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**THE PRODUCTION AND
CHARACTERIZATION OF ASYMMETRIC
SOMATIC HYBRIDS
BETWEEN
ARABIDOPSIS THALIANA
AND BRASSICA NAPUS**

by

Brigitte J. Bauer-Weston, B.Sc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of

MASTER OF SCIENCE

Department of Biology

Carleton University
Ottawa, Ontario

December 14, 1990

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in partial fulfillment of the requirements
for the degree Master of Science.

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Chairman, Dept. of Biology

A handwritten signature in black ink, appearing to be 'J. L.', written over a horizontal dotted line.

Thesis Supervisor

To Erol and to my parents,

for your love, understanding and support.

Science is nothing but developed perception,
interpreted intent, common sense rounded out
and minutely articulated.

Santayana - The Life of Reason

ABSTRACT

Cell suspension-derived protoplasts of a chlorsulfuron resistant (GH50) strain of Arabidopsis thaliana cv. Columbia were X-irradiated at 0, 60 and 90 kRad, in an effort to direct the elimination of GH50 donor chromosomes in fusion products. After irradiation, GH50 protoplasts were fused, using polyethylene glycol, to stem epidermal strip derived-protoplasts of Brassica napus cv. Westar followed by selection at various levels of chlorsulfuron. Resistant heterofusion colonies were regenerated in vitro and then transferred to soil. Of 17 regenerated hybrids, eight were successfully established in the greenhouse where they flowered. These eight hybrids displayed male sterile flowers with abnormal petal and pistil structures. According to chlorsulfuron rooting and spray assays, the hybrid 3:B (GH50-60 kRad + Westar) demonstrated a 100 fold greater tolerance level than the non-resistant Westar parent. Zymograms for phosphoglucumutase, esterase and peroxidase showed the presence of parental isozymes in all of the hybrids tested. Hybridity was also verified for glucosinolate content and for the nuclear-encoded ribosomal RNA genes. The chloroplast genome in the hybrids was derived solely from the Westar parent, while some evidence for mitochondrial DNA recombination was obtained. The degree of GH50 genome elimination in irradiated-donor hybrids was not conclusively determined.

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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. With the advent of protoplast culture and fusion technology these sexual incompatibilities have been bypassed and the production of somatic hybrids from distantly related parents has become a reality.

Somatic hybridization can be defined as the joining of two non-gametophytic cells by protoplast fusion. A successful fusion usually results in a random cytoplasmic and nuclear combination of the two parental cells. Following this mixing, viable fusion products can often be regenerated into hybrid plants. This method of hybridization offers the unique possibility of combining and mixing nuclear and cytoplasmic genes from very different, unrelated sources (parents) in a single fusion product.

Often the maintenance of two completely unrelated genomes in a hybrid is not desired since this condition has been associated with morphological and cytogenetic abnormalities causing sterility, and other aberrant characteristics. To produce more viable, morphologically normal hybrids, one of the parental genomes can be partially or completely eliminated by irradiation. In this manner, a specific trait native to the irradiated genome may eventually be transferred into the genome of the recipient. This is accomplished without

thaliana callus-derived protoplasts using PEG. These fusion products were cultured and eventually 31 hybrid cell clones were isolated and cytologically analyzed. Gleba and Hoffman were able to regenerate hybrid callus tissue into 'Arabidobrassica' plants, that failed to root (Gleba and Hoffmann, 1980). Morphologically these hybrids demonstrated many differences from their parents. On their leaf surface they portrayed abnormal leaf hair structures, while their flowers demonstrated partially transformed pistils and ovaries (phyllody), and sometimes even the absence of petals or anthers. All hybrid plants were self sterile and some grotesque morphological abnormalities were also observed in shoot and leaf structures (Gleba and Hoffmann, 1979).

Since the pioneering work of Gleba and Hoffmann, there have been numerous reports on protoplast fusion and plant regeneration from fusion products in Brassica (Table 2).

LITERATURE REVIEW

A. PROTOPLAST ISOLATION AND CULTURE

A protoplast represents the cellular contents bounded by the plasmamembrane. Although protoplasts were originally isolated by mechanical methods they are now commonly isolated by enzymatic hydrolysis which has made it feasible to obtain them in very large numbers (Cocking, 1960).

Protoplasts lend themselves to a large number of manipulations, such as the uptake of cell organelles, of foreign genetic material, and of microorganisms. For all of these procedures, osmotic stability must be maintained to protect the protoplast against osmotic swelling and bursting. A combination of metabolically active stabilizers (i.e. glucose, sorbitol, sucrose) along with metabolically inert stabilizers (i.e. mannitol) are used to enhance protoplast viability both in the enzyme solution and in the protoplast culture medium (Vasil and Vasil, 1980).

The formation of a cell wall around a cultured protoplast may occur more rapidly in cell culture-derived protoplasts than in mesophyll-derived protoplasts (Williamson et. al., 1977). The deposition of the first cellulose microfibrils can occur anywhere from 20 minutes to 24 hours post-isolation

(Williamson et. al., 1977; Fowke, 1978). These initial microfibrils are loosely organized at the surface of the plasmamembrane and later in the culture procedure, but before the first division, they become more organized. It has been demonstrated, in tobacco cultures, that protoplasts only require exogenous hormones after the first division (Cooke and Meyer, 1981), which usually occurs between two and seven days. The exact timing, again, depends on the protoplasts' tissue source. Differentiated tissue sources require a longer culture period before undergoing the first division (Gamborg and Miller, 1973).

Many protoplast culture and plant regeneration studies have been undertaken with cruciferous species including both Brassica and Arabidopsis (Table 1).

The development of reliable plant regeneration protocols from protoplast cultures has provided the prerequisites for engineering improved plant varieties. Especially in the economically important Brassicas, such technology can and is being used to regenerate transformed plants, somatic hybrid plants, and plants with novel cytoplasms.

In Brassica, plants have been successfully regenerated from protoplasts derived from mesophyll, hypocotyl, stem cortex and root cells (Table 1). These studies have involved the use of a variety of different protoplast culture techniques to achieve plant regeneration. Culture systems have been continuously improved. For example, the one-step method of Chuong and

coworkers (Chuong et. al., 1985) was based on a medium containing Ficoll and this method has been applied to stem cortex protoplasts by Klimaszewska and Keller (1987). The agarose support system for culturing B. napus protoplasts has also become a standard technique (Millam et. al., 1988).

The regeneration of plants from Arabidopsis protoplasts was first realized in 1980 when Xuan and Menczel regenerated in vitro plantlets from cell suspension-derived protoplasts, and this has been recently repeated (Ford, 1990).

**TABLE 1: EXAMPLES OF PLANT REGENERATION STUDIES
FROM CULTURED PROTOPLASTS IN BRASSICA
AND ARABIDOPSIS**

SPECIES	SOURCE OF PROTOPLASTS	REFERENCE
<u>Brassica</u>		
<u>B. napus</u>	mesophyll	Kartha et. al., 1974
<u>B. napus</u>	mesophyll	Newell, 1984
<u>B. napus</u> <u>B. oleracea</u>	mesophyll	Jourdan & Earle, 1989
<u>B. oleracea</u>	mesophyll	Bidney et. al. 1983
<u>B. napus</u> <u>B. oleracea</u>	hypocotyl	Glimelius, 1984
<u>B. napus</u>	hypocotyl	Barsby et. al. 1986
<u>B. napus</u>	stem cortex	Klimaszewska & Keller, 1987
<u>B. napus</u> <u>B. oleracea</u>	root	Xu et. al. 1982
(CULTIVAR) <u>Arabidopsis thaliana</u>		
Columbia	cell suspension	Xuan & Menezel 1980
Wassilewskija	cell suspension	Ford, 1990

B. SOMATIC HYBRIDIZATION

Somatic hybridization is a method employed for the genetic modification of plants by combining genomes of sexually incompatible species through protoplast fusion. It can serve as a potential method for crop improvement since it facilitates the transfer of genetic information from a wild species into a cultivated crop (Cocking, 1988). A number of novel somatic hybrids have been produced whose genetic composition has been shown to contain numerous combinations of the two parental genomes.

Various fusogens have been employed in an attempt to optimize the somatic hybridization process. Examples of such fusogens include: polyethylene glycol (PEG), alkaline calcium solutions, dextran, polyvinyl alcohol, synthetic phospholipids and electrical stimulation. Polyethylene glycol is the most widely used and the most effective due to the large number of protoplasts which can be simultaneously fused with a relatively low toxicity (Gleddie et. al., 1985).

At the early stages of protoplast fusion a product called a heterokaryon is formed. At this level, the two parental nuclei have not yet fused but the cytoplasmic components are mixed. Karyogamy, the formation of one hybrid nucleus, occurs either at interphase or during the first mitotic division. The fusion of two cytoplasms does not necessarily ensure a fusion of the nuclear envelopes. Studies on fusions between different species have shown a

tremendous variation in fusion frequency (Harms, 1985). These variations along with unknown causative factors make it difficult to settle on a single, simplified fusion model.

Nuclear segregation is dependent upon such factors as the level of differentiation of the tissue source, the physiological state of the parental protoplasts, and the cell cycle stage. Protoplasts from different tissue sources have been shown to differ in their cell cycle phase, therefore when two different protoplasts are fused together, chromosome pulverization of one of the genomes may occur (Harms, 1985). The ploidy level of the parental protoplasts may also affect the sequential loss of chromosomes from one partner. Gross genomic imbalances may occur if the partners differ greatly in their chromosome number or if the fusion event involves more than two protoplasts. These imbalances have been shown to sometimes correlate with a loss in fertility in somatic hybrids (Keller et. al., 1982). It is important to note, though, that multiple fusion events have a lower frequency of regenerating into plants due to developmental disadvantages (Harms, 1985).

Unlike sexually produced zygotes, where the cytoplasm is often maternally derived, fusion products initially contain a mixture of both parental cytoplasm. Cytoplasmic segregation in fusion products often occurs after mitosis and usually results in one parental cytoplasm predominating. Cytoplasmic DNA recombination, as sometimes seen between two different mitochondrial DNAs, occurs prior to cytoplasmic segregation (Evans, 1984).

Theoretically, assuming that the higher plant cell possesses only two extranuclear genophores, the fusion of two genomically different protoplasts may yield 8 different combinations of nuclear and extranuclear gene systems. This calculation only considers viable genome combinations and excludes the possibility of cytoplasmic DNA recombination (Gleba and Sytnik, 1984).

Numerous procedures for the identification and selection of somatic hybrid cells, organs and plantlets have been developed. Over the years, several complementation systems have also been generated. Such systems are usually based on the principle of fusing two protoplasts, each carrying a different selectable marker. For example, one parent can be a recessive auxotroph while the other parent carries a conditionally lethal mutant. The fusion products which carry both genomes should be able to survive the double selection system (Harms, 1985).

Treatment with metabolic inhibitors, such as iodoacetate, can be used as a selection system by blocking essential metabolic functions in the parental lines (Cocking, 1988). Visual identification at the cellular level, if the two parental protoplast populations can be distinguished visually, is another method for isolating fusion products. Parental protoplasts can be stained with specific vital fluorescent dyes and heterokaryons emitting two fluorescence wavelengths have been sorted using a Fluorescence Activated Cell Sorter (FACS) (Gleddie et. al., 1985).

At the regenerated plant level, morphological traits intermediate to the parents can sometimes be used as a selective characteristic for identifying somatic hybrids. Various biochemical assays, such as isozyme analyses and DNA analyses using diagnostic markers, have also been successfully employed to determine hybridity.

Theoretically, somatic hybrids should have a chromosome number reflecting that of the sum of the parents (Evans, 1984). In some intergeneric fusion studies, such as those between B. campestris and A. thaliana (Gleba and Hoffmann, 1978), amphidiploid chromosome numbers were observed in the hybrid plants. Other fusion studies where distantly related parental genomes were integrated and completely maintained are those of Solanum tuberosum + Lycopersicon esculentum (Melchers et. al., 1978) and Sinapis turgida + B. oleracea (Toriyama et. al., 1987). Some symmetric fusions, though, have resulted in fusion products containing a range of novel nuclear and cytoplasmic mixtures. These mixtures can be obtained by nuclear and/or cytoplasmic segregation of the parental chromosomes following fusion (Evans, 1984).

The production of the first somatic hybrid was reported by Carlson et. al. (1972) and involved the hybridization of two different Nicotiana species. Six years later, Gleba and Hoffman produced the first intergeneric cruciferous hybrids between Arabidopsis thaliana and Brassica campestris (Gleba and Hoffmann, 1978). B. campestris leaf-derived protoplasts were fused to A.

thaliana callus-derived protoplasts using PEG. These fusion products were cultured and eventually 31 hybrid cell clones were isolated and cytologically analyzed. Gleba and Hoffman were able to regenerate hybrid callus tissue into 'Arabidobrassica' plants, that failed to root (Gleba and Hoffmann, 1980). Morphologically these hybrids demonstrated many differences from their parents. On their leaf surface they portrayed abnormal leaf hair structures, while their flowers demonstrated partially transformed pistils and ovaries (phyllody), and sometimes even the absence of petals or anthers. All hybrid plants were self sterile and some grotesque morphological abnormalities were also observed in shoot and leaf structures (Gleba and Hoffmann, 1979).

Since the pioneering work of Gleba and Hoffmann, there have been numerous reports on protoplast fusion and plant regeneration from fusion products in Brassica (Table 2).

TABLE 2: A SUMMARY OF PEG-MEDIATED PROTOPLAST FUSION STUDIES INVOLVING BRASSICA

FUSION PARTNERS	RHP	REFERENCE
<u>B. napus</u> karyoplast + <u>B. napus</u> cytoplasm	No	[Spangenberg & Schweiger, 1986]
<u>B. napus</u> triazine res. + <u>B. napus</u> CMS	Yes	[Yarrow et. al., 1986]
<u>B. napus</u> haploid atrazine resistant + <u>B. napus</u> haploid CMS	Yes	[Chuong et. al., 1988a] [Chuong et. al., 1988b]
<u>B. napus</u> + <u>B. campestris</u>	No	[Menczel & Wolfe, 1984]
<u>B. napus</u> atrazine resistance + <u>B. oleracea</u> Ogura CMS	Yes	[Jourdan et. al., 1989]
<u>B. oleracea</u> + <u>B. campestris</u>	No	[Glimelius et. al., 1986]
<u>B. oleracea</u> + <u>B. campestris</u>	Yes	[Terada et. al., 1987] [Schenk & Roebbelen, 1982] [Sundberg et. al., 1986]
<u>B. oleracea</u> Ogura CMS + <u>B. campestris</u> atrazine res.	Yes	[Robertson et. al., 1987]
<u>B. oleracea</u> + <u>B. campestris</u>	Yes	[Sundberg et. al., 1987]
<u>B. oleracea</u> + <u>Sinapis turgida</u>	Yes	[Toriyama et. al., 1987]
<u>A. thaliana</u> + <u>B. campestris</u>	Yes	[Gleba & Hoffmann, 1978, 1979, 1980]
<u>Di. lotaxis harra</u> + <u>B. napus</u>	Yes	[Klimaszewska & Keller, 1988]
<u>Eruca sativa</u> + <u>B. napus</u>	Yes	[Fahleson et. al., 1988]
<u>Glycine max</u> + <u>B. napus</u>	No	[Kantha et. al., 1974]

RHP= Regenerated Hybrid Plants

C. ASYMMETRIC FUSION

In an effort to produce viable and morphologically sound regenerants, which express a particular characteristic of interest, and to increase the use of protoplast fusion for crop improvement, methods for inactivating and/or eliminating one of the nuclear genomes have been developed. Two successful methods used to induce preferential chromosome loss are: chemical inactivation and high doses of irradiation.

Certain chemicals, such as iodoacetate can prevent protoplasts from dividing. When a mitotically inactive protoplast is fused to a dividing protoplast, the non-dividing nucleus is eventually diluted out (Dudits, 1988). High doses of X- or gamma-irradiation are used to induce genome instability by damaging the DNA. In theory, the irradiated protoplast would be rendered non-dividing and the damaged DNA would be preferentially eliminated. Before this elimination could occur, recombination of the chromosomes, or of a few genes, would take place during the transient coexistence period of the two combined genomes (Dudits, 1988). In this manner, only specific selectable traits of the donor could be transferred to the recipient without the rest of the donor genome being maintained.

In plant cells, the nucleus appeared to be the most radiosensitive structure, the larger the nuclear volume, or DNA content, the more radiosensitive the cell (Sparrow et. al., 1961). Differences in radiation

damage were also correlated to ploidy level. A Japanese group working on Nicotiana species determined that haploid cells were more sensitive to gamma-ray irradiation than diploid cells (Balito et. al., 1989).

Some of the damage produced by irradiation is in the form of single strand breaks, which are incurred by splitting the sugar-phosphate bond of the DNA molecule (Veleminsky and Gichner, 1978). The presence of hydrogen atoms during radiolysis can also increase nuclear damage by reacting with purine and thymine base groups in the DNA (Holroyd and Glass, 1969). In many studies, oxygen has also been shown to catalyze the effect of the ionizing particles on the genetic material (Veleminsky and Gichner, 1978; Conger et. al., 1972).

To repair this induced damage, plants have several inherent mechanisms which are similar to those found in other organisms. In Nicotiana, Lathyrus, barley and carrot a photoreactivation of UV-induced pyrimidine dimers has been discovered. The excision of dimers and the repair of single strand breaks have also been proven for many plant species. Due to these mechanisms, along with a variation in the location of the induced damage, it is difficult to consistently predict the relation of the number of breaks to the genetic effect on the plant (Veleminsky and Gichner, 1978).

In 1969, Ammirato and Steward demonstrated that some of these irradiation-characteristics were not solely products of nuclear damage, but instead could be due to the toxic byproducts produced when media

components were irradiated. When unirradiated plant cells were placed in an irradiated sucrose-containing medium, they displayed a decrease in division rate and abnormal development in the sequence from cells to plantlets. Some of these heat-stable sucrose radiolysis products were found to be formic acid and other acidic reduction agents (Ammirato and Steward, 1969). Gamma-irradiation studies on embryo formation in orange ovular callus tissue agreed with Ammirato's and Steward's findings. Low level irradiation of just the medium induced the same level of embryogenesis as low level irradiation of the callus (Spiegel-Roy and Kochba, 1973).

Studies on the effects of irradiation on the cell have inspired the use of ionizing radiation for asymmetric protoplast fusion. In many asymmetric fusion experiments, irradiation seems to induce the recombination of the nuclear genomes, while in other studies a decrease in the formation of nuclear hybrids occurs. A decrease in nuclear recombination is usually correlated with an early elimination of the irradiated nuclear genome and a mixing of the organelles from the two parents (Gleba and Sytnik, 1984).

A fusion product which experiences a complete loss of one of the parental genomes, yet a combination or segregation of the parental cytoplasms is known as a cybrid. Such cybrids are extremely useful for the investigation of traits native to either chloroplast or mitochondrial genomes. Irradiation of protoplasts from one of the parents facilitates the production of such cybrid plants (Cocking, 1988). Interspecific Nicotiana cybrids were produced by X-

irradiating an alloplasmic-male-sterile donor and fusing it to a chlorophyll deficient recipient. The regenerated hybrids contained only the nuclear genome of the chlorophyll deficient parent and expressed both male sterility and chlorophyll deficiency (Fluhr et. al., 1984).

The relationship between the degree of asymmetry in the chromosome constitution of the hybrids and the level of irradiation was studied by Yamashita and coworkers. The donor protoplast, B. campestris, was X-irradiated at different levels ranging from 10 kRad to 80 kRad prior to fusion with B. oleracea. In many of the hybrids there was an increase in chromosome elimination as the dose increased from 10 kRad to 30 kRad. Some hybrids, though, showed no evidence of chromosome elimination at these levels. At higher doses, of 60 kRad and 80 kRad, a further increase in donor genome elimination was not observed (Yamashita et. al., 1989).

Similarly, Imamura and coworkers also found substantial amounts of tobacco DNA in fusion products derived from heavily irradiated Nicotiana tabacum + Hyoscyamus muticus (Imamura et. al., 1987). Other intergeneric fusion studies, such as that between gamma-irradiated Atropa belladonna + Nicotiana chinensis (Gleba et. al., 1983), and X-irradiated Petroselinum hortense + Daucus carota (Dudits et. al., 1980) showed a directional elimination of the donor genome but the degree of elimination could not be correlated to an increase in the radiation level.

Asymmetric fusion studies have also been successfully employed to transfer marker genes, which can be used as selection systems for genotypes carrying agronomically important traits. Hygromycin resistance has been transferred from a disease-resistant B. nigra strain into a sensitive strain of B. napus. Hygromycin resistant fusion products which also expressed disease resistance were selected (Sacristan et. al., 1989). The agronomic trait has also been directly transferred into the recipient parent from an irradiated donor, as shown by the transfer of blackleg (Phoma lingam) resistance from B. juncea into B. napus (Sjodin and Glimelius, 1989). Cytoplasmic male sterility (CMS), another agronomically valuable trait, has also been successfully transferred into many genera, including the Brassicacs (Barsby et. al., 1987) and the Nicotianas (Fluhr et. al., 1984).

Successfully regenerated hybrid plants from asymmetric fusion studies in Brassica are listed in Table 3.

TABLE 3: HYBRID BRASSICA PLANTS PRODUCED BY ASYMMETRIC PROTOPLAST FUSION

FUSION PARTNERS	IRRAD TYPE	IRRAD LEVEL	REF
Studies on Nuclear Transfer			
<u>B. nigra</u> * + <u>B. napus</u> hygromycin res.	X-ray	20-30 kRad	Sacrsitan et. al. 1989
<u>Eruca sativa</u> * + <u>B. juncea</u>	γ -ray	22.5-41 kRad	Sikdar et. al., 1990
<u>B. juncea</u> * + <u>B. napus</u> <u>B. nigra</u> * <u>B. carinata</u> * Phoma lingam res.	X-ray	7-20 kRad	Sjodin & Glimelius 1989
<u>B. campestris</u> * + <u>B. oleracea</u>	X-ray	10-80 kRad	Yamashita et. al. 1989
Studies on Cytoplasmic Transfer			
<u>B. napus</u> * + <u>B. napus</u> Polima CMS	X-ray	30 kRad	Barsby et. al., 1987
<u>B. napus</u> * + <u>B. napus</u> Ogura CMS	X-ray	5.7 kRad	Menczel et. al. 1987

* = irradiated fusion partner
 CMS = cytoplasmic male sterility
 IRRAD = irradiation

D. PARENTAL SPECIES

i- Brassica napus

Brassica napus (Brassicaceae family) ($2n=38$) is a major crop species which is grown extensively in Canada as an oilseed known as rapeseed, and in specific instances as canola. B. napus is an amphidiploid hybrid between Brassica oleracea ($2n=18$) (broccoli, cauliflower, cabbage) and Brassica campestris ($2n=20$) (turnip rape) (U, 1935).

According to the Canadian Seeds Regulations, the term 'canola' refers to the cultivars of Brassica napus or Brassica campestris that contain less than 2% erucic acid in oil, and less than 30 umoles glucosinolates per gram of dry, oil free solid component (meal) (Canadian Grain Commission). The oil-free portion of the seed, referred to as canola meal, contains high levels of essential amino acids, minerals and vitamins. This meal contains approximately 36% protein (Brauer and Roebbelen, 1989), and serves as an excellent feed for poultry, swine and ruminants (Canadian Canola Research Summary). Previous to the reduction of glucosinolate levels, rapeseed meal caused negative side effects when ingested by monogastric animals (Brauer and Roebbelen, 1989).

Canola breeding programs have been in existence for many years. In Canada, emphasis is being placed on increasing both the protein and the oil

content of the seed, without decreasing overall seed yield. Other objectives have been to breed cultivars with enhanced disease resistance (blackleg, white rust) and insect resistance (flea beetle, diamond back moth). Herbicide resistance and the production of vigorous hybrid varieties are also objectives of many breeding programs (McVetty et. al., 1987).

ii)-Arabidopsis thaliana

Arabidopsis thaliana (L.) Heynh ($2n = 10$) is also a member of the mustard family (Brassicaceae). It has become a model system for studying plant molecular biology due to its short life cycle, small size, small genome size and the considerable classical genetic information available on this species. Its short generation time, of approximately six weeks and the small amount of space occupied by a single plant, enhances the capability of carrying out numerous genetic studies within a given time frame and within a restricted space (Pang and Meyerowitz, 1987).

With a haploid genome size of 70 000 kb, Arabidopsis has one of the smallest genomes among flowering plants (Meyerowitz and Pruitt, 1984). This small genome size, though, has no correlation to organismal complexity (Bennett and Smith, 1976). Instead, its size is due to a simpler organization of gene families compared to similar gene families from other plants (Bowman et. al., 1988). Approximately 14% of the DNA consists of highly

repetitive sequences, 25% consists of middle repetitive sequences and more than 50% consists of single copy sequences. All of these characteristics, along with a low level of cytosine methylation make this species amenable to mutagenesis, cloning and gene identification studies (Leutwiler et. al., 1984).

In 1983, Koorneef and coworkers published a linkage map of the Arabidopsis nuclear genome. They assigned 76 loci to five linkage groups, corresponding to the five chromosomes. Several years later, a Restriction Fragment Length Polymorphism (RFLP) map, consisting of 90 single-copy molecular markers, was constructed (Chang et. al., 1988). While such useful maps were being constructed, other research teams were cloning and characterizing genes from both wild type and mutant phenotypes. Biochemical mutants were isolated with minimal difficulty due to the ease with which large numbers of seed and progeny could be screened. Recently isolated mutants include photorespiration mutants (Somerville and Ogren, 1982), male sterile lines (Moffatt and Somerville, 1987), mutants resistant to amino acid analogues (Estille and Lehle, 1987; Verbruggen and Jacobs, 1987), nitrate reductase mutants (Crawford et. al., 1986), alcohol dehydrogenase mutants (Dolferus and Jacobs, 1984) and herbicide resistant strains (Haughn and Somerville, 1986).

E. SULFONYLUREA RESISTANCE IN PLANTS

Sulfonylurea is a class of herbicide produced by Ciba-Geigy and DuPont (Chaleff, 1988). This compound, because it only inhibits the branched chain amino acid biosynthetic pathway (Ray, 1984; LaRossa and Falco, 1984), has a very low mammalian, avian and aquatic toxicity level (Sweetser et. al., 1982; Canadian Centre for Occupational Health and Safety). Both sulfometuron methyl (Oust) and chlorsulfuron (Glean) (Figure 1) are examples of sulfonylurea herbicides produced by DuPont (Chaleff and Mauvais, 1984).

Since the early 1980s, studies on chlorsulfuron's mode of action have been undertaken. It has been firmly established that chlorsulfuron, along with other sulfonylurea herbicides and imidazolinone herbicides inhibit one of the early steps in leucine, isoleucine and valine biosynthesis (Mifflin and Cave, 1972; Reynolds, 1986; Shaner et. al., 1984). The enzyme which is affected by this inhibitor is acetolactate synthase (ALS) or acetohydroxyacid synthase (AHAS). Both terms are synonymous for the enzyme which binds α -ketobutyrate, plus thiamine pyrophosphate and FAD to render α -aceto- β -hydroxybutyrate, a precursor of L-isoleucine. The same enzyme catalyzes a parallel reaction where pyruvate plus thiamine pyrophosphate and FAD, are converted into α -acetolactate, a precursor of L-valine (Zubay, 1983). An abridged version of this pathway can be found in Figure 2.

**FIGURE 1: STRUCTURE OF THE CHLORSULFURON
MOLECULE**

**FIGURE 2: AMINO ACID BIOSYNTHETIC PATHWAYS AND
RESPECTIVE CHEMICAL INHIBITORS**

Relevant Abbreviations:

CS = chlorsulfuron
SM = sulfometuron methyl
AC = AC 243, 997

From growth inhibition studies, chlorsulfuron has been shown to arrest the cell cycle by inhibiting isoleucine and valine biosynthesis (Rost, 1984; Ray, 1982a, 1982b). These experiments implicate these two amino acids in the indirect control of the plant cell cycle, yet not in cytodifferentiation (Reynolds, 1986).

To further study the nature of sulfonylurea resistance, ALS was isolated from different organisms and characterized at molecular and cytological levels. In Escherichia coli and Salmonella typhimurium, ALS is a tetramer composed of two large and two small subunits in an $\alpha_2\beta_2$ structure with approximate molecular weights of 60 kDa and 9 to 17 kDa respectively (deFelice et. al., 1982; Eoyang and Silverman, 1984). To date, three ALS isozymes have been determined for these two species (Friden et. al., 1985; Wek et. al., 1985). In certain fungi, ALS has been isolated from the mitochondrion (Ryan et. al., 1974), while in higher plants it is always localized in the chloroplast (Jones et. al., 1985).

In yeast, only one functional ALS gene has been found which bears little homology to genes encoding either one of the bacterial subunits. In B. napus, at least four ALS genes have been isolated and characterized (Lee et. al., 1988; Rutledge et. al., 1990). One single copy ALS gene has been isolated from Arabidopsis thaliana (Haughn and Somerville, 1986) and two ALS genes from Nicotiana tabacum (Charest et. al., 1989). These higher plant genes are

homologous with genes encoding the bacterial large ALS subunit, but bear no homology with the genes for the small subunit (Mazur et. al., 1987).

In plants, ALS is encoded by a nuclear gene which also codes for a transit peptide on the N-terminal (Haughn et. al., 1988; Wiersma et. al., 1989). According to recent models, RNA molecules are transcribed from the nuclear ALS gene, processed and then modified by polymerization of additional AMP residues to the 3' end. These RNAs are translated, in the plant cell cytoplasm, into precursor proteins, and they are subsequently transported to the chloroplast. Chloroplasts usually carry a high negative charge on their surface, which along with specific precursor binding sites within the envelope, aid in binding the target sequence to the chloroplast (Pfisterer et. al., 1982). The transport of the precursor polypeptide into the organelle is facilitated by the N-terminus extension, however this mechanism is not yet fully understood (Hooper, 1984). After entering the chloroplast, the precursor ALS protein is proteolytically cleaved by a protease, and assembled into a mature protein (Gibbs, 1979). The ALS polypeptide then may partake in the branched chain amino acid biosynthetic pathway within the chloroplast. Whether all of the necessary amino acid biosynthetic enzymes can be found in the chloroplast, is still under investigation (Halliwell, 1981).

Plants expressing chlorsulfuron tolerance do so either by detoxifying the herbicide into an inactive product or by altering the structure of their ALS protein so that it can no longer associate with the chlorsulfuron molecule

(Sweetser et al., 1982). Many cereal crops possess a natural resistance to the herbicide (Durner and Boeger, 1988). Wheat, wild oats, barley and annual bluegrass rapidly metabolize chlorsulfuron, through hydroxylation, to a non-toxic O-glycoside. Broadleaf plants, on the other hand show little or no metabolism of the chemical, as demonstrated by carbon-labelling experiments (Sweetser et. al., 1982). Sensitive dicots, besides their inability to break down the herbicide, suffer from a toxic accumulation of the ALS substrate, α -ketobutyrate (LaRossa et. al., 1987; Danchin et. al., 1984). A number of research projects have focussed on the production of mutations within the ALS coding region of plants (Conner et. al., 1988; Knowlton et. al., 1988; Yadav et. al., 1986; Haughn and Somerville, 1987; Chaleff, 1988).

In 1984, Chaleff and coworkers developed the first sulfonylurea resistant dicot by using tobacco cell culture (Chaleff and Ray, 1984). Two years later, a chlorsulfuron resistant strain of Arabidopsis thaliana was selected by Haughn and Somerville via chemical mutagenesis of seeds. The nature of the mutation was later deduced to be a single base mutation within the ALS coding region, resulting in an amino acid substitution (Haughn et. al., 1988). This rendered the mutated phenotype 100-fold more resistant to the herbicide than the wild type strain (Haughn and Somerville, 1986). Using this well characterized gene, B. napus was successfully transformed via Agrobacterium-mediated gene transfer and transformants expressed the herbicide-resistant phenotype (Wiersma et. al., 1989).

MATERIALS AND METHODS

A. PLANT MATERIAL

i) Arabidopsis thaliana c.v. Columbia

The Arabidopsis thaliana c.v. Columbia GH50 donor plant material was a cell suspension culture initiated from leaf callus tissue by S. Gleddie (1989). Seeds of the GH50 line, from which plants were grown and leaf callus was obtained, had been kindly provided by G. Haughn, and was derived from a mutant line with resistance to sulfonylurea herbicides (Haughn and Somerville, 1986).

The GH50 cell suspension cultures were subcultured on a weekly basis. Twenty mL of spent medium were removed from the confluent cell culture and the remaining cell volume was evenly transferred into three sterile 125 mL Ehrlenmeyer flasks. Each flask contained 40 mL of freshly prepared medium (2% sucrose, 2 mg/L 2,4-dichlorophenoxyacetic acid (Sigma) (2,4 D), 1X modified Murashige and Skoog salts (MS) (Murashige and Skoog, 1962) at pH 5.8, prior to autoclaving at 15 psi for 23 minutes). Cultures were maintained on a gyratory shaker at 150 rpm, with an ambient temperature of 25°C, and a fluorescent-incandescent light intensity of $15 \mu\text{Em}^{-2}\text{s}^{-1}$ during a 16 hour photoperiod.

The GH50 plants carry a single base mutation, 870 nucleotides downstream from the transcriptional initiation site of the single copy acetolactate synthase (ALS) gene (Figure 3). This mutation results in a single amino acid change (Figure 4) which confers resistance to sulfonylurea herbicides, such as chlorsulfuron.

To insure that the cell suspension maintained and expressed this trait, suspensions were periodically cultured under selective pressure. Every second week, GH50 cell suspensions were transferred to fresh medium containing 0.1 $\mu\text{g/mL}$ of chlorsulfuron.

**FIGURE 3: ACETOLACTATE SYNTHASE GENE MAP IN
BRASSICA**

**FIGURE 4: ACETOLACTATE SYNTHASE AMINO ACID
SEQUENCE**

ii) Brassica napus cv. Westar

The recipient fusion partner, Brassica napus L. spp. oleifera cv. Westar was grown from seed (K. Downey, Agriculture Canada, Saskatoon) in the greenhouse at 25°C, 70% humidity, and with a supplementary fluorescent-incandescent light source with a 16 hour photoperiod. Plants which were older than three weeks (from day of germination) were fertilized once a week with a liquid 20:20:20 fertilizer. Seedlings were watered with tap water on a daily basis. Plants were grown in a soil mixture consisting of one part soil, three parts peat, one part sand, one part perlite and 1/20th part lime.

B. PROTOPLAST ISOLATION AND CULTURE

i) Arabidopsis thaliana c.v. Columbia GH50
Cell Suspensions

For the isolation of GH50 protoplasts, three to four day old cell suspension cultures were filtered through a sterile 88 μm nylon filter. A wet cell mass of approximately 1.5 g was dispensed into 18 mL of a filter sterilized enzyme solution (1% cellulase Onozuka R-10 (Yakult Honsha Co.), 1% macerozyme R-10 (Yakult Honsha Co.), 0.4 M mannitol, 10 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5 mM 2[N-Morpholino]ethanesulfonic acid (MES) (Sigma) at pH 5.8). The

cells were agitated at 100 rpm, on a reciprocal shaker, for 18 hours at 25°C in the dark.

Following digestion, protoplasts were filtered through a 44 μm nylon filter and subsequently centrifuged at 50 g for six minutes. Protoplasts were washed twice with an MES wash solution (0.4 M mannitol, 10 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5 mM MES, pH 5.8) and then resuspended in NLN culture medium (Nitsch and Nitsch, 1967; Lichter, 1981; modified by N. Long and A. Atanassov, Agriculture Canada, Ottawa, Ontario) (0.4 M sucrose, 0.03 g/L glutathione, 0.8 g/L L-glutamine, 0.1 g/L L-serine, 0.5 mg/L 2,4-D, 0.5 mg/L 1-naphthalene acetic acid (NAA), 0.05 mg/L 6-benzylamino purine (BAP), 2% Ficoll (Sigma), 1X Nitsch and Nitsch salts (1967), pH 5.8 and filter sterilized) at a final density of 1.0×10^5 protoplasts per mL of medium. A protoplast suspension volume of 1.5 mLs was added to 15 x 60 mL sterile petri dishes, each containing a two mL solidified agarose sublayer (0.4 M sucrose, 0.5 mg/L 2,4 D, 0.5 mg/L NAA, 0.05 mg/L BAP, 0.6% Seaplaque agarose (FMC Bio-Products), pH 5.8.

Protoplast culture dishes were incubated for 14 days in the dark at 28°C. Cultures were then supplemented with 0.5 mL of C medium with an osmoticum of 0.2 M mannitol (Pelletier et. al., 1983) and then transferred to a growth room with a very low fluorescent-incandescent light intensity ($6.4 \mu\text{Em}^{-2}\text{s}^{-1}$), 25°C, and a 16 hour photoperiod. After four days, the light intensity was increased to $10 \mu\text{Em}^{-2}\text{s}^{-1}$, and on day 21, cultures were supplemented

with 0.5 mL of C medium with no osmoticum, and the light intensity increased to $45 \mu\text{Em}^{-2}\text{s}^{-1}$. Half a mL of C medium was added on a weekly basis until colonies were large enough ($= > 0.5 \text{ mm}$ in diameter) to be transferred to Shoot Regeneration Medium (SRM) (0.1 M mannitol, 1% sucrose, 0.2 mg/L zeatin (Sigma), 1X modified MS salts, 0.6% Seaplaque agarose, pH 5.8).

ii) Brassica napus c.v. Westar

Westar protoplasts were isolated from stem epidermal strips which were carefully excised from surface sterilized inflorescence stems of plants which had just started to bolt, as described by Klimazewska and Keller (1987). Strips were conditioned in a 1X modified MS salt solution (2% sucrose, pH 5.5) at 4°C for 24 hours in the dark. Strips were then digested in an enzyme solution (same solution as that used for GH50 cell digestion) for 18 hours at 28°C on a reciprocal shaker (40 rpm) in the dark.

Following digestion, protoplasts were gently spun down at 30 g for six minutes and subsequently floated on a cushion of 0.6 M sucrose (pH 5.8 with KOH) which was covered in a few milliliters of MES wash solution. After centrifugation at 50 g for 10 minutes, viable protoplasts were collected from the meniscus between the sucrose bed and the MES wash layer. Protoplasts were washed once with MES wash solution, and then resuspended in NLN culture medium to a final density of 1.0×10^5 protoplasts per mL. The

protoplast suspension was plated out over an agarose sublayer in the same manner as previously described. Culturing conditions were identical to those already outlined for GH50 protoplasts.

C. X-IRRADIATION KILLING CURVES OF GH50 PROTOPLASTS

GH50 protoplasts, in enzyme solution, were subjected to X-irradiation at doses ranging from 0 krad to 80 krad. After 16 hours of digestion, petri dishes containing the partially digested GH50 protoplasts, were X-irradiated 5.0 cm from the source of a Picker Scanray X-Ray machine (AC 128 Automatic) at a constant setting of 240 kV and five mA. Different dosages were obtained by varying the time of exposure, as illustrated in Table 4.

**TABLE 4: X-Ray Exposure Times for GH50 Protoplasts
at 240 Kilovolts and 5.0 Milliamps**

APPROX. kRAD	ACTUAL kRAD	TIME (minutes)
0	0.0	0
8	8.21	12
10	10.26	15
11	10.95	16
12.5	12.62	18
20	19.84	29
30	29.76	44
40	39.67	58
80	80.03	117

Following irradiation, protoplasts were isolated and cultured as previously described. Protoplasts treated at each X-ray dosage were plated out in triplicate. The viability of the protoplasts was monitored using one of two methods.

i) Fluorescein Diacetate

Protoplasts were mixed at a 1:1 ratio with a freshly prepared 1:50 dilution of fluorescein diacetate (FDA) stock solution (5mg/1.0 ml of acetone, stored at 20°C). Protoplasts, in stain solution, were incubated in the dark for five minutes at room temperature. They were then observed under an inverted fluorescence microscope (Zeiss, West Germany) using both phase contrast optics and a barrier filter (487709) for fluorescence microscopy. Fluorescing protoplasts were counted and compared to the total number of protoplasts visualized under phase contrast. Protoplast cultures were regularly assessed in this manner over a two week period.

The percentage of protoplasts undergoing cell division was monitored visually at 200 X magnification using a simple inverted light microscope (PhotoZoom, Bausch and Lomb). These rates were also assessed on a regular basis over a period of two weeks.

ii) Tritiated Leucine Incorporation Studies

The second method, for determining the viability of X-irradiated protoplasts, monitored the incorporation of tritiated leucine into protoplasts over a period of ten days.

GH50 protoplasts were isolated and cultured over agarose sublayers in a multiwell plate. Immediately after plating out, cultures were fed with 1 μ Ci of ^3H -leucine per mL of culture suspension. Each sample was cultured in triplicate.

^3H -leucine fed controls were also plated out in triplicate. These controls consisted of: NLN culture medium, silver nitrate (0.75 mM) inactivated protoplasts and unirradiated protoplasts.

^3H -leucine incorporation was determined by washing protoplasts onto a 3C Millipore glass microfibre filter with a solution of ten percent trichloroacetic acid (TCA), followed by a solution of five percent TCA. These filters were submersed in ten mLs of scintillation cocktail (CytoScint, ICN Biomedicals Inc.) and vortexed for fifteen seconds. The amount of radioactivity (CPM) in each scintillation vial was determined by scintillation counting (Beckman LS 8000; 2 channel program for ^3H). Each vial was read for a period of ten minutes.

Aliquots of protoplasts to which ^3H -leucine was not added were cultured in the same manner. These were used for total protein determination (Biorad

Assay) using Bovine Serum Albumin as a standard. These experiments were repeated twice.

D. CHLORSULFURON KILLING CURVES

i) Westar Protoplasts

Following isolation, Westar protoplasts were plated out over agarose sublayers containing different concentrations of chlorsulfuron. The chlorsulfuron stock (100 μg of chlorsulfuron per ml of sodium phosphate buffer, pH 7.5) was filter sterilized and added to the molten agarose sublayers, after the agarose had been autoclaved. The final chlorsulfuron concentrations, of the agarose sublayers, were: 0, .001, .01, .1, and 1 $\mu\text{g}/\text{ml}$.

The protoplasts were cultured in triplicate for 30 days. Following this culture period, colonies with a diameter greater or equal to 0.5 mm were counted and the plating efficiency for each sample was determined. Colonies from the 0 $\mu\text{g}/\text{ml}$ control plates were transferred to SRM and incubated for another 30 days at 25°C under a light intensity of 55 $\mu\text{Em}^{-2}\text{s}^{-1}$ and with a 16 hour photoperiod. After one month, the number of colonies which had regenerated shoots were counted, and the frequency of shoot formation was assessed.

ii) GH50 Protoplasts

GH50 protoplasts were isolated, and then cultured on agarose sublayers containing the following concentrations of chlorsulfuron: 0, .1, 1.0, 2.5, and 5.0 $\mu\text{g/ml}$. Protoplast viability was monitored over a ten day period using the tritiated leucine incorporation method from section C ii).

E. FUSION AND SELECTION OF HETEROKARYONS

After Westar and GH50 protoplast isolation (section B), protoplasts were washed separately with a high calcium solution (0.2% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.5% KCl, pH 6.9 prior to autoclaving). Protoplasts were counted using a Rosenthal haemocytometer and then gently pelleted at 30 g for six minutes. The supernatant was carefully discarded and the GH50 protoplast pellet was carefully mixed with the Westar protoplast pellet at a 1:1 ratio. An equal volume of 25% polyethylene glycol (PEG) (6000 MW (Sigma); in a solution of 3% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, pH 6.9 and filter sterilized) was slowly added to the mixed protoplasts. The mixture was gently agitated and then allowed to stand for fifteen minutes. The PEG was eluted with 0.4 M mannitol (pH 5.8) in 0.5 mL aliquots at two minute intervals over 50 minutes. The diluted fusion mixture was gently centrifuged at 30 g (six min.), and the supernatant discarded. The pellet was resuspended in NLN culture medium and the fusion products were

plated onto agarose sublayers at a density of 5.0×10^5 protoplasts per mL. One and a half mLs of suspension were plated out into each 15 x 60 mm petri dish.

Culture dishes were incubated in the dark at 28°C and 70% humidity for a period of 14 days. The subsequent culturing procedure is outlined in Section B, of this chapter.

Fusion products were subjected to various concentrations of chlorsulfuron selection (.001, 0.01 and 0.1 $\mu\text{g/mL}$) and also to different selection procedures. Fusion products and control dishes were selected at day 0, day 5, and day 14 of the protoplast culture period. Surviving colonies were transferred to SRM, without selection, and incubated at $55 \mu\text{Em}^{-2}\text{s}^{-1}$, 25°C, and a 16 hour photoperiod. Colonies were transferred to fresh SRM every two weeks until they formed shoots. Regenerated shoots were excised using a sterile scalpel and forceps, and placed onto Root Regeneration Medium (RRM) (2% sucrose, 0.1 mg/L NAA, 1X modified MS salts, 0.35% gelrite [Kelco], pH 5.8) in 20 x 120 mL petri dishes. These dishes were cultured under the same conditions as the SRM plates. Shoots were subcultured on a monthly basis until they produced roots.

The rooted plantlets were carefully cut out of the solidified medium, to prevent damage to any roots, and transplanted into sterile baby food jars (50 x 350 mm) containing 35 mL of solidified RRM. These were cultured, with the jar lid slightly loosened, under the same environmental conditions as

previously described. Plantlets were maintained and cloned by excising shoots from rooted plantlets and transferring these shoots to fresh RRM in sterile baby food jars.

Rooted in vitro plantlets were transferred to soil and grown in the greenhouse. This was done most successfully by washing the roots clean of the RRM with tap water and transplanting them into a mist chamber (25°C, 95% humidity, and a 16 hour photoperiod) containing vermiculite: turface: perlite (1:1:1) for one week. Hardened plantlets were then transplanted into soil. The entire culture procedure is illustrated in Figure 5.

Controls included homofusions, such as Westar + Westar and GH50 + GH50, plated out over agarose sublayers containing different chlorsulfuron concentrations. These were plated out at densities of 5.0×10^5 protoplasts per mL. Unfused protoplasts were also cultured simultaneously. Some of these latter controls were also subjected to herbicide selection, while others were not (Table 5).

**FIGURE 5: GENERAL PROCEDURE FOR THE
PRODUCTION, ISOLATION AND
REGENERATION OF
BRASSICA + ARABIDOPSIS
FUSION PRODUCTS**

PRODUCTION OF ASYMMETRIC ARABIDOPSIS HYBRIDS

TABLE 5: Summary of Fusion and Control Experiments

Sample	Level of CS in Agarose ($\mu\text{g/ml}$)	Approximate Plating Density (protoplast/ml)	Selection Protocol (day)
<u>Fusions</u>			
W x GH150-60 kRad	0.01, .001	5.0×10^5	day 0
W x GH150-60 kRad	0.01, .001	5.0×10^5	day 5
W x GH150-60 kRad	0.01	5.0×10^5	day 14
W x GH150-90 kRad	0.01, .001	5.0×10^5	day 0
W x GH150-90 kRad	0.01, .001	5.0×10^5	day 5
W x GH150-90 kRad	0.01	5.0×10^5	day 14
W x GH150-0 kRad	0.1	5.0×10^5	day 0
W x GH150-0 kRad	0.1	5.0×10^5	day 5
W x GH150-60 kRad	0.1	5.0×10^5	day 0
W x GH150-60 kRad	0.1	5.0×10^5	day 5
W x GH150-90 kRad	0.1	5.0×10^5	day 0
W x GH150-90 kRad	0.1	5.0×10^5	day 5
<u>Controls</u>			
W x W	.001, .01, 0.1	5.0×10^5	day 0
W x W	.001, .01, 0.1	5.0×10^5	day 5
W x W	.01	5.0×10^5	day 14
GH150 x GH150 *	.001, .01, 0.1	5.0×10^5	day 0
GH150 x GH150 *	.001, .01, 0.1	5.0×10^5	day 5
GH150 x GH150 *	.01	5.0×10^5	day 14
W	0, .001, .01, .1	1.0×10^5	day 0
W	0, .001, .01, .1	1.0×10^5	day 5
W	0, .01	1.0×10^5	day 14
GH150 *	.001, .01, 0.1	1.0×10^5	day 0
GH150 *	.001, .01, 0.1	1.0×10^5	day 5
GH150 *	.01	1.0×10^5	day 14

abbreviations:

W = Westar

CS = chlorsulfuron

* = cultures irradiated at 60 and 90 kRad

F. HYBRID ANALYSIS

i) Morphological Analysis

The flower morphology of the hybrids was assessed by recording the petal colour, the number of petals, sepal colour, the number of sepals, the number of anthers, stigma morphology and the length of the corolla. A minimum of three separate regenerants were analyzed for each hybrid.

The detailed nature of the upper leaf surface of greenhouse hardened hybrids was compared to the upper leaf surface of the parents using Scanning Electron Microscopy (SEM). Leaves were freshly excised and immediately placed onto the cryostage of the electron microscope (Hexland Cryotrans CT 1000). The leaf samples were cooled to -80°C to sublime condensation, and then coated with gold. Samples were then cooled again to -150°C and placed in the path of the electron beam of an AMR 1000 A SEM.

ii- Rooting Assay

The level of herbicide resistance, in the hybrids, was assessed using a rooting assay. Magenta boxes were filled with 50 mLs of RRM supplemented with one of the following concentrations of chlorsulfuron: 0, 0.001, 0.01, 0.1,

and 1.0 $\mu\text{g/ml}$. Shoots, from previously rooted plantlets, were excised aseptically and placed onto the RRM.

Magenta boxes were incubated at 55 $\mu\text{Em}^{-2}\text{s}^{-1}$, 25°C, and a 16 hour photoperiod for 21 days, when they were assessed for their ability to form roots and for the number of new leaves produced.

iii- Herbicide Spray Assay

One month old greenhouse acclimatized hybrids and parents, which had not yet bolted, were sprayed with different levels of chlorsulfuron (0.5% TWEEN, in distilled water). All plants were sprayed with the same concentration of herbicide. Only the volume, which each species received, varied. The amount of liquid needed to completely wet all leaf surfaces on the parental and the hybrid plants was determined by averaging the volume of water plus surfactant sprayed from a used hairspray non-aerosol pump onto a minimum of five plants.

The herbicide concentrations used were 0, .25, 0.5 and 1.0 $\mu\text{g/ml}$. Plants were sprayed in a fumehood and then transferred to a growth chamber (285 $\mu\text{mEm}^{-2}\text{s}^{-1}$, 16 hour photoperiod, 70% humidity, 25°C day temperature, 15°C night temperature) where they remained for fourteen days. After two weeks, plants were analyzed for their height, number of new leaves formed, and for the number of flower bolts they had produced.

iv) Glucosinolate Analysis

One to 1.5 grams of leaf tissue was harvested both from greenhouse grown and acclimatized plants and from *in vitro* regenerants. The leaf tissue was immediately placed into a tube containing five milliliters of cold distilled water. The tubes were boiled for ten minutes, allowed to cool to room temperature and centrifuged at 100 g for fifteen minutes. The supernatant was aseptically removed and the glucosinolates were desulphated and analyzed using reversed-phase high-performance liquid chromatography (HPLC) as described by Minchition (Minchition et. al., 1982).

The HPLC analysis was performed by Dr. J. GrootWassink at the Plant Biotechnology Institute, National Research Council in Saskatoon.

v) Isozyme Analysis

Various isozyme staining methods were utilized. These staining methods, along with the type of tissue used and the concentration of polyacrylamide in the running gel, are outlined in Table 7. Three different protein extraction buffers (Table 6) were also compared using the isozyme staining protocol for glutamate-oxaloacetate transaminase (GOT) (Thorpe et. al., 1987). For all of the isozyme analyses, the following protocol was utilized.

a- Protein Extraction

Leaf, root or stem tissue was carefully harvested from young (one to two month old) Westar and GH50 plants grown from seed, in the greenhouse. Tissue was also taken from young putative hybrid plants which had been transferred from in vitro conditions to the green- house, one month earlier.

Between 0.1 and 0.2 grams of tissue were immediately placed into 0.5 mLs of ice-cold extraction buffer (30 mM Dithiothreitol (J.T. Baker), 0.2 M Tris.HCl, pH 8.5). The tissue was ground with a pestle along with a pinch of sea sand (Fisher Scientific) on ice. The samples were spun at 12 000 g for fifteen minutes at 4°C. The supernatant was carefully removed and stored on ice while a protein determination assay (BioRad) was performed to determine the total protein concentration.

b- Preparation of the Polyacrylamide Gel Electrophoresis (PAGE) Apparatus

Samples consisting of 15 µg of protein and 15% of loading dye (50% sucrose, 0.1% bromophenol blue) were loaded into the wells of a vertical BioRad Protean II mini-gel apparatus, which was packed on ice. Samples were electrophoresed at 200 volts (constant) for approximately 45 minutes, or until the dye front had migrated to the bottom of the gel. The running gel was carefully removed from the stacking gel and subjected to one of the staining methods in Table 6.

TABLE 6: Isozyme Staining Protocols, Modifications and Tissue Type

Isozyme	Stain Reference	% PAG*	Tissue
ACP	Thorpe et. al. 1987	6.25	leaf
ACP	Thorpe et. al. 1987	5.0	leaf
ADH	Thorpe et. al. 1987	6.25	leaf
EST	Thorpe et. al. 1987	6.25	leaf
EST	Wetter and Dyck 1983	5.0	leaf
			stem
			root
EST	Wetter and Dyck 1983	6.25	leaf
			stem
			root
GDG	Thorpe et. al. 1987	5.0	leaf
		6.25	leaf
GOT	Thorpe et. al. 1987	6.25	leaf
		8.0	leaf
			stem
			root
		10.0	leaf
			stem
			root
GPI	Thorpe et. al. 1987	5.0	leaf
		6.25	leaf
LAP	Thorpe et. al. 1987	5.0	leaf
		6.25	leaf
MDH	Thorpe et. al. 1987	5.0	leaf
		6.25	leaf
+ 0.2 mg NAD /mL running PAG		6.25	leaf
PER	Schleicher and Schuell 1988	6.25	stem
			root
PGM	Thorpe et. al. 1987	5.0	leaf
		6.25	leaf
+ 0.2 mg NADP/mL running PAG		6.25	leaf
SAD	Thorpe et. al. 1987	6.25	leaf

abbreviations: ACP = acid phosphatase

ADH = alcohol dehydrogenase

EST = esterase

GDG = glutamate dehydrogenase

GOT = glutamate-oxaloacetate transaminase

GPI = glucose phosphate isomerase

LAP = leucine aminopeptidase

MDH = malate dehydrogenase

PER = peroxidase

PGM = phosphoglucomutase

SAD = shikimic acid dehydrogenase

*PAG = polyacrylamide gel

vi) Southern Hybridization

Genomic DNA was extracted from the leaf tissue of the parents and of the hybrids by grinding in Tris.EDTA buffer. Following a proteinase K digestion for 30 minutes, samples were extracted with phenol:chloroform. DNA was dissolved in TE buffer (10mM Tris, 1 mM EDTA, pH 8.0), quantitated using UV spectrophotometry (Manniat, 1982), and stored at -20°C.

Ten µg of DNA were used for each restriction enzyme reaction. DNA was digested with different restriction enzymes (BRL) (one unit per µg of DNA) along with an assay buffer (BRL), 25 mM Spermidine (Sigma), 2 µl of BSA (BRL) per 100 µl of reaction mix, 20 µg RNase per 100 µl of reaction mix (pancreatic RNase A), and one unit of enzyme per µg of DNA.

Digested DNA was precipitated using 95% ethanol, resuspended in sterile, distilled water and run on 0.8%, or 2% agarose gels. After 16 hours at 40 volts, the gel was photographed and blotted onto a Nytran (Schleicher and Schuell) support membrane using the method developed by Southern (Southern, 1975). The DNA was fixed onto the membrane using a two minute exposure of long wavelength UV light, and then prepared for hybridization with a radiolabelled DNA probe.

a) Hybrid Determination Using a Wheat Ribosomal DNA Probe

Genomic DNA was restricted with HindIII, EcoRI, BamHI and PstI and hybridized to a wheat ribosomal DNA probe, PTA 71 (Gerlach and Bedbrook, 1979).

b) The Nature of the Acetolactate Synthase Gene

To determine the nature of the acetolactate synthase gene in the fusion products, genomic DNA was digested with the following enzymes: XbaI, EcoRI, BamHI, and HindIII (BRL). ALS probes were isolated from the pGH1 plasmid (Haughn et. al., 1988) by digesting with XbaI and NcoI to render four fragments which varied in size from 0.9 kilobases to 3.7 kilobases. The 0.9 kb and the 1.1 kb isolated fragments were random primed, and hybridized to the Nytran blots using the method employed by Rutledge and coworkers (1990). Blots were washed at specific stringencies and exposed to X-ray film.

c) Mitochondrial and Chloroplast DNA Analysis

Genomic DNA was isolated and digested as described in section F vii. The following restriction enzymes were used for this study: TaqI, HaeIII, BamHI, XbaI and SacI (BRL). The chloroplast DNA probe was pBa1-9 (Aviv et. al., 1984) and the mitochondrial DNA probes were ATP-6 (Bonen and Bird, 1988), cox II 5'end (Bonen et. al., 1984) and cytochrome b (Boer et. al., 1985). Probes were random primed with ^{32}P -CTP (Feinberg and Vogelstein, 1983) and hybridized to the Nytran membranes.

RESULTS

A. CHARACTERIZATION OF CHLORSULFURON RESISTANCE IN PARENTAL SPECIES

i) Brassica napus cv. Westar

To determine the tolerance level of B. napus (Westar) strip derived-protoplasts, they were plated over agarose sublayers containing 0, .001, .01, 0.1 and 1.0 $\mu\text{g/ml}$ chlorsulfuron.

Initially, the effect of the herbicide on protoplast viability was assessed using fluorescein diacetate (FDA). This labour intensive method of viability determination was inconsistent and it failed to show significant differences in viability with increasing levels of herbicide. From observations taken with phase contrast optics, visual differences could be detected in protoplast cultures at different herbicide concentration. As the herbicide concentration increased, so did the proportion of dead or unhealthy protoplasts. Interestingly, even at 1.0 $\mu\text{g/ml}$ of chlorsulfuron, a few mitotic divisions were still observed, yet no microcolonies were formed.

Untreated Westar protoplasts usually underwent their first mitotic division on day 3 of the culture period. It is important to note, though, that there was much variation from experiment to experiment. After 30 days in culture,

Westar protoplasts developed into distinct, firm colonies. These colonies were counted and the plating efficiency calculated in the following manner:

$$\% \text{ Plating Efficiency} = \frac{\text{total No. of colonies}}{\text{total No. of protoplasts}} \times 100\%.$$

(only colonies greater or equal to 0.5 mm were counted)

This simple method was adopted to determine the effect of increasing levels of herbicide on the ability of a Westar culture to regenerate colonies.

From a sample of ten plates (two experiments) it was calculated that cultures lacking chlorsulfuron had a plating efficiency of 0.056% with a standard deviation of 0.027%. For cultures grown on chlorsulfuron levels of .001 $\mu\text{g/ml}$ or higher, the plating efficiency was 0%.

ii) Arabidopsis thaliana cv. Columbia GH50

To establish the optimal level of selection for this suspension culture, the rate of growth was determined at different chlorsulfuron concentrations. GH50 cell suspensions were cultured in triplicate at 0, 0.1, 1.0, 2.5, and 5.0 $\mu\text{g/ml}$ of chlorsulfuron. The cumulative results of these experiments (Figure 6) also served as a preliminary indicator of the possible herbicide concentrations necessary for selecting fusion products of Westar + GH50. Concentrations up to 1.0 $\mu\text{g/ml}$ chlorsulfuron did not appear to affect the relative growth rates of GH50 suspension cultures.

**FIGURE 6: GROWTH RATE OF GH50 CELL SUSPENSION
CULTURES AS A FUNCTION OF
CHLORSULFURON LEVEL OVER TIME**

Relative Growth Rates were plotted to standardize slight variations in the initial seeding volume of each culture.

$$\text{Relative Growth Rate} = \frac{\text{cell volume at day (x)}}{\text{cell volume at day (0)}}$$

The initial cell pellet volume ranged from 1.9 mls to 3.2 mls in 40 mls of culture medium.

The Standard Deviation for each time point is represented by vertical error bars.

The sample size (n) varied between 9 and 21 flasks (minimum of three separate experiments) per time point.

After 30 days of culture, GH50 protoplasts did not produce individual colonies which were easy to count. They consistently produced a thin, aggregated cell layer at the surface of the medium and under these circumstances, it was extremely difficult to count individual colonies or to calculate plating efficiencies. As an alternative method for determining viability, tritiated leucine was fed to freshly isolated GH50 protoplasts and its incorporation monitored over a period of approximately 10 days. The incorporation of tritiated leucine into protein was negatively affected by increases in chlorsulfuron concentration (Figure 7). A concentration of 2.5 $\mu\text{g}/\text{ml}$ resulted in approximately a 50% reduction in leucine incorporation when compared to cultures grown without chlorsulfuron. GH50 protoplasts treated with 0.1 $\mu\text{g}/\text{ml}$ chlorsulfuron did not demonstrate a decrease in incorporated leucine until day 13.

**FIGURE 7: PROTEIN SYNTHESIS IN GH50
 PROTOPLASTS AS A FUNCTION OF
 HERBICIDE CONCENTRATION**

On day 0 of the protoplast culture period, GH50 protoplasts were fed with specifically labelled tritiated leucine. Each condition (various chlorsulfuron levels) was plated out in triplicate, and all of the dishes had an initial plating density of 1×10^5 protoplasts per mL of medium. The amount of incorporated tritium was measured on day 3, 5, 8 and 13 of the culture period. Measurements were taken in counts per minute (CPM).

Control cultures were fed with silver nitrate to determine the metabolic activity in inactivated protoplasts.

METABOLISM OF GH50 PROTOPLASTS AT VARIOUS CS LEVELS

n = 3 for each data point

B. GH50 PROTOPLASTS AT VARIOUS LEVELS OF X-IRRADIATION

i) Fluorescein Diacetate

GH50 protoplasts were X-irradiated in the enzyme solution at levels ranging from 0 to 30 kRad. After irradiation, the protoplasts were thoroughly washed and plated in culture medium. Small aliquots were removed and FDA stained at regular intervals over a two week period. From visual observations, there did not appear to be much variation in the proportion of fluorescing protoplasts at the various radiation levels (Figure 8). On day 0, the number of fluorescing protoplasts, compared to the total number of protoplasts, did not seem to vary between the 10, 13 and 20 kRad treatments (Figure 8). Even on day 2 of culture, there was little difference between protoplasts irradiated with 30 kRad and unirradiated protoplasts. The only difference between the two levels may have been the intensity of the fluorescence. The varying intensities of the fluorescence staining made it more difficult to clearly identify metabolically active protoplasts. To eliminate any error incurred by false judgement, all protoplasts which demonstrated any degree of fluorescence were considered to be viable. On numerous occasions it was also noted that ruptured protoplasts, along with debris particles, fluoresced brightly when stained. These were not counted. Such findings may lead one to wonder whether metabolically inactive yet intact FDA-stained protoplasts emit a fluorescence, and if so, to what extent would these false positives affect the overall percent viability.

**FIGURE 8: FLUORESCEIN DIACETATE STAINED GH50
PROTOPLAST CULTURES, X-IRRADIATED
AT DIFFERENT LEVELS**

All three micrograph sets demonstrated GH50 protoplasts several hours after protoplast isolation.

Micrographs 1,3 and 5 were pictures of FDA stained protoplast cultures viewed with phase contrast optics, while micrographs 2,4 and 6 were the same preparations viewed with a fluorescence barrier filter.

1 & 2. Micrograph of GH50 protoplasts irradiated
with 10 kRad (day 0).

Magnification: 250 X

3 & 4. Micrograph of GH50 protoplasts irradiated
with 13 kRad (day 0).

Magnification: 250 X

5 & 6. Micrograph of GH50 protoplasts irradiated
with 20 kRad (day 0).

Magnification: 250 X

Viability curves, regardless of the level of irradiation (Figure 9), appeared to follow the exact same pattern as that of the unirradiated culture. Between day 2 and day 3, all of the cultures had demonstrated an increase (positive slopes) in percent viability. Between day 5 and day 6 all of the cultures displayed a drastic decrease in viability as seen by the steep negative slopes.

The first mitotic division in GH50 protoplast cultures occurred on the second or third day after isolation. This observation (Table 7) was uniform for all levels of X-irradiation.

ii) Tritiated Leucine Incorporation

GH50 protoplasts, which had been X-irradiated from 0 to 30 kRads, were treated with tritiated leucine immediately after isolation. To determine metabolic activity as a function of X-ray level, cultures were sampled over a period of 12 days. This experiment was repeated twice and each condition within an experiment was done in triplicate. The amount of leucine incorporated between identical samples in different experiments varied to such a great degree that the data has not been included. By comparing the leucine incorporation curves from the two experiments, no consistent trends could be established.

$$\% \text{ viability} = \frac{\text{No. of fluorescing protoplasts}}{\text{total No. protoplasts}} \times 100\%$$

Legend Abbreviations: avg. = average value
St. Dev. = standard deviation

Sample sizes ranged from n=3 to n=12

**TABLE 7: THE DIVISION RATE OF X-IRRADIATED GH50
PROTOPLASTS AS DETERMINED BY PHASE CONTRAST
MICROSCOPY**

TIME day	AVERAGE PERCENT DIVISION ($\bar{x}$ Avg. $\pm$ $\bar{x}$ S.D.)					
	0 kRad	8 kRad	10 kRad	13 kRad	20 kRad	30 kRad
0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
1	1.4 $\pm$ 2.2	1.5 $\pm$ 3.4	1.7 $\pm$ 3.1	0.6 $\pm$ 1.1	3.4 $\pm$ 5.4	0.7 $\pm$ 1.5
2	9.2 $\pm$ 5.6	11.1 $\pm$ 9.8	7.2 $\pm$ 6.3	5.1 $\pm$ 5.2	2.8 $\pm$ 3.1	1.4 $\pm$ 2.2
3	4.1 $\pm$ 3.6	2.9 $\pm$ 4.1	7.4 $\pm$ 5.8	3.3 $\pm$ 4.6	9.7 $\pm$ 10.0	9.7 $\pm$ 11.5
4	5.0 $\pm$ 2.8	4.7 $\pm$ 4.6	5.4 $\pm$ 4.1	1.9 $\pm$ 3.2	6.7 $\pm$ 4.8	2.7 $\pm$ 3.4
5	4.0 $\pm$ 0.7	1.3 $\pm$ 1.3	4.7 $\pm$ 0.8	3.8 $\pm$ 0.6	3.3 $\pm$ 0.7	4.4 $\pm$ 5.7
6	4.4 $\pm$ 1.7	3.9 $\pm$ 3.9	4.0 $\pm$ 2.8	1.2 $\pm$ 1.2	5.1 $\pm$ 3.9	9.8 $\pm$ 9.8
7	8.8 $\pm$ 1.4	-	7.0 $\pm$ 2.4	-	12.1 $\pm$ 2.1	-
14	0.8 $\pm$ 0.6	1.5 $\pm$ 0.9	0.3 $\pm$ 0.5	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0

Abbreviations: Avg = average
S.D. = standard deviation

The sample size (n) varied from 3 to 15 for each time point.
Each n was an average value for one experiment, therefore the values in
this table represent averages taken from 3 to 15 different experiments.

C. WESTAR + GH50 FUSION EXPERIMENTS

Both Westar and GH50 protoplasts were digested for a period of 18 to 20 hours on a rotary shaker in the dark. This timing was crucial since overdigestion of the cells would cause them to lyse and hence decrease the final protoplast yield. The optimal digestion time was determined using a calcofluor white staining procedure which was taken at various time points during the digestion. A 15 hour digest (Figure 10) left approximately 25% of the cells undigested. It was at approximately this stage (between 15 and 18 hours) that the GH50 protoplasts were irradiated in the enzyme solution. The irradiation treatment (90 kRad) normally took 117 minutes. By the time the treatment was completed (usually 17 to 20 hours) all of the cells had been converted to protoplasts

Following irradiation, GH50 protoplasts were washed and fused to Westar protoplasts using polyethylene glycol (PEG) (Figure 11). In general, Westar protoplasts could be distinguished from GH50 protoplasts by their larger size and by the presence of green chloroplasts. Following fusion, protoplasts were cultured for thirty days prior to transfer to a shoot regeneration medium (SRM). Within this protoplast culture period, various selection schemes were undertaken. At day 30, the number of colonies produced by each fusion event was counted (Figure 12). From these culture plates, plating efficiencies for each event were calculated and compared to the plating efficiencies of the control cultures containing

unselected Westar protoplasts. In Fusion Experiment 1, the selection level ($.001 \mu\text{g/ml}$ of chlorsulfuron) was not stringent enough since some Westar homofusion colonies, selected on day 0 of the protoplast culture period, managed to grow. At day 30 these homofusion control escapes were transferred to SRM where they browned and died. The other Westar homofusion control dishes which were subjected to selection later in the protoplast culture period (day 4) did not produce any escape colonies. This was certainly not indicative of a selection trend since there were only two replicates for each selection condition.

The selection levels for Fusion Experiments 2, 3 and 4 were increased to $.01$, $.01$ and $0.1 \mu\text{g/ml}$ respectively, in order to prevent further escapes and in an attempt to isolate fusion products with increased levels of resistance. The plating efficiencies of the fusion products for the last three experiments usually ranged between $.01\%$ and 0.1% . The plating efficiencies of fusion and unselected Westar control cultures from the first experiment were in the range of $.001\%$ to $.01\%$, ten fold lower than the preceeding experiments. The plating efficiencies of the fusion cultures were always slightly lower than the plating efficiencies of the Westar control cultures. In general though, the size of the fusion product colonies were larger than the Westar colonies. GH50 plating efficiencies were not calculated due to the fact that cultures did not produce individual colonies.

**FIGURE 10: CALCOFLUOR WHITE STAINED GH50
CELL DIGEST**

Simultaneous phase contrast and fluorescence microscopy of GH50 cells
digested for 15 hours in enzyme solution.

Magnification: 100 X

**FIGURE 11: POLYETHYLENE GLYCOL
MEDIATED WESTAR + GH50 FUSION**

In a 1:1 ratio of Westar to GH50 protoplasts, hetero-aggregates, homo-
aggregates and multiple aggregation events were observed.

All colonies greater or equal to 0.5 mm in diameter were transferred to SRM (Figure 12, plate 3). Smaller colonies, even after day 30, were allowed to increase in size and then were transferred to the SRM. 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 the SRM. The proportion of fusion product calli which regenerated shoots was much lower, ranging from 4% for regenerants selected at .001 $\mu\text{g/ml}$ to 0.9% for regenerants selected at 0.1 $\mu\text{g/ml}$. Calli which had regenerated shoots were separated from other calli and subcultured in individual SRM dishes. In many cases the shoots were very vitrified or waterlogged. This posed a problem since vitrified shoot tissue did not readily develop roots when placed on root regeneration medium (RRM). Vitrified material usually required many *in vitro* passages before it lost the waterlogged characteristics, and subsequently produced roots.

GH50 calli were unable to regenerate shoots on SRM, as shown by Figure 13 where GH50 is compared to a Westar + GH50-60 kRad fusion product after 30 days of culture.

Sometimes a single callus produced many different shoots, over the culture period. To distinguish between different regenerants, a letter-number system was employed which enabled one to differentiate between fusion experiments, regenerating calli and between individual shoots

produced by a single colony. Arabic numbers from 1 to 4 were used to represent the fusion experiment. Each colony which successfully produced a shoot was identified with a letter while those regenerating more than one shoot were named with a letter followed by a roman numeral, referring to the individual shoot.

Often the putative hybrid shoots had to be subcultured many times onto fresh RRM in petri dishes before developing any roots. Once a stable root system had formed, putative hybrids were transferred to RRM in baby food jars where they continued to grow, or in magenta boxes for ease of photography, Figure 15.

**FIGURE 12: FUSION PRODUCTS AND CONTROLS
IN PROTOPLAST CULTURE DISHES AND
ON SHOOT REGENERATION MEDIUM**

- 1) Herbicide selected (0.001 $\mu\text{g/ml}$) fusion product and control cultures at day 30 post isolation.

Upper left dish contains fusion products from a 60 kRad X-irradiated GH50 + Westar fusion.

Upper right dish contains a homofusion of Westar + Westar.

Lower left dish contains selected Westar protoplasts.

Lower right dish contains fusion products from a 90 kRad X-irradiated GH50 + Westar fusion.

- 2) Homofusion controls at day 30, also selected with 0.001 $\mu\text{g/ml}$ of chlorsulfuron.

Left dish contains GH50-60 kRad + GH50-60 kRad.

Middle dish contains GH50-90 kRad + GH50-90 kRad.

Right dish contains Westar + Westar (day 0 selection). These colonies were escapes due to selection conditions which were not stringent enough.

- 3) Fusion products and control calli at day 30 on shoot regeneration medium.

Upper left dish contains a GH50-60 kRad homofusion control which never regenerated shoots.

Upper middle and left dishes contain GH50-90 kRad + Westar calli.

Lower left dish contains a GH50-60 kRad + Westar callus regenerating a shoot.

Lower right dish contains unselected Westar control calli, some regenerating shoots.

1

2

3

**FIGURE 13: A HOMOFUSION CONTROL AND A FUSION
PRODUCT AT DAY 30 ON SHOOT
REGENERATION MEDIUM**

Top: homofusion callus, GH50-60 kRad + GH50-60 kRad, does not produce shoots.

Bottom: fusion callus, GH50-60 kRad + Westar, demonstrates the initiation of a regenerating shoot.

Plantlets grew more vigorously in loosely capped jars rather than in magenta boxes. The reasons for this are obviously related to gas exchange. In Nicotiana tabacum, ventilation or gas exchange may have been a key factor in determining plant growth. In very early experiments on fusion systems in tobacco (this data is not presented in thesis), in vitro seed germinated-tobacco plantlets were transferred to RRM in magenta boxes which had been sealed either with two layers of parafilm or two layers of Handi-Wrap. Of all the plantlets tested, the ones wrapped in parafilm consistently demonstrated a retarded growth (Figure 14).

From four Westar + GH50 fusion experiments, a total of 17 putative hybrids were regenerated in vitro (Table 8). To date, eight of those putative hybrids have been transferred into a greenhouse environment and have matured, flowered and produced pods.

**FIGURE 14: IN VITRO GERMINATED TOBACCO
PLANTS IN MAGENTA BOXES SEALED
WITH PARAFILM AND WITH HANDI-WRAP**

Left: Parafilm wrapped magenta box

Right: Handi-Wrap wrapped magenta box

**FIGURE 15: REGENERATED PUTATIVE HYBRID
SHOOTS ON ROOT REGENERATION
MEDIUM**

Left: GH50-90 kRad + Westar.

Center: GH50-60 kRad + Westar.

Middle: several vitrified GH50-60 kRad + Westar shoots

**TABLE 8: PUTATIVE HYBRIDS PRODUCED FROM
WESTAR + GH50 FUSIONS**

HYBRID	X-RAY LEVEL (kRad)	SELECTION LEVEL (cs) ($\mu\text{g/ml}$)	DAY OF SELECTION
1:Ai	90	.001	0
1:Aii	90	.001	0
1:Aiii	90	.001	0
1:Aiv	90	.001	0
1:Bi	60	.001	0
1:Bii	60	.001	0
1:C	60	.001	5
2:A	60	.01	0
2:Bi	60	.01	0
2:Bii	60	.01	0
2:C	60	.01	0
3:A	60	.01	14
3:B	60	.01	14
3:C	60	.01	5
4:A	0	.1	5
4:B	0	.1	0
4:C	90	.1	5

abbreviations: cs = chlorsulfuron

Putative hybrids which had successfully developed roots were transferred to a high humidity mist bed in the greenhouse. During this acclimatization period approximately one half of the 17 rooted putative hybrids (Table 8) browned and died, yet were still maintained on RRM in vitro. Putative hybrids 1:Ai, 1:Aii, 1:Aiii, 1:C, 2:Bi, 2:Bii, 3:B and 3:C were successfully established in the greenhouse.

D. CHARACTERIZATION OF FUSION PRODUCTS:

i- Morphological Analysis

The putative hybrids which were established in the greenhouse were visually and microscopically analyzed for specific morphological characteristics. All eight regenerated plants were intermediate in size to their parents (Figure 17). Upon closer morphological examination, it was observed that they had flowers and leaves of intermediate size and shape in comparison to GH50 and Westar leaves (Figure 16).

Using a dissecting microscope the flower structure was more closely scrutinized. The flowers from protoplast-derived Westar control plants demonstrated an unaltered flower morphology when compared to flowers from Westar plants grown from seed. The flowers from the putative hybrids, though, demonstrated tremendous variation from plant to plant and aberrations were also observed on the same plant. This deviation was most clearly shown by the differences in petal number and pistil number and structure within one plant (Table 9).

The only characteristic which remained unchanged both in the parents and in the putative hybrids was the sepal number. Both sepal length and corolla length were fairly consistent amongst the different regenerants, but were intermediate between the measurements recorded for the parents. The petal colour in fusion product flowers varied from yellow to white.

2

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

The only putative hybrid which possessed one morphologically normal pistil was 3:B. The other fusion regenerants displayed morphological abnormalities in their female reproductive organs. Some had abnormal pistils which exhibited leaf-like structures topped with a stigma-like organ. Other aberrant pistils had opened up revealing the ovaries attached to appendages within. All the plants, except 3:B, produced one normal pistil along with one to three abnormal ones.

Six stamens were found in both GH50 and Westar parents. All of the putative hybrids lacked this male reproductive organ. In a few regenerants vestigial filaments were observed, yet they lacked the anther.

FIGURE 16: LEAF AND FLOWER MORPHOLOGY OF THE 3:B HYBRID COMPARED TO ITS PARENTS

The large leaf and flower on the left belong to the Westar parent, the intermediately sized structures in the middle are taken from a greenhouse acclimatized 3:B putative hybrid, and the small leaf and flower on the right belong to the GH50 parent.

FIGURE 17: GREENHOUSE-HARDENED PUTATIVE HYBRIDS ALONG WITH GREENHOUSE GROWN FUSION PARTNERS

The putative hybrids, 3:B (middle left) and 2:Bi (middle right) are flanked by GH50 (left) and Westar (right). All the plants, except 2:Bi, have bolted and flowered.

The morphology of the upper leaf surface of mature leaves was closely examined under a scanning electron microscope. Figure 18 represents the upper leaf surface of the parents at different magnifications. Westar grown in the greenhouse (micrographs 1, 2 and 3) did not produce any leaf hairs, or trichomes. Instead, Westar produced a thick waxy cuticle on its leaf surface. It was observed, though, that Westar grown in vitro produced non-branching trichomes, but as soon as these plants had acclimatized to the greenhouse they lost their hairs and developed a waxy cuticle. GH50, both in vitro and in the greenhouse, produced tri-branched trichomes, as depicted in micrograph 5, and a minimal amount of wax (micrograph 6).

The greenhouse grown hybrids, in Figures 19 and 20, all produced a heavy waxy cuticle with no evidence of leaf hair formation. Interestingly, the physical structure of the wax particles differed amongst the hybrids.

**Table 9: FLOWER MORPHOLOGY OF PUTATIVE HYBRID PLANTS
AND OF THE PARENTS**

	PETAL COLOR	PETAL NO.	COROLLA LENGTH (mm)	SEPAL COLOR	SEPAL NO.	SEPAL LENGTH (mm)	ANTHER NO.	PISTIL NO.
Westar	y	4	12.0-14.0	g *	4	6.0-7.0	6	1
rWestar	y	4	14.0-15.0	g *	4	7.5-8.0	6	1
GH50	w	4	2.0-2.5	g	4	1.5	6	1
1:Aii	ly	6	4.5-6.0	g	4	4.5-5.0	0	1+ (lab)
1:Aiii	ly	6	4.5-5.5	y-g	4	4.5-5.0	0	1+ (lab)
1:C	w-g	4	5.5-6.0	lg	4	5.5-6.0	0	2+ (0-1ab)
2:Bi	y-g	5	5.5-6.5	y-g	4	4.0-5.0	0	1+ (1-2ab)
2:Bii	ly	6-8	5.5-6.0	g	4	4.0-6.0	0	1+ (2ab)
3:C	w	4-6	6.0-7.0	g	4	5.0-6.0	0	2-3ab
3:B	y	5	5.5-6.5	y-g	4	4.0-4.5	0	1

Data is based on measurements and observations taken from three plants of each variety, and at least two flowers per plant.

Notes: * as the flower matures the sepals change from a green to a predominant yellow color

rWestar = Westar plant regenerated from
protoplasts

ab = abnormal morphology

y = yellow, w = white,
ly = light yellow, w-g = white-green

**FIGURE 18: SCANNING ELECTRON MICROSCOPY OF
THE UPPER LEAF SURFACE FROM
GREENHOUSE GROWN WESTAR AND GH50**

Westar: Micrograph 1: Hairless leaf surface at 50 X
magnification.
Micrograph 2: 500 X magnification.
Micrograph 3: An evenly dispersed layer of
of wax particles.

GH50: Micrograph 4: Trichomes on leaf surface at
10 X magnification.
Micrograph 5: A higher magnification (100 X)
of a single trichome.
Micrograph 6: An open stomate surrounded by
very few and unevenly dispersed
wax particles.

**FIGURE 19: SCANNING ELECTRON MICROSCOPY OF
THE UPPER LEAF SURFACE FROM
GREENHOUSE ACCLIMATIZED
PUTATIVE HYBRIDS: 1:Ai AND 1:Aii**

- 1a: Micrograph 7: A 50 X magnification of the
hairless leaf surface.
Micrograph 8: Magnification of 500 X.
Micrograph 9: A thick waxy layer surrounding
a partially open stomate (3000 X).
- 1b: Micrograph 10: A hairless leaf surface at 100 X.
Micrograph 11: Magnification at 500 X.
Micrograph 12: Globular shaped wax particles
around and on a closed stomate at
3000 X.

**FIGURE 20: SCANNING ELECTRON MICROSCOPY OF
THE UPPER LEAF SURFACE FROM
GREENHOUSE ACCLIMATIZED
PUTATIVE HYBRIDS: 1:Aiii AND 1:Aiv.**

- 1c: Micrograph 13: Relief of upper leaf surface at
50 X.
Micrograph 14: Hairless leaf surface with closed
stomata at 500 X.
Micrograph 15: Closed stomate with a relatively sparse
wax coating (3000 X).
- 1d: Micrograph 16: A hairless, irregular leaf
surface at 50 X.
Micrograph 17: Mostly open stomata with
pronounced and profuse wax
particles, at 500 X.
Micrograph 18: An open stomate with very
cylindrical wax particles
(3000 X).

ii- Chlorsulfuron Resistance

a) Rooting Assay

To determine the level of resistance in putative hybrids, excised shoots were transferred to magenta boxes containing RRM at 0, .001, .01, 0.1 and 1.0 $\mu\text{g/ml}$ of chlorsulfuron (Figure 21). After 21 days, the number of new leaves formed and the presence or absence of roots were recorded. The number of new leaves formed were calculated in the following manner:

$$\text{No. New Leaves} = \text{No. Leaves (day 21)} - \text{No. Leaves (day 0)}.$$

Two methods of measuring herbicide resistance were compared (Figure 22). Judging from the graphs alone, root formation appeared to be a more sensitive measure than leaf formation. This was assumed from the observation that the two most herbicide-sensitive putative hybrids (1:Ai and 1:Aii), according to the root formation assay, did not show the same sensitivity when using leaf formation as a criterion. For all of the fusion regenerants depicted in Figure 22, the general trend was a decrease in the number of leaves with an increase in herbicide concentration. From these line graphs it was difficult to determine which putative hybrid had the highest resistance. All of the plants were at least ten fold more resistant than the Westar control.

FIGURE 21, A & B: IN VITRO HERBICIDE ROOTING ASSAY

Putative hybrids 2:A (F8:2) and 3:B (F9:3) after 21 days on root regeneration medium supplemented with 0, .001 (A), .01 and 0.1 (B) $\mu\text{g/ml}$ of chlorsulfuron.

At .001 $\mu\text{g/ml}$ the Westar parent no longer produces roots, and at .01 $\mu\text{g/ml}$ the leaves and stem brown completely.

**FIGURE 22: IN VITRO NEW LEAF FORMATION AND
THE FREQUENCY OF ROOT DEVELOPMENT
AS AN INDICATOR OF HERBICIDE
RESISTANCE**

Error bars represent high and low values for samples exposed to the same culture conditions.

Hybrid 1: Ai vs Westar

In vitro Leaf Formation as a Function of Herbicide Level

In vitro Root Formation as a Function of Herbicide Level

Hybrid 1: Aii vs Westar

In vitro Leaf Formation as a Function of Herbicide Level

In vitro Root Formation as a Function of Herbicide Level

Hybrid 1: C vs Westar

In vitro Leaf Formation as a Function of Herbicide Level

In vitro Root Formation as a Function of Herbicide Level

Hybrid 2:A vs Westar

In vitro Leaf Formation as a Function of Herbicide Level

In vitro Root Formation as a Function of Herbicide Level

Hybrid 2:Bii vs Westar

In vitro Leaf Formation as a Function of Herbicide Level

In vitro Root Formation as a Function of Herbicide Level

Hybrid 3:B vs Westar

In vitro Leaf Formation as a Function of Herbicide Level

In vitro Root Formation as a Function of Herbicide Level

Using root formation as a scale, the 3:B regenerant was the most resistant plant, having 100 X or greater resistance than Westar. Putative hybrids 1:C and 2:A also demonstrated resistance levels of 100 fold or greater than Westar.

b) Spray Assay

To determine how well the in vitro assay would relate to an actual herbicide field application, parents and the putative hybrid 3:B were subjected to a greenhouse spray assay. Initially, the amount of liquid needed to thoroughly wet the parents and 3:B was calculated. One month old Westar plants needed approximately 15 mls of a dilute solution of Tween 80 (surfactant) to completely wet both upper and lower leaf surfaces. One month old GH50 plants required approximately three mls of wetting solution, while one month old greenhouse acclimatized 3:B plants required eight mls.

The concentration of chlorsulfuron needed to kill the Westar parent also had to be determined. Chlorsulfuron concentrations ranging from 0 to 0.25 $\mu\text{g/ml}$ were sprayed onto one month old Westar plants. After two weeks it was determined that 0.25 $\mu\text{g/ml}$ of chlorsulfuron stunted Westar growth by initially causing chlorosis of the apical meristem. This concentration did not completely kill the plant, therefore, the dose of chlorsulfuron was increased.

Westar plants sprayed with increasing levels of chlorsulfuron showed a reduction in height (Figure 23), an abrupt decrease in leaf formation (Figure 24) and an abrupt drop in the number of flower stems produced (Figure 25). GH50 plants, on the other hand, remained relatively unaltered by the increasing herbicide concentrations. Initially they experienced a slight decrease in height when sprayed with a low concentration of the sulfonylurea herbicide ($0.25 \mu\text{g/ml}$), but were affected by increasing chlorsulfuron levels. New leaf formation increased with herbicide application, regardless of the concentration.

**FIGURE 23: THE EFFECT OF DIFFERENT LEVELS OF
HERBICIDE APPLICATION ON THE
HEIGHT OF HYBRID 3:B AND OF THE
PARENTS**

Error bars represent the standard deviations calculated from numerous samples ($n \geq 4$) of the parental plants.

No error bars can be calculated for 3:B since $n=1$.

Height was measured from the soil-stem interface to the top of the apical meristem.

4-n<11 for Westar and GH50, n-1 for 3 B

**FIGURE 24: THE EFFECT OF DIFFERENT LEVELS OF
HERBICIDE APPLICATION ON THE LEAF
FORMATION OF HYBRID 3:B AND
OF THE PARENT PLANTS**

Error bars represent the standard deviations calculated for the samples ($n \geq 4$) of the parental plants.

No standard deviation could be calculated for 3:B ($n=1$).

Data was collected 14 days after topical application of the herbicide.

Relative leaf formation = (No. of leaves, day 14) - (No. of leaves, day 0)

4<n<11 for Westar and GH50, n=1 for 3 B

**FIGURE 25: THE EFFECT OF DIFFERENT LEVELS OF
HERBICIDE APPLICATION ON THE
FLOWERING OF THE HYBRID 3:B AND
OF THE PARENT PLANTS**

Error bars represent standard deviations calculated from samples ($n \geq 4$) of the parental plants.

No standard deviations could be calculated for 3:B ($n=1$).

Plants had not bolted prior to spraying. At 14 days post-spray, lateral and primary shoots were counted for Westar and hybrid 3:B plants. Only shoots directly emerging from the rosette were counted for GH50.

4-n=11 for Westar and GH50, n=1 for 3 B

The results for the putative hybrid 3:B were derived from one experiment only, hence data interpretations are by no means conclusive. According to this experiment (Figure 26), the 3:B plant demonstrated no change in flowering with increasing herbicide levels. The height of the plant also showed very little increase if any, regardless of the treatment. Increasing the treatment from 0.5 $\mu\text{g/ml}$ to 1.0 $\mu\text{g/ml}$ caused a halt in new leaf formation, yet this may have been an anomaly due to the small sample size.

From the data presented, it could only be postulated that 3:B showed little toxic effect to topical applications of chlorsulfuron solutions up to a concentration of 1.0 $\mu\text{g/ml}$. In summary, 3:B was resistant to twice the chlorsulfuron concentration needed to completely defoliate and kill the Westar parent (0.5 $\mu\text{g/ml}$) in a period of 14 days.

To determine how these spray levels compared to chlorsulfuron application rates used in the field, the amount of active ingredient applied to a plant had to be determined. For a Westar plant sprayed with 1.0 $\mu\text{g/ml}$ of herbicide, the final chlorsulfuron dose amounted to 15 μg . According to a study done on winter varieties of rapeseed (Lauten, 1985), the optimal plant density, at harvest time, which rendered the largest seed yield was 100 plants per square meter. Using this number it was calculated that a total of 1×10^6 plants were able to grow in one hectare. If each one month old Brassica plant in this hectare were evenly covered with 15 μg of chlorsulfuron, then the amount of active ingredient needed to treat an entire hectare was 15 grams.

**FIGURE 26: HERBICIDE SPRAY ASSAY ON PARENTS
AND ON THE HYBRID 3:B.**

The level of herbicide application was increased from 0.25 $\mu\text{g}/\text{ml}$ of chlorsulfuron (top), to 0.5 $\mu\text{g}/\text{ml}$ (middle) and 1.0 $\mu\text{g}/\text{ml}$ of chlorsulfuron (bottom).

Both GH50 and the hybrid 3:B did not show any apparent deleterious effects from the treatment.

iii- GLUCOSINOLATE ANALYSIS

Boiled leaf extracts were sent to the laboratory of Dr. J. GrootWassink, Plant Biotechnology Institute, Saskatoon, Saskatchewan, for a qualitative analysis of the glucosinolate profile of 15 putative hybrids. A compiled version of the HPLC profiles was assembled into a compressed illustration in Figure 27. The leaf extract from the Westar parent possessed very few (seven) and very discrete glucosinolate peaks. GH50 displayed 13 peaks in total, most of which had a very pronounced height. The intermediate glucosinolate profiles of the fusion products could be more precisely visualized in Table 10, where each peak was recorded as a function of its relative retention time. Westar only displayed three diagnostic peaks which were not expressed in GH50. The other parent, GH50, on the other hand, had nine original peaks which were not found in Westar. Using these diagnostic peaks as markers the following putative hybrids, which expressed both Westar and GH50 specific glucosinolate peaks, were identified as: 1:C, 2:A, 2:Bi, 2:Bii, 3:A, 3:B and 4:A.

**FIGURE 27: QUALITATIVE GLUCOSINOLATE ANALYSIS
OF HYBRID AND PARENTAL LEAF TISSUE**

Glucosinolate leaf extracts were derived from leaves excised from upper, middle and lower parts of mature plants.

None of the hybrids expressed all of the glucosinolate peaks found in GH50.

DESULPHATED GLUCOSINOLATE ANALYSIS OF PARENTS AND HYBRIDS USING REVERSED-PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY

TABLE 10: THE DIFFERENT GLUCOSINOLATE PEAKS AS A FUNCTION OF THEIR RETENTION TIMES RELATIVE TO THE STANDARD

RRT (min)	PARENTS		PUTATIVE HYBRIDS										
	WES	GH50	1:A I	1:A II	1:A III	1:B	1:C	2:A	2:B I	2:B II	3:A	3:B	4:A
-12	GW	GW	GW	GW	GW	GW	GW	GW	GW	GW	GW	GW	
-11				UN		UN		UN	UN	UN			UN
-10 .5	GW	GW	GW	GW	GW	GW	GW	GW	GW	GW	GW	GW	GW
-10											UN	UN	
-9 .5	GW	GW					GW	GW	GW	GW	GW	GW	GW
-9													UN
-8	W							W		W			
-4 .5		G	G	G	G		G	G	G	G			G
-3						UN						UN	
-2	W								W		W	W	
-1			UN						UN	UN			UN
0	--	--	--	--	--	--	--	--	--	--	--	--	--
.5								UN	UN	UN			UN
1.5		G										G	G
2		G											
2.5		G	G	G	G	G	G	G	G	G		G	
3	W						W				W		W
4												UN	UN
4.5	GW	GW	GW		GW		GW					GW	GW
5		G	G	G	G	G	G	G	G	G	G	G	G
5.5								UN	UN			UN	
8		G	G	G	G	G	G		G	G	G	G	
9			UN		UN		UN			UN		UN	UN
10		G										G	
11 .5		G				UN					UN		
15			UN		UN		UN				UN		UN
16		G								G		G	

ABBREVIATIONS: RRT : Relative Retention Time = peak RT - standard RT

G : peaks common to the GH50 parent only

W : peaks common to the Westar parent only

GW : peaks common to both parents

UN : peaks uncommon to the parents

The results discussed so far were derived from a single extraction experiment. For comparison, leaves were harvested both from in vitro regenerants and from greenhouse-hardened regenerants to determine whether they possessed different glucosinolate profiles (not shown). Qualitatively, all of the peaks, except one at retention time 9.90 min. were identical. Quantitatively, the peaks from in vitro leaf tissue were of greater height and area than the peaks from the greenhouse Westar.

The glucosinolate profiles of GH50 parents grown at very high and low light intensities were also analyzed (not shown). Both profiles had identical peak positions relative to the standard, and the height and area of peaks from both assays were also well conserved. Both GH50 profiles expressed 11 peaks, the same number of peaks expressed by GH50 leaf tissue in the previous assay (Figure 27).

iv- ISOZYME ANALYSIS

From 11 different isozymes which were tested, only three produced well resolved bands which positively identified hybrid bands in protein extracts from selected putative hybrids. These three isozymes were: phosphoglucumutase, esterase and peroxidase.

Some isozyme staining recipes did not produce a clear banding pattern with leaf tissue. This was the case for one of the esterase staining protocols

(Wetter and Dyck, 1983). For this recipe, three different tissue sources were compared. Root tissue produced more pronounced patterns than the leaf or stem tissue. Upon closer examination, the most distinguishing factor between all of the tissues was the band intensities. The migration patterns of the various tissues remained relatively unaltered.

The phosphoglucumutase (PGM) isozyme analysis successfully demonstrated hybrid banding patterns among the hybrid plants which had combined bands from both Westar and GH50 parents. The Westar parent had three major fast moving bands and three slower moving bands. GH50 had one slow migrating band and two faster moving bands. This band number and intensity was verified in three previous PGM analyses (not shown). The parental mix as well as all of the hybrid protein extracts exhibited seven major bands (Figure 28).

The esterase isozyme banding pattern shown in Figure 29 was obtained from root-derived protein extracts. The Westar parent possessed one relatively fast moving major band and the GH50 parent possessed two relatively slow moving major bands. Interestingly, the parental mix produced three bands (two from GH50 and one from Westar) while the hybrids revealed one Westar band along with one or two intermediate migrating bands which were unique. The hybrids may have expressed the two GH50 bands, but on this gel they were very poorly stained. The hybrids expressed the fast migrating Westar band along with faster migrating minor bands, which

were native to the Westar parent as revealed in previous esterase assays (not shown).

The peroxidase isozyme study in Figure 30 was also successful in demonstrating the expression of all parental isozymes in the hybrids. GH50 exhibited one intermediate-migrating band and the Westar parent exhibited two slow-migrating bands along with one fast migrating band. All of the hybrids revealed four peroxidase bands which had the exact same migration distances as those of the parents.

**FIGURE 28: PHOSPHOGLUCOMUTASE ISOZYME
ANALYSIS ON LEAF TISSUE**

1 = Westar	5 = 2b
2 = Parental Mix	6 = 3b
3 = 1a	7 = 4a
4 = 1b	8 = GH50

**FIGURE 29: ESTERASE ISOZYME ANALYSIS ON ROOT
TISSUE**

(lanes 1 to 8, same as above)

**FIGURE 30: PEROXIDASE ISOZYME ANALYSIS ON LEAF
TISSUE**

1 = GH50	5 = 1b
2 = 4a	6 = 1a
3 = 3b	7 = Parental Mix
4 = 2b	8 = Westar

v- Southern Blot Analyses

a) The Hybrid Nature of Fusion Products Using Wheat Ribosomal DNA Probe

TaqI, EcoRI or BamHI digested DNA of the parents all demonstrated differential banding patterns when probed with PTA 71 (a wheat ribosomal DNA probe) (Table 11). In Figure 31, both parental BamHI and EcoRI digests are illustrated. The BamHI digested GH50 parent (At) had three unique bands, varying between 1.0 and 1.5 kb, which were not present in the

TABLE 11: THE DIFFERENCE IN BANDING PATTERN BETWEEN *B.napus* and *A.thaliana* DNAs DIGESTED WITH TEN DIFFERENT RESTRICTION ENZYMES AND PROBED WITH RADIOLABELLED DNA PROBES

DNA PROBE	XbaI	HindIII	TaqI	EcoRI	BamHI	HaeIII	PstI	Sall	PvuII	KpnI
ALS 0.9 kb		pr	pr		pr			pr	pr	pr
ALS 1.1 kb		y	pr		pr					
ALS 2.2 kb					pr					
ALS 5.5 kb		y	y		y		y	y	y	y
ATP-6			y		y	y				
CYT B			y							
COX II 5			y							
pIIa 1-9		y			y					
Brassica cp				y						
Brassica mt		y		y						
PTA-71			n	y	y	y				
napin		pr	pr		pr	pr		pr	pr	pr

y - different banding pattern between parent DNAs
n - no difference in banding pattern between parent DNAs
pr - poor resolution

Westar parent (Bn). The EcoRI fragments ranging from 1.0 to 2.0 kb were the most diagnostic since they displayed the greatest variation in banding patterns between the parents (Figure 31). When the hybrid plant BamHI digests were probed with PTA 71 they displayed unique GH50 bands (Figure 33), however the same hybrid DNAs digested with EcoRI displayed unique Westar bands (Figure 32).

b) Acetolactate Synthase Gene

The 1.1 kb ALS Arabidopsis DNA fragment was isolated by digesting the pGH1 vector with EcoRI and NcoI restriction endonucleases. This 1.1 kb fragment, which codes for a region upstream from the transit peptide sequence, was radiolabelled and hybridized to parental and hybrid DNA digested with XbaI, HindIII and EcoRI.

The ALS 1.1 kb probe showed a strong hybridization to GH50 DNA and a very weak hybridization to Westar DNA (Figure 34). The lower 5.5 kb Arabidopsis band was present in the hybrids 1:C (1g), 3:B (3b) and 4:A (4a), but there was no hybridization to this region for Westar DNA. Hybrids 2:A (2a) and 2:Bi (2b) may also have expressed this band, but due to poor resolution this was not conclusive.

FIGURE 31: THE ANALYSIS OF PARENTAL GENOMIC DNA WITH A WHEAT RIBOSOMAL DNA PROBE (PTA-71)

The blot on the left illustrates the BamHI digested DNA. The blot on the right shows the EcoRI digest.

**FIGURE 32: PTA-71 ANALYSIS OF ECORI DIGESTED
PARENTAL AND HYBRID GENOMIC DNA**

The 4.5 kb band in this blot corresponds to the 4.1 kb band in the EcoRI digest of Figure 31. This discrepancy in base length is due to an error factor in the calculation of the fragment length, which was derived by comparing the migration distance of the fragment to the migration distances of λ HindIII molecular markers.

**FIGURE 33: PTA-71 ANALYSIS OF BAMHI DIGESTED
PARENTAL AND HYBRID GENOMIC DNA**

**FIGURE 34: THE ANALYSIS OF GH50 SPECIFIC
ACETOLACTATE SYNTHASE BANDS IN
PARENTAL AND HYBRID GENOMIC DNA**

c) The Relative Amount of GH50 DNA in the Hybrids

Using EcoRI digested genomic GH50 DNA as a probe, the relative amounts of hybridization were determined for both the parents and the hybrids (Figure 35). Compared to the GH50 lane (At), the Westar parent demonstrated a relatively weak hybridization to the GH50 genome. The hybrids all had much stronger hybridization intensities than the Westar parent, indicating that a significant amount of GH50 DNA was maintained in the fusion products. The hybrid 4:A (lane 4a) displayed more intensive hybridization than the other hybrids. The GH50 lane, when subjected to the same autoradiogram exposure time as the other lanes, rendered a completely black lane with no distinguishable bands. For this reason, the GH50 sample depicted in Figure 35 was only exposed to one tenth the length of exposure.

d) Chloroplast Genomes of the Hybrids

Genomic DNA from the parents and from the hybrids was hybridized to two chloroplast specific DNA probes. A hybridization between a tobacco chloroplast probe, pBa 1-9, and XbaI digested genomic DNA resulted in a banding pattern in which all of the hybrids shared the same bands as the Westar parent (Figure 36). The same result was also obtained for EcoRI digested DNA hybridized to the same probe (Figure 38). When Westar chloroplast DNA was restricted with EcoRI, radiolabelled and hybridized to

EcoRI parental and hybrid total DNA digests, all of the hybrids again demonstrated the same banding pattern as the Westar parent (Figure 37).

A control Southern blot was performed to determine how well the tobacco chloroplast DNA probe hybridized to total genomic DNA, as compared to isolated chloroplast DNA. Purified Westar chloroplast DNA (gift of S. Gleddie) was restricted and probed with pBa 1-9. Both genomic DNA and chloroplast DNA rendered identical banding patterns, indicating that the tobacco chloroplast probe can be used to detect chloroplast-specific Westar sequences in total genomic DNA digests.

FIGURE 35: THE RELATIVE AMOUNT OF GH50 SPECIFIC SEQUENCES IN THE PARENTS AND IN THE HYBRIDS

Total genomic GH50 DNA was random-primed and used as a probe to estimate the relative amounts of GH50 specific sequences in EcoRI digested hybrid plant DNAs.

Bn

1a

1b

1c

1g

2a

2b

2c

2d

2e

2f

2g

FIGURE 36: ANALYSIS OF XBAI RESTRICTED GENOMIC DNA WITH A TOBACCO CHLOROPLAST DNA PROBE (pBA 1-9)

**FIGURE 37: ANALYSIS OF ECORI DIGESTED GENOMIC
DNA WITH LABELLED BRASSICA
CHLOROPLAST DNA**

**FIGURE 38: ANALYSIS OF ECORI DIGESTED GENOMIC
DNA WITH A TOBACCO CHLOROPLAST DNA
PROBE (pBA 1-9)**

e) Composition of Mitochondrial Genomes in Hybrids

Three wheat mitochondrial genes were hybridized to total genomic DNA of Westar, GH50 and the hybrids. All of the hybrids displayed unique bands when hybridized with the wheat ATP-6 gene probe. BamHI digested genomic DNA of Westar, GH50 and the hybrids was also probed with the wheat ATP-6 gene (Figure 39). Hybrid 2:Bii (lane 2c) was the only hybrid plant which demonstrated the same banding pattern as one of the parents (GH50 in this hybrid's case). The other hybrids showed unique single or double bands larger than 14 kb (Figure 39).

Another mitochondrial probe, the 5' end of the wheat Cox II gene, was used to illustrate the combination of Westar and GH50 fragments among the hybrid plant DNA digests (Figure 40). Due to the poor resolution of the autoradiogram, a diagrammatic illustration was reproduced from the original (Figure 41). Hybrids 1:Ai (lane 1a), 2:A (lane 2a), 2:Bi (lane 2b), 2:C (lane 2d), 3:B (lane 3b) and 4:A (lane 4a) all displayed bands from both Westar and GH50 parents.

FIGURE 39: ANALYSIS OF BAMHI DIGESTED GENOMIC DNA WITH AN ATP-6 MITOCHONDRIAL DNA PROBE

FIGURE 40: ANALYSIS OF TAQI DIGESTED GENOMIC DNA WITH A COX II 5' MITOCHONDRIAL DNA PROBE

FIGURE 41: A SCHEMATIC REPRESENTATION OF THE
BANDING PATTERN OF TAQI DIGESTED
GENOMIC DNA PROBED
WITH COX II 5' DNA

DISCUSSION

A. CHLORSULFURON RESISTANCE IN PARENTAL SPECIES

The chlorsulfuron tolerance level of each parent had to be determined in order to select an appropriate herbicide concentration which, when administered to the protoplast cultures, would kill the Westar parents yet favour the growth of Westar + GH50 fusion products expressing the resistance gene. Westar protoplast cultures developed into distinct colonies, hence, viability (as a function of herbicide concentration) could be easily determined. Since GH50 did not produce these distinct colonies, this method of viability determination could not be employed, therefore, initial chlorsulfuron tolerance studies were performed on a cell suspension culture (Figure 6).

According to the cell growth rates, measured by calculating the relative increase in cell volume, herbicide concentrations up to and including 1.0 $\mu\text{g/ml}$ did not appear to inhibit an increase in cell mass. The curves for the inhibition of protoplast protein synthesis, as determined by the incorporation of tritiated leucine, on the other hand, displayed a decrease in metabolism (negative slope) at 0.1 $\mu\text{g/ml}$ (Figure 7). This 10 fold increase in

chlorsulfuron sensitivity from one assay (Cell Growth Rate) to the next (Protein Synthesis) could be due to the fact that protoplasts are more sensitive than cells. Another possible explanation may be the sensitivity of the assay, since by measuring the pelleted cell volume, both dead and living cells are incorporated into the final value, while by measuring the protein synthesis only the living cells are taken into consideration. After Haughn and Somerville (1986) isolated the chlorsulfuron resistant mutant, GH50, they characterized the effect of chlorsulfuron on acetolactate synthase (ALS) enzyme activity. At 1.0 $\mu\text{g/ml}$ of chlorsulfuron they observed a 50% decrease in enzyme activity compared to untreated tissue. In the present study, a 40% decrease in protein synthesis was observed in cultures treated with 0.1 $\mu\text{g/ml}$ and 1.0 $\mu\text{g/ml}$ over 13 days, while untreated GH50 cultures demonstrated approximately a 40% increase in protein synthesis.

B. GH50 PROTOPLASTS AT VARIOUS LEVELS OF X-IRRADIATION

The effect of different levels of X-irradiation on GH50 protoplasts was determined by a vital staining technique (Figure 8 and 9), by calculating the division rate (Table 7) and by measuring the level of ^3H -leucine incorporation. To compare the accuracy of each method as a determinant for protoplast viability, one must define what is meant by viability. If protoplast viability were defined as a protoplast's ability to divide, then the division rate would

be the most effective measurement. If protoplast viability were defined as metabolic activity then a ^3H -leucine incorporation should be used, since it initially measures protein biosynthesis. If membrane activity is being studied, then the presence of membrane esterase activity which can be visualized by FDA staining (Widholm, 1972) is the most suitable.

A major drawback of the FDA-staining protocol was that it sometimes demonstrated active esterase activity in dead and dying protoplasts. It also failed to show any differences between differentially X-irradiated cultures. The division rate and leucine incorporation measurements theoretically should be the best indicators of viability. From the division rates (Table 7) and the ^3H -leucine incorporation rates (not shown) the degree of variability was so great that no consistent trend could be determined. This variability was probably not due to sampling since replicates within one experiment did not vary significantly. Extreme variations were observed between identical X-ray treatments from different experiments as evident from the large standard deviations (Table 7). A possible cause for this may be due to the fact that the amount of X-irradiation delivered to a cell can be controlled, but the extent of nuclear damage and the type of genes which are damaged cannot always be regulated.

C. WESTAR + GH50 FUSION

Prior to the fusion event, GH50 protoplasts were X-irradiated at 60 or 90 kRad. After the initial background work (Section B) it was shown that even high levels of irradiation, such as 80 kRad, could not be used to metabolically shut down GH50 protoplasts. The X-ray treatment, though, would still be used to direct the elimination of the donor's chromosomes (Gleba et. al., 1988). Any GH50 colonies which did form in the protoplast fusion culture dishes would not be able to regenerate any further, since the shoot regeneration medium used could not induce shoot regeneration in Arabidopsis (Figure 13), only in B. napus (Klimaszewska and Keller, 1987). Different hormones have been deemed necessary for the differentiation of Arabidopsis callus tissue into shoots (Ceddie, 1989; Ford, 1990).

Out of 17 hybrids, only one managed to establish themselves in the greenhouse. The inability of some of the hybrids to acclimatize to a greenhouse environment may have been due to vitrification. It was generally observed that highly vitrified plant tissue generally browned and died when placed in a high humidity mist bed. Another cause may have been a genetic alteration which may have prevented the regenerant from surviving without a hormone and carbohydrate source. The addition of extra donor chromosomes may also have had deleterious effects on the regenerant's capability to acclimatize.

D. CHARACTERIZATION OF FUSION PRODUCTS

i- Morphological Characterization

A total of 17 hybrid plants were regenerated from four fusion experiments. Each of the hybrids had morphological or biochemical features which distinguished them from the parental species. Only the eight hybrids which were successfully established in the greenhouse flowered and eventually developed pods. These flowers were closely examined and shown to portray very abnormal characteristics (Table 9).

The corolla lengths of the hybrids were intermediate in size to the corolla lengths of the two parents, while the hybrid petal colour varied from yellow, in hybrid 3:B, to white-green in hybrid 1:C. The number of petal-like structures ranged from six to eight, while the parents each had four petals. All of the regenerants possessed four morphologically normal looking sepals. The hybrid flowers examined were male sterile and all, except 3:B, had abnormal female reproductive structures. These abnormal petal and pistil arrangements along with an absence of the stamen, displayed some similarity to *pistillata* mutants which were characterized in *Arabidopsis* (Bowman et. al., 1989). The *pistillata* mutation, which has been linked to a recessive mutant allele on chromosome 5 (Koorneef et. al., 1983), affects the development of all floral organs except the sepals. Some of the ovaries from the hybrids

exhibited more than two carpels and sometimes even unfused carpel margins, just like the *Arabidopsis pistillata* ovary which was characterized by Bowman.

The Arabidobrassica hybrids which were developed in 1978 by Gleba and Hoffmann, through symmetric hybridization, also demonstrated bizarre floral abnormalities such as a lack of anthers or petals, transformed floral structures and a petal colour varying from predominantly white to light-yellow and sectionally yellow. All of these hybrids were self sterile (Gleba and Hoffmann, 1978, 1979). From visual observations in the present study, the X-ray pretreatment of the donor protoplasts did not appear to produce fusion products containing fertile flowers, as was hoped. Instead, all of the regenerants analyzed lacked anthers (male sterile) and most had abnormally shaped pistils. Whether the cause of these aberrations was due to: a combination of two sets of genes directing flower development, an incomplete elimination or inexpression of some flower development genes, or a mutation, possibly caused by the X-irradiation, in floral specific genes cannot be shown from these studies. The only unaffected floral structure was the sepals.

The upper leaf morphology of the greenhouse-grown hybrids was more similar to the upper leaf surface of the Westar parent than to the GH50 parent. Of the four Arabidobrassica plants examined with the scanning electron microscope (SEM), none had trichomes and all exhibited a thick waxy cuticle just like Westar (Figure 18, 19, 20). Westar and the hybrids, when grown *in vitro*, all produced leaf hair structures which were lost after

acclimatization in a greenhouse environment. The production of different wax structures on the leaf surface of the hybrids could either have been caused by environmental factors or by possible genetic changes. Of all the hybrids examined, the physical appearance of the wax particles on 1:Ai (Figure 19) had the closest resemblance to the irregular plate-shaped wax structures on the Westar parent (Figure 18). Hybrids 1:Aii, 1:Aiii and 1:Aiv had wax particles in the shape of globules, flat dispersed plates and short rods with polygons, respectively.

In a recent study on insect resistance, Eigenbrode and researchers (1990) studied different surface waxes on B. oleracea to determine which wax type inhibited the larval stages of the diamondback moth. They concluded that flat, reduced surface waxes, which gave the leaf a glossy appearance, were the most effective in deterring these insect pests. The wax surface structure of the hybrid 1:Aiii resembled the glossy surface waxes observed by Eigenbrode et al. (1990), suggesting a closer study of the factors causing the different surface waxes and the possibility of isolating insect-resistant Arabidobrassica hybrids.

In the study by Gleba and Hoffmann (1979), only leaves from in vitro hybrids were examined. These waxless leaves were often deformed showing no upper or lower surfaces. Some of the in vitro plantlets, though, did demonstrate trichome structures which were intermediate to the tri-branched Arabidopsis hairs and the single haired Brassica campestris parent (Gleba and Hoffmann, 1979 and 1980).

ii- Chlorsulfuron Resistance

The presence of the mutant acetolactate gene in a regenerated asymmetric fusion product can be verified at the molecular level, but the expression of such a gene can be determined physiologically by studying the hybrid's herbicide tolerance. This tolerance level was measured by an in vitro rooting assay and by an in vivo topical spray assay.

a) Rooting Assay

For this in vitro assay, both the number of new leaves formed and the hybrid's ability to produce roots were evaluated (Figure 22). Even though the number of new leaves produced over a three week period decreased with increasing levels of chlorsulfuron, it was extremely difficult to determine which hybrids expressed a greater tolerance level. The hybrid's ability to produce roots at increasing herbicide concentrations was a more sensitive measurement, clearly demonstrating that 3:B, 1:C and 2:A expressed the highest tolerance levels (100 fold or greater than the Westar parent)(Figure 22). The other hybrids also demonstrated an increased herbicide tolerance when compared to the Westar parent. From studies on pea root cultures, it was shown that chlorsulfuron inhibits cell division by inhibiting valine and isoleucine biosynthesis (Ray, 1984; Rost, 1984). Hence, the most actively dividing cells, such as the lower stem cells which are stimulated to

differentiate and divide by the naphthaleneacetic acid (NAA), are initially more sensitive to the herbicide than the cells which are not actively dividing.

b) Spray Assay

How a plant reacts to a chemical in vitro may differ from its reaction in vivo. For this reason, it was interesting to record how the most resistant hybrid from the rooting assay, 3:B, reacted to a topical application of the herbicide. At a spray level of 0.5 $\mu\text{g/ml}$ the Westar parent was completely killed, while the hybrid 3:B and the GH50 parent remained relatively unaffected. Interestingly, at all levels of chlorsulfuron, GH50 appeared to put more energy into leaf formation and less into an increase in height. At 1.0 $\mu\text{g/ml}$ the hybrid 3:B produced no new leaves and no increase in height was observed, but an fluorescence was still produced. In the in vitro rooting assay, this same level of chlorsulfuron prevented root formation in 85% of the 3:B explants tested.

From a calculation shown in the Results chapter (section D ii), the herbicide threshold level, 1.0 $\mu\text{g/ml}$, was correlated to an approximate field application value of 15 g/ha. The Westar plants were irreversibly inhibited by a single spray application equivalent to 3.7 g/ha (.25 $\mu\text{g/ml}$). These hypothetical field application levels are not entirely representative of what is actually sprayed in the field, since the volume or dose of herbicide which a

crowded field plant receives may not coincide with the wetting volume used in a greenhouse spray assay.

In 1988, Swanson and coworkers isolated chlorsulfuron resistant B. napus plants from microspores and from protoplasts which were resistant to spray assay levels of 6 g/ha, or a tolerance level 10 to 100 fold greater than the non-resistant B. napus plants (Swanson et. al., 1988). Unfortunately, the methodology for their equivalent field spray level calculation was not outlined. According to a brochure published by DuPont (Glean) a field application level of 11.3 g of active ingredient (chlorsulfuron) per hectare is sufficient to control a wide variety of weeds, including crucifers such as volunteer rapeseed, wild mustard and stinkweed. It is evident that the calculated g/ha unit for greenhouse spray tests is not an accurate measure for determining the coinciding field spray level. Unless certain variables are standardized for the calculation of the representative field spray level, researchers should employ herbicide concentrations or the amount of herbicide sprayed onto each plant as an indicator of spray level.

iii- Glucosinolate Analysis

Glucosinolates are plant products which, upon maceration of the plant tissue, are hydrolyzed by the myrosinase enzyme into volatile byproducts such as isothiocyanate, nitrile or thiocyanate. These toxic byproducts are

responsible for the flavour and pungency of cruciferous vegetables, especially mustard, radish and horseradish. In order to increase the palatability of rapeseed protein so that it can be used as a feed source, plant breeders have developed double low rapeseed varieties having low glucosinolate levels in the protein portion of the seed (Sang et. al., 1984).

To analyze the types and amounts of glucosinolates present in plant tissue, researchers have developed a method of desulphating glucosinolates, and then separating them using reversed-phase high-performance liquid chromatography (Minchin et. al., 1982). This method of analysis was used to produce the glucosinolate profiles in Figure 36. As expected, the Westar parent had very few peaks since it is a double low rapeseed variety. The other parent, GH50, demonstrated a large number of different glucosinolate peaks. The hybrids, 1:B, 1:C, 2:A, 2:Bii, 3:A, 3:B and 4:A, had a combination of Westar and GH50 peaks, yet most of the peaks originated from the GH50 parent. Numerous peaks uncommon to both parents were also observed in the glucosinolate profiles of the regenerants (Table 10). The nature or origin of these novel peaks could not be determined from this study.

This analysis is only a brief overview of the qualitative nature of the glucosinolates present in the leaf tissue of *Arabidobrassica* hybrids. It serves as a tool to provide evidence that the hybrids express genes inherited from both parents. A quantitative analysis cannot be accurately obtained from this study, but does require further study.

iv- Isozyme Analysis

Isozymes, because they are the ultimate product of gene expression, serve as excellent genetic markers. The expression of phosphoglucumutase (PGM) (Figure 28), esterase (Figure 29) and peroxidase (Figure 30) was employed to verify the hybrid nature of the *Arabidobrassica* fusion products. Nine other isozyme staining assays (Table 2) were tested on electrophoresed protein extracts isolated from leaf tissue.

Five hybrid plants, which were assayed for the PGM enzyme, displayed bands from both Westar and GH50 parents. This was an expected result since PGM is a monomeric enzyme. From linkage studies of isozyme loci in *B. napus*, three different loci coding for PGM were identified. Of these, two were inherited from *B. campestris* and one was inherited from *B. oleracea* (Chen et. al., 1989). The combination of alleles from these three loci produced the slow moving region (PGM-2), consisting of three major bands, and the fast moving region (PGM-1), also consisting of three major bands (Figure 40). Linkage studies for the *Arabidopsis* parent have not yet been published for PGM, therefore, the number of loci and their location in the genome are unknown. The five hybrids expressed the six major PGM-1 and PGM-2 Westar bands along with the two fast-migrating GH50 bands, and very weakly expressed the slow-migrating GH50 band. For this isozyme assay, all

of the GH50 alleles were expressed in the hybrid extracts, hence, none were lost through the asymmetric fusion and culture procedure.

The esterase staining pattern of the hybrids (Figure 29) revealed bands which were not initially present in either of the parents. For the Westar parent, only one fast migrating band was strongly expressed, but from the previous parental esterase assay using root tissue (not shown) four fast-moving Westar bands were present. These three faster Westar bands were strongly expressed by all of the hybrids. The two very slow-moving GH50 bands were also weakly expressed by the hybrid root tissue, suggesting that all of the parental esterase alleles were present in the hybrids. The intermediate-migrating hybrid bands, though, were not expressed in the parental tissue in Figure 29. Whether these bands were caused by a dimeric esterase isozyme or whether these alleles were actually present yet not expressed in one of the parental tissues cannot be proven by this study. One can only postulate, by comparing Figure 29 to previous esterase root protein migration patterns, that the intermediate bands belong to the GH50 esterase allele family, yet are not expressed by GH50 (for whatever physiological reason) in Figure 29.

Originally, three different tissues were tested with Wetter and Dyck's esterase isozyme staining protocol (Wetter and Dyck, 1983). This study revealed a variation in the staining intensity of many of the bands between leaf, stem and root tissue. The migration distances of the bands remained relatively constant for all of the tissues, therefore, one can postulate that

certain esterase isozymes are expressed to a greater degree in some tissues than in others. For this reason the isozyme banding intensity or level of expression of certain alleles by the root tissue was more diagnostic than that produced by leaf or stem tissue.

Numerous research projects have utilized the esterase enzyme system as a marker for studying the nature of interspecific and intergeneric hybrids, and for distinguishing between species of the same genera. Studies involving B. napus have given a variety of results. Yadava and coworkers discovered variations in esterase banding patterns between different Brassica species (Yadava et. al., 1979), while a Korean group of researchers found a variation in esterase isozyme patterns between electrophoresed root, hypocotyl and cotyledon tissue from B. napus and B. campestris (Nam et. al., 1989). Both Sacristan, et. al. (1989) and Yamashita and coworkers used esterase zymograms to verify the hybrid nature of their Brassica somatic hybrids (Yamashita et. al., 1989). Using callus tissue as a protein source, Gleba and Hoffmann found that their Arabidobrassica hybrids expressed some Arabidopsis and some Brassica bands. Their hybrids, even though they possessed a complete set of chromosomes from each parent, did not express all of the parental esterase alleles (Gleba and Hoffmann, 1978). This may lend some proof to the suggestion that the expression of certain protein alleles is tissue dependent and sensitive to environmental factors.

The peroxidase enzyme study (Figure 30) clearly demonstrated the expression of all of the parental bands in the five hybrid protein extracts. A similar result was obtained by Gleba and Hoffmann in their analysis of the hybrid callus tissue using a peroxidase assay system. The hybrids expressed all of the Brassica and all of the Arabidopsis bands (Gleba and Hoffmann, 1978).

The three isozyme systems (PGM, esterase and peroxidase) verified the hybrid nature of the fusion product regenerants. A preferential elimination of the GH50 bands was not observed, hence, evidence for an asymmetric fusion product could not be obtained from these protein studies.

v- Southern Blot Analysis

a) The Hybrid Nature of the Fusion Products Using a Wheat Ribosomal DNA Probe

The wheat ribosomal DNA probe, PTA 71, is a full length rDNA repeat unit which includes the structural genes for 26 S, 18 S, and 5.8 S ribosomal RNA as well as spacers. In plant species the number of ribosomal gene repeats is usually in the order of 10^4 per genome, making this probe a very sensitive marker for characterizing nuclear DNA enriched with ribosomal RNA genes (Gerlach and Bedbrook, 1979). Due to its strong hybridization to nuclear plant DNA, PTA 71 has been effectively used to diagnose the

nuclear origin of interspecific somatic hybrids of Medicago (Thomas et. al., 1990), and tobacco (Sproule et. al., in preparation).

To determine whether the Arabidobrassica fusion regenerants possessed nuclear genes from both parents, genomic DNA was restricted both with EcoRI (Figure 32) and BamHI (Figure 33) and hybridized to PTA 71. The hybrid plants' DNA all appeared similar to Westar DNA when cut with EcoRI (Figure 32). In the BamHI digest (Figure 33), most of the bands were indistinguishable between the parents, except for a region between 1.0 and 1.5 kb. This banding region was diagnostic for the BamHI digested hybrid DNAs (Figure 33), which all displayed identical banding patterns to the GH50 parent. The presence of Westar-like bands in one digest and the presence of GH50-like bands in a different enzyme digest may suggest that the hybrids possess nuclear-encoded ribosomal RNA genes from both parents. The possibility of an incomplete digestion of the genomic DNA samples may also explain some of the discrepancies observed between the parental banding patterns in Figures 31, 32 and 33.

b) Acetolactate Synthase Gene

The presence of a 5.5 kb GH50-specific ALS band in 1:C (lane 1g), 2:A (lane 2a), 3:B (lane 3b) and 4:A (lane 4a) indicated that the Arabidopsis ALS gene was maintained in the regenerants (Figure 34). This 5.5 kb fragment corresponded to the 5.8 kb fragment which Haughn and Somerville had

isolated and sequenced from the ALS locus of chlorsulfuron resistant Arabidopsis (line GH50) (Haughn et. al., 1988). This 5.8 kb fragment contains the ALS coding and transit peptide sequences along with flanking regions at the 3' and 5' ends.

The regenerants which actively expressed the GH50 ALS gene, as determined by the chlorsulfuron rooting and spray assays, were also the ones which displayed the 5.5 kb gene fragment. The most resistant hybrids (3:B, 2:A and 1:C) may have maintained the GH50 chromosome, or a chromosome fragment thereof, which contained all the necessary regulatory sequences. The actual fate of the GH50 ALS locus, though, can only be determined through a cytogenetic analysis of the hybrid genomes.

c) Relative Amount of GH50 DNA in the Hybrids

A cytogenetic analysis would provide some more data on the number of GH50 chromosomes actually maintained in the somatic hybrids, but due to time constraints this study was not undertaken. The amount of GH50 DNA transferred to the regenerants was only estimated by determining the relative amount of hybridization between total DNA isolated from the regenerants and the GH50 genome, relative to the amount of hybridization of the GH50 genome to the Westar total DNA (Figure 35). An equal amount of digested genomic DNA was loaded into each of the wells of the agarose gel. After hybridizing the electrophoresed DNA to a genomic GH50 DNA probe, the

hybrid 4:A (lane 4a) demonstrated the strongest band intensity. Interestingly, this somatic hybrid was produced by fusing an unirradiated GH50 protoplast(s) to a Westar protoplast(s). Irradiating the GH50 protoplast prior to fusion may in fact direct the elimination of the donor genome in the Arabidobrassica fusion products. Due to the small sample size such a finding is not conclusive, however it does correlate with other asymmetric fusion studies.

In a 1988 study on Nicotiana plumbaginifolia + Atropa belladonna somatic hybrids, Gleba and coworkers found that irradiation of the donor parent before fusion was necessary to produce highly asymmetric nuclear hybrid plants. The degree of asymmetry, or elimination of the irradiated genome, though, could not be determined by varying the level of irradiation (Gleba et. al., 1988).

d) Chloroplast Genomes of 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. In the present study, Southern Blot analyses of the Arabidobrassica somatic regenerants indicated that the chloroplast genome was inherited solely from the Westar parent (Fig. 36, 37, 38). No evidence for chloroplast recombination was observed using either the Brassica chloroplast DNA probe or this heterologous tobacco chloroplast DNA probe. In an

asymmetric fusion study between two different strains of B. napus, only the chloroplasts from the unirradiated recipient were present in the fusion products (Menczel et. al., 1987). Also in intergeneric fusions, such as Nicotiana + Daucus, the chloroplast genome of the hybrids was derived from the unirradiated recipient (Smith et. al., 1989). In some symmetric intergeneric fusion studies, such as Nicotiana + Petunia, the chloroplasts of the hybrids were inherited only from the Nicotiana parent (Pental et. al., 1986). It is interesting to note that in the latter two intergeneric fusions and in the present study the eliminated chloroplast genome originated from undifferentiated cell culture-derived protoplasts. Even the unirradiated GH50 donor-fusion event (lane 4a) demonstrated only Westar derived chloroplast DNA. This may indicate that chloroplast segregation in somatic hybrids does not just depend on the irradiation of a donor protoplast, but also on the species which are being fused and on the state of differentiation of the tissue source from which protoplasts are obtained.

In a few cases, chloroplast recombination, following protoplast fusion, has been observed (Medgyesy et. al., 1985; Kumashiro et. al., 1989; Fluhr et. al., 1984), however the majority of somatic hybrids possess only one chloroplast type (Glimelius et. al., 1981; Gleddie et. al., 1983). A possible explanation for this sorting out is the demonstration that cells with mixed plastid types have been shown to have reduced regeneration capacity relative to those with only one plastid type (Hirai et. al., 1988).

e) Mitochondrial Genomes of the Hybrids

The fate of the parental mitochondrial genomes was analyzed by probing total DNA of the hybrid plants with mitochondrial-specific gene probes. The proper controls were also performed to ensure that the bands visualized in Figures 39 and 40 were mitochondrial specific and not due to hybridization with a homologous nuclear sequences. Most of the Arabidobrassica hybrids demonstrated some Westar-specific and some GH50-specific bands (Figure 40). Some hybrids also showed novel non-parental bands when probed with ATP-6 (Figure 39). Even the hybrid 4:A, which had originated from a symmetric fusion event, displayed mitochondrial bands from both parents (Figure 41, lane 4a). This may indicate that recombination of mitochondrial genomes among somatic hybrids may not be solely induced by irradiation, but perhaps by other factors not investigated in this study. Another possibility for the differential banding pattern observed in the hybrids may have been due to the presence of multiple subgenomic mitochondrial DNAs which were formed by various interactions between recombinationally active repeats present in the plant mitochondrial DNA (Bonen and Bird, 1988). This possibility, though, was not investigated.

In symmetric hybridization studies within Brassica, the mitochondrial genome of regenerants often was derived from one parent only (Jourdan et. al., 1989; Chuong et. al., 1988). On the other hand, an asymmetric fusion involving two strains of B. napus resulted in a mitochondrial recombination

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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 Arabidopsis (GH50) chlorsulfuron resistance acetolactate synthase gene. Of the six regenerated hybrids tested for chlorsulfuron resistance, three had maintained (Southern Blot analysis of the ALS gene) and expressed (Rooting and Spray Assays) this nuclear-encoded gene. Other genomic traits were also transferred from GH50 into the fusion products, as demonstrated by the isozyme, glucosinolate, and ribosomal DNA analyses.

The effect of the irradiation dose on these fusion products, though, was more difficult to deduce since the symmetric fusion hybrid, 4:A, often displayed the same protein and DNA profiles as the asymmetric fusion hybrids. The only evidence for preferential genome elimination, from the irradiated-donor fusion hybrids, was obtained from the chloroplast genome study and from the DNA-DNA hybridization study using GH50 genomic DNA as a probe. Results indicated the presence of less Arabidopsis DNA in the asymmetric hybrids than in the symmetric hybrid (4:A). The degree of genome elimination, though, can only be conclusively determined from a thorough cytogenetic analysis of the hybrids.

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.

**Science is a first-rate piece of
furniture for a man's upper chamber,
if he has common sense on the ground floor.**

Holmes

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