THE PRODUCTION AND CHARACTERIZATION OF ASYMMETRIC SOMATIC HYBRIDS BETWEEN Brassica napus AND Arabidopsis thaliana.

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INTRODUCTION

The production of intergeneric and interspecific hybrid plants by sexual crossing is often prevented by physical and/or biological barriers to the reproductive process. These sexual incompatibilities may be bypassed by the application of somatic hybridization techniques (Evans, 1984). In order to transfer limited amounts of genetic information, donor cells (protoplasts) are irradiated to enhance chromosome elimination. Fusion of such irradiated protoplasts with protoplasts of the non-irradiated recipient has been referred to as 'asymmetric somatic hybridization'.

This presentation reports the results of research undertaken to produce asymmetric somatic hybrids between Arabidopsis thaliana and Brassica napus. A major objective of the project was to assess the efficiency of transferring a selectable marker gene, from Arabidopsis to Brassica. A. thaliana serves as an ideal donor for such a study due to the extensive molecular characterization of its small genome and the availability of many mutants (Meyerowitz and Pruitt, 1984). One such gene, namely the acetolactate synthase gene (ALS), has been altered via mutagenesis to confer sulfonylurea resistance (Haughn and Somerville, 1986). In the present study, protoplasts of a sulfonylurea resistant strain of A. thaliana were irradiated and fused to protoplasts of the economically important B. napus, with the objective of successfully transferring this herbicide resistance into canola.

MATERIALS AND METHODS

Plant Material

The Arabidopsis thaliana cv. Columbia mutant donor plant material, termed 'GH50', was a cell suspension culture initiated from leaf callus by S. Gleddie. Seeds of the GH50 line had been originally provided by G. Haughn, and were derived from a mutant line expressing resistance to sulfonylurea herbicides, such as chlorsulfuron (cs)(Glean, DuPont).

The recipient fusion partner, Brassica napus L. spp. oleifera cv. Westar was grown from seed in the greenhouse.

Protoplast Isolation

For the isolation of GH50 protoplasts, three to four day old cell suspension cultures were digested in enzyme solution as detailed in Bauer-Weston (1990). While still in the enzyme solution, protoplasts were X-irradiated at 0, 60 and 90 kRad using a Picker Scanray X-Ray machine (AC 128 Automatic). Protoplasts were then filtered and washed with an MES wash solution (Bauer-Weston, 1990). Westar protoplasts were isolated from stem epidermal strips according to Klimazewska and Keller (1987).

Protoplast Fusion, Culture and Selection

Pelleted GH50 and Westar protoplast pellets were mixed in a 1:1 ratio, and fused in 0.5 mL of 25% polyethylene glycol (PEG-6000 MW (Sigma), 3% $CaCl_2.2H_20$, pH 6.9) for

15 minutes. The PEG was diluted with 12 mL of 0.4 M mannitol (pH 5.8), gently spun down and resuspended in modified NLN culture medium as described by Bauer-Weston (1990). Westar and GH50 homofusion controls were also prepared simultaneously.

Fusion products were subjected to various concentrations of cs selection (0.001, 0.01 and 0.1 μg/mL) on day 0, day 5 and day 14 of the protoplast culture period. Surviving colonies, 0.5 mm in diameter or greater, were transferred to shoot regeneration medium (SRM) (Klimazewska and Keller, 1987) without selection pressure. Regenerated shoots were excised and placed onto root regeneration medium (RRM) (2% sucrose, 0.1 mg/L NAA, 1X modified MS salts (Murashige and Skoog, 1962), 0.35% gelrite (Kelco), pH 5.8). Rooted plantlets were transferred to soil and grown in the greenhouse.

Hybrid Analysis

Morphological Analysis- The flower morphologies of the hybrids were assessed visually, while the nature of the upper leaf surface of greenhouse acclimatized hybrids was examined using scanning electron microscopy (AMR 1000 A SEM).

Rooting Assay- The level of in vitro herbicide resistance was assessed by supplementing RRM with cs concentrations in the range of 0, 0.001, 0.01, 0.1 and 1.0 μg/mL. Shoots, from previously rooted hybrid and control Westar plants, were excised and placed onto the RRM selection for 21 days, and then assessed for their ability to form roots.

Herbicide Spray Assay- One month old greenhouse acclimatized hybrids and parent plants, which had not yet bolted, were sprayed with a sufficient volume of 0, 0.25, 0.5 and 1.0 μg/mL cs (with 0.5% TWEEN in distilled water) sufficient to wet the plant's entire leaf surface. The plants were scored for their change in height, number of new leaves formed and for the number of inflorescences produced two weeks post-spray.

Isozyme Analysis- Leaf or root tissue was taken from one month old greenhouse grown or acclimatized Westar, GH50 and hybrid plants. Approximately 0.2 g of tissue was ground in extraction buffer (30 mM Dithiothreitol, 0.2 M Tris.HCl, pH 8.5), and the supernatant was resolved on a 6.25% non-denaturing polyacrylamide gel. Separating gels were stained for esterase, peroxidase and phosphoglucomutase (PGM) activities (Bauer-Weston, 1990).

Chloroplast DNA Analysis- Purified genomic DNA was restricted with BamHI, XbaI, and EcoRI, separated on an agarose gel, and blotted onto a Nytran (Schleicher and Schuell) support membrane. The membrane was hybridized to a ^{32}P radiolabelled chloroplast DNA probe, pBa1-9 (Aviv et al., 1984) and to radiolabelled B. napus chloroplast DNA. As a control, isolated B. napus chloroplast DNA was run alongside B. napus genomic DNA.

Acetolactate Synthase Gene Analysis- ALS probes were isolated from plasmid pGHI (Haughn et al., 1988) by digesting with XbaI/NcoI to render four fragments which varied in size from 0.9 to 3.7 kb. The 1.1 kb XbaI/NcoI fragment was random primed, and hybridized to Nytran filters containing restricted genomic hybrid and parental DNA.

RESULTS

During the PEG fusion process, hetero-aggregates, homo-aggregates and multiple aggregations were observed between the GH50 and Westar protoplasts, the latter generally being larger in size and containing green plastids. The level of selection used in the protoplast cultures, following fusion, was increased to 0.01 and 0.1 μg/mL cs, since several escape Westar homofusion colonies survived selection at 0.001 μg/mL cs. The plating efficiency of fusion products which did survive the selection was in the range of 0.01% to 0.1%. Surviving colonies were transferred onto SRM lacking cs selection. At this stage of development, most of the colonies browned and died. Others increased in size to produce large clusters of green, sometimes purple, calli. On average, 14.5% of the unselected Westar control calli produced shoots after 30 days on SRM. The proportion of fusion product calli

which regenerated shoots was much lower, ranging from 4% for regenerants selected at .001 μg/mL to 0.9% for regenerants selected at 0.1 μg/mL.

From four Westar + GH50 fusion experiments, a total of 17 hybrids were regenerated in vitro. To date, eight of those hybrids (A to H) have been transferred into a greenhouse environment and have matured, flowered and produced pods.

Morphological Analysis

All eight greenhouse-acclimatized hybrids were intermediate in size, and leaf shape to their parents. The flowers from protoplast-derived untreated Westar control plants demonstrated an unalterred flower morphology when compared to flowers from Westar plants grown from seed. The flowers from the hybrids, though, demonstrated intermediate petal colour, variation in petal number, abnormal pistil morphologies, and all lacked anthers. The only floral characteristic which remained unchanged, when compared with the parents, was the sepal number. The sepal and corolla lengths were consistent amongst the different regenerants, yet were intermediate in length between the measurements recorded for the parents.

According to scanning electron microscopy (SEM) analyses of greenhouse grown Westar and hybrid Arabidobrassica plants, the upper leaf surfaces did not have any leaf hairs, yet had a thick waxy cuticle. On the other hand, upper leaf surfaces of Westar plants grown in vitro demonstrated trichomes when observed under a dissecting microscope. Greenhouse GH50 plants produced tribranched trichomes on their upper leaf surface, with very little wax deposition.

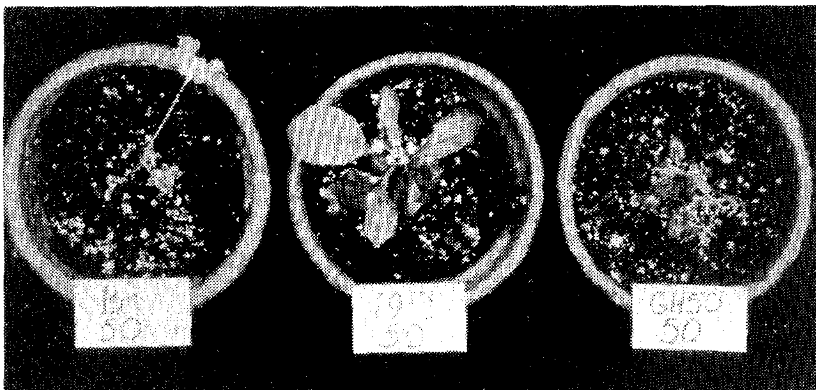
Rooting Assay

Using root formation as an indicator of resistance, the hybrids all demonstrated at least a ten fold greater cs tolerance than the Westar control. Hybrids L, D and H rooted at cs levels 100 times higher than the level which Westar rooted at.

Herbicide Spray Assay

The results of the greenhouse herbicide spray assay are still preliminary since they have only been performed once. From this experiment, hybrid H plants tolerated a ten times greater herbicide spray level than the Westar control plants. H showed no decrease in height, leaf formation or in the number of inflorescences produced when sprayed with 0.25 μg/mL, 0.5 μg/mL and 1.0 μg/mL cs (Figure 1).

Figure 1. Hybrid H (F9:3) and Parents (Bn and GH50) Two Weeks Post-Spray with 0.5 μg/mL Chlorsulfuron



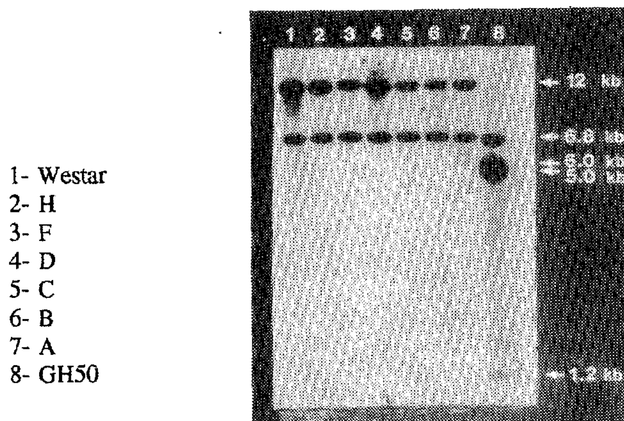
Isozyme Analysis

From 11 different isozymes which were tested, only esterase, peroxidase and PGM staining protocols produced well resolved bands which positively identified hybrid bands in protein extracts. The protein extracts for the peroxidase and PGM isozyme analyses were derived from leaf tissue, while root tissue protein extract produced the clearest banding pattern for the esterase isozyme analysis.

Chloroplast DNA Analysis

Southern blots performed on genomic DNA with two chloroplast specific DNA probes, pBa1-9 and total Brassica napus chloroplast DNA, always demonstrated a banding pattern in which all of the hybrids shared the same bands as the Westar parent (Figure 2). A control Southern blot illustrated identical banding patterns between genomic Westar DNA and chloroplast Westar DNA when probed with pBa1-9.

Figure 2. BamHI Digested Parental and Hybrid DNA Hybridized to pBa1-9



Analysis of Acetolactate Synthase Gene

The ALS 1.1 kb probe showed a strong hybridization to GH50 genomic DNA and a very weak hybridization to Westar DNA. A lower 5.5 kb GH50 specific band was present in the hybrids L, H and O, yet there was no hybridization to this region in the Westar DNA lane.

DISCUSSION

High doses of X- or gamma-irradiation are used to induce genome instability by damaging the DNA. In theory, the irradiated protoplast would be renderred non-dividing and the damaged DNA would be preferentially eliminated. Before this elimination occurs, recombination of the chromosomes, or of a few genes, could take place during the transient coexistence period of the two combined genomes (Dudits, 1988).

From the 17 Arabidobrassica hybrids produced, more than half of them originated from a fusion event involving a 60-kRad irradiated GH50 parent. The regenerants which demonstrated a relatively high cs resistance (Rooting and Herbicide Spray Assays; ALS Gene Analysis) were all derived from a 60-kRad irradiated GH50 donor, or from an unirradiated GH50 donor protoplast. One can postulate that the higher X-ray dose, 90 kRad, may have destabilized the GH50 genome to such a degree that even after the selection was removed,

and shoot regeneration induced, the fusion product continued to eliminate GH50 chromosomes. In this manner, a cs resistant callus may have lost the cs^r ALS gene after the selective pressure was removed, and subsequently may have regenerated a non-resistant shoot. This scenario can only be presumed since the sample size is not large enough to conclusively determine a correlation between dose and genome elimination. Also, a cytogenetic analysis of the hybrids is presently being undertaken, and only with this crucial piece of evidence can the effects of donor irradiation on Arabidobrassica somatic hybrids be more clearly understood.

The true hybrid nature of the regenerants was verified using a number of different morphological and biochemical analyses. These analyses were compared to a somatic hybridization study performed in 1978 between Brassica campestris + Arabidopsis thaliana (Gleba and Hoffmann, 1978), in which unirradiated protoplasts of A. thaliana were fused to B. campestris protoplasts. Regenerants from this study were all self-sterile and possessed a full set of chromosomes from each parent. They also developed deformed leaves with no distinguishable upper or lower surfaces, yet sometimes demonstrated intermediate trichome structures (Gleba and Hoffmann, 1979). These morphological analyses were only performed on in vitro plants, since their regenerants were not able to develop roots. The Arabidobrassica hybrids produced in the present study were morphologically more normal in their development and leaf structure, except for hybrid 4:A which was derived from an unirradiated GH50 parent. Donor-irradiation of the GH50 parent, though, did not appear to direct the production of fertile flowers in GH50 + Westar regenerants.

Using callus tissue as a protein source, Gleba and Hoffmann (1978) also verified the hybrid nature of their regenerants using esterase and peroxidase zymograms. Both of these isozymes, as well as PGM, were successfully used in the present study to demonstrate GH50 and Westar specific bands in the hybrids.

Chloroplasts are maternally inherited in most plant species, however, by protoplast fusion two different types of chloroplasts can be present in a single fused cell. This, though, is a rare event. In a few cases, chloroplast recombination, following protoplast fusion, has been observed (Fluhr et al., 1984), but the majority of somatic hybrids possess only one chloroplast type (Gleddie et al., 1983). In this study, Southern blot analyses of the Arabidobrassica hybrids indicated that the chloroplast genome was inherited solely from the Westar parent (Figure 2). No evidence for chloroplast recombination was observed using any of the chloroplast DNA probes.

CONCLUSION

This study has demonstrated the production of novel X-irradiated A. thaliana + B. napus somatic hybrids, using a model herbicide selection system to screen for regenerants expressing the GH50 cs^r ALS gene. Of all the hybrids tested for cs resistance, three had maintained and expressed this nuclear encoded gene. The effectiveness of asymmetric somatic hybridization as a method for introducing a novel trait into a regenerant is dependent upon the gene or genes which are being transferred, and on the ability to select in vitro. This method is very useful for transferring molecularly uncharacterized traits, and for multigenic traits. One of the limitations of this hybridization system is the lack of control over the amount of DNA transferred from the donor to the recipient cell. Even by irradiating a donor with high levels of X-irradiation, the amount of donor DNA lost is still partially dependent on other factors, many of which are not known. Further investigation into the determinants responsible for nuclear elimination and recombination in somatic cells should be pursued in order to exploit this technology to produce morphologically sound and fertile hybrids.

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