LABORATORY AND FIELD PERFORMANCE OF TRANSGENIC BRASSICA PLANTS EXPRESSING CHIMERIC 2S ALBUMIN GENES

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INTRODUCTION

The seed cake resulting after the extraction of oil from Brassica napus could heretofore be used in animal feeds in only limited quantities. This limitation was in part due to the presence of antinutritional factors (glucosinolates). Progress using traditional breeding methodologies has resulted in lines in which glucosinolate levels are significantly reduced. A second limitation for its use in monogastric animal feeds has been the amino acid composition of the protein in rapeseed meal. In particular, increases in the levels of methionine, lysine, and tryptophan would make rapeseed meal more competitive with the meal from other species such a soybean.

Recent advances in recombinant DNA and plant transformation technologies have provided an alternative method to transfer specific traits between species. Once in the desired species breeding techniques can be used to move the trait to different lines; the two techniques thus complement each other. One could envision at least two approaches to introducing genes encoding lysine and/or methionine rich proteins into Brassica. Genes from other species with the desired characteristics could be transferred directly, perhaps with modifications to the regulatory sequences where needed. Alternatively, existing genes known to be expressed in Brassica or related species could be modified. The latter approach is more risky, as modified seed storage proteins may not be stably accumulated (Hoffman et al., 1988), but is more flexible in that different types of modifications can be introduced, depending on the need.

The 2S albumin storage proteins provide a system in which both approaches can be applied. 2S albumins are small (13kD) storage proteins consisting of two subunits linked by disulfide bridges (Youle and Huang, 1981). In Brassica they contribute about 24% of total seed protein (Murphy and Cummins, 1989). The 2S albumin of Bertholletia excelsa (Brazil nut) is unusual in containing 18% methionine (Ampe et al., 1986). One approach taken in the present work is to transfer this gene to Brassica using regulatory signals from a 2S gene from a closely related species, Arabidopsis thaliana (Krebbers et al., 1988). This was done to avoid the problems associated with understanding differences in expression between the multitude (12-16) of Brassica 2S albumins genes (Scofield and Crouch, 1987; Josefsson et al., 1987); in Arabidopsis only four genes are present (Krebbers et al., 1988).

Vandekerckhove et al. (1989) demonstrated that the structure of the 2S albumin is such that it will tolerate

modifications between the sixth and seventh cysteine residues. The goal of this work was not only to demonstrate that modified 2S albumins could stably be expressed, but that a peptide embedded in a 2S albumin could be recovered in significant quantities. Their results demonstrated that up to 200 nmol of peptide per gram of seed could be recovered from both Arabidopsis and Brassica plants (Krebbers and Vandekerckhove, 1990). If a 25 amino acid peptide were produced in this way, over 1 kg of peptide could be isolated per hectare of canola grown, suggesting that this might be a viable method for peptides needed in large quantities. As discussed below, modified 2S albumins containing substitutions of this length have since been produced.

De Clercq et al. (1990b) reasoned that just as peptide encoding sequences could be introduced, so might it be possible to introduce extra methionine codons into the gene. This resulted in the expression of modified 2S albumins containing increased levels of methionine. The longest substitution/ insertion produced was 24 amino acids, of which 12 were methionine. The presence of the modified 2S albumin was demonstrated by isolating part of the inserted peptide. The yields obtained were comparable to that obtained by Vandekerckhove et al. (1990) with a shorter biological peptide, demonstrating that length of substitution does not necessarily affect yields. However, the expression levels were not high enough to measure significant changes of methionine contents in the meal; the reasons for this are considered below.

The molecular behavior of chimeric or wild type 2S albumin genes introduced into transgenic plants has been studied recently (De Clercq et al., 1990a,b; Vandekerkhove, 1989). Less work has been done on the field performance of such plants. The remainder of this manuscript will briefly review the molecular data, report on the expression of a new modified 2S albumin containing lysine, and summarize results concerning the performance of such plants in the field.

MATERIALS AND METHODS

Molecular Techniques, Plant Transformation

The methods used for chimeric gene constructions and transformation of Brassica napus Drakkar or Westar plants have been extensively described elsewhere (De Clercq et al., 1990b; De Block et al., 1989). The same techniques were used to produce the new chimeric gene enriched in lysine presented in this paper.

Field trial protocols

The layout of the field trials was designed to allow a regular agronomic comparison between several independent lines. Each trial included at least three repetitions in a randomized block design. Culture and harvest procedures were as close to standard practices as possible. However, small adaptations were included in order to work in accordance with minimizing the potential risks for involuntary dissemination. The trials, which were conducted in a wide range of countries (Sweden, U.K., Belgium, France and Canada), were all done on the basis of a proposal to the appropriate authorities for a small scale release of genetically modified organisms and all

procedures were carried out in accordance with the authorization given.

Seed Analysis

Standard quality analysis for oilseed rape was performed, including determination of humidity, oil content and composition, and glucosinolate content and composition. In addition the amino acid composition was determined.

RESULTS AND DISCUSSION

Molecular Analysis of Transgenic Plants

The molecular data on the expression of chimeric, modified and wild type Arabidopsis 2S albumin genes in tobacco, Arabidopsis, and Brassica plants are described at length in papers by Vandekerkhove et al., (1989), De Clercq et al., 1990a,b and Guerche et al. 1990. That data necessary to understand the background to the work presented here can be summarized as follows.

1. 2S albumin genes introduced into transgenic plants are developmentally regulated, being expressed only in seeds following a normal temporal profile.

2. Wild type 2S albumins undergo correct posttranslational processing (which is complex for 2S albumins; see Krebbers et al., 1988) and are correctly targeted to the protein storage vacuoles in tobacco. It is assumed that this is also the case for modified 2S albumins in Brassica, but for technical reasons this has not been rigorously demonstrated.

3. Modified 2S albumins containing substitutions encoding a biological peptide as well as three different substitutions containing methionine have been stably expressed. An antibacterial peptide has also been produced (A. De Clercq, EK, and JV, unpublished data) as well as one containing extra lysine residues presented below, bring to six the total number of stable modified 2S albumins stably expressed in transgenic plants.

4. Chimeric fusions of the methionine rich Brazil nut 2S albumin to Arabidopsis promoters are stably expressed in all three species (for tobacco, see also Altenbach et al., 1989).

5. Neither in the methionine substitutions nor in the Brazil nut chimeric fusions could an increase in methionine content be measured in the seed cake. This was linked to two factors. First, small shifts in methionine content are difficult to measure accurately. Secondly, the promoter used in all the constructions (that of at2S1) was shown to be expressed in the embryo axis but not in the cotyledons of the developing embryo. Preliminary data from experiments using another promoter (at2S2) suggest that much higher levels of expression can be achieved (A. Da Silva and EK, unpublished observations).

Expression of a Modified 2S Albumin Containing Lysine Residues

Increasing the lysine content of canola meal is of equal or greater importance to that of increasing the methionine content. No lysine rich 2S albumins have been described, and thus the strategy of modifying existing 2S albumins presented itself. The charged nature of the lysine residue might have been expected to present extra problems. The construction was designed so as to minimize possible disruption to the normal structure. The sequence of the modified region is shown in figure 1.

C	P	T	L	**K**	G	A	A	**K**	A	V	**K**	M	*	E	**K**	Q	**K**	**K**
C	P	T	L	**K**	Q	A	A	**K**	A	V	R	L		Q	G	Q	H	Q
P	E	Q	V	**K**	**K**	M	*	Y	**K**	T	A	**K**	H	L	P	N	V	C
P	M	Q	V	R	**K**	I		Y	Q	T	A	**K**	H	L	P	N	V	C

The peptide sequence of the modified (top line of each pair) and wild type Arabidopsis 2S albumin in the region between the sixth and seventh cysteine residues. Lysine residues are shown in bold. The peptide fragment used to screen for the modified 2S albumin using the methods described in De Clercq et al. (1990b) are flanked by asterisks (*). No further modifications were made in the sequence of the original gene, at2S1 (Krebbers et al., 1988).

Figure 1

The original construct was made using the at2S1 promoter. During the period that plant regeneration was underway it became clear that this promoter would not give sufficient levels of expression to significantly alter amino acid content, for the reasons discussed above. Thus no attempt was made to measure lysine content in the seed cake. Instead, new constructs using the at2S2 promoter have been made. These are currently being analyzed.

Field Performance of Transgenic Canola Plants

As discussed above, the original transgenic plants were made with a promoter not capable of providing the expression levels necessary to give a measurable change in amino acid content. Plants transformed with constructs based on the stronger at2S2 promoter will be field tested in the coming season. It was of interest, however, to determine what the effects of the transformation and regeneration process, as well as the expression (at whatever level) of an extra storage protein gene would have on the agronomic performance of the plants and the quality of the seed produced. Multiple site field trials were thus done using plants transformed with the at2S1-Brazil nut chimeric construct. The agronomic parameters measured included establishment, date of flowering, height of the plants at flowering and yield. There was a slight delay in the development of the transgenic plants which could be traced back to the poor seed quality resulting from an accelerated production procedure. This was confirmed by additional germination tests and additional comparisons with control material. Analysis of all the quality parameters of the field harvested seeds revealed statistically indistinguishable results for both the transformed and control plants. As expected, amino acid analysis showed no significant increase in the targeted amino acids yet. These

results are encouraging as they suggest that the transformation and regeneration procedures do not result in poor agronomic performance or changes in seed quality relative to normal plants.

Summary and Prospects

The first parts of the research program described above were directed towards determining in chimeric storage protein genes, like the Arabidopsis-Brazil nut fusions, or modified Arabidopsis 2S albumin genes like those containing extra methionine or lysine encoding sequences, could be stably expressed at both the mRNA and protein levels in Brassica. An essentially positive result was obtained, both for the chimeric proteins and modified 2S albumins containing either essential amino acids or biological peptides. The second phase, currently underway, is directed towards enhancing the expression levels so that significant alterations in seed cake amino acid composition can be obtained, or, in the case of biological peptides, to make the process even more economical. However, even at the levels obtained so far the process is of interest, and further experiments to confirm the feasibility of using canola as a production systems for biological peptides are under way. Preliminary laboratory results suggest that it should be possible to increase expression levels. In parallel, the transgenic plants resulting from the first phase were used in field experiments to determine if their overall agronomic performance and seed quality differed from normal plants. The first results suggest that this is not the case; expression of a specific extra protein in the seed fraction of oilseed rape did not appear to introduce other performance penalties.

In a third phase of the program, the lines transformed with highly expressing methionine or lysine rich genes will be used in breeding programs to move them into modern commercial lines. In some cases it may be of interest to combine them with other new traits, such as male sterility. In general, evaluation of the kind discussed in this contribution will be necessary for any trait introduced by genetic engineering; demonstration not just of the expression of the trait of interest but overall performance, using the normal evaluation procedures for any new plant variety, will be required. In order to combine biotechnology with breeding, such procedures will be of the utmost importance.

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