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USE OF CELL AND MOLECULAR GENETIC MANIPULATION TO IMPROVE PASTURE PLANTS

D. W. R. White
Grasslands Division, DSIR, Palmerston North

Abstract

Cell culture and genetic engineering techniques can be used to develop improved pasture plants. To utilise these methods we have developed procedures for regenerating plants from tissue cultures of perennial ryegrass and white clover. In both, the plant genotype influences regeneration capacity. There was significant genetic variation among regenerated perennial ryegrass plants in a wide range of characteristics. Most of the regenerants were resistant to crown rust and this trait was highly heritable. This rust resistance is being used to breed a new ryegrass cultivar. A system for introducing cloned genes into white clover is described. This capability is being used to incorporate genes with the potential to improve nutritional quality and pest resistance. Other possibilities for engineering genetic improvements in white clover, genes conferring herbicide tolerance and resistance to white clover mosaic virus, are briefly outlined.

Keywords: *Lolium perenne, Trifolium repens,* cell culture, somaclonal variation, crown rust resistance, transformation, cloned genes, nutritional quality, proteinase inhibitors, B.t. toxins, pest resistance, WCMV. viral cross-protection, herbicide *tolerance, Agrobacterium, Bacillus thuringiensis.*

INTRODUCTION

Cell culture and gene transfer techniques can be used to make genetic improvements in economically important plants (White 1983). The basis of these techniques is the capability to regenerate whole plants from cultured cells. During a period of unorganized proliferation in culture, plant cells can undergo extensive permanent genetic alteration. The frequency of variation generated in cell culture can be very high and is reflected in the genetic variation among regenerated plants (Larkin and Scowcroft 1981). Plants regenerated from cell culture have been termed 'somaclones', and variation among them 'somaclonal variation'. In this article I describe a high level of heritable crown rust resistance obtained among somaclones of perennial ryegrass.

Recently, efficient procedures for transferring one or a few cloned genes into higher plants have been developed. Use has been made of the naturally occurring capacity of the soil bacterium *Agrobacterium* to transfer some of its genes to plant cells. Normally this causes a disease known as a crown gall. A specific portion (T-DNA) of a large extrachromosomal replicon (plasmid) containing genes for hormone independent growth is transferred into the plant cell and integrated into the plant genome. Transfer of the T-DNA is mediated by genes on a separate part of the same plasmid. This sytem has been modified so that normal plants can be regenerated from transformed cells. Transformants can be selected by introducing a bacterial gene encoding antibiotic (kanamycin) resistance. This gene has been modified by recombinant DNA techniques to that it is constitutively expressed in plant cells. This ability to engineer the level and tissue specificity of gene expression of an introduced cloned gene is crucial to the use of molecular genetics for plant improvement.

Here I describe the development of a method for introducing cloned genes into white clover and review some of the prospects for improving this species using gene manipulation techniques.

MATERIALS AND METHODS

Perennial ryegrass tissue culture

Immature seed was collected from field grown, open pollinated, spaced plants of a late flowering perennial ryegrass selection, usually about 14 days after anthesis, and surface

sterilized. Immature embryos were dissected out and placed on Murashige and Skoog (1962) medium (MS) containing 2 mg/L, 2,4-D and 0.01 mg/L 6-benzyl aminopurine (BAP), to induce callus formation, Cultures were maintained at 28°C in continuous light and transferred to fresh callusing medium or to regeneration medium (MS with 0.01 mg/L BAP), at 3-week intervals.

Testing for crown resistance

Mass collections of crown rust were made from perennial ryegrass plants growing in field plots at DSIR, Grasslands Division, Palmerston North. Urediospores for the screening tests were diluted 1:30 (w/w) with talc and used as a dry inoculum. Twenty highly susceptible Grasslands Ruanui plants were used as controls. The glasshouse-grown plants were subjected to high humidity overnight to achieve infection and scored for infection type 2 and 3 weeks after inoculation. The crown rust infection type classification used is given in Table 1.

White clover transformation

A white clover genotype selected for its capacity for regenerating from cell culture (White 1984), was used as a recipient in genetic transformation experiments. Foreign cloned gene introduction was accomplished using an *Agrobacterium tumefaciens* mediated binary vector strategy (White and Greenwood 1987). Essentially, the desired gene constructs were cloned into a binary vector (Bevan 1984), mobilized into *A. tumefaciens* by conjugation and incorporated into the white clover genome by inoculating surface-sterilized stolon segments. Growth of transformed cells was selected on kanamycin containing medium and regenerated plants were checked for the presence of the integrated DNA sequence (White and Greenwood 1987).

Table 1: Infection type classification of crown rust (*Puccinia coronata*) on perennial ryegrass

0.	no symptoms.
1.	chlorotic flecking but no pustule development.
2.	chlorotic flecking with occasional small pustules.
3.	small pustules with associated chlorisis.
4.	large pustules with associated chlorosis low density.
5.	high density of large pustules with associated chlorosis.

RESULTS AND DISCUSSION

Somaclonal variation in perennial ryegrass

Most plant regeneration from immature embryo callus of perennial ryegrass is by the formation of somatic embryos (i.e. somatic embryogensis) and the frequency of regeneration is influenced by the maternal genotype (White, in preparation). After the first subculture most of the plants regenerated are albinos. An exception to this pattern was obtained from a callus line initiated from a single immature embryo which regenerated plants by shoot formation. From this callus line (SR1) 104 somaclones were regenerated during a 12-week culture period. Analysis in glasshouse and field trials has established that there is significant variation among these somaclones, but not within the clones, for a range of growth, morphological and developmental characters. Surprisingly, all of the clones examined had a normal chromosome number and were fertile (White, Easton and Whelan, in preparation). This somaclonal variation in the absence of karyotypic abnormality is an exception to the high incidence of polyploidy and aneuploidy which often accompanies somaclonal variation (Skirvin 1978, Larkin and Scowcroft 1981).

Preliminary glasshouse and field observations indicated that most of the somaclones were resistant to crown rust. Glasshouse inoculation experiments confirmed that a major portion of the somaclones were highly resistant to crown rust infection (Figure 1 a). However, there was also considerable variation in infection type score among the somaclones, although only a few were classified as susceptible (infection types 4 & 5). All the 20 susceptible control plants developed infection type 5 symptoms. While this result shows that

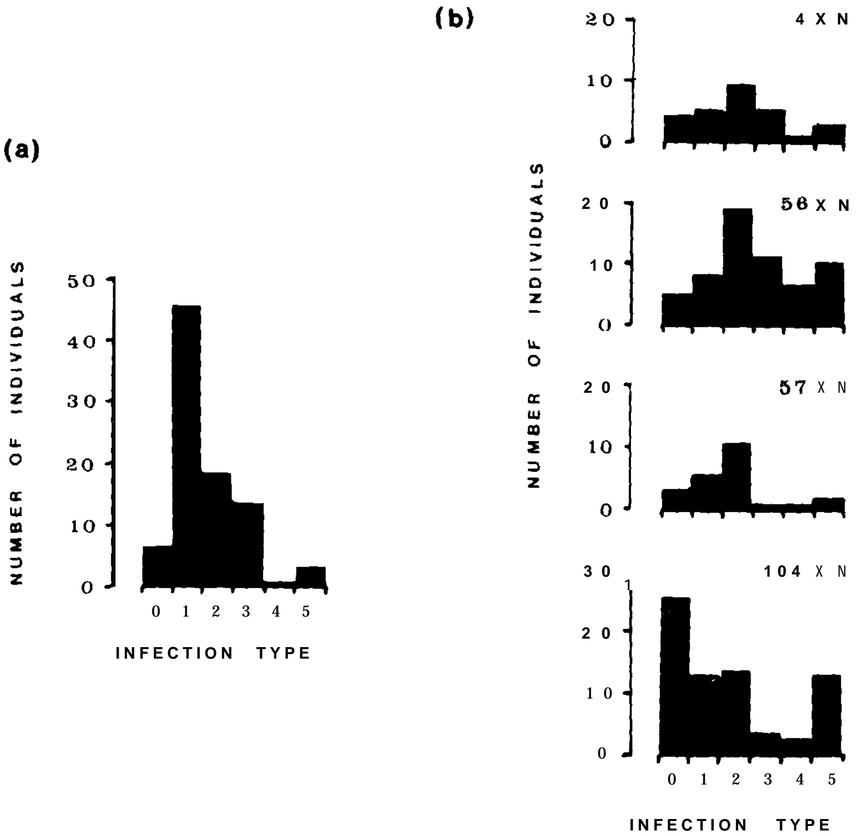

Figure 1. Frequency distribution of infection type among SR1 perennial ryegrass somaclones and some progeny tested with a mass inoculum of *Puccinia coronata*. *(a)* Variation in crown rust resistance among 80 somaclones. (b) Four somaclones (numbers 4, 56. 57 and 102, having infection type scores of 0, 1, 3 and 1, respect&y), was emasculated and crossed with a single susceptible (Infection type 5) Nui (N) genotype.

variation in crown rust resistance can be generated in perennial ryegrass by callus culture, the absence of the parental genotype (the immature embryo used to initiate the SR1 culture) did not enable us to conclude whether this variation resulted in an increase or a decrease in rust resistance. To answer this question callus was initiated from immature inflorescences of three of the SR1 somaclones (primary somaclones), which differed in crown rust infection type score, and regenerated secondary somaclones were screened for variation in rust resistance (Skipp and White this proceedings). This study showed that both increases and decreases in the level of rust resistance occur during the callus culture of perennial ryegrass SR1 genotypes. An unexpected result was the generation of a fine leaved "turf type" crown rust resistant genotype. This type of alteration in one step is unlikely to occur in a conventional breeding programme.

To study the heritability of the resistance obtained in the primary somaclones, progeny and parents of four crosses were screened for their infection type response to a mass collection of crown rust urediospores. Four SR1 somaclones, varying in habit, had previously been emasculated and crossed with a single Grasslands Nui pollen parent. Subsequently it

was determined that the somaclone parents, 4, 56, 57 and 102 had infection type scores of 0, 1, 3 and 1 respectively and that the Nui parent was susceptible (infection type 5). The distribution of infection type scores among the four progenies is shown in Figure 1 b. These distributions show a high level of heritability for the somaclone rust resistance, The lack of a bimodal distribution indicates that a number of genes contribute to the resistance, There have been reports of both dominant and recessive genes for resistance to crown rust in perennial ryegrass, see Hayward (1977).

These results demonstrating somaclonal variation in perennial ryegrass, together with the high heritability of the crown rust resistance obtained, suggest that the process of tissue culture induced variability can be used to improve the efficiency of breeding heterozyous, outbreeding species, Some of the most resistant clones are at present being used to breed a new crown rust resistant perennial ryegrass cultivar.

The use of callus culture to promote genetic exchange between the genomes of perennial ryegrass x tall fescue hybrids, and to generate stable amphidiploids, is also being examined (Scott and White this proceedings).

Introducing cloned foreign genes into white clover

White and Greenwood (1987) have developed a system for introducing cloned genes into white clover. This system utilises the natural gene transfer capacity of *Agrobacterium*, a modified Ti-plasmid binary vector containing a selectable kanamycin resistance gene and a white clover genotype which can be regenerated from cell culture as a recipient. At present the main limitation in the transformation of white clover is a requirement for the prior selection of a plant genotype with the capacity for regeneration from cell culture (White and Greenwood 1986). An introduced kanamycin resistance resistance gene was expressed in transformed white clover cells and in transgenic plants. Also the integration of foreign DNA sequences into the white clover genome was demonstrated.

Given that we can: (1) clone genes from a variety of organisms, (2) manipulate gene expression using recombinant DNA techniques and (3) introduce cloned genes into white clover, how can these techniques be used to contribute to the genetic improvement of white clover? Until recently a paucity of cloned agronomically useful genes was a major constraint. However, in the past year the following interesting prospects have developed.

Improved nutritional quality. It has been demonstrated that addition of sulphur amino acids, cysteine and methionine, to the abomasum of sheep (i.e. bypassing the rumen) can substantially increase wool growth (Reis 1967). However, there is a barrier to supplementing the sheep's diet with sulphur rich proteins because most plant proteins are broken down in the rumen. Recently CSIRO researchers cloned a pea albumen 1 gene (PA1) which encodes a seed storage protein rich in sulphur-containing amino acids. This protein is relatively resistant to breakdown in the rumen (T. J. Higgins pers. comm.) but would be digested in the intestine. Normally the PA1 gene is only expressed during seed development. However, a number of light regulated DNA sequences have been defined (Simpson et *al.* 1986) which when fused to a foreign gene coding sequence will give expression of that gene in the leaves of transgenic plants. The CSIRO group are attempting to obtain expression of the PA1 gene in the foliage of lucerne and sub clover. At present we are introducing the PA1 gene into white clover.

Pest resistance. The bacterium *Bacillus thuringiensis* produces protein toxins (St. toxins) active against insects. These B.t. toxins are solubilized and cleaved in the insect gut to produce the active form of the toxin. Different strains of the bacterium produce toxins with different specificities, and strains active against some members of the Lepidoptera (moths), Coleoptera (beetles) and Diptera (blackflies, etc.) have been isolated. Vaeck et a/. (1987) have engineered the expression of a cloned fragment of a B.t. gene active against tobacco hornworm in transgenic tobacco plants. The transformed plants produced enough of the toxin

to kill tobacco homworm larvae and provide protection. If strains of B.t. active against grass grub, porina or clover casebearer can be isolated then this approach might be used to produce white clover resistant to these pests.

Another possible group of cloned genes which might be used to reduce insect damage to plants are the wound-inducible proteinase inhibitors. These proteins act to inhibit the activity of insect and microbial proteinases (but not plant proteinases) and thereby disrupt the digestion of proteins in the insect gut. Alteration of either the site or level of expression of an introduced proteinase inhibitor gene might deter insect feeding. Recently a cloned cowpea trypsin inhibitor (CpTi) gene was introduced into tobacco and conferred resistance to a range of insect pests (Agricultural Genetics Company, UK). To examine this prospect we are introducing a modified potato proteinase inhibitor II gene (Keil et *al.* 1986), into white clover.

Viral resistance. Inoculation of plants with mild strains of viruses can provide cross-protection against subsequent infection with a virulent strain of the virus. Although most plant viruses are RNA viruses a complementary DNA (cDNA) copy can be made and cloned. This allows the introduction of parts of the virus which, when expressed might provide cross-protection against infection by the intact virus. Abel et al. (1986) demonstrated this concept when they introduced a cloned cDNA for the coat protein gene of tobacco mosaic virus (TMV), under the control of a strong promoter, into tobacco. Transgenic plants resisted TMV infection.

Although white clover mosaic virus (WCMV) does not usually produce symptoms on white clover, infection is widespread and reduces yield and N_2 fixation. There is no known source of resistance. The recent cloning and DNA sequencing of a complete WCMV cDNA (R. Forster pers. comm.) provides a means of testing this concept in white clover.

Herbicide tolerance. The broad spectrum herbicide glyphosate acts by blocking the biosynthesis of aromatic amino acids in plants and bacteria. The activity of a single enzyme, enolpyruvyt shikimate-3-phosphate synthase (ESPS), is inhibited by the herbicide. The expression of a mutant bacterial enzyme, selected for its insensitivity to glyphosate inhibition, in transgenic plants gives some tolerance to the herbicide (Comai et *al.* 1985). Normally the plant enzyme is transferred to the chloroplast, and when the mutant enzyme is engineered so that it accumulates in the chloroplasts of transgenic plants a higher level of tolerance is obtained (della Cioppa et *a/* 1986). The introduction of this glyphosate resistant gene into white clover could solve the problem of contamination of new cultivar seed crops with buried seed.

In conclusion there is now clear evidence that cell and molecular genetic manipulation techniques, particularly those of gene cloning and introduction, will have a major impact on the improvement of pasture plants.

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