THE PRODUCTION AND DEVELOPMENT OF TRANSGENIC PLANTS

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ABSTRACT

Significant advances have been made in the production and development of transgenic plants. A range of agronomically important characters have been modified including disease resistance, pest resistance and seed storage products. Studies are underway to determine the stability of transgene expression and inheritance. Methods of transgene regulation and to remove antibiotic resistance markers are being assessed. The potential of using transposon tagging for gene isolation in *B.napus* is being evaluated.

INTRODUCTION

Since the last international rapeseed meeting in Saskatoon there have been advances in the efficiency of producing transgenic plants and in the range of gene constructs used. The plant characters being modified include pest resistance, disease resistance, herbicide tolerance, male sterility and fatty acid content of the oil. Internationally, various transgenic lines are in trials leading to the production of transgenic commercial varieties. Transformation is providing many new opportunities to modify rapeseed and to use a wide array of genes from outside the *Brassica* gene-pool. The aim in this paper is to review some of the developments in the production of transgenic plants and to focus specifically on studies presented at the meeting.

TRANSFORMATION

There have been various reports of transformation in *B. napus*, *B. rapa* and *B. juncea* over the past few years. *Agrobacterium tumefaciens* is used widely, and several research groups use *A. rhizogenes*. The success of transformation is influenced by the plant genotype and the characteristics of the vector constructs. There is some interest in using DNA uptake into protoplasts and in the particle accelerator, but *Agrobacterium* transformation is usually preferred (for review see Dale and Irwin 1993).

Transgene stability

Transgene instability is a common feature in primary transformed plants (T_0) and sometimes in the early sexual generations (Finnegan and Elroy 1994). There is also variation between independently transformed plants in transgene expression. In a research programme (Jones *et al.*, 1995) to study transgene stability in *B. napus*, a range of constructs has been used which contain constitutive promoters (*35S* from cauliflower mosaic virus, *nos* from *Agrobacterium*) and embryo specific promoters (oleosin and

cruciferin from *B. napus*) regulating the coding sequences for *gus* (β-glucuronidase) and *bar* (resistance to the herbicide Basta). For all constructs, transgenic plants were observed that had some loss of expression and some physical loss of at least part of the construct. Some transgenic lines containing the embryo specific promoters showed an alteration in tissue specificity of transgene expression and this abnormal expression was also observed in the T_1 generation.

In other studies with *B. napus* there is evidence that background genotype can affect the expression and structural stability of certain constructs (Irwin and Dale unpublished).

The long term stability and utility of transgenes will eventually be assessed in transgenic plant varieties used in commercial production. It is expected that transgenes will be as stable as resident genes, when transgenic lines have gone through the standard selection and evaluation trials used in conventional breeding. In practise it is usual to produce >100 independently transformed plants with a given construct to provide enough transgenic lines to be able to select for the desired transgene copy number (usually one copy), level of transgene expression, structural stability and tissue specificity.

Selectable markers for transformation

In the commonly used transformation methods, only a small proportion of cells become transformed, so it is important to use a selectable marker gene. The most frequently used marker gene is *nptII*, which confers resistance to the antibiotic kanamycin. The long term consequence of using a selectable marker gene in this way, is that the transgenic plant variety will also contain the antibiotic resistance gene. There have been several reviews of the evidence for and against the presence of the *nptII* gene in finished plant varieties. The conclusion of these studies has been that the presence of the *nptII* gene is of little or no consequence for human health or the environment (Flavell *et al.*, 1992; Nap *et al.*, 1992). However questions are still asked about the advisability, or the likely public reaction to the inclusion of the *nptII* gene in commercialized transgenic plant varieties. In the USA the Food and Drug Administration (FDA) has allowed the commercial use and human consumption of Flavr Savr tomatoes carrying the *nptII*, but the reaction to the use of this gene in edible plants in Europe is still unclear. In the UK, the Advisory Committee on Novel Foods and Processes (ACNFP) has recently stated that researchers developing food GMO's (Genetically Modified Organisms) should be encouraged to develop and use alternatives to antibiotic resistance markers.

One method of eliminating the *nptII* gene from transgenic varieties is to transform the plant with a mixture of two lines of *Agrobacterium*, one carrying the selectable marker gene (eg *nptII*) and the other carrying the gene of interest. The requirement is for the plant to become transformed with both *Agrobacterium* strains, and for the two T-DNA's (transforming DNA's) to be inserted at different chromosomal locations. Backcrossing to non-transgenic lines should then give some progeny containing the gene of interest but without the *nptII* gene.

In work reported by Bade and Damm (1995), *nptII* was used as a selectable marker gene and *gus* as a reporter gene. Following transformation with a mixed

inoculum of two *Agrobacterium* lines and selection for kanamycin resistance, 30-50% of the regenerated plants were *gus* positive and therefore cotransformed. More than 50% of these cotransformed lines gave progeny that were *gus* positive and kanamycin susceptible. This suggests that in about half of the lines the *gus* and *nptII* genes were unlinked (or not tightly linked) and could be separated by sexual genetic recombination. Molecular analysis is currently being used to confirm whether the kanamycin susceptible lines do not contain *nptII* sequences.

TRANSGENES MODIFYING AGRONOMICALLY IMPORTANT CHARACTERS

Seed storage proteins

Anti-sense strategies are being evaluated for modifying a wide range of characters. mRNA coded by the anti-sense version of a particular gene is thought to bind with the mRNA coded by the sense version of the resident gene, and cause reduction in the specific protein gene product. This does not provide a complete explanation, however, because the introduction of the sense version of a transgene has also been observed to result in a reduction in the corresponding protein gene product.

To study the effect of anti-sense DNA on protein production, an anti-sense gene for cruciferin has been inserted into *B. napus* (Kohno-Murase *et al.*, 1995). This has resulted in a reduction in the amount of cruciferin in seeds, and was accompanied by an increase in napin production. The transgenic lines contained increased levels of the three essential amino acids: lysine (10%), methionine (8%) and cysteine (32%). The amount of the protein oleosin was unaffected.

Fungal disease resistance

Chitin is a major component of the cell walls of most fungi (except oomycetes). Plants frequently produce hydrolytic enzymes such as chitinase as a defence mechanism against fungal diseases (Zhu et al., 1994; Bowles, 1990). Various attempts have been made to determine whether the over production of chitinase in plants will give enhanced levels of resistance to fungal diseases. A chitinase gene with a 35S promoter and a nopaline synthase terminator has been incorporated into an inbred line of *B. napus* using *A. rhizogenes* mediated transformation (Grezes-Besset *et al.*, 1995). Progeny from the T_3 generation (third generation of selfing) were grown in two locations in France and challenged with four fungal pathogens: *Alternaria brassicae, Cylindrosporium concentricum, Phoma lingam* and *Sclerotinia sclerotiorum*. When compared with the non-transgenic control plants the authors report increased resistance to all four pathogens. Trials are now in progress to determine comparative yields.

Viral disease resistance

The incorporation of a viral coat protein gene can give protection against the same or a related virus, and there are now many examples where this resistance is being evaluated in transgenic plants (Dale *et al.*, 1993). The way the resistance operates is not fully understood, but it may involve process similar to cross protection that has been used for virus disease control for many years, where inoculation with an

innocuous virus offers protection against a pathogenic virus.

In an attempt to study resistance to the widely occurring beet yellows virus (BWYV), the coat protein gene along with other relevant sequences have been transformed into *Nicotiana benthimiana* and *B. napus* (Laucke *et al.*, 1995). Data on inoculation of these plants with the virus will be reported later.

Insect resistance

Various strategies are being considered for making plants more resistant to insect pests (Dale *et al.*, 1993). Before transgenic plants can be evaluated in field experiments and before they can be used commercially, it is necessary to assess whether a pest resistance strategy has any impact on non-target organisms. Because oilseed rape crops are frequently a major source of nectar and pollen used by honey bees, it is important to determine the impact of insect resistance strategies on honey bees.

Protease inhibitors (PI) are known to provide plants with enhanced resistance to feeding insects. In an attempt to determine whether PI's have any impact on honey bees a cysteine PI from rice has been introduced into *B. napus* under control of the 35S promoter (Grallien *et al.*, 1995). Transgenic plants along with non-transgenic control plants were exposed to honey bees (*Apis mellifera mellifera*) in various ways. No differences were observed in the numbers of visits by honey bees between the transgenic and non-transgenic control line (variety Drakkar). There was evidence of higher sucrose concentration in nectar from the transgenic plants, but no change in glucose and fructose concentrations. Tests were also made with sugar solutions containing added synthetic protease inhibitors. The data so far suggest that the PI gene in *B. napus* probably has an insignificant effect on bees. However the gene construct used gave a rather low level of PI gene expression.

TRANSGENE REGULATION

Gene constructs generally contain a coding sequence for a gene of interest along with a promoter on the same DNA insert. May *et al.*, (1995) propose the incorporation of a promoter on one transgene construct and the gene of interest on another gene construct. Using a trans-activation system (from yeast) they propose to combine promoters and coding sequences by sexual hybridization. This could have the advantage that a wide array of promoters could be tested with particular coding sequences (following hybridization) without having to prepare separate constructs with every promoter/coding sequence combination. The system is currently in the early stages of evaluation, but evidence, from transient expression studies using particle bombardment of tissues from various species, suggests that the trans-activation may work. Studies to incorporate the elements of the system by stable transformation are underway.

GENE ISOLATION

One of the limiting factors to the use of transformation for modifying crop

plants in the future will be the availability of cloned genes for important plant characters. Endogenous transposable elements have been used to isolate genes from maize, and maize transposable elements are being introduced into several plant species for the isolation of genes from them. The well characterised Ac/Ds element system from maize has been found to transpose in various species, including tomato, potato and rice.

Separate constructs containing an immobilized Ac element and others with a Ds element have been introduced into different *B. napus* plants (McKenzie and Dale, 1995). The reason for having the constructs in different plants is to enable the two elements to be combined in the same plant (to activate transposition) and separated again into different plants (to inactivate transposition) by sexual hybridization.

Transposition of Ds elements needs to be confirmed by molecular analysis in *B. napus*, but has recently been confirmed to occur in *B. oleracea.*

CONCLUSIONS

Significant advances have been made in the research, development and use of transformation in rapeseed. During the next 4 years we expect to see further substantial advances in research along with the commercialisation and use of transgenic varieties containing several modified characters.

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