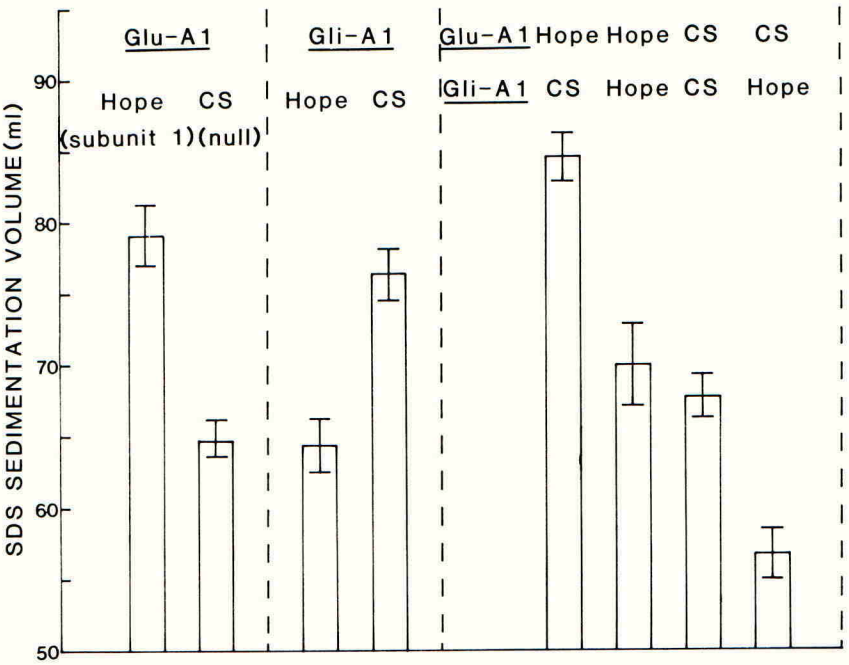

Fig.2. Effect of allelic variation at the Glu-1 and Gli-1 loci on sedimentation volume in progeny of the cross CS x CS (Hope 1A).

Relating HMW glutenin composition to the bread-making quality of varieties

The relationship between individual HMW subunits of glutenin and sedimentation volume is now sufficiently well known to estimate the contribution of this group of proteins to the bread-making qualities of varieties. To do this, individual HMW subunits were each assigned a score as shown in Table 1 which reflects their effect on sedimentation volume in segregating populations (Payne et al., 1984a). The overall HMW glutenin subunit quality score for a variety can be calculated by simply adding together the scores of the individual subunits. Thus Maris Huntsman, a hard milling feed wheat bred at the PBI, has the subunit composition of null, 6 +

TABLE 1

Quality scores assigned to individual or pairs of HMW glutenin subunits

Score	Chromosome		
	1A	1B	1D
4	-	-	5+10
3	1;2*	17+18;7+8	-
2	-	7+9	2+12;3+12
1	null	7;6+8	4+12

8, and 2 + 12, giving it an overall score of 1 + 1 + 2 = 4. By contrast, Monopol, a very good bread-quality wheat from Germany, has subunits 1, 7 + 9 and 5 + 10 and thus a score of 3 + 2 + 4 = 9. The minimum score value for a variety is normally 3 and the maximum, 10.

The majority of the winter wheats recommended for growing in West Germany were analysed by SDS-PAGE to determine their HMW glutenin subunit composition. From this their HMW quality scores were calculated and compared to the official German estimation of the bread-making quality of these varieties (Anon, 1983) which ranged from A9 (very good) to C1 (very bad). For the 56 varieties studied a highly significant correlation between the two estimates of quality of $r = 0.561$ was obtained. In a related study, the HMW glutenin subunit quality scores of 65 varieties grown in the UK were compared with the bread-quality scores determined by the Flour, Milling and Baking Research Association at Chorleywood (Stevens et al., 1984). Four categories of quality are given, from A (good) to D (very bad). The correlations between the two sets of variables, 0.621, was similar to that obtained for the German wheats, even though only five varieties were common to both National Lists and the frequencies of occurence of the various alleles for the two sets of varieties were different. Calculation of r^2 indicates that a little over one third of the variation in the bread-making quality of both German and British-grown varieties can be accounted for by the variation in composition of HMW glutenin subunits. Of the remaining half to two thirds of the bread-quality variation, some will be undoubtedly due to variation in the composition of other proteins of the endosperm and presumably also to components other than proteins. The major component affecting quality is likely to be variation in grain protein content however, caused by differences in yield potential between varieties (Pushman & Bingham, 1976).

Introduction of novel HMW glutenin subunits

Ancient wheat populations (i.e. landraces) of primitive farming from many parts of the world have been screened for novel HMW glutenin subunits by SDS-PAGE. Several new alleles have been found for all the Glu-1 loci and the genes for about six of them are being transferred separately into the genome of the Dutch spring wheat, Sicco, by repetitive backcrossing. Their effect on yield, disease resistance and bread-making quality will then be assessed in comparison with Sicco. The novel subunits include subunit 2.2, found in several old Japanese varieties (Payne et al., 1983a) and coded by the Glu-1 locus on chromosome 1D. Circumstantial evidence suggests that it arose from a mutation of subunit 2, causing its molecular weight to increase by up to one third. Another example of a 1D-encoded allele being transferred has an intermediate mobility between subunits 2.2 and 2. It occurs in many landraces of Iran but is infrequent in the landraces of neighbouring countries and has never been detected elsewhere. Finally, rare landraces from all countries have one of a pair of either 1B or 1D-encoded HMW subunits deleted. The corresponding genes are also being transferred to Sicco. Preliminary results show that a reduction in the percentage of HMW subunits to total protein in flour by the insertion of such 'silent' genes into genotypes, is very harmful to bread-making quality (Payne et al., 1983b), altnough it may be valuable for biscuit making. Assessment of the effects of these novel subunits will be made when the backcrossing programme has been completed.

Apart from hexaploid landraces of wheat there are extensive collections of wild relatives such as diploid Triticum and Aegilops species. Because of their great antiquity they exhibit much greater variation in storage proteins than does bread wheat (Law & Payne, 1983). Amongst the Aegilops species alone there are numerous HMW glutenin subunits not found in bread wheat. Unfortunately their genes cannot be incorporated into the genome of wheat by standard crossing procedures because of the presence of the "pairing homoeologous" gene (Ph) on chromosome 5B of wheat which prevents homoeologous chromosomes from pairing and hence recombining (Riley & Chapman, 1958). However by using lines which lack chromosome 5B, the genes for two HMW subunits from Ae. umbellulata are being transferred into the genome of Chinese Spring wheat. Ae. umbellulata contains the U genome as opposed to the ABD genomes of bread wheat and its Glu-1 locus occurs on chromosome 1U, which is homoeologous to chromosomes 1A, 1B and 1D. An important advantage of using this diploid species is that a set of CS - Ae. umbellulata chromosome substitution lines have been developed. In each of these lines, a single chromosome pair from Ae. umbellulata has been transferred to the genome of CS with the concomitant loss of a homoeologous chromosome pair. In preliminary studies it was shown that the CS(1A)1U substitution line (i.e. chromosome 1U substituted for 1A) had a superior bread-making quality, in terms of SDS-sedimentation volume, to euploid CS, whereas CS(1B)1U was similar and CS(1D)1U greatly inferior (Law et al., 1984). It was therefore decided to try and transfer the Glu-1 locus from chromosome 1U, onto chromosomes 1A and 1B in different lines.

The first hurdle to overcome is to induce pairing and hence recombination between chromosomes 1U and 1A and between 1U and 1B. To achieve this the CS(1A)1U and CS(1B)1U substitution lines were first crossed with CS nullisomic 5B tetrasomic 5D, a line completely lacking 5B chromosomes and hence the Ph locus. The next generation plants, all containing one chromosome each of chromosomes 5B, 1U and either 1A or 1B per diploid nucleus, were backcrossed with CS nulli 5B tetra 5D. The resulting grains were analysed by electrophoresis of half grains to determine which contained chromosome 1U. The embryo-ends of the grains containing 1U were grown and the plants examined cytologically at meiosis. Plants lacking chromosome 5B were recognised by the presence of multivalents, an indication that extensive homoeologous pairing was taking place. These were then crossed with Chinese Spring nulli 5D tetra 5B to restore the chromosome balance of these two group 5 chromosomes to normality and to genetically fix genes introduced from chromosome 1U by restoring the Ph gene. Half grains were again analysed by SDS-PAGE and used this time to select individuals containing the HMW glutenin subunits of Ae. umbellulata but missing the alien gliadins coded on the opposite arm of chromosome 1U. Plants obtained from these selections are currently being back-crossed to euploid Chinese Spring, and, with the aid of genetically linked markers, lines will be selected that have the desired HMW subunits but associated with as few other genes from Ae. umbellulata as possible (Faridi, Miller, Payne & Law, unpublished work).

DISCUSSION

Bread-making is a very complex process involving many flour components, including starch, lipid and protein, as well as added ingredients such as salt and sometimes oxidisers and emulsifiers. The quality of bread made from the flour of a given variety is however liable to be limited by a

single component or a limited number of components in the flour. In the UK and many other countries, insufficient elasticity of the dough is generally recognised as the major limiting factor which determines bread-making quality. The elasticity of flour doughs is caused by glutenin. Thus, the major, short-term goal of our research programme has been to determine which glutenin subunits that occur in commercial varieties inpart to glutenin long chain length and high elasticity. Many of them have now been identified, at least with respect to HMW glutenin subunits, and wheat breeders at the PBI are bringing together the best of the "good-quality" subunits coded at each of the three Glu-1 loci by incorporating single-seed descent procedures and electrophoresis of half grains into traditional breeding programmes.

At present it is not possible to predict whether glutenin elasticity will still be the limiting factor in making bread from UK-grown winter wheats when optimisation of HMW subunits present in modern cultivars has been achieved. In case it is not, research is continuing in two separate directions. The first is to transfer genes for novel HMW glutenin subunits, from hexaploid landraces in the short term and from wild diploid species in the long term, into commercial wheats. The hope is that one or more of them will have a biochemical structure which imparts strong elasticity to glutenin. The second direction is to relate allelic variation at the Gli-1 loci, which include genes coding for LMW glutenin subunits, to variation in elasticity. This will be achieved partly be analysing random lines and partly by exploiting the published work of Sozinov & Poperelya (1980).

Very recently a spring wheat, Solitaire, was developed at the PBI which is high in elasticity and is thus strong mixing. This is more easy to achieve in spring than winter wheats because the former are higher in grain protein content and there is a direct relationship between protein content and dough strength. Solitaire has a mixing requirement that is much closer to Canadian wheats than is Avalon, a bread-quality winter wheat that is currently widely grown in British agriculture. Its flour does not bake into such a good loaf as Avalon however, because it does not appear to have the extensibility to match its high strength. This variety is thus probably unique amongst modern, British wheats in not having glutenin strength as its limiting factor for bread-making. This variety will be exploited by crossing it with other genotypes to determine the effect of allelic variation at the Gli-1 and Gli-2 loci on extensibility. The long-term goal is to obtain varieties which produce highly elastic, highly extensible doughs that will be much more similar to Canadian wheats than those currently available, and which will greatly reduce the need to import North American wheat for British bread grists.

Future impact of molecular biology

The molecular biology of wheat endosperm storage proteins is being studied extensively by several research groups on both sides of the Atlantic. Clones of tissue-specific genes active in the developing endosperm have been prepared for several varieties. From them, storage-protein genes have been identified and partial or complete sequences of most of the storage-protein groups have been published (see Shewry & Miflin, 1985). The complete nucleotide sequences of two HMW glutenin subunit genes have also been described, one coding for the chromosome 1D-encoded subunit 12 (Thompson et al., 1985) and the other, derived from chromosome 1A, which is apparently not transcribed or translated (Forde et

al., 1985).

Nucleotide sequencing is revealing new insights into the structure of HMW subunits of glutenin. Each protein molecule contains three domains. Two are small and include the C- and N-termini. They contain virtually all the hydrophilic amino-acid residues in the molecule, including the cysteine residues, at least some of which are responsible for glutenin formation by the creation of inter-subunit disulphide bonds. The remaining domain makes up the majority of the molecule; it is extremely rich in glutamine, proline and glycine residues and effectively consists of repeated sequences. Computer predictions and biophysical measurements suggest that its secondary structure is primarily in the form of β-turns, like the animal protein elastin, and it is probable that this domain imparts much of glutenin's elasticity (Tatham et al., 1985). Variation in the length of the central domain of HMW glutenin subunits also appears to be responsible for the numerous allelic variants detected by SDS-PAGE (Harberd et al., 1986).

More HMW glutenin subunit genes are currently being sequenced and a further, judicious, selection of genes for sequencing should enable the differences observed between glutenin subunits and bread-making quality to be understood in biochemical terms. Although all this research may be regarded as retrospective to the genetic findings on quality differences, it may be possible in future for the molecular biologist to predict which of the many HMW glutenin subunits in wild species related to wheat are most likely to confer strong elasticity to glutenin. The geneticist can then transfer the genes for these subunits into commercially grown bread wheats by the techniques currently being devised to introduce Ae. umbellulata genes.

The major benefits from molecular biology however, as discussed previously (Flavell et al., 1984), are only likely to come once transformation of wheat becomes a reality. Genetic experiments have shown that as well as quality differences between HMW subunits there are also major differences caused by the relative quantities of these subunits. Thus subunits 1 and 2* are both superior to the null allele of Glu-1 on chromosome 1A (Payne et al., 1984a) and near-isogenic lines with deleted HMW glutenin subunits have a greatly inferior bread making quality to the recipient parent (Lawrence and Shepherd, quoted by Payne et al., 1983b). Perhaps one of the first transformation experiments of applied significance will therefore be the insertion of HMW subunit genes already present in the wheat genome. Problems associated with the derepression of introduced, alien genes at the correct time and in the appropriate tissue, should not arise. Hopefully this approach of increasing gene dosage will increase the proportion of nitrogen in the grain which in the form of HMW glutenin subunits, thereby increasing the elastic properties of the flour.

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MOLECULAR MARKERS OF SELF-INCOMPATIBILITY IN *BRASSICA*

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ABSTRACT

Molecular cloning of self-incompatibility sequences in *Brassica* provides breeders with markers for the identification of S alleles in vegetative tissue. The feasibility of screening at the seedling stage for S-related restriction fragment polymorphisms is discussed. The use of S-locus specific glycoproteins and DNA polymorphisms should enhance our ability to exploit self-incompatibility systems towards crop improvement.

INTRODUCTION

The self-incompatibility locus, or S-locus, controls a very crucial step in the life cycle of many flowering plant species, namely the interaction between the haploid male gametophyte and the diploid somatic tissue of the pistil. By its action, this locus has far-reaching effects on the population structure and evolution of angiosperm species. At the practical level, self-incompatibility has been used extensively in breeding programs of some crop plants, Crucifers in particular. For all its importance however, the phenomenon of self-incompatibility itself and its control by the S-locus are poorly understood. An elucidation of the molecular basis of incompatibility specificity and of the ensuing pollen rejection is of both scientific and practical interest. Basic questions concerning the origin of the extensive S-locus polymorphisms, the evolutionary relationships of various incompatibility systems, the expression of S-loci, and the cell-to-cell recognition phenomenon need to be addressed, and should translate into relevant practical applications in a wide range of crop plants. Until recently, the absence of well defined molecular markers of self-incompatibility has severely hampered scientific advances and limited the potential practical exploitation of this system. It is the aim of this paper to describe molecular parameters of the self-incompatibility system of *Brassica oleracea* and to present data supporting some potential applications stemming from their use.

CLASSICAL METHODS OF S-ALLELE IDENTIFICATION

Compatibility/incompatibility reactions are traditionally assayed on pollinated flowers by one of two methods: the number of seed set is counted following maturation, or pollen-tube growth is monitored by fluorescence microscopy following staining with decolorized aniline blue (Linskens & Esser 1957; Martin 1959). Of the two, the latter method is the more rapid. New S alleles are identified by assaying self-incompatibility response in crosses to a set of defined tester lines. In breeding programs, phenotypes are determined in F_1 plants in backcrosses to the parental homozygotes, and in the F_2 generation in a series of diallel crosses among F_2 plants. Although quite effective in many cases, these pollination methods have a number of drawbacks. They are quite tedious, time-consuming, and the outcome can be affected by both environmental factors and by genetic background. In a sporophytic self-incompatibility system such as the *Brassica* system, their interpretation in addition is complicated by such allelic interactions as dominance-recessiveness,

competition leading to mutual weakening, and different pollen-specific and stigma-specific interactions (Thompson & Taylor 1966; Ockendon 1982). All of the above combine in making the crucial identification of homozygotes difficult.

PROTEIN MARKERS OF SELF-INCOMPATIBILITY

The analysis of self-incompatibility in Brassica was greatly aided by the identification in stigma extracts, first of S-specific antigens (Nasrallah & Wallace 1967), and later of S-locus specific glycoproteins (SLSG) (Nasrallah et al 1972; Hinata & Nishio 1978; Roberts et al 1979; Nasrallah & Nasrallah 1984). For almost half of the fifty or so known S-alleles, SLSGs have been detected on isoelectric focusing (IEF) gels, either as single bands or as a complex of isoforms (Fig. 1).

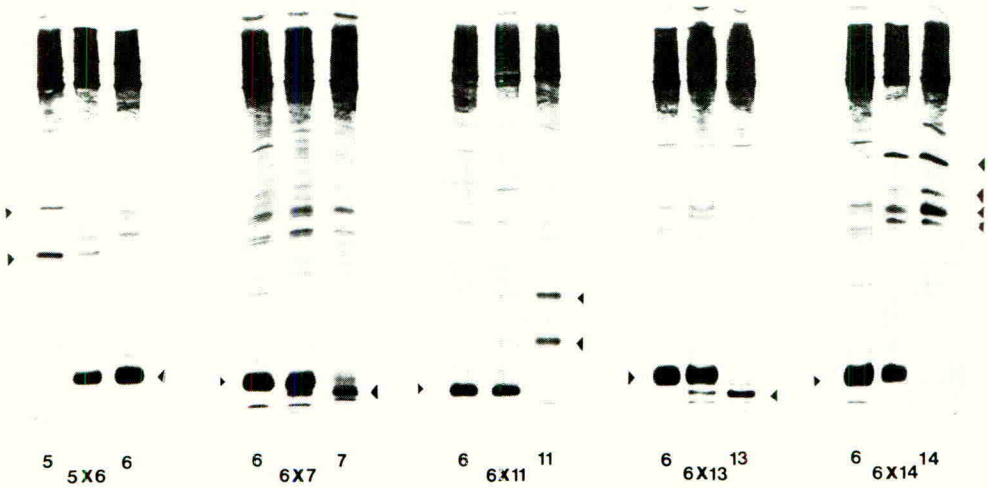

Fig. 1. Isoelectric focusing separation of stigma extracts from a number of allele homozygotes and the corresponding heterozygotes. Numbers refer to S allele designation. Arrows indicate the allele-specific SLSG bands.

Several lines of evidence suggest that these glycoproteins play an important role in self-incompatibility. Firstly, the mobilities of these molecules, particularly on IEF gels, vary in stigma extracts derived from Brassica strains with different S alleles. Significantly, SLSG

polymorphism is characteristic of a particular S-allele irrespective of the genetic background into which it is placed. Secondly, the time at which these proteins accumulate in the developing stigma correlates well with the onset of the incompatibility reaction in the stigma. Thirdly, these molecules are synthesized in the papillar cells of the stigma surface, the site of the initial contact between the pollen and stigma (Nasrallah et al 1985a). Fourthly, self-compatibility resulting from the action of unlinked modifier genes is corelated with lower levels of these molecules (Nasrallah 1974). Finally, the inheritance of these SLSGs correlates perfectly with the segregation of S alleles in genetic crosses. Comparisons of the IEF patterns of stigma homogenates from the homozygous parental genotypes and the corresponding F_1 heterozygote have revealed the contribution of both alleles to the heterozygous pattern in all allelic combinations analyzed to date (Fig. 1). Segregation of the IEF patterns was followed in individual plants of the F_2 generation for a number of allelic combinations. In crosses involving the S_6 and S_{14} alleles for example, only IEF patterns typical of the parental and F_1 genotypes were obtained, and the S_{14} isoforms segregated together as a unit. In one experiment, out of a total of 48 F_2 plants examined, 10 exhibited the S_6 parental pattern (referred to as group I), 24 exhibited the hybrid S_6S_{14} pattern (referred to as group II), and 14 the parental S_{14} pattern (referred to as group III) (see Table 1). The incompatibility relationships of these F_2 plants were determined by performing diallel crosses on four progenies each consisting of 12 plants. For each progeny, the plants were crossed in all of the 144 possible combinations and found to fall into three groups; the members within each group behaved identically on the basis of their pollen reaction as viewed by fluorescence microscopy. Pollen from group I plants was incompatible with groups I and II flowers but compatible with group III flowers; group II pollen was incompatible with groups I, II and III flowers; and group III pollen was compatible with groups I and II flowers but not with group III flowers. The identical responses of group I and group II plants when used as female parents points to the dominance of the S_6 allele in the stigma. In the pollen however, S_6 and S_{14} are codominant. The genotype assignment from IEF patterns is in agreement with phenotype assignment from fluorescence microscopy.

TABLE 1

Segregation of incompatibility phenotypes deduced from diallel analysis of twelve plants in each of four F_2 progenies.

F_2 progeny	Incompatibility Group		
1	I	II	III
1	3	4	5
2	3	5	4
3	2	8	2
4	2	7	3
Totals	10	24	14

In view of this strict correspondence and of the fact that codominant expression of the two alleles in the stigma at the IEF level seems to be the rule, the screening of a segregating population by isoelectric focusing should prove of general usefulness in assigning S-genotypes. It is simple and circumvents the time-consuming task of performing diallel pollinations. Furthermore, in the occasional cases in which diallel pollinations cannot directly distinguish between dominant homozygotes and heterozygotes, as a result of poor pollen viability for example, IEF should allow rapid and unambiguous genotype assignment. Likewise, the study of the frequency and distribution of self-incompatibility alleles in wild populations will no doubt be aided considerably by the use of electrophoretic markers.

DNA MARKERS

Two characteristics of SLSGs have provided us with a strategy for isolating nucleic acid sequences encoding these molecules (Nasrallah et al 1985b): 1) they cannot be detected in vegetative tissues, but accumulate to relatively high levels during stigma development; 2) the rate of their synthesis increases during stigma development and reaches maximal levels at one day prior to anthesis, coincidentally with the acquisition of the incompatibility response (Nasrallah et al 1985a).

Stigmas were isolated from buds at one day prior to anthesis and poly A^+ RNA purified. A cDNA library was then prepared from this RNA in pBR322 by standard procedures. Stigma-specific clones were identified from approximately 2,000 recombinant clones by differential hybridization to cDNA probes prepared from stigmas and seedling mRNA. One such cDNA clone was used in the analysis described below. The regulation of the gene corresponding to the cloned sequences was analyzed on Northern blots of RNA isolated from leaves, seedlings, and stigmas at various stages of development. As expected for sequences encoding SLSG, no homologous message was detected in leaf and seedling tissue, while the synthesis of the homologous transcripts in stigmas was shown to closely parallel the synthesis of SLSG. The most direct proof that the cloned cDNA sequences did indeed encode SLSG came from the demonstration that, when inserted into expression vectors, they could direct the synthesis of fusion proteins that reacted with antibodies raised against authentic stigma SLSG.

We have used the cloned SLSG sequences to analyze homologous sequences in the Brassica genome. Polymorphisms in restriction fragments sufficient to account for the extreme natural variability of the S locus were uncovered. Of twenty different S-allele homozygotes analyzed, each exhibited unique restriction fragment polymorphisms. More importantly, these polymorphisms have been shown, in a total of over 100 F_2 plants analyzed, to segregate with SLSG polymorphism, and with self-incompatibility phenotype as determined by pollination analysis and microscopic monitoring of pollen-tube behavior (see Table 1). In all cases, the S locus genotype can be correctly inferred from the pattern of the homologous genomic restriction fragments. Representative patterns are shown in Figure 2 for the S_6 and S_{13} homozygous lines and for the hybrid S_6S_{13} heterozygous pattern. It may be seen that the band denoted by an arrow is found in S_6S_6 homozygous individuals, and that this band is replaced by that marked with an arrow in the $S_{13}S_{13}$ homozygotes. S_6S_{13} heterozygotes show signals which are essentially the sum of the two patterns seen in the respective homozygotes.

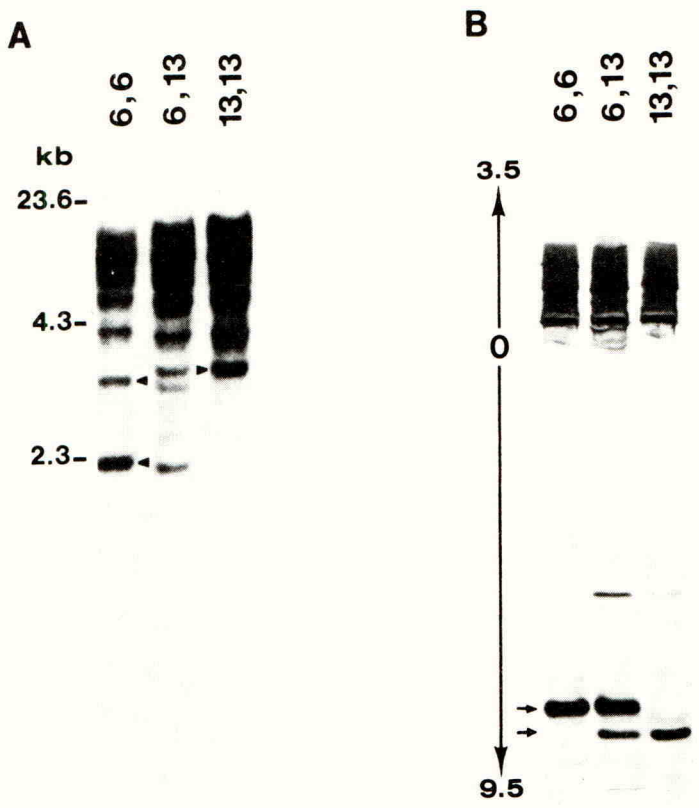

Fig. 2. Segregation of S-related restriction site polymorphisms (A) and of the corresponding SLSG polymorphisms (B) in crosses involving S_6 and S_{13}. 6,6: S_6 homozygote; 13,13: S_{13} homozygote; 6,13: S_6S_{13} heterozygote. Arrows indicate the allele-specific Bam H_1-Pst I restriction fragments in (A) and the Coomassie Blue-stained allele-specific isoelectric focusing bands in (B).

PROSPECTS FOR CROP IMPROVEMENT

Molecular markers are now available for the self-incompatibility locus of Brassica. Our analysis of twenty-five of the known S-allele homozygotes demonstrates that each exhibits unique SLSG and S-related DNA polymorphisms. It should therefore be possible to establish a catalogue in which each allele is defined by its SLSG pattern (Hinata & Nishio 1980) and by its S-related DNA polymorphism. New S alleles and S genotypes may then be identified in reference to such a catalogue, and thus expedite the selection of desirable plants for further analysis.

Both protein and DNA markers provide for convenient and rapid analysis of self-incompatibility phenotypes. SLSG patterns can be identified only at the flowering stage, and the time required for screening is therefore dependent on plant generation time. S-related DNA patterns, on the other

hand, can be deduced from genomic DNA samples prepared rapidly from a small amount of vegetative tissue. Screening can therefore be accomplished very early in seedling development at the cotyledon stage. It must be cautioned however that although genetic background has clearly no bearing on SLSG patterns, its effect on S-related DNA patterns has not been tested. It is even likely that at least some of the restriction fragments wil be affected by the nature of contiguous DNA.

In any case, the availability of cloned S-locus sequences should allow the introduction of stable S alleles by transformation into elite lines of Brassica oleracea and related species. Here again, the molecular markers of self-incompatibility will be useful in the identification of transformants modified in their incompatibility specificity.

The practical applications stemming from an understanding of self-incompatibility systems in other species are many. Examples are the introduction of self-incompatibility sequences into important crop plants such as rapeseed, potato and tomato for hybrid seed production, or transformation with manipulated sequences to prevent the expression of self-incompatibility in stone fruit plants for example to improve fruit set.

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