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Biotechnology of Cereals

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Introduction: Cereal production and utilization

Cereals are the most important crops in the world in terms of area under cultivation, yield and contribution to the diet of man and his livestock. In 1991, over 700 million hectares were cultivated, with total yields of about 1900 million tonnes. Only three species account for about 80% of the total: wheat (550 million tonnes), rice (520 million tonnes) and maize (480 million tonnes) (FAO, 1988). Other widely grown cereals are (in order of decreasing production) barley, sorghum, oats, millets (various species, the most important of which is pearl millet) and rye. The great success of cereals is due to a number of factors, including their high yields, ease of harvest, technological properties and the availability of a range of species and cultivars to suit almost all climatic conditions. Their tolerance of a wide range of water status is illustrated by Tef, which is adapted to the semi-arid conditions of Ethiopia, and deep water rice, which will grow in water several metres deep. Similarly, their tolerance of temperature ranges from rye, which is particularly suited to the continental climate of Northern Europe and Canada, to a range of minor millets and rice, which flourish in the tropics.

The major harvested organ of cereals is, of course, the grain, although the leaves and stems are harvested for silage and forage in some countries. The seed is, in botanical terms, a caryopsis, in which the seed wall (testa) becomes fused with the fruit wall (pericarp). It consists of two organs, the triploid endosperm and diploid embryo. The endosperm is the major storage organ,

Abbreviations: ACCase, acetyl CoA carboxylase; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CaMV, cauliflower mosaic virus; CAT, chloramphenicol acetyltransferase; cDNA, complementary DNA; CMS, cytoplasmic male sterility; es, embryo-derived suspension; ftp, fertile transgenic plants; HMG, 3 hydroxy 3 methylglutaryl; IEF, isoelectric focusing; is, inflorescence-derived suspension; ISL, inflorescence sheath leaf; LUX, luciferase; mc, mesophyll cells; M_r, relative molecular mass; mRNA, messenger RNA; ms, microspore-derived suspension; PAT, phosphinothricin phosphotransferase; PEG, polyethylene glycol; PVC, polyvinyl chloride; RFLP, restriction fragment length polymorphism; sc, stably transformed callus; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; T-DNA, transferred DNA; T_R-DNA, right border of the DNA; tc, transient expression; tp, transgenic plants.

Biotechnology and Genetic Engineering Reviews — Vol. 11, December 1993
0264-8725/93/11/79-145 $20.00 + $0.00

and differentiates to form two tissues – a central starchy endosperm surrounded by an aleurone layer. The starchy endosperm is a dead tissue in the mature grain, while the aleurone layer (which consists of one layer of cells in wheat but up to three in barley) remains live and acts as a source of hydrolytic enzymes during germination. The embryo consists of the embryonic axis (including the plumule and radicle), which will grow to form the seedling, and a single storage cotyledon (scutellum). In the case of wheat, the starchy endosperm forms the white flour, and the aleurone and embryo the bran and germ, respectively. A longitudinal section of a typical cereal grain (wheat) is shown in *Figure 1*.

Plant seeds contain three major groups of storage compounds, the proportions of which vary between and within different botanical groups. In the cereals, the major storage compound is always starch, which usually accounts for 60–70% of the grain dry weight. The other two groups, protein and lipids, are relatively minor components, accounting for about 8–12% and 2–8% of the dry weight, respectively (Bewley and Black, 1978). The variation in the content of lipid, from about 2% in wheat and barley to 8% in oats and 5–9% in maize, is of considerable interest, as it indicates the potential for manipulating the balance of carbon partitioning between starch and lipid. Maize is already grown as an oil crop, and increasing the proportions of oil in other cereals could lead to a change in the balance of utilization.

Although proteins only account for a relatively small proportion of the cereal grain, they have been of overwhelming importance in determining the pattern of utilization. The total amount of protein present in the cereal grains harvested by humankind far exceeds that in the more protein-rich (up to 40% dry weight) legume seeds, and consequently they form the major source of dietary protein for humans and their livestock. In addition, they have a major impact on the properties of the grain for utilization in the food industry, and in particular in the properties of wheat for making bread and baked goods, pasta and noodles, and other food products.

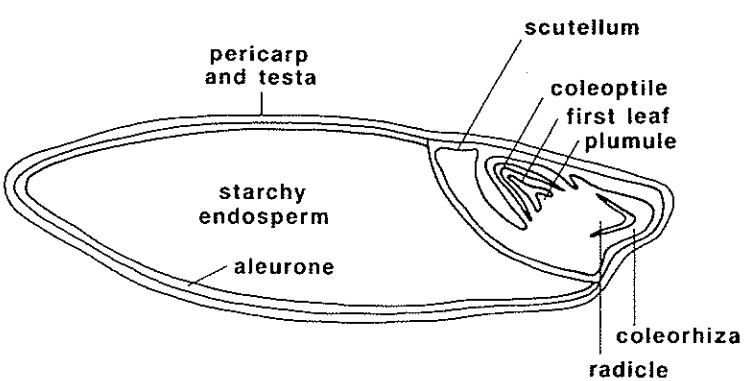


Figure 1. Longitudinal section of a typical cereal grain (wheat).

Targets for manipulation

Cereals have been grown by humans for about 9000 years (Harlan, 1978) and have undoubtedly been selected for improved characteristics (either consciously or unconsciously) over most of this period. In addition, the application of the scientific principles of plant breeding over the last century has led to tremendous improvements in yield, agronomic performance and aspects of quality. However, improvement using this classical approach is limited by the available range of genetic variation in the crop itself or in related species that can be readily crossed. Biotechnology offers the opportunity to introduce variation beyond that normally available, as well as providing tools to maximize the efficiency of classical plant breeding. However, before looking at aspects of cereal biotechnology in detail, we will first briefly discuss the main targets for manipulation.

GRAIN YIELD

Increases in yield have always been a major aim of plant breeders. Since the mature grain consists mainly of starch, attention has been focused on the pathway of starch synthesis and its control (see Preiss, 1988). However, we do not fully understand the role of source–sink relations in determining cereal grain yield (Wardlaw, 1990), and biotechnology offers an opportunity to explore this in detail (see, for example, the elegant studies of Willmitzer and co-workers on source–sink relations in tobacco: Stitt, von Schaewen and Willmitzer, 1990; Sonnewald *et al.*, 1991). A further factor which may also affect yield is endosperm cell number. Chojecki, Bayliss and Gale (1986) demonstrated that the yield of wheat was correlated with endosperm DNA content and cell number, confirming previously reported correlations between kernel size and endosperm cell number in wheat (Brochlehurst, 1977; Radley, 1978; Singh and Jenner, 1982) and barley (Schacherer and Beringer, 1984). It is therefore of considerable importance to understand the control of cell division in the developing cereal endosperm, and in particular the transition between the phases of cell division and cell expansion (the latter phase being associated with storage compound synthesis).

RESISTANCE TO BIOTIC STRESSES

Resistance to pests and pathogens is a major target for plant breeders, with attention focused on a range of fungal, bacterial and viral diseases, and on insect and nematode pests. This is important both for intensive arable agriculture in the developed world and for lower-input farming systems in less developed countries. In the former, the growth of cereal monocultures and intensive use of chemical control methods facilitates the development and spread of new strains and species of pathogen, leading to a constant battle between the crop and plant breeder on the one hand and the pest or pathogen on the other. The exploitation of natural resistance mechanisms is of equal importance in lower-input systems where economic and other factors pre-

clude the extensive use of agrochemicals. In addition, one should not ignore the environmental benefits of decreased pesticide use resulting from the exploitation of natural and engineered defence mechanisms, especially in high-input systems.

We still understand very little about the natural mechanisms of plant resistance to pests and pathogens, and in particular about gene-for-gene resistance to fungal pathogens. Nevertheless, strategies have been used with varying degrees of success to confer resistance in transgenic plants. These include the use of viral coat protein genes (Beachy, 1988) or satelite DNA (Harrison, Mayo and Baulcombe, 1987; Gerlach, Llewellyn and Haselhoff, 1987) to confer resistance to viruses, and of bacterial genes encoding insecticidal proteins (*Bacillus thuringiensis* toxins) to confer insect resistance (Koziel *et al.*, 1993).

In addition, a number of plant proteins, which may be expressed constitutively or induced on infection or feeding, have been demonstrated to confer resistance to pests or pathogens either *in vitro* or in transgenic plants. These include inhibitors of hydrolytic enzymes (α-amylases and proteases), β-glucanases, endochitinases, protein synthesis inhibitors, lectins, thionins and membrane-permeabilizing proteins. The first such protein to be used in transgenic plants, and the most well known, is the cowpea trypsin inhibitor, which was shown to confer resistance to tobacco budworm in transgenic tobacco (Hilder *et al.*, 1987). Other proteins may confer resistance to fungal or bacterial pathogens, or even broad-spectrum resistance to a range of such organisms. Some of these proteins are derived from cereals and are therefore attractive targets for eventual incorporation into transgenic cereal plants.

An alternative approach to confering resistance in transgenic plants is to transfer genes for the production of defence-related chemicals. Because such chemicals are synthesized by multigene pathways, this is a more formidable challenge than the transfer of single genes for disease resistance proteins as discussed above. However, Hain *et al.* (1993) have recently reported enhanced disease resistance of tobacco to the fungal pathogen *Botrytis cinerea* by inserting two stilbene synthase genes which resulted in the synthesis of phytoalexins.

These and other strategies for enhancing crop resistance to microbial pathogens are discussed in a recent review article by Lamb *et al.* (1992). However, there is no reason to assume that such engineered sources of pest and pathogen control will prove to be any more durable than many chemical control methods used at present, as they will almost certainly lead to the evolution of resistant forms of pests and pathogens (May, 1993).

RESISTANCE TO ABIOTIC STRESSES

In global terms, the most important abiotic stress is mineral toxicity, although this is not always appreciated in developed countries. It has been estimated that nearly a quarter of the world's soils are affected by mineral stress (Clark, 1982), the major problems being salinity, aluminium, manganese and boron. Heavy metal toxicity also occurs under some circumstances, notably copper

(see Manyowa and Miller, 1991). The most successful approach to this problem in cereals is to exploit natural variation in tolerance, either in the crop species itself or in wild relatives (see Manyowa and Miller, 1991). An interesting illustration of the potential of genetic engineering to improve stress tolerance is the recent report by Tarczynski, Jensen and Bohnert (1993) that the introduction of bacterial mannitol-1-phosphate dehydrogenase into tobacco plants results in the accumulation of mannitol and increased tolerance of high salinity.

Tolerance to high and low temperatures (the latter including frost damage) and to waterlogging are also of some importance, and have been studied in cereals. High temperature effects in cereals include the appearance of 'heat shock' proteins as in other systems. In addition, high temperatures during grain filling in wheat result in decreased quality for bread-making, which may result in part from specific effects on gluten protein gene expression (Blumenthal *et al.*, 1990).

NUTRIENT USE EFFICIENCY

The current concern about the environmental consequences of intensive agricultural practices includes recognition of the need to reduce the use of fertilizers and the levels of nitrate in groundwaters. This has stimulated interest in nitrogen use efficiency, and in the mechanisms of nitrate uptake, assimilation and utilization. Cloned genes for nitrate assimilation (nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase) are available from several species and are currently being used to explore the control of nitrogen metabolism in transgenic plants (see Vaucheret *et al.*, 1992). Similar studies could lead to improved nitrogen use efficiency in cereals.

GRAIN QUALITY AND COMPOSITION

There has been increased emphasis on grain composition and quality over the past decade, partly because the increased yields of cereals in the developed world, and the spread of wheat cultivation within the European Community, have led to new opportunities for utilization. Recent studies have indicated that it should be possible to manipulate many aspects of grain composition and quality, some of which are discussed in detail later.

HERBICIDE RESISTANCE

Engineered resistance to herbicides is perhaps the most widely discussed and emotive issue in plant genetic engineering, because it is seen as establishing dependence on the intensive use of herbicides. In fact, if managed properly, it should ultimately reduce the herbicide dosage by allowing more efficient use. Resistance to several major classes of herbicide has been achieved by two basic strategies: by manipulating the amount or sensitivity of the target enzyme, or by introducing genes for detoxification. Furthermore, the latter

approach has been successfully used with genes from bacterial as well as plant sources. This work has, of course, been facilitated by detailed studies of the target enzymes and of the detoxification pathways. It has been discussed in a number of recent articles (see, for example, Gasser and Fraley, 1989; Hartnett *et al.*, 1991; Botterman *et al.*, 1991; O'Keefe, Lenstra and Omer, 1992), and will not be elaborated here.

So far, the only herbicide-resistant cereal to be marketed is maize, and this has been produced by mutant selection rather than genetic engineering. However, the development of reliable methods of cereal transformation will undoubtedly lead to rapid exploitation of the strategies that have been successfully developed for other plants.

Development and application of methods

Three strategies have been adopted to improve cereals. The first is plant breeding, which involves crossing contrasting genotypes to generate new combinations of genes, followed by selection of the most suitable of these. In addition, exotic genotypes or closely related (i.e. crossable) wild species may be used as sources of specific genes (for example, for resistance to pests or abiotic stress). This approach does not involve biotechnology, and will not be discussed further.

The second strategy is still based on classical plant breeding, but elements of biotechnology (tissue culture, regeneration) are used to make the process more efficient and to allow genes to be incorporated from wild relatives that cannot readily be crossed. The third strategy is genetic engineering: the insertion of specific genes to improve aspects of resistance, agronomic performance, quality or yield.

In the following sections, we will discuss progress in the latter two strategies, highlighting achievements and identifying problems and limitations.

TISSUE AND CELL CULTURE

Plant tissue culture is based on two principles: first, that plant cells may be cultured axenically *in vitro* and, second, that they are inherently totipotent, so that given the correct conditions they are able to regenerate to form new plants. In herbaceous dicot species, regenerable tissue cultures can be established from a variety of plant parts, including relatively mature organs. However, in most monocots the process is more difficult, as only immature tissues which are to some degree still meristematic can be induced to proliferate *in vitro* (Vasil, 1987). This poor capacity for dedifferentiation in culture is correlated with a wound response in which damaged areas are 'sealed-off' rather than being 'repaired' by *de novo* cell division forming a wound callus (Kahl, 1982). Among the monocots, members of the grass family (*Poaceae*) are especially determinate, their cells becoming committed to a particular developmental pathway soon after emergence from the meristem (see Morrish, Vasil and Vasil, 1987). These characteristics have

necessitated the development of specific methods for the culture and manipulation of cereals *in vitro* (Vasil, 1988; Lazzeri, Kollmorgen and Loerz, 1990).

Meristem culture

As with other plant species, it is possible to culture isolated shoot apices of cereals and to propagate them indefinitely (Dalton and Dale, 1985). In most cereals there is limited potential for vegetative multiplication, as they are efficiently propagated by seed, but there may be occasions where it is of interest to clone individual genotypes *in vitro* (e.g. the multiplication of transformants before transfer to soil, or of lines with reduced fertility). Additionally, *in vitro* tiller cultures may be maintained at low temperatures for germplasm storage (Dale and Webb, 1985).

Callus culture

Cereal callus cultures are initiated by plating explanted tissue on medium containing a synthetic auxin, usually 2,4-D. Cell proliferation is induced and a heterogeneous callus is typically produced, containing both embryogenic tissue competent for regeneration and non-embryogenic tissue. The former is characteristically slow-growing, compact and distinctly organized, whereas the latter tends to be faster-growing, more friable and disorganized. Regeneration may occur spontaneously from embryogenic tissues, but is usually stimulated by a reduction in the auxin content of the medium, and possibly by the addition of cytokinin.

The choice of explant is the major factor determining culture success. The most responsive tissues are immature embryos/scutella (before the onset of starch deposition), immature inflorescences, apical meristems and seedling leaf base meristems. In contrast with most dicots, cereal leaf tissues are generally determinate and non-responsive *in vitro*. One exception is inflorescence sheath leaves (ISLs), which appear to retain more developmental plasticity (Barcelo *et al.*, 1991). ISL cultures also demonstrate the rule that in cereal tissue cultures, as in other systems, increasing levels of cellular competence are required for proliferation, root formation and embryogenesis/regeneration, with these different competencies being successively lost as tissues mature (Barcelo *et al.*, 1992).

Apart from explant type and ontogeny, several other factors are important in determining response in culture. A major element is donor plant genotype. There is great variation in the culturability of different genotypes within all cereal species (Duncan *et al.*, 1985; Luehrs and Loerz, 1988; Vasil, 1987). Culture response is clearly under genetic control and there has been some success in breeding for improved performance *in vitro* (Armstrong, Romero-Severson and Hodges, 1992 and references therein). As well as genotype, donor plant conditions (and thus the physiological status of the explant), culture medium composition and culture environment also influence response.

Regeneration from cereal tissue cultures occurs primarily by embryogen-

esis, with somatic embryos, similar in morphology to zygotic embryos, being formed from the surfaces of embryogenic callus. In some cases, however, regeneration appears to occur via organogenesis, with shoot apical meristems being formed superficially on callus tissues. As organogenesis is usually also associated with embryogenesis, the shoots observed may actually develop from the apical meristems of 'disorganized' somatic embryos.

Cereal cultures generally show the greatest capacity for plant regeneration during the first passage in culture, with this capacity declining over subsequent subcultures. This effect may to some extent be overcome by visual selection for embryogenic tissues at each subculture (Redway, Vasil and Vasil, 1990), but remains a limitation to the use of long-term cultures. Loss of regeneration competence is frequently correlated with increasing disorganization of cultured tissues and with the accumulation of cytological aberrations, but the underlying causes of the process are not fully understood (see pp. 107–108). Regenerable callus cultures can now be produced in all the important cereal species and the majority of genotypes can be cultured and regenerated, albeit with varying efficiencies.

The major uses for cereal callus cultures are as starting material for suspension and protoplast cultures and as regenerable target tissues for transformation. Callus cultures may also be used for the *in vitro* selection of mutants, and for the generation of somaclonal variants.

Suspension culture

Cell suspensions are produced by transferring callus tissue to liquid medium and shaking the culture to ensure aeration. Although the process is simple, primary embryogenic cereal callus does not adapt readily to liquid conditions and it is generally necessary to select for specific callus phenotypes which allow more efficient suspension initiation (Shillito *et al.*, 1989; Redway, Vasil and Vasil, 1990). In order to avoid long-term selection for embryo-derived callus suitable for suspension establishment, some workers have used microspore-derived callus as starting material (Datta, Datta and Potrykus, 1990; Jaehne, Lazzeri and Loerz, 1991). However, this approach requires an efficient anther or microspore culture system (see below). Early-stage cereal suspensions are typically slow-growing and heterogeneous, with large cell aggregates. They become finer and more homogeneous during the establishment phase and stable suspensions are usually composed of small aggregates (0.1–2.00 mm in diameter) of isodiametric, cytoplasm-rich cells.

Cereal suspensions do not produce somatic embryos in the liquid medium, but must be plated onto solid medium to test their regeneration capacity, where they first form embryogenic callus, which then develops somatic embryos. With their highly disorganized nature and fast growth rate (doubling times typically 4–7 days), suspension cultures tend to lose regeneration capacity relatively quickly (see below), and it is unusual for a suspension to remain regenerable for more than 1 year. This problem can to some extent be overcome by cryopreserving embryogenic lines and re-initiating them when the 'parent' line is no longer regenerable (Shillito *et al.*, 1989), but in most

cases it is still necessary to establish new cell lines regularly.

Embryogenic cell suspensions capable of plant regeneration have been produced in all the major cereals (*see Table 1*). The process is most efficient in japonica rice, and probably least efficient in wheat and barley. In addition, there is considerable genotypic variation within each species in the ease and reproducibility of suspension establishment.

Cell suspensions are attractive experimental tools, as they are fast-growing, easy to maintain, have an indefinite life-span and can provide large numbers of easily accessible, relatively homogeneous cells. Their major disadvantage, as discussed above, is that they have a relatively short 'window' of competence for plant regeneration. Today, the most important applications of cereal suspensions are as sources of protoplasts (see below) and as targets for transformation (see below). They may also be useful for *in vitro* selection purposes (see below) and in physiological, biochemical and molecular studies. However, because cell suspensions do not have the structural organization of the parent plant and are grown under highly artificial conditions, they may not always be good models for investigating plant processes. Nevertheless, where cell suspensions do retain important characteristics of the parent plant/tissue (e.g. normal or mutant storage protein profiles: Tewes *et al.*, 1991; Schaeffer, Sharpe and Dudley, 1992), they can prove valuable as it is possible to make highly controlled manipulations of nutritional and growth factors and to study their effects.

Protoplast culture

Protoplasts can be isolated from cereal cells using the same methods as those used for herbaceous dicot species, but it is much more difficult to induce these protoplasts to divide. Regeneration from cereal protoplasts was a major objective in plant cell culture for some 15 years and it was not until 1986 that the production of fertile cereal (rice) plants from protoplasts was reported

Table 1. Regenerable suspension cultures of cereals

Species	Common name	Reference[a]
Hordeum vulgare	Barley	Jaehne, Lazzeri and Loerz (1991) Funatsuki, Loerz and Lazzeri (1992)
Hordeum chilense × *Triticum durum*	Tritordeum	Barcelo *et al.* (in press)
Oryza sativa ssp. *indica*	Indica rice	Lee *et al.* (1989) Datta *et al.* (1990)
Oryza sativa ssp. *japonica*	Japonica rice	Fujimura, Sakurai and Akagi (1985) Kyozuka, Mayasami and Shimamoto (1987)
Pennisetum americanum	Pearl millet	Vasil and Vasil (1980)
Sorghum vulgare	Sorghum	Wei and Xu (1990)
× *Triticosecale*	Triticale	Stolarz and Loerz (1986)
Triticum aestivum	Bread wheat	Wang and Nguyen (1990) Redway, Vasil and Vasil (1990)
Zea mays	Maize	Prioli and Sondahl (1989) Morocz *et al.* (1990)

[a] Selected references, not necessarily first reports.

(Toriyama, Hinata and Sasaki, 1986). The fact that protoplasts isolated from differentiated cereal tissues very rarely divide reflects the extreme determinacy of cereal cells, as discussed above. Furthermore, it is difficult to obtain adequate yields of protoplasts from meristematic tissues and those protoplasts which are obtained are fusogenic and difficult to handle. The preferred source of dividing cereal protoplasts is cell suspensions or, less frequently, fast-growing friable callus. The most efficient protoplast isolations and the highest plating efficiencies are obtained from long-established suspensions which grow very fast and have friable cell aggregates. Such suspensions have, however, usually lost the ability to regenerate plants, and embryogenic suspensions are required for the isolation of competent protoplasts. The development of methods for the production of embryogenic suspensions of various cereal species has been followed by reports of the regeneration of plants from protoplasts derived from them (*see Table 2*). The efficiency of protoplast regeneration in a particular species is dependent on the ease of establishing suspensions, which means that at present the process is only widely reproducible in japonica rice, and relatively few laboratories worldwide have produced fertile plants from maize, wheat or barley protoplasts. As experience grows, however, it is clear that techniques are being improved and that reproducible systems will be developed for more species.

The use of techniques such as alginate embedding (Tricoli, Hein and Carnes, 1986) and the use of nurse cultures (Funatsuki, Loerz and Lazzeri,

Table 2. Regeneration from cereal protoplasts

Species	Common name	References[a]	Source[b]
Hordeum vulgare	Barley	Jaehne, Lazzeri and Loerz (1991)	ms
		Funatsuki, Loerz and Lazzeri (1992)	es
Hordeum chilense × *Triticum durum*	Tritordeum	Barcelo *et al.* (in press)	es
Oryza sativa ssp. *indica*	Indica rice	Lee (1989)	es
		Su, Rudert and Hodges (1992)	ms
Oryza sativa ssp. *japonica*	Japonica rice	Kyozuka, Mayasami and Shimamoto (1987)	es
		Wu and Zapata (1992)	pc
		Gupta and Pattanayak (1993)	mc
Pennisetum americanum	Pearl millet	Vasil and Vasil (1980)	is
Sorghum vulgare	Sorghum	Wei and Xu (1990)	is
Triticum aestivum	Bread wheat	Vasil, Redway and Vasil (1990)	es
		Ahmed and Sagi (1993)	es
Triticum durum	Durum wheat	Yang *et al.* (1989)	es
Zea mays	Maize	Shillito *et al.* (1989)	es
		Prioli and Sondahl (1989)	es

[a] Selected references, reporting fertile plant regeneration where achieved.
[b] ms, microspore-derived suspension; es, embryo-derived suspension; mc, mesophyll cells; is, inflorescence-derived suspension.

1992) can improve cereal protoplast culture efficiency significantly and some workers have re-tested the possibility of using these methods for culturing protoplasts isolated directly from cereal organs. It has been shown that it is possible to obtain macroscopic calli from mesophyll protoplasts of oat (Hahne, Fleck and Hahne, 1989) and of *Tritordeum* (Barcelo, pers. comm.), and recently plants have been regenerated from mesophyll protoplasts isolated from rice leaf base and sheath (Gupta and Pattanayak, 1993).

Cereal protoplasts are used quite extensively in physiological and biochemical studies, but their main importance in biotechnology is as tools for genetic manipulation. Before cell wall reformation occurs, they are easily fused, either by chemical or electrical stimuli, allowing a range of somatic cell genetic techniques, including hybridization, organelle transfer or partial genome transfer (see below). In addition, protoplasts may easily be induced to take up DNA from surrounding solutions, again in response to electrical or chemical stimuli. Protoplast transformation is discussed below.

HAPLOIDY

Among the different *in vitro* genetic manipulation techniques used in plants, haploid induction and embryo rescue have the most direct applications in breeding programmes. Haploid plants themselves are not of practical use, but doubled haploids, resulting from spontaneous or induced chromosome duplication, constitute a single step to homozygosity and have value in breeding and genetic research.

Doubled haploids allow breeders to produce completely homozygous genotypes from heterozygous parents in a single generation. The uses of double haploid production in breeding depend largely on whether a particular crop is in- or out-breeding. Varieties of inbreeding species, including small-grain cereals such as wheat, barley and rice, are usually single homozygous genotypes. In this case, doubled haploids could become new cultivars in a single step by 'fixing' the product of a cross without the need for backcrossing. Alternatively, the doubled haploid may be used in further crosses.

In out-breeding crops, such as maize, doubled haploid lines may be used as the parents for hybrid production instead of the true-breeding lines conventionally produced by several generations of inbreeding (for discussions of haploid breeding systems, see Snape, 1989; Petolino, 1992; Pickering and Devaux, 1992).

There are three major routes for the production of doubled haploid plants. The first is the use of genetic systems, such as the haploid initiator *hap* gene in barley (Hagberg and Hagberg, 1987), which produces haploids through disturbances in zygote development. These systems will not be discussed as they do not involve biotechnological techniques. The second route, which is used principally in barley but also functions in wheat, is the *bulbosum* system (Kasha and Kao, 1970), in which a cross between *Hordeum bulbosum* and a cultivar is made, followed by rescue of the embryo (see below) which becomes haploid due to the elimination of the *H. bulbosum* chromosomes after fertilization. The third route is via the culture of gametophytic tissues

(i.e. anthers or microspores, ovaries or ovules), which under appropriate conditions will proliferate and regenerate haploid or doubled haploid plants. In the cereals, anther/microspore culture is generally practised rather than ovary/ovule culture, as the latter require much more labour to dissect them out of the donor plant and the efficiency of haploid production is low (Pickering and Devaux, 1992; Castillo and Cistué, 1993).

The production of plants from microspores involves a drastic developmental switch in which the normal course of microspore development towards a pollen grain is diverted into the formation of an embryo or callus tissue. The precise trigger(s) causing this diversion are not understood, but it is postulated that the isolation and culture procedures lead to the depletion of factors required to maintain normal pollen development, allowing the switch from the gametophytic to a sporophytic pathway (Dunwell, 1992).

The first cereal haploids were produced in the early 1970s from anther cultures, but efficiencies were extremely low and high frequencies of albino plants occurred (see Dunwell, 1985, for a review). In the intervening years, considerable effort has been devoted to improving the methodology, particularly in the major crops, and today haploid techniques are routinely applied in maize, rice, wheat and barley breeding (Petolino, 1992; Zhang, 1989; Devaux, 1992).

Although, as previusly discussed, the underlying mechanisms of microspore embryogenesis have yet to be understood, many of the major factors influencing microspore response *in vitro* have been identified. Donor plant genotype is of primary importance. Response is clearly under genetic control and it is possible to transfer associated genes via crossing (e.g. Petolino and Thompson, 1987). Recently, genes affecting androgenetic response have been mapped by restriction fragment length polymorphism (RFLP) analysis in order to improve haploid breeding efficiency (Cowen *et al.*, 1992). After the genotype, the physiological status of the donor plants and the pretreatments (temperature, preculture) of the spikes containing microspores are the most important factors which influence microspore development and subsequent response in culture. Similarly, the stage at which spikes are taken for culture is significant, microspores at different developmental stages responding differently and yielding different frequencies of albino and haploid versus doubled haploid plants. In addition to these biological parameters, medium composition (particularly carbon source and growth regulators) and the physical culture environment have profound effects. Apart from the basic response, that is, the diversion to an embryogenic or callus formation pathway, other components of the process, such as chromosome doubling, plantlet formation and the ratio of green : albino plants, are all influenced by these parameters, giving in total a highly complex and interactive system, albeit one which is used successfully. Pickering and Devaux (1992) have made a comprehensive review of the factors affecting barley anther culture response, and a similar spectrum of factors operates in other cereal species.

Although anther culture remains the most widely used dihaploid production technique, it is also possible to produce plants from the culture of

isolated microspores (Kasha, Cho and Ziauddin, 1992). The method is less labour-intensive than plating individual anthers and offers greater opportunities to understand the pathway of microspore embryogenesis without the influence of anther (tapetal) tissue. In addition, the technique allows the manipulation of large populations of uniform haploid cells, making it attractive for transformation, *in vitro* mutation and selection. Although high levels of response are currently only being obtained in model genotypes, efficiencies are constantly being improved.

Cultivars derived from doubled-haploid techniques have now been released in all of the major cereals (for examples, see Zhang, 1989; Pickering and Devaux, 1992). As the methodology improves, the efficiency will increase and the costs should decrease in comparison with more conventional techniques, leading to wider applications.

EMBRYO RESCUE/WIDE CROSSING

Plant breeders have produced improved cereals by combining desirable genes from the natural gene pool. However, the variability within several of the major cereals has been reduced as landraces have been replaced by pure line varieties (Frankel, 1970). In order to offset this effect and to provide supplementary genetic variability, breeders now look to the incorporation of genes from wild progenitor species or from other genera. Where wild species are closely related to the cultivated species (e.g. *Hordeum vulgare* and *H. spontaneum*), standard sexual crosses may be made to introgress genetic diversity for agronomic characteristics (Nevo, 1992). Where wild species are more distantly related or where attractive genetic variation exists in other genera, then standard crosses will not be possible and enabling technology will be needed for gene transfer. The best established technique for alien gene transfer (wide crossing) is that of hybrid embryo rescue, but two more recent techniques which may have applications are *in vitro* fertilization and somatic hybridization (protoplast fusion).

Embryo rescue

Barriers to the crossability of two species may exist at a number of levels, either pre- or post-fertilization (see Kalloo and Chowdhury, 1992). In cereals, it is often desirable to make wide crosses between distantly related species within a genus, or between species in related genera (Islam and Shepherd, 1991). In these cases, it is common for fertilization to take place, but for endosperm development to fail so that the hybrid embryo starves. In such situations, the hybrid plant may be recovered by embryo rescue, in which the developing embryo is excised from the caryopsis and cultured *in vitro*. In this procedure, the female parent is emasculated before pollen maturity and pollen from the male parent is applied when the stigmas are receptive. The efficiency of seed set may be improved by the addition of GA_3 at intervals after pollination, or by the application of 2,4-D as a pre-treatment before pollination. Immature embryos are isolated from enlarged caryopses as

shortly before endosperm degeneration as possible, and are then transferred either to culture medium or to a nurse endosperm (Khush and Brar, 1992).

Athough the major factors controlling seed set in wide crosses are genetic, other factors such as the growth environment and physiological status of the parental plants are also of importance. Furthermore, frequencies of seed set may be good while plant recovery frequencies are low (Bothmer *et al.*, 1989), due to poor embryo germination. This may result from the operation of a number of post-fertilization barriers, and several procedures such as alteration of the genomic ratios of the parents, or the induction of chromosomal exchanges, have been developed to overcome such blocks (Khush and Brar, 1992).

Embryo rescue techniques have allowed the introgression of many important agronomic characteristics from wild species into cultivated cereals, and have great potential. Within the Triticeae, new genomic combinations have been made to produce fertile synthetic amphiploids (homozygous products of chromosome doubling of interspecific or intergeneric hybrids). In most cases, amphiploids derived from cultivated species show inferior performance to the cultivated parent, but two exceptions are *triticale* and *tritordeum*. Hexaploid *triticale* (AABBRR) is an amphiploid between tetraploid wheat and rye. It has a number of superior agronomic characteristics and is an accepted cereal crop, particularly in Eastern Europe. *Tritordeum* (HHAABB) is a more recently developed amphiploid between *Hordeum chilense* and tetraploid wheat (Martin and Sanchez-Monge, 1982). It has several interesting characteristics, including high protein content, pathogen resistances and drought and salinity tolerance (see Barcelo *et al.*, 1993).

Rather than transferring whole genomes, the purpose of most wide crosses is the transfer of small chromosomal segments or single genes from a wild relative into a crop species. This can be achieved by 'chromosome engineering' techniques, which start with hybridization and then involve a series of cytogenetic manipulations to give an elite line containing an alien segment.

In wheat, wide crossing has been used to transfer numerous genes from other cereals or wild relatives, including genes conferring resistances to pests and pathogens, tolerance to abiotic stresses such as salinity, and improved nutrient use efficiency (see Gale and Miller, 1987; Islam and Shepherd, 1991). Similar transfers have been performed in barley, although to a lesser extent (see Fedak, 1992).

A limitation in many of these gene transfer experiments has been the lack of markers on the cereal or alien chromosomes. The development of RFLP maps for the major cereals is changing this situation and will increase the efficiency of introgression of useful alien genes into cereal crops.

In vitro *fertilization*

Where pre-fertilization barriers to wide hybridization exist, a number of techniques may be applied, such as removal of the stigma followed by direct application of pollen to the style, or *in vitro* pollination of isolated ovaries (Zenkteler, 1990; Khush and Brar, 1992). The logical extension to these

techniques is to carry out the fertilization *in vitro*, using isolated sperm and egg cells. This has long been a goal of plant cell biologists and has recently been achieved in maize (Kranz, Bautor and Loerz, 1991). The process involves the enzymic isolation of egg cells, which are then fused with mechanically isolated sperm cells using an electric pulse. The fusion products are cultivated in a feeder-cell system and will develop into multicellular structures. Further development was limited in the early experiments, but fertile hybrid maize plants have now been recovered (Kranz and Loerz, 1993). In this system, the frequency of egg + sperm cell fusions is high (~80%), as is the frequency of zygotic cell division (80%+) (Kranz, Bautor and Loerz, 1991). Furthermore, high fusion frequencies have also been achieved between maize egg cells and *Coix* pollen (E. Kranz, pers. comm.), indicating that the technique may have further applications in making wide crosses. At present, it offers a powerful research tool to study the fertilization process in plants.

Somatic hybridization

The final technique to overcome crossability barriers, particularly sexual incompatibility, is somatic cell fusion, using isolated protoplasts. The fusion of protoplasts is achieved either by the use of polyethylene glycol (PEG) under conditions of high pH and high calcium (Ozias-Akins, Ferl and Vasil, 1986), or by electrofusion using a short DC pulse after the alignment of protoplast pairs in an AC field (Terada *et al.*, 1987). Protoplast fusion offers the opportunity to make genetic manipulations which are difficult to achieve by other means. First, it is possible to make 'full' somatic hybrid plants from nuclear and cytoplasmic fusion of the two partners. Second, it is possible to create 'cybrid' plants (i.e. cytoplasm transfer) by inactivating the cytoplasm of one partner (via treatment with iodoacetamide) and the nucleus of the other partner (via X-irradiation), followed by fusion. Third, it is possible to achieve partial genome transfer (asymmetric hybridization) by both inactivating the cytoplasm and pulverizing the nucleus of the donor cells. The products are then selected for the transfer of fragments the donor genome into the recipient nucleus. To recover somatic hybrid plants, it must be possible to regenerate from callus produced from protoplast fusion. As discussed above, protoplast regeneration is efficient in only a few cereals and this has limited the application of somatic hybridization.

Somatic hybrid cell lines have been produced from a number of intergeneric and interspecific combinations (e.g. *Pennisetum* × *Panicum*, Ozias-Akins, Ferl and Vasil, 1986; *Triticum* × *Pennisetum*, Vasil, Ferl and Vasil, 1988; *Zea* × *Triticum*, Wang *et al.*, 1993; *Hordeum vulgare* × *H. bulbosum*, H. Funatsuki, pers. comm.; *H. vulgare* × *H. marinum*, X.-H. Wang, pers. comm.), but hybrid plants have only been recovered in rice.

Thus somatic hybrid plants have been obtained between rice and barnyard grass (*Echinochloa* sp.): Terada *et al.*, 1987), between rice and several wild *Oryza* species (Hayashi, Kyozuka and Shimamoto, 1988) and between rice cultivars (Toriyama *et al.*, 1988). Rice hybrid plants have also been produced,

with the purpose of transferring cytoplasmic male sterility (CMS) cytoplasms into cultivars for hybrid seed production (Akagi *et al.*, 1989; Yang *et al.*, 1989; Kyozuka, Kaneda and Shimamoto, 1989). These experiments demonstrate that protoplast fusion can potentially be used to manipulate agronomic characters, but cereal protoplast culture techniques will need to be improved for these methods to have wider application.

TRANSFORMATION

For genetic engineering to be possible, we need to be able to introduce and stably integrate foreign genes which will modify the characteristics of the recipient plant. In most dicotyledonous species, this can be achieved by *Agrobacterium* transformation in which the T-DNA of the bacterium acts as the vector for the gene transfer (see Ooms, 1992, for review). Cereals are not natural hosts for *Agrobacterium* and consequently there is the need to apply alternative transformation techniques. A spectrum of different methods have been tried and three direct gene transfer methods – cell electroporation, protoplast transformation and particle bombardment – have now yielded fertile transgenic plants.

In this section, we will first survey the various techniques used for cereal transformation (*see Table 3*), then discuss the DNA constructs used, selection for transformed cells, transgene integration and stability in cereals, and finally consider the current situation regarding application of the technology.

Whole plant transformation methods

The attraction of whole plant methods is that an *in vitro* regeneration step is avoided and the technology is relatively simple, requiring no specialized equipment, but only a supply of plasmid DNA. The two approaches which have received most attention are macroinjection and pollen transformation.

De la Pena, Lorz and Schell (1987) injected plasmid DNA containing the *nptII* gene into immature floral tillers of rye and recovered three kanamycin-resistant plants which, in Southern blots, appeared to contain the appropriate

Table 3. Transformation methods tested in cereals

Method	Results
Whole plant level; macroinjection, pollen tube pathway, topical DNA applications, etc.	No good evidence for stable, integrative transformation
DNA uptake into imbibing seeds, embryos	Transient gene expression
Agrobacterium transformation, agroinfection	Transient gene expression, systemic virus multiplication
Microinjection	Transient gene expression, stably transformed somatic cells
Silicon carbide fibre injection	Stably transformed tissues
Cell electroporation	Fertile transformed plants
Protoplast transformation	Fertile transformed plants
Particle bombardment	Fertile transformed plants

npt fragments. However, transmission of the transgene to progeny was not reported. This first report prompted many groups to test the macroinjection method, but although there were several reports of the detection of sequences hybridizing to the input DNA, both in primary transformants and progeny, transgene expression was often undetectable. Furthermore, there was no clear proof for the stable integration of these sequences into genomic DNA, so that mechanisms for extrachromosomal maintenance of input DNA or endophyte transformation have been invoked (Rogers and Rogers, 1992; Konstantinov, Mladenovic and Denic, 1991).

Pollen transformation methods aim to transform pollen cells either before fertilization or shortly after fertilization when the gametic cells or zygote have no cell walls and may therefore be accessible to exogenous DNA. There has been a long-standing interest in gametophytic transformation of cereals (De Wet *et al.*, 1986) and in the mid- to late 1980s several workers claimed success using such methods in maize (Ohta, 1986), wheat (Picard *et al.*, 1988) and rice (Luo and Wu, 1988). These reports generated much interest and many laboratories attempted to repeat the experiments. The initial results proved dificult to reproduce, however, and no study has produced clear evidence for stable transformation using these methods. A frequent observation has been that the very high nuclease activity associated with germinating pollen degrades the applied DNA within a few minutes of application (Roeckel *et al.*, 1988; Booy, Krens and Huizing, 1989).

A common feature of the 'successful' gametophytic transformation experiments has been that Southern analyses suggest the presence of sequences corresponding to the input DNA, but that expression of the marker genes cannot be detected. This observation is consistent with degradation of the plasmid DNA before uptake and integration and suggests that pollen transformation could become a viable technique if a suitable DNA protection method can be devised.

DNA uptake into seeds/embryos

The cell membranes of dry tissues are disorganized and show increased permeability. During the process of imbibition, a very large water potential gradient exists between the hydrating tissue and the external solution and the rapid cell expansion leads to cell wall disruption. These conditions could be expected to facilitate the uptake of macromolecules into cells, and consequently a number of workers have examined the imbibition of dry tissues in DNA solutions as a potential transformation method. In early experiments, foreign DNA uptake could only be demonstrated by detecting its physical presence, leading to ambiguous results. The advent of heterologous marker genes has made detection far more sensitive and in 1989 Toepfer and co-workers demonstrated that dry cereal embryos could take up plasmid DNA from an imbibition buffer and that transient gene expression could be measured. Embryos are still viable after treatment and will germinate to produce normal plants. Although the method would therefore appear to have potential for stable transformation, this has not yet been achieved despite

large-scale experiments. It may be that exogenous DNA is only taken up by damaged cells that ultimately do not survive, but it is more likely that uptake is confined to somatic cells of the embryos, which will not proliferate further, and that meristematic cells are not accessible to transformation. It has been shown that desiccated somatic embryos can also be transiently transformed by the imbibition method (Senaratna *et al.*, 1991), but there are no reports of stable transformation.

Agrobacterium *transformation/agroinfection*

For most plants, the most efficient transformation method is the use of the *Agrobacterium* vector system (Ooms, 1992). Cereals are classed as non-hosts to *Agrobacterium*, as they are not infected *in vivo* and show no response to inoculation *in vitro*. However, a series of investigations has shown that *Agrobacterium* can attach to cereal cells (Mooney and Goodwin, 1991), that cereal cells produce factors which induce *Agrobacterium* virulence genes (Schlaeppi and Hohn, 1992), and that the bacterium can transfer its T-DNA to cereal cells, leading to expression of genes contained within the T-DNA sequence.

Despite these indications of cereal × *Agrobacterium* compatibility, a reproducible method for stable transformation has yet to be developed. Attempts have been made to transform cereals at the whole plant level by pipetting Agrobacteria into flowers at the time of fertilization, but with mixed results. Hess, Dressler and Nimmrichter (1990) reported inheritance of the transferred genes in progeny of wheat treated using this method, while Langridge *et al.* (1992) made similar experiments in wheat, barley and maize but found no evidence for transmission of transgenes. They suggested that this result and the anomalous integration patterns observed could be explained by the transformation of endophytic bacteria present in the tissues of treated plants.

Relatively more effort has been applied to the transformation of cereal tissues with *Agrobacterium* under *in vitro* conditions. A variety of different culture systems have been used, including sterile seedlings (Graves and Goldman, 1986), shoot apices (Gould *et al.*, 1991) and embryos (Raineri *et al.*, 1990; Mooney *et al.*, 1991). All of these reports presented biochemical evidence for transformation, and most also presented molecular evidence for transgene integration. However, in no case was transmission of transgenes to progeny clearly demonstrated, and the experiments have not been repeated by independent workers. There is, therefore, still some doubt about the validity of this approach.

While the stable transformation of cereals by *Agrobacterium* is still questionable, the evidence that gene transfer occurs is now very good, largely as a result of the use of the agroinfection technique (Grimsley *et al.*, 1987), in which viral sequences capable to replication in cereal cells are cloned into the *Agrobacterium* T-DNA. This creates an extremely sensitive assay for T-DNA transfer, as a single viral molecule has the potential to replicate and establish a systemic infection which may easily be detected. Agroinfection has enabled

factors affecting T-DNA transfer to cereal cells to be investigated. It has been shown that the *Agrobacterium* strain (Marks *et al.*, 1989), bacterial virulence genes (Grimsley *et al.*, 1989), recipient cell type (Grimsley *et al.*, 1988) and developmental stage (Schlaeppi and Hohn, 1992) are all important factors.

As the viral molecules transferred via agroinfection do not integrate into the host cell chromosomes, the method does not lead to stable transformation. However, it may be possible to use the system to transfer a transposable element which might subsequently transfer the gene of interest into chromosomal DNA. The first step in the process, the excision of a transposable element from a T-DNA-introduced viral vector, has recently been described (Shen and Hohn, 1992).

A recent positive development in the *Agrobacterium*-mediated transformation of cereals is the finding that bacterial strains containing multiple copies of *virG* genes transiently transform rice seedling tissues at enhanced frequencies (Li *et al.*, 1992). Although the authors do not report stable transformation, the results suggest that it may be possible to engineer *Agrobacterium* to improve its compatibility with cereal cells.

Microinjection

Microinjection of DNA into embryos or germline cells is an efficient transformation method in a number of animal systems and there have been repeated attempts to apply this approach to plants, in particular to cereals. To transform plant cells via microinjection, it must be possible to manipulate and culture single cells at high efficiency, the cell wall must be penetrated by the microcapillary needle, DNA delivery must avoid the vacuole, there must be a high frequency of DNA integration, and the injected cells must survive and subsequently proliferate and regenerate. Such conditions are satisfied by few plant cell culture systems, and in cereals it is particularly dificult to culture isolated cells at low density. These technical difficulties have meant that there has been little success in stably transforming cereals by microinjection despite major efforts and early optimism (Potrykus, 1989, 1990). The technique may, however, have applications in studying the developmental biology of cereals, as it is relatively easy to achieve transient gene expression after microinjection (e.g. Toyoda *et al.*, 1990). For example, it is possible to study cell lineages from maize apical meristems by following the transient expression of marker genes which can be retained over several cell divisions (G. Neuhaus, pers. comm.). Similarly, the newly developed *in vitro* fertilization technique for maize (Kranz and Loerz, 1993), the developing zygote is amenable to microinjection, which might allow the 'marking' of cells to study early events in embryo development.

DNA delivery via silicon carbide fibres

A variation on injection techniques for cell transformation is the use of microscopic fibres which can be coated with DNA and then penetrate a cell, thus effecting transformation. This method has been demonstrated in various

animal systems and in yeast cells using asbestos or silicon carbide fibres and has recently been shown to function in plant cells (Kaeppler *et al.*, 1990). Fibres of diameter ~0.6 μm and length 10–80 μm are used, and transformation is dependent on vortexing the fibre/DNA/cell mixture to achieve cell penetration. In the grasses, the method has resulted in transient gene expression in maize and *Agrostis* suspension cells (Kaeppler *et al.*, 1990; Asano, Otsuki and Ugaki, 1991) and in stable transformation of maize suspension cells (Kaeppler *et al.*, 1990). The attraction of the technique is its simplicity, and it could become an important tool in cereal biotechnology if it proves applicable to regenerable tissues as suggested by Kaeppler *et al.* (1992).

Cell electroporation

Transformation via electroporation of intact cells (electroinjection) was initially attempted by many workers, and was generally concluded not to work. However, there is now clear evidence for cereal transformation by this method. Dekeyser *et al.* (1990) reported transient gene expression in leaf base tissues of rice, wheat and barley using a procedure involving prolonged incubation of the explants in buffer containing plasmid DNA before electroporation. Subsequently, transient expression has been reported from electroporated microspores, suspension cells (Fennel and Hauptmann, 1992) and immature embryos (Songstad *et al.*, 1993) of maize. The utility of the technique for stable transformation has been demonstrated by D'Halluin *et al.* (1992), who electroporated immature maize embryos or embryogenic callus in the presence of a plasmid containing the *neo* gene and obtained fertile kanamycin-resistant plants. Transmission and Mendelian segregation of the transgene in progeny was confirmed. Electroinjection has also been used to obtain stably transformed rice plants by the treatment of germinating seeds (Li *et al.*, 1991).

The mechanism of transformation by electroporation is not yet understood. It is possible that the electric pulse makes both the cell wall and plasma membrane porous to DNA, or that DNA uptake through the wall (via pre-existing pores or areas of damage) is passive and that the electric pulse simply perforates the membrane. The latter mechanism is supported by the observation that pre-incubation of tissues with the plasmid DNA is important, and by the report that in rice small cell groups may also be transformed by PEG treatment (Lee *et al.*, 1991).

The advantages of cell electroporation are that regenerable tissues such as immature scutella and embryogenic callus are accessible to transformation and that the procedure does relatively little damage to treated cultures. It is now of great interest to determine whether the technique can be applied to other cereals such as wheat and barley.

Protoplast transformation

Protoplast transformation was the first direct gene transfer method to be used in plants (Paszkowski *et al.*, 1984), and the first method used to transform a member of the grass family (Potrykus *et al.*, 1985). The principle is simple: isolated protoplasts are suspended in a buffer solution containing plasmid DNA and the protoplast plasma membranes transiently disrupted by a chemical (PEG) or electrical (electroporation) stimulus, allowing DNA uptake. The protoplasts are then either cultured for a few days before harvesting to assess transient gene expression, or are allowed to proliferate before selection with a toxin to identify transformed lines expressing a marker gene conferring resistance.

The advantages of protoplast transformation are that very large numbers ($>10^7$) of cells can be handled with ease, the transformation process (whether mediated by PEG or electroporation) is simple, with similar protocols being suitable for a wide range of species, and the selection of small protoplast-derived microcalli (typically 10–100 cells) is efficient.

The main disadvantage of protoplast transformation is that regeneration from protoplasts in many species, and particularly cereals, is difficult (see above). A second problem for use of the technique in cereals is that cell suspensions must be used as source material to obtain protoplasts with plating efficiencies high enough for transformation to be feasible. These cultures (and thus their protoplasts) lose regeneration potential relatively quickly and are also prone to cytological aberrations (i.e. somaclonal variation), which may result in variant plants being regenerated (see below). However, despite the difficulties associated with cereal protoplast culture, stably transformed cell lines have been produced via protoplast transformation in all the major

Table 4. Protoplast-mediated transformation in cereals

Species	Common name	References[a]	Level of transformation[b]
Hordeum vulgare	Barley	Lazzeri *et al.* (1991)	sc
Hordeum chilense × *Triticum durum*	Tritordeum	Barcelo *et al.* (in press)	te
Oryza sativa ssp. *indica*	Indica rice	Datta *et al.* (1989)	ftp
		Rathore, Chowdhury and Hodges (1993)	ftp
Oryza sativa ssp. *japonica*	Japonica rice	Shimamoto *et al.* (1989)	ftp
		Battraw and Hall (1992)	ftp
Pennisetum americanum	Pearl millet	Hauptmann *et al.* (1987)	te
Sorghum vulgare	Sorghum	Battraw and Hall (1991	sc
Triticum aestivum	Bread wheat	Mueller *et al.* (1992)	sc
Zea mays	Maize	Rhodes *et al.* (1988)	tp
		Omirulleh *et al.* (1993)	ftp

[a] Selected references, not necessarily first reports.
[b] te, transient expression; sc, stably transformed callus; tp, transgenic plants; ftp, fertile transgenic plants.

cereals, and the method has yielded fertile transgenic plants of maize and of japonica and indica types of rice (*see Table 4*).

Both of the techniques for inducing DNA uptake by protoplasts, PEG treatment and electroporation, have been used successfully in cereals and neither is consistently superior. PEG transformation is cheap, as it requires no specialized equipment, but rigorous washing of sensitive protoplasts may be necessary to achieve good division frequencies. Electroporation usually employs buffer solutions having lower toxicity to protoplasts (although toxic ions may be liberated during the pulse discharge: Tada, Sakamoto and Fujimura, 1990), but some protoplasts are damaged by the voltages needed to achieve membrane poration. In addition, the technique requires special equipment for pulse generation.

The efficiency of stable transformation of cereal protoplasts may be high (e.g. $1.0–4.5 \times 10^{-4}$: Hayashimoto, Li and Murai, 1990), but the production of transgenic plants is invariably limited by the frequency of regeneration from transgenic calli. At present, protoplast transformation can be considered a usable and routine procedure only in rice (japonica varieties, and in fewer indica varieties) and in a single highly-selected maize line (Omirulleh *et al.*, 1993). Significant improvements must be made in the culture of protoplasts of other cereals for the method to have wider applicability.

Particle bombardment

The development of particle bombardment (biolistic) transformation technology (Klein *et al.*, 1987) has been of major significance for plant genetic engineering, as it has enabled the transformation of species which are not infected by *Agrobacterium* and for which protoplast regeneration procedures are not efficient.

The principle of the method is that dense microscopic particles, usually tungsten or gold, are coated with DNA and then accelerated into plant cells, through cell walls, allowing transient gene expression, or possibly stable transformation, to take place. The first generation of 'particle guns' used gunpowder charges as the propulsive force (Klein *et al.*, 1987), but although these devices could be effective there were problems with reproducibility due to variation in the charges used. The second generation of particle guns includes electrostatic, pneumatic and compressed gas propulsion systems. These newer guns give more controlled and reproducible particle delivery and have improved transformation efficiency (for reviews on transformation by particle bombardment, see Klein *et al.*, 1992; Christou, 1992).

In particle bombardment, the DNA transfer process is essentially genotype- and tissue-independent, giving the method great flexibility. It can be used to obtain transient gene expression in any plant tissue which can be introduced into the bombardment chamber. To obtain transgenic plants, however, particles must be targeted to regenerable cells, which must survive the delivery and proliferate allowing the transformed cells to be selected from non-transformed tissues. To achieve this at acceptable efficiency requires co-optimization of the physical parameters of particle delivery and the

regeneration of the target tissue. Several physical and chemical factors, such as particle type, size and density, DNA precipitation method, particle acceleration speed, etc, influence transformation efficiency (see Morrish *et al.*, 1992), but the critical factor is that the DNA is delivered to cells which are competent for regeneration. In cereals, such cells are found principally in embryogenic cell cultures. In the first reports of cereal transformation via bombardment, cell suspensions were targeted (Klein *et al.*, 1988). Subsequently, several groups produced transgenic maize plants by bombarding embryogenic suspensions (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990). This approach is, however, limited by the need to establish embryogenic suspensions and the fact that suspension-derived regenerants are prone to be variant. More recently, attention has focused on the bombardment of callus cultures (wheat: Vasil *et al.*, 1992), or of primary explants such as embryos (rice: Christou, Ford and Kofron, 1992; wheat: Weeks, Anderson and Blechl, 1993) or inflorescences (*tritordeum*: Barcelo *et al.*, in press) (*Figure 2*). Primary cultures have the advantages that they are highly embryogenic and that the short culture periods involved should limit the incidence of somaclonal variation. There are also other target tissues for bombardment in cereals, such as exposed meristems or microspore cultures, but these are technically more difficult to prepare than primary explants such as embryos and there is as yet no report of transgenic plants from these sources.

Today, particle bombardment is the most efficient of the various methods

Table 5. Particle gun-mediated transformation in cereals

Species	Common name	References[a]	Level of transformation[b]
Avena sativa	Oats	Somers *et al.* (1992)	ftp
Hordeum vulgare	Barley	Mendel *et al.* (1989)	te
		Kartha *et al.* (1989)	te
Hordeum chilense × *Triticum durum*	Tritordeum	Barcelo *et al.* (in press)	ftp
Oryza sativa ssp. *indica*	Indica rice	Christou, Ford and Kofron (1991)	ftp
		Christou, Ford and Kofron (1992)	ftp
Oryza sativa ssp. *japonica*	Japonica rice	Cao *et al.* (1992)	ftp
		Christou, Ford and Kofron (1992)	ftp
Pennisetum glaucum	Pearl millet	Taylor and Vasil (1991)	te
Sorghum vulgare	Sorghum	Hagio, Blowers and Earle (1991)	sc
Triticum aestivum	Bread wheat	Vasil *et al.* (1992)	sc
		Vasil *et al.* (1992)	ftp
		Weeks, Anderson and Blechl (1993)	ftp
Zea mays	Maize	Klein *et al.* (1988)	sc
		Gordon-Kamm *et al.* (1990)	ftp

[a] Selected references, not necessarily first reports.
[b] te, transient expression; sc, stably transformed callus; ftp, fertile transgenic plants.

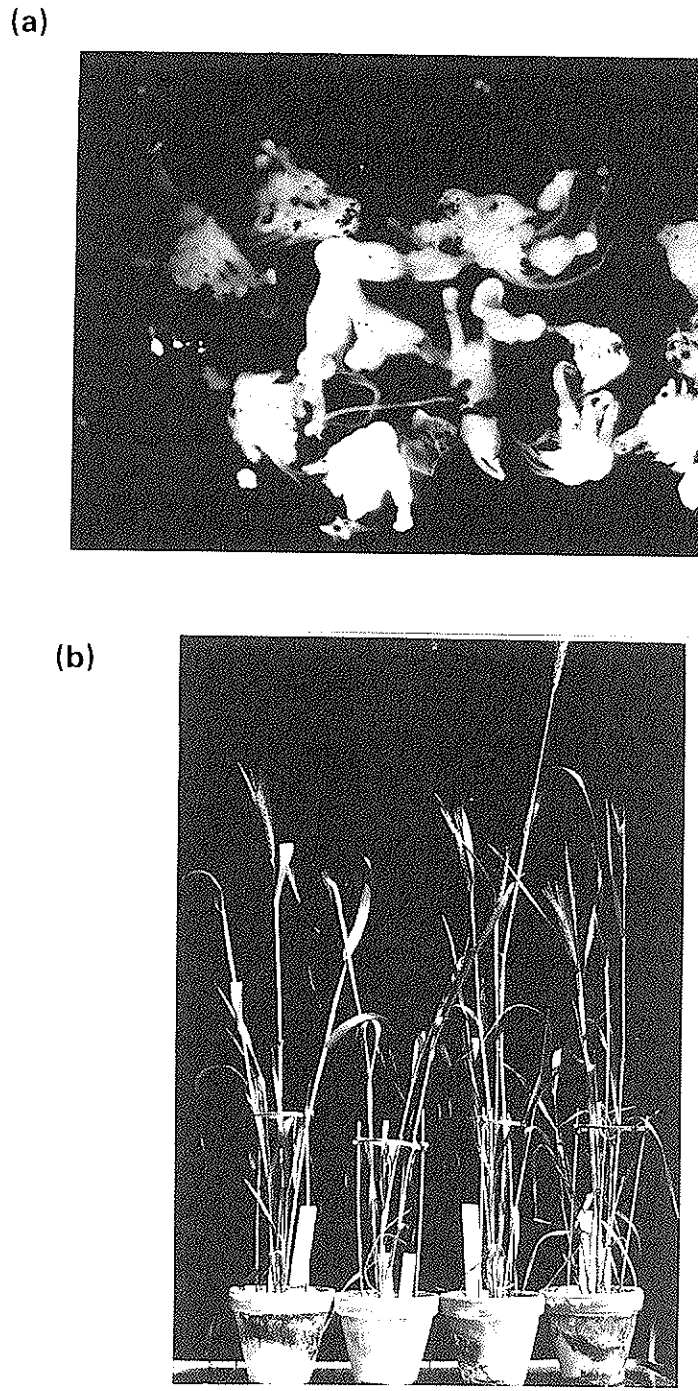


Figure 2. (a) Transient expression of the *gus* reporter gene (seen as dark spots) in bombarded immature inflorescences of *tritordeum*. (b) Mature and fully fertile transformed plants of *tritordeum*.

for cereal transformation. It has already been used to produce fertile transgenic plants in several of the major cereals (*Table 5*), and it is certain that more species will shortly be added to this list.

DNA constructs

The plasmid vectors used for direct gene transfer to plants typically contain an expression cassette comprising a promoter region, a transcription iniation site and a multilinker sequence with unique restriction sites, allowing the insertion of the desired coding sequences (selectable and/or scorable markers, genes of interest), followed by a polyadenylation signal. The components of these vectors which have received most attention in attempts to improve transformation efficiency are promoter sequences, and the selectable and scorable markers.

To achieve high-level expression of inserted transgenes, it is necessary to use promoters which are active in the recipient cells. A number of bacterial and viral promoters have been used for transformation of dicot species, for example, the *Agrobacterium* opine and T_R-DNA gene 5′ sequences or the cauliflower mosaic virus (CaMV) 35S sequence. Of the numerous promoters tested, the CaMV 35S promoter has given high-level constitutive expression in many species and this promoter is also the most commonly used in cereals: maize (Rhodes *et al.*, 1988), rice (Shimamoto *et al.*, 1989), barley (Lazzeri *et al.*, 1991) and wheat (Vasil *et al.*, 1991). It is observed, however, that the levels of expression from the CaMV 35S promoter are generally lower in cereals than in dicot species, especially in transient expression assays (e.g. Hauptmann *et al.*, 1987). Consequently, efforts have been made to identify or construct more active promoters for cereals.

In transient expression assays, the introduction of monocot intron sequences into the transcriptional units of constructs has been shown to increase activity. Several different introns incuding those from the maize *adh1* (alcohol dehydrogenase) gene (Callis, Fromm and Walbot, 1987), the maize *shrunken1* gene (Vasil *et al.*, 1989) and the rice *act1* (actin) gene (McElroy *et al.*, 1990) have been shown to be effective.

The choice of a 'strong' promoter for stable transformation is less clear cut and the effect of the insertion site within the recipient genome (position effects) becomes a major factor deciding transgene expression level. Furthermore, there are clearly differences in the levels of expression of the promoters currently available between different cereal species. For example, in rice the maize *adh1* promoter gives good levels of expression (Zhang and Wu, 1988), whereas it is only weakly active in wheat (Vasil *et al.*, 1992). Typically, expression levels are lowest in barley and wheat, are higher in maize and are highest in rice (authors' personal observations). One promoter which has given high levels of expression in a number of different cereals is the rice actin 1 promoter/actin 1 intron sequence (McElroy *et al.*, 1990).

There has been some success in constructing composite transcriptional units to give improved expression in cereals. Last *et al.* (1991) combined an anaerobic response element from the maize ADH gene with multiple

enhancer elements from the *Agrobacterium* octopine synthase gene, the *adh1* intron and a truncated *adh1* promoter, and obtained levels of expression in wheat up to 50 times those obtained from the CaMV 35S promoter. More recently, Omirulleh *et al.* (1993) have combined two CaMV 35S enhancer elements with a truncated wheat α-amylase promoter to achieve high levels of expression in transgenic maize plants.

Various different scorable marker genes have been used in plant systems, including chloramphenicol acetyltransferase (CAT) (Fromm, Taylor and Walbot, 1985, radiochemical assay), luciferase (LUX) (Ow, Jacobs and Howell, 1987, luminescence assay) and β-glucuronidase (*uidA*, GUS) (Jefferson, 1987, histochemical or fluorometric assays). The latter marker is the most popular because it is easy to assay and allows the visualization of transgene expression *in situ*. Selectable marker genes are considered below.

Selection of transformed tissues

An ideal selection system should give good discrimination between transformed and non-transformed cells, allowing few escapes, and should have no influence on regeneration from transformed tissues. A number of compounds, and their associated resistance genes, have been tested in plants, but only a few, generally antibiotic or herbicide markers, are in common use (*see Table 6*). Cereals, particularly in culture, show higher levels of resistance to selection agents than most dicot species, meaning that high concentrations of the compounds must be used and that the timing and maintenance of selection pressure are critical. In recent experiments, the most popular selectable markers for cereals have been the *nptII* (neomycin phosphotransferase) gene (kanamycin or G418 selection) or the *bar* (phosphinothricin phosphotransferase, PAT) gene (phosphinothricin, Basta or Bialaphos selection). The latter system has the advantage that the herbicides Basta or Bialaphos may be used for selection at the whole plant level, facilitating the selection of plants containing the transgene in segregating progeny generations.

Transgene expression and stability

As the production of transgenic cereal plants is a relatively new development, there are limited published data on the stability and heritability of transgenes in plants. Several studies made at the callus level and on primary (T_0) plants have, however, given information on integration and expression patterns. As observed in other species, direct gene transfer to cereals appears to involve random integration into the recipient genome. It is common for multiple copies of the transforming plasmid to be integrated, which may vary from low numbers (e.g. 1–2: maize, D'Halluin *et al.*, 1992; 1–6: rice, Battraw and Hall, 1992; 1–10: rice, Hayashimoto, Li and Murai, 1990) to high numbers (e.g. 1–20+: oats, Somers *et al.*, 1992; 50–100: rice, Datta *et al.*, 1990). Plasmids are commonly integrated as head-to-tail concatamers of two or more molecules and there are frequently sequence rearrangements which give restric-

Table 6. Selection systems used in cereal transformation

Selection agent	Resistance gene	Reference[a]	Species
Kanamycin	*nptII*	D'Halluin *et al.* (1992)	Maize
	Neomycin phosphotransferase	Battraw and Hall (1992)	Rice
G418	*nptII*	Battraw and Hall (1992)	Rice
		Barcelo *et al.* (in press)	Tritordeum
Hygromycin B	*hpt.aphIV*	Hayashimoto, Li and Murai (1990)	Rice
	Hygromycin phosphotransferase	Walters *et al.* (1992)	Maize
Phosphinothricin (Basta, Bialaphos)	*bar*	Gordon-Kamm *et al.* (1990)	Maize
	Phosphinothricin Acetyltransferase	Vasil *et al* (1992)	Wheat
		Rathore, Chowdhury and Hodges (1993)	Rice
Chlorsufuron	*ILV2, ilvG*	Fromm *et al.* (1990)	Maize
Glyphosate	*EPSP synthase* 5-enolpyruvylshikimate phosphate synthase	Vasil *et al.* (1991)	Wheat

[a] Selected references.

tion fragments larger or smaller than expected (Gordon-Kamm *et al.*, 1990; Battraw and Hall, 1992; D'Halluin *et al.*, 1992). Integration sites are generally at single or closely linked loci (e.g. Spencer *et al.*, 1992; D'Halluin *et al.*, 1992), but integrations at multiple and unlinked loci are also found (Hayashimoto *et al.*, 1990; Rathore, Chowdhury and Hodges, 1993). The factors determining the complexity of integration pattern are not understood. Variables such as culture system, DNA conformation and delivery method may have an influence, but the observation that a range of integration patterns (varying from single copies to multiple insertions and multiple copies with rearrangements) may be found within a single experiment (Rathore, Chowdhury and Hodges, 1993), suggests that cell-to-cell variation in factors such as cell cycle stage, activity of DNA repair mechanisms or endogenous nuclease activity may also be involved.

When levels of transgene activity are assessed in independent transformants, there is typically wide variation (e.g. Omirulleh *et al.*, 1993), but this variation is rarely correlated with the number of copies integrated (e.g. Gordon-Kamm *et al.*, 1990). Variation in expression levels may be explained by the influence of the surrounding genomic environment, and the lack of correlation between copy number and expression levels suggests a significant frequency of silent transgenes. The occurrence of inactive insertions becomes apparent in co-transformation experiments where two independent transgenes are supplied on different plasmids. It is common for co-integration frequencies to be high (e.g. 77%, Gordon-Kamm *et al.*, 1990), but co-expression frequencies are variable (e.g. 18%, Gordon-Kamm *et al.*, 1990; 30%, Rathore, Chowdhury and Hodges, 1993; 70%, Barcelo *et al.*, in press; 75%, Somers *et al.*, 1992). In some cases, transgene inactivation may result from rearrangements occurring during transformation or from mechanisms

such as promoter methylation (Matzke *et al.*, 1989) and co-suppression by multiple transgene copies (DeCarvalho *et al.*, 1992).

Several studies have now examined the heritability of transgenes in maize and rice, the cereals in which transformation procedures are most developed. In maize, transgenes usually segregate as single dominant alleles, even when multiple copies are present, indicating either linkage of the copies or that only single copies are active (Spencer *et al.*, 1992; Walters *et al.*, 1992; D'Halluin *et al.*, 1992). However, instances of non-Mendelian inheritance were reported in all of these studies, with some integrated copies of the transgenes appearing to be unstable. Similarly, in rice, Rathore, Chowdhury and Hodges (1993) found both Mendelian and non-Mendelian segregation ratios in T_1 and T_2 populations.

Transgene integration via direct gene transfer is at present an uncontrolled process and this has implications for transgene expression and stability. Further work is needed to understand its mechanisms in order to be able to manipulate the process to produe plants with predictable and stable transgene expression.

An observation common to virtually all the studies on transgenic cereals cited is the occurrence of phenotypically abnormal and infertile plants. These aberrations are not associated with the transformation process, but result from culture-induced variation. This phenomenon is discussed below.

Applications

Cereal transformation is too recent a procedure for there to be many examples of practical application. The majority of experiments performed to date have been directed at establishing gene transfer techniques and at identifying suitable selectable and scorable markers and promoter sequences, but there is increasing use of cereal transformation as a research tool. Transient expression systems, either via protoplast transformation or particle bombardment, are particularly suitable for gene expression analysis, with bombardment giving the possibility of examining tissue-specific expression in intact organs. Examples of these applications include analyses of intron sequence function (Callis, Fromm and Walbot, 1987), organelle targeting with heterologous sequences (Teeri *et al.*, 1989), anthocyanin biosynthesis genes (Klein, Roth and Fromm, 1989), α-amylase promoter specificity (Lee *et al.*, 1991) and regulation (Salmenkallio *et al.*, 1990; Jacobsen and Close, 1991), and analysis of phytochrome gene photoregulation (Bruce *et al.*, 1989).

There is limited scope for analyses of gene expression in stably transformed callus, as the undifferentiated callus state is an artificial condition which does not constitute a good model for the organized plant. The ability to produce transgenic plants, however, offers a great opportunity for analysing gene function via the introduction of heterologous genes or the over-expression or suppression of homologous sequences. Several studies have investigated spatial expression patterns of heterologous and homologous promoters in cereals (Zhang and Wu, 1988; Battraw and Hall, 1990; Terada, Nakayama

and Iwabuchi, 1993; Omirulleh *et al.*, 1993; Zhang *et al.*, 1991; Tada *et al.*, 1991) using fusions with the GUS gene.

The first examples of applied genetic engineering of cereals are now starting to appear. Much of this work is carried out by the major agrochemical and plant breeding companies, so the actual state of progress is significantly in advance of that which has been published. There are several reports of herbicide-resistant cereals: rice (Datta *et al.*, 1992; Cao *et al.*, 1992; Rathore, Chowdhury and Hodges, 1993), maize (Gordon-Kamm *et al.*, 1990; Fromm *et al.*, 1990), wheat (Vasil *et al.*, 1992) and oats (Somers *et al.*, 1992). In most cases, the herbicide resistance genes have been introduced to act as selection markers, although there are a number of situations where herbicide-resistant cereals may solve agronomic problems, such as where weeds have developed resistance to conventional selective herbicides, or where engineered resistant plants would allow the use of a 'benign' molecule such as Glyphosate instead of toxic molecules such as Atrazine (Gressel, 1992).

A rather less controversial application of transformation technology is the engineering of pest resistance. The field testing of transgenic maize expressing a *Bacillus thuringiensis* crystal protein gene has recently been reported (Koziel *et al.*, 1993; see also Chapter 6, this volume). In this work, an elite maize inbred was transformed, via particle bombardment, with an engineered crystal protein gene under the control of either the CaMV 35S promoter or of two tissue-specific promoters from maize (minimizing transgene expression in seeds). The transgenic plants were protected in the field against very high infestations of the European corn borer, a major pest of maize in North America and Europe.

This experiment demonstrates that cereal genetic engineering is now a reality, and in the next few years many of the targets identified in other sections of this review will be approached using this strategy.

SOMACLONAL VARIATION

A feature of plant cell and tissue culture is that regenerated plants may be variant, that is, they may differ in some characteristics from the parent plant. This variation may be stable and heritable or may be unstable and show non-Mendelian inheritance ('epigenetic' variation). This phenomenon is termed 'somaclonal variation' (Larkin and Scowcroft, 1981) and has been observed in all groups of plants which have been regenerated *in vitro* (for reviews, see Karp, 1991; Peschke and Phillips, 1992). The causes of somaclonal variation are not fully understood, but a number of the factors involved have been identified.

Variation is associated with *de novo* regeneration from dedifferentiated and disorganized tissue; plants regenerated from cultured meristems are rarely variant, indicating that the normal controls which maintain genetic fidelity in the whole plant operate *in vitro* in isolated meristems. Some variation is undoubtedly present in the explants used to establish cultures, as plant somatic tissues commonly contain polyploid or aneuploid cells. However, the frequency of variation increases with culture age. This may result

simply from the accumulation of mutations/epigenetic changes over time, or from faster proliferation of variant cells so that they form an increased proportion of the culture. The variation increases as cultures become progressively less organized and grow rapidly, so that established fast-growing cell suspensions typically have high frequencies of aberrant cells.

A number of different mechanisms of somaclonal variation have been identified, including chromosomal aberrations such as polyploidy and aneuploidy, chromosomal breakage, activation of transposable elements, DNA methylation changes, single base pair changes and gene amplifications and deletions (for bibliography, see Karp, 1991; Peschke and Phillips, 1992).

Somaclonal variation has both positive and negative aspects for cereal biotechnology. In transformation, all the currently proven methods involve an *in vitro* culture step (see above) and somaclonal variation is a problem in these systems. The ideal transformation system is one in which elite cultivars can be modified by the introduction of specified genes, but remain otherwise unchanged. The most obvious marker for the occurrence of variation *in vitro* is loss of regeneration competence, which correlates closely with the accumulation of cytological aberration. Such cultures regenerate fewer plants and those that are produced become progressively more variant with lower fertility (see Morrish, Vasil and Vasil, 1987). The impact of this process is seen in several of the first reports of cereal transformation, where regeneration from transgenic cell lines was inefficient and the transgenic plants were frequently infertile (e.g. Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990; Vasil *et al.*, 1992). To avoid these problems, primary cultures have been transformed in more recent experiments (Koziel *et al.*, 1993; Barcelo *et al.*, in press).

There has been some interest in the harnessing of somaclonal variation as a source of novel variation for cereal improvement (Larkin and Scowcroft, 1981), but the general view today is more cautious. However, there are several reports of useful variation arising from tissue culture, such as improved protein content (Schaeffer, Sharpe and Cregan, 1984; Ryan and Scowcroft, 1987), dwarf stature (Galiba *et al.*, 1985) and modified flowering time (Sun *et al.*, 1983; Larkin *et al.*, 1984). In addition, there has also been some success with *in vitro* selection experiments which have, for example, yielded plants resistant to pathotoxins (Rines and Luke, 1985; Pauly, Shane and Gengenbach, 1987), resistant to an insect toxin (Zemetra *et al.*, 1993), with increased freezing tolerance (Lazar *et al.*, 1988) and with increased levels of specific amino acids (Miao, Zhuang and Hu, 1988) (see review in Lazzeri *et al.*, 1991). Overall, however, attempts to exploit somaclonal variation in cereals have not been very successful. Most of the variation observed is deleterious and that which is useful is frequently neither novel nor genetically stable, and therefore difficult to use in seed-propagated crops.

Although somaclonal variation is today generally seen in a negative light, it is still a subject worthy of study. With a better understanding of its underlying mechanisms, it should be possible to reduce its impact where it is unwanted and even to use the process to direct genetic change.

Application of biotechnology to grain composition and quality

The quality of a cereal grain is, of course, related to the end use. The major traditional end uses are as food or feed, either consumed directly or after processing. The quality for these uses is largely determined by the grain proteins, so we will initially discuss the properties of the individual proteins and strategies for their manipulation. In this we will focus on the use of barley for livestock feed and of wheat for bread-making, although we will also discuss other uses of these two cereals. We will then discuss wider opportunities for manipulating the proportions and compositions of the starch and lipid fractions of the grain, and for producing high-value chemicals for pharmaceutical and other purposes.

MANIPULATING THE PROTEIN QUALITY OF BARLEY AND WHEAT

Cereal grain proteins

Cereal grain proteins can be broadly divided into two types: storage and non-storage. The latter include structural and metabolic proteins, such as enzymes and components of cell walls and membranes. Storage proteins can be defined as those that are synthesized specifically to provide a store of nitrogen, sulphur and carbon for seed germination and seedling growth. They account in wheat and barley for 30–60% of the total grain nitrogen (depending on nutrient availability), and are stored in dense membrane-bound deposits called protein bodies. In most cereals (with the exception of oats and rice), the major storage protein fraction is insoluble in water or dilute salt solutions but soluble in alcohol–water mixtures.

These alcohol-soluble proteins (called prolamins) are characterized by high levels of proline and glutamine, about 20 mol % and 30 mol % respectively in total prolamin fractions from barley and wheat, and about 20 mol % and 10 mol % respectively in maize and sorghum. In all cereals, the prolamins are complex polymorphic mixtures of proteins, which range widely in their M_rs (from about 30 000 to 90 000 in barley and wheat and 14 000 to 27 000 in maize) and amino acid compositions (the combined proportions of proline and glutamine ranging from about 25 to 70 mol %). However, in all cases, they are low in one or more nutritionally essential amino acids. This results in low nutritional quality of the whole grain when used as food for humans or as feed for monogastric livestock (such as pigs and poultry). The first limiting amino acid in barley, wheat and maize is lysine, followed by threonine in wheat and barley and tryptophan in maize.

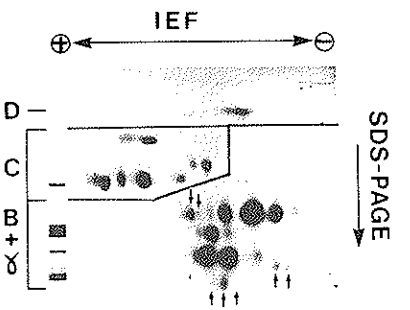
The prolamins are synthesized only in the starchy endosperm of the developing grain. Although they form the major protein fractions in most species, other storage proteins may also be present, either in the starchy endosperm or the aleurone and embryo, or both. These include albumins and globulins related to those stored in seeds of many dicotyledonous species.

The nutritional quality of barley

The nutritional quality of barley is limited by the levels of lysine and threonine: about 3.1 and 3.3 g per 100 g of protein, respectively, compared with WHO recommended levels of 5.5 and 5.0 g per 100 g, respectively (Ewart, 1967; FAO, 1973). The significance of this deficiency for human nutrition is uncertain, but it is probably unlikely to pose problems except perhaps for rapidly growing children living solely on cereals. The situation with livestock is more clear-cut, and it is necessary to supplement cereals with sources of lysine and threonine in order to obtain maximum growth of rapidly growing pigs and poultry. These sources include fish meal, pure amino acids or soybean meal. There is, of course, no problem with ruminants, as they can digest the bacterial flora of the rumen to provide essential amino acids.

The prolamin storage protein fraction of barley is called hordein. It is a complex mixture of over 20 proteins, which vary in precise number, proportions and properties (i.e. M_r and pI) between different genotypes (*Figures 3, 4*). These components are classified into two major groups (B and C hordeins) and two minor groups (D and γ-hordeins) (*Figure 3*). All of these groups are severely deficient in lysine, although D hordein is unusually rich in threonine (~8 mol %) (*Figure 3*). B and C hordeins account for about 80% and 10–20% of the total hordein fraction, respectively (Kirkman, Shewry and Miflin, 1982). Both consist of a number of proteins, which are encoded by families of about 20–30 closely linked genes at the *Hor* 1 (C hordein) and *Hor* 2 (B hordein) loci located on chromosome 5 (1H) (see Shewry *et al.*, 1990). Extensive restriction fragment polymorphism is observed, reflecting the protein polymorphism (*Figure 4*).

D and γ-hordeins each account for a small proportion of the total hordein fraction. D hordein is a single band on SDS-PAGE separations, and appears



Group	Composition (mol%)				
	Glx	Pro	Cys	Lys	Thr
B	35	21	2·5	0·5	2·1
C	41	31	0	0·2	1·0
D	30	12	1·5	1·1	8·1
γ	32	16	2·7	1·6	3·1

Figure 3. Two-dimensional IEF/SDS-PAGE of barley hordeins, and the proportions of glutamate + glutamine (Glx), proline (Pro), cysteine (Cys), lysine (Lys) and threonine (Thr) in the individual groups. The electrophoretic separation of hordeins from cv Carlsberg II is taken from Kreis *et al.* (1983). The arrows indicate the γ-type hordeins which have similar M_rs to the B hordeins.

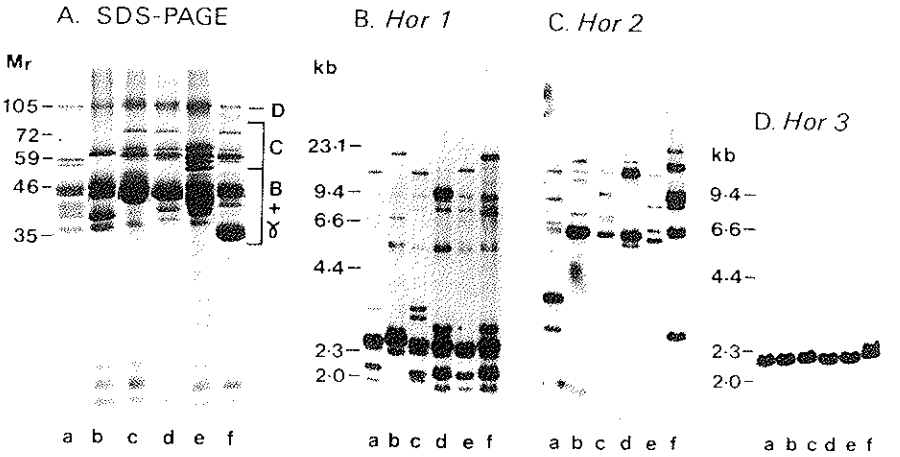


Figure 4. Polymorphism in hordein polypeptides and genes. (A) SDS-PAGE analyses of total reduced and pyridylethylated hordein fractions from the cultivars Athos (a), Keg (b), Jupiter (c), Hoppel (d), Igri (e) and Sundance (f). (B, C and D) Total genomic DNAs from the same six cultivars, digested with *Hin*dIII and probed with cDNA clones related to C hordein (B), B hordein (C) and D hordein (D). Rearranged from Bunce *et al.* (1986) and taken from Shewry (1992).

to be encoded by a single copy gene (*Figure 4*) at the *Hor* 3 locus on the long arm of chromosome 5 (1H) (Shewry *et al.*, 1983; Bunce *et al.*, 1986). γ-Hordeins consist of several minor bands with similar M_rs to B hordeins (*Figures 3,4*), and are encoded by a small multigene family, probably located at the *Hor* 5 locus which is closely linked to *Hor* 1 (Shewry and Parmar, 1987; Shewry *et al.*, 1990).

A number of partial and complete cDNAs and/or genes for hordeins have been isolated. These demonstrate a high degree of structural homology between members of a group, but greater differences between the groups. However, it is clear from sequence comparisons that all hordeins have some degree of structural relatedness (as discussed by Shewry, 1993), indicating a common evolutionary origin.

We have discussed the complexity of the hordein fraction in some detail, as this has important implications for selecting strategies for improvement. First, the low contents of lysine in the individual hordein polypeptides may be significant in relation to the packaging of the proteins as hydrated solid within the protein bodies. It is not known whether the lysine content could be increased sufficiently to have an impact on the nutritional quality of the grain without affecting this or other important biological properties. However, studies of maize zeins indicate that at least some additional lysine residues may be tolerated (Lending, Wallace and Larkins, 1992). Second, the existence of complex multigene families means that any inserted hordein genes will be expressed in the presence of many other actively expressed hordein genes. Consequently, the inserted genes would need to have exceptionally strong promoters or be expressed in lines in which other hordein genes are inactivated or deleted in order to have a significant impact on the

lysine content of the whole fraction. Because of these considerations, the direct manipulation of hordein genes to increase the lysine contents of the encoded proteins has never been seriously considered as a strategy for barley improvement.

A more promising strategy is suggested by analyses of mutant high lysine barley lines. A number of such mutants were reported in the 1970s, mostly resulting from treatment with chemical or physical mutagens. In almost all cases, the high lysine phenotype results from a decrease in the proportion of hordein, with increases in the proportions of other more lysine-rich protein fractions (see Shewry *et al.*, 1987b). In addition, the decreased proportion of prolamins is usually associated with a reduced amount of starch, and consequently lower yield. Because of this there has been limited success in incorporating the mutant high lysine genes present in these lines into high-yielding cultivars for commercial use (but see Munck, 1992).

Although most high lysine mutants were produced by mutagenesis, the first to be described was a spontaneous mutant identified by screening the world barley collection held by the USDA (Munck *et al.*, 1970). This is a primitive line (land race) from Ethiopia, and was named Hiproly. Although it has not been possible to incorporate the high lysine gene from Hiproly (designated *lys*) into high-yielding cultivars, molecular and biochemical analyses of the mutant phenotype have proved to be very valuable.

Hiproly has about 30% more lysine than normal cultivars, accompanied by a modest decrease in the proportion of hordein (~10–20%: see Shewry *et al.*, 1987b). The latter is clearly not sufficient to account for the increased lysine content, and the answer lies in the presence of increased amounts of specific salt-soluble proteins. Four such proteins account for about half of the increased lysine content, and for 17% of the total grain lysine in Hiproly compared with about 7% in normal cultivars (Hejgaard and Boisen, 1980). These are β-amylase (5.0 g % lys), protein Z (7.1 g % lys) and chymotrypsin inhibitors CI-1 (9.5 g % lys) and CI-2 (11.5 g % lys). CI-1 and CI-2 are clearly of most interest because of their very high contents of lysine, and Hejgaard and Boisen (1980) showed that the amounts of these two proteins were increased about 6- to 8-fold in high lysine lines derived from Hiproly, as compared with the normal parents.

CI-1 and CI-2 were initially purified at the Carlsberg Laboratory and complete or partial amino acid sequences determined (Svendsen, Boisen and Hejgaard, 1982; Svendsen, Martin and Jonassen, 1980). They showed that both proteins exist in isoforms, and this was confirmed by cDNA cloning which demonstrated that both were encoded by two sub-families of mRNAs and genes (Williamson *et al.*, 1987; Williamson, Forde and Kreis, 1988). The major isoform of CI-2 consists of 84 residues with an M_r of about 9400, while two equally abundant isoforms of CI-1 consist of 83 residues with M_rs of about 8800 and 9000 (*Figure 5*). Neither protein contains cysteine residues, or appears to be synthesized with a signal peptide. Nevertheless, there is clear evidence from immunogold labelling that CI-2 is located in protein bodies and in vesicles derived from the Golgi apparatus (Rasmussen, Munck and Ullrich, 1990). Both proteins are encoded by small multigene families (3–4 copies)

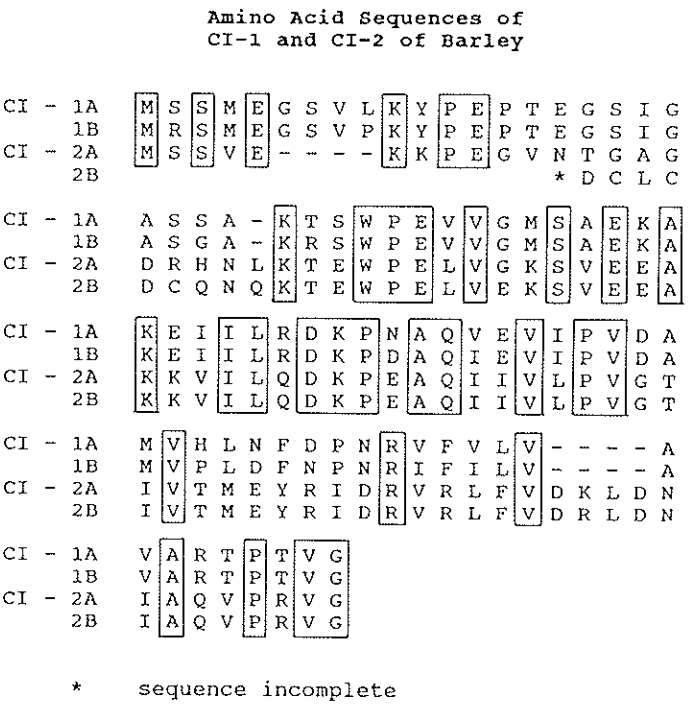


Figure 5. The amino acid sequences deduced from cDNA clones for barley chymotrypsin inhibitors CI-1 and CI-2. In each case, two sub-families of clones were identified, and were designated A and B. Note the high contents of lysine residues (K) and the absence of cysteine. Based on data in Williamson *et al.* (1987) and Williamson, Forde and Kreis (1988). Standard single letter abbreviations for amino acids are used.

located on the short arm of chromosome 5 (1H) (Cannell *et al.*, 1992).

Because of its small size and lack of disulphide bonds, CI-2 has been an attractive target for structural and protein engineering studies. Its three-dimensional structure has been determined (alone and in complex with subtilisin) by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, and its inhibitory constant and specificity have been altered by engineering of the reactive loop region that interacts with serine proteases (see Campbell, 1992).

The biological roles of CI-1 and CI-2 are not known, but they may play a role in natural defence against pests and pathogens. This suggestion is supported by the fact that related inhibitors present in tomato are induced by damage or feeding (Graham *et al.*, 1985).

An analysis of Hiproly demonstrates that substantial increases in the amounts of CI-1 and CI-2, which are sufficient to have an impact on the lysine content of the whole grain, can be tolerated without adverse effects on grain development or digestibility. Also, it is possible that such increases may also provide enhanced protection against pests and pathogens. In Hiproly, these increases are achieved by the action of a *trans*-acting 'regulatory' gene, and the incorporation of this gene into cultivars has been limited by pleiotropic effects on grain yield. An alternative strategy would be to increase the

amount of CI-2 by inserting additional gene copies, either under control of their own promoter or, preferably, a stronger promoter which also confers seed specificity.

A gene for CI-2 has been isolated, and its control of expression studied using chimaeric genes with the GUS reporter (Peterson *et al.*, 1991; Shewry and Kreis, 1992; Kreis and Shewry, 1992). This has shown that 1100 bp of 5′ upstream sequence is sufficient to confer tissue-specific expression in transgenic tobacco plants, but that 263 bp of 5′ sequence is insufficient. It is unlikely, however, that the CI-2 promoter would be used to drive expression if additional CI-2 genes were inserted into barley. This is because it would be advantageous to use a stronger promoter, for example from the D hordein gene of barley or from one of the homologous HMW subunit genes of wheat. D hordein accounts for about 2–4% of the total hordein fraction, and is encoded by a single gene (see above). Similarly, each HMW subunit is encoded by a single gene and accounts (on average) for about 2% of the total extractable grain protein (Halford *et al.*, 1992b). D hordein gene expression has not been studied in detail, but several studies of HMW subunit gene expression have been made using transgenic tobacco and transient expression in protoplast systems. These have shown that only 280 bp of DNA immediately upstream of the transcription start site is sufficient to confer temporal and endosperm-specific gene expression (Halford *et al.*, 1989). It should, therefore, be possible to fuse the 5′ upstream sequence from an HMW subunit or D hordein gene with the coding sequence of CI-2 in order to obtain high levels of tissue-specific expression in transgenic barley plants. It is difficult to calculate how many such copies of CI-2 genes would need to be inserted to have a significant impact on grain quality, as it is not possible to predict the precise levels of expression that would be achieved.

We consider that increasing the level of expression of lysine-rich proteins such as CI-2 is the most promising approach to increasing the lysine content of cereals such as barley, wheat and maize, and that this will be more readily achieved by the insertion of additional gene copies under the control of strong promoters than by attempting to increase the levels of expression of endogenous genes. The latter will depend on identifying the molecular basis for the control of gene expression, and in particular the role of *trans*-acting factors that are presumably produced by regulatory genes such as the *lys* gene of Hiproly.

The malting quality of barley

The major food use of barley is for malting, and the subsequent use of the malt for brewing or distilling. Malting quality is therefore a prime consideration for plant breeders, but only gradual improvements have been made using classical approaches. This is probably because malting quality is a complex character, depending on the interactions of a number of individual characters expressed both during grain development and during the malting process. The former result in differences in endosperm structure and composition (e.g. amounts of β-glucans, hordein storage proteins and β-amylase), while the

latter relate mainly to the production of hydrolytic enzymes (e.g. α-amylases, β-glucanases and proteases) and their effects on the endosperm cells and their storage components. These characters and their role in malting quality are still incompletely understood, and we will not attempt a detailed discussion here. Instead, we will focus on one character whose role has been the subject of considerable discussion and speculation. This is the amount, composition and spatial distribution of the hordein storage proteins.

It has long been known that high levels of grain protein are disadvantageous for malting, due to a negative correlation with hot water extract (Bishop, 1930a). Bishop (1930a) also showed that this was not solely due to mere dilution of starch, but that increases in respiration and rootlet growth also contributed to malting losses. Since the major effect of high grain nitrogen is an increase in the proportion of hordein storage proteins (Bishop, 1930b; Kirkman, Shewry and Miflin, 1982), this implies that a high level of hordein is a disadvantage. However, Bishop (1930a) and subsequent workers also appreciated that the magnitude of this effect varied with the variety. It was not until the late 1970s that improved methodology and increased knowledge of the structure and genetics of hordeins allowed this genetic effect to be examined in more detail.

The first studies of this type were reported by Baxter and Wainwright (1979a), who compared 16 varieties which varied in malting quality. They suggested that varieties with better quality had lower proportions of fast-moving (on electrophoresis at low pH) B hordein polypeptides. However, Shewry *et al.* (1980) were unable to confirm these observations using SDS-PAGE analyses of a wider range of malting and non-malting varieties of barley (28 in total). Similarly, Riggs *et al.* (1983) found no clear correlation between hordein pattern and malting quality in a collection of 84 cultivars, although they used an acid pH electrophoresis system similar to that used by Baxter and Wainwright (1979a).

Baxter and Wainwright (1979b) also drew attention to a further characteristic of hordein that could be relevant to malting quality – the proportion of the proteins present in high M_r polymers stabilized by disulphide bonds. We now know that these polymers consist predominately of D and B hordeins, but that some γ-hordeins may also be present. Baxter and Wainwright (1979b) extracted hordeins in two fractions: with 70% ethanol followed by the same solvent with a reducing agent (2-mercaptoethanol). They showed that the proportions of hordein present in these two fractions varied with genotype and environment, and that the second fraction (which presumably consisted mainly of polymers) was degraded less during malting than the first (mainly monomeric) fraction. Furthermore, the spent grains contained a higher proportion of polymeric hordeins than malt, suggesting that some hordeins became associated by disulphide bonds during mashing. The authors suggested that these polymeric hordeins could entrap starch granules resulting in poor extraction. Shewry *et al.* (1981) also showed that the proportion of aggregated hordeins was slightly greater in cultivars of low malting quality than in good-quality cultivars, while Smith and Lister (1983) showed a clear inverse correlation between malting quality and the amount of gel protein

(which consists predominately of polymers of D and B hordeins). They also suggested that the amount of gel protein was determined by the proportion of D hordein, which was synthesized at a disproportionately higher level in poor-quality cultivars as the grain nitrogen increased. This implies that an improvement in quality could be achieved by down-regulating the synthesis of D hordein, which appears a realistic target since only one D hordein gene per haploid genome appears to be present.

A further factor which may influence malting quality is the spatial distribution of hordein within the endosperm. Millet, Montembault and Autran (1991) compared the distribution of monomeric and polymeric hordeins in pearling fractions prepared from one good-quality and one poor-quality cultivar of barley, and showed that a higher proportion of the hordein was present in the peripheral layers of the poor-quality cultivar. In addition, a higher proportion of the hordein was present in polymers stabilized by disulphide bonds. It is not yet known whether similar differences are present between other good- and poor-quality cultivars, but it raises the intriguing possibility that hordeins may limit the rate of endosperm modification during malting of poor-quality cultivars by forming an insoluble gel around the periphery of the endosperm, thus hindering the diffusion of α-amylase and other hydrolytic enzymes.

So far, hordein has been discussed in relation to adverse, or at best neutral, effects on the quality for malting, brewing and distilling. However, there is one respect in which hordein may play a positive role, in conferring foam retention and cling (adhesion to the glass) to beer. These are important quality parameters for the consumer, and appear to be related to the presence of hydrophobic peptides (Bamforth, 1985a,b) that have similar amino acid compositions to hordeins (Dale, Young and Brewer, 1989; Dale and Young, 1989). However, their precise origins and structures remain to be determined.

It is clear from this brief discussion that the relationship between hordein and the quality of barley for malting, brewing and distilling is complex, and may involve the interactions of genotypic and environmental factors operating during grain development and germination with chemical reactions occurring during the subsequent technological processes (e.g. mashing). In terms of hordein composition, these factors may affect the proportions and properties of the individual polypeptides, their assembly into disulphide-stabilized polymers and their spatial distribution within the grain. Furthermore, it is probable that the same proteins have negative effects on some aspects of quality (e.g. modification and hot water extract) and positive effects on others (foam stability and cling). Since hordein is only one of a number of factors which affect quality for malting, brewing and distilling, it is not surprising that only slow progress has been made in effecting improvements by plant breeding. Genetic engineering will undoubtedly provide opportunities to manipulate some of the individual characters that contribute to quality (e.g. the proportion of D hordein), but we still have insufficient understanding of these characters at the biochemical and molecular levels to assess which approaches would be most likely to succeed.

The bread-making quality of wheat

Bread-making is one of humankind's oldest technologies, dating back to at least ancient Egyptian times. In addition, wheat gluten (corresponding to the prolamin fraction) was one of the earliest plant proteins to be studied, being first isolated in 1745 (Beccari, 1745), and has been the subject of a vast volume of research over the past century. Despite this effort, we have only recently started to gain an understandng of the detailed structures of the gluten proteins and their role in grain technology.

The wheat prolamins are deposited in the developing grain in protein bodies, which become disrupted during the later stages of endosperm maturation to form a proteinaceous matrix surrounding the starch granules. When a dough is made, these proteins form a cohesive network which can be isolated in a form that is substantially pure by washing to remove starch granules and water-soluble components. Gluten plays a key role in bread-making, by enabling the entrapment and retention of the carbon dioxide produced by fermentation to give a light porous crumb structure which is fixed by baking. The ability to do this depends on a combination of two physical properties: elasticity and extensibility (viscous flow). A precise balance of these is essential, and the poor quality of many wheats (including some UK wheats) is related to insufficient elasticity.

The gluten proteins of wheat are usually classified into two groups: the gliadins are single monomeric proteins, while the glutenins consist of individual subunits that are assembled into high M_r polymers stabilized by inter-chain disulphide bonds. The individual gliadin monomers and glutenin polymers also interact by strong non-covalent forces, notably hydrogen bonds and hydrophobic interactions. These two fractions also have functional significance, as the glutenins are associated with elasticity and the gliadins with extensibility.

Electrophoretic analyses of total prolamin fractions from bread wheat show a very high degree of polymorphism, with at least 50 individual proteins revealed by two-dimensional IEF/SDS-PAGE under reducing conditions (*Figure 6*). The degree of polymorphism is considerably greater than that observed in barley (cf. *Figures 3 and 6*), which is at least partly due to the allohexaploid nature of bread wheat. Bread wheat has three genomes (called A, B and D), each consisting of seven pairs of chromosomes. Because these genomes are derived from related species of wild grass, there is much repetition of genetic information, with related genes often present at similar positions on the homoeologous (homologous but non-pairing) chromosomes of the three genomes. Thus in bread wheat the gliadins and glutenin subunits are encoded by loci on all three pairs of the group 1 (1A, 1B, 1D) and group 6 (6A, 6B, 6D) chromosomes (see Payne, 1987). It is of interest that the group 1 chromosomes are homeologues of chromosome 5 (1H) of barley, which is the location of all the hordein genes (Shewry *et al.*, 1990).

As in barley, it is possible to classify the individual gluten proteins into several groups. The gliadins are classified into three groups, α-, γ- and ω-, in order of decreasing mobility when separated by electrophoresis at low pH.

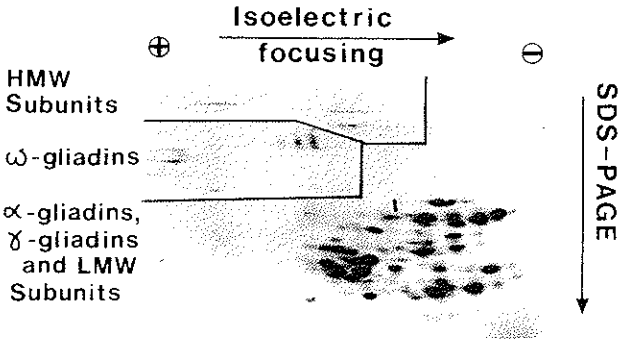


Figure 6. Two-dimensional IEF/SDS-PAGE of total prolamins from wheat cv Chinese Spring. The electrophoretic separation is from Shewry *et al.* (1987a).

The ω- and γ-gliadins are homologues of C and γ-hordeins, respectively, and are encoded by genes on the group 1 chromosomes. In contrast, the α-gliadins have no precise homologues in barley, and are encoded by genes on the group 6 chromosomes. The glutenin subunits are similarly divided into groups, but on the basis of mobility by SDS-PAGE. The HMW subunits are homologues of barley D hordein, while the major group of LMW subunits is most closely related to B hordeins. These two groups of proteins are also encoded by genes on the group 1 chromosomes.

There is no doubt that all these proteins contribute to the structure and functionality of wheat gluten, but two lines of evidence indicate that one group is particularly important in determining dough quality. This is the HMW subunits of glutenin. Cultivars of bread wheat contain three, four or five individual HMW subunits, which are divided into high M_r x-types and low M_r y-types (Payne, Holt and Law, 1981). They are encoded by single loci on the long arms of chromosomes 1A, 1B and 1D, each locus consisting of two genes: one x-type and one y-type (Harberd, Bartels and Thompson, 1986). Specific silencing of one, two or three of these genes results in the observed variation in HMW subunit number (*Figure 7*). Thus cultivars always contain 1Dx, 1Dy and 1Bx subunits, sometimes 1By and 1Ax subunits, but never 1Ay subunits. In addition to variation in subunit number, there is also allelic variation in the structures of the individual subunits, which is observed as differences in their mobilities in SDS-PAGE. A number of correlative studies have shown that this allelic variation in subunit number and mobility is correlated with differences in bread-making quality, providing the first line of evidence that the HMW subunits play a major role in determining gluten functionality (see Payne, 1987). On the basis of detailed comparisons, it has been possible to assign 'bread-making scores' to all the individual HMW subunits (Payne *et al.*, 1987). However, the most striking correlations are with the presence or absence of a 1Ax subunit, and with allelic variation in the subunit pair encoded by chromosome 1D (1Dx + 1Dy).

The second line of evidence for the importance of the HMW subunits comes from biochemical studies. It has long been known that good quality is associated with a high proportion of high M_r (above about 1 million) glutenin

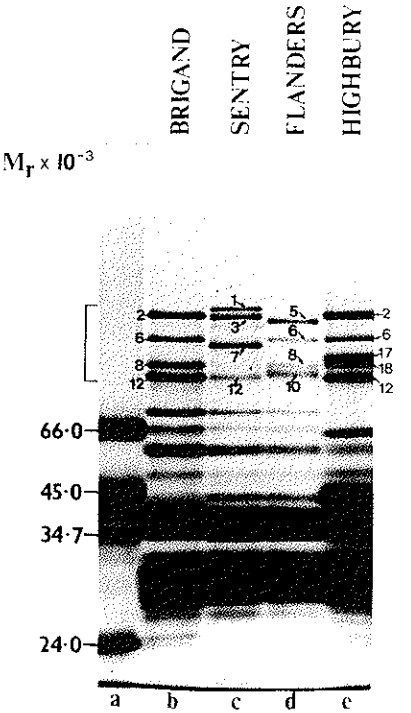


Figure 7. SDS-PAGE of total prolamins of four cultivars of wheat, illustrating variation in the number and mobilities of the HMW subunits (numbered according to Payne and Lawrence, 1983). Taken from Shewry and Tatham (1989).

polymers, and biochemical studies demonstrate that such polymers are enriched in HMW subunits (Field *et al.*, 1983). In contrast, the LMW subunits are present in polymers of high and low M_r.

In order to understand the role of the HMW subunits in gluten structure and functionality, it is necessary to determine the structures of the individual proteins, their interactions with other glutenin subunits to form polymers, and the properties and interactions of the polymers that are formed. Although our knowledge is still incomplete, studies carried out over the last decade are now providing valuable information on these subjects (see Shewry, Halford and Tatham, 1989, 1992).

A total of nine genes encoding different HMW subunits have been isolated and characterized (see Shewry *et al.*, 1989; Halford *et al.*, 1992). These include the complete family of six genes from cv Cheyenne: expressed genes encoding subunits 1Ax2*, 1Bx7, By9, 1Dx5 and 1Dy10, and a silent gene of the 1Ay type (*Figure 8*). In addition, genes for the allelic subunits 1Ax1, 1Dx2 and 1Dy12 have been isolated from other cultivars.

Analysis of the sequences of the encoded proteins show that they have similar structures, consisting of between about 480 and 700 residues, with M_rs of about 67 500–88 000 (see Shewry, Halford and Tatham, 1989). All consist of three structural domains – a long repetitive central domain which is flanked by shorter non-repetitive domains at the *N*-terminus (81–104 residues) and

C-terminus (42 residues in all proteins). The repetitive domains range in size from about 480 to 700 residues, and are largely responsible for the differences in M_rs of the whole proteins. The x-type and y-type proteins have similar structures (*Figure 8*), but differ in their detailed repeat structure. Whereas the y-type subunit repeats are based on hexapeptide (PGQGQQ) and nonapeptide (GYYPTSLQQ) motifs, the x-type subunits contain an additional tripeptide motif (GQQ) and the consensus nonapeptide motif differs in the substitution of P for L at position 7. In addition, the two subunit types differ in their number of cysteine residues. Whereas a single cysteine is present in the C-terminal domains of all subunits, only three cysteines are present in the N-terminal domains of the x-type subunits compared to five in the y-type. Additional cysteine residues are also present within the repeats of some subunits only (*Figure 9*).

A combination of predictive hydrodynamic and spectroscopic studies indicate that the repetitive sequences form an unusual supersecondary structure, a loose spiral structure based on repetitive β-turns, giving an extended rod-like conformation to the whole molecule (see Shewry, Halford and Tatham, 1989). This has recently been confirmed by the application of scanning tunnelling microscopy to image a purified protein fraction (Miles *et al.*, 1991). This showed aligned rods of diameter about 19.5 Å, with diagonal striations (presumably representing the turns of the spiral) with a periodicity of about 15 Å. The N- and C-termini were not imaged in this study, but are predicted to be globular with high contents of α-helix.

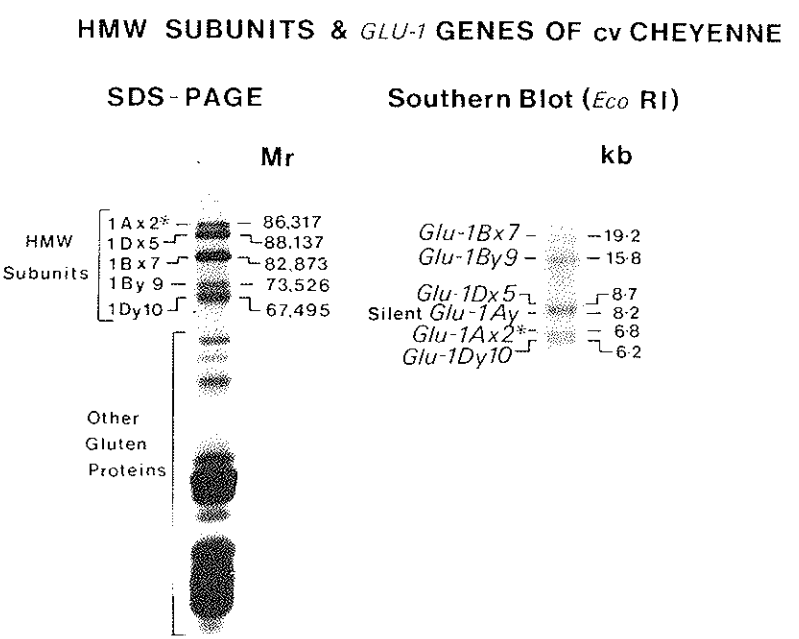


Figure 8. The HMW subunit proteins, separated by SDS-PAGE, and the *Glu-1* genes, determined by Southern blotting, of wheat cv Cheyenne. Taken from Shewry, Halford and Tatham (1989).

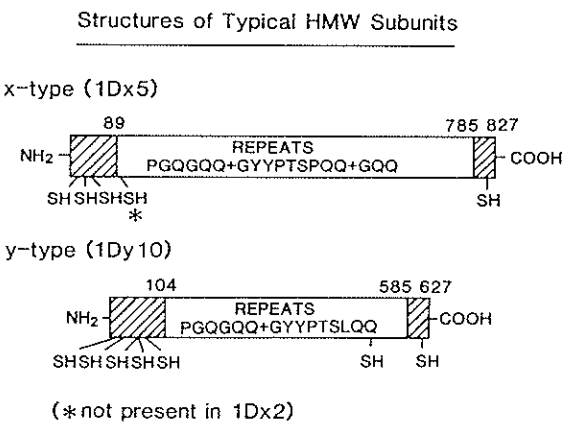


Figure 9. The structures of typical x-type and y-type HMW subunits. Based on sequences reported in Anderson *et al.* (1989).

What are the implications of HMW subunit structure for the formation and properties of glutenin polymers? An important factor is, of course, the number and distribution of cysteine residues. The presence of most of the cysteine residues in the *N*- and *C*-terminal domains would be expected to lead to predominantly head-to-tail polymers, with cross-links and branches arising from the additional cysteines present in the *N*-terminal domains and in the repetitive domains of some subunits. Despite impressive attempts to unravel the disulphide structure of wheat glutenin (Köhler, Belitz and Wieser, 1991, 1993; Tao, Adalsteins and Kasarda, 1992), we still know very little about the precise patterns of disulphide bond formation of the HMW subunits, and in particular about which cysteine residues form intra-chain or inter-chain disulphide bonds. There is, of course, the added complication that it is theoretically possible for the same cysteine residue to form an intra-chain bond (as reported by Köhler, Beltiz and Wieser, 1993), or an inter-chain bond with an identical subunit (as reported by Köhler, Belitz and Wieser, 1991), with a different type of subunit (x or y: as reported by Tao, Adalsteins and Kasarda, 1992), with a different subunit of the same type, or with an LMW subunit. It is probable that the number and distribution of such bonds will affect the elasticity of the glutenin polymers, in the same way that the degree of cross-linking determines the elastic modulus of rubbers (Flory, 1953). Although it has been proposed that the β-spiral conformation also contributes to glutenin elasticity (Tatham, Shewry and Miflin, 1984), recent studies cast doubt on this (Belton *et al.*, in press).

Assuming that glutenin cross-linking is the major determinant of glutenin elasticity (and hence bread-making quality), what can we say about the quality differences associated with allelic variation in HMW subunit composition? We believe that these arise from quantitative and qualitative effects. The former will be related to differences in the number of expressed genes, and in their relative levels of expression. Halford *et al.* (1992b) compared the total amounts of HMW subunit protein in cultivars with four and five expressed genes, showing that this represented about 8 and 10% of the total

extracted protein, respectively. The five subunit cultivars all had subunit 1Ax1 or 1Ax2*, whose presence has been shown to be associated with good quality. The implication is that the increased amount of HMW subunit protein in these cultivars may lead to a greater amount of high M_r polymers, with a resulting effect on quality (see above). Although this study indicated that each 1Ax gene accounted for about 2% of the total extracted proteins, there were statistically significant differences between the total amounts of HMW subunit protein in cultivars with subunits 1Ax2* and 1Ax1, and in cultivars where these subunits were expressed in the presence of subunits 1Bx7 + 1By9 or 1Bx7 + 1By8. These results, and those of studies in other laboratories, indicate that differences in HMW subunit amount also occur due to variation in the level of gene expression. These differences would also be expected to result in differences in quality.

Qualitative effects on wheat quality may arise from differences in the structures of allelic subunits. The most notable variation of this type is between the allelic pairs of subunits 1Dx5 + 1Dy10 (good quality) and 1Dx2 + 1Dy12 (poor quality). Because recombination between the genes encoding 1Dx and 1Dy subunits is very rare, it is not possible to conclude whether the quality correlations are due to differences between the allelic 1Dx subunits (1Dx2 and 1Dx5), the allelic 1Dy subunits (1Dy10 and 1Dy12) or the allelic pairs. However, comparisons of the amino acid sequences of these four subunits show only one striking difference between the two pairs of alleles. This is the presence of a cysteine residue towards the *N*-terminal end of the repetitive domain of subunit 1Dx5 (*see Figure 9*), which is not present in subunit 1Dx2. This could lead to a higher degree of cross-linking of the glutenin polymers, and hence greater gluten elasticity.

It is clear from the brief summary presented here that we now know a considerable amount about the structures of the individual HMW subunits, and their potential roles in gluten structure and functionality. It should, therefore, be relatively straightforward to design strategies to manipulate gluten structure to improve quality. The two obvious strategies to increase the elasticity of gluten would be to manipulate the amounts or structures (i.e. cross-linking) of the high M_r glutenin polymers, by increasing the total amount of HMW subunit protein or by introducing additional cross-linking sites (i.e. cysteine residues). However, more subtle alterations to the structure of glutenin could also be made to optimize the quality for other end uses (e.g. noodles, biscuits, wafers and cakes), by manipulating the amounts and/or properties of other gluten proteins such as the gliadins or LMW subunits.

Other uses of wheat and gluten

Wheat is, of course, used for many other food products in addition to bread. These include baked goods (biscuits, cakes and pastries), noodles and pasta, and various batters, breadings and coatings. In addition, isolated gluten is used as a binder and filler, for example in processed meat products and pet foods. All of these uses depend on the unusual physical properties of the

gluten proteins, but the precise balance of properties that is required differs. For example, less elastic gluten is required for biscuits, pastries and cakes than for bread-making, and it is usual to make these products with weak varieties.

A considerable volume of research has been carried out on the quality of wheats for pasta-making, including the identification of protein markers which can be followed in plant-breeding programmes (similar to the HMW subunits of glutenin and bread-making quality) (see MacRitchie, DuCros and Wrigley, 1990). The quality requirements for other food applications are less well understood, and vary considerably between different products (for example, different types of biscuit).

Understanding the molecular basis for the physical properties of gluten, and in particular for the balance between elasticity and extensibility, will undoubtedly facilitate the use of wheats for all these products, and will almost certainly also reduce the reliance on chemical improvers to produce the required properties. However, as with cereal starches (see below), the most important impact may well be on the development of new uses, particularly for isolated gluten.

Wheat gluten is readily separated from the other major grain component, starch, in industrial separation plants. However, whereas the starch finds a number of uses (see below), most of the gluten is used in baking, either to fortify poor-quality flours or for speciality products that require a high content of gluten. The latter include wholemeal and high-fibre breads, and hamburger buns. The other major use of isolated gluten is as a binder, most notably in pet foods.

At present, the supply of gluten and the demand from the bread-making and other industries are pretty well in balance, but several factors could result in a surplus of gluten for other uses. For example, further improvement in the intrinsic bread-making quality of European wheats would require less gluten for fortification, while more gluten would also be produced as a by-product of the substitution of wheat starch for imported maize starch. This could lead to the use of gluten to produce textured protein products, or to produce protein emulsifiers or foaming agents. Once again, a detailed understanding of gluten structure and functionality will be critical for the development of such uses, whether based on genetic engineering to produce specific cultivars or on downstream processing of isolated gluten (for example, by partial enzymic digestion or partial deamidation by acid hydrolysis).

Novel protein products

There has been considerable discussion about using plant storage organs to produce high-value chemicals, including biologically active peptides and proteins. The feasibility of this has been demonstrated by the production of functional antibodies in the leaves of transgenic tobacco plants (obtained by crossing plants independently transformed with genes encoding gamma or kappa immunoglobulin chains) (Hiatt, Cafferkey and Bowdish, 1989), and by the elegant studies of Krebbers, Vandekerckhove and co-workers at Ghent

(see Krebbers, Bosch and Vandekerckhove, 1992; Krebbers *et al.*, 1993). The Ghent group have used a 2S albumin storage protein from *Arabidopsis* as host for two biologically active peptides: the six residue peptide Leu-enkephalin and magainin, a 28 residue antibacterial peptide from *Xenopus*. This was done by engineering the cDNA to replace a variable loop region with sequence encoding the biological peptide, flanked by cleavage sites to facilitate purification. The cDNAs were then expressed in seeds of transgenic *Arabidopsis* or oilseed rape, the 2S albumins purified and the peptide excised. Yields of up to 200 nmol per g seed were obtained which would correspond to about 1 kg of a 25 residue peptide per hectare of oilseed rape (Krebbers *et al.*, 1993). However, the authors consider that yields up to five times greater could be obtained using stronger promoters.

Although Krebbers and co-workers have used oilseed rape for their studies, it is clear that a similar approach could be used with cereals. In fact, cereals would probably be the preferred hosts because of their high yields and the ease of harvest and processing (compared to oilseed rape, where up to about 30% of the seeds can be lost before or during harvest). However, the extent to which this approach is finally adopted will probably depend on regulatory and economic factors rather than biological constraints.

MANIPULATING STARCH CONTENT AND COMPOSITION

Starch composition and synthesis

Because starch is the major storage compound in cereal grains, it is an obvious target for manipulation, in order to increase its total amount (and hence yield) or its composition and properties.

Starch is not a single compound but a mixture of two complex polymers called amylose and amlyopectin. Amylose is a linear chain of between 1000 and 10 000 D-glucopyranosyl units, with α1-4 linkages. Amylopectin is also composed of D-glucopyranosyl residues, but has a highly branched structure due to the presence of α1-6 linkages as well as α1-4 linkages. The average chain length between branches is about 20–25 and the total degree of polymerization about 1000. The properties of starches are determined to a large extent by the proportions of these polymers (which vary between ~70–85% amylopectin and ~15–30% amylose in cereals), their precise structures (i.e. chain lengths and degrees of branching) and their organization within the granule. These properties include the density, gelatinization temperature and crystallinity index, and affect the utilization in the food and other industries. The basic enzymology of starch synthesis has been reviewed by several authors (Preiss, 1988; Smith and Martin, in press) and is summarized in *Figure 10*.

It appears that ADP glucose is the major building block for starch synthesis, being produced by the reaction of glucose-1-phosphate with ATP. This reaction, catalysed by ADP glucose pyrophosphorylase (an enzyme consisting of two different subunits), is the first committed reaction in starch synthesis and is considered to be an important regulatory step. The glucosyl

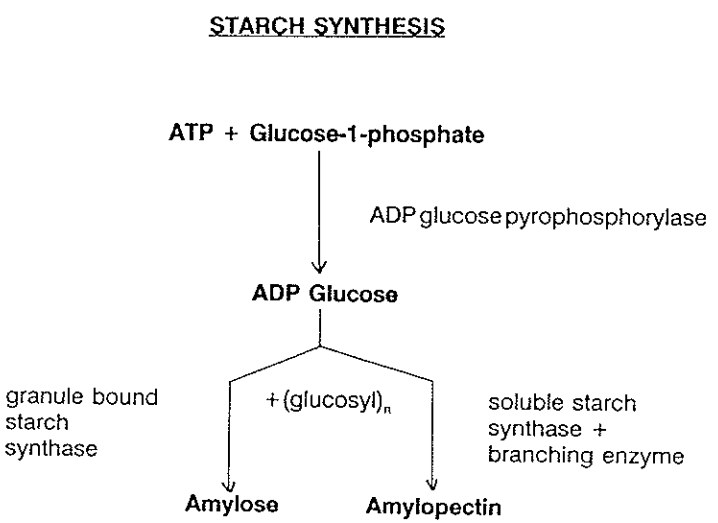


Figure 10. Summary of the enzymology of starch synthesis.

unit is then transferred to an α-glucan primer, forming an α1-4 linkage. This reaction is catalysed by starch synthases which occur in two classes (soluble and granule-bound) and, in most species, in two forms within each class. Analyses of mutant lines (see below) indicate that the bound forms are mainly responsible for the synthesis of amylose and the soluble forms for the synthesis of amylopectin. Finally, the branch points in amylopectins are introduced by branching enzyme (which again exists in several forms), which hydrolyses α1-4 bonds and transfers short oligosaccharides to form α1-6 linkages. Recent studies have also shown that debranching enzyme is active in developing endosperms of maize, and it has been suggested that the precise structure of amylopectin results from an equilibrium between the activities of branching and debranching enzymes (Preiss, 1988).

Genes and/or cDNAs have been cloned for several of these enzymes, either from cereals or from other species. The cereal enzymes which have been cloned include the two subunits of ADP glucose pyrophosphorylase (rice, wheat) and forms of granule-bound starch synthase (maize, barley and wheat) (see Smith and Martin, in press). Mutant genes encoding some starch biosynthetic enzymes have also been identified, and these result in changes in the composition of starch and a reduction in the total amount. These mutations also result in compensatory increases in soluble sugars, and could provide an opportunity for the diversion of carbon into alternative pathways, for example lipid synthesis. The feasibility of blocking starch synthesis by genetic engineering has been elegantly demonstrated by Willmitzer and colleagues, who used antisense technology to eliminate the activity of one of the two subunits of ADP glucose pyrophosphorylase from potato tubers (see Smith and Martin, in press). The result was an absence of starch and the accumulation of sucrose.

Manipulating starch synthesis in cereals

The potential to increase the accumulation of starch in developing cereal endosperms is uncertain, due to lack of information about the control of flux through the pathway and the relative importance of source and sink activities. However, Smith and Martin (in press) have suggested that ADP glucose pyrophosphorylase may be the best target, using the bacterial enzyme which consists of only one subunit compared to two in higher plants.

As discussed above, several types of mutants with altered starch composition have been described. The *waxy* genes of maize, barley and rice have increased amylopectin, and may encode the granule-bound starch synthase I. In contrast, the *amylose extender* (*ae*) genes of maize and barley result in high amylose, and may encode forms of branching enzyme. A further mutant of maize, called *sugary 1*, contains about 25% dry weight of phytoglycogen, a water-soluble glucan which is more highly branched than amylopectin. This may result from decreased activity of debranching enzyme disturbing the equilibrium between the formation and removal of branches. These mutants are important in relation to genetic engineering, as they demonstrate that changes in the amounts of single enzyme proteins can have major effects on starch composition. This is despite the fact that most of the enzymes occur in more than one form.

It is anticipated, therefore, that the composition of cereal starches could be altered by the insertion of genes to either increase the activities of intrinsic enzymes or introduce novel activities, or by the use of antisense technology to reduce the activities of other enzymes. For example, the proportion of amylopectin could be increased by reducing granule-bound starch synthase and/or introducing glycogen branching enzyme, while the proportion of amylose could be increased by reducing branching enzyme and/or increasing debranching enzyme. The consequences of such changes for seed development and germination are not known, but the occurrence of *waxy*, *amylose extender* and *sugary* mutants demonstrates that wide variation in starch composition can be tolerated.

Current and novel end uses of cereal starches

Assuming, therefore, that the composition of starch could be readily altered by genetic engineering, it is of interest to consider what types of alterations would be required. At present, about two-thirds of the starch produced in Western Europe is used in the food and drink industries, and most of this for enzymic hydrolysis to give syrups for confectionery and soft drinks. In this case, engineering could possibly be used to make the polymer more susceptible to enzymic digestion (Kennedy, Cabalda and White, 1988). Starch with a normal amylose : amylopectin ratio also finds a range of uses in the food industry, both in the unmodified state and after chemical modification (e.g. oxidation, esterification and phosphorylation), while starches from *waxy* and *amylose extender* types are also produced for specific uses (Dunn, 1985). The functional properties of these starches are determined by their physical

properties such as gelatinization temperature, density, crystallinity index, freeze–thaw stability and resistance to water separation. These properties could possibly be optimized by genetic engineering, reducing the need for chemical modifications with associated legal constraints concerned with food safety (Galliard, 1985).

Although the food industry is currently the major consumer of starch, there is no doubt that the greatest opportunity for increased consumption lies in non-food products. At present, these account for only about a third of starch production, with the paper and board and the chemical and pharmaceutical industries being the main users. Munck, Rexen and Haastrup (1988) identified three areas in which increased use of starch can be expected.

The first is in the cellulose industry. The growing demand for paper and packaging will lead to increased use of non-traditional fibres (e.g. from recycled products and straw) which are shorter than the traditional hardwood fibres. Laboratory tests show that up to 30% starch can be incorporated to increase the strength and maintain a low cost. The second area is in the production of chemicals and pharmaceuticals. In this case, starch will be used as a substrate for fermentation, and similar considerations apply as for production of syrups for the food and drink industry. Some bulk chemicals are already produced by starch fermentation (e.g. ethanol, glycerol, acetone), but the range can be expected to increase. The third, and perhaps the most exciting, of the potential new outlets is in the production of synthetic polymers. Starch can be used as a filler (e.g. in PVC) or as a graft co-polymer with synthetic polymers. It can also be used for the production of phenol resin as a substitute for formaldehyde, and to produce polyols which find uses in various products including polyurethane foams.

The extent to which such applications take off will, of course, depend on a number of factors – environmental as well as economic. They have the advantages that they will allow the utilization of agricultural surpluses and conserve fossil fuels in favour of a renewable resource, while the products and by-products will be more biodegradable and biocompatible. In addition, the cosmetic, pharmaceutical and chemical products can be expected to be non-irritant and non-allergenic, with the consumer appeal of being 'natural'. Developing a sound understanding of the control of starch synthesis and composition in cereals will undoubtedly make an important contribution to such developments.

REDESIGNING GRAIN COMPOSITION

Despite the wide range of morphological variation in cereals and their adaptation to a range of climatic zones and environmental extremes, there is very little variation in grain composition. Thus protein accounts on average for about 11–12% of the grain dry weight, and starch for about 75% of the dry weight in barley, rye, wheat and maize, but slightly less in sweetcorn and oats. These minor differences in starch content are associated with differences in total lipids, from about 2–3% dry weight in barley, wheat and rye to 5% in maize, 8% in oats and 9% in sweetcorn. Although these proportions vary to a

certain extent within species (for example, in relation to nitrogen nutrition), this is limited when compared with the range of variation between seeds of other species. For example, protein can account for up to almost 40% of dry weight in soybean and cotton, and lipid for up to 50% in peanut, oilseed rape and oilpalm, and over 60% in castor bean and brazil nut. Similarly, the proportion of starch varies from less than 10% of dry weight in brazil nut, to 15–30% in temperate oilseeds (rape, cotton, soybean) and over 75% in cereals (see Bewley and Black, 1978). At present, maize is the only cereal that is used to produce oil, although oats could also be used if problems of stability could be eliminated.

The protein content of cereals is regulated to some extent by nutrition, as higher levels of nitrogen fertilization result in increased levels of grain protein. For example, the total nitrogen content of field-grown barley in the UK has been reported to vary between 1.27 and 2.0% dry weight (Kirkman, Shewry and Miflin, 1982). However, even excessive fertilization would not result in the production of protein to a level of 40% of dry weight, as in soybean. It appears, therefore, that there are two levels of control on cereal grain protein content: an environmental control operating within genetically determined limits. Recent studies indicate that the expression of cereal prolamin genes is controlled by the levels of free amino acids in the endosperm, so we may need to increase the transport of amino acids into the grain if we wish to increase the protein content beyond the current levels (Balconi *et al.*, 1991; Müller and Knudsen, 1993). This would clearly require an understanding of the nitrogen economy of the whole plant, including the mechanisms of uptake, assimilation and long-range transport. It may therefore still be some time before we have sufficient understanding to manipulate cereal grain protein content. However, of much greater potential value, and of more immediate interest, is the manipulation of cereals to produce larger amounts of lipids.

At present, a range of plant lipids are required for specific food and non-food uses, coming principally from two types of plant: the tropical palms (oilpalm and coconut) and cocoa, and a range of temperate species (e.g. sunflower, cotton, soybean, oilseed rape, safflower, linseed and various minor species). A wide range of species are used because of their characteristic oils and their adaptation to different climates. The palms are used in particular to provide short chain fatty acids for soaps and detergents, and cocoa to provide specific types of triacylglycerol for chocolate and confectionery, but both have a limited geographical range. Similarly, the major temperate oilseeds also have limited ranges, and do not produce the full spectrum of oils required for food and industrial applications. Finally, a number of minor species are currently of interest in that they produce oils with specific pharmaceutical or industrial applications, but these have poor agronomic characteristics and low yields (Röbbelen, 1991). The production of such oils in cereals would open new opportunities for the industrial use of plant products. Thus it would be of value to produce cereals with a range of lipids for specific end uses as well as high levels of total lipid.

Triacylglycerols (triglycerides) consist of three fatty acid molecules esteri-

fied to the three OH groups of glycerol. Their properties depend on the structures of the individual fatty acids (chain length, number and positions of double bonds, presence of other groups such as hydroxyl and epoxy) and on their spatial distribution on the glycerol molecule. Most triacylglycerols from plant sources have high proportions of C16 and C18 fatty acids, with zero or one double bond in the former and zero to three in the latter. However, fatty acids of longer or shorter chain length and with more double bonds or other modifications occur in some seed oils and have specific industrial or food applications.

The first reaction in fatty acid synthesis is the carboxylation of acetyl CoA to give malonyl CoA, catalysed by acetyl CoA carboxylase (ACCase). This is a key regulatory step in animal systems, the enzyme activity being controlled by phosphorylation and dephosphorylation (Davies *et al.*, 1992). The phosphorylation reaction is catalysed by a specific protein kinase which also regulates the activity of HMG CoA reductase, which catalyses the first reaction in terpenoid synthesis (Davies *et al.*, 1992). In plants, ACCase and the initial elongation (by the addition of 2 carbon units) and desaturation reactions occur in the plastid, to give fatty acids with up to 18 carbon atoms and one double bond. Further elongation and desaturation reactions, other modifications (e.g. hydroxylation) and triacylglycerol assembly then take place in the endoplasmic reticulum (Slabas *et al.*, 1993; Stymne and Stobart, 1993).

ACCase is an obvious target for attempts to increase the flux of carbon into fatty acids, and several laboratories are making good progress in characterizing the enzyme and isolating corresponding DNA clones from oilseeds. However, an alternative approach is to assume that the flux is controlled not by the total amount of enzyme protein, but by regulation of its activity by reversible phosphorylation. MacKintosh *et al.* (1992) have recently purified the plant HMG CoA kinase and shown that this also phosphorylates human ACCase but not plant ACCase. The regulation of both enzymic activities by a single kinase would in any case be unlikely, since ACCase and HMG Co reductase have different subcellular locations in plants (in the plastid and endoplasmic reticulum, respectively). However, the presence of a separate and specific ACCase kinase in plants cannot be ruled out.

At Long Ashton, we are studying a family of plant protein kinases which may also play a regulatory role in carbon metabolism in the developing endosperm. These are related to the *Sucrose Non-Fermenting 1 (SNF1)* gene of yeast, which controls a carbon catabolite repression system (Celenza and Carlson, 1986). Thus in the presence of glucose, the *SNF1* gene acts in concert with other regulatory genes to repress the synthesis of a number of enzymes including invertase, and *snf1* mutants cannot grow on substrates such as sucrose, maltose, galactose, melibiose, glycerol and ethanol. We have shown that a cDNA prepared using rye endosperm mRNA can functionally complement the *snf1* mutant of yeast, enabling it to grow with glycerol as a substrate (Alderson *et al.*, 1991). In addition, barley contains at least two families of *SNF1*-related genes, one of which appears to be expressed in all tissues, and the other only in aleurones and starchy endosperms (Halford *et*

al., 1992a; Hannappel *et al.*, in prep.). The roles of these genes are not known, but the endosperm-specific form may well play a role in regulating carbon metabolism in the developing seed.

Any attempt to increase lipid synthesis, for example by increasing the synthesis and/or activation of ACCase, may well need to be accompanied by a decrease in the activity of starch synthesis by using an available mutant or down-regulating ADP glucose pyrophosphorylation) in order to provide an adequate supply of carbon skeletons. In addition, it is not known whether the activities of the elongases, desaturases, etc, that catalyse subsequent steps in fatty acid synthesis, would also need to be increased. Finally, there may also be an opportunity to produce lipids rich in specific fatty acids by incorporating enzyme systems from other species, for example the 14-hydroxylase from castor bean to synthesize ricinoleic acid for industrial use, or the $\triangle 6$ desaturase from evening primrose or borage to synthesize γ-linolenic acid for pharmaceutical use. These and other opportunities are discussed in a number of recent reviews of oilseed manipulation (see, e.g. Stobart, Stymne and Shewry, 1992; Slabas *et al.*, 1993; Safford, 1993; Lapinskas, 1993).

Acknowledgement

We are grateful to Dr Pilar Barcelo (Universität Hamburg) for providing the photographs used in *Figure 2*.

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