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PETITION FOR DETERMINATION OF NONREGULATED STATUS OF GLYPHOSATE TOLERANT SUGARBEET LINE 77

The undersigned submits this Petition of 7 CFR 340.6 to request that the Administrator, APHIS, determine that Glyphosate Tolerant Sugarbeet Line 77 not be regulated under 7 CFR 340.

Submitted by

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CBI DELETED VERSION

PETITION FOR DETERMINATION OF NONREGULATED STATUS FOR GLYPHOSATE TOLERANT SUGARBEET LINE 77

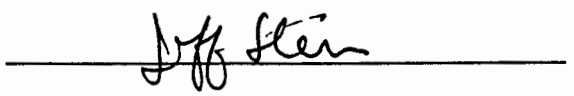
Novartis Seeds and Monsanto Company are submitting this petition for determination of nonregulated status to the United States Department of Agriculture, Animal Plant Health Inspection Service for sugarbeet which is tolerant to glyphosate. This petition requests a determination from APHIS that the glyphosate tolerant sugarbeet line 77 (GTSB77) and any descendants derived from traditional breeding methods between this line and other sugarbeet lines, and any descendants derived from traditional breeding of this line with other transgenic sugarbeet lines that have also received a determination of nonregulated status, no longer be considered regulated articles under regulations in 7 CFR part 340

GTSB77 has been genetically engineered with a gene from *Agrobacterium* sp. strain CP4 that expresses an enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Padgette *et al*, 1993a). This enzyme, similar to EPSPS enzymes from other sources, catalyses the conversion of shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) into 5-enolpyruvylshikimate-3-phosphate (EPSP), an intermediate in the production of aromatic amino acids (Hermann, 1983; Haslam, 1974). The catalytic activity associated with the EPSPS protein from *Agrobacterium* strain CP4, unlike EPSPS enzymes from other sources, is not inhibited by glyphosate (N-phosphonomethylglycine). Sugarbeet plants (e.g., GTSB77) that express this form of EPSPS are tolerant to treatment with glyphosate, while sugarbeet plants lacking this form of the gene are not. In addition, GTSB77 also expresses the *uidA* (GUS; β-D-glucuronidase) gene from *E. coli*, which served as a selectable marker during the plant transformation process (Jefferson *et al.*, 1987; Raju *et al.*, 1991), and a novel protein known as protein 34550 with no known biologocal activity.

Glyphosate, the active ingredient in Roundup®, is a post-emergent, systemic herbicide that is currently used worldwide for the non-selective control of a wide variety of annual and perennial weeds. Due to its broad activity on nearly all species of plants, in-crop uses of Roundup are limited. The tools of genetic engineering enable the development of crop lines that are tolerant to the herbicide (Barry *et al.*, 1992; Padgette *et al*, 1996). The availability of glyphosate-tolerant sugarbeet will enable farmers to utilize Roundup herbicide for the effective control of weeds and take advantage of the environmental and safety characteristics of this herbicide.

CERTIFICATION

The undersigned certifies that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes relevant data and information known to the petitioner which are unfavorable to the petition.



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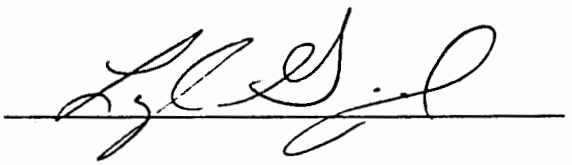

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ABBREVIATIONS

aad	Gene encoding spectinomycin and streptomycin resistance
bp	Nucleotide base pairs
CaMV	Cauliflower mosaic virus
CP4 EPSPS	EPSPS from *Agrobacterium* sp. strain CP4
CTP	Chloroplast transit peptide (CTP1, CTP2)
E9 3'	3' transcriptional termination sequence of pea rbcS E9 gene
ELISA	Enzyme-linked immunosorbent assay
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FMV	Figwort mosaic virus
fwt	Fresh weight
GOX	Glyphosate oxidoreductase from *Ochrobactrum anthropi*
gox	Gene coding for GOX
GUS	ß-D-glucuronidase from *E. coli*
gus	see *uidA*
Kb	Nucleotide kilobase pairs
kD	Kilodaltons
LB	Left border
NOS 3'	3' transcriptional termination sequence from nopaline synthase
NPTII	Neomycin phosphotransferase II
nptII	Gene coding for NPTII
OECD	Organization for Economic Cooperation and Development
ori-V	Bacterial origin of replication
ori-322	Bacterial origin of replication
PCR	Polymerase chain reaction
RB	Right border
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
T-DNA	Transfer DNA
uidA	Gene (also coded *gus*) encoding the GUS protein

PETITION FOR DETERMINATION OF NONREGULATED STATUS FOR GLYPHOSATE-TOLERANT SUGARBEET LINE 77

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I. Rationale for Development of Glyphosate Tolerant Sugarbeet Line 77

The products which are the subject of this application are seeds of glyphosate-tolerant sugarbeets and seeds of any progeny (inbred or hybrid) derived from GTSB77 by conventional breeding. This application addresses safety issues associated with the environmental release and commercial production of GTSB77 in the United States and Europe, as well as processing and eventual food and feed use of the derived products. Seeds of GTSB77 will be marketed as new varieties of sugarbeets (*Beta vulgaris*), and the products obtained from these beets will be introduced into commerce as any other new variety.

GTSB77 has been genetically engineered with a gene from *Agrobacterium* sp. strain CP4 that expresses enolpyruvylshikimate-3-phosphate synthase (EPSPS). The CP4 EPSPS gene is flanked by the figwort mosaic virus (FMV) promoter, and a chloroplast transit peptide (CTP) from *Arabidopsis thaliana* and the pea (*Pisum sativum*) E9 3' terminator. The CP4 EPSPS, like other EPSPS enzymes, catalyses the conversion of shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) into 5-enolpyruvylshikimate-3-phosphate (EPSP), an intermediate in the production of aromatic amino acids (Hermann, 1983; Haslam, 1974). Unlike other EPSPS enzymes that are inhibited by glyphosate, the catalytic activity of CP4 EPSPS is unaffected by glyphosate, and plants expressing CP4 EPSPS are tolerant to Roundup (Padgette *et al.* 1996).

GTSB77 has also contains the *uidA* (GUS; β-D-glucuronidase) gene from *E. coli* with an enhanced 35S promoter from cauliflower mosaic virus (CaMV) and E9 3' terminator from pea. The *uidA* gene expresses the GUS protein which served as a scorable marker during the plant transformation process (Jefferson *et al.*, 1987; Raju *et al.*, 1991). A truncated version of the glyphosate oxidoreductase (*gox*) gene from *Ochrobactrum anthropi* sp. is also present in GTSB77, but expresses a non-functional enzyme designated protein 34550. This gene utilizes the figwort mosaic virus (FMV) promoter, and a chloroplast transit peptide (CTP) from *Arabidopsis thaliana*.

The nature of the product and the objective of the genetic modification are to improve weed management practices in sugarbeets. Weed management is regarded as an expensive, labor intensive, and in some cases complicated operation necessary for optimal production efficiency of sugarbeets. No single currently approved herbicidal ingredient offers the broad-spectrum weed control afforded by glyphosate. Instead, farmers must resort to using multiple herbicides in several applications at highly variable cost and performance efficiency.

GTSB77 has been field tested at numerous sites across the U.S., under USDA permits or notifications[1](Appendix VIII), with no indications of toxicity toward insects, birds, or other species, and no detectable adverse environmental impact. In addition, EPSPS enzymes are already present in plants (including sugarbeet) and microorganisms. Furthermore, β-

[1] USDA Permit Nos. 96-031-01, 96-057-03, 96-061-01, 96-309-01, 96-361-02, 97-029-02, and USDA Notification Nos. 97-169-03, 97-182-08, 97-190-02, 98-035-01, 98-050-02, 98-057-01, 98-072-11, 98-079-11.

glucuronidase (GUS) is found in mammals and many microorganisms. Both are ubiquitous in nature and present in food and feeds.

GTSB77 has been demonstrated to be substantially equivalent to the parental variety. It is being crossed into adapted varieties by traditional breeding methods, and will be grown in the same geographic regions and with the same practices as current varieties. The herbicides that are currently available for sugarbeets do not provide broad spectrum weed control compared to glyphosate. Most current herbicides are effective for control of annual grasses, while glyphosate controls annual grasses, annual broadleaves, and perennial weed species.

The use of GTSB77 for sugarbeet production would enable a farmer to use glyphosate herbicide for effective control of weed pests while receiving the benefits of its environmental safety characteristics. These new glyphosate-tolerant sugarbeets can positively impact current agronomic practices by: 1) offering farmers broad-spectrum weed control, 2) allowing the use of an environmentally acceptable herbicide, 3) enhanced flexibility to treat weeds "as needed", 4) offering less dependence on use of pre-emergent herbicides, and 5) providing cost-effective weed control due to the reduced price of glyphosate herbicide compared to competitive products accepted for use in sugarbeets. These seeds may consist of inbred or hybrid lines developed using conventional breeding methods. Seeds of GTSB77 will be marketed as new varieties of sugarbeets, and the products obtained from these sugarbeets will be introduced into commerce as any other new variety of sugarbeets.

Cultivated *B. vulgaris* varieties are not invasive, are weakly competitive outside cultivated areas, and possess few weedy characteristics. Data included in this Petition demonstrate that GTSB77 are substantially equivalent to non-modified beets except in regards to tolerance to glyphosate. Furthermore, volunteer or bolting plants are readily managed using numerous agricultural practices including other herbicides, hand weeding, and cultivation.

II. BIOLOGY AND PRODUCTION OF SUGARBEET

A. Economics and Use of Sugarbeet

Sugarbeet has a history of safe use; sugar and other processed fractions are consumed in many human food products or animal feeds. Currently, sugarbeet is the major sugar crop grown in temperate regions of the world. Total worldwide sugar production in 1996 is estimated at 123 million tons. Commercial sugarbeet production in the United States occurs in 13 states, with the majority grown in North Dakota, Minnesota, Michigan, Wyoming, California, and Colorado. These states account for 85% of the total area cultivated with beets in the country and almost 85% of the total United States sugarbeet production. The sugarbeets have an estimated total value of $1.3 billion to the country's beet farmers.

The overall contribution of the growing, harvesting, and post-harvest processing of sugarbeets to U.S. employment amounts to 21,800 full-time jobs and 57,300 seasonal jobs, with a the total wage bill estimated to be $553.2 million (1993 figures).

Sugar is a multi-purpose carbohydrate that contributes significantly to the flavor, aroma, texture, color and body of a variety of foods. Sugar helps bread rise by acting as a food source for the yeast. In all baked products, sugar contributes to the flavor and crust color as well as prolonged shelf life. In addition to being an important component in jams and jellies, sugar is a contributor to bulk, texture and body of ice cream, beverages, baked goods, and other products.

In addition to processing pure sugarbeet sugar, sugar factories also produce a by-product known as dried sugarbeet pulp. This pulp can be produced and shipped in many forms, including plain dried, molasses dried, and pelleted. These fractions are used for feed for dairy cattle, feeding cattle, and sheep. In the western US growing region, livestock (cattle and sheep) infrequently (<1% of total acres) graze on sugarbeet tops that remain in the fields following harvest.

Another important by-product is sugarbeet molasses, a viscous liquid containing about 48% saccharose, which cannot be crystallized. Sugarbeet molasses is used for production of yeast, chemicals, pharmaceuticals, as well as in the production of mixed cattle feeds.

B. Taxonomy

Sugarbeet has been grown as a food crop for more than 150 years, and is taxonomically classified as follows:

a) Family name: Chenopodiaceae
b) Genus: *Beta*
c) Species: *vulgaris*
d) Subspecies *vulgaris*
e) Cultivar line: A1012
f) Common name: sugarbeet

For the taxonomic division of the genus *Beta* see Table 2.1.

C. Phenotypic and Genetic Traits

Sugarbeet (*Beta vulgaris* subsp. *vulgaris*) is usually propagated by seeds; natural reproduction from vegetative tissue is not known. It is normally diploid with 2n = 2x = 18 chromosomes. Artificially induced autotetraploid sugarbeet (2n= 4x = 36) were introduced into sugarbeet breeding in Europe in the early 1940's and gave rise to so called polyploid or anisoploid sugarbeet varieties, consisting of a mixture of tetraploid, triploid (2n = 3x = 27) and diploid plants. Beginning in the mid - 1960's these varieties were largely replaced by pure triploid hybrid varieties. Currently, both diploid and triploid hybrid varieties are sold commercially.

The development of hybrid varieties in sugarbeet was made possible by the discovery of cytoplasmic male sterility [(CMS); Owen, 1945] and the subsequent development of hybrid breeding techniques (Owen, 1948). CMS in sugarbeet is the result of interaction between nuclear genes and changes in the mitochondrial genome (Powling, 1982; Halldén *et al*, 1990).

Sugarbeets produce a perfect flower consisting of a tricarpelate pistil surrounded by five stamens and a perianth of five narrow sepals. Petals are absent. Flowers are located on the terminal portions of the main axes and the lateral branches of the inflorescence. Sugarbeet seed is generally found as a cluster or ball formed by the aggregation of two or more flowers. This aggregation of two or more true seeds forms the multigerm beet seed. In plants homozygous for the recessive gene m (Savitsky, 1952), flowers occur singly and a monogerm seed is formed. Since its discovery in 1948, the gene m has been broadly introduced. The availability of monogerm seed and effective sugarbeet herbicides have made the mechanization of sugarbeet production possible.

Most sugarbeet genotypes are strongly self-incompatible and set few or no seeds when grown under strict isolation. Self-incompatibility is caused by four interacting S-loci, each carrying two S-alleles (Larsen, 1977a; 1978). "Pseudo-compatibility " or "pseudo-self-fertility" may occur due to a breakdown of the incompatibility mechanism. The degree of self-incompatibility is influenced by genotype as well as by environmental factors, most notably temperature (Larsen, 1977b).

Sugarbeet is largely wind-pollinated with insects playing a minor role (Cooke and Scott, 1993). Since the pollen can be carried by the wind over long distances, breeding stock and commercial seed production fields must be isolated by distance (see Part D, this chapter).

Sugarbeet is normally biennial and develops a large succulent root the first year and a seed stalk the second year. The root crop is usually sown in the spring and harvested in the autumn the same year. For seed production, small plants known as stecklings are produced in the first season. The following season they are transplanted into the field where seed production will take place. A period of low temperature is required to induce a change from the vegetative to the reproductive stage. The length of thermal induction is genotype dependent. As day length is also important for flower induction, the term "photo-thermal flower induction" is used, especially when biennial genotypes are induced to flower and set seed in the first year through manipulation of temperature and day length. The

genetics of bolting resistance in biennial beets is still unclear. Some studies suggest that it is governed by several genes with different degrees of dominance (Le Cochec and Soreau, 1989), while others suggest that it is largely recessive (Mc Farlane *et al.*, 1948)

The majority of wild Mediterranean *Beta* beets are annuals, but biennial types also occur. North Atlantic *B. maritima* types are normally perennial. The annual growth habit is governed by a dominant gene B (Abegg, 1936), which causes plants that carry it to run to seed very quickly under conditions of long days and reasonably high temperatures.

D. Potential for Genetic Transfer and Exchange with Other Organisms

Sugarbeet is predominantly wind pollinated and the pollen can travel shorter or longer distances depending on the windforce, humidity and temperature. Pollen trapping experiments conducted in England showed that 900 meters downwind of its release point, pollen concentration had fallen to 0.5% of that at the release point (Dark, 1971).

According to the OECD beet seed scheme of October 10, 1988, basic seed production must be at least 1000 meters distance from any pollen source of the genus *Beta.* For production of certified seed, the minimum isolation distance varies from 300 meters to1000 meters, depending on the chromosome number of the intended pollinator and the chromosome number of a neighboring pollen source.

In the United States, the majority of sugarbeet seed production takes place in Oregon. For certified seed production, a minimum isolation distance of 3,200 feet (approximately 1,000 meters) between sugarbeets with different backgrounds is required, and at least 8,000 feet (approximately 2,500 meters) from other *Beta* species

Typically, in seed production areas the pollinator stecklings and CMS stecklings are planted with 2 and 4-8 rows respectively. After flowering and pollen dispersal, the pollinator plants are removed in order to optimize seed quality. When the seed starts to mature, the seed-bearing plants are often cut and placed on the stubble or treated with a herbicide to have improved and synchronous ripening. In most instances, the seed are then harvested directly in the field with a combine.

The wild relatives of sugar beet originated in Asia Minor but some forms are widely distributed throughout the Mediterranean. All cultivated beets (both leaf-beets and those with swollen roots) are likely to have originated from wild maritime beets through simple selection by man. Sugarbeet (*Beta vulgaris* ssp. *vulgaris*) is the sole or main crop for sugar production in the temperate zones of the northern hemisphere. Since the Second World War sugarbeet has also been grown as a winter crop in countries with warmer climates such as Morocco, Algeria, Tunisia, Egypt, Syria, Iraq and Iran. Sugarbeet is not reported to be a weed in the US (WSSA, Composite List of Weeds, 1994) and is not reported to be a serious weed in other countries where it is grown.

Sugarbeet hybridizes freely with all wild members of the section *Beta* (Table 2.1), and the resulting hybrids are normally fully fertile. Of the wild relatives that can interbreed with sugarbeet, only *B. vulgaris* ssp. *maratima* and *B. vulgaris* ssp. *macrocarpa* are present in the US, and these isolated populations are limited to California (see Section

VII.C.2). These wild species (*B.v.* ssp. *maritima* and ssp. *macrocarpa*) are not recognized as being serious weeds in the US (WSSA, Composite List of Weeds, 1994).

Holms (1979, *A Geographic Atlas of World Weeds*) lists *B. vulgaris* (without distinguishing between the various wild subspecies) as a serious weed in Egypt, a common weed in Iraq, Israel and Portugal, and a weed of unknown importance in the US, Morocco, Afghanistan, Australia and Mexico. Global distribution of the wild members of the section *Beta* as reported by Terrell (1986, *A Checklist of Names for 3,000 Vascular Plants of Economic Importance.* USDA Agric. Handb. 505) are listed in Table 2.1.

Wild annual *Beta* beets (primarily *B.vulgaris* ssp. *maritima*) grow as weeds in fields or on wasteland in many parts of the Mediterranean area. Stray pollen from such weed beets had very limited possibilities for contaminating seed crops since these were well protected by an abundance of their own pollen. However, with the introduction of hybrid varieties, where 75% of the plants in the seed production fields are male sterile, pollen contamination from wild species can be a problem, especially in triploid seed production, since the tetraploid male parent plants usually open their flowers and release pollen later in the morning than do diploids (Scott and Longden, 1970). Thus, the diploid male sterile flowers may susceptible to fertilization by stray pollen. According to the OECD beet seed scheme, a seed production field is certified only if there is assurance that there are no volunteer plants of the genus *Beta.* As a consequence, breeders in Europe have moved seed production away from areas with known weed beet populations, and test the seed from every seed grower for the presence of crosses between sugar beet and annual weed beet. In these tests, all seed lots with a frequency of over 0.2% annual hybrids are discarded.

Artificial hybrids can be produced (with difficulty) with the species in the section *Corollinae*. However, such hybrids are highly sterile and set few seed when back-crossed to sugarbeet. Artificial hybrids between sugarbeet and members of the section *Procumbentes* normally die at the seedling stage. They can be saved by grafting onto sugarbeet and may then develop into vigorous plants. These hybrids are almost completely sterile and set few seed upon back-crossing. No hybrids between cultivated beets and *B. nana* of section *Nanae* have been reported.

In conclusion, within the family Chenopodiacea, all crosses between cultivated sugarbeet and species from sections other than *Beta*, are highly improbable.

Table 2.1 Taxonomic division of the genus Beta (based on DeBock, 1986)

SPECIES	CHROMOSOME NUMBER (2n)	DISTRIBUTION[1]
Section 1: Beta (syn: vulgares)		
B. vulgaris L.	18	Global (cultivated)
B. maratima L.	18	N. Africa, Portugal, Spain, Egypt Israel, Jordan, Syria, Turkey, Albania Belgium, Bulgaria, Denmark, France Germany, Greece, Ireland, Italy, Netherlands Sweden, U.K., Yugoslavia
B. macrocarpa Gus.	18, 36	N. Africa, Spain, Israel, Jordan Greece, Italy, Portugal
B. atriplicifolia Rouy	18	Europe
B. patula Ait.	18	Portugal
B. orientalis Roth.	18	India (cultivated)
Section 2: Corollinae		
B. macrorhiza Stev.	18	
B. lomatogona Fish et Mey.	18, 36	
B. corolliflora Zos.	36	
B. trigyna Wald et Kit.	45, 54	
B. intermedia Bunge	36	
B. foliosa Hausskn.	?	
Section 3: Nanae		
B. nana Bois. Et Held.	18	
Section 3: Patellares		
B. procumbens Chr. Sm.	18	
B. webbiana Moq.	18	
B. patellaris Moq.	36	

[1] From Terrell, E.E. 1986. A Checklist of Names for 3,000 Vascular Plants of Economic Importance. U.S.D.A. Agric. Handb. 505.

III. MOLECULAR BIOLOGY AND GENETIC ANALYSIS OF GTSB77

A. Description of Vector PV-BVGT03 and Method of Transformation

GTSB77 was produced by transforming a proprietary sugarbeet line (A1012) with plasmid PV-BVGT03, a disarmed *Agrobacterium tumefaciens* double border plant transformation vector (Figure 3.1). The plasmid contains (1) the C-terminal 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) gene from *Agrobacterium*, (2) the *uidA* gene, from *E. coli,* encoding a β-D-glucuronidase (GUS) protein, (3) a glyphosate oxidoreductase (*gox*) gene from *Ochrobactrum anthropi,* and (4) a neomycin phosphotransferase (*nptII*) gene from *E. coli*, all within the right and left borders of the vector. In addition, the plasmid contains a bacterial selectable marker gene (*spc/str)* as well as origins of replication (*ori-V* and *ori-322*) necessary for replication and maintenance of the plasmid PV-BVGT03 in bacteria. More detail regarding the genetic elements in vector PV-BVGT03 is presented in Table 3.1.

A disarmed *Agrobacterium tumefaciens* plant transformation system was used to produce GTSB77 (*Euphytica* 94: 83-91, 1997). This plant transformation system is well documented to transfer and stably integrate T-DNA into a plant's nuclear chromosome (White, 1989; Howard *et al.*, 1990). Only those DNA sequences within the left and right border sequences [CP4 EPSPS, *uidA* (GUS), *gox*, and *nptII*] are expected to be transferred and integrated into the plant chromosome.

Following transformation, Agrobacterium cells were eliminated by incubating plant tissue with cefotaxime (0.5g/L; 3X 60'). Transformed tissue was selected and plants regenerated in the presence of glyphosate (1 mM) as well as cefotaxime (0.5g/L) to ensure elimination of Agrobacterium cells (*Euphytica* 94: 83-91, 1997).

B. Origin of Donor Genes and Regulatory Sequences

1. The *cp4 epsps* gene

The *cp4 epsps* gene cassette consists of the figwort mosaic virus (FMV) promoter, a chloroplast targeting sequence from *Arabidopsis thaliana*, the *cp4 epsps* coding region from *Agrobacterium* sp. strain CP4, and a 3' nontranslated region from pea which directs polyadenylation. This gene codes for the protein CP4 EPSPS, which catalyses the conversion of shikimate-3-phosphate (S-3-P) and phosphoenolpyruvate (PEP) into 5-enolpyruvylshikimate-3-phosphate (EPSP), an intermediate in the production of aromatic amino acids (Hermann, 1983; Haslam, 1974). The CP4 EPSPS protein is highly resistant to inhibition by glyphosate, the active ingredient in the Roundup herbicide.

The original gene sequence from *Agrobacterium* was modified to create a synthetic gene, which allows for higher expression in plants. Bacterial genes, such as those from *Agrobacterium,* have several features that reduce their ability to function efficiently in plants. These features include potential polyadenylation sites that are often rich with A+T nucleotides, a higher G+C nucleotide percentage than that frequently found in dicotyledonous plant genes, concentrated stretches of G and C nucleotide residues, and codons that may not be found frequently in dicotylendonous plant genes.

2. The *gus* gene

The *gus* (*uidA*) gene cassette contains the enhanced 35S promoter from the cauliflower mosaic virus, the *uidA* coding region for the β-D-glucuronidase protein from *E. coli*, and the 3' nontranslated region from pea which directs polyadenylation. This gene serves as a marker during the plant transformation process.

3. The *gox* gene

The *gox* gene cassette consists of the figwort mosaic virus promoter, a chloroplast targeting sequence from *Arabidopsis thaliana*, the *gox* coding region from *Ochrobactrum anthropi*[1], and a 3' nontranslated region of the nopaline synthase gene, which directs polyadenylation. When expressed, the function of the glyphosate oxidase (GOX) enzyme is to metabolize glyphosate (N-phosphonomethylglycine), the active ingredient in Roundup herbicide, to an inactive form. As with the *cp4 epsps* gene above, the original *gox* gene sequence from *Ochrobactrum anthropi* was modified to create a synthetic gene which allows for higher expression in plants.

4. The *nptII* gene

The neomycin phosphotransferase II gene is from transposon Tn5. The NPTII enzyme coded by this gene confers resistance to selected aminoglycoside antibiotics and is used as a plant selectable marker (Beck *et al.*, 1982). However, this gene was not transferred into the sugar beet genome because of the truncation of the insertion event within the *gox* gene in PV-BVGT03.

5. The chloroplast transit peptide genes (CTP1 and CTP2)

Targeting of the CP4 EPSPS and GOX protein to the chloroplast has been shown to be critical to achieving the greatest levels of tolerance to glyphosate (della-Cioppa *et al.*, 1987). The *ctp2* sequence from the *Arabidopsis thaliana epsps* gene is fused to the 5-prime end of *cp4 epsps* to enhance tolerance, while *ctp1*, the sequence encoding the chloroplast transit peptide derived from the small subunit of rubisco from *A. thaliana,* was fused to the 5-prime end of the *gox* gene. For functionally active proteins, these peptides are rapidly digested immediately after import into the chloroplast. Similar signal peptides are present in all plants and are of no toxicological concern.

6. Genetic elements beyond the borders of the T-DNA

The following elements are present on the plasmid PV-BVGT03, but are outside of the borders of the T-DNA, and are hence not expected to be transferred into the sugarbeet genome.

- *ori-V*: a 0.4 Kb origin of replication segment derived from the broad-host range plasmid RK2 is located just outside the left border of PV-BVGT03.

- *aad*: a 0.79 Kb gene isolated from transposon Tn7 is located just outside the right border of PV-BVGT03. This gene encodes the enzyme streptomycin adenyltransferase that allows the selection of transformed bacteria on culture medium containing spectinomycin or streptomycin.

- *ori-322*: a 0.6 Kb segment which provides an (1) origin of replication for maintenance of the PV-BVGT03 plasmid in *E. coli* and (2) a site for the conjugational transfer into the *Agrobacterium tumefaciens* cells is located between the *aad* gene and the *ori-V* gene.

C. Southern Hybridization Analysis of GTSB77

[1] A previous designation was *Achromobacter* sp. strain LBAA.

Methodology

Total DNA was extracted from sugarbeet tissue using the QIAGEN DNeasy™ Plant Mini Kit according to the manufacturer's instructions. DNA (10 μg) was digested with the appropriate restriction enzymes, fractionated by electrophoresis in 0.6% agarose gels and transferred to Hybond-N$^+$ membranes (Amersham) by capillary blotting in 20xSSC transfer buffer (Sambrook *et al*, 1989). Hybridizations were conducted using probe fragments generated by PCR amplification of the corresponding sequences in plasmid PV-BVGT03, and radio-labeled using the AlkPhos Direct labeling kit from Amersham, according to manufacturer's instructions.

1. The *cp4 epsps* and right border region

Using specific restriction endonucleases and DNA probes, it is possible to reliably estimate the number of T-DNA inserts in a transformed plant's genome. In order to estimate the number of *cp4/epsp* inserts, two restriction enzymes were chosen that either restrict at a single site within the *cp4/epsps* coding region (*BclI*) or at a single site flanking (3-prime) the *cp4/epsps* coding region (*NcoI*) (Figure 3.1). There are no *BclI* restriction sites 5-prime to the *cp4/epsps* coding region (within the right T-DNA border); the most proximal 5-prime *BclI* will be in the sugarbeet chromosomal DNA. Digesting GTSB77 genomic DNA with *BclI* and using a *cp4/epsps*-specific hybridization probe (nucleotides 356-1147; Figure 3.1), representing sequences within the *cp4/epsps* coding region, a unique band (greater than 1.8 kb) should be visible for each *cp4/epsps* sequence present in GTSB77. The data from the Southern blot shows one band at approximately 3.2 kb (Figure 3.2). Similarly, there are no *NcoI* restriction sites 5-prime to the *cp4/epsps* codong region within the right T-DNA border. Digesting GTSB77 genomic DNA with *NcoI* and using a *cp4/epsps*-specific hybridization probe, a unique band (greater than 3.7 kb) should be visible for each *cp4/epsps* sequence present in GTSB77. The data from the Southern blot shows a band at approximately 5.7 kb (Figure 3.2). While a faint band at approximately 7.0 kb is also present, we attribute this band to incomplete digestion of the sugarbeet genomic DNA. The Southern blot data from both restriction digests suggests a single insert of this portion of the T-DNA.

2. The *gus* gene

The presence of the *uidA* gene in the GTSB77 genome was confirmed by Southern hybridization. Digestion of genomic DNA with *Xba*I, *BamHI*, or *HindIII* and using a *uidA*-specific probe (nucleotides 3177-4218; Figure 3.1), representing sequences within the *uidA* coding region, yielded single hybridizing bands (Figure 3.3).

3. The *gox* gene

In order to elucidate the number of gox inserts and integrity of the left border region, GTSB77 genomic DNA was restricted separately with *XbaI*, *BamHI*, and *HindIII*. The enzyme *HindIII* restricts between the E9 3' terminator 3-prime to the GUS gene and the 5-prime end of the FMV promoter (Figure 3.1). By digesting GTSB77 genomic DNA with *HindIII* and using a *gox*-specific hybridization probe (nucleotides 6489-6916; Figure 3.1), representing sequences within the *gox* coding region, a unique band (greater than 4.4 kb) should be visible for each *gox* insert. The data from the Southern blot reveal one band of approximately 2.0 kb, indicating a single insert of this portion of the T-DNA (Figure 3.4). The enzyme *XbaI* restricts at a single location between the 3-prime end of the FMV promoter and the 5-prime end of the gox gene. Digesting GTSB77 genomic DNA with *XbaI* and using an identical *gox*-specific hybridization probe, a unique band (greater than 3.7 kb) should be visible for each *gox* insert. The data from the Southern blot indicate one band of approximately 6.8 kb, supporting the *HindIII* restriction data that indicates a single insert of this portion of the T-DNA. There was no hybridization of the gox probe to the DNA digested with *BamHI*.

The observation that the *gox*-homologous DNA present in the genome of GTSB77 is smaller than expected suggested that the left portion of T-DNA might not have integrated as a complete entity. In order to elucidate the exact nature of the inserted DNA, the nucleotide sequence of the flanking DNA sequences to the inserted DNA was determined.

A Lambda FIXII phage library of GTSB77 genomic DNA was probed with both *cp4/epsps* and *gox* probes. Of 25 of the initial plaques pulled from the library, two hybridized to both the *cp4/epsps* and the *gox* probe. The DNA from one of these was recloned and the nucleotide sequence of the adjacent sugarbeet DNA determined, revealing the junction sites of the sugarbeet genome and the integrated DNA. The right border junction of the integrated DNA was at bp 15116 (Figure 3.1), between the 25 bp right border and the FMV promoter. The left border junction of the integrated DNA was at bp 7372, *within* the coding region of the *gox* gene, 897 basepairs downstream of the *gox* gene start codon (Figures 3.1, 3.5). Downstream (3-prime) of the *gox* gene fragment (within the sugarbeet genomic DNA), two translational stop codons located 130 and 234 bp from the junction were identified. In addition, a *HindIII* site was found 231 bp downstream from the junction site (~2.0 kb from the FMV promoter *HindIII* site) as well as a transcription termination signal (AATAAA) 650 bp from the junction point. Based upon these data, it is apparent that the complete DNA insert within the left and right T-DNA borders present in the transformation vector PV-BVGT03 is not present in the genome of GTSB77. This resulted in a truncated form of the *gox* gene, which is fused to sugarbeet genomic DNA.

4. Other PV-BVGT03 sequences

To determine whether sequences outside of the T-DNA border region of PV-BVGT03 had been transferred into the genome of GTSB77, total DNA was digested with appropriate restriction enzymes, and subject to Southern hybridization using either a PCR-generated DNA probe homologous to the entire *oriV* sequence present in plasmid PV-BVGT03 (nucleotides 9906-11912; Figure 3.1), or a probe homologous to the entire *ori322/aad* sequence present in plasmid PV-BVGT03 (nucleotides 12571-14980; Figure 3.1). There was no hybridization between either of these probes and GTSB77 genomic DNA, indicating that these sequences were not transferred (or stably integrated) into the sugarbeet genome (Figures 3.6, 3.7).

D. Mendelian Inheritance

Glyphosate tolerance in other commercial crops (e.g., soybean, canola, and cotton) transformed with a similar *cp4 epsps* gene is inherited as a dominant trait; a single copy (allele) of the introduced *cp4 epsps* confers whole plant tolerance to glyphosate. Novartis Seeds' plant breeders have conducted numerous backcrosses and selfing (utilizing conventional breeding techniques) with the original GTSB77 line. The inheritance of the introduced DNA in the progeny from these crosses is monitored phenotypically at the whole plant level by application of glyphosate herbicide and/or performing *in vitro* β-D-glucuronidase (GUS) assays. Data from these analyses provide further evidence of the number of loci as well as the stability of the introduced DNA. The results from a typical analysis are presented in Table 3.2. The number of GUS-positive plants (222) in the F2 generation is very close to the expected value (213) for a single locus (as predicted by the Southern analysis) acting in a dominant fashion. Further, the number of glyphosate-tolerant plants in the F3-generation (derived from selfed-F2) were also as predicted for a single-dominant locus. These results prove that the single T-DNA insert present in GTSB77 is inherited as a single locus in a stable manner.

Table 3.1. Summary of the Genetic Elements in PV-BVGT03

Genetic Element	Size (Kb)	Function
Right Border	0.03	A 25 nucleotide sequence that acts as the initial point of DNA transfer into plant cells originally isolated frompTiT37 (Depicker et al., 1982).
P-FMV	0.67	The 35S promoter from a modified figwort mosaic virus used to drive expression of CP4 EPSPS and *gox* genes (Shepard *et al.*, 1987; Richins *et al.*, 1987; Gowda *et al.*, 1989).
AEPSPS/CTP2	0.31	The N-terminal chloroplast transit peptide sequence from the *Arabidopsis thaliana* EPSPS gene (Richins *et al.*, 1987; Gowda *et al.*, 1989; Sanger *et al.*, 1993).
CP4syn	1.36	The C-terminal 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) gene from *Agrobacterium* sp. strain CP4 (Padgette *et al.*, 1993a).
E9 3'	0.63	The 3' end of the pea rbcS E9 gene which provides the polyadenylation sites for the CP4 EPSPS and GUS genes (Coruzzi *et al.*, 1984; Morelli *et al.*, 1985).
P-35S	0.62	The cauliflower mosaic virus (CaMV) promoter (Odell *et al.*, 1985) with the duplicated enhancer region (Kay *et al.*, 1985) used to drive expression of the GUS and *nptII* genes.
GUS:1	1.81	The *uidA* gene from *E. coli* encoding a β-D-glucuronidase or GUS protein (Jefferson *et al.*, 1986).
E9 3'	0.63	The 3' end of the pea rbcS E9 gene which provides the polyadenylation sites for the CP4 EPSPS and GUS genes (Coruzzi *et al.*, 1984; Morelli *et al.*, 1985).
P-FMV	0.67	The 35S promoter from a modified figwort mosaic virus used to drive expression of CP4 EPSPS and *gox* genes (Shepard *et al.*, 1987; Richins *et al.*, 1987; Gowda *et al.*, 1989).
CTP1	0.17	The N-terminal chloroplast transit peptide sequence from the small subunit 1A of rubisco from *A. thaliana* (Timko *et al.*, 1988).
GOXsyn	1.30	The glyphosate oxidoreductase (*gox*) gene isolated from *Achromobacter* sp. strain LBAA (Barry *et al.*, 1994).
NOS 3'	0.26	The 3' nontranslated region of the nopaline synthase gene from *Agrobacterium* which terminates transcription and directs polyadenylation (Fraley *et al.*, 1983).
P-35S	0.62	The cauliflower mosaic virus (CaMV) promoter (Odell *et al.*, 1985) with the duplicated enhancer region (Kay *et al.*, 1985) used to drive expression of the GUS and *nptII* genes.
KAN	0.80	The neomycin phosphotransferase II gene from Tn5. This enzyme confers resistance to aminoglycoside antibiotics and used as a plant selectable marker (Beck *et al.*, 1982).
NOS 3'	0.26	The 3' nontranslated region of the nopaline synthase gene from *Agrobacterium* which terminates transcription and directs polyadenylation (Fraley *et al.*, 1983).
Left Border	0.03	A 25 nucleotide sequence that delimits the T-DNA transfer and acts as the endpoint of DNA transfer into plant cells. It was originally isolated frompTiA6 (Barker et al., 1983).
ori-V	0.39	origin of DNA replication, originally isolated from plasmid RK2; permits plasmid replication in *Agrobacterium*. (Rogers *et al.*, 1987).
ori-322	0.63	Origin of replication isolated from the plasmid pBR322; permits plasmid replication in *E. coli* (Sutcliffe, 1979).
Spc/Str	0.79	The bacterial gene encoding the Tn7 AAD 3" adenyltransferase conferring spectinomycin and streptomycin resistance to bacterial cells that carry the plant vector (Fling *et al.*, 1985).

Table 3.2. Segregation of glyphosate-tolerant sugarbeet obtained from GTSB77

Generation	Number of glyphosate-tolerant plants (*RR* or *Rr*)	Number of non-tolerant plants (*rr*)
BC_1, F2 [1] -actual results: -expected results:	 222 [3] 213	 62 71
BC_1, F3 [2] -actual results: -expected results:	 25 [3] 25.5	 9 8.5
Expected proportions (Mendelian inheritance)	75 %	25 %

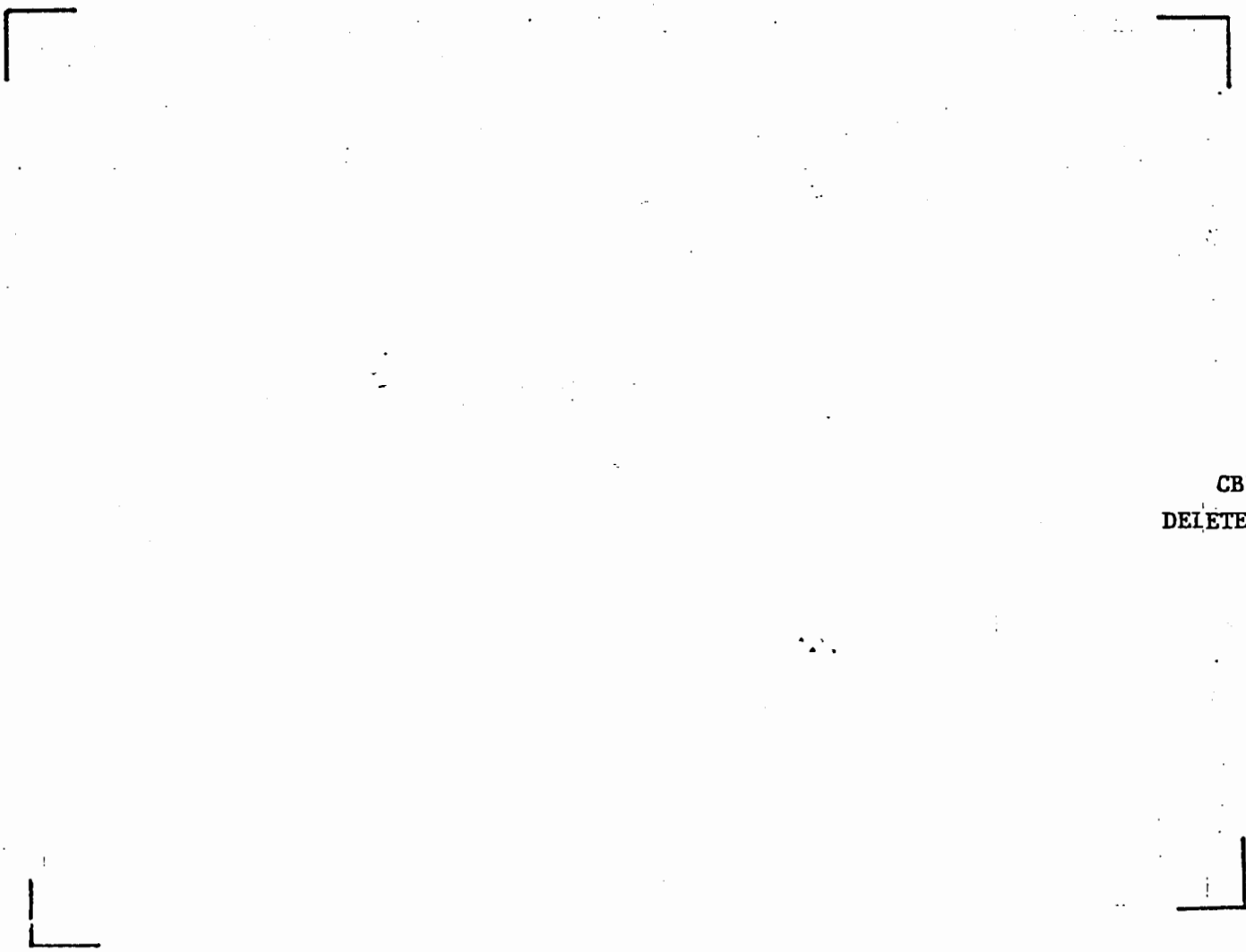
[1] Selection of plants made with the GUS test.

[2] Selection of plants made with an application of Roundup herbicide, applied at the anticipated label rate (1 liter/acre)

[3] Chi-square probability: 21 %.

Figure 3.1. Plasmid map of PV-BVGT03 with restriction sites

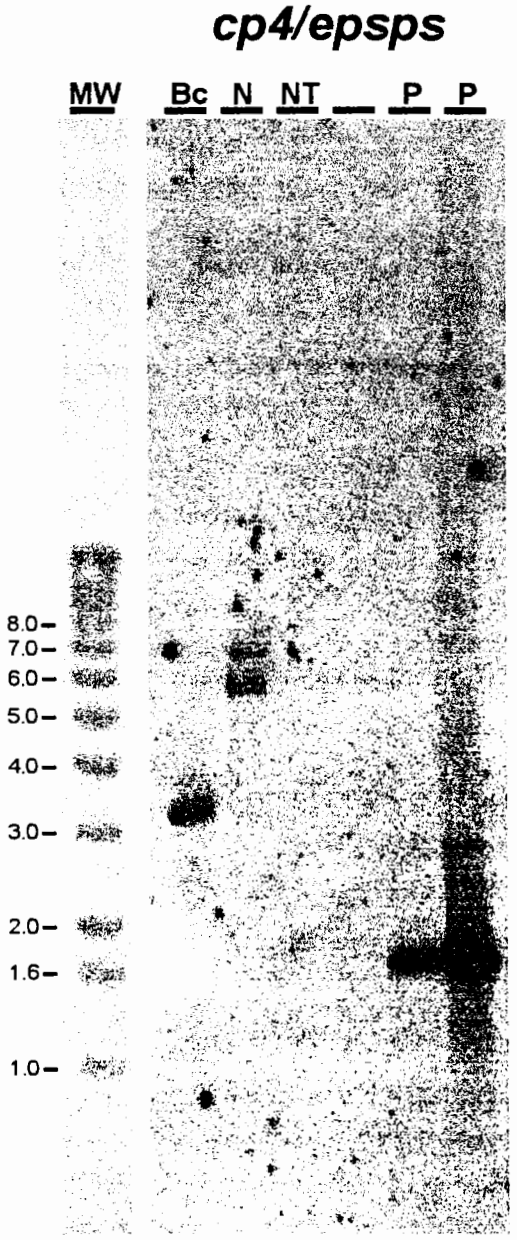
CBI DELETED



CBI
DELETED

Figure 3.2

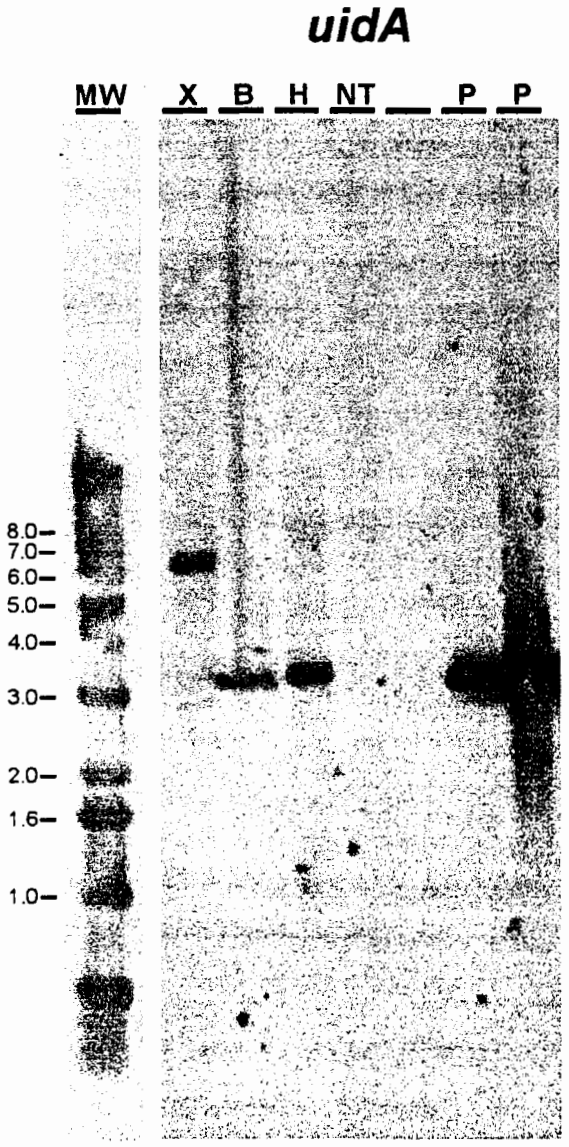
Southern blot analysis of GTSB77 using *cp4/epsps* sequence as probe



Probe: *cp4/epsps* sequence from vector PV-BVGT03(nucleotides 356-1147, Figure 3.1).
Lane Bc: GTSB77 DNA (10 ug) digested with restriction enzyme *Bcl* I.
Lane N: GTSB77 DNA (10ug) digested with restriction enzyme *Nco* I.
Lane NT: Non-transformed control sugarbeet DNA digested with restriction enzyme *Nco*I.
Lane P: Plasmid PV-BVGT03 digested with restriction enzyme *Eco*RI.
MW: 1 Kb molecular weight standard.

Figure 3.3

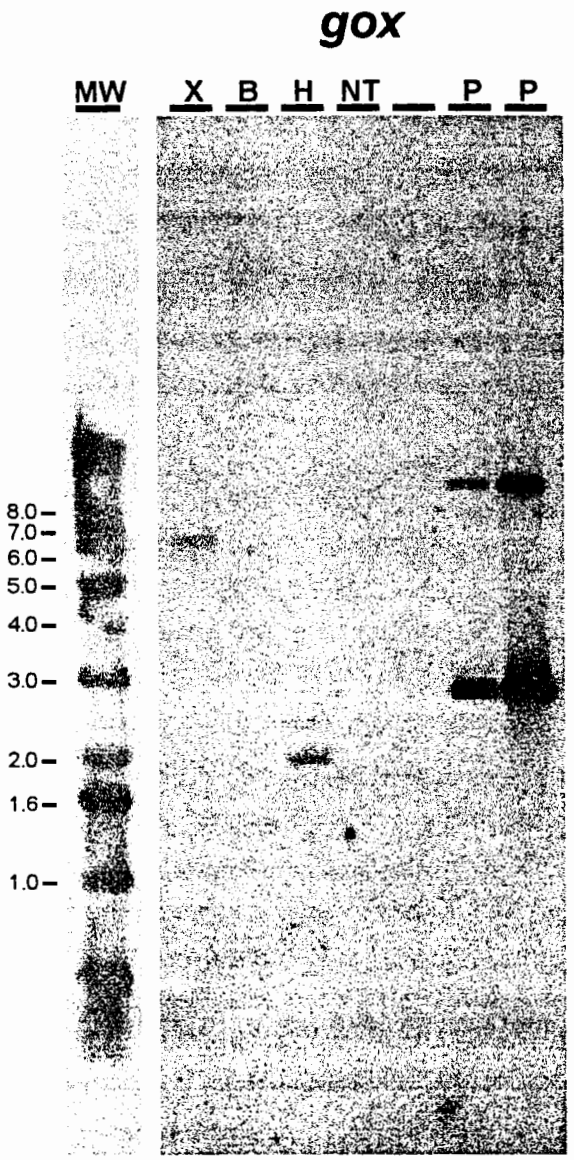
Southern analysis of GTSB77 using *uid*A (*gus*) sequence as probe



Probe: *uid*A (*gus*) sequence from vector PV-BVGT03(nucleotides 3177-4218, Figure 3.1).
Lane X: GTSB77 DNA (10 ug) digested with restriction enzyme *Xba* I.
Lane B: GTSB77 DNA (10 ug) digested with restriction enzyme *Bam*HI.
Lane H: GTSB77 DNA (10ug) digested with restriction enzyme *Hin*d III.
Lane NT: Non-transformed control sugarbeet DNA digested with restriction enzyme *Bam*HI.
Lane P: Plasmid PV-BVGT03 digested with restriction enzyme *Bam*HI.
MW: 1 Kb molecular weight standard.

Figure 3.4

Southern blot analysis of GTSB77 using *gox* sequence as probe



Probe: *gox* sequence from vector PV-BVGT03 (nucleotides 6489-6916, Figure 3.1).
Lane X: GTSB77 DNA (10ug) digested with restriction enzyme *Xba* I.
Lane B: GTSB77 DNA (10 ug) digested with restriction *Bam*HI.
Lane H: GTSB77 DNA (10 ug) digested with restriction enzyme *Hin*dIII.
Lane NT: Non-transformed control sugarbeet DNA digested with restriction enzyme *Bam*HI.
Lane P: Plasmid PV-BVGT03 digested with restriction enzyme *Bam*HI.
MW: 1 Kb molecular weight standard

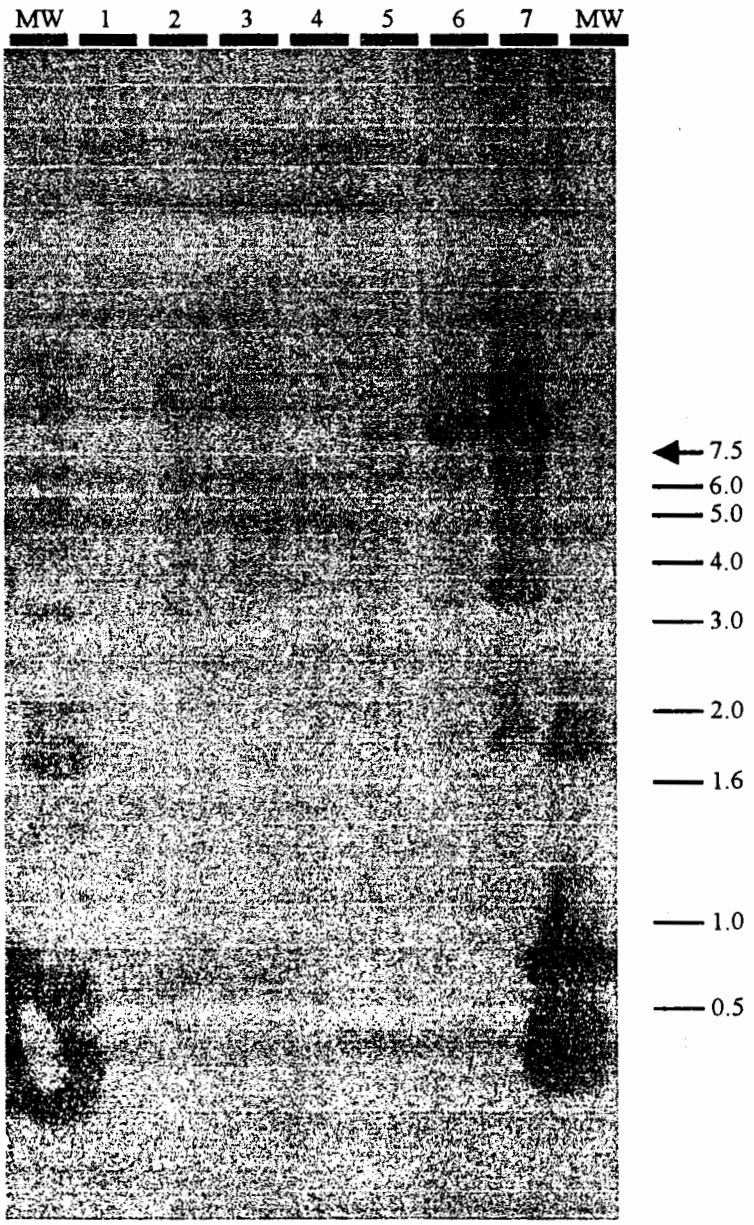
Figure 3.5. The sequence of the *gox-fusion* gene past the stop codon.

atggcttcc tctatgctct cttccgctac tatggttgcc tctccggctc aggccactat ggtcgctcct
ttcaacggac ttaagtcctc cgctgccttc ccagccaccc gcaaggctaa caacgacatt acttccatca
caagcaacgg cggaagagtt aactgcatgc aggtgtggcc tccgattgga aagaagaagt ttgagactct
ctcttacctt cctgacctta ccgattccGG TGGTCGCGTC AACTGCATGC AGGCCATGGC TGAGAACCAC
AAGAAGGTTG GTATCGCTGG AGCTGGAATC GTTGGTGTTT GCACTGCTTT GATGCTTCAA CGTCGTGGAT
TCAAGGTTAC CTTGATTGAT CCAAACCCAC CAGGTGAAGG TGCTTCTTTC GGTAACGCTG GTTGCTTCAA
CGGTTCCTCC GTTGTTCCAA TGTCCATGCC AGGAAACTTG ACTAGCGTTC CAAAGTGGCT TCTTGACCCA
ATGGGTCCAT TGTCCATCCG TTTCAGCTAC TTTCCAACCA TCATGCCTTG GTTGATTCGT TTCTTGCTTG
CTGGAAGACC AAACAAGGTG AAGGAGCAAG CTAAGGCACT CCGTAACCTC ATCAAGTCCA CTGTGCCTTT
GATCAAGTCC TTGGCTGAGG AGGCTGATGC TAGCCACCTT ATCCGTCACG AAGGTCACCT TACCGTGTAC
CGTGGAGAAG CAGACTTCGC CAAGGACCGT GGAGGTTGGG AACTTCGTCG TCTCAACGGT GTTCGTACTC
AAATCCTCAG CGCTGATGCA TTGCGTGATT TCGATCCTAA CTTGTCTCAC GCCTTTACCA AGGGAATCCT
TATCGAAGAG AACGGTCACA CCATCAACCC ACAAGGTCTC GTGACTCTCT TGTTTCGTCG TTTCATCGCT
AACGGTGGAG AGTTCGTGTC TGCTCGTGTT ATCGGATTCG AGACTGAAGG TCGTGCTCTC AAGGGTATCA
CCACCACCAA CGGTGTTCTT GCTGTTGATG CAGCTGTTGT TGCAGCTGGT GCACACTCCA AGTCTCTTGC
TAACTCCCTT GGTGATGACA TCCCATTGGA TACCGAACGT GGATACCACA TCGTGATCGC CAACCCAGAA
GCTGCTCCAC GTATTCCAAC TACCGATGCT TCTGGAAAGT TC[7372]**CGGTCCAA ATTTGTTTAC ATTGTGTCCA**
AATTTCGGCT GATTTGGACT TCCCTAGCTA TGCCAACTAA GCTAATAAAA AACATGAAAC AACAATTACA
AACTGTCGAG CACACCTTCT ACAAACTAGC TTAGATTTCT ATTGGAAGTT ACAAAACAGT AAAACTACCA
ATAGGATACT AAATTAAACA TATTAAACTA TTACTCCTCA AAAGCTTGTA CATTTGCAAA AGAAATGATG
GTTGCCCAAA AGCTTCAAAG

The sequence of the *gox-fusion* gene from the ***atg*** of the CTP1 past the stop codon (***TAG***) found in the sugar beet DNA. Sequences from sugar beet are in bold and the site where the fusion begins, bp 7372 is identified. Sequences underlined identify the locations of PCR primers used to clone the *gox-fusion* gene into *E. coli*.

Figure 3.6

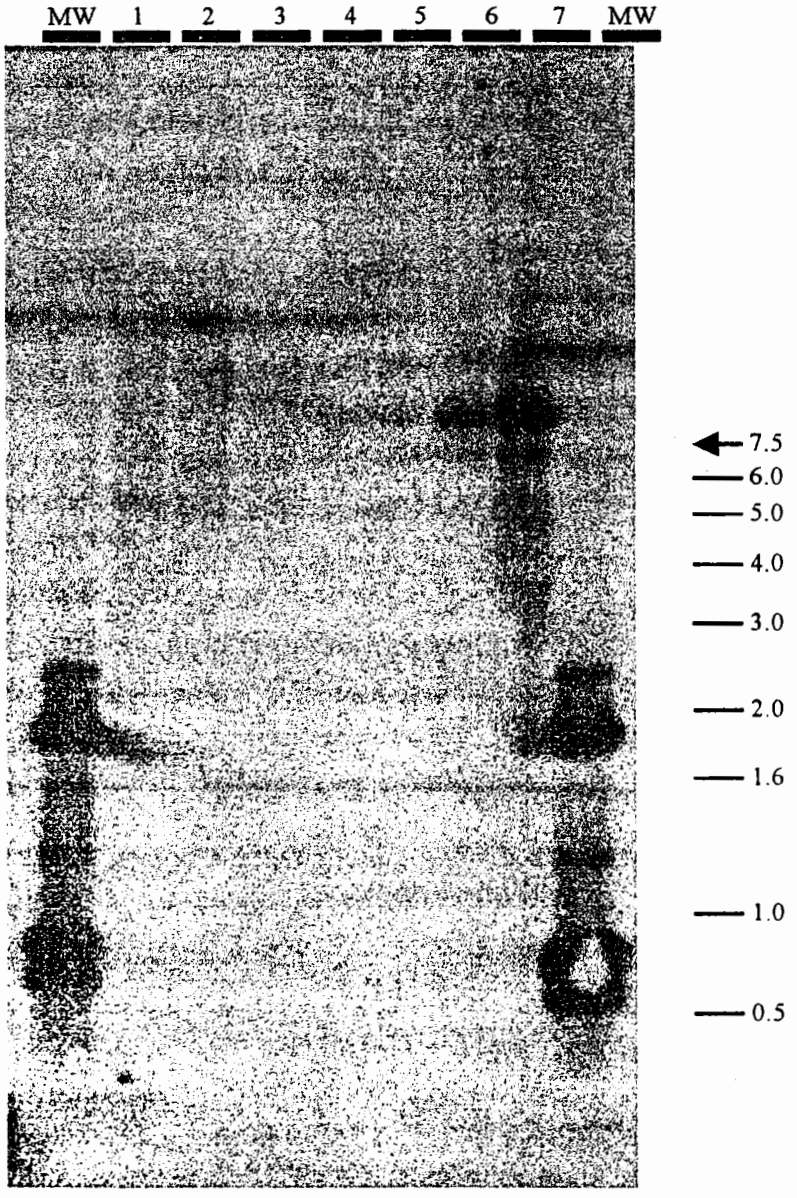
Southern analysis of GTSB77 using *ori-322* sequence as probe



Probe: *ori-322* sequence from vector PV-BVGT03 (nucleotides 12571-14980, Figure 3.1).
Lane 1: GTSB77 DNA (10 ug) digested with restriction enzyme *EcoRI*.
Lane 2: GTSB77 DNA (10 ug) digested with restriction enzyme *HindIII*.
Lane 3: Non-transformed control sugarbeet DNA digested with restriction enzyme *EcoRI*.
Lane 4: Empty lane.
Lanes 5-7: Increasing amounts of PV-BVGT03 DNA digested with *EcoRI*.
MW: 1Kb molecular weight standard.

Figure 3.7

Southern analysis of GTSB77 using *ori-v* sequence as probe



Probe: *ori-v* sequence from vector PV-BVGT03 (nucleotides 9906-11912, Figure 3.1).
Lane 1: GTSB77 DNA (10 ug) digested with restriction enzyme *EcoRI*.
Lane 2: GTSB77 DNA (10 ug) digested with restriction enzyme *HindIII*.
Lane 3: Non-transformed control sugarbeet DNA digested with restriction enzyme *EcoRI*.
Lane 4: Empty lane.
Lanes 5-7: Increasing amounts of PV-BVGT03 DNA digested with restriction enzyme *EcoRI*.
MW: 1 Kb molecular weight standard.

IV. EXPRESSION LEVELS OF TRANSGENES IN GTSB77

A. The CP4 EPSPS and β-D-Glucuronidase (GUS) Proteins

In 1995, field trials were conducted at six locations in Europe (Italy, Spain, United Kingdom, Denmark, Belgium, and France) that represent major sugarbeet production areas. Representative samples of leaf tissue and brei (root tissue processed using standard methods of the sugarbeet industry for analysis of roots) were collected from these sites and analyzed using ELISA assays for CP4 EPSPS and GUS. Results from these analyses, presented in Table 4.1, indicate that expression of CP4 EPSPS and GUS was highest in the tops and lowest in the brei.

In 1996, field trial samples from five locations in the US [MN (2 sites), ND, CA, ID] and six locations in Europe were analyzed according to the same methods as in 1995. Since early leaf tissue (6 – 12 leaf stage) is not used in commerce and because levels of CP4 EPSPS and GUS were in the same range as those for top and brei in 1995 study, this material was not analyzed in 1996. The results from these analyzes are presented in Tables 4.1 and 4.2. Similar to the 1995 study in Europe, the levels of both proteins were higher in tops than in brei. No meaningful differences between the ranges and mean levels of CP4 EPSPS and GUS in GTSB77 were observed over the two years of sampling in Europe. This is consistent with stable insertion and expression of the CP4 EPSPS and *uidA* gene over generations. No detectable CP4 EPSPS or GUS was observed in negative control top or root tissue.

B. Truncated Gox Protein

As described in Chapter III of this Petition, nucleotide sequencing of the left border region of the transgene insert in GTSB77 revealed a truncated *gox* gene fused to sugarbeet DNA. Sequence analysis also revealed (1) an in-frame translational stop codon 130 bp, and (2) a transcription termination signal 650 bp from the *gox*-sugarbeet junction. Western blot analysis using a polyclonal antibody to the GOX protein demonstrated a unique band in protein extracts from GTSB77 that was absent in extracts similarly prepared from non-transgenic sugarbeet. This protein (34550) had an apparent molecular weight of approximately 46 kD, slightly larger than the GOX protein. Several unsuccessful attempts were made to purify sufficient plant-expressed protein in order to obtain N-terminal sequence information. These data would have elucidated whether the plant expressed Gox-sugarbeet fusion protein had the CPT1 transit peptide at its amino terminus. In order to identify the nature of the plant-expressed protein in more detail, the *gox*-sugarbeet fusion (lacking the chloroplast transit peptide sequence CTP1) was PCR-cloned from the genome of GTSB77 into an *E. coli* protein-expression vector. In addition, a second expression vector was constructed that fused the CTP1 sequence (as present in the original transformation vector) to the 5-prime portion of the PCR-cloned *gox*-sugarbeet fusion. The insert in both plasmids was confirmed by nucleotide sequence analysis.

Protein extracts were prepared from each expression system and analyzed by SDS PAGE and western blot. The protein encoded by the *gox-fusion* gene had an apparent molecular weight much lower than the plant-produced protein, while the protein resulting from the

CTP1-gox-fusion gene had an approximate molecular weight similar to the protein detected in GTSB77. This analysis suggests that the plant-expressed protein was likely composed of the 89 amino acids of CTP1, 299 amino acids from the N-terminus of the GOX protein, and 43 amino acids encoded by sugarbeet DNA.

1. Antibody avidity and quantitation of protein 34550

Antibody DR1, although produced against an intact GOX protein, was anticipated to immunoreact to protein 34550 (as expressed in GTSB77) since (1) protein 34550 contains 299 amino acids of the N-terminus of the GOX protein (~71% of the total amino acids of the complete GOX protein), and (2) DR1 is a polyclonal antibody. In order to utilize DR1 to quantitate the amount of protein 34550 present in GTSB77, the avidity of DR1 to protein 34550 relative to the intact GOX protein in a western blot had to be established.

Replicate extracts of protein 34550 expressed by *E. coli* were separated by SDS PAGE along with standards of the GOX protein. Protein was visualized with coomassie colloidal blue, and the quantity of protein 34550 in the extracts was determined using the BioImage Visage 2000 image analysis system with GOX protein as the standard. These same extracts were also diluted and analyzed by western blot followed by image analysis (gel scanning) again using GOX protein as a standard and DR1 for detection. Using the levels determined from the image analysis of the coomassie stained gel, the concentration of protein 34550 loaded in the western blot was calculated. The results of this calculation were then compared to the results obtained from image analysis of the western blot versus the GOX standard. Dividing the theoretical or known level of protein 34550 by the observed level, the avidity factor the GOX antibody was determined. To ensure accuracy, a mean from three extracts was used to compute the avidity value. The results indicate that DR1 is estimated to have 2.036-fold higher avidity for GOX as it has for protein 34550. Hence, a band corresponding to approximately 8 ng of protein 34550 in a western blot detected using DR1 will be approximately the same intensity as 4 ng of the GOX standard.

Using 2.036 as the avidity of DR1 for protein 34550, gel scanning of the western blot indicated 5.5 ng of protein 34550 per 2.86 mg of plant tissue extracted and loaded into the western blot. Dividing the amount of protein 34550 (5.5 ng) by the weight of GTSB77 tissue extracted (2.86 mg) gives an estimated expression level of protein 34550 in GTSB77 of 3.92 ng/mg fresh weight.

2. Enzymatic activity

The assay developed for the detection of GOX enzymatic activity measures the conversion of glyphosate or IDA (iminodiacetic acid) into glyoxylate (Padgette *et al.*, 1994). Glyoxylate is detected by conversion to its 2,4-dinitrophenyl hydrazone derivative whose production is monitored at 520 nm after adjustment to a basic pH. Quantitation is determined using a standard curve of known amounts of glyoxylate derivatized in the same manner. An extract of protein 34550 was analyzed for GOX enzymatic activity. The freshly prepared extract showed no evidence of oxidation of glyphosate.

3. Other considerations

The *in vitro* digestibility, oral toxicity, and allergenic potential of the novel proteins expressed in GTSB77 have been evaluated. The rapid degradation and lack or toxicity in an acute oral toxicity study in mice suggest that these novel proteins will be digested as conventional dietary protein. The results from these evaluations are provided in Appendix I.

Table 4.1. Summary of Expression Levels in GTSB77 Tissues in 1995 and 1996 European field trials.

Tissue Type	CP4 EPSPS Protein (μg/mg tissue fresh weight)		GUS Protein (μg/mg tissue fresh weight)	
	1995	1996	1995	1996
Leaf (6-12 leaf stage[1])				
mean[2]:	0.145	n.a.[5]	0.0020	n.a.[5]
range[2]:	0.130-0.179		0.0008-0.0036	
Top[3]				
mean[2]:	0.285	0.190	0.0030	0.0034
range[2]:	0.249-0.370	0.134-0.273	0.0024-0.0036	0.0021-0.0061
Brei[4]				
mean[2]:	0.054	0.063	0.0006	0.0005
range[2]:	0.046-0.064	0.050-0.076	0.0004-0.0008	0.00008-0.0006

[1] The youngest fully developed leaf was sampled by either taking a section of the leaf or obtaining the whole leaf. Samples were immediately frozen between dry ice to maintain the stability of the sample until analysis.

[2] In 1995, the mean was calculated using n=6, where each of the 6 sites provided a single sample for analysis. The range reported is for 6 values. In 1996, the mean was calculated using n=12, where each of the 6 sites provided a single sample for analysis, each sample was analysed in duplicate. The range reported is for 12 values.

[3] The youngest fully developed leaf was sampled by taking the whole leaf of 30 plants immediately before harvest. Samples were immediately frozen between dry ice to maintain the stability of the sample until analysis. These leaf samples are representative of tops.

[4] Brei was prepared using a sugarbeet saw. Samples were immediately frozen between dry ice to maintain the stability of the sample until analysis.

[5] n.a., not analyzed.

Table 4.2. Summary of Expression Levels in GTSB77 Tissues in 1996 United States field trials.

Tissue Type	CP4 EPSPS Protein (μg/mg tissue fwt)	GUS Protein (μg/mg tissue fwt)
Top[1]		
mean[2]:	0.172	0.00278
range[2]:	0.126-0.193	0.00235-0.00335
Brei[3]		
mean[2]:	0.047	0.00039
range[2]:	0.032-0.060	0.00028-0.00055

[1] A composite of young leaves were pooled before harvest based on identification from a qualitative ELISA. Samples were immediately frozen between dry ice to maintain the stability of the sample until analysis. These leaf samples are representative of tops.

[2] The mean was calculated using n=10, where each of the 5 sites provided a single sample for analysis, each sample was analysed in duplicate. The range reported is for 10 values.

[3] Brei was prepared using a sugarbeet saw. Samples were immediately frozen between dry ice to maintain the stability of the sample until analysis.

V. AGRONOMIC PERFORMANCE OF GTSB77

Summary
The agronomic performance of GTSB77 was compared to non-transgenic sugarbeet in order to ascertain whether any unintended changes had occurred as a result of the transformation process or the expression of novel proteins. Evaluations included both laboratory studies as well as numerous field trials under a diverse set of geographical and environmental conditions. Parameters evaluated include disease susceptibility, sensitivity to fungicides, insecticides, and herbicides, plant morphology, and vigor. The results from these studies indicate that except for tolerance to glyphosate, GTSB77 is indistinguishable from non-transgenic sugarbeet.

A. Germination rate (emergence), seed dormancy, and invasiveness

Results from field trials indicate no differences in germination and emergence of GTSB77 compared to non-transgenic control (Table 5.1).

Overwintering capacity (frost /cold tolerance) of GTSB77 sugarbeet seed and plant tissue has been evaluated. Observations of fields (following harvest) in which GTSB77 had been cultivated indicate no germination of sugarbeet seed. Results from whole plant studies also indicate that the overwintering capacity nor competitiveness (invasiveness) of GTSB77 has not been altered relative to non-transgenic sugarbeet (Appendices 2 - 5).

B. Vegetative vigor

As a hybrid crop, the vegetative vigor of sugarbeet is dependent on the genetic composition of the parental lines. Commercial varieties are produced by crossing a monogerm cytoplasmic male sterile line (CMS) and a multigerm pollinator (the glyphosate-tolerant trait can be introduced from either parent in the cross). Field trials have been conducted with populations segregating for the glyphosate-tolerant trait. Plants exhibiting glyphosate tolerance are indistinguishable from the controls (glyphosate-sensitive) in terms of growth rate, general appearance, and final yield. In addition, observations by plant breeders during European and North American field trials indicate that GTSB77 is indistinguishable from non-transgenic sugarbeet with regards to susceptibility to predation by insects, as well as to diseases and abiotic factors (Table 5.1; Appendix VIII).

Table 5.1

Summary of field monitoring results: Comparison of agronomic properties of GTSB77 and non-transgenic sugarbeet

Data collected from field trials performed in Belgium, Denmark, France, Italy, Spain, and the United Kingdom during the 1995 growing season. Except for the glyphosate-tolerance studies, all comparisons performed in the absence of glyphosate treatment.

Parameter	Time of Assessment	Transgenic lines	Non-transgenic lines	Comments
Susceptibility to Fungicides				
Thirame	seed treatment	No	No	
Cyproconazole	foliar	No	No	
Carbendazime	foliar	No	No	
Flutriafol	foliar	No	No	
Flusilazole	foliar	No	No	
Susceptibility to Herbicides (Active ingredients)				
Glyphosate	post-emergence	No	Yes	The Roundup Ready gene made the transgenic line tolerant to glyphosate.
Glyphosate	groundkeepers	No	Yes	Treated on small beet (groundkeepers).
Glyphosate	stecklings	No	Yes	Treated on small vernalised plantlets (stecklings).
Glyphosate	bolters	No	Yes	Treated on beet bolters.
Glyphosate	large beet	No	Yes	Treated on well developed beet in August.
Chloridazon	pre-emergence	No	No	
Metamitron	pre-emergence	No	No	
Ethofumesate	post-emergence	No	No	Some phytotoxicity in some trials (burning).
Clopyralid	post-emergence	No	No	
Cycloxydime	post-emergence	No	No	
Desmediphame	post-emergence	No	No	
Fluazifop-p-butyl	post-emergence	No	No	
Lenacile	post-emergence	No	No	Some retarding in few trials.
Phenmediphame	post-emergence	No	No	
Tialiate	post-emergence	No	No	
Triflusulfuron	post-emergence	No	No	Marbling noted on leaves in few trials.
Prosulfocarbe	pre-emergence	Yes	Yes	
Isoxaben	pre-emergence	Yes	Yes	
Isoxaben	groundkeepers	No	No	Treated on small beet (groundkeepers).
Isoxaben	stecklings	No	No	Treated on vernalised plantlets (stecklings).
Methabenzthiazuron	pre-emergence	No	No	Slight retarding effect on few beet.
Trifluraline	pre-emergence	Yes	Yes	

Parameter	Time of Assessment	Transgenic lines	Non-transgenic lines	Comments
Susceptibility to Herbicides (Active Ingredients)				
Gluphosinate-ammonium	post-emergence	Yes	Yes	
Gluphosinate-ammonium	groundkeepers	Yes	Yes	Treated on small beet (groundkeepers). A lot of regrowth.
Gluphosinate-ammonium	stecklings	Yes	Yes	Treated on vernalised plantlets (stecklings).
Gluphosinate-ammonium	bolters	Yes	Yes	Treated on beet bolters.
Paraquat	post-emergence	Yes	Yes	
Paraquat	groundkeepers	Yes	Yes	Treated on small beet (groundkeepers).
Paraquat	stecklings	Yes	Yes	Treated on vernalised plantlets (stecklings).
Metsulfuron methyl	post-emergence	Yes	Yes	
Metsulfuron methyl	groundkeepers	Yes	Yes	Treated on small beet (groundkeepers).
Metsulfuron methyl	stecklings	Yes	Yes	Treated on vernalised plantlets (stecklings).
Metsulfuron methyl	bolters	Yes	Yes	Treated on beet bolters.
Metsulfuron methyl	large beet	Yes	Yes	Treated on well developed beet in August.
Metsulfuron methyl + thifensulfuron	large beet	Yes	Yes	Treated on well developed beet in August.
tribenuron	large beet	Yes	Yes	Treated on well developed beet in August.
Triasulfuron+fluoroglycofen+IPU	post-emergence	Yes	Yes	
Dichlorprop	post-emergence	Yes	Yes	
Dichlorprop	groundkeepers	Yes	Yes	Treated on small beet (groundkeepers). A lot of regrowth.
Dichlorprop	stecklings	Yes	Yes	Treated on vernalised plantlets (stecklings).
Dichlorprop	bolters	Yes	Yes	Treated on beet bolters. Typical plant hormonal effects. Some plants not killed.
Diflufenicanil + IPU	post-emergence	Yes	Yes	
Diflufenicanil + IPU	groundkeepers	Yes	Yes	Treated on small beet (groundkeepers).
Diflufenicanil + IPU	stecklings	Yes	Yes	Treated on vernalised plantlets (stecklings).
Diflufenicanil + IPU	large beet	Yes	Yes	Treated on well ddeveloped beet in August.
Metazachlore	post-emergence	No	No	Slight retarding effect on few beet.
Dicamba	post-emergence	Yes	Yes	Typical plant hormonal effects, beet stopped in development.
Dicamba	groundkeepers	Yes	Yes	Treated on small beet (groundkeepers). A lot of regrowth.
Dicamba	stecklings	Yes	Yes	Treated on vernalised plantlets (stecklings). A lot of regrowth.
Dicamba	bolters	Yes	Yes	Treated on beet bolters. Typical plant hormonal effects. Some plants not killed.
Dicamba	large beet	Yes	Yes	Treated on well developed beet in August.
Triclopyr	bolters	Yes	Yes	Treated on beet bolters. New branch formation.
Aclinofen	large beet	Yes	Yes	Treated on well developed beet in August.

Parameter	Time of Assessment	Transgenic lines	Non-transgenic lines	Comments
Germination	4 weeks after drilling	83 - 92 %	67 - 90 %	Variable germination recorded for different trials. The seed quality was inferior for some seedlots (low thousand kernel weights and not calibrated). Early breeding material was used for some trials.
Vigor (0-10)	2-4 leaf stage	5-6	5-6	For material of type MSF1 X OType.
		8	8	For 2 way hybrids.
		9	9	For 3 way hybrids.
	6-8 leaf stage	6-7	6-7	For MSF1 x OType.
		8	8	For 2 way hybrids.
		9	9	For 3 way hybrids.
	8-10 leaf stage	6-7	6-7	For MSF1 x OType.
		8	8	For 2 way hybrids.
		9	9	For 3 way hybrids.
Morphology				
Leaves	cotyledon	normal	normal	Shape, colour.
	2-4 leaves	normal	normal	Shape, colour.
	4-8 leaves	normal	normal	Shape, colour.
	Canopy closure	normal	normal	Shape, colour for 2 way hybrid and 3 way hybrid.
	Canopy closure	narrow, wilting	narrow, wilting	The MSF1x OType material was suffering from drought in some locations. (some locations in UK, France, Belgium and Italy).
Roots	Thinning	normal	normal	Shape, size.
	Harvest	normal	normal	Shape, colour, size.
	Harvest	branched	branched	In 1 Belgian and 1 UK location because of soil structure. (Compaction through heavy machinery).

Parameter	Time of Assessment	Transgenic lines	Non-transgenic lines	Comments
Susceptibility to insects				
Aphids		Normal	Normal	
Beet flea beetle		Normal	Normal	
Wireworm		Normal	Normal	
Susceptibility to slugs		Normal	Normal	
Susceptibility to nematodes				
Heterodera schachtii		Susceptible	Susceptible	Nematode infestations were tried to be avoided for these trials.
Susceptibility to virusses				
Rhizomania (BNYVV)		Susceptible	Susceptible	Locations were selected to be free of Rhizomania.
Beet curly top (BCTV)		Susceptible	Susceptible	
Virus Yellows (BYV/BMYV)		Normal	Normal	Preventive treatments with insecticides were used to control aphids populations.
Susceptibility to deseases				
Powdery mildew		Normal	Normal	
Downey mildew		Normal	Normal	
Alternaria leaf spot		Normal	Normal	
Ramularia leaf spot		Normal	Normal	
Cercospora leaf spot		Susceptible	Susceptible	1 Italian location was heavily infested with Cercospora.
Pythium root rot		Normal	Normal	
Phoma root rot		Normal	Normal	
Susceptibility to insecticides				
Deltamethrine	foliar treatment	No	No	
Imidaclopride	seed treatment	No	No	
Aldicarbe	In furrow	No	No	
Tefluthrine	seed treatment	No	No	
Susceptibility to Anthraquinone	seed treatment	No	No	

VI. COMPOSITIONAL ANALYSES OF GTSB77

Summary
A comparative study of the major components of GTSB77 and non-transgenic controls was performed. The composition of root and top (above ground) tissue, harvested from field trials conducted in Europe and the United States, was determined. Proximates (total ash, fiber, protein and dry matter), and carbohydrates were determined for both tissues, as well as total fat content in top tissue. Levels of saponin, the principle toxicant in *Beta vulgaris* root and top tissue, were determined, as well as quality components [polarization (% sucrose), invert sugar, sodium, potassium, amino nitrogen] in roots.

The results of compositional analytical studies conducted on root and top from GTSB77 compared to control sugar beet and literature values establishes that GTSB77 is substantially equivalent to non-transformed sugarbeet in terms of nutrient (proximate and quality components) and saponin composition.

A. Proximates and quality component analysis

Comparison of proximate values from GTSB77 and control (non-transformed) sugarbeet revealed little differences (Tables 6.1 – 6.6), and the levels measured fell within reported literature ranges.

Analyses of processed root samples (brei) for sugar content (polarization), sodium, potassium, amino nitrogen and invert sugar were conducted in triplicate. The values from these tests were statistically analyzed and no differences between GTSB77 and controls were observed (Tables 6.7 – 6.9).

B. Saponin analysis of sugarbeet

Saponins are triterpenoid glycosides constituents of plants from a number of monocot and dicot families including numerous food and feed crops (Oakenfull and Sidhu, 1989). Enzymatic hydrolysis of the glycoside results in the conversion to the saponin aglycone, or sapogenin. Sapogenins are known to interact with cell membrane sterols to form pores that lead to cytoplasm leakage and cell death. The predominant sapogenin in sugarbeet is oleanolic acid. Analysis for saponins in sugarbeet usually consists of liberation of the oleanolic acid, which is quantified on an HPLC (Schiweck *et al.*, 1991).

Root and top tissue from GTSB77 and non-transformed beet were analyzed for saponins by HPLC (Schiweck *et al.*, 1991). The data from that analysis indicate that there are no statistically measurable differences between GTSB77 and control tissue (Table 6.10 – 6.11).

Table 6.1. Proximate Analyses of Top Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature range [2]
	Mean [3]	Range [3]	Mean [3]	Range [3]	
Crude Ash [4]	21.69	14.10-25.78	20.56	15.82-25.87	11.5-34.4
Crude Fibre [5]	10.52	9.59-11.70	10.64	9.03-12.40	5.9-15.9
Crude Protein [6]	15.56	12.88-16.88	16.13	13.69-17.81	8.4-23.2
Crude Fat [7]	2.22	1.47-3.17	2.19	1.43-3.07	0-4.7
Dry Matter [8]	14.37	12.95-16.43	13.99	12.76-16.50	16.0-20.0
Soluble Carbohydrates [9]	49.98	45.03-61.41	50.52	46.06-57.94	38.3-64.5

1 Tissue samples were collected from field studies conducted at various locations in Europe in 1995.
2 See reference DLG, 1991.
3 n=6, all analyses were conducted in triplicate, and all values are given on a dry matter basis except dry matter.
4 Crude ash was determined using an oven method # EF L 155/13 p.430 12/7-71 modified.
5 Crude fibre was determined using the Weende method # EF L 344/36-3726/11-92.
6 Crude protein was determined using a total nitrogen value determined using a Kjeldahl method (# EF L 179/9-10 22/7-93 modified) multiplied by 6.25.
7 Crude fat was determined using a soxhlet method # EF L 15/29-30 18/1-84 modified.
8 Dry matter was determined using an oven method # EF 71/393/EOF; L 279/7 p.858-61 20/12-71.
9 Carbohydrate Calculation was based on Plantedirecktoratet bek. #19 13/1-92.

Table 6.2. Proximate Analyses of Top Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature range [2]
	Mean [3]	Range [3]	Mean [3]	Range [3]	
Crude Ash [4]	21.99	18.70-24.79	22.51	18.17-26.84	11.5-34.4
Crude Fibre [5]	9.18	8.46-9.84	9.34	7.83-10.14	5.9-15.9
Crude Protein [6]	13.00	9.45-16.24	13.24	9.73-16.25	8.4-23.2
Crude Fat [7]	2.56	2.06-3.26	2.51	2.06-3.08	0-4.7
Dry Matter [8]	14.79	11.93-17.41	14.89	11.99-17.25	16.0-20.0
Soluble Carbohydrates [9]	53.27	49.78-55.13	52.39	48.92-55.03	38.3-64.5

1 Tissue samples were collected from field studies conducted at various locations in Europe in 1996.
2 See reference DLG, 1991.
3 n=6, all analyses were conducted in triplicate, and all values are given on a dry matter basis except dry matter.
4 Crude ash was determined using an oven method # EF L 155/13 p.430 12/7-71 modified.
5 Crude fibre was determined using the Weende method # EF L 344/36-3726/11-92.
6 Crude protein was determined using a total nitrogen value determined using a Kjeldahl method (# EF L 179/9-10 22/7-93 modified) multiplied by 6.25.
7 Crude fat was determined using a soxhlet method # EF L 15/29-30 18/1-84 modified.
8 Dry matter was determined using an oven method # EF 71/393/EOF; L 279/7 p.858-61 20/12-71.
9 Carbohydrate Calculation was based on Plantedirecktoratet bek. #19 13/1-92.

Table 6.3. Proximate Analyses of Top Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature
	Mean [3]	Range [3]	Mean [3]	Range [3]	range [2]
Crude Ash [4]	20.60	18.3-24.3	21.6	16.2-28.2	11.5-34.4
Crude Fibre [5]	8.46	6.11-10.4	8.76	6.56-10.7	5.9-15.9
Crude Protein [6]	16.1	10.5-18.4	14.7	10.0-18.3	8.4-23.2
Crude Fat [7]	0.79	0.73-1.03	0.92	0.76-2.16	0-4.7
Dry Matter [8]	15.3	13.9-16.5	16.3	14.9-19.6	16.0-20.0
Soluble Carbohydrates [9]	54	47.0-62.3	53.1	45.0-61.4	38.3-64.5

1 Tissue samples were collected from field studies conducted at various locations in the USA in 1996.
2 See reference DLG, 1991.
3 Values are taken from analyses of samples from 5 sites (n=5) for line #77 and for control, with the exception of the ash analyses conducted in duplicate for line #77 (n=10). All values are given on a dry matter basis except dry matter.
4 Crude ash was determined using method AOAC Official Ash Method 923.03, 1990, modified.
5 Crude fibre was determined using AOAC method 962.09, 1990, modified.
6 Crude protein was determined via total nitrogen determination. (AOAC Official Methods 992.03 and 990.03, 1995, modified.
7 Crude fat was determined using AOAC Official Method 960.39, 1990, modified.
Means include all available data some of which are below the limit of detection of the assay. The range highlights the lowest detectable value.
8 Dry matter was determined using an oven method. (AOAC Official Method 925.45, 1990).
9 Carbohydrates were calculated by difference using the fresh weight-derived data.

Table 6.4. Proximate Analyses of Root Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature range [2]
	Mean [3]	Range [3]	Mean [3]	Range [3]	
Crude Ash [4]	5.47	4.58-6.26	6.62	4.76-9.02	3.3-17.7
Crude Fibre [5]	4.10	2.76-5.01	3.96	3.28-4.72	3.4-7.4
Crude Protein [6]	6.28	3.41-9.54	5.60	2.43-8.04	1.2-12.4
Dry Matter [7]	19.40	17.8-22.6	21.10	19.4-22.6	23.00
Soluble Carbohydrates [8]	84.1	80.3-87.2	84.1	79.0-88.1	67.3-90.9

1 Tissue samples were collected from field studies conducted at various locations in the USA in 1996.
2 See reference DLG, 1991.
3 Values are taken from the analyses of samples from 5 sites (n=5) for line #77 and for the control, with the exception of the ash analyses conducted in duplicate for 2 of the 5 sites for line #77 (n=7). All values are given on a dry matter basis except dry matter.
4 Crude ash was determined using method AOAC Official Ash Method 923.03, 1990, modified.
5 Crude fibre was determined using AOAC method 962.09, 1990, modified.
6 Crude protein was determined via total nitrogen determination. (AOAC Official Methods 992.03 and 990.03, 1995, modified.
7 Dry matter was determined using an oven method. (AOAC Official Method 925.45, 1990).
8 Carbohydrates were calculated by difference using the fresh weight-derived data.

Table 6.5. Proximate Analyses of Root Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature range [2]
	Mean [3]	Range [3]	Mean [3]	Range [3]	
Crude Ash [4]	3.42	2.71-4.94	3.40	2.66-5.08	3.3-17.7
Crude Fibre [5]	4.10	3.47-5.22	3.97	3.09-5.33	3.4-7.4
Crude Protein [6]	6.25	4.81-8.19	6.25	4.94-7.88	1.2-12.4
Dry Matter [7]	20.46	14.05-23.48	20.45	13.57-23.12	23.00
Soluble Carbohydrates [8]	86.25	81.65-88.89	86.34	81.69-88.72	67.3-90.9

1 Tissue samples were collected from field studies conducted at various locations in Europe in 1995.
2 See reference DLG, 1991.
3 n=6, all analyses were conducted in triplicate, and all values are given on a dry matter basis except dry matter.
4 Crude ash was determined using an oven method # EF L 155/13 p.430 12/7-71 modified.
5 Crude fibre was determined using the Weende method # EF L 344/36-3726/11-92.
6 Crude protein was determined using a total nitrogen value determined using a Kjeldahl method (# EF L 179/9-10 22/7-93 modified) multiplied by 6.25.
7 Dry matter was determined using an oven method # EF 71/393/EOF; L 279/7 p.858-61 20/12-71.
8 Carbohydrate Calculation was based on Plantedirecktoratet bek. #19 13/1-92

Table 6.6. Proximate Analyses of Root Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature range [2]
	Mean [3]	Range [3]	Mean [3]	Range [3]	
Crude Ash [4]	2.53	1.95-3.22	2.51	2.09-3.35	3.3-17.7
Crude Fibre [5]	4.19	3.87-4.60	4.15	3.88-4.62	3.4-7.4
Crude Protein [6]	4.26	3.02-5.44	4.30	3.02-5.18	1.2-12.4
Dry Matter [7]	23.88	19.18-26.37	23.93	19.53-26.22	23.00
Soluble Carbohydrates [8]	89.01	87.12-91.06	89.03	87.59-90.87	67.3-90.9

1 Tissue samples collected from field studies conducted at various locations in Europe in 1996.
2 See reference DLG, 1991.
3 n=6, all analyses were conducted in triplicate, and all values are given on a dry matter basis except dry matter.
4 Crude ash was determined using an oven method # EF L 155/13 p.430 12/7-71 modified.
5 Crude fibre was determined using the Weende method # EF L 344/36-3726/11-92.
6 Crude protein was determined using a total nitrogen value determined using a Kjeldahl method (# EF L 179/9-10 22/7-93 modified) multiplied by 6.25.
7 Dry matter was determined using an oven method # EF 71/393/EOF; L 279/7 p.858-61 20/12-71.
8 Carbohydrate Calculation was based on Plantedirecktoratet bek. #19 13/1-92.

Table 6.7. Quality Analyses of Root Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature range [2]
	Mean [3]	Range [3]	Mean [2]	Range [3]	
Polarization[4]	14.36	8.40-17.43	14.48	7.89-17.18	10.8-20.7
Sodium[5]	1.68	0.50-3.08	1.77	0.40-3.50	0.35-5.48
Potassium[6]	5.28	4.55-5.87	5.29	4.22-5.95	4.19-10.2
Invert Sugar[7]	1.66	0.32-3.69	1.76	0.35-4.24	0.3-2.7
Amino Nitrogen[8]	2.84	2.01-4.00	2.88	1.98-3.93	0.93-5.14

1 Tissue samples collected from field studies conducted at various locations in Europe in 1995.
2 See reference Märländer *et al.*, 1996 and Smed *et al.*, 1996.
3 n=6, all analyses were conducted in triplicate.
4 Polarization is reported as g/100g root fresh weight, and was determined using a polarimeter, ICUMSA method Sugar Analysis 1979, Proc. 1990.
5 Sodium is reported as mmol/ 100g root fresh weight, and was determined using an SMA method Technicon, technical publication THO-0160-10.
6 Potassium is reported as mmol/100g root fresh weight, and was determined using an SMA method Technicon, technical publication THO-0160-10.
7 Invert Sugar is reported as mmol/100g root fresh weight, and was determined using an SMA method Technicon, technical publication THO-0160-10.
8 Amino nitrogen is reported as mmol/100g root fresh weight, and was determined using ICUMSA method Sugar Analysis 1979 modified.

Table 6.8. Quality Analyses of Root Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature range [2]
	Mean [3]	Range [3]	Mean [3]	Range [3]	
Polarization[4]	17.26	13.79-19.37	17.33	14.12-19.41	10.8-20.7
Sodium [5]	0.46	0.26-0.82	0.54	0.20-0.82	0.35-5.48
Potassium [6]	4.89	4.12-6.01	5.03	3.97-6.38	4.19-10.2
Invert Sugar [7]	0.40	0.29-0.54	0.39	0.28-0.53	0.3-2.7
Amino Nitrogen[8]	1.60	0.67-2.84	1.63	0.76-2.48	0.93-5.14

1 Tissue samples collected from field studies conducted at various locations in Europe in 1996.
2 See reference Märländer et Al., 1996 and Smed et al., 1996.
3 n=6, all analyses were conducted in triplicate.
4 Polarization is reported as g/100g root fresh weight, and was determined using a polarimeter, ICUMSA method Sugar Analysis 1979 Proc.1990.
5 Sodium is reported as mmol/ 100 g root fresh weight and was determined using an SMA method Technicon, Technical publication THO-0160-10.
6 Potassium is reported as mmol/ 100 g root fresh weight and was determined using an SMA method Technicon, technical publication THO-0160.10.
7 Invert Sugar is reported as mmol/ 100 g root fresh weight and was determined using an SMA method Technicon, technical publication THO-0160-10.
8 Amino nitrogen is reported as mmol/100 g root fresh weight, and was determined using ICUMSA method Sugar Analysis 1979, modified.

Table 6.9. Quality Analyses of Root Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature
	Mean [3]	Range [3]	Mean [3]	Range [3]	range [2]
Polarization[4]	14.80	12.9-17.1	14.6	12.7-16.2	10.8-20.7
Sodium[5]	1.53	0.96-2.28	1.54	1.26-1.92	0.35-5.48
Potassium[6]	8.17	6.79-11.7	8.02	6.73-11.5	4.19-10.2
Amino Nitrogen[7]	5.56	2.66-7.62	5.67	3.37-7.19	0.93-5.14

1 Tissue samples collected from field studies conducted at various locations in the USA in 1996.
2 See reference Märländer *et al.*, 1996 and Smed *et al.*, 1996.
3 n=5, all analyses were conducted in triplicate.
4 Polarization is % sucrose and was determined using a "Pro-Pol" short-path polarimeter.
5 Sodium is reported as mmol/ 100g root fresh weight, and was determined spectroscopically using a Model FP-2 flame photometer.
6 Potassium is reported as mmol/100g root fresh weight, and was determined spectroscopically using a Model FP-2 flame photometer.
7 Amino nitrogen is reported as mmol/100g root fresh weight, and was determined by fluorescence after derivitization of the filtrate with orthophthalic dicarboxaldehyde.

Table 6.10. Saponin Analyses of Root and Top Tissue from GTSB77[1]

Tissue	Control Sample		GTSB77		Literature
	Mean [3,4]	Range [3]	Mean [3,4]	Range [3]	range [2]
Roots	215	111-304	208	128-260	75-965
Tops	175	125-242	215	98-358	50-600

1 Tissue samples collected from field studies conducted at various locations in the USA in 1996.
2 See reference Lüdecke *et al.*, 1958.
3 Values are taken from analyses of samples from 5 sites (n=5) for line #77 and for control. Values are given on a mg/kg fresh weight basis.
4 Saponin method was based on a published method. (J. Agric. Food Chem. 1994, 42, 279-282 (Ridout, et al., 1994)).

Table 6.11. Saponin Analyses of Root and Top Tissue from GTSB77[1]

	Control Sample		GTSB77		Literature
Analysis	Mean [3,4]	Range [3]	Mean [3,4]	Range [3]	range [2]
Roots 1995	151	72-233	137	60-261	75-965
Tops 1995	116	52-193	103	51-165	50-600
Roots 1996	529	304-999	484	293-846	75-965
Tops 1996	478	115-727	353	139-564	50-600

1 Tissue samples were collected from field studies conducted at various locations in Europe in 1995-1996.
2 See reference Lüdecke et al., 1958.
3 n=5, all analyses were conducted, and values are given on a mg/kg fresh weight basis.
4 Saponin method was based on a published method. (J. Agric. Food Chem. 1994, 42, 279-282 (Ridout, et al., 1994)).

VII. ENVIRONMENTAL CONSEQUENCES OF INTRODUCTION OF GTSB77

A. Introduction

Glyphosate (N-phosphonomethylglycine), the active ingredient in Roundup® herbicide, is a post-emergent, systemic herbicide which is active on virtually all plants. Currently, it is one of the most widely used herbicides in the world for the control of a wide variety of annual and perennial weeds. Because of its lack of selectivity, in-crop uses of glyphosate are limited. Although considerable effort has been directed at developing glyphosate tolerant crops using classical breeding techniques such as selection and mutation, these efforts have been unsuccessful. Utilising the most recent advances in genetic engineering, Novartis Seeds and Monsanto have been successful in developing crops that are tolerant to glyphosate (Barry *et al.*, 1992; Padgette *et al.*, 1996). The availability of glyphosate tolerant crops such as sugarbeet will enable farmers to utilize glyphosate-based herbicides for the effective control of weeds and take advantage of the environmental and safety characteristics of this herbicide.

Extensive field studies have shown that glyphosate-tolerant sugarbeet is equivalent to non-modified cultivated beet in all aspects evaluated, except for the expression of GUS protein and low levels of the CP4 EPSPS protein (providing tolerance to glyphosate) (see Chapter V). The cold sensitivity of GTSB77 has been studied in Denmark (Appendix 2) and Belgium (Appendix 3), and the results indicate that it does not differ from traditional beet. Other fitness experiments (greenhouse and field) demonstrated that competitiveness and establishment with glyphosate-tolerant beet plants and an inter-specific hybrid with wild beet are unchanged and no competitive advantage was provided by this trait (Appendices 4, 5). GTSB77 and bolters were shown to be susceptible to the mechanical and herbicide treatments used currently to control weed beet. Furthermore, plants of *Beta* species are not generally treated with glyphosate because they are not perceived as weeds outside of cropping systems. Based on all experimental evidence, gene flow from GTSB77 will be unaltered relative to traditional sugarbeet, and the glyphosate tolerance trait would confer no selective advantage to hybrids with wild beet.

Introduction of glyphosate-tolerance into sugarbeet production will impact some weed management practices. This chapter will describe the practices that could be affected by the introduction of GTSB77 and the use of glyphosate in weed management programs. The questions of hybridization and gene transfer between beet species, the control of volunteer beet tolerant to glyphosate, the potential development of weed resistance to glyphosate and the introduction of other glyphosate-tolerant crops are addressed.

B. The Impact of Weeds On Sugarbeet Crop and Seed Production

In its early stages of growth, sugarbeet is very slow in development and consequently demonstrate poor competitiveness with faster developing weeds. In order to optimize yield, it is essential to keep the crop weed-free until canopy closure. Sugarbeet crops are particularly sensitive to weed competition in the first six to eight weeks after planting. The presence of a few weeds per square meter can reduce yield and quality substantially. A

recent study showed that yields from sugarbeet plots containing a high population of weeds were reduced by 80 - 85% compared to the weed-free sugarbeet plots of the same variety (Longden, 1989). There has been little reduction in herbicide use in sugarbeet fields because of the crops' sensitivity to competition from weeds, and the high value of the crop. There is a need to provide options to sugarbeet producers for highly effective weed control (May, 1996).

Breeding of sugarbeet varieties is a complex and long process as sugarbeet is a biennial plant, and the end-product is a 2- or 3-way (two or three parents) hybrid. In the basic seed (seed from the parental components from which the commercial hybrids are produced) production fields single cross seed is produced. The single cross plants, being male sterile, are crossed with a pollinator to give rise to the 3-way hybrid, which is sold to farmers. Purity of the basic seed (or parental seed) is extremely important to the seed companies since it largely determines the purity of the hybrid seedlot. Seed purity is controlled by the Federal Seed Act to ensure extremely high standards for seed sold to farmers. Control measures are employed to minimize outcrossing from sugarbeet to related species and from related species into sugarbeet in seed multiplication areas. Further, basic seed multiplication areas are selected to minimize the presence of these wild relatives.

C. Weed Species Common in Sugarbeet Fields

1. Non-*Beta* Species

A wide range of weeds is present in sugarbeet production fields in North America. The most common weeds, which infest sugarbeet fields, include:

Amaranthus retroflexus (redroot pigweed)
Chenopodium album (common lambsquarter)
Kochia scapania (kochia)
Sataria species (foxtail grass)
Solanum species (night shades)
Avena fatua (wild oats)
Cyperus esculentus (yellow nutsedge)
Convolvulus arvensis (field bindweed)
Cuscuta pentagona (dodder)
Polygonum persicaria (smart weed)
Ambrosia artemisii folia (ragweed)
Cirsium arvense (canada thistle)
Malva neglecta (mallow)
Polygonum convolvulos (buckwheat)
Brassica kaber (mustard)
Polygonum aviculare (prostrate knotweed)
Amaranthus blitoides (protrate pigweed)
Solanum tuberosum (volunteer potatoes)
Cereal volunteers

2. *Beta* Species

Wild beet is defined as species within the Section *Beta* (DeBlock, 1986) and growing outside of managed areas. In the United States, there are two locations, both in the state of California, where wild beet populations are known to exist. One population (the Milpitas wild beet) persists in the San Francisco Bay area, a region where there is no sugarbeet production. A second population, taxonomically classified as *Beta vulgaris* subspecies *macrocarpa*, is found in the Imperial Valley and is considered a weed problem. In the opinion of Drs. Panella and Llewellyn (Appendix 7), it is highly unlikely that gene flow between cultivated sugarbeet (e.g., GTSB77) and *B. vulgaris* spp. *macrocarpa* (or any other wild beet species) would occur in the United States. Their opinion was based upon a number of factors, including temporal differences in flower development, very low fertility and overall poor fitness of interspecific hybrids. Recent studies evaluating *Beta* species in southern California may indicate low but detectable introgression of genes from *B. vulgaris* spp. *vulgaris* into *B. vulgaris* spp. *macrocarpa* (Dr. Detlef Bartsch, UC – Riverside, personal communication). However, the overall conclusion is that gene flow between these two species is a rare occurrence, and any gene flow between GTSB77 and wild beet would be readily managed using herbicides other than glyphosate.

In the United States, large-scale sugarbeet seed multiplication takes place mainly in the Willamette Valley of Oregon and the Salt Lake Basin of Utah. The area for hybrid seed production is very limited; annually approximately 3,000 to 5,000 acres in Oregon and 300 to 500 acres in Utah. Wild beet species are not known to be present in this geographical region, thus gene flow between wild and cultivated beet is not expected to occur (Appendix 7).

Weed beet are defined as undesirable beet species (within the *Beta* Section) occurring in managed areas. While not a management issue in the United States, weed beet is one of the most significant weed problems in European sugarbeet production. There are five potential sources of weed beet:

- **Weed beet contamination of beet seedlots**

Weed beet seed can be present in sugarbeet seedlots in Europe as a result of contaminating pollen from wild annual relatives [*B. vulgaris* (biennial) x *B. vulgaris* spp.*maritima* (annual)] during seed production. These weed beet (interspecific hybrids) will bolt, and if not controlled, will flower and produce seed during the season (Deprez, 1980). Contaminating weed beet seeds can also be a source of volunteer beet in rotational crops.

- **Bolting sugar beet**

Weed beet can arise from bolting beet plants in uncontaminated seedlots. Bolting sensitive beet cultivars give rise to weed beet in commercial beet fields when vernalisation effectively converts beet from a biennial to an annual plant. Early drilling of the bolting-sensitive varieties, with low vernalisation requirements and cold conditions during early stages of development will lead to the production of bolting beet plants. If these bolters are not removed before seedset, the seed they produce can also become a source of future weed beet. The more recently developed varieties are generally less susceptible to bolting.

- **Groundkeepers**

Weed beet can arise from the second generation of the beet crop, also called groundkeepers. Groundkeepers are vegetative tissue (small roots) left in the field after harvest, which will flower in the next season if not controlled. After harvesting, tops with a large attached root fraction can also give rise to bolters in the following year.

- **Volunteer beet**

Groundkeepers and tops could be considered volunteers. Another source of volunteer beet is seed remaining dormant in a field from a previous season (beet seed bank). Longevity of buried weed beet seeds can exceed a decade. If the seedbed is disturbed and the seed are brought to the surface where the conditions may be suitable for germination, a weed beet would result (Clarke, 1993; Longden 1993).

Volunteer beet can arise in sugarbeet crops due to the presence of contaminating weed beet seed from sugarbeet seed production fields. Establishing sugarbeet seed production fields is done in two ways: direct drilling and transplanting stecklings. Fields used for seed production and using the direct drilling system are in 5-10 year rotations. The length of this rotation is important to assure purity by minimizing weed beet. Volunteer beet coming from seed scattering from the previous beet seed production and occurring between the seed production rows is readily controlled manually. However, some of the volunteer beets within rows are not controlled if phenotypically indistinguishable from the sugarbeet in production.

Stecklings (small vegetative sugarbeet plants) are produced by drilling seed in areas free of volunteer beet. The plants grow to a required vegetative stage and are then transplanted to the seed production multiplication areas. The fields drilled to produce the stecklings are never contaminated with seed scatter, as the plants never reach the reproductive phase. In the seed production area where the stecklings are transported, the fields used for seed multiplication are not re-used for beet seed production for 4-5 years. The earlier reuse of these fields, compared to the direct drilled multiplication fields, is due to the better control of volunteer beet (contamination of seed production fields from earlier beet seed production in same field). Any volunteer sugarbeet in these fields would be distinguished by their dissimilar developmental stage from the transplanted stecklings and easily removed by mechanical means. This is the standard procedure employed by breeders in order to maintain high purity seedlots.

Despite these precautions taken by the seed companies, crosses between *B. vulgaris* spp. *vulgaris* and related species (in Europe) and volunteers can take place and seedlots can be contaminated. The contamination of the sugarbeet hybrid may be observed as a bolting plant in a commercial sugarbeet crop. During the production of herbicide-tolerant sugarbeet seed, the herbicide tolerance trait could outcross to the related population and subsequently incross in the seed production fields in subsequent years. This possibility has been suggested by research on the origin of weed beet in the farmers' field (Boudry *et al*, 1993). In practice, 0.1% is the limit of contamination that is accepted by seed breeders.

The frequency of bolting is evaluated in all countries during variety testing for official listing and commercialization. Breeding companies do *a priori* testing for bolting occurrence as a quality control prior to using a seedlot for commercial purposes.

D. Current Weed Control Practices In The United States and Europe

Control of volunteer and related beet in areas surrounding sugarbeet seed production fields is well organized by contract farmers in order to ensure high purity seedlots. On a regular basis, a 0.5 mile zone around the production areas is scouted for volunteer and related beet and these are removed manually.

Farmers who multiply sugarbeet seed on a contract basis are selected by seed companies on the basis of their ability to produce pure seed. The standard industry practice is to mechanically eradicate any seedlings before establishing the next sugarbeet production field. In Europe, pre-harvest herbicide treatments are sometimes used in seed multiplication fields. Glyphosate has been tested intensively for this purpose, but is not the current herbicide of choice. Diquat is preferred for this usage because of its fast burndown characteristics.

In sugarbeet crops, related species are not controlled with sugarbeet herbicides because the chemicals cannot distinguish the beet from the weed beet. Introduction of GTSB77, if managed properly, will provide a new tool for farmers to control volunteer beet during the growing season. With careful monitoring and improved management practices, including avoidance of early drilling, removal of bolters during the growing season, and improved bolting-tolerant varieties, the appearance of tolerant weed beet will be minimized.

Weed control in sugarbeet is complicated because there is no single program that can be broadly applied. Chemical and mechanical methods are used in various combinations depending on the types of weeds present, their density and stage of development. Multiple applications of various herbicide combinations starting at the cotyledon to early two-leaf stage of sugarbeet are utilized. Current beet herbicide programs usually consist of a pre-emergence treatment followed by 2 to 4 post-emergence applications with a mixture of active ingredients.

E. Impact of GTSB77 and Glyphosate on Weed Management Practices

1. Use of glyphosate with GTSB77

Except for the introduced trait of glyphosate tolerance, GTSB77 is indistinguishable from traditional sugarbeet lines in all other agronomic traits. Other than the additional choice of glyphosate to control weeds, we anticipate that all other management practices employed by commercial sugarbeet producers will not be altered (e.g., GTSB77 will continue to be susceptible to the herbicides currently used in rotational crops; see Chapter V, Table 5.1).

Glyphosate use on glyphosate-tolerant crops varies according to the weed control needs of each particular crop, as well as the environmental conditions in which it is being grown. Sugarbeet is slow growing at the early developmental stages, requiring 8-10 weeks to reach canopy closure. During this time period, weeds have a large window to emerge and establish. The window of herbicide application for GTSB77 is similar to that of the currently used sugarbeet herbicides (from drilling to canopy closure). The use of glyphosate will be dictated by types of weeds, their frequency, and stage of development. The first treatment of glyphosate is envisioned to occur following weed germination. As this herbicide has no residual activity in soil, any newly germinated weeds following this initial treatment will need to be controlled with a subsequent glyphosate treatment. Two or three sequential treatments have proven to give full weed control in sugarbeet field trials. In many cases two sequential treatments, the first at an early crop stage (2-4 leaf stage of the beet crop) and the second shortly before canopy closure, using a rate of 1 quart per acre will provide full weed control. A grower may elect to apply three to four sequential treatments, within the same application window, because of weed control needs. The rates in a four-application program will be four times a rate of 1 quart per acre. The total amount of glyphosate applied in crop will not exceed 4 quarts per acre in a season. Lower rates can be used, depending on environmental conditions and weed populations, and there will be the flexibility in the timing of application to provide optimal weed control. There will be sufficient flexibility to allow adaptation to specific farmer needs.

Glyphosate-tolerant sugarbeet varieties will offer growers access to a new weed management tool in sugarbeet crops and afford beet producers a number of proven benefits in weed management, including:

- **Broad spectrum weed control**

Glyphosate controls both broad leaf weeds and grasses. Tough weeds (e.g., *Polygonum convulvulus, Kochia scaparia, Cirsium arvense)*) can be controlled with sequential treatments. Of particular importance will be effective control of volunteer potatoes, one of the most significant weed problems in some sugarbeet producing areas.

- **Flexibility in weed control**

Weeds will be controlled on an "as needed" basis. Applications will only be required if weed infestations reach a level that typically results in yield reduction. Sequential applications of glyphosate have been shown to effectively control weeds difficult to control using currently available herbicides.

- **Excellent crop safety**

When used according to the label recommendations, glyphosate will provide high selectivity on GTSB77-derived varieties while providing excellent weed control.

- **Flexibility in the timings of herbicide applications**

Unlike existing programs for weed control, glyphosate controls weeds at nearly all stages of development. Hence, timing of application becomes less critical.

- **Glyphosate is environmentally sound**

This herbicide has been used for more than 20 years in various conditions and applications, and is recognized for its lack of persistence, low risk of groundwater contamination and safety toward fauna. Compared to the current sugarbeet production herbicide-systems, the use of glyphosate will allow farmers to use an environmentally sound herbicide (Goldburg *et al.*, 1990; Malik *et al.*, 1989)

- **Rotational flexibility**

Since glyphosate has no residual activity in the soil, any crop may be planted following the use without risk of crop injury.

- **Cost-effective weed control**

The cost of weed control with glyphosate will be competitive with that of alternative weed control programs on sugarbeet.

2. Potential for Transfer of the Glyphosate-tolerance Trait to Related Species

Hybridization is the transfer of genetic information between plants. The likelihood of hybridization is a function of the sexual compatibility (the ability of two plants to form stable hybrids) and the barriers that exist to pollen transfer (e.g., distance, synchronicity, pollen competition, etc.). Gene flow from cultivated beet to wild relatives and vice versa has occurred for centuries. Viable pollen of *Beta vulgaris* spp. *vulgaris* and sexually compatible species is known to travel long distances depending on wind velocity, temperature and humidity. Although *Beta* pollen moves readily, there are very few relatives that will form viable hybrids. All evidence demonstrates that *B. vulgaris* spp. *vulgaris* only interbreeds freely with specific members of the Chenopodiaceae within the *Beta* section (de Bock, 1986). Hybridization between sugarbeet, *B. vulgaris* spp. *vulgaris*, and its wild relatives *B.vulgaris* spp. *maritima, B. vulgaris* spp. *atriplicifolia* (both present in Europe), and *B. vulgaris* spp. *macrocarpa (*present in Europe and U.S.) (Abe, *et al.*, 1987; Ford-Lloyd, 1986; de Bock, 1986 and Horsney & Arnold, 1979) was confirmed by experiments conducted in the BRIDGE study, a study funded by the European Community (BRIDGE, (1993) Appendix 6). These wild relatives were selected as they represent the western European wild *Beta* species of any significance (Ford-Lloyd, 1991).

B. vulgaris spp. *maritima* is widely distributed from the Asiatic steppes and East India to the Canary Islands and up to the North Sea coastline in Europe. *B. vulgaris* spp. *atriplicifolia* and *B. vulgaris* spp. *macrocarpa* are confined in distribution to the Mediterranean region (Hulten & Fries, 1986). Hybrids between *B. vulgaris* spp. *macrocarpa* and *B.vulgaris* spp. *vulgaris* have caused weed problems in sugarbeet fields in Europe (McFarlane, 1975), but as previously indicated, genetic barriers were detected between those two species, resulting in partial pollen sterility and embryo abortion in the hybrid (Abe, *et al.,* 1986; Appendix 7). Lower hybridization levels between *B.vulgaris* spp. *vulgaris* and *B. vulgaris* spp. *macrocarpa* were also found in the BRIDGE study. These wild relatives of beets are not prevalent in the U.S. and gene flow between these wild species and cultivated beets is not where sugarbeets are grown commercially.

In the BRIDGE study, glyphosate-tolerant beet pollen dispersal was also evaluated (Appendix 6). It was concluded that pollen dispersal declines rapidly with distance from the pollen source, with most pollen trapped within 30 meters from the source. It was still possible to occasionally trap pollen at 100 meters (maximum distance used in these trials). Although these experiments employed relatively small pollen sources, the literature also indicates pollen transport (wind/insects) at distances up to 1000 meters (Dark, 1971). The movement of pollen from GTSB77 was specifically studied in the BRIDGE project and neither differences between several transformation events, or a difference between a glyphosate- tolerant pollen source and non-transgenic *B.vulgaris* was recorded.

The observations from this study indicate that distances up to 1000 meters are needed to ensure that wild beet pollen contamination in beet seed production fields is kept to a minimum. This spatial separation will ensure minimal outcrossing from the sugarbeet seed production crop into wild species. These procedures include minimum isolation distances between production fields (from 300 to 1000 meters depending on the beet ploidy levels) as well as monitoring of the areas around the sugarbeet seed production fields for the presence of wild relatives or volunteers. Farmers that are contracted as seed multipliers are obliged to monitor the area around their production fields as a condition of their seed production contract.

3. GTSB77 and glyphosate use in rotation crops

The principal rotation crops in sugarbeet seed multiplication areas are wheat and vegetable crops. Winter wheat is the most common crop to be planted in a field used for beet seed production. Late germinating beet seedlings will not survive the winter or are controlled with traditional herbicide programs in autumn or spring. Volunteers from GTSB77 can be controlled using active ingredients, other than glyphosate, currently employed in the rotational crops.

Volunteer beet may be present in crops grown in rotation with commercial sugarbeet crops. These volunteers result from uncontrolled weed beet in the beet crop that were allowed to set seed, from groundkeepers, or from the tops of harvested beet. However, sugarbeets are cold sensitive and do not easily survive winter, and data generated in Appendices 2 - 3) indicate that GTSB77 are equally sensitive to cold temperatures as other beet. In the unlikely event that a glyphosate-tolerant sugarbeet successfully overwinters, this will not present a new management problem for growers, as glyphosate is not used to control volunteer beet in rotation crops.

In spring drilled rotational crops, volunteer beet will be controlled by normal weed control practices used for these crops, such as

- minimizing soil incorporation of the crop debris, to allow any beet seed to germinate before tillage
- eradication of volunteers by the application of herbicides other than glyphosate.

4. GTSB77 and pre-plant situations

Field preparation prior to the drilling of a spring- or an autumn-sown crop include destruction of existing vegetation and soil cultivation. Glyphosate treatment is commonly used for pre-plant clean-up, often in combination with cultivation prior to seedbed preparation. Existing beet volunteers at pre-plant are fully controlled by cultivation. No special chemical treatments are used to control beet volunteers in pre-plant situations. Autumn germinated volunteer beet will seldom survive the winter cold periods. Spring germinated volunteer beet will be controlled by tillage. In exceptional cases, a mixture of glyphosate with a complementary active ingredient, such as methylsulfuron methyl, can be considered to control volunteer beet tolerant to glyphosate.

5. Other situations

Beet can occasionally be found along roadsides due to losses during transportation of harvested beet. These beet are not a problem since plants of the *Beta* section are not considered invasive and the beet decompose over time or are mechanically destroyed by the mowing procedures used to maintain roadsides. Where herbicides are authorized, glyphosate can be mixed with low rates of other herbicides.

F. Potential for the Development of Weed Resistance to Glyphosate

Today there exist some 109 herbicide-resistant weed biotypes, with over half of them resistant to the triazine family of herbicides (Holt and Le Baron, 1990; Le Baron, 1991; Shaner, 1995). Resistance to herbicides has usually developed because of (1) the selection pressure exerted by the repeated use of herbicides with a single target site and a specific mode of action, (2) long residual activity with the capacity to control weeds year-long, and (3) frequent applications without rotation to other herbicides or cultural control practices. Using these criteria and based on current use data, glyphosate is considered to be a herbicide with a low risk for weed resistance (Benbrook, 1991). Nonetheless, it has been questioned whether the introduction of crops tolerant to a specific herbicide, such as glyphosate, may lead to the occurrence of weeds resistant to that particular herbicide. This concern is based on the assumptions that the use of the herbicide will be increased significantly, and possibly that it will be used repeatedly in the same location. However, other increases in glyphosate use over the previous years have been more significant than the projected increase associated with the introduction of Roundup Ready crops in the U.S. Although it cannot be stated that evolution of resistance to glyphosate will not occur, the development of weed resistance to glyphosate is considered unlikely because:

1. Weeds and crops are inherently not tolerant to glyphosate, and the long history of extensive use of glyphosate has not resulted in resistant weeds. Glyphosate has been used for over 20 years in various preplant, directed, spot and postharvest weed management systems with no verified cases of weed resistance (Holt *et al.*, 1993; Dyer, 1994). A preliminary report was recently presented that discussed annual ryegrass (*Lolium* sp.) seeds collected from a field that, upon germination, demonstrated a rate-related tolerance to glyphosate (Pratley *et al.* 1996). This observation merits further investigation. Insufficient data were reported to define the factors contributing to the observed phenomenon and Monsanto has entered into a collaborative research agreement with Charles Sturt University of Wagga Wagga in Australia

to further investigate these results. Since the source of the 'sensitive' biotype used by Pratley (Pratley *et al.*, 1996) was from a different location than the 'resistant' biotype, their genetic relatedness is unclear and additional research to address this question is being initiated.

2. Glyphosate has many unique properties, such as its mode of action (glyphosate is unrelated to triazines and has a differing mode of action from any other herbicide on the market today), chemical structure, limited metabolism in plants, lack of residual activity in the soil and its relatively quick break down by microorganisms in the soil (Malik *et al.*, 1989).

3. Selection for glyphosate resistance using whole plant and cell/tissue culture techniques, including mutagenesis, was largely unsuccessful, and unlikely to be duplicated under normal field conditions. Similarly, the complex genetic transformations required for the development of glyphosate tolerant crops (e.g. modified gene, unique promoters, transit peptide, etc...) would be unlikely to be duplicated in nature to yield glyphosate resistant weeds (Bradshaw *et al.*, 1997).

G. Management of GTSB77 and Phosphinothricin-tolerant Sugarbeet

The commercial availability of sugarbeet varieties resistant to other active ingredients, such as phosphinothricin, raises questions of the likelihood of sugarbeet volunteers possessing a combination of two different herbicide resistance genes, and how such volunteers would be managed by growers.

The most likely location for inadvertent creation of multi-herbicide tolerant sugarbeet is in the seed multiplication areas. However, isolation measures and other production management strategies that are employed to ensure the high purity seedlots will also serve to minimize contamination between sugarbeet seed multiplication fields involving different herbicide tolerance traits.

The consequences of the occurrence of any sugarbeet volunteers with resistance to glyphosate and phosphinothricin are expected to be no different to the consequences of sugarbeet volunteers tolerant to glyphosate alone. Currently, phosphinothricin is not employed to any significant degree for the control of sugarbeet volunteers, and none of the management practices described previously for the control of glyphosate-tolerant sugarbeet volunteers involve the use of phosphinothricin.

STATEMENT OF GROUNDS UNFAVORABLE

Novartis Seeds and Monsanto Company are unaware of any conditions that are unfavorable to this request for non-regulated status of Glyphosate Tolerant Sugarbeet Line 77

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Appendix I. Toxicity and allergenicity assessment of the CP4 EPSPS, GUS, and protein 34550.

I. TOXICITY ASSESSMENT OF THE CP4 EPSPS PROTEIN

Introduction

The CP4 EPSPS protein has a well characterized catalytic function in plants, bacteria and fungi (the shikimate pathway is not present in mammals). The only known function of CP4 EPSPS protein is to catalyze the conversion of one molecule of shikimate-3-phosphate and phosphoenolpyruvate into 5-enolpyruvylshikimate-3-phosphate, an intermediate in the shikimate pathway to aromatic amino acids. The CP4 EPSPS protein has been shown to be functionally and structurally similar to EPSPS proteins typically found in foods and feeds derived from plant and microbial sources (Padgette *et al.,* 1993a), including (1). the reaction it catalyzes, (2) the amino acid sequence similarity to other EPSPS enzymes, (3) the homology of the active site residues, and (4) 3-dimensional structure. Studies of the temperature dependence of CP4 EPSPS demonstrate that the enzymatic activity is eliminated after 15 minutes incubation at 65°C, indicating that the activity is likely to be lost during processing of beets. In addition, CP4 EPSPS rapidly loses activity at a pH typically encountered in the mammalian digestive tract.

Digestion of CP4 EPSPS Protein in Simulated Gastric and Intestinal Fluids

CP4 EPSPS was shown to be rapidly degraded by the components of the mammalian digestive system, greatly minimizing any potential for this protein to be absorbed by the intestinal mucosa. The data demonstrate a half-life for CP4 EPSPS of less than 15 seconds in the gastric system and less than 10 minutes in the intestinal system, based on Western blot analysis (solid food has been estimated to empty from the human stomach by about 50% in two hours, while liquid empties 50% in approximately 25 minutes).

In the unlikely event that CP4 EPSPS protein remained intact following exposure to gastric conditions, it would be rapidly degraded in the intestine. Greater than 50% of CP4 EPSPS protein was degraded in the simulated intestinal system in less than 10 minutes (Western blot analysis). This compares with transit times through the intestine (for radiolabelled chromate, which is not absorbed) of 4 to 10 hours for the first products to appear in the faeces and 68 to 165 hours for the remainder to be detected. Based upon the data from simulated gastric and simulated intestinal fluid model systems, the CP4 EPSPS protein is predicted to be readily degraded in the mammalian digestive tract.

Acute Mouse Gavage Study with CP4 EPSPS Protein

An acute mouse gavage study with CP4 EPSPS protein as the test material was performed to directly assess any potential toxicity associated with the protein. Results from this study indicate that the CP4 EPSPS protein is not toxic. Purified *E. coli*-produced CP4 EPSPS protein, demonstrated to be equivalent to the beet-produced CP4 EPSPS, was administered by gavage to mice in an acute toxicity test. There were no treatment-related adverse effects in mice administered CP4 EPSPS protein by oral gavage at dosages up to 572 mg/kg. There were no statistically significant differences in body weight, cumulative body weight, or food consumption between the vehicle or bovine serum albumin protein control groups, and CP4 EPSPS protein-treated groups. This dose (572 mg/kg) represents an approximate 1300-fold safety margin relative to the highest potential human consumption (based on U.S.

data) of CP4 EPSPS if the protein were expressed in soybean, corn, tomato, and potato (assuming no loss of CP4 EPSPS due to processing). Calculation of a safety factor based on consumption of protein from GTSB77 would increase the safety margin significantly due the very low level of protein consumed upon use of sugarbeets as food or feed.

II. TOXICITY ASSESSMENT OF THE GUS PROTEIN

Introduction

The GUS protein form *E. coli* is a visible marker frequently used to evaluate putative transformation events and gene promoters in plant biotechnology. The GUS protein is an acid hydrolase that catalyzes the cleavage of certain β-glucuronides. The biochemistry and catalysis of this protein has been thoroughly studied. Based upon published results, and the data presented below, it is concluded that the protein has a specific catalytic function without any association with human toxicity and/or allergenic potential.

The assessment of human safety of the GUS protein was designed like that of the assessment of CP4 EPSPS consisting of three types of experiments including protein characterization, digestive fate, and acute oral toxicity studies in mice. Relatively large amounts of protein were obtained from an *E. coli* expression system . This material was fully characterized and shown to be a suitable substitute for plant-produced GUS protein The following section summarizes the results of numerous experiments that demonstrate that GUS, like CP4 EPSPS protein, is not toxic and presents no significant risk to human and animal safety.

Digestion of GUS Protein in Simulated Gastric and Intestinal Fluids

The GUS protein, whether added to simulated gastric (SGF) or intestinal fluids, was readily degraded. Within 15 seconds of exposure to SGF, there is no detectable GUS protein by either Western blot or enzymatic activity. After 2 h in SIF, a very faint band was observed in the Western blot and the protein had lost approximately 91% of its original enzymatic activity. The rapid degradation of GUS in both SGF and SIF suggests that this protein will degrade readily in the mammalian digestive tract. The levels of GUS protein in GTSB77 are extremely low (0.003 μg/mg leaf tissue and 0.0006 μg/mg root tissue). Based on these results and the results in this study, it is concluded that GUS protein, if ingested by humans, will degrade in the digestive tract readily. Hence, the presence of low levels of GUS protein in GTSB77 constitutes a negligible risk to human health.

Acute Mouse Gavage Study with GUS Protein

An acute mouse gavage study using GUS protein as the test material was performed to directly assess any potential toxicity associated with the protein. The GUS protein used in this evaluation was over-produced and purified from *E. coli*, characterized, demonstrated to be equivalent to the beet-produced GUS and administered by gavage to mice.

There were no treatment-related adverse effects in mice administered GUS protein by oral gavage at dosage up to 69 mg/kg, (this dose represents a >1000-fold safety margin relative to the highest potential human consumption, based on U.S. data of GUS if the protein were expressed in soybean and beet, assuming no loss of GUS due to processing. Calculation of a safety factor based on consumption of protein from GTSB77 would increase the safety margin significantly due the very low level of protein consumed by humans. There were no statistically significant differences in body weight, cumulative body weight, or food consumption between the vehicle or bovine serum albumin protein control groups and GUS protein-treated groups. Several minor pathologic changes were observed at necropsy which were randomly distributed among all treatment groups and are commonly observed in the strain of mice used by the testing laboratory.

III. TOXICITY ASSESSMENT OF PROTEIN 34550

Digestion of Protein 34550 in Simulated Gastric and Intestinal Fluids

The effect of incubation in simulated gastric (SGF) or simulated intestinal fluid (SIF) on the integrity of protein 34550 was examined. Analysis of incubation treatments was performed by detection protein bands after SDS-PAGE using colloidal blue staining or by western blotting. Protein 34550 was degraded within 15 seconds in SGF and within 60 seconds of incubation in SIF. The data shows that no intermediate stable fragments larger than 2 kDa were generated by the digestion in SGF or SIF.

Acute Mouse Gavage Study with Protein 34550

Protein 34550, enriched from fermentation cultures of *E. coli*, was used as a test material in an acute oral gavage study in mice. The highest dosage level was approximately 80-fold higher than the exposure level that a lactating cow would receive from consumption of fresh sugarbeet tops containing the levels (4 ppm) of protein 34550 present in the leaves.

There were two control groups in the study. One group was administered a comparable amount of *E. coli* protein prepared in the same manner as the test material. The hollow vector control is an *E. coli* transformed with a plasmid that contains the same genetic elements as the test material except for the gene of interest. The second control group received the buffer vehicle at the same volume given to mice dosed with protein 34550 and the hollow vector control.

No mortality occurred during the 12-day study, nor were any adverse clinical signs observed. There were no effects on body weight or food consumption.

IV. ASSESSMENT OF THE ALLERGENIC POTENTIAL OF THE CP4 EPSPS AND GUS PROTEINS, AND PROTEIN 34550

Although large quantities of a vast variety of proteins are consumed in diets each day, rarely do any of these tens of thousands of proteins elicit an allergenic response. Although there are no predictive assays available to assess the allergenic potential of proteins, the

physicochemical profile of the CP4 EPSPS and GUS proteins provides a basis for assessing the allergenicity by comparing them to known protein allergens. A key parameter contributing to the allergenicity of food allergens appears to be stability to gastrointestinal tract, especially stability to acid proteases (e.g., pepsin) found in the stomach. Protein allergens must be stable to the peptic digestion and the acid conditions of the stomach system if they are to reach and pass through the intestinal mucosa where an immune reponse can be initiated. Another significant factor contributing to the allergenicity of proteins is their high concentrations in foods that elicit an allergenic response.

Most allergens are present as major protein components in the specific food, including allergens in milk, soybeans and peanuts. In contrast, CP4 EPSPS, the most abundant of the novel proteins expressed in GTSB77, is present at very low levels [approximately 0.05% of fresh weight of the root and approximately 0.3% fresh weight of the top. The CP4 EPSPS, GUS, and protein 34550 were shown to be extremely labile to digestion by the proteases present in the mammalian digestive system. This supports the prediction that the CP4 EPSPS, GUS, and protein 34550 will not survive the peptic and tryptic conditions of the mammalian digestive system.

The genes for CP4 EPSPS and GUS were obtained from sources not known to be allergenic. The CP4 EPSPS protein was initally obtained from the naturally occurring soil-borne and plant-symbiotic bacterium *Agrobacterium sp.* strain CP4. The *uidA* gene was originally obtained from *E. coli*, a bacterium normally found in the intestine of mammals. No incidence of allergy have has been reported for either bacterium. Thus, both CP4 EPSPS and GUS are unlikely to encode allergens since the organisms from which they were obtained are not allergenic.

CP4 EPSPS and GUS proteins show no significant sequence similarity to any known protein allergens. CP4 EPSPS shows a high degree of amino acid sequence similarity to EPSPS proteins produced in other crop plants. In contrast, CP4 EPSPS showed no significant homology to any of the 219 amino acid sequences reported for the allergens in the three current protein data bases (GenBank, PIR, and SWISSPROT databases). There was no greater homology of the native CP4 EPSPS to any of the 219 amino acid sequences for the allergenic proteins than for a scrambled sequence of the same amino acids that comprise the CP4 EPSPS.

Similar to the CP4 EPSPS and GUS proteins, the amino acid sequence of protein 34550 was compared with sequences from known allergens using public domain genetic databases (GenBank, EMBL, PIR, and SwissProt). Protein 34550 shares no significant sequence homology with known allergens.

V. HOMOLOGY OF CP4 EPSPS, GUS, AND TRUNCATED GOX SEQUENCES TO KNOWN PROTEIN TOXINS

The CP4 EPSPS, GUS, and protein 34550 and the deduced amino acid sequence resulting from the truncated insertion event do not demonstrate substantial amino

acid sequence homology when compared to 1,935 known protein toxins present in the PIR, EMBL, SwissProt and GenBank protein databases. The analysis of the homology of these amino acid sequences to known protein toxins was based on the fact that patterns of amino acid sequence or regions of strong homology shared between two or more proteins may provide insight to the biological significance of a protein. The deduced amino acid sequences of CP4 EPSPS, GUS, and protein 34550 and the deduced sequence derived from the Left Border region of the insertion event were compared to peptide sequences identified as "toxins" from all available protein databases, to identify if each had any meaningful sequence homology with known toxins. Results from this search indicate that, using the best methods available today, there are no biologically significant homologies between CP4 EPSPS, GUS, and protein 34550 sequences and the deduced sequence derived from the truncated insertion event and the protein sequences of all known toxins in the available protein databases.

Appendix II. Report on results from frost resistance trials with sugarbeets (*Beta vulgaris* L.) transformed with glyphosate resistance genes

REPORT ON RESULTS FROM FROSTRESISTANCE TRIALS WITH SUGARBEETS (Beta vulgaris L.) TRANSFORMED WITH GLYPHOSATERESISTANCE GENES.

Summary.

To investigate whether the introduction of transgenes into sugarbeets could induce a better frostresistance, progenies of different transgenic plants were transplanted to a restricted area at MARIBO SEED in Holeby and left in the field to overwinter in the winter 1992/93. On 1 May 1993 the fieldtrial was finished, and it was concluded, that all plants both controls and transgenic plants were dead.

During the same winter trials were performed indoor in freezers and climate chambers on sugarbeet progenies containing the same constructs as the in fieldtrials. These trials were terminated on 1 March 1993. It was concluded, that all plants both controls and transgenic plants from the treatment -5 degrees Celsius in four weeks were dead. All plants from the treatments zero degrees or +5 degrees Celsius in four weeks survived most in good condition.

Introduction.

When a transgene is introduced into a plant genome using Agro- bacterium tumefaciens as vector, the position of the transgene on the chromosomes is beyond our control. It has been reported (Anon. 1991), that the expression of the transgene can vary. In some instances the transgene was not expressed to a measurable degree although southern analysis proved, that it was present in the plant tissue. Position determined influence by transgenes on the expression of native genes has been observed (Anon. 1993, Steen et al. 1993).

So far it has not been seen, that the introduction of constructs into sugarbeet genome could influence the native genes in such a way, that it would cause the general expression of a new an unexpected trait in the plants. Nevertheless it is important constantly to screen new constructs, because the presence of such general features could prevent the use of certain constructs or delay the programmes.

The aim of the present investigation is to elucidate whether constructs including transgenes coding for glyphosateresistance also influences frostresistance.

Material and methods.

a) Field trials.

In the autumn 1992 the following positypes and a control were transplanted into a restricted area in the field belonging to MARIBO SEED, fenced with chickenwire and left to overwinter:

NUMBER OF PLANTS	POSITYPE/REGNO	CONSTRUCT	COMMENTS
75	A1012.BG001B-1972-01		Nontransgenic control
8	A1012.EQ007A-1172-01	pMON I	GUS negative
32	A1012.EQ007A-1172-01	pMON I	GUS positive
5	A1012.EQ009A-1171-01	pMON II	GUS negative
43	A1012.EQ009A-1171-01	pMON. I	GUS positive
6	A1012.EQ012A-1171-00	pMON I	GUS negative
16	A1012.EQ012A-1171-00	pMON I	GUS positive
6	A1012.EQ013A-1171-00	pMON I	GUS negative
9	A1012.EQ013A-1171-00	pMON I	GUS positive
25	A1012.EQ039A-1270-00	pMON VI	No marker genes
25	A1012.EQ043A-1270-01	pMON IV	GUS positive

The positypes tested are all progenies (F1 or F2) of transformed plants.

On 1 May the trial was terminated, and in accordance with our scoring system the plants were grouped into 3 groups: Good vigour, bad vigour, dead.

The winter 1992/93 was not very severe, but in January and February we did record black frost down to 8 degrees Celsius.
In March and April night frosts down to 4 degrees Celsius alternated with high daytemperatures and sunshine.

b) Indoor trials.

The indoor freezing trials included the following positypes:

NUMBER OF PLANTS	POSITYPE/REGNO	CONSTRUCT	COMMENTS
36	A1012.BG001B-1972-01		Nontransgenic control
36	A1012.EQ009A-1171-01	pMON II	
36	A1012.EQ043A-1271-01	pMON. IV	
24	A1012.EQ066A-1271-01	pMON VI	

The positypes tested are all progenies (F1) of transformed plants.

The plants were exposed to one of 3 temperatures (-5,0,+5) in 4 weeks. Thereafter all plants were treated alike, which means 2 weeks at 12 degrees and then at 20-24 degrees, until it could be concluded, if the plants were alive.

The temperature treatments were carried out the way mentioned below:

TREATMENT	PLACE	COMMENTS
-5 degrees	Freezer	With front door, no light during experiment.
0 degrees	Refrigerators	With top lids replaced by transparent plastic, daylight.
+5 degrees	Climaterooms	Artificial light.

The experiment was performed in the period 5 January to 1 March. The layout was a block design with 3 replications. For the -5 degree treatment the replications were 3 different shelves in the freezer. For the other treatments the replications were made in different places.

On 1 March the trial was terminated, and in accordance with our scoring system the plants were grouped into 3 groups: Good vigour, bad vigour, dead.

Results.

a) Field trials.

On 1 May it was stated, that all plants, transgenic as well as controls, were dead. There had been no regrowth of leaves at all, and the roots had started to rot. No physical damage from hares or other animals was shown.

b) Indoor trials.

On 1 March the following results from this trial were noted:

POSITYPE/REGNO	TREATMENT	PLANTS WITH GOOD VIGOUR	PLANTS WITH BAD VIGOUR	DEAD PLANTS
A1012.BG001B-1471-00				
	-5 degrees	0	0	12
	0 degrees	11	1	0
	+5 degrees	12	0	0
A1012.EQ009A-1171-00				
	-5 degrees	0	0	12
	0 degrees	10	2	0
	+5 degrees	12	0	0
A1012.EQ043A-1271-01				
	-5 degrees	0	0	12
	0 degrees	11	1	0
	+5 degrees	11	1	0
A1012.EQ066A-1271-01				
	-5 degrees	0	0	12
	0 degrees	11	1	0
	+5 degrees	11	1	0

Conclusions.

Based on the two sets of data from these experiments it can be concluded, that regarding frost resistance measured under the conditions in question, there is no difference between the nontransgenic control and the transgenic positypes tested with respect to this trait. Furthermore it can be concluded, that the use of the constructs included in these experiments, does not in general influence on the frostresistance of transformed plants neither in positive nor in negative sense.

Literature.

Anon. 1991, Reports on 1990 trials with transgenic glyphosate tolerant sugar beet.

Anon. 1993, Reports on 1992 trials with transgenic glyphosate tolerant sugar beet.

Steen et al. 1993, Personal communication.

REPORT ON RESULTS FROM INDOOR FREEZING TRIALS 1993/94 WITH SUGAR BEET (Beta vulgaris L.) TRANSFORMED WITH GLYPHOSATE-TOLERANCE GENES.

Summary.

This trial is a continuation of trials made in 1992/93 at MARIBO Seed in Holeby. The aim is to evaluate if beets transformed with glyphosate-tolerance genes have achieved better frost resistance than their origin. Progenies of different transgenic plants were potted and placed indoor in freezers and climate chambers. The trial was terminated on 5 January 1994. We concluded, that all plants of both controls and transgenic plants from the treatment -5 degrees Celsius in four weeks were dead. 50% of the plants from the treatments zero degrees survived, and from +5 degrees Celsius all plants survived.

Introduction.

The expression of genes inserted into sugar beet by means of Agrobacterium tumefaciens seems to be position dependent (Anon. 1991, Anon. 1993a). If transgenes could also influence the native genes in such a way, that it would cause a general expression of new and unexpected traits in the plants, is yet to be seen. Nevertheless it is important constantly to screen new constructs, because the presence of such general features could prevent the use of certain constructs or genes.

The aim of this experiment is to confirm results from earlier experiments (Anon. 1993b), and to test other positypes for frost resistance.

Material and methods.

The indoor freezing trial is made on F1 hybrids including the following positypes:

NUMBER OF PLANTS	POSITYPE/REGNO	CONSTRUCT	COMMENTS
30	K1047.ZZ003B-1091-00		Nontransgenic control
30	A1012.EQ055A-1170-01	pHON. III	
30	A1012.EQ064A-1270-01	pHON. IV	
30	A1012.EQ066A-1270-01	pHON. VI	Repeat from 1992/93
30	A1012.EQ077A-1370-00	pHON V	

All the positypes have been sprayed with Roundup prior to this experiment. Therefore all the plants except the control harbour the transgenes. The plants from each positype were divided in 3 groups. The groups were exposed to one of 3 temperatures -5, 0 or +5 degrees Celsius, in 4 weeks. Thereafter all plants were placed in a greenhouse 1 week at 20-24 degrees.

The equipment used in the experiment was:

TREATMENT	EQUIPMENT	COMMENTS
-5 degrees	Freezer	With top lid replaced by transparent plastic, Daylight.
0 degrees	Freezer	With top lids replaced by transparent plastic, Daylight.
+5 degrees	Climate chamber	Artificial light.

The experiment was performed from 6 December 1993 to 5 January 1994. On 5 January the plants were examined and scored for vigour after the following scale - good vigour, bad vigour, dead.

Results.

On January 5 the following results from this trial were noted:

POSITYPE/REGNO	TREATMENT	PLANTS WITH GOOD VIGOUR	PLANTS WITH BAD VIGOUR	DEAD PLANTS
K1047.ZZ003B-1091-00				
	-5 degrees	0	0	10
	0 degrees	0	3	7
	+5 degrees	10	0	0
A1012.EQ055A-1170-01				
	-5 degrees	0	0	10
	0 degrees	0	9	1
	+5 degrees	9	1	0
A1012.EQ064A-1270-01				
	-5 degrees	0	0	10
	0 degrees	0	1	9
	+5 degrees	8	2	0
A1012.EQ066A-1270-01				
	-5 degrees	0	0	10
	0 degrees	0	4	6
	+5 degrees	9	1	0
A1012.EQ077A-1370-00				
	-5 degrees	0	0	10
	0 degrees	2	8	0
	+5 degrees	10	0	0

All plants died at -5 degrees, and all plants survived at +5 degrees although not all in good shape. At zero degrees the picture is more variable. We had problems keeping the temperature constant in the zero degrees' freezer, and the randomization of pots in the freezer seemed to be somewhat biased with more A1012.EQ077 plants near the top, where the temperature was higher, and more A1012.EQ064 and K1047.ZZ003 plants near the bottom, where the temperature was lower. This variation in temperature can explain the variation in surviving plants, and it shows, that the critical temperature for frost damages in sugar beet is just below the freezing point.

Conclusions.

The four positypes transformed with 4 different constructs showed the same reaction as the non-transgenic control to a range of temperatures. The positype A1012.EQ066A was tested under the same conditions in 1992/93 (Anon. 1993b), and this experiment confirms the previous results. We therefore can conclude, that based on 2 years experiments, we have found no difference between the non-transgenic control and the transgenic positypes tested with respect to frost resistance.

Literature.

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Anon. 1993a, Reports on 1992 trials with transgenic glyphosate tolerant sugar beet. Reports to The National Agency for Environmental Protection.

Anon. 1993b, Report on results from frostresistance trials with sugarbeets (Beta vulgaris L.) transformed with glyphosateresistance genes. Report to The National Agency for Environmental Protection.

REPORT FROM 1994/95 FREEZING AND FROSTRESISTANCE TRIALS WITH ROUNDUP READY™ SUGAR BEET (*BETA VULGARIS* L.).

DANISCO SEED - DENMARK

Plants were grown under controlled temperature conditions and in field experiments to access whether the introduction of the Roundup Ready (RR) gene in sugar beet does influence the ability to survive cold temperatures.

Indoor freezing trials, Denmark 1994/95.

Progenies of different transgenic lines (derived from T9100152) were potted and placed indoor in freezer and climate chambers. The plants were divided into three groups with an equal number of plans of each line. The three groups were placed in a freezer (- 5°C) and in climate chambers (0°C and 5°C) respectively. The plants were randomised within each group and stored 4 weeks.

After cold storage the plants were placed in greenhouse, 16 hours with 22°C and 8 hours with 16°C. After one week the plants were scored either dead or alive.

Table 1: *Number of plants alive and dead after 4 weeks cold storage in freezer and cold chamber*

Line	- 5 degrees		0 degrees		+ 5 degrees	
	dead	live	dead	live	dead	live
Marathon	8	0	2	9	0	15
# 77 triploid	8	0	9	2	0	15
# 77 diploid	8	0	3	8	0	15

The results of storage of line # 77 (T9100152), both di- and triploid, showed no significant differences between the # 77 and a correspondent commercial hybrid (Marathon). The results were that 0 %, 45 % and 100 % of the modified line survived - 5°C, 0° C and 5° C respectively compared to the survival of the commercial line, which survived 0 % , 82 % and 100 %.

Frost resitance field trial, Denmark 1994/95.

Selected plots form field experiments conducted in Denmark 1994 were not destroyed before winter. The plots were spread out on a relatively big area. The maximum distance between the plots was 75 meters. Glyphsate tolerant beet had been sprayed during spring with Roundup.

The number of plants in each plot was counted before winter, December 1994. The following spring and after the beets had began to grow, the number of living and dead plants was counted on April 17 1995. At this relatively late time it was estimated that the number of dead plants would not increase. The temperature conditions in the winter 94/95 were relatively mild. During the testing time there was no period with long-lasting constant hard frost. The lowest temperature was - 10° C for one single night. The meteorological registration shows several consecutive nights with temperatures below freezing point. In table 2 the results of over-wintering is showed. Percentage of plants alive in April was very similar between the modified lines and a commercial variety.

Table 2: *The number of plants in December 1994 and number of dead plants in April 1996.*

Line		Number of plants Dec. 94	Number of dead plants 17.04.95	% plants alive 17.04. 96
Marathon	Commercial variety	49	7	86
		54	6	89
Marathon	Total	103	13	87
# 77	Roundup Ready	100	17	83

Conclusion.

The e::periments under controlled conditions (freezer and climate chamber) with line # 77 (derived from T9100152) indicate, that the glyphosate tolerant beet do not survive cold temperatures better than normal beet. An e::periment in the field run during winter 1994/95 in Denmark showed that 83 % plants of a modified line derived from T9100152 survived and 87 % plants of a commercial line survived which again indicated no differences between a modified and normal line.

Appendix III. Report on winter survival of beet groundkeepers. A two-season comparison of glyphosate tolerant and non-transgenic groundkeepers.

Monsanto

Winter survival of beet groundkeepers. A two-season comparison of glyphosate tolerant and non-transgenic groundkeepers.

Franc-Waret, Belgium 1994/95 - 1995/96.

Ivo Brants

Method

Groundkeepers, small roots of beet from 2-4 cm in diameter, were retained from the trials harvested in 1994 and 1995. Transgenic roots, derived from transformants T9100152, 203121 and A5/15, were compared to appropriate non-transgenic material for their ability to survive under winter conditions. The roots were transplanted in the field in small plots (8 roots/plot). We also included Roundup® treated transgenic roots for comparison with untreated transgenic roots. The trial design was identical for the two seasons of experiments.

Results

The mild winter in 1994/95 resulted in some survival of beet root. In spring (March 15, 1995) counts of surviving plants were made, roots producing any new green leaf were considered to be alive. There was no difference in survival rates between non-transgenic and glyphosate-tolerant groundkeepers. No difference was recored between glyphosate treated and untreated tolerant beet.

The winter of 1995/96 was more severe and all beet, irrespective of their origin were killed before the assessment carried out on March 20, 1996.

Conclusion

Field data, collected during two seasons in Belgium 1994-1996, demonstrate that the cold sensitivity of Roundup Ready™ beet has not been altered as compared to the non-transgenic control beet lines. Treatments with Roundup® on Roundup Ready™ beet did not alter the cold sensitivity. These data confirm earlier work reported in the EEC-funded BRIDGE studies (1990-1993).

Table

Survival scores of groundkeepers in spring 1995 and spring 1996

Number of groundkeepers alive

1995 : March 15th

Glyphosate treatment	T9100152	NT1	203121	NT2	A5/15	NT3
Untreated	2	3	1	2	3	3
Treated (3 x 2 l/ha)	1	—	2	—	2	—
Treated (3 x 4 l/ha)	2	—	0	—	3	—

1996 : March 20th

Glyphosate treatment	T9100152	NT1	203121	NT2	A5/15	NT3
Untreated	0	0	n.a.	n.a.	0	0
Treated (3 x 3 l/ha)	0	—	n.a.	—	0	—
Treated (2 x 6 l/ha)	0	—	n.a.	—	0	—

— = no plants available as non-transgenic beet are killed with Roundup treatments
Glyphosate treatments with Bioforce (360 gram a.e./l)
n.a. = plant material not included in the experiment

T9100152 = Roundup Ready beet line derived from transformation T9100152
NT1 = comparable non-transgenic line for T9100152
203121 = Roundup Ready beet line derived from transformation 203121
NT2 = comparable non-transgenic line for 203121
A5/15 = Roundup Ready beet line derived from transformation A5/15
NT3 = comparable non-transgenic line for A5/15

Appendix IV. Report on the competitive ability of transgenic sugarbeet.

Competitive ability of transgenic sugar beet

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Introduction

The introduction of transgenic plants to field conditions limits the possibilities of confinement. Therefore, a simple protocol for testing transgenic plants at the greenhouse level is necessary to assess the potential hazard of plants before release. So far, there are no examples on potentially dangerous plants, but public concern about the consequences of releasing transgenic plants necessitates that no future release includes undesired effects.

An ecological concern in the introduction of transgenic plants is the possible creation of a new weed (OECD. 1986). New abilities to invade natural and cultivated ecosystems or a general increase in the fitness of the transgenic plant or its hybrids with wild relatives may result in an increased weediness of the plants. The success of a transgenic plant depends on the ability to establish and grow under different growth conditions, and the ability to utilize resources in competition with other species. In the life cycle of an annual plant (Fig. 1) the important phases are germination, establishment, vegetative growth, flowering, reproductive growth, seed dispersal and seed survival in the soil (seed bank) (Fredshavn & Poulsen, 1994a).

Results

In 1992 we made experiments with a hybrid between transgenic sugar beet, (Beta vulgaris L. ssp. vulgaris), and sea beet, (Beta vulgaris L. ssp. maritima), provided by MARIBO Seed, Denmark. The introduced genes provided tolerance to the herbicide glyphosate (Roundup Ready™ genes from Monsanto). NPTII (kanamycin resistance) and GUS (betaglucuronidase) genes were included as markers. The transgenic diploid hybrid, of which half the population actually carries the introduced genes, was compared to a nontransformed diploid sugarbeet and a wild seabeet. The three Beta ssp. were planted in both monocultures and binary mixtures in different density combinations under greenhouse and field conditions. The monoculture yields of aboveground biomass were relatively similar for all three subspecies, with an insignificant superiority of the hybrid in the greenhouse and of the seabeet in the field. However, a high monoculture yield is not linked to good competitiveness, as shown in Fig. 2. The predicted monoculture yields of the three subspecies at a density of 64 plants m^{-2} in the field and 300 plants m^{-2} in the greenhouse are shown in full lines, whereas the corresponding yields of the same plants when grown in mixture with an identical density of sugarbeet plants are shown in dotted lines. The reduction in yield from the monoculture yield to the yield in mixed population is a measure of competitiveness. Sugar beet and the hybrid were least influenced by competition, whereas the wild seabeet suffered most. The growth conditions in the field were very different from the ideal conditions of the greenhouse, and the colder temperatures and eight weeks of no rainfall in the field resulted in yields less then 25 percent of the greenhouse yields. Probably the drier conditions in the field were responsible for the better relative yield of seabeet in the field, as seabeet is more drought tolerant than sugarbeet.

In a later greenhouse experiment, it was shown that the ranking in competitiveness of the

three *Beta* components: sugarbeet, hybrid and seabeet, corresponded to the ranking of the three components with regard to shade tolerance. Barley and oilseed rape were also included in this experiment,as well as a genetically modified parental sugar beet. Barley and oilseed rape was significantly less affected by shade than the *Beta* species, and the transgenic sugarbeet was more affected than the other sugar beet subspecies (Fig. 3). This result is consistent with the relative competitiveness of the same species, with barley and oilseed rape being the strongest competitors and sea beet and the transgenic sugar beet being the poorest competitors.The results indicate that under the greenhouse conditions, competitiveness is related to the tolerance of shading, and that above ground competition for light was probably the most important factor in this experiment. There was no significant relation between competitive behavior and tolerance to low temperatures in this experiment.

Another greenhouse experiment on the relation between shade tolerance and competition with barley was performed using eight transgenic lines of sugar beet and one nontransformed male sterile sugar beet line. The plant material was provided by MARIBO Seed, Denmark, and represented different constructs and transformation events of glyphosate tolerance genes (Roundup Ready™ genes from Monsanto). The sugar beets were grown in three replicates in monocultures and in combination with barley. The plants were harvested after 56 days. Light intensity and barley competition significantly influenced the aboveground yield, but no difference in mean biomass production per plant within the transgenic lines or between the transgenic lines and the nontransformed sugar beet was detected.

All *Beta* ssp. are poor competitors compared to other plant species, which only allows *Beta* ssp. to grow in habitats without competition, e.g. seashores and well kept agricultural fields. Sea beet is adapted to the unfavorable growth conditions on the seashore because of its salt and drought tolerance. Sugar beet plants are never found in natural habitats in Denmark. In order to change this pattern of distribution and invasiveness, the increase in competitiveness of transgenic sugar beet compared to nontransformed sugar beets would have to be much more dramatic than found in this experiment.

Discussion

Our results show that the transgenic lines of both sugar beet behaved similar to nontransgenic varieties under the range of growth conditions we used. Similar results have been put forward by Arnoldo et al. (1992) and Crawley et al. (1993) in their field experiments with transgenic oilseed rape (kanamycin resistance and glufosinate tolerance). Furthermore De Greef et al. (1989) found the same results in field experiments with transgenic tobacco and potato (both glufosinate resistant).

A pre-release evaluation of the competitiveness of transgenic plants could involve greenhouse tests against a range of commercially grown varieties. Pure stands will provide information on the growth behavior, the yield potential and the yield density relationship. Mixtures with a standard competing species will provide information on the relative competitiveness. If the transgenic line fall within the same range as the existing commercial varieties there is no reason to believe that the competitive behavior of the transgenic plants has been changed.

Our experiments on transgenic plants are carried out under controlled conditions in greenhouses and arable fields. The experiments are simple and reproducible, and it is possible to compare the results found for different plant species. Other projects used natural habitats, with the possibility to give a more precise description on the actual behavior in the chosen habitats (Crawley *et al.*, 1993). But the unforeseen growth conditions and the difficulties to

influence these, makes it difficult to relate the
results to other species and habitats. The magnitude of the problems concerning the release of transgenic plants depends on the new gene, the nature of the plant and the habitat where it is introduced (OECD, 1986). The direct influence of the genes we have used in our experiments on future changes in the distribution of crops and their hybrids in natural habitats should not cause serious concerns, as herbicide resistance is not advantageous in wild habitats. Changes in tolerance to abiotic factors e.g. shading, low temperatures, draught, low pH, salinity etc. may be of greater concern, as they may be expected to influence the distribution of the plant also in natural habitats. As shown in our experiments, the growth conditions (the habitats) plays an important role. Plants are different in invasiveness, but habitats are just as different in their invasibility (Crawley, 1987).

Concluding remarks

It is possible to test a transgenic plant in critical phases of the life cycle and compare it with a range of existing nontransformed varieties, and thus detect any principal changes in growth behaviour. Each plant species has its own growth characteristics which enables it to grow under the natural and cultivated conditions where it is found. This information is important when deciding what characteristics of the transgenic plants are to be compared to the nontransformed varieties.

Changes in growth conditions affect the potential production of the species, and the competitive relationship between the species. The growth conditions, transgenic plants are tested under should therefore be well characterised, and preferably a set of standard growth conditions should be used.

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Fig. 1. Important phases in the life cycle of an annual plant. At flowering, the dispersal of pollen may result in a hybridization with wild relatives.

Fig. 2. The predicted monoculture yield per m^2 of a transgenic hybrid between sugarbeet and seabeet compared to the yield of the nontransformed parental subspecies under greenhouse (a) and field (b) conditions. Plant densities were 300 plants m^{-2} (a) and 64 plants m^{-2} (b). Full lines are the monoculture yields, and the broken lines show the reduced yield in mixtures with identical densities of sugarbeet plants.

Fig. 3. The correllation between biomass yield under full light conditions (100%) and 50% reduced light. A multiple regression analysis showed that the ranking in shade tolerance was: barley, oilseed rape, sugar beet, transgenic hybrid, transgenic sugar beet and sea beet, with barley as the most tolerant. This result was consistent with the found ranking of competitiveness in greenhouse experiments.

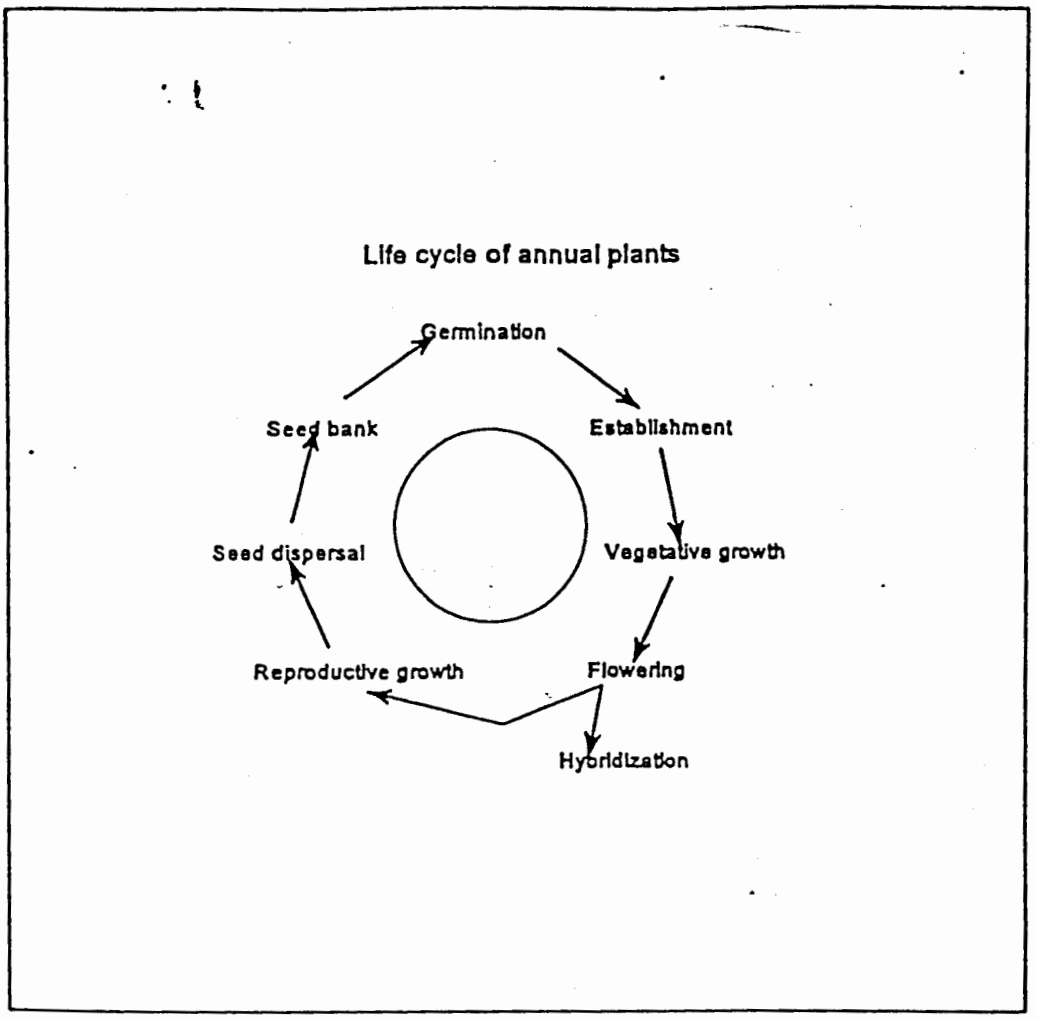
Life cycle of annual plants
Germination
Establishment
Vegetative growth
Flowering
Hybridization
Reproductive growth
Seed dispersal
Seed bank

Fig. 1.

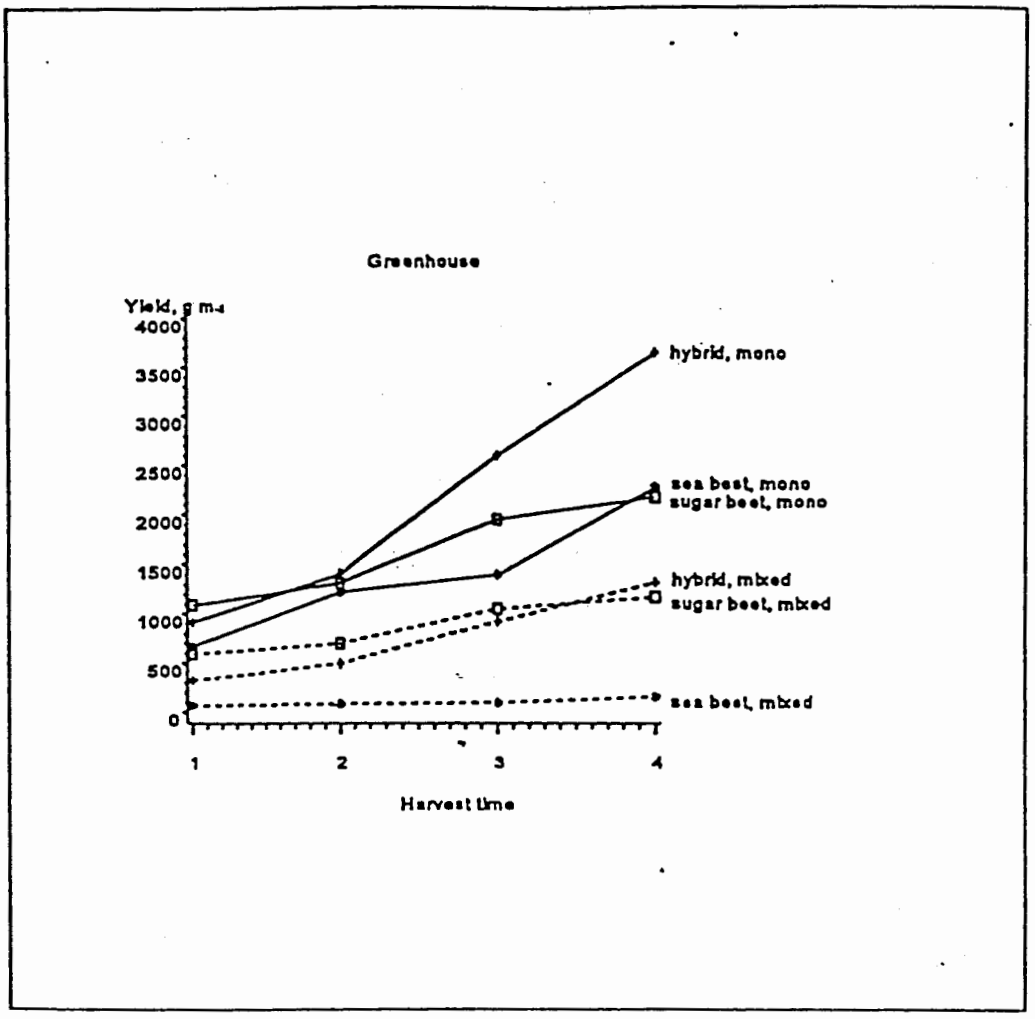
Greenhouse
Yield, g m-2
4000
3500
3000
2500
2000
1500
1000
500
0
1
2
3
4
Harvest time
hybrid, mono
sea beet, mono
sugar beet, mono
hybrid, mixed
sugar beet, mixed
sea beet, mixed

Fig. 2a.

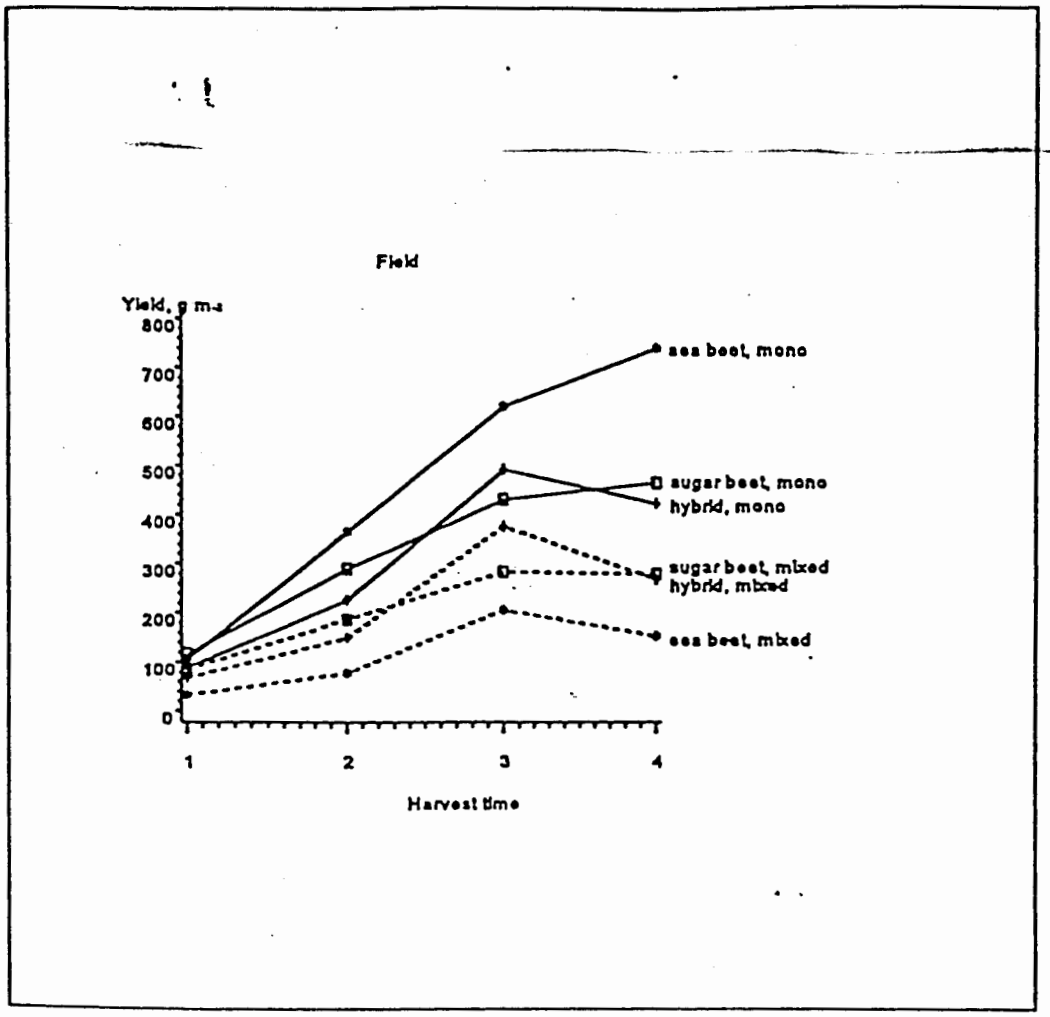
Field
Yield, g m-2
800
700
600
500
400
300
200
100
0
1
2
3
4
Harvest time
sea beet, mono
sugar beet, mono
hybrid, mono
sugar beet, mixed
hybrid, mixed
sea beet, mixed

Fig. 2b.

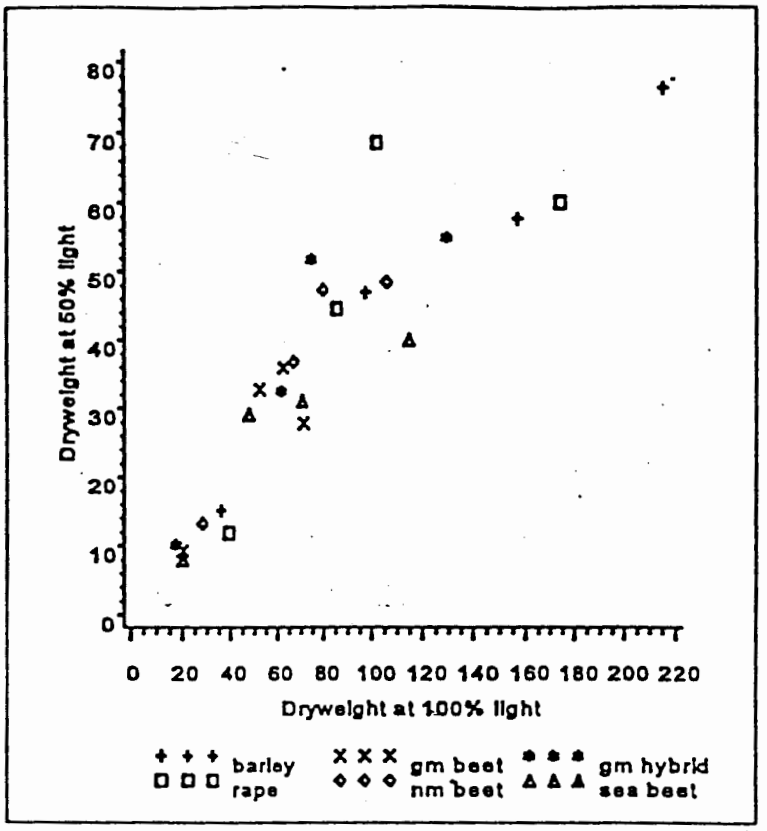
Dryweight at 60% light
0
10
20
30
40
50
60
70
80
0 20 40 60 80 100 120 140 160 180 200 220
Dryweight at 100% light
barley
rape
gm beet
nm beet
gm hybrid
sea beet

Fig.3.

Appendix V. Competition studies of hybrids between seabeet (*Beta maritima* L.) and transgenic sugarbeet (Beta vulgaris L.)

Competition studies of hybrids between seabeet (*Beta maritima* L.) and transgenic sugarbeet (*Beta vulgaris* L.)

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Running title: Competition studies of hybrids between seabeet (*Beta maritima* L.) and transgenic sugarbeet (*Beta vulgaris* L).

Keywords: *Beta vulgaris*, *Beta maritima*, genetically modified, glyphosate resistance, remote sensing, bootstrap

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Abstract

A field experiment using a factorial design with a geometric series of densities and four harvest times was evaluated as a method to describe the competitive ability and growth behavior of beets with genetically engineered genes. A mixture of 50% transgenic and 50% non-transgenic hybrids between seabeet and a transgenic glyphosate tolerant sugarbeet was compared with pure lines of sugarbeet and seabeet to determine if the hybrid had a higher biomass production or a substantially increased competitive ability than the non-transgenic parental types. An inverse linear competition model measuring interspecific competition could adequately describe above ground biomass from harvest 2, 3 and 4. The confidence limits of the parameters was determined by the bootstrap method. The mixture of transgenic and non-transgenic hybrids did not produce more biomass than did sugarbeet. The hybrids were more competitive than the parental types, but did not exceed the expected level caused by hybrid vigor. Remote sensing was used to detect differences in the relative vegetation index of the *Beta* ssp.

Introduction

Glyphosate tolerant sugarbeets have been field tested in Denmark, France, England and Belgium since 1990. During this period different transformation events (positypes) with different gene constructs, coding for glyphosate tolerance, have been evaluated (Steen & Pedersen, 1993). Glyphosate tolerant sugarbeet is one of the pioneers of herbicide tolerant crops produced by genetically engineering, and consequently it has received much agronomic and ecological attention. One of the ecological concerns is that if the gene is introduced into natural populations of the natural relative, then it could increase fitness of these hybrids, which in turn could cause a decrease in the variability of germplasm in the natural populations (Keeler & Turner, 1991). Another concern is that engineered genes could escape from the crop plant into a wild relative and perhaps create a future weed problem (Ellstrand, 1988). The likelihood of one gene creating weediness is however small because weediness is a trait from many genes (Keeler, 1989).

Beta spp. are generally considered to be rather self-incompatible wind pollinators, and gene escape from cultivated *Beta vulgaris* to wild relatives can occur if a wild flowering relative is present close to a flowering and fertile transgenic sugarbeet (Madsen, 1994). However, there are yet no documented cases of genes responsible for natural tolerance to herbicides being transferred from crop plants to weedy relatives (Duke et *al.*, 1991; Dyer et *al.*, 1993). A herbicide tolerance gene is unlikely to offer any advantage in fitness outside arable land, because there is no selection pressure in favor of herbicide tolerant plants in a natural population (Clausen, 1989; Crawley, 1993). In this context, fitness is described as reproductive success, or the proportion of genes an individual leaves in the gene pool of a population. Fitness is therefore the "currency" of natural selection, which is characterized by several components such as germination, establishment, survival during vegetative stage, seed

production etc. One of the major components that determines the survival during the vegetative stage is growth rate and competition from neighboring plants (Holt, 1990).

Bartsch et al. (1994) tested the competitiveness of three Beta genotypes against different densities of *Chenopodium album* and found that the transgenic virus and glufosinate resistant genotype had a lower biomass production and a lower relative competitiveness than did the unaltered original breeding line. A hybrid cultivar, showed the highest biomass production of the genotypes in each competitive density, which was explained by the hybridization effect.

In this study we primarily test the growth and competitive ability of hybrids between seabeet and a transgenic glyphosate tolerant sugarbeet (heterozygous for the transgene) against the non-transgenic parental types sugarbeet and seabeet respectively to determine if a field experiment using a factorial design with a geometric series of densities can be used as a method to describe the competitive ability and growth behavior of hybrids between seabeets and future commercial transgenic sugarbeet varieties. Secondary we investigate if these hybrids are substantially superior to sugarbeets or seabeets in growth and competitive ability, without selection pressure from glyphosate.

Materials and methods

Plant material

Stecklings of a glyphosate tolerant self-incompatible sugarbeet (the gene was inserted into a diploid pollinator-line used in commercial seed production) and stecklings of seabeets, (from seed collected on natural habitats along the Great Belt in Denmark) were vernalized for 16 weeks, and then crossed in small pollen proof greenhouses. Each greenhouse contained one transgenic sugarbeet and two seabeets, and the hybrid seeds were harvested from the

transgenic self-incompatible sugarbeet. Approximately 50% of the hybrids were glyphosat tolerant, because the maternal transgenic sugarbeet was heterozygous for the transgene. Th non-transgenic sugarbeet material was a diploid outcrossed line with similar behavior an vigor as a commercial variety, and the seabeet material came from the same source as th paternal parents of the hybrids. The hybrid, sugarbeet and seabeet seed were sown i paperpots and transplanted into the field at the cotyledon stage.

Experimental design

The experiment was placed on a heavy clay soil (26% clay and 1.6% humic acids). The thre *Beta* populations were transplanted in a factorial design with a geometric series of densitie (Cousens, 1991) into the field on 21–26 May. The experiment was irrigated until 6 June. n rain fell in June, while 24 mm of rain fell between 1–14 July. The average daily ai temperatures were 17°C in June and 18°C in July. Pesticides were applied when needed, an a mixture of phenmedipham, ethofumesate and metamitron was used to control weeds. Th three *Beta*-types were grown in monoculture at three densities of 16, 32 and 64 individual per m^2 and in binary mixed populations with the following densities, 16:16, 16:64, 32:32 64:16 and 64:64. The plot size of the harvest plots varied with plant density to ensure sufficient number of harvested plants per density combination (harvested individuals > 20 pe plot and type). There were four harvest times in three replications during the experiment o 29 June, 27 July, 24 August and 28 September. Number of plants per population was counte and above ground biomass (fresh weight) was measured. Dry weight was recorded after 2 hours at 80°C.

Remote sensing

The relative vegetation index *RVI* is based on spectral analysis, and correlates to leaf area of grain crops (Petersen, 1987). *RVI* was measured on a 0.25–0.5 m² area per plot 10 times during the growth season with a portable remote sensing equipment (Skye SKR 1800 sensors) measuring reflectance from the vegetation at 650 nm (R=red wave length) and at 810 nm (IR=infra red wave length). *RVI* is calculated as IR relative to R (*RVI=IR/R*). Reflectance from bare soil is dependent primarily on moisture and humic acids (Petersen, 1987) and to avoid differences of reflectance due to soil characteristics a net reflectance was used. $RVI_{net}=RVI_{veg}-RVI_{soil}$.

Data analysis and statistical models

The yield per plant, w_1 and density relationship of mixed populations of two biotypes at densities N_1 and N_2, can be described by an inverse linear model where:

$$w_1 = \frac{A}{1 - \frac{A}{B}(N_1 + cN_2) - N_m)} \qquad (1)$$

Where A denotes the maximum biomass per plant at density N_m. B is the maximum biomass per m² at infinite density in monoculture. c is the substitution rate and denotes the number of individuals of subspecies 1 necessary to substitute one individual of subspecies 2 and N_m is an arbitrarily chosen density of 16 plants per m² in order to avoid extrapolation from the observed data (Spitters, 1983; Cousens, 1991; Fredshavn, 1993).

The variance was stabilized with the Transform-Both-Sides technique used with a Box and Cox procedure with a power transformation (Box and Cox, 1964; Carrol & Ruppert, 1988). A Lack-Of-Fit test (Weisberg, 1982) was used to determine if the model could adequately describe data.

The parameters in equation (1) are per definition strictly positive, however, ne values are often included in the confidence limits. Bootstrap is a method to overcor problem as this method does not require any normality assumption of the parameter Bootstrap method uses the empirical distribution of the residuals to generate new data which the model is fitted a large number of times which reflects the true distributic provides confidence limits with a more correct biological interpretation (Jensen, 1991

The effective density of the mixture can be defined as (Jensen, 1993):

$$N_{eff} = N_1 + cN_2$$

N_{eff} is useful in describing the decline in biomass for the subspecies as the effective c progresses.

Differences in relative vegetation index of the *Beta* types during the growth sea estimated by an analysis of variance, with RVI_{art} as the response variable, and Beta ty density as class variables.

Results and discussion

The experiment showed that the chosen design (factorial with a geometric series of den in the field was functional to determine the competitive ability and growth behav different *Beta* types. However, competition between the mixtures planted at higher de is required to determine substitution rates and the dry conditions in the beginning season caused a severely reduced growth in all three *Beta* types which made it impossi determine the substitution rates at harvest 1 with adequate precision. The dry con stopped growth before the individual plants were able to utilize the same soil moistu thereby compete for this resource.

The Lack-of-Fit test (Weisberg, 1982) did not reject the hypothesis that the model was applicable to the data for harvest 2, 3 and 4. For these harvest times the model described the data, which is best shown by plotting the yield per plant against the effective density N_e (fig. 1). The individual parameters at harvest 2 and 3 (Table 1) show no large consistent differences in the A and B parameters between the different combinations, which makes the decline in biomass per plant as N_e increases similar for all combinations. At harvest 4 the hybrid had the highest weight per plant A at the lowest density but the lowest maximum biomass production B. It can thus be concluded, that the weight per plant at the lowest density (A) and the maximum biomass production per m² (B) were rather similar for the hybrid, sugarbeet and seabeet under these field conditions.

If the substitution rate c is significantly different from 1, it can be concluded that there is difference in competitive ability of the two subspecies. Table 1 shows that the hybrid was a significantly stronger competitor than sugarbeet in a sugarbeet population at harvest 2, 3 and 4, and the hybrid was a significantly stronger competitor than seabeet in a seabeet population at harvest 2 and 4. Sugarbeet was a significantly stronger competitor than seabeet in a seabeet population at harvest 2 and 4. Finally, seabeet was a significantly weaker competitor in a hybrid population at harvest 2 and 4.

The substitution rates c (Table 1 and fig. 3) were rather constant over the harvest times with exception of the hybrid as competitor in seabeet at harvest 4 (this c has a very large confidence interval and is therefore unreliable).

The *Beta* types can be ranked after the substitution rates follows: Hybrid > sugarbeet > seabeet, which means that the hybrid was the most competitive and seabeet was the least competitive type. The superior competitive ability in the hybrids is not surprising. First, the hybrids are favorized by heterosis effect from the different genetic background in the parents.

which is similar to results found in the competition study conducted by Bartsch *et al.* (1994). Second, the model is only fitted on above ground biomass, and do not account for the high root production in sugarbeet later in the season, and third the seabeet suffered from the disadvantage, that the experiment was conducted on a heavy clay soil which is not the natural habitat for this species. On basis of these substitution rates, it cannot be concluded that the hybrids are substantially superior to sugarbeets or seabeets in growth behavior and competitive ability, without selection pressure from glyphosate. However, there is a small but significant higher competitive ability in the hybrid compared with sugarbeet and seabeet.

The relative vegetation index (RVI_{ab}) was not significantly different for the sugarbeet and the hybrid at any stage during the growth season. Seabeets had a lower *RVI* than the other two *Beta* types during certain growth periods (19–36 and 79–92 days after planting in the field) (fig. 4). The RVI_{ab} data recorded at the harvest times showed a 93 percent correlation (linear model of 2'nd degree) with fresh weight per m^2. Sugarbeet, seabeet and the hybrid had a similar relationship from RVI to biomass, which means that the same link function can be used to describe the relationship for all three *Beta* types. Consequently, remote sensing techniques may be a useful non-destructive tool, to detect differences in the biomass of *Beta* species during the growth season.

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Table 1. Summary of regression of biomass (g dry weight) per plant on densities in binary mixtures of hybrids, sugarbeets and seabeets. Bootstrap 95% confidence intervals in parenthesis.

har-vest	Spe-cies 1	Spe-cies 2	*A*	*B*	*c*
2.	sugar-beet	hybrid	12.4 (9.9-15.5)	420 (300-679)	1.81 (1.13-3.09)
	sugar-beet	sea-beet	12.2 (9.9-15.5)	382 (282-585)	0.75 (0.34-1.41)
	sea-beet	hybrid	12.0 (8.6-20.8)	228 (151-492)	2.04 (1.05-4.91)
	sea-beet	sugar-beet	11.5 (8.7-15.2)	269 (186-441)	2.21 (1.38-3.84)
	hybrid	sugar-beet	14.8 (11.9-17.8)	294 (225-392)	0.72 (0.41-1.14)
	hybrid	sea-beet	13.7 (10.7-17.0)	309 (230-444)	0.44 (0.18-0.87)
3.	sugar-beet	hybrid	18.8 (15.6-24.5)	627 (455-1081)	1.77 (1.07-3.25)
	sugar-beet	sea-beet	18.5 (15.7-23.9)	591 (443-946)	0.91 (0.50-1.68)
	sea-beet	hybrid	17.6 (12.1-30.1)	515 (287-1876)	1.42 (0.52-5.65)
	sea-beet	sugar-beet	19.4 (13.4-31.3)	656 (356-2485)	2.37 (0.98-9.73)
	hybrid	sugar-beet	15.1 (11.3-20.6)	896 (474-3490)	0.79 (0.11-3.73)
	hybrid	sea-beet	19.0 (14.9-25.2)	584 (408-1000)	0.41 (0.05-1.01)
4.	sugar-beet	hybrid	30.2 (20.8-49.7)	616 (433-1000)	2.26 (1.42-3.94)
	sugar-beet	sea-beet	35.0 (23.2-59.7)	560 (414-834)	0.88 (0.50-1.49)
	sea-beet	hybrid	22.3 (15.0-42.1)	772 (415-3880)	3.99 (1.77-20.77)
	sea-beet	sugar-beet	21.7 (16.0-32.5)	755 (471-1912)	2.96 (1.64-7.91)
	hybrid	sugar-beet	40.4 (28.4-59.7)	493 (399-639)	0.65 (0.40-1.01)
	hybrid	sea-beet	42.9 (23.6-73.0)	471 (328-723)	0.50 (0.22-0.98)

Fig. 1. Yield per plant (g dry weight) of sugarbeet versus the effective density $N_{e,m}$ where N is a combination of sugarbeet and hybrid densities.

Fig. 2. Yield per plant (g dry weight) of the hybrids versus the effective density $N_{e,-}$ where N is a combination of hybrid and sugarbeet densities.

Fig. 3. Substitution rates over time for the different density combinations of hybrids. sugarbe ets and seabeets. The symbols are as follows:

○ = Hybrid as competitor in a sugarbeet population.

+ = seabeet as competitor in a sugarbeet population.

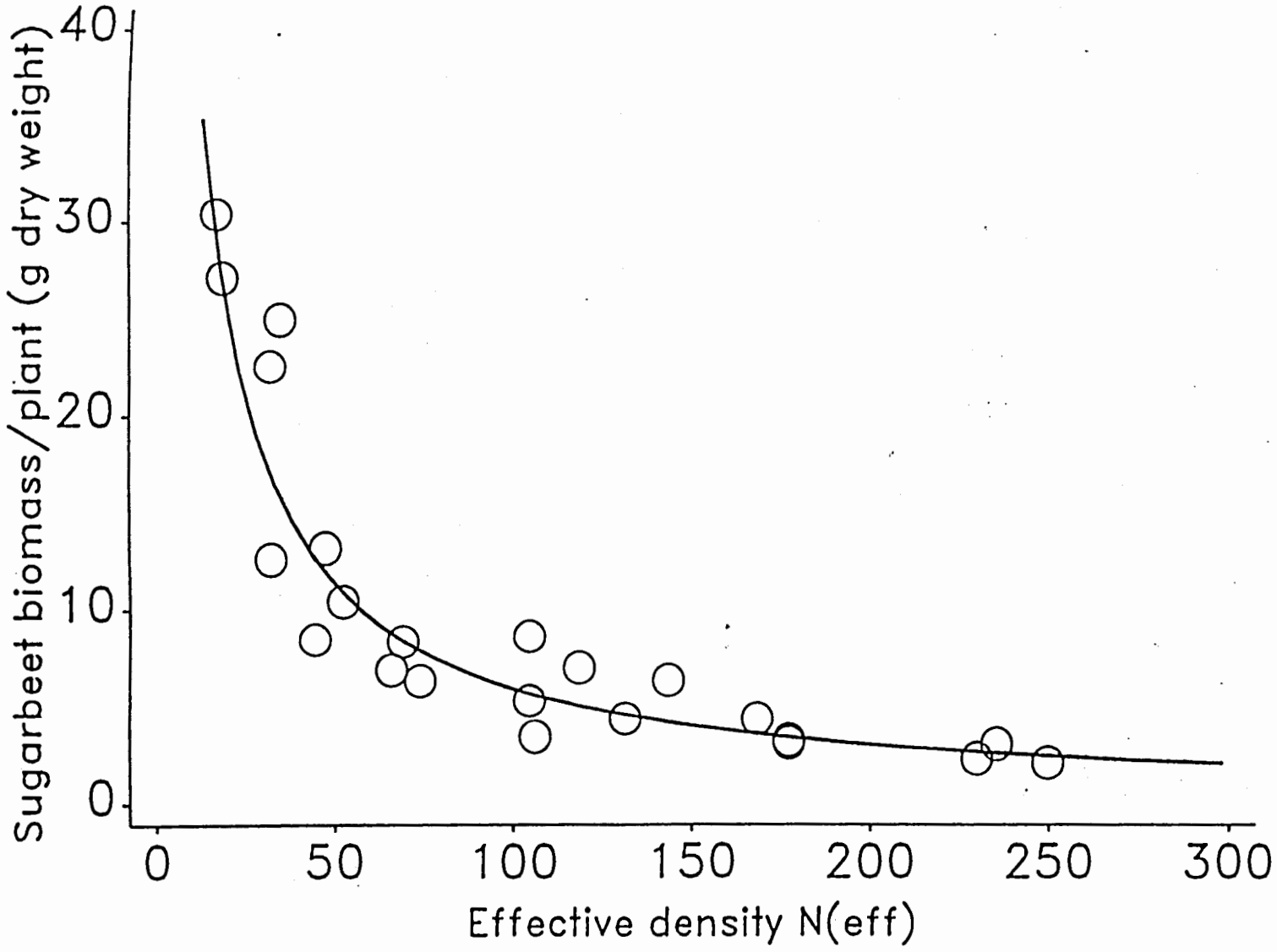
□ = hybrid as competitor in a seabeet population.

◆ = sugarbeet as competitor in a seabeet population.

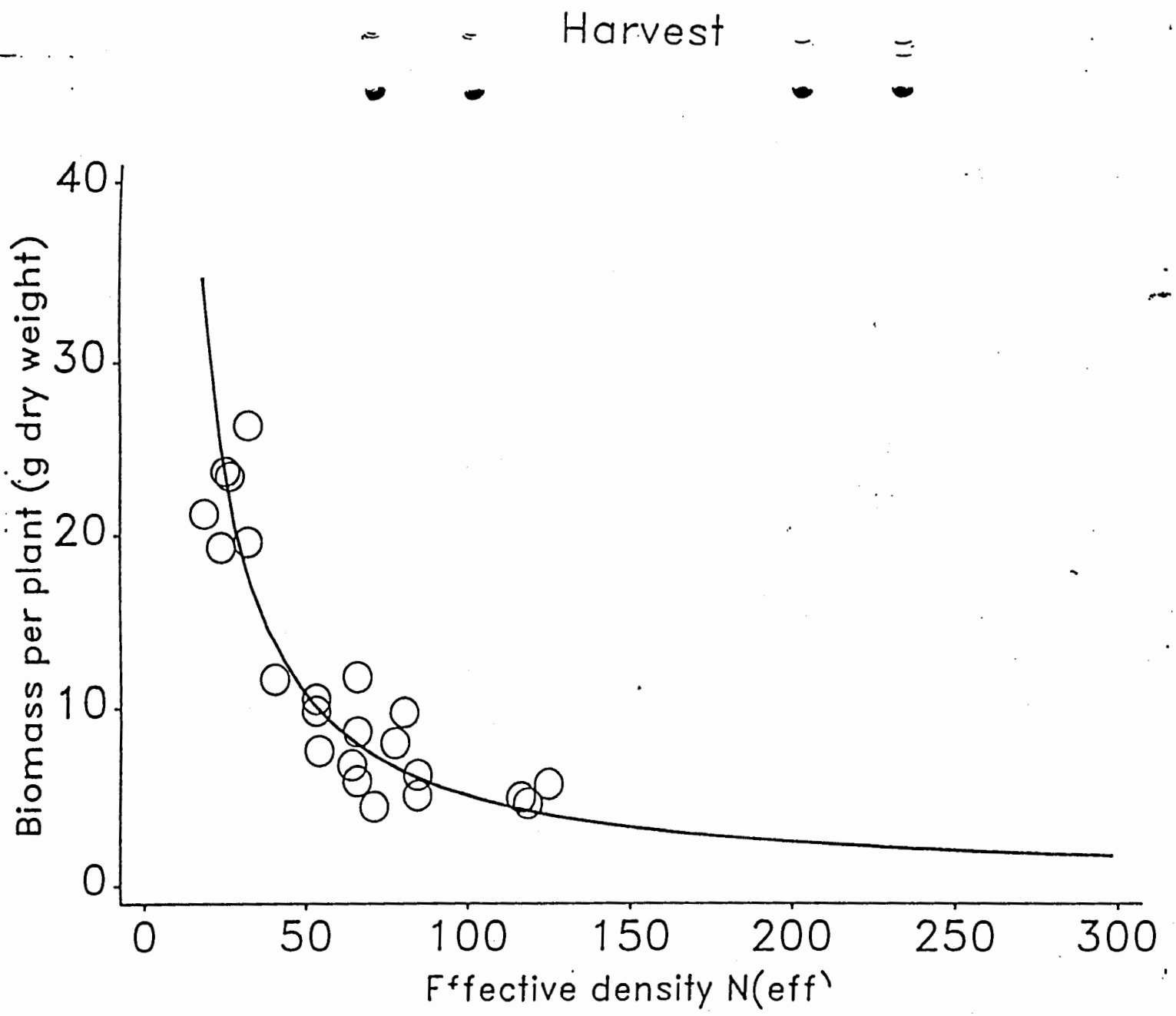
▼ = sugarbeet as competitor in a hybrid population.

▲ = seabeet as competitor in a hybrid population.

Fig. 4. The relative vegetation index ($\hat{RVI}_{,t}$) over the growth season for monocultures of the hybrid = ○. sugarbeet = △ and seabeet = □ at 64 plants per m².


Sugarbeet biomass/plant (g dry weight)
0
10
20
30
40
0
50
100
150
200
250
300
Effective density N(eff)

Substitution rate (c)
0
1
2
3
4
5
1
3
2


Harvest
Biomass per plant (g dry weight)
40
30
20
10
0
0
50
100
150
200
250
300
Effective density N(eff)

Net Relative Vegetation Index
0
2
4
6
8
10
0
20
40
60
80
100
120

Appendix VI. Safety assessment of the deliberate release of two model transgenic plants, oilseed rape and sugar beet.

SAFETY ASSESSMENT OF THE DELIBERATE RELEASE OF TWO MODEL TRANSGENIC PLANTS, OILSEED RAPE AND SUGAR BEET

BRIDGE PROJECT BIOT CT-910298
ANNUAL REPORT - 1991/92

SAFETY ASSESSMENT OF THE DELIBERATE RELEASE OF GENETICALLY MODIFIED SUGAR BEET

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ABSTRACT

The transfer of introduced genes to wild relatives is one of the questions raised by the use of genetically modified sugar beet. While the significance of such a gene transfer must be evaluated on a gene-by-gene basis, a study of gene transfer using genetic markers can provide useful information on sugar beet pollen movement and hybridisation with related species under field conditions.

In collaboration with Maribo, Monsanto conducted the first field experiment with flowering transgenic sugar beet in Belgium in 1991, following approval from the Belgian authorities responsible for the field release of genetically modified plants. The objective of the study was to test experimental protocols designed to study sugar beet pollen movement and hybridisations with *Beta maritima*. The pollen donor was a sugar beet breeding line, typical of the type used in basic seed production, containing genes for both Roundup® tolerance and ß-glucuronidase (GUS) activity. Plants were vernalised to induce flowering, and transplanted to the field in spring. Some 80 flowering plants were maintained in an area of about 10 square metres. Pollen movement was monitored by the use of single row plots of male sterile *Beta vulgaris* plants downwind from the pollen source, at distances of 25, 50 and 75 metres. Mixtures of *B. maritima* genotypes were planted in five directions at the same distance as the male sterile *B. vulgaris*. Flowering was well synchronised during July and August, and harvesting was carried out in late September. Plants were harvested individually and analysed for GUS activity to detect gene transfer. A subsample of 1.000 transgenic sugar beet seeds, 4.200 seeds harvested from CMS plants, and 17.500 seeds harvested from *B. maritima*

vulgaris var. *conditiva*.

In the experiment with *B. macrocarpa* it was difficult to synchronize the flowering of the two species since *Beta vulgaris* var. *conditiva* began flowering 18 days after *B. macrocarpa*. Such differences in flowering-time creates a reproductive barrier that makes gene transfer from *B. vulgaris* to this wild relative less frequent.

From these experiments we can conclude that outcrossing from *Beta vulgaris* to *B. maritima*, *B. atriplicifolia* and *B. macrocarpa* is generally low, but actual gene flow can occur beyond 75 m from the pollen source. In relation to gene escape from genetically engineered plants, this means that gene escape from cultivated transgenic sugarbeets to wild relatives can occur if a wild flowering relative is present in the near surroundings of a flowering and pollen fertile transgenic sugarbeet. A study on fitness of the hybrids between a transgenic *B. vulgaris* and a wild relative, can possibly reveal if an escaped engineered gene will persist in a population of wild *Beta* spp. and if the wild relative will become more "weedy".

Thirty plants of *B. vulgaris* var. *conditiva* were randomly placed on a circle shaped area of 120 cm in diameter. The wild *Beta* spp. were transplanted into belts placed at right angles to the pollen source. The belts widened with distance to fit the same arc of a circle (Fig. 2.1). The wild *Beta* spp. were planted east of the pollen source because westerly wind is predominant in Denmark. The three experiments were conducted in sugarbeet fields at Lolland in the southern part of Denmark and received the same fertilizer and pesticide treatments as the sugarbeet fields.

In 1991 *B. maritima* was transplanted to different distances from the pollen source in three eastern directions at 9.4, 25, 50 and 75 meters. As a control, four vernalized and male sterile *B. vulgaris* plants were planted at 200 m east of the pollen source. In 1992 *Beta macrocarpa* and *B. atriplicifolia* were each transplanted to a circle of radius 1.8 m surrounding the pollen source and to four distances in one eastern direction at 9.4, 25, 50 and 75 meters from the pollen source (Fig. 2.1 and Table 2.1). The two experiments were seperated by a distance of 5 kilometers to prevent pollen exchange between the experiments.

Table 2.1. Pollen dispersal experiments from *Beta vulgaris* var. *conditiva* to *Beta* - species - distance from pollen source to belt of wild *Beta* spp., number of plants in belts and length of the plant belt per position.

Beta spp.	direction	distance from pollen source per direction	length of belt per distance per direction	plants per belt
Beta maritima	ENE, E and ESE	9.4	1.25	15
	ENE, E and ESE	25	3.75	45
	ENE, E and ESE	50	6.25	75
	ENE, E and ESE	75	10	120
Beta macrocarpa and *Beta atriplicifolia*	Circle around	0.3	9.4	38
	E	9.4	1.25	15
	E	25	3.75	45
	E	50	6.25	75
	E	75	10	120

The flowering period of *B. vulgaris* var. *conditiva* was adjusted to correlate with the flowering of the wild *Beta* spp. by an extra transplanting 5–8 weeks later and by cutting back the seed stems in the species with the earliest bolting. Diploid plants were used in order to facilitate synchronization in flowering between the species (Scott & Longden, 1979).

The seed stems were harvested in August, weathered and then threshed. The seed weight of the *B. vulgaris* var. *conditiva* was used to calculate the number of fertile flowers in the pollen source (Table 2.2) by dividing the total seed yield from the pollen source planting with the measured Thousand-Kernel-Weight. A random sample of seed from each belt and species were sown in trays and grown to plants at the 2–4 leaf stage after which the percentages of red coloured plants were recorded as crossing frequencies (each sample consisted of 163–2462 plants).

Table 2.2. Pollen dispersal experiments from *Beta vulgaris* var. *conditiva* to *Beta* species. Flowering period and wind direction in favour of outcrossing.

Beta species	Trans-planted	Start of flowering	End of flo-wering	Harve-sted	Number of flowers in pollen source	Wes-terly wind %
Beta maritima	1 May 91	28 June 91[1]	13 August 91[4]	26 Au-gust 91	19.900	67
Beta ma-crocarpa	12 May 92	1 June 92[2]	27 July 92	4 August 92	33.300	36
Beta atri-plicifolia	12 May 92	22 June 92[3]	17 August 92[4]	27 Au-gust 92	6.400	48

[1]The flowering period of *Beta vulgaris* var. *conditiva* was 7 June – 13 August 1991.
[2]The flowering period of *Beta vulgaris* var. *conditiva* was 18 June – 4 August 1992.
[3]The flowering period of *Beta vulgaris* var. *conditiva* was 7 June – 15 August 1992.
[4]End of main flowering, the seed stems continued to set new flowers until harvest time.

2.3. Results and discussion

The actual gene flow in 1991 from *Beta vulgaris* var. *conditiva* to *B. maritima* was small at all distances (Fig. 2.2), but even at 75 m from the pollen source, crossing frequencies of 0.06–0.31% were recorded. The male sterile *B. vulgaris* planting had a crossing frequency of 0.79% with the *B. vulgaris* var. *conditiva*, which confirms that fertile pollen can be dispersed to at least 200 m. In sugarbeet seed production, 0.02 % is considered as the maximal acceptable level of crossings from wild annual *Beta* spp. into a sugarbeet seed field (Boeskov, personal communication). Analogous, 0.02% could be the maximal level to be considered negilible in the reverse process, namely crossing from *B. vulgaris* to *B. maritima*. According to this threshold level there is measureable geneflow at 75 m distance in all three eastern directions (Fig. 2.2). Consequently, our proposed hypothesis, that actual gene flow beyond 50 m distance is negligible from *B. vulgaris* to *B. maritima*, is rejected. It is not surprising to find relative high crossing frequencies between the two species since *B. maritima* is in general considered to be the ancestor of *B. vulgaris*, and morphological studies of Danish *B. maritima* populations revealed traces from hybridization with cultivated beets (Rasmusson, 1932). Furthermore, the wind

conditions (Table 2.2), and the synchronization of flowering with *B. vulgaris* var. *conditiva* provided excellent opportunities for crossings between the species in 1991.

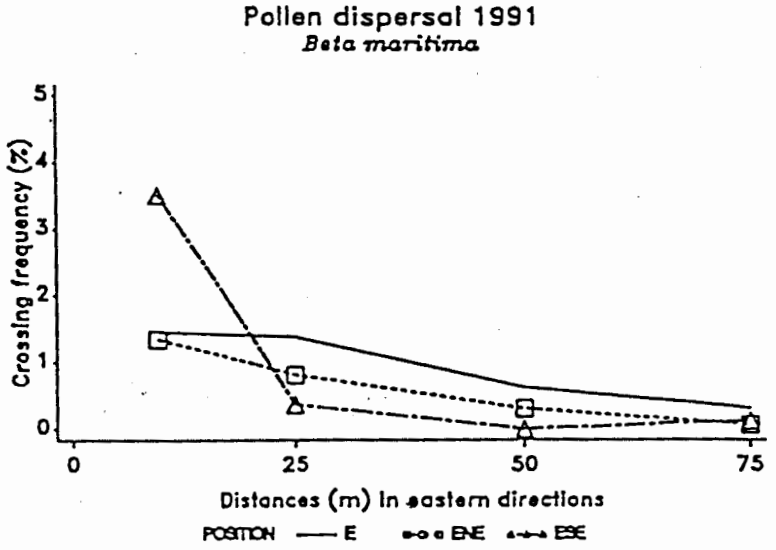


Fig. 2.2. Pollen dispersal from *Beta vulgaris* var. *conditiva* to *B. maritima* - crossing frequency (%) as function of distance from *B. vulgaris* var. *conditiva*.

In 1992, a circle of the wild species surrounding the pollen source was included in the experimental design (Fig. 2.1) in order to determine crossing frequencies in the other wind directions. Crossing frequencies in the circle ranged from 2.4–29.5 percent in *B. atriplicifolia* and from 1.1–4.2 percent in *B. macrocarpa* (Fig. 2.3). Based on the total outcrossing from *B. vulgaris* var. *conditiva* to the circle of *B. atriplicifolia* and *B. marcrocarpa* only 13% and 7% respectively, of the outcrosses in the circle happened in eastern direction. The frequency of days with westerly winds during flowering (Table 2.2) appears higher than the actual gene flow in eastern direction, which can be explained by the fact that the main flowering took place in a period of predominant south-easterly winds (Fig. 2.3). Crosses in eastern direction could not be found beyond 25 m in *B. atriplicifolia* and 9.4 m in *B. macrocarpa* (Fig. 2.4) which supports the proposed hypothesis, but on the other hand wind conditions in 1992 were not favourable to this experimental design, which makes it difficult to make any generalizations based on these two experiments.

Table 4. GUS +ve percentages of germinating seeds harvested from Beta maritima and Beta vulgaris as a function of genotype, distance from the pollen source and direction

a. Genotype

Beta vulgaris - Pollen source	57%
Beta vulgaris - male sterile	13%
Beta maritima Y 1123*	4%
Beta maritima Y 1124	7%
Beta maritima Y 1126	5%
Beta maritima Y 1128	2%
Mean of Beta maritima	5%

b. Distance

Genotype	Centre	0 m	25 m	50 m	75 m
Beta vulgaris - male sterile	47.5		13.4	4.6	3.0
Beta maritima Y 1123	34.8	20.8	0	0	0.8
Beta maritima Y 1124	-	18.1	0	0	0
Beta maritima Y 1126	-	22.1	0	0	0
Beta maritima Y 1128	n.a	23.1	0	1.5	0
Mean of Beta maritima	34.8	20.2	0	0.27	0.21

c. Direction*

Genotype	SW	NW	N	NE	E	SE
Beta vulgaris - male sterile	-	-	7.1	-	5.7	-
Beta maritima Y 1123	0	0	-	4.8	0	0
Beta maritima Y 1124	0	0	-	0	0	0
Beta maritima Y 1126	0	0	-	0	0	0
Beta maritima Y 1128	0	1.2	-	0	0	0
Mean of Beta maritima	0	0.3	-	0.6	0	0

n.a = no seeds harvested in the field because of plant death

- = no plants planted in this position

* = Includes only 25, 50 and 75 metre distances (centre and 0m excluded)

Table 5. GUS+ percentages of Beta maritima as a function of distance and direction from the pollen source

GENOTYPE	DIRECTION	Distance from source				SURROUNDING CROP
		0 m	25 m	50 m	75 m	
Beta maritima Y 1123	SW	7	n.a	0	0	Sugar beet
	NW	39	0	0	0	Triticale
	NE	100	0	0	8	Sugar beet
	E	-	0	0	0	Barley
	SE	n.a	n.a	0	0	Barley
Beta maritima Y 1124	SW	21	0	0	0	Sugar beet
	NW	15	0	0	0	Triticale
	NE	15	0	0	0	Sugar beet
	E	-	0	0	0	Barley
	SE	0	n.a	0	0	Barley
Beta maritima Y 1126	SW	10	0	0	0	Sugar beet
	NW	0	0	0	0	Triticale
	NE	25	0	0	0	Sugar beet
	E	-	0	0	0	Barley
	SE	37	0	0	0	Barley
Beta maritima Y 1128	SW	0	0	0	0	Sugar beet
	NW	n.a	n.a	3	0	Triticale
	NE	n.a	0	0	0	Sugar beet
	E	-	n.a	0	0	Barley
	SE	33	0	0	0	Barley

n.a = no seeds harvested in the field because of plant death
- = no plants planted in this position

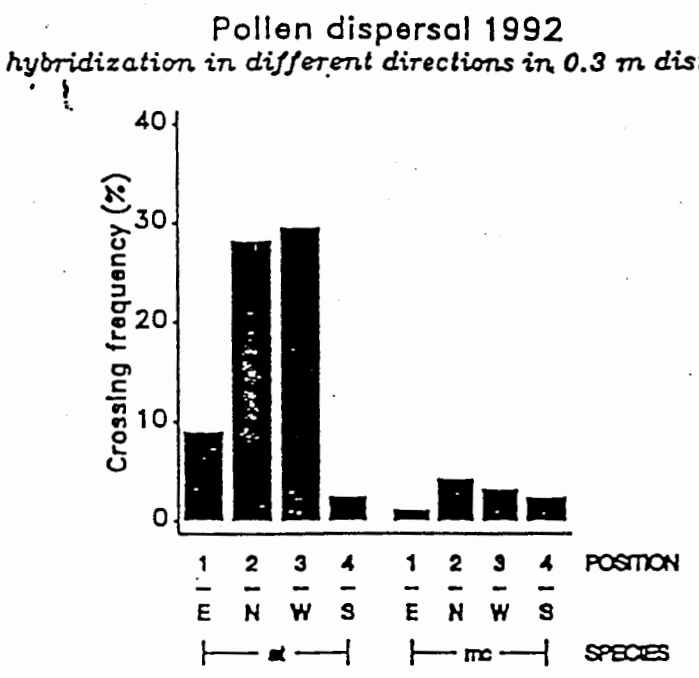


Fig. 2.3. Outcrossing frequencies (%) in different directions from *Beta vulgaris* var. *conditiva* to circle of *B. atriplicifolia* (at) and *B. macrocarpa* (mc).

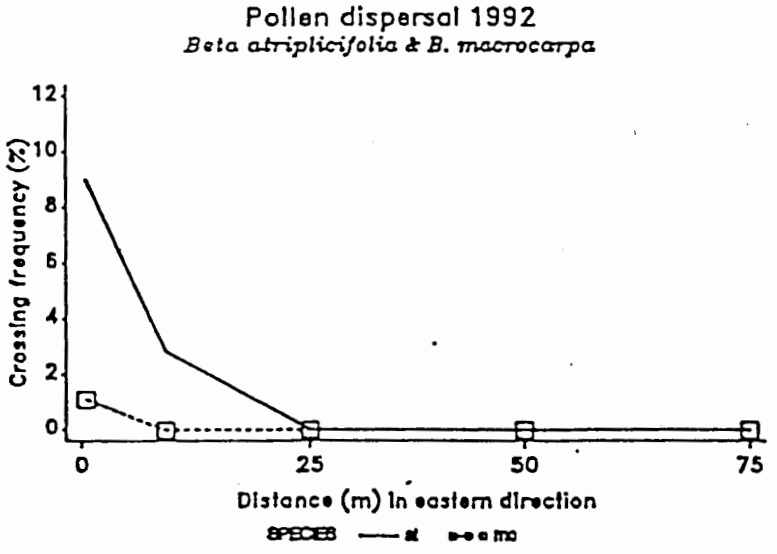


Fig. 2.4. Pollen dispersal from *Beta vulgaris* var. *conditiva* to *B. atriplicifolia* (at) and *B. macrocarpa* (mc) - crossing frequency (%) as function of distance from *Beta*

Appendix 1 : Details of plant survival, bolting, and flowering dates of Beta maritima and Beta vulgaris plants.

Line	Color code	Bolters cut back : No. of plants	Bolting (50% of plants)	First anthers (50% of plants)	Plant mortality	End of flowering
LINE 1	*B. maritima*					
Y1123	Yellow	1 : 9	8/7/91	16/7/91	0 : 9	harvest
Y1124	Red	2 : 8	8/7/91	16/7/91	0 : 9	harvest
Y1126	Blue	1 : 9	8/7/91	16/7/91	0 : 9	harvest
Y1128	Green	0 : 9	8/7/91	16/7/91	3 : 9	harvest
LINE 2	*B. maritima*					
Y1123	Yellow	0 : 4	4/7/91	25/7/91	0 : 4	harvest
Y1124	Red	0 : 4	4/7/91	16/7/91	0 : 4	harvest
Y1126	Blue	1 : 4	4/7/91	16/7/91	0 : 4	harvest
Y1128	Green	0 : 4	10/7/91	16/7/91	0 : 4	harvest
LINE 3	*B. maritima*					
Y1123	Yellow	0 : 1	N.A.	N.A.	0 : 1	N.A.
Y1124	Red	0 : 1	4/7/91	16/7/91	0 : 1	harvest
Y1126	Blue	0 : 1	8/7/91	25/7/91	0 : 1	harvest
Y1128	Green	0 : 1	4/7/91	25/7/91	0 : 1	harvest
LINE 4	*B. maritima*					
Y1123	Yellow	0 : 1	8/7/91	25/7/91	0 : 1	harvest
Y1124	Red	0 : 1	> 13/8/91	> 13/8/91	0 : 1	harvest
Y1126	Blue	0 : 1	4/7/91	8/7/91	0 : 1	harvest
Y1128	Green	0 : 1	4/7/91	25/7/91	0 : 1	harvest
LINE 5	*B. maritima*					
Y1123	Yellow	0 : 4	8/7/91	16/7/91	0 : 4	harvest
Y1124	Red	1 : 4	8/7/91	25/7/91	0 : 4	harvest
Y1126	Blue	2 : 4	8/7/91	16/7/91	0 : 4	harvest
Y1128	Green	0 : 4	8/7/91	25/7/91	0 : 4	harvest
LINE 6	*B. maritima*					
Y1123	Yellow	0 : 9	8/7/91	16/7/91	0 : 9	harvest
Y1124	Red	1 : 9	8/7/91	16/7/91	0 : 9	harvest
Y1126	Blue	2 : 9	8/7/91	16/7/91	0 : 9	harvest
Y1128	Green	0 : 9	25/7/91	25/7/91	1 : 9	harvest
LINE 7	*B. maritima*					
Y1123	Yellow	0 : 1	10/7/91	16/7/91	0 : 1	harvest
Y1124	Red	0 : 1	16/7/91	25/7/91	0 : 1	harvest
Y1126	Blue	1 : 1	4/7/91	8/7/91	0 : 1	harvest
Y1128	Green	0 : 1	N.A.	N.A.	1 : 1	N.A.

LINE 8	*B. maritima*					
Y1123	Yellow	0 : 5	8/7/91	16/7/91	0 : 5	harvest
Y1124	Red	0 : 4	4/7/91	10/7/91	0 : 4	harvest
Y1126	Blue	2 : 4	4/7/91	8/7/91	0 : 4	harvest
Y1128	Green	0 : 3	16/7/91	25/7/91	2 : 3	harvest
LINE 9	*B. maritima*					
Y1123	Yellow	1 : 9	> 13/8/91	> 13/8/91	0 : 9	harvest
Y1124	Red	2 : 9	> 13/8/91	> 13/8/91	0 : 9	harvest
Y1126	Blue	2 : 9	1/8/91	1/8/91	0 : 9	harvest
Y1128	Green	0 : 9	> 13/8/91	> 13/8/91	0 : 9	harvest
LINE 10	*B. maritima*					
Y1123	Yellow	0 : 1	25/7/91	25/7/91	0 : 1	harvest
Y1124	Red	0 : 1	N.A.	N.A.	0 : 1	N.A.
Y1126	Blue	1 : 1	8/7/91	25/7/91	0 : 1	harvest
Y1128	Green	0 : 1	1/8/91	1/8/91	0 : 1	harvest
LINE 11	*B. maritima*					
Y1123	Yellow	0 : 4	16/7/91	25/7/91	0 : 4	harvest
Y1124	Red	0 : 3	16/7/91	25/7/91	0 : 3	harvest
Y1126	Blue	1 : 5	8/7/91	16/7/91	0 : 5	harvest
Y1128	Green	0 : 4	25/7/91	1/8/91	0 : 4	harvest
LINE 12	*B. maritima*					
Y1123	Yellow	0 : 9	1/8/91	1/8/91	0 : 9	harvest
Y1124	Red	0 : 9	13/8/91	13/8/91	0 : 9	harvest
Y1126	Blue	2 : 9	1/8/91	1/8/91	0 : 9	harvest
Y1128	Green	1 : 9	1/8/91	1/8/91	0 : 9	harvest
LINE 13	*B. maritima*					
Y1123	Yellow	0 : 1	> 13/8/91	> 13/8/91	0 : 1	harvest
Y1124	Red	0 : 1	10/7/91	16/7/91	0 : 1	harvest
Y1126	Blue	0 : 1	10/7/91	16/7/91	0 : 1	harvest
Y1128	Green	0 : 1	N.A.	N.A.	1 : 1	N.A.
LINE 14	*B. maritima*					
Y1123	Yellow	1 : 4	8/7/91	1/8/91	0 : 4	harvest
Y1124	Red	1 : 4	16/7/91	16/7/91	0 : 4	harvest
Y1126	Blue	1 : 4	8/7/91	25/7/91	0 : 4	harvest
Y1128	Green	0 : 4	8/7/91	1/8/91	0 : 4	harvest
LINE 15	*B. maritima*					
Y1123	Yellow	0 : 9	25/7/91	25/7/91	0 : 9	harvest
Y1124	Red	0 : 9	16/7/91	25/7/91	0 : 9	harvest
Y1126	Blue	3 : 9	8/7/91	16/7/91	0 : 9	harvest
Y1128	Green	0 : 9	1/8/91	1/8/91	0 : 9	harvest
LINE 16	CMS	2 : 4	4/7/91	10/7/91	0 : 4	harvest
LINE 17	CMS	0 : 17	4/7/91	16/7/91	0 : 17	harvest
LINE 18	CMS	0 : 38	4/7/91	16/7/91	0 : 38	harvest
LINE 19	CMS	2 : 4	4/7/91	8/7/91	0 : 4	harvest

LINE 20	CMS	3 : 17	10/7/91	16/7/91	0 : 17	harvest
LINE 21	CMS	5 : 36	10/7/91	16/7/91	0 : 36	harvest
C1	*B. maritima*					
Y1123	Yellow	0 : 1	10/7/91	16/7/91	0 : 1	harvest
Y1124	Red	0 : 1	4/7/91	10/7/91	0 : 1	harvest
Y1126	Blue	0 : 1	4/7/91	16/7/91	0 : 1	harvest
Y1128	Green	0 : 1	8/7/91	25/7/91	0 : 1	harvest
C2	*B. maritima*					
Y1123	Yellow	0 : 1	10/7/91	25/7/91	0 : 1	harvest
Y1124	Red	0 : 1	10/7/91	1/8/91	0 : 1	harvest
Y1126	Blue	0 : 1	> 13/8/91	> 13/8/91	0 : 1	harvest
Y1128	Green	0 : 1	N.A.	N.A.	0 : 1	N.A.
C3	*B. maritima*					
Y1123	Yellow	0 : 1	8/7/91	10/7/91	0 : 1	harvest
Y1124	Red	0 : 1	4/7/91	10/7/91	0 : 1	harvest
Y1126	Blue	0 : 1	4/7/91	16/7/91	0 : 1	harvest
Y1128	Green	0 : 1	N.A.	N.A.	0 : 1	N.A.
C4	*B. maritima*					
Y1123	Yellow	0 : 1	N.A.	N.A.	0 : 1	N.A.
Y1124	Red	0 : 1	8/7/91	25/7/91	0 : 1	harvest
Y1126	Blue	0 : 1	4/7/91	16/7/91	0 : 1	harvest
Y1128	Green	0 : 1	> 13/8/91	> 13/8/91	0 : 1	harvest
C5	*B. maritima*					
Y1123	Yellow	0 : 2	10/7/91	1/8/91	0 : 2	harvest
Y1128	Green	0 : 2	N.A.	N.A.	2 : 2	N.A.
C6	*B. maritima*					
C6,1	CMS	0 : 1	4/7/91	8/7/91	0 : 1	13/8/91
C6,2	CMS	0 : 1	4/7/91	16/7/91	0 : 1	13/8/91
C6,3	CMS	0 : 1	4/7/91	8/7/91	0 : 1	13/8/91
C6,4	CMS	0 : 1	4/7/91	8/7/91	0 : 1	13/8/91
C7	*B. maritima*	0 : 80	4/7/91	10/7/91	1 : 80	13/8/91

NOTE = X:Y signifies X plants out of Y plants
N.A. = No flowering or plant killed

Appendix II. Germination and GUS+ percentages of seeds harvested from Beta maritima plants

Plot Location	Beta maritima - Y1123						Beta maritima - Y 1124						Beta maritima - Y 1126						Beta maritima - Y 1128					
	Seeds Planted	No. of plants	Germ. %	No. of GUS+	GUS+ % of seeds	GUS+ % of plants	Seeds Planted	No. of plants	Germ. %	No. of GUS+	GUS+ % of seeds	GUS+ % of plants	Seeds Planted	No. of plants	Germ. %	No. of GUS+	GUS+ % of seeds	GUS+ % of plants	Seeds Planted	No. of plants	Germ. %	No. of GUS+	GUS+ % of seeds	GUS+ % of plants
CENTRE	400	23	6	8	2	35													n.a.	n.a	n.a	n.a	n.a	n.a
0 m - SW	200	29	15	2	1	7	200	76	38	16	8	21	200	42	21	4	2	10	67	4	6	0	0	0
25 m - SW	n.a.	n.a	n.a.	n.a.	n.a.	n.a.	200	0	0	0	0	0	200	6	3	0	0	0	200	2	1	0	0	0
50 m - SW	200	4	2	0	0	0	200	3	2	0	0	0	200	1	0.5	0	0	0	200	4	2	0	0	0
75 m - SW	1000	34	3	0	0	0	600	17	3	0	0	0	400	14	4	0	0	0	400	22	6	0	0	0
0 m - NW	200	18	9	7	4	39	200	13	7	2	1	15	200	6	3	0	0	0	n.a.	n.a	n.a.	n a.	n a	n a
25 m - NW	n<200	0	0	0	0	0	200	29	15	0	0	0	200	34	17	0	0	0	n.a.	n.a	n.a.	n a.	n.a	n a
50 m - NW	200	39	20	0	0	0	200	23	12	0	0	0	200	28	14	0	0	0	200	38	19	1	0 5	3
75 m - NW	200	41	21	0	0	0	200	16	8	0	0	0	200	55	28	0	0	0	200	46	23	0	0	0
0 m - NE	100	1	1	1	1	100	200	47	24	7	4	15	200	24	12	6	3	25	n.a.	n.a.	n.a.	n.a	n.a	n a
25 m - NE	200	6	3	0	0	0	n<200	0	0	0	0	0	200	4	2	0	0	0	200	2	1	0	0	0
50 m - NE	200	3	2	0	0	0	200	14	7	0	0	0	600	28	5	0	0	0	200	14	7	0	0	0
75 m - NE	1000	12	1	1	0.1	8	600	27	5	0	0	0	600	20	3	0	0	0	600	28	5	0	0	0
0 m - E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25 m - E	200	4	2	0	0	0	n<200	0	0	0	0	0	400	40	10	0	0	0	n.a.	n.a.	n.a.	n.a	n a	n.a
50 m - E	200	6	3	0	0	0	200	11	6	0	0	0	400	68	17	0	0	0	200	4	2	0	0	0
75 m - E	200	10	5	0	0	0	200	31	16	0	0	0	200	22	11	0	0	0	200	8	4	0	0	0
0 m - SE	n.a	n.a.	n.a.	n.a.	n.a.	n.a.	200	2	1	0	0	0	200	41	21	15	8	37	100	9	9	3	3	33
25 m - SE	n.a	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a	n.a.	n.a.	n a.	n.a.	200	10	5	0	0	0	180	9	5	0	0	0
50 m - SE	200	16	8	0	0	0	200	21	11	0	0	0	200	40	20	0	0	0	200	6	3	0	0	0
75 m - SE	200	33	17	0	0	0	200	10	5	0	0	0	200	17	9	0	0	0	200	24	12	0	0	0
All directions																								
0 m	500	48	9.6	10	2.0	20.8	800	138	17.3	25	3.1	18.1	800	113	14.1	25	3.1	22.1	167	13	7.8	3	1 8	23 1
25 m	400+	10	4.0	0	0.0	0.0	400+	29	7.3	0	0.0	0.0	1200	94	7.8	0	0.0	0.0	680	13	2 2	0	0 0	0 C
50 m	1000	67	6.7	0	0.0	0.0	1000	72	7.2	0	0.0	0.0	1600	166	10.3	0	0.0	0.0	1000	65	6 6	1	0 1	1 5
75 m	2600	130	5.0	1	0.04	0.8	1800	101	5.6	0	0.0	0.0	1600	128	8.0	0	0.0	0.0	1600	128	8 0	0	0 0	0 0

GUS+ = Plants demonstrating β-glucuronidase activity
n.a = no seeds harvested in the field due to plant death
- = no plants planted in this position

Chapter 2.

Pollen Dispersal from *Beta vulgaris* to Wild Relatives (*Beta* spp.)

Summary

Actual gene flow by pollen dispersal from *Beta vulgaris* to *B. maritima*, *B. atriplicifolia* and *B. macrocarpa* was experimentally determined using red colour as a marker gene. The outcrossing frequency to *B. maritima* was 0.06–0.31 percent at 75 m distance from *B. vulgaris*. Outcrossing beyond 25 m into populations of *B. atriplicifolia* and *B. macrocarpa* could not be found.

2.1. Introduction

Sugarbeet (*Beta vulgaris*) was the first genetically engineered crop plant to be tested in Danish fields (Clausen, 1989) and as a consequence much attention has been directed towards risk assessment with emphasis on pollen dispersal from sugarbeet. One concern is, that engineered genes will escape from the crop plant into a wild relative and perhaps create a future weed problem (Ellstrand, 1988), even though the likelihood of one gene creating weediness is small, since weediness is a trait from many genes (Keeler, 1989). Another concern is, that if the new genes are introduced into natural populations of wild relatives then the fitness of these hybrids could be increased, which in turn could cause a decrease in variability of germplasm in these populations (Keeler & Turner, 1991). Both concerns are based on the assumption that the genes in question offer an adaptive advantage for the hybrid, otherwise the genes will probably not persist in the population (Ellstrand & Hoffman, 1990). The possibilities for escape of a specific gene from a crop by pollen dispersal is determined by the cytoplasmatic location of the gene (nuclear or plastids), presence of wild relatives, synchronous flowering, competition between pollen and the distance to which pollen may travel to effect fertilization (Ellstrand, 1988).

This paper deals with gene flow through pollen dispersal from *Beta vulgaris* to three wild relatives *B. maritima* (seabeet), *B. macrocarpa* and *B. atriplicifolia*, all members of the section *vulgares* within the genus *Beta* (DeBock, 1986). Reproductive barriers have not been recognized between *B. vulgaris*, *B. maritima* and *B. atriplicifolia* (Abe, Yoshikawa & Tsuda, 1986). Hybrids between *B. macrocarpa* and sugarbeets can cause weed problems in sugarbeet fields in California (McFarlane, 1975), but there seems to be genetic barriers between *B. vulgaris* and *B. marcrocarpa* occasionally resulting in partial pollen sterility and embryo abortion in the hybrid (Abe et al., 1986). *Beta maritima* is widely distributed from the Asiatic steppes and East India to the Canary Islands and up to the North Sea coast line in Europe while *B. atriplicifolia* and *B. macrocarpa* are confined in distribution to the Mediterranean area (Hultén & Fries, 1986). *B. atriplicifolia* and *B. macrocarpa* are both known to be annual whereas *B. maritima* changes from annual biotypes in the Mediterranean area to biannual/perennial biotypes in Northern Europe (Doney,

1991). In Denmark the only wild relative to sugarbeet is *B. maritima*, which has been reported present for less than 200 years. The first observed specimen dates back to 1806 and since then it has been dispersed along the coast line of the Great Belt and some of the southern islands (Nørgaard, 1959). The Danish *B. maritima* population is rather homogeneous (Doney, 1991) and shows a higher degree of self-fertility than most cultivated beets (Rasmusson, 1932; Tjebbes, 1933).

Potential gene flow by pollen is defined as the depositions of pollen from a source as a function of distance (Levin & Kerster, 1974). Most of the pollen from *B. vulgaris* is deposited close to the source, but windy conditions can carry a minor fraction at least 800 m away (Jensen & Bøegh, 1942). Some seed growers associations have specified a distance of isolation of minimum 3.200 m for *B. vulgaris* to maintain varietal purity (Levin & Kerster, 1974). *Beta* spp. are generally considered wind pollinators but numerous insects contribute in pollinating the *Beta* flowers (Free et *al.*, 1975)

In this study we propose and test the hypothesis that the actual gene flow, meaning the incidence of fertilization (Levin & Kerster, 1974) from *Beta vulgaris* to its wild relatives, is almost negligible beyond 50 m from a *B. vulgaris* source.

2.2. Materials and methods

Before 1993, open flowering transgenic *Beta vulgaris* was prohibited in Denmark, therefore *B. vulgaris* var. *conditiva* (redbeet) was used as a marker plant to determine outcrossing from *B. vulgaris* to wild *Beta* spp. in these experiments. The red colour in *B. vulgaris* var. *conditiva* is a dominant trait with high degree of expression and practically all crosses with this variety will become red coloured (Pedersen, 1944).

Biannual *Beta vulgaris* var. *conditiva* and perennial *B. maritima* (selected from natural habitats in Denmark) were sown in paperpots and vernalized for 14 weeks to induce flowering prior to being transplanted to the field. Annual *B. macrocarpa* and *B. atriplicifolia* were sown in paperpots six weeks prior to being transplanted into the field.

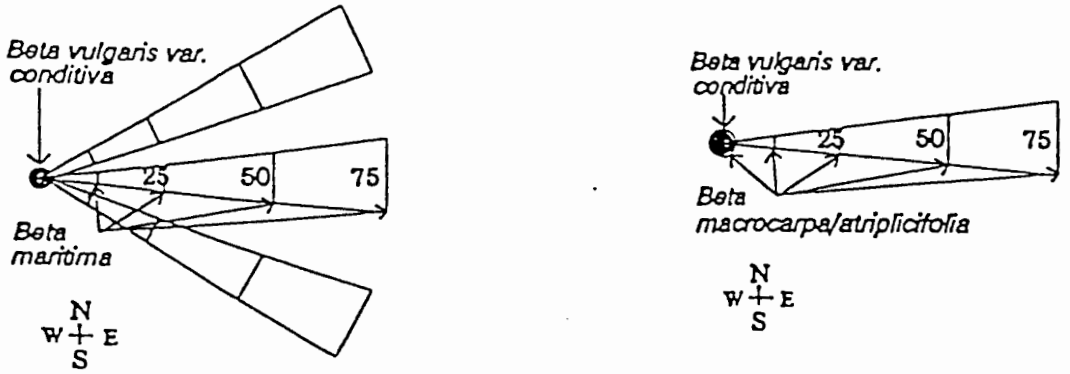


Fig. 2.1. Pollen dispersal from *Beta vulgaris* var. *conditiva* to *B. maritima*, 1991 and *B. macrocarpa* and *B. atriplicifolia*, 1992 - experimental design.

plants were analysed. Germination levels were generally low, and 3.250 GUS assays were performed.

The analyses indicated that expression of GUS in the progeny of the segregating pollen donor was 57%, compared to the maximum possible of 89%, possibly because of hybridisation by *Beta maritima* or by non-transgenic *Beta vulgaris* pollen from the central plot. On the CMS plants at distance zero, 48% of the germinating seeds were GUS positive (maximum of 67% possible). The comparable frequency of outcrossing to the CMS plants decreased with distance from the transgenic pollen source, to 14%, 5% and 3% of the germinated seeds at distances of 25, 50 and 75 metres respectively. The level of germination of seeds harvested from *Beta maritima* plants was extremely low. Of more than 15.000 seeds harvested at distances of greater than 25 metres, some 1.000 seeds germinated. Only two of these seeds were confirmed to be GUS positive; one located at 25 metres from the pollen source and the other at 75 metres.

A similar trial is now in progress in Belgium, with modifications to the area of transgenic plants and to the size of the pollen-trap plots (100 m^2). Additional studies will be conducted to explain the low germination of *Beta maritima* seeds, as well as testing alternative methods for detecting gene transfer, including Roundup® tolerance.

INTRODUCTION

The transfer of introduced genes to wild relatives is one of the questions raised by the use of genetically modified sugar beet. While the significance of such a gene transfer must be evaluated on a gene-by-gene basis, a study of gene transfer using genetic markers can provide useful information on sugar beet pollen movement and hybridisation with related species under field conditions. The first of a series of such field experiments was conducted in 1991 within the scope of BRIDGE. The objective of the study was to test experimental protocols designed to study sugar beet pollen movement and hybridisations with *Beta maritima* using Roundup® tolerant beet (*Beta vulgaris)* as the source of pollen.

PLANT MATERIAL

A transgenic O-type line, typical of the type used in basic seed production, was used as the pollen donor in the study. Transformation was carried out using *Agrobacterium tumefaciens* containing the vector pMON638. Besides the Roundup® tolerance gene, this construct contains the ß-glucuronidase (GUS) marker gene. The offspring from the selfing of the original transgenic plant were screened for GUS enzyme activity, and all GUS negative plants were discarded. A sample of the remaining plants was used as the pollen donor in the experiment.

The donor plant population, therefore, contained plants both heterozygous and homozygous for the transgenes. The pollen released from the population was expected to segregate in a 2:1 ratio (positive to negative) for both Roundup® tolerance and for GUS expression.

Beta vulgaris (transgenic O-type and non-transgenic male sterile lines) and *Beta maritima* were sown at the end of November, 1990 in a confined growth room. The *Beta maritima* seed had an extremely poor germination percentage. At the four-leaf stage, in mid-December, the plants were moved into a cold room (4C and 24 hours light) for vernalisation. At the end of March, 1991 an 8 hour night period was introduced while maintaining the temperature at 4C. In the beginning of April, 1991 the plants were relocated from the cold room into a growth room and brought gradually, over a 7 day period, up to 14C (85 %RH and 16 hours daylength).

Beta maritima plants were collected from four different locations in Denmark. The genotypes were coded Y1123, Y1124, Y1126 and Y1128. Seed was multiplied in Denmark, grown to seedlings and vernalised to induce flowering. The vernalised seedlings were shipped to Belgium, where they were maintained under the same growth room conditions as *Beta vulgaris* plants.

EXPERIMENTAL DESIGN

All plants were transplanted to the field in April 1991. The transgenic *Beta vulgaris* plants (pollen source) were planted in a central square of 2.5 x 2.5 metres. Plants were arranged in a grid with inter-plant spacing of 50 cm. Non-transgenic *Beta maritima* and male sterile *Beta vulgaris* plants were also included in the central square to measure outcrossing to these species at zero distance. *Beta maritima* seedlings were planted in five directions, at distances of 25, 50 and 75 metres from the central pollen source. The male sterile *Beta vulgaris* (CMS) line was used as 'pollen trap' and planted in two downwind directions at 25, 50 and 75 metres from the pollen source. The receptor plots were single-row plots 1, 4 and 9 metres in length at the distances of 25, 50 and 75 metres respectively. Inter-plant spacing was 25 cm. The receptor plots were planted amongst the surrounding rotation crops, sugar beet, barley or triticale.

The experimental field design, with explanatory notes, is provided in Figures 1 and 2.

The *Beta maritima* genotypes were transplanted at two dates, with a two-week interval. An equal number of the four genotypes were included in each receptor plot, and each plant was individually identified and monitored. *Beta vulgaris* (transgenic and CMS) were planted together with the second planting date of *Beta maritima*. All plants were individually fenced to protect against rabbit damage. On June 3 the most advanced *Beta maritima* (mainly genotype Y1126) and the CMS-line started bolting, while the transgenic line had not. To ensure synchronicity of

flowering these early bolters were cut back to delay flowering. Bolting and flowering dates of each plant were recorded. Some of the *Beta maritima* and *Beta vulgaris* plants did not establish successfully following transplantion, and perished prior to flowering. Variation in ability to establish was observed among the different *Beta maritima* lines with the highest mortality rate found for the line Y1128.

HARVESTING

The flowering of the transgenic pollen source was complete in mid August. Harvesting was started in the third week of September, 1991. The *Beta maritima* plants were still flowering at the time of harvest. The receptor plants were harvested by plot and by genotype (of *Beta maritima*). The plants were placed in bags and dried by ventilation prior to threshing and subsequent analysis. A sample (39 plants) of the transgenic plants from the central plot was harvested for confirmation of expression of the marker gene. The *Beta maritima* and male sterile *Beta vulgaris* plants in the central plot were also harvested for evaluations of outcrossing at zero distance.

RESULTS AND DISCUSSION

Bolting and flowering

A summary (Figure 3) and details (Appendix I) of bolting and flowering dates are provided. The transgenic pollen source was shedding pollen from early July until mid-August, and there was generally good synchronicity with the flowering of the receptor plants. Flowering of the CMS plants coincided with the early phase of flowering of the transgenic beet. Flowering of most of the *Beta maritima plots* continued beyond the flowering of *Beta vulgaris* until the harvest in September/ October. Each receptor plot was exposed to transgenic pollen during a period of 2-3 weeks. For all plots considered collectively, exposure to pollen extended over a 5-6 week period. The bolting and flowering dates of the *B. maritima* plants were found to be independent of the planting date. *B. maritima* plants growing in a triticale or barley crop flowered later, and were more variable in timing than those grown amongst sugar beet. The *B. maritima* plants were also more vigorous when growing in the sugar beet field and less vigorous in the cereal crops.

The field trial design was favourable for pollen interception. The major wind direction was from the west (42% of the time recorded) during the flowering period of the pollen source. The receptor plots were largely located in a zone from SE to NW (Figure 1), so wind conditions were favourable for the purpose of the experiment.

Pollen movement and outcrossing

A subsample of 1.000 transgenic seeds harvested from *Beta vulgaris* plants, 4.200 seeds harvested from CMS plants and ca. 17. 500 grains harvested on *Beta maritima* plants were tested for germination. Of these, a total of 3.250 seedlings were assayed for ß-glucuronidase activity to assess pollen movement and outcrossing.

Since the pollen donor plants (transgenic *B.vulgaris*) were still segregating the degree of segregation was estimated by GUS assays on subsamples of the harvested seeds. The results of the GUS assays carried out on seeds harvested on the pollen source indicate that 57 % of these contained the marker gene (Table 1). The theoretical maximum value is estimated at 89%. Since the plants were not bagged the deviation was most likely due to pollination by *Beta maritima* plants in the central plot. Contamination from the surrounding sugar beet field is excluded as bolters were manually removed before flowering. The germination level of the transgenic sugar beet plants was low (35 %) but this is not unusual as selfed OT lines often have low vigor and high inbreeding depression.

Germination of the seeds harvested from the CMS plants in the central core was 71%. The seeds are expected to be mainly hybrids between *B.vulgaris* (pollen donor) and the male sterile *B. vulgaris*. GUS analyses demonstrated that 48 % were GUS positive (Table 1), compared with an expected 66 %, since the pollen produced by the donor was segregating in a 2:1 ratio for the genetic marker. At distances of 25, 50 and 75 meters from the centre the proportion of GUS positive plants was respectively 14, 5 and 3 % (mean of two downwind directions), indicating that pollen moves over distances of at least 75 metres, but with significantly decreasing frequency. Germination of the hybrid seed ranged from 16 to 54 %. The lower germination, compared with the central plot, may indicate a higher frequency of hybridisation with the nearby *B.maritima* plants. If so, *B.vulgaris x B.maritima* hybrids would appear to be less competitive than *B.vulgaris x B.vulgaris* hybrids.

The germination levels of the seed harvested on the *Beta maritima* plants were variable and generally very low. Average germination percentages for the four *Beta maritima* genotypes were in the range of 7-10% (Table 2a). These data are consistent with data available from Maribo, where it was found that *B.maritima* seeds grown in their natural environment in Denmark only germinate at a frequency of 20 %. There were no clear effects of the genotype of *B.maritima*, nor the distance of the *B.maritima* plants from the centre plot (Tables 2 b,c). Interestingly, germination levels were higher for seeds harvested on *B.maritima* plants growing in isolation among triticale (NW) and barley (SE) crops (Table 3). This may reflect a higher level of vigour of *Beta maritima* hybrids (or selfed progeny) compared to *Beta vulgaris* x *Beta maritima* hybrids. To develop a better understanding of the origin of the low germination of seeds harvested on *Beta maritima* plants, crosses between various *B. vulgaris* and *B. maritima* plants will be conducted in future experiments.

The interpretation of the GUS assay results were difficult because of the low germination levels of seed harvested from *Beta maritima* plants (see Appendix II). GUS analyses could only be performed on germinated seeds, and the frequency of GUS positive plants is expressed in reference to the number of germinated seeds and not to the total number of seeds. Seeds harvested from *Beta maritima* plants at the edges of the central plot (zero distance) were found to be 20.2% GUS positive on average, confirming the occurrence of natural hybridisation between *Beta vulgaris* and *Beta maritima* under favourable conditions (high pollen load). The frequency of hybridisation was similar for the four genotypes, ranging from 18.1 to 22.1% (Tables 4 and 5) . The frequency of outcrossing to the *Beta maritima* genotype Y1123, which was completely surrounded by the *B.vulgaris* of the central plot was 35%. This frequency is lower than for the comparable CMS receptor plants in the central plot (48%), indicating pollination between *Beta maritima* plants or self pollination (although *B. maritima* is considered to be mainly self-incompatible).

GUS analyses of seeds (1002 seeds) collected at distances of 25 metres or more from the central plot, indicated only two hybridisation events between *Beta vulgaris* and *Beta maritima*, involving two different *Beta maritima* genotypes, Y1128 and Y1123. Hybridisations occurred at distances of 50 and 75 metres from the pollen source, in NW (triticale) and NE (sugar beet) directions respectively. This represents an overall outcrossing frequency of 0.2% at distances from 25 to 75 metres, compared with the corresponding frequency of 20.2% at zero distance.

CONCLUSIONS

The conclusions that can be drawn from the first of the sugar beet outcrossing studies conducted under BRIDGE are :

- the experimental design employed is suitable for detecting sugar beet pollen movement and outcrossing to *Beta maritima*.
- since the number of analyses which can be performed with GUS is limited, alternative methods, such as Roundup® treatments, should be developed to allow screening larger numbers of plants in future experiments. A larger source of pollen should also be employed to increase pollen pressure.
- with the specific experimental design, pollen pressure decreased significantly from the pollen source to distances of up to75 metres.
- hybridisation between *Beta vulgaris* (O-type parent) and *Beta maritima* occurred naturally under field conditions, but the frequency of hybridisation also decreased significantly with distance from the pollen source.

- The transfer of introduced genes will probably occur with a frequency equal to that for "endogenous" genes, so risk assessment should focus on whether wild beet receiving specific genes are likely to develop a competitive advantage.

ACKNOWLEDGEMENTS

The authors wish to thank Per Steen, Maribo Seed, Denmark for making the plant material available for the experiment, and H.C Pederson, Maribo Seed Denmark for performing the GUS analyses on the donor plants and the harvested seed.

Notes with Figure 1. FIGURE 1: NOT INCLUDED, DETAILED FIELD MAP.

Numbers 1 to 21 represent the locations of harvested samples of *Beta maritima* and male sterile *Beta vulgaris*. At distances of 0 and 25 metres from the pollen source a one metre single row plot of *Beta maritima* was planted, at 50 metres a four metre long row and at 75 metres a 9 metre long row. Within a row plants of the four *Beta maritima* species were alternated, with an inter-plant distance of 25 cm.

Lines 9,11,12,17 and 18 were situated in a winter barley crop, lines 14, 15, 20 and 21 were planted in a triticale crop. All of these lines were substantially less developed than the other lines (1,2,3,4,5,6,7,8,10,13,16 and 19 and the center plot) which were planted in a commercial sugar beet field.

Notes with Figure 2.

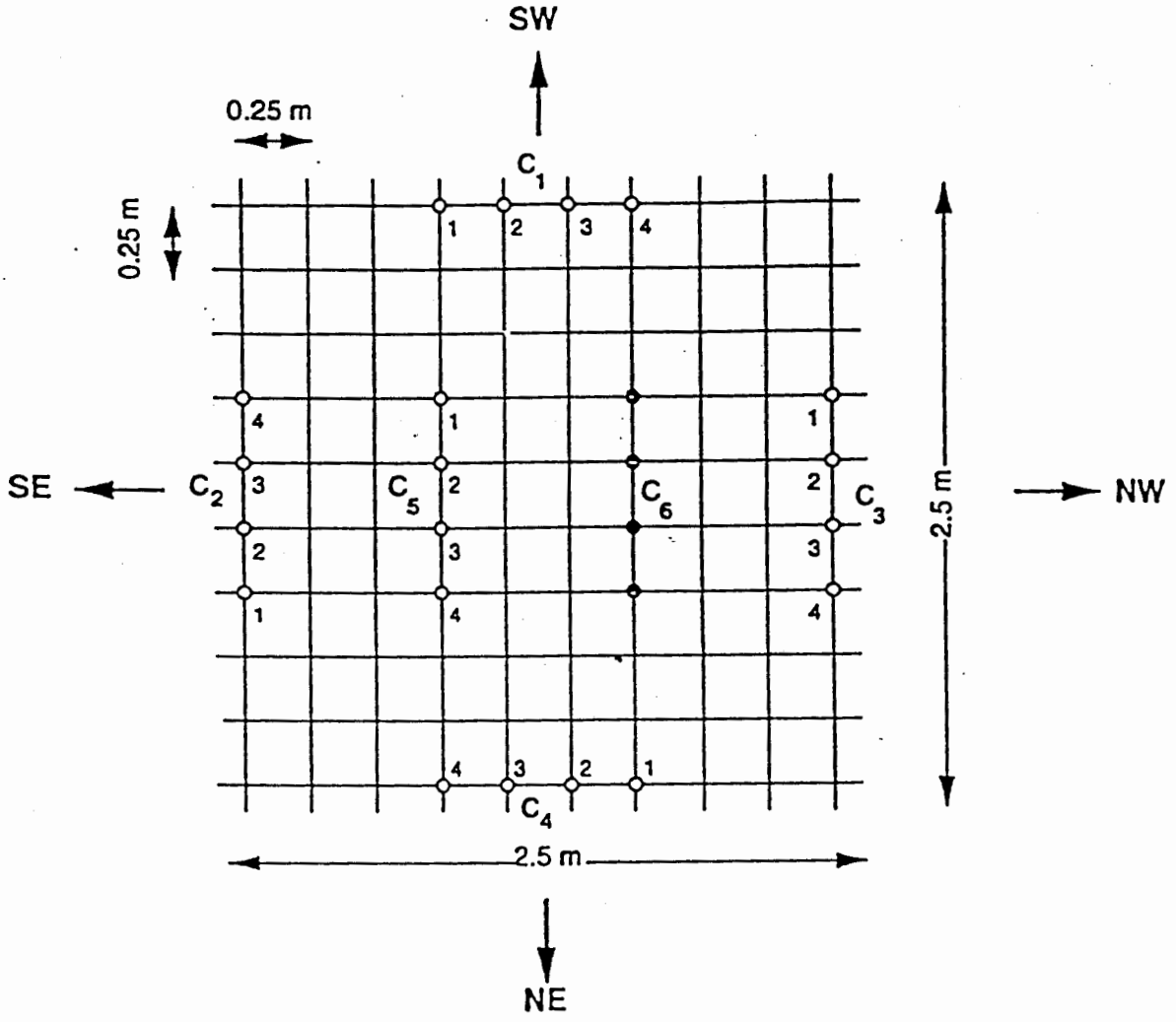
C1, C2, C3 and C4 are *Beta maritima* plants planted in the border of central plot (0 metre distance).

C5 = Two plants each of *Beta maritima* Y1123 and Y1128 planted in the middle of the center plot (Y1124 and Y1126 were exhausted);

C6 = Male sterile *Beta vulgaris* (CMS) plants in the middle of the center plot.

All other plants in the central plot are transgenic sugar beet plants (OT).

Figure 2 : Details of the central block containing the transgenic pollen source



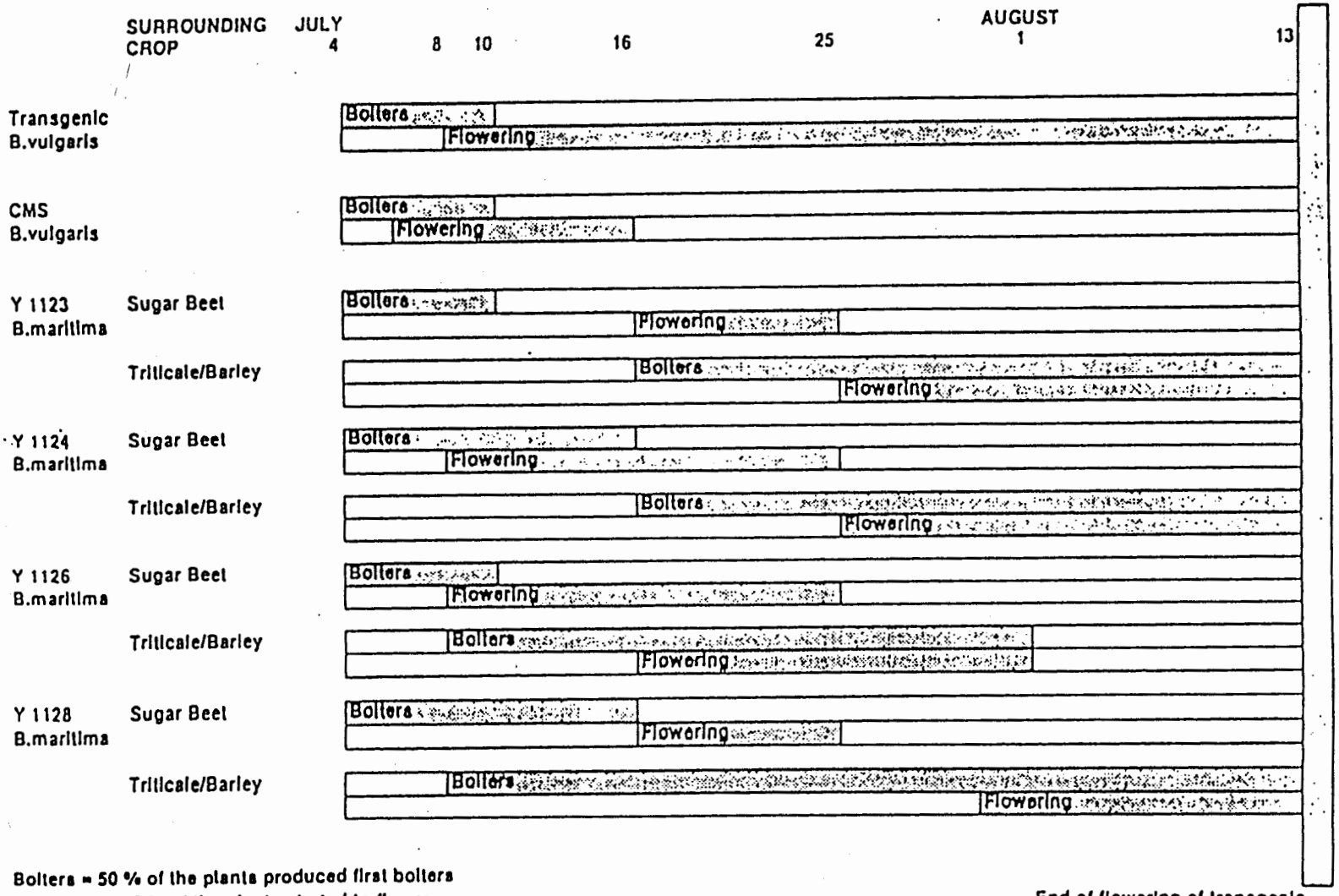
= Beta vulgaris OT

= Beta maritima

= Beta vulgaris CMS

1 = Y 1123
2 = Y 1124
3 = Y 1126
4 = Y 1128

Figure 3. Flowering synchronicity between the different Beta species



Bolters = 50 % of the plants produced first bolters
Flowering = 50 % of the plants started to flower

End of flowering of transgenic pollen source

The range of the dates includes variability occurring between distance * orientation combinations.

Table 1. Germination and GUS+ percentages of seeds harvested from Beta vulgaris plants (pollen donor and male sterile CMS)

Plot Location	Transgenic Beta vulgaris						Male sterile Beta vulgaris (CMS)					
	Seeds Planted	No. of plants	Germ. %	No. of GUS+	GUS+ % of seeds	GUS+ % of plants	Seeds Planted	No. of plants	Germ. %	No. of GUS+	GUS+ % of seeds	GUS+ % of plants
CENTRE	1000	354	35	202	20	57	800	564	71	268	34	48
25 m - N							400	171	43	23	6	14
50 m - N							400	218	54	11	3	5
75 m - N							800	203	25	8	1	4
25 m - E							400	106	27	14	4	13
50 m - E							600	172	29	7	1	4
75 m - E							800	128	16	2	0.3	2

GUS+ = Plants demonstrating B-glucuronidase activity

Table 2. Germination of seeds harvested from Beta maritima and Beta vulgaris as a function of genotype, distance from the pollen source and direction.

a. Genotype

Beta vulgaris - Pollen source	35%
Beta vulgaris - male sterile	38%
Beta maritima Y 1123	6%
Beta maritima Y 1124	9%
Beta maritima Y 1126	10%
Beta maritima Y 1128	7%
Mean of Beta maritima	8%

b. Distance

Genotype	Centre	0 m	25 m	50 m	75 m
Beta vulgaris - male sterile	71	-	35	41	20
Beta maritima Y 1123	6	10	4	7	5
Beta maritima Y 1124	-	17	7	7	6
Beta maritima Y 1126	-	14	8	10	8
Beta maritima Y 1128	-	8	2	7	8
Mean of Beta maritima	6	14	6	8	6

c. Direction*

Genotype	SW	NW	N	NE	E	SE
Beta vulgaris - male sterile	-	-	37	-	22	-
Beta maritima Y 1123	3	14	-	2	3	13
Beta maritima Y 1124	2	12	-	4	7	8
Beta maritima Y 1126	3	20	-	3	13	11
Beta maritima Y 1128	3	21	-	4	3	7
Mean of Beta maritima	3	16	n.a.	3	8	9

n.a = no seeds harvested in the field because of plant death

- = no plants planted in this position

* = Includes only 25, 50 and 75 metre distances (centre and 0m excluded)

Table 3. Germination of Beta maritima as a function of distance and direction from the pollen source

GENOTYPE	DIRECTION	Distance from source				SURROUNDING
		0 m	25 m	50 m	75 m	CROP
Beta maritima Y 1123	SW	15	n.a	2	3	Sugar beet
	NW	9	0	20	21	Triticale
	NE	1	3	2	1	Sugar beet
	E	-	2	3	5	Barley
	SE	n.a	n.a	8	17	Barley
Beta maritima Y 1124	SW	38	0	2	3	Sugar beet
	NW	7	15	12	8	Triticale
	NE	24	0	7	5	Sugar beet
	E	-	0	6	16	Barley
	SE	1	n.a	11	5	Barley
Beta maritima Y 1126	SW	21	3	1	4	Sugar beet
	NW	3	17	14	28	Triticale
	NE	12	2	5	3	Sugar beet
	E	-	10	17	11	Barley
	SE	21	5	20	9	Barley
Beta maritima Y 1128	SW	6	1	2	6	Sugar beet
	NW	n.a	n.a	19	23	Triticale
	NE	n.a	1	7	5	Sugar beet
	E	-	n.a	2	4	Barley
	SE	3	5	3	12	Barley

n.a = no seeds harvested in the field because of plant death
- = no plants planted in this position

Appendix VII. **Letter from Dr. Lee Panella, USDA-ARS Sugarbeet Research to Novartis Seeds regarding the potential for outcrossing from cultivated sugarbeet into wild species in the United States.**

United States Department of Agriculture
Agricultural Research Service
Northern Plains Area
Crops Research Laboratory
1701 Center Ave.
Ft. Collins, Colorado
80526

Dr. Lee Panella
USDA-ARS Sugarbeet Research

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FAX (970) 482-2909
EMAIL lpanella@lamar.colostate.edu

December 29, 1997

Dr. Gerald M. Simantel
Novartis Seeds, Inc.
Sugarbeets - NAFTA
1139 Sugarmill Rd
Longmont, CO 80501

Dear Dr. Simantel,

I have been giving a lot of consideration to your request to comment on the occurrence and potential problems with "weed beet" in the United States, especially in relation to the potential for hybridization between herbicide-resistant sugar beet and any weed beet. I have contacted both public and private weed scientists and researchers who work with sugar beet in all of the sugar beet growing regions of the United States to assure that I was properly informed before commenting. Let me give you a little background for those who may not be as familiar with sugar beet as yourself.

Normally sugar beet (*Beta vulgaris* subspecies *vulgaris*) is a biennial crop that remains vegetative and forms a fleshy taproot as a storage organ (the agronomic crop) in the first year. The plant must undergo a period of cold temperature vernalization before it can enter its reproductive phase, and, in the second year, the sugar beet uses the stored sugar to produce a flower stalk and set seed. If the spring weather is especially cool, some of the sugar beet plants may vernalize in the seedling stage and bolt - i.e., put up a flower stalk it the first year, and sometimes these will set a little seed. These seed have the potential to become weeds in following crops. Additionally, some of the wild relatives of sugar beet, especially those in the subspecies *maritima* that are sexually compatible (i.e. can form fertile hybrids) with sugar beet, have an annual reproductive cycle. These would have the potential to become weeds, and, indeed, are a serious weed problem in parts of Europe where they are native. None of those are, however, native to the United States, and the only area in which they might be present is California. I do not know of any other plant species (outside of *Beta vulgaris* ssp.) in the United States that are sexually compatible with sugar beet.

There have been reports in the literature of sugar beet that has bolted and produced plants from seed the following year. In our rotations, however, sugar beet is generally planted only every third year

and is easily controlled by most broadleaf herbicides, indeed, if the weather conditions are right, even some of those herbicides that are registered for use on sugar beet can cause considerable damage. Our winter weather in most sugar beet growing areas will not allow the root to survive, and any plants produced by seed from bolters do not persist long in the environment. Sugar beet has been cultivated in the Northern High Plains and many other parts of the United States for well over 100 years, and, in that time, no weed beet problem has ever occurred. And we have much better varieties, with fewer bolters, than was the case even thirty years ago.

The story in California is a little bit different due to the climate and historical introduction of cultivated beet by the Spanish and Portugese. I have talked with Dr. R. T. Lewellen, an ARS geneticist who has worked with sugar beet at the USDA-ARS Salinas Research Station for many years. He is familiar with the situation there, and what he reports agrees with what I have seen reported in the literature and heard from others.

There is a wild beet population, the so-called Milpitas wild beet, in the San Francisco Bay area. It is most likely a mixture of escaped and annualized cultivated beet, introduced by the early Spanish settlers, with escaped sugar beet from the early sugar beet culture in this area (began in the last half of the 1800s). This is, however, an area in which sugar beet are no longer grown.

There is also a population of wild weed beets in the Imperial Valley of California. These are another subspecies of *Beta vulgaris*, *B. vulgaris* spp. *macrocarpa*. They are thought to have been introduced in the early settlement of Imperial Valley from the Canary Islands by Portugese immigrants. They are a weed problem in this area. Dr. Lewellen has, however, done some research on this species and it is his opinion that it does not outcross to sugar beet. The are a number of factors supporting this conclusion. First, these plants bolt and flower too early to hybridize with sugar beet - their seed has usually matured before sugar beet bolts and flowers in May to June. *Macrocarpa* is not sexually compatible in crosses with sugar beet. In F_1 hybrids made by Dr. Lewellen, the plants were mostly pollen sterile, and the F_2 plants had very disturbed genetic ratios and growth habit. He feels that they would not survive in nature. Additionally, this population of *B. v.* spp. *macrocarpa* is totally self-fertile. Even in the greenhouse, crosses of *B. v.* spp. *macrocarpa* and sugar beet could only be made with sugar beet as the female, either using self-sterile or male sterile sugar beet plants. In nature, this would not happen because the flowering period of bolted sugar beet comes much later in spring than that the flowering of *B. v.* spp. *macrocarpa*.

Dr. Lewellen feels that any of the wild beets reported in this area that are not the specific *B. v.* spp. *macrocarpa* types were derived from seed from early bolting sugar beet, and these have not been a persistent weed problem. Similarly other annual beets that are periodically reported in California are probably the result of shattered seed of easier bolting, overwintered sugar beet. These do not seem to persist in nature - the exception being the Milpitas wild beet. Some wild beets in California are thought to have arisen from imported Southern European seed, where in the European seed fields, sugar beet was outcrossed to wild weedy beets of *B. v.* spp. *maritima*. Again, with the exception of the Milpitas beet, these have not persisted in nature. Similar wild weedy beets are not known to

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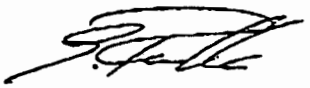
Research, Education and Economics
Agricultural Research Service

occur in Oregon where the USA commercial sugar beet seed is grown.

Finally, as Dr. Lewellen notes, after more than 100 years of sugar beet production and breeding programs in the Salinas Valley of California, where winter planted sugar beet has often bolted and produced hard seed, no wild beet problem is known. Nor has there been obvious outcrosses of wild beets into their seed isolation plots used to make line increases and experimental hybrids.

For these reasons, I believe there is very little risk of a herbicide-resistant sugar beet hybridizing with a weed beet population. And in the remote possibility that this would happen, I do not think that there would be a potential weed problem. There are no persistent wild beet populations known anywhere in the United States, outside of the one in the San Francisco Bay area, where sugar beet is no longer grown. Sugar beet can be controlled with many classes of herbicides and even a weed beet population resistant to a couple of herbicides, if it could persist under our climatic conditions, could be easily controlled before it became a serious weed problem.

Sincerely yours,



Lee Panella
Chair, Sugarbeet Crop Germplasm Committee

Appendix VIII. U.S. Field Trial Reports

Summary Report to the Field Release of Transgenic Sugarbeet Line 77 (GTSB77) Expressing Resistance to the Herbicide Glyphosate.

Date of Report: June 1, 1998

Permit Number: 96-031-01r

Applicant:

[CBI DELETED

]

Dates of Release: June, 1996

Dates of Termination: September, 1996

Sites of Release (States/Number per State): Minnesota/1

Purpose of Release
To evaluate weed control and crop tolerance with glyphosate herbicide applied to sugarbeet (Beta vulgaris) containing the gene which confers resistance to the herbicide glyphosate. Samples were collected for sugar and quality analysis.

Results
Glyphosate herbicide applied three times at the recommended rate of 2.0 pints/A provided complete control of all weeds throughout the growing season. No herbicide symptoms were expressed by the transgenic sugarbeets.

Observations
This plot was observed several times a week, as it was located on the research station. The area planted to transgenic sugarbeet was less than 0.25 acres. The transgenic sugarbeet plant population was two plants per square foot.

Herbicide Tolerance:
Crop tolerance was very good with the recommended use rate of glyphosate; no resistant plants had any symptoms of the herbicide. Transgenic beets were tolerant to other herbicides currently registered in sugarbeets.

Insect Susceptibility:
No differences were observed between the transgenic sugarbeets and nearby commercial fields. Beneficial insects were noted in this trial.

Disease Susceptibility:
Disease resistance in tranformed sugarbeet is not different from its non-transformed counterpart. Observations in the transgenic sugarbeet trial indicate disease tolerance similar to surrounding commercial fields.

Weather Related Conditions:
Near normal weather conditions prevailed throughout the growing season.

Physical Characteristics:
The transgenic sugarbeet plants were observed from emergence through maturity. No differences were observed between the transformed sugarbeets and their non-transformed counterparts or commercial sugarbeets.

Weediness Characteristics:
Growth rate and growth habit were identical in both transgenic and non-transgenic plants.

Means of Plant Destruction:
Destruction of the plants was performed by means of cultivation; both disking and rototilling procedures were utilized.

Time and Methods of Monitoring for Volunteers:
The site was observed throughout the 1997 growing season. No volunteer sugarbeet plants have been observed.

Number of Volunteers Observed and Action Taken:
If volunteer sugarbeet plants are observed, counts will be taken and all volunteer sugarbeet plants will be destroyed by mechanical means, removed by hand, or destroyed with herbicides other than glyphosate.

Summary Report to the Field Release of Transgenic Sugarbeet Line 77 (GTSB77) Expressing Resistance to the Herbicide Glyphosate.

Date of Report: June 1, 1998

Permit Number: 96-309-018r

Applicant:
[CBI DELETED
]

Dates of Release: March, 1997

Dates of Termination: July, 1997

Sites of Release (States/Number per State): Colorado/1, Oregon/4

Purpose of Release
To produce experimental hybrids and seed increase of transformed sugarbeets (Beta vulgaris) containing the gene which confers resistance to the herbicide glyphosate.

Results
The seed productions were successful; no glyphosate was applied to these sites.

Observations
These sites were observed on a weekly basis from planting until harvest at a minimum. The area planted to transgenic sugarbeet was less than 0.25 acres at each location. The transgenic sugarbeet plant population averaged two plants per square foot.

Herbicide Tolerance:
No glyphosate herbicide was applied to any of these sites. The transgenic sugarbeets were tolerant to herbicides currently registered for use on sugarbeets.

Insect Susceptibility:
No differences were observed between the transgenic and non-transgenic sugarbeets at any of the sites. Beneficial insects were noted feeding on the transgenic plants.

Disease Susceptibility:
No differences were observed between the transgenic and non-transgenic sugarbeets at any of the sites.

Weather Related Conditions:
Near normal growing conditions prevailed throughout the growing season.

Physical Characteristics:
Transgenic sugarbeet plants were observed from transplanting through maturity. No differences were observed between transformed and non-transformed sugarbeet plants thoughout the growing season.

Weediness Characteristics:
Growth rate and growth habits were identical in both transgenic and non-transgenic plants.

Means of Plant Destruction:
Following seed harvest, the plants were destroyed by mechanical means; both disking and rototilling procedures were utilized.

Time and Methods of Monitoring for Volunteers:
The sites were observed throughout the remainder of the summer on a weekly basis and monthly during the winter. All sites were rototilled the following spring and continue to be monitored on a weekly basis.

Number of Volunteers Observed and Action Taken:
Volunteer plants, transgenic and non-transgenic were removed by mechanical means, rototilling and physical removal with a hoe on the procedures being utilized.

Summary Report to the Field Release of Transgenic Sugarbeet Line 77 (GTSB77) Expressing Resistance to the Herbicide Glyphosate.

Date of Report: June 1, 1998

Permit Number: 96-361-028r

Applicant:

[CBI DELETED

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Dates of Release: April and May, 1997

Dates of Termination: September and October, 1997

Sites of Release (States/Number per State): Colorado/3, Idaho/2, Minnesota/8, Montana/1, Nebraska/2, North Dakota/4, Oregon/1, Wyoming/2

Purpose of Release

To evaluate weed control, crop tolerance and variety performance with glyphosate herbicide applied to sugarbeet (Beat vulgaris) containing the gene which confers resistance to the herbicide glyphosate. Samples were collected for sugar and qualtiy analysis.

Results

Glyphosate herbicide applied three times at the recommended rate of 2.0 pints/A provided complete control of all weeds throughout the growing season. No herbicide symptoms were expressed by any of the transgenic sugarbeet varieties. The varieties performed in an expected manner.

Observations

These trials were observed on a regular basis throughout the growing season. Each location was observed a minimum of two times/ month. The area planted to transgenic sugarbeet was less than 0.25 acres/site. The trangenic sugarbeet plant population averaged two plants per square foot.

Herbicide Tolerance:

Crop tolerance was very good with the recommended use rate of glyphosate, no resistant plant had any lasting symptoms of the herbicide. At one location, the sugarbeets laid down for a short period following glyphosate application, beets recovered rapidly and appeared normal the remainder of the growing season. Transgenic sugarbeets were tolerant to other herbicides currently registered in sugarbeets.

Summary Report to the Field Release of Transgenic Sugarbeet Line 77 (GTSB77) Expressing Resistance to the Herbicide Glyphosate.

Date of Report: June 1, 1998

Permit Number: 96-361-028r

Applicant: Gerald Simantel
Hilleshög Mono-Hy Inc.
11939 Sugarmill Road
Longmont, CO 80501

Dates of Release: April and May, 1997

Dates of Termination: September and October, 1997

Sites of Release (States/Number per State): Colorado/3, Idaho/2, Minnesota/8, Montana/1, Nebraska/2, North Dakota/4, Oregon/1, Wyoming/2

Purpose of Release
To evaluate weed control, crop tolerance and variety performance with glyphosate herbicide applied to sugarbeet (Beat vulgaris) containing the gene which confers resistance to the herbicide glyphosate. Samples were collected for sugar and qualtiy analysis.

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Herbicide Tolerance:
Crop tolerance was very good with the recommended use rate of glyphosate, no resistant plant had any lasting symptoms of the herbicide. At one location, the sugarbeets laid down for a short period following glyphosate application, beets recovered rapidly and appeared normal the remainder of the growing season. Transgenic sugarbeets were tolerant to other herbicides currently registered in sugarbeets.

Insect Susceptibility:
No differences were observed at any locations between the transgenic sugarbeets and non-transgenic sugarbeets growing at the same site or nearly commercial fields. Beneficial insects were noted at a number of the trial sites.

Disease Susceptibility:
Disease resistance in transformed sugarbeets is not different from its non-transgenic counterpart. Both transformed and non-transformed couterparts were entered into various disease nurseries providing no significant differences. Observations in the transgenic sugarbeet trial indicates disease tolerance is similar to non-transformed and commercial sugarbeets.

Weather Related Conditions:
Near normal weather conditions prevailed throughout the growing season at most locations. Dry soil conditions during emergence reduced the stands at several locations. All trials were carried to harvest.

Physical Characteristics:
The trangenic sugarbeet plants were observed from emergence through maturity. No differences were observed between the transformed and non-transformed sugarbeet counterparts or commercial fields.

Weediness Characteristics:
Growth rate and growth habit were identical in both transgenic and non-transgenic plants.

Means of Plant Destruction
Destruction of the plants was performed by means of cultivation, both disking and/or rototilling procedures were utilized.

Time and Methods of Monitoring for Volunteers:
The sites were observed during the winter following harvest, no volunteers were observed. Observation of these sites will continue throughout the 1998 growing season. No volunteers have been detected to date.

Number of Volunteers Observed and Action Taken:
If volunteer sugarbeet plants are observed, counts will be taken and all volunteer sugarbeet plants will be destroyed by mechanical means, removed by hand or destroyed with herbicides other than glyphosate.

Individual Site Information

Stanislaus County, CA
Planting Date: May 23, 1997

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. Trials were monitored June 24, July 23, August 20, and September 23, 1996.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.
Initial monitoring revealed five percent insect damage in both the transgenic and non-transgenic plants. Monitoring on August 20, 1996 revealed 80%-100% insect damage due to diabrotica and beet army worm in both the transgenic and non-transgenic plants. Trials were monitored June 24, July 23, August 20, and September 23, 1996.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Trials were monitored June 24, July 23, August 20, and September 23, 1996.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. Trials were monitored June 24, July 23, August 20, and September 23, 1996.

Weld County, CO
Planting Date: June 1, 1996
Harvest Date: November 11, 1996

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. Trials were monitored July 3, August 5, September 1, and October 8, 1996.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. Trials were monitored July 3, August 5, September 1, and October 8, 1996.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Trials were monitored July 3, August 5, September 1, and October 8, 1996.

Field Monitoring for Weediness Characteristics: The field trial observation July 3, 1996 revealed that germination of transgenic plants was different from non-transgenic plants due to late planting and dry conditions after planting. There were no differences noted in the germination stages when trials were monitored August 5, September 1, and October 8, 1996.

Power County, ID
Planting Date: May 20, 1996

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants

Twin Falls County, ID
Planting Date: May 24, 1996
Harvest Date: October 17, 1996

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. Trials were monitored June 27, 1996.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. Trials were monitored June 27, 1996.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Trials were monitored June 27, 1996.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. The trials were monitored June 27, 1996.

Saginaw County, MI
Planting Date: May 28, 1996

Field Monitoring for Disease Susceptibility: The transgenic plants did have a higher incidence of disease than the non-transgenic plants, exhibiting cercospora leaf spots but it was no different than adjacent fields of sugarbeet. Trials were monitored September 20, 1996.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. Trials were monitored September 20, 1996.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Trials were monitored September 20, 1996.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. Trials were monitored September 20, 1996.

Reville County, MN
Planting Date: May 29, 1996
Harvest Date: September 27, 1996

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. Trials were monitored June 25, August 2, August 30, and September 27, 1996.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. Trials were monitored June 25, August 2, August 30, and September 27, 1996.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Trials were monitored June 25, August 2, August 30, and September 27, 1996.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. Trials were monitored June 25, August 2, August 30, and September 27, 1996.

Polk County, MN
Planting Date: May 28, 1996
Harvest Date: September 6, 1996

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants.

Clay County, MN
Planting Date: May 28, 1996

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. Trials were monitored June 18, July 24, and August 13, 1996.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. Trials were monitored June 18, July 24, and August 13, 1996.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Trials were monitored June 18, July 24, and August 13, 1996.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. Trials were monitored June 18, July 24, and August 13, 1996.

Scotts Bluff County, NE
Planting Date: May 21, 1996
Harvest Date: October 9, 1996

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.

Field Monitoring for Plant Growth Characteristics: Some transgenic plants did exhibit differences in appearance, looking abnormal, when compared to non-transgenic plants.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants.

Richland County, ND
Planting Date: May 29, 1996
Harvest Date: October 7, 1996

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants; both exhibited cercospora leaf spot at final harvest. Trials were monitored October 4, 1996.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. Trials were monitored October 4, 1996.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Trials were monitored October 4, 1996.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. Trials were monitored October 4, 1996.

Hockley County, TX
Planting Date: June 3, 1996
Harvest Date: October 30, 1996

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. Both groups exhibited necrotic areas. Trials were monitored July 1, July 29, August 27, September 23, and October 23, 1996.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. Trials were monitored July 1, July 29, August 27, September 23, and October 23, 1996.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Trials were monitored July 1, July 29, August 27, September 23, and October 23, 1996.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. Trials were monitored July 1, July 29, August 27, September 23, and October 23, 1996.

1996 SUGARBEET FIELD RELEASE
USDA 96-063-01R/MONS # 96-041PR
FINAL REPORT

Ramona G. Edwards
Monsanto Company

The purpose of this residue study is to supply data on the glyphosate residue levels that will likely result in or on sugarbeet raw agricultural commodities (beet and tops) as a result of the application of Roundup Ultra® herbicide according to label directions for current uses plus the proposed label directions for topical applications afforded by the use of glyphosate sugarbeet plants.

Location	County	State
[CBI DELETED]	Fresno	CA
	Tulare	CA

These trials were cancelled.

1997 SUGARBEET FIELD RELEASE
USDA 97-029-02R/MONS # 97-039PR
FINAL REPORT

Ramona G. Edwards
Monsanto Company

The purpose of these field trials are efficacy studies for weed control and crop tolerance.

Site

Location	County	State
[CBI DELETED	Yolo	CA
	Canyon	ID
	Twin Falls	ID
	Saginaw	MI
	Ingham	MI
	Polk	MN
	Chippewa	MN
	Scottsbluff	NE
	Scottsbluff	NE
	Cass	ND
	Cass	ND
	Goshen	WY
]	Park	WY

Individual Site Information

Yolo County, CA

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.

Field Monitoring for Plant Growth Characteristics: The transgenic plants did not exhibit differences in appearance or growth when compared to non-transgenic plants.

Field Monitoring for Weediness Characteristics: The transgenic plants did not exhibit differences in germination when compared to non-transgenic plants.

Canyon County, ID

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.

Field Monitoring for Plant Growth Characteristics: The transgenic plants did not exhibit differences in appearance or growth when compared to non-transgenic plants.

Field Monitoring for Weediness Characteristics: The transgenic plants did not exhibit differences in germination when compared to non-transgenic plants.

Twin Falls County, ID

Planting Date: April 28, 1998

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.

Field Monitoring for Plant Growth Characteristics: The transgenic plants did not exhibit differences in appearance or growth when compared to non-transgenic plants.

Field Monitoring for Weediness Characteristics: The transgenic plants did not exhibit differences in germination when compared to non-transgenic plants.

Saginaw County, MI
Planting Date: April 28, 1998

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.

Field Monitoring for Plant Growth Characteristics: The transgenic plants did exhibit differences in appearance or growth when compared to non-transgenic plants. The transgenic line had poor growth early in the season. This was overcome within two months. Yield was not reduced in either line.

Field Monitoring for Weediness Characteristics: The transgenic plants did not exhibit differences in germination when compared to non-transgenic plants.

Ingham County, MI
Planting Date: April 28, 1998

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.

Field Monitoring for Plant Growth Characteristics: The transgenic plants did exhibit differences in appearance when compared to non-transgenic plants. Until mid summer the transgenic line had very poor growth early in the season and stayed small compared to the non transgenic line.

Field Monitoring for Weediness Characteristics: The transgenic plants did not exhibit differences in germination when compared to non-transgenic plants.

Polk County, MN
Planting Date: May 20, 1998
Harvest Date: October 1, 1998

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. Crops were monitored June 16, June 28, July 7, July 15, July 25, and August 14, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. Crops were monitored June 16, June 28, July 7, July 15, July 25, and August 14, 1997.

Field Monitoring for Plant Growth Characteristics: The transgenic plants did not exhibit differences in appearance or growth when compared to non-transgenic plants. Crops were monitored June 16, June 28, July 7, July 15, July 25, and August 14, 1997.

Field Monitoring for Weediness Characteristics: The transgenic plants did not exhibit differences in germination when compared to non-transgenic plants. Crops were monitored June 16, June 28, July 7, July 15, July 25, and August 14, 1997.

Chippewa County, MN
Planting Date: May 20, 1998

Field Monitoring for Disease Susceptibility: The transgenic plants did have a higher incidence of Cerocospera leaf spot disease than the non-transgenic plants.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.

Field Monitoring for Plant Growth Characteristics: The transgenic plants did not exhibit differences in appearance when compared to non-transgenic plants.

Field Monitoring for Weediness Characteristics: The transgenic plants did not exhibit differences in germination when compared to non-transgenic plants.

Scottsbluff Country, NE
Planting Date: April 22, 1998
Harvest Date: September 24, 1998

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. Trials were monitored May 21, June 5, June 10, June 26, July 10, August 8, and October 29, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.
Trials were monitored May 21, June 5, June 10, June 26, July 10, August 8, and October 29, 1997.

Field Monitoring for Plant Growth Characteristics: The transgenic plants did not exhibit differences in appearance when compared to non-transgenic plants. Trials were monitored May 21, June 5, June 10, June 26, July 10, August 8, and October 29, 1997.

Field Monitoring for Weediness Characteristics: The transgenic plants did not exhibit differences in germination when compared to non-transgenic plants. Trials were monitored May 21, June 5, June 10, June 26, July 10, August 8, and October 29, 1997.

Scottsbluff Country, NE
Planting Date: April 22, 1998
Harvest Date: September 24, 1998

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. Trials were monitored May 21, June 5, June 10, June 26, July 10, August 8, and October 29, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.
Trials were monitored May 21, June 5, June 10, June 26, July 10, August 8, and October 29, 1997.

Field Monitoring for Plant Growth Characteristics: The transgenic plants did not exhibit differences in appearance when compared to non-transgenic plants. Trials were monitored May 21, June 5, June 10, June 26, July 10, August 8, and October 29, 1997.

Field Monitoring for Weediness Characteristics: The transgenic plants did not exhibit differences in germination when compared to non-transgenic plants.
Trials were monitored May 21, June 5, June 10, June 26, July 10, August 8, and October 29, 1997.

Cass County, ND
Planting Date: May 16, 1998
Harvest Date: October 1, 1998

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. Trials were monitored June 9, June 30, July 9, July 19, July 24, and August 11, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.
Trials were monitored June 9, June 30, July 9, July 19, July 24, and August 11, 1997.

Field Monitoring for Plant Growth Characteristics: The transgenic plants did not exhibit differences in appearance when compared to non-transgenic plants. Trials were monitored June 9, June 30, July 9, July 19, July 24, and August 11, 1997.

Field Monitoring for Weediness Characteristics: The transgenic plants did not exhibit differences in germination when compared to non-transgenic plants. Trials were monitored June 9, June 30, July 9, July 19, July 24, and August 11, 1997.

Cass County, ND
Planting Date: May 30, 1998
Harvest Date: September 23, 1998

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. Trials were monitored June 18, July 11, and August 12, and September 4, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.
Trials were monitored June 18, July 11, and August 12, and September 4, 1997.

Field Monitoring for Plant Growth Characteristics: The transgenic plants did not exhibit differences in appearance when compared to non-transgenic plants. Trials were monitored June 18, July 11, and August 12, and September 4, 1997.

Field Monitoring for Weediness Characteristics: The transgenic plants did not exhibit differences in germination when compared to non-transgenic plants. Trials were monitored June 18, July 11, and August 12, and September 4, 1997.

Goshen County, WY
Planting Date: April 23, 1998
Harvest Date: September 29, 1998

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.

Field Monitoring for Plant Growth Characteristics: The transgenic plants did exhibit differences in appearance when compared to non-transgenic plants. Weed counts, crop stand counts and visual crop injury ratings were made June 26, 1997. Slight injury was evident with Roundup treatments initiated at the cotyledoary leaf stage. Initial sugarbeet stands were not adequate in any treatment and decreased three to thirty nine percent by harvest in all treatments except the hand weeded check. Sugarbeet yields in the hand weeded check were lower than in herbicide treated plots because of late weed removal in this treatment.

Field Monitoring for Weediness Characteristics: The transgenic plants did not exhibit differences in germination when compared to non-transgenic plants.

Park County, WY
Planting Date: April 24, 1998
Harvest Date: September 26, 1998

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.

Field Monitoring for Plant Growth Characteristics: The transgenic plants did exhibit differences in appearance when compared to non-transgenic plants. Weed counts, crop stand counts and visual crop injury ratings were made June 17, 1997. Slight to moderate injury was evident with Roundup treatments applied at the cotyledoary leaf stage. All Roundup treatments reduced initial sugarbeet stands eighteen to fifty-seven percent compared to the untreated check. Plots began being hoed June 24, 1997 with hoe times closely related to weed control and lowest with Roundup treatments intiated at the 4-Leaf stage.

Field Monitoring for Weediness Characteristics: The transgenic plants did not exhibit differences in germination when compared to non-transgenic plants.

1997 SUGARBEET FIELD RELEASE
USDA 97-190-02N/MONS # 97-219XR
FINAL REPORT

Ramona G. Edwards
Monsanto Company

Location	County	State
[CBI DELETED]	Marion	OR

Marion County, OR
Planting Date: September 1997
Harvest Date: March 1998

This bi-annual crop was planted in an outdoor nursery until they reached the reproductive stage. They were not evaluated for any agronomic traits, i.e., growth, and weediness characteristics or for disease or insect susceptibly. The location changed for the second stage and they are in a commercial field under USDA 98-037-04N.

98-173-01p

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14 August, 1998

Dr. James White
USDA-APHIS
4700 River Road
Riverdale, MD 20737-1237

Subject: Glyphosate-Tolerant Sugarbeet Line GTSB77 supplemental information

Dear Dr. White:

The attached information is in response to your letter of July 24, 1998 regarding deficiencies in Petition 98-173-01p for glyphosate-tolerant sugarbeet line 77 (GTSB77).

The following modification to the Petition should be made to address Point 11 (as number in the August 7, 1998 letter from Jeff Stein).

Point 11: modified pages 5-7 are enclosed to address the issue of weedy *Beta* sp. distribution.

Please let me or Jeff Stein know if you have any questions on this information.

Sincerely,

Raymond Dobert

Raymond Dobert, Ph.D.
Regulatory Affairs Manager
Monsanto Company

8/19/98

Novartis Seeds, Inc.
Seeds Biotechnology Research Unit
3054 Cornwallis Road
Research Triangle Park, North Carolina 27709-2257
Telephone 919-541-8683 Telefax 919-541-8535

Jeffrey Stein
Senior Regulatory Affairs Manager

August 7, 1998

Dr. James White
USDA-APHIS
4700 River Road
Riverdale, MD 20737-1237

Dear Dr. White,

RE: Petition No. 98-173-01p

This letter (and enclosures) is in response to your letter of July 24, 1998 regarding deficiencies discovered during your review of the above noted Petition for non-regulated status for glyphosate tolerant sugarbeet line 77 (GTSB77). Please note the following additions and/or modifications to the Petition, provided in the same order as indicated in your letter (attached):

Point1: the narrative description of plasmid PV-BVGT03 has been added to page 1. Please find enclosed modified versions of page 1 – 2.

Point 2: a modified Page 3 is enclosed

Point 3: a modified Table 3.2 (page 13) is enclosed (see legend)

Point 4: a modified Section III (pages 8 – 11) is enclosed to address these deficiencies. In particular, please see pages 10 – 11.

Point 5: please see the modified page 10 (enclosed). In particular, see paragraph 1. Legends for modified Figures 3.2 (page 15), 3.3 (page 16), 3.4 (page 17), 3.6 (page 19), and 3.7 (page 20) now indicate the amount of DNA loaded in each lane.

Points 4, 6 & 7: please find enclosed new versions (six original copies of each) of Figure 3.2 (page 15), Figure 3.3 (page 16), and Figure 3.4 (page 17), Figure 3.6 (page 19), and Figure 3.7 (page 20). The corresponding text in Section III (pages 8 – 11) has also been modified.

Point 8: An analysis of the 43 amino acid residues from sugarbeet which were found to be in frame with the truncated *gox* gene in GTSB77 (protein 34550) was conducted. The sequence (see

I. Rationale for Development of Glyphosate Tolerant Sugarbeet Line 77

The products which are the subject of this application are seeds of glyphosate-tolerant sugarbeets and seeds of any progeny (inbred or hybrid) derived from GTSB77 by conventional breeding. This application addresses safety issues associated with the environmental release and commercial production of GTSB77 in the United States and Europe, as well as processing and eventual food and feed use of the derived products. Seeds of GTSB77 will be marketed as new varieties of sugarbeets (*Beta vulgaris*), and the products obtained from these beets will be introduced into commerce as any other new variety.

GTSB77 has been genetically engineered with a gene from *Agrobacterium* sp. strain CP4 that expresses enolpyruvylshikimate-3-phosphate synthase (EPSPS). The CP4 EPSPS gene is flanked by the figwort mosaic virus (FMV) promoter, and a chloroplast transit peptide (CTP) from *Arabidopsis thaliana* and the pea (*Pisum sativum*) E9 3' terminator. The CP4 EPSPS, like other EPSPS enzymes, catalyses the conversion of shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) into 5-enolpyruvylshikimate-3-phosphate (EPSP), an intermediate in the production of aromatic amino acids (Hermann, 1983; Haslam, 1974). Unlike other EPSPS enzymes that are inhibited by glyphosate, the catalytic activity of CP4 EPSPS is unaffected by glyphosate, and plants expressing CP4 EPSPS are tolerant to Roundup (Padgette *et al.* 1996).

GTSB77 has also contains the *uidA* (GUS; β-D-glucuronidase) gene from *E. coli* with an enhanced 35S promoter from cauliflower mosaic virus (CaMV) and E9 3' terminator from pea. The *uidA* gene expresses the GUS protein which served as a scorable marker during the plant transformation process (Jefferson *et al.*, 1987; Raju *et al.*, 1991). A truncated version of the glyphosate oxidoreductase (*gox*) gene from *Ochrobactrum anthropi* sp. is also present in GTSB77, but expresses a non-functional enzyme designated protein 34550. This gene utilizes the figwort mosaic virus (FMV) promoter, and a chloroplast transit peptide (CTP) from *Arabidopsis thaliana*.

The nature of the product and the objective of the genetic modification are to improve weed management practices in sugarbeets. Weed management is regarded as an expensive, labor intensive, and in some cases complicated operation necessary for optimal production efficiency of sugarbeets. No single currently approved herbicidal ingredient offers the broad-spectrum weed control afforded by glyphosate. Instead, farmers must resort to using multiple herbicides in several applications at highly variable cost and performance efficiency.

GTSB77 has been field tested at numerous sites across the U.S., under USDA permits or notifications[1](Appendix VIII), with no indications of toxicity toward insects, birds, or other species, and no detectable adverse environmental impact. In addition, EPSPS enzymes are already present in plants (including sugarbeet) and microorganisms. Furthermore, β-

[1] USDA Permit Nos. 96-031-01, 96-057-03, 96-061-01, 96-309-01, 96-361-02, 97-029-02, and USDA Notification Nos. 97-169-03, 97-182-08, 97-190-02, 98-035-01, 98-050-02, 98-057-01, 98-072-11, 98-079-11.

glucuronidase (GUS) is found in mammals and many microorganisms. Both are ubiquitous in nature and present in food and feeds.

GTSB77 has been demonstrated to be substantially equivalent to the parental variety. It is being crossed into adapted varieties by traditional breeding methods, and will be grown in the same geographic regions and with the same practices as current varieties. The herbicides that are currently available for sugarbeets do not provide broad spectrum weed control compared to glyphosate. Most current herbicides are effective for control of annual grasses, while glyphosate controls annual grasses, annual broadleaves, and perennial weed species.

The use of GTSB77 for sugarbeet production would enable a farmer to use glyphosate herbicide for effective control of weed pests while receiving the benefits of its environmental safety characteristics. These new glyphosate-tolerant sugarbeets can positively impact current agronomic practices by: 1) offering farmers broad-spectrum weed control, 2) allowing the use of an environmentally acceptable herbicide, 3) enhanced flexibility to treat weeds "as needed", 4) offering less dependence on use of pre-emergent herbicides, and 5) providing cost-effective weed control due to the reduced price of glyphosate herbicide compared to competitive products accepted for use in sugarbeets. These seeds may consist of inbred or hybrid lines developed using conventional breeding methods. Seeds of GTSB77 will be marketed as new varieties of sugarbeets, and the products obtained from these sugarbeets will be introduced into commerce as any other new variety of sugarbeets.

Cultivated *B. vulgaris* varieties are not invasive, are weakly competitive outside cultivated areas, and possess few weedy characteristics. Data included in this Petition demonstrate that GTSB77 are substantially equivalent to non-modified beets except in regards to tolerance to glyphosate. Furthermore, volunteer or bolting plants are readily managed using numerous agricultural practices including other herbicides, hand weeding, and cultivation.

II. BIOLOGY AND PRODUCTION OF SUGARBEET

A. Economics and Use of Sugarbeet

Sugarbeet has a history of safe use; sugar and other processed fractions are consumed in many human food products or animal feeds. Currently, sugarbeet is the major sugar crop grown in temperate regions of the world. Total worldwide sugar production in 1996 is estimated at 123 million tons. Commercial sugarbeet production in the United States occurs in 13 states, with the majority grown in North Dakota, Minnesota, Michigan, Wyoming, California, and Colorado. These states account for 85% of the total area cultivated with beets in the country and almost 85% of the total United States sugarbeet production. The sugarbeets have an estimated total value of $1.3 billion to the country's beet farmers.

The overall contribution of the growing, harvesting, and post-harvest processing of sugarbeets to U.S. employment amounts to 21,800 full-time jobs and 57,300 seasonal jobs, with a the total wage bill estimated to be $553.2 million (1993 figures).

Sugar is a multi-purpose carbohydrate that contributes significantly to the flavor, aroma, texture, color and body of a variety of foods. Sugar helps bread rise by acting as a food source for the yeast. In all baked products, sugar contributes to the flavor and crust color as well as prolonged shelf life. In addition to being an important component in jams and jellies, sugar is a contributor to bulk, texture and body of ice cream, beverages, baked goods, and other products.

In addition to processing pure sugarbeet sugar, sugar factories also produce a by-product known as dried sugarbeet pulp. This pulp can be produced and shipped in many forms, including plain dried, molasses dried, and pelleted. These fractions are used for feed for dairy cattle, feeding cattle, and sheep. In the western US growing region, livestock (cattle and sheep) infrequently (<1% of total acres) graze on sugarbeet tops that remain in the fields following harvest.

Another important by-product is sugarbeet molasses, a viscous liquid containing about 48% saccharose, which cannot be crystallized. Sugarbeet molasses is used for production of yeast, chemicals, pharmaceuticals, as well as in the production of mixed cattle feeds.

B. Taxonomy

Sugarbeet has been grown as a food crop for more than 150 years, and is taxonomically classified as follows:

a) Family name: Chenopodiaceae
b) Genus: *Beta*
c) Species: *vulgaris*
d) Subspecies *vulgaris*
e) Cultivar line: A1012
f) Common name: sugarbeet

For the taxonomic division of the genus *Beta* see Table 2.1.

genetics of bolting resistance in biennial beets is still unclear. Some studies suggest that it is governed by several genes with different degrees of dominance (Le Cochec and Soreau, 1989), while others suggest that it is largely recessive (Mc Farlane *et al.*, 1948)

The majority of wild Mediterranean *Beta* beets are annuals, but biennial types also occur. North Atlantic *B. maritima* types are normally perennial. The annual growth habit is governed by a dominant gene B (Abegg, 1936), which causes plants that carry it to run to seed very quickly under conditions of long days and reasonably high temperatures.

D. Potential for Genetic Transfer and Exchange with Other Organisms

Sugarbeet is predominantly wind pollinated and the pollen can travel shorter or longer distances depending on the windforce, humidity and temperature. Pollen trapping experiments conducted in England showed that 900 meters downwind of its release point, pollen concentration had fallen to 0.5% of that at the release point (Dark, 1971).

According to the OECD beet seed scheme of October 10, 1988, basic seed production must be at least 1000 meters distance from any pollen source of the genus *Beta.* For production of certified seed, the minimum isolation distance varies from 300 meters to1000 meters, depending on the chromosome number of the intended pollinator and the chromosome number of a neighboring pollen source.

In the United States, the majority of sugarbeet seed production takes place in Oregon. For certified seed production, a minimum isolation distance of 3,200 feet (approximately 1,000 meters) between sugarbeets with different backgrounds is required, and at least 8,000 feet (approximately 2,500 meters) from other *Beta* species

Typically, in seed production areas the pollinator stecklings and CMS stecklings are planted with 2 and 4-8 rows respectively. After flowering and pollen dispersal, the pollinator plants are removed in order to optimize seed quality. When the seed starts to mature, the seed-bearing plants are often cut and placed on the stubble or treated with a herbicide to have improved and synchronous ripening. In most instances, the seed are then harvested directly in the field with a combine.

The wild relatives of sugar beet originated in Asia Minor but some forms are widely distributed throughout the Mediterranean. All cultivated beets (both leaf-beets and those with swollen roots) are likely to have originated from wild maritime beets through simple selection by man. Sugarbeet (*Beta vulgaris* ssp. *vulgaris*) is the sole or main crop for sugar production in the temperate zones of the northern hemisphere. Since the Second World War sugarbeet has also been grown as a winter crop in countries with warmer climates such as Morocco, Algeria, Tunisia, Egypt, Syria, Iraq and Iran. Sugarbeet is not reported to be a weed in the US (WSSA, Composite List of Weeds, 1994) and is not reported to be a serious weed in other countries where it is grown.

Sugarbeet hybridizes freely with all wild members of the section *Beta* (Table 2.1), and the resulting hybrids are normally fully fertile. Of the wild relatives that can interbreed with sugarbeet, only *B. vulgaris* ssp. *maratima* and *B. vulgaris* ssp. *macrocarpa* are present in the US, and these isolated populations are limited to California (see Section

VII.C.2). These wild species (*B.v.* ssp. *maritima* and ssp. *macrocarpa*) are not recognized as being serious weeds in the US (WSSA, Composite List of Weeds, 1994).

Holms (1979, *A Geographic Atlas of World Weeds*) lists *B. vulgaris* (without distinguishing between the various wild subspecies) as a serious weed in Egypt, a common weed in Iraq, Israel and Portugal, and a weed of unknown importance in the US, Morocco, Afghanistan, Australia and Mexico. Global distribution of the wild members of the section *Beta* as reported by Terrell (1986, *A Checklist of Names for 3,000 Vascular Plants of Economic Importance.* USDA Agric. Handb. 505) are listed in Table 2.1.

Wild annual *Beta* beets (primarily *B.vulgaris* ssp. *maritima*) grow as weeds in fields or on wasteland in many parts of the Mediterranean area. Stray pollen from such weed beets had very limited possibilities for contaminating seed crops since these were well protected by an abundance of their own pollen. However, with the introduction of hybrid varieties, where 75% of the plants in the seed production fields are male sterile, pollen contamination from wild species can be a problem, especially in triploid seed production, since the tetraploid male parent plants usually open their flowers and release pollen later in the morning than do diploids (Scott and Longden, 1970). Thus, the diploid male sterile flowers may susceptible to fertilization by stray pollen. According to the OECD beet seed scheme, a seed production field is certified only if there is assurance that there are no volunteer plants of the genus *Beta.* As a consequence, breeders in Europe have moved seed production away from areas with known weed beet populations, and test the seed from every seed grower for the presence of crosses between sugar beet and annual weed beet. In these tests, all seed lots with a frequency of over 0.2% annual hybrids are discarded.

Artificial hybrids can be produced (with difficulty) with the species in the section *Corollinae*. However, such hybrids are highly sterile and set few seed when back-crossed to sugarbeet. Artificial hybrids between sugarbeet and members of the section *Procumbentes* normally die at the seedling stage. They can be saved by grafting onto sugarbeet and may then develop into vigorous plants. These hybrids are almost completely sterile and set few seed upon back-crossing. No hybrids between cultivated beets and *B. nana* of section *Nanae* have been reported.

In conclusion, within the family Chenopodiacea, all crosses between cultivated sugarbeet and species from sections other than *Beta*, are highly improbable.

Table 2.1 Taxonomic division of the genus Beta (based on DeBock, 1986)

SPECIES	CHROMOSOME NUMBER (2n)	DISTRIBUTION[1]
Section 1: Beta (syn: vulgares)		
B. vulgaris L.	18	Global (cultivated)
B. maratima L.	18	N. Africa, Portugal, Spain, Egypt Israel, Jordan, Syria, Turkey, Albania Belgium, Bulgaria, Denmark, France Germany, Greece, Ireland, Italy, Netherlands Sweden, U.K., Yugoslavia
B. macrocarpa Gus.	18, 36	N. Africa, Spain, Israel, Jordan Greece, Italy, Portugal
B. atriplicifolia Rouy	18	Europe
B. patula Ait.	18	Portugal
B. orientalis Roth.	18	India (cultivated)
Section 2: Corollinae		
B. macrorhiza Stev.	18	
B. lomatogona Fish et Mey.	18, 36	
B. corolliflora Zos.	36	
B. trigyna Wald et Kit.	45, 54	
B. intermedia Bunge	36	
B. foliosa Hausskn.	?	
Section 3: Nanae		
B. nana Bois. Et Held.	18	
Section 3: Patellares		
B. procumbens Chr. Sm.	18	
B. webbiana Moq.	18	
B. patellaris Moq.	36	

[1] From Terrell, E.E. 1986. A Checklist of Names for 3,000 Vascular Plants of Economic Importance. U.S.D.A. Agric. Handb. 505.

below) was compared to the SwissProt (GenBank+EMBL+DDBJ+PDB) protein sequence database using the BLAST search engine. No sequences with any homology were found.

An analysis of the 258 nucleotides that were sequenced from sugarbeet which are downstream of the truncated *gox* gene in GTSB77 was also conducted. The sequence (see below) was compared to the GenBank nucleotide sequence database using the BLAST search engine. The results of this analysis (see attached query summary) indicated that the span with the greatest homology (23 of 24 nucleotides) was from a sea urchin ectodermal gene. The longest consecutive stretch of sequence homology was 20 nucleotides derived from the *Homo sapiens* chromosome 16. These small stretches of homology are not considered to be biologically significant.

Protein Sequence:
SLAMP TKLIKNMKQQ LQTVEHTFYK LAXISIGSYK TVKLPIGY

Nucleotide Sequence:

[CBI DELETED

]

TAG indicates the putative stop codon

Point 9: please find enclosed a modified page 26. The language under Section A been modified to more accurately reflect the data in Table 5.1

Point 10: modified versions of Tables 6.1 – 6.11 (pages 33 - 43) are enclosed.

Point 11: Ray Dobert will be forwarding to you, under separate cover, a response to this point

Point 12: The footnote on page 1 has been modified, and now lists all release permits and notifications for this sugarbeet event.

Point 13: these questions have been addressed in the modified text on page 8.

Also enclosed is a modified Table 3.1 (page 12), as you requested.

Please do not hesitate to contact me with any questions.

Sincerely,

Jeff Stein
Novartis Seeds

III. MOLECULAR BIOLOGY AND GENETIC ANALYSIS OF GTSB77

A. Description of Vector PV-BVGT03 and Method of Transformation

GTSB77 was produced by transforming a proprietary sugarbeet line (A1012) with plasmid PV-BVGT03, a disarmed *Agrobacterium tumefaciens* double border plant transformation vector (Figure 3.1). The plasmid contains (1) the C-terminal 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) gene from *Agrobacterium*, (2) the *uidA* gene, from *E. coli,* encoding a β-D-glucuronidase (GUS) protein, (3) a glyphosate oxidoreductase (*gox*) gene from *Ochrobactrum anthropi,* and (4) a neomycin phosphotransferase (*nptII*) gene from *E. coli*, all within the right and left borders of the vector. In addition, the plasmid contains a bacterial selectable marker gene (*spc/str)* as well as origins of replication (*ori-V* and *ori-322*) necessary for replication and maintenance of the plasmid PV-BVGT03 in bacteria. More detail regarding the genetic elements in vector PV-BVGT03 is presented in Table 3.1.

A disarmed *Agrobacterium tumefaciens* plant transformation system was used to produce GTSB77 (*Euphytica* 94: 83-91, 1997). This plant transformation system is well documented to transfer and stably integrate T-DNA into a plant's nuclear chromosome (White, 1989; Howard *et al.*, 1990). Only those DNA sequences within the left and right border sequences [CP4 EPSPS, *uidA* (GUS), *gox*, and *nptII*] are expected to be transferred and integrated into the plant chromosome.

Following transformation, Agrobacterium cells were eliminated by incubating plant tissue with cefotaxime (0.5g/L; 3X 60'). Transformed tissue was selected and plants regenerated in the presence of glyphosate (1 mM) as well as cefotaxime (0.5g/L) to ensure elimination of Agrobacterium cells (*Euphytica* 94: 83-91, 1997).

B. Origin of Donor Genes and Regulatory Sequences

1. The *cp4 epsps* gene

The *cp4 epsps* gene cassette consists of the figwort mosaic virus (FMV) promoter, a chloroplast targeting sequence from *Arabidopsis thaliana*, the *cp4 epsps* coding region from *Agrobacterium* sp. strain CP4, and a 3' nontranslated region from pea which directs polyadenylation. This gene codes for the protein CP4 EPSPS, which catalyses the conversion of shikimate-3-phosphate (S-3-P) and phosphoenolpyruvate (PEP) into 5-enolpyruvylshikimate-3-phosphate (EPSP), an intermediate in the production of aromatic amino acids (Hermann, 1983; Haslam, 1974). The CP4 EPSPS protein is highly resistant to inhibition by glyphosate, the active ingredient in the Roundup herbicide.

The original gene sequence from *Agrobacterium* was modified to create a synthetic gene, which allows for higher expression in plants. Bacterial genes, such as those from *Agrobacterium,* have several features that reduce their ability to function efficiently in plants. These features include potential polyadenylation sites that are often rich with A+T nucleotides, a higher G+C nucleotide percentage than that frequently found in dicotyledonous plant genes, concentrated stretches of G and C nucleotide residues, and codons that may not be found frequently in dicotylendonous plant genes.

2. The *gus* gene

The *gus* (*uidA*) gene cassette contains the enhanced 35S promoter from the cauliflower mosaic virus, the *uidA* coding region for the β-D-glucuronidase protein from *E. coli*, and the 3' nontranslated region from pea which directs polyadenylation. This gene serves as a marker during the plant transformation process.

3. The *gox* gene

The *gox* gene cassette consists of the figwort mosaic virus promoter, a chloroplast targeting sequence from *Arabidopsis thaliana*, the *gox* coding region from *Ochrobactrum anthropi*[1], and a 3' nontranslated region of the nopaline synthase gene, which directs polyadenylation. When expressed, the function of the glyphosate oxidase (GOX) enzyme is to metabolize glyphosate (N-phosphonomethylglycine), the active ingredient in Roundup herbicide, to an inactive form. As with the *cp4 epsps* gene above, the original *gox* gene sequence from *Ochrobactrum anthropi* was modified to create a synthetic gene which allows for higher expression in plants.

4. The *nptII* gene

The neomycin phosphotransferase II gene is from transposon Tn5. The NPTII enzyme coded by this gene confers resistance to selected aminoglycoside antibiotics and is used as a plant selectable marker (Beck *et al.*, 1982). However, this gene was not transferred into the sugar beet genome because of the truncation of the insertion event within the *gox* gene in PV-BVGT03.

5. The chloroplast transit peptide genes (CTP1 and CTP2)

Targeting of the CP4 EPSPS and GOX protein to the chloroplast has been shown to be critical to achieving the greatest levels of tolerance to glyphosate (della-Cioppa *et al.*, 1987). The *ctp2* sequence from the *Arabidopsis thaliana epsps* gene is fused to the 5-prime end of *cp4 epsps* to enhance tolerance, while *ctp1*, the sequence encoding the chloroplast transit peptide derived from the small subunit of rubisco from *A. thaliana,* was fused to the 5-prime end of the *gox* gene. For functionally active proteins, these peptides are rapidly digested immediately after import into the chloroplast. Similar signal peptides are present in all plants and are of no toxicological concern.

6. Genetic elements beyond the borders of the T-DNA

The following elements are present on the plasmid PV-BVGT03, but are outside of the borders of the T-DNA, and are hence not expected to be transferred into the sugarbeet genome.

- *ori-V*: a 0.4 Kb origin of replication segment derived from the broad-host range plasmid RK2 is located just outside the left border of PV-BVGT03.

- *aad*: a 0.79 Kb gene isolated from transposon Tn7 is located just outside the right border of PV-BVGT03. This gene encodes the enzyme streptomycin adenyltransferase that allows the selection of transformed bacteria on culture medium containing spectinomycin or streptomycin.

- *ori-322*: a 0.6 Kb segment which provides an (1) origin of replication for maintenance of the PV-BVGT03 plasmid in *E. coli* and (2) a site for the conjugational transfer into the *Agrobacterium tumefaciens* cells is located between the *aad* gene and the *ori-V* gene.

C. Southern Hybridization Analysis of GTSB77

[1] A previous designation was *Achromobacter* sp. strain LBAA.

Methodology

Total DNA was extracted from sugarbeet tissue using the QIAGEN DNeasy™ Plant Mini Kit according to the manufacturer's instructions. DNA (10 μg) was digested with the appropriate restriction enzymes, fractionated by electrophoresis in 0.6% agarose gels and transferred to Hybond-N⁺ membranes (Amersham) by capillary blotting in 20xSSC transfer buffer (Sambrook *et al*, 1989). Hybridizations were conducted using probe fragments generated by PCR amplification of the corresponding sequences in plasmid PV-BVGT03, and radio-labeled using the AlkPhos Direct labeling kit from Amersham, according to manufacturer's instructions.

1. The *cp4 epsps* and right border region

Using specific restriction endonucleases and DNA probes, it is possible to reliably estimate the number of T-DNA inserts in a transformed plant's genome. In order to estimate the number of *cp4/epsp* inserts, two restriction enzymes were chosen that either restrict at a single site within the *cp4/epsps* coding region (*BclI*) or at a single site flanking (3-prime) the *cp4/epsps* coding region (*NcoI*) (Figure 3.1). There are no *BclI* restriction sites 5-prime to the *cp4/epsps* coding region (within the right T-DNA border); the most proximal 5-prime *BclI* will be in the sugarbeet chromosomal DNA. Digesting GTSB77 genomic DNA with *BclI* and using a *cp4/epsps*-specific hybridization probe (nucleotides 356-1147; Figure 3.1), representing sequences within the *cp4/epsps* coding region, a unique band (greater than 1.8 kb) should be visible for each *cp4/epsps* sequence present in GTSB77. The data from the Southern blot shows one band at approximately 3.2 kb (Figure 3.2). Similarly, there are no *NcoI* restriction sites 5-prime to the *cp4/epsps* codong region within the right T-DNA border. Digesting GTSB77 genomic DNA with *NcoI* and using a *cp4/epsps*-specific hybridization probe, a unique band (greater than 3.7 kb) should be visible for each *cp4/epsps* sequence present in GTSB77. The data from the Southern blot shows a band at approximately 5.7 kb (Figure 3.2). While a faint band at approximately 7.0 kb is also present, we attribute this band to incomplete digestion of the sugarbeet genomic DNA. The Southern blot data from both restriction digests suggests a single insert of this portion of the T-DNA.

2. The *gus* gene

The presence of the *uidA* gene in the GTSB77 genome was confirmed by Southern hybridization. Digestion of genomic DNA with *Xba*I, *BamHI*, or *HindIII* and using a *uidA*-specific probe (nucleotides 3177-4218; Figure 3.1), representing sequences within the *uidA* coding region, yielded single hybridizing bands (Figure 3.3).

3. The *gox* gene

In order to elucidate the number of gox inserts and integrity of the left border region, GTSB77 genomic DNA was restricted separately with *XbaI*, *BamHI*, and *HindIII*. The enzyme *HindIII* restricts between the E9 3' terminator 3-prime to the GUS gene and the 5-prime end of the FMV promoter (Figure 3.1). By digesting GTSB77 genomic DNA with *HindIII* and using a *gox*-specific hybridization probe (nucleotides 6489-6916; Figure 3.1), representing sequences within the *gox* coding region, a unique band (greater than 4.4 kb) should be visible for each *gox* insert. The data from the Southern blot reveal one band of approximately 2.0 kb, indicating a single insert of this portion of the T-DNA (Figure 3.4). The enzyme *XbaI* restricts at a single location between the 3-prime end of the FMV promoter and the 5-prime end of the gox gene. Digesting GTSB77 genomic DNA with *XbaI* and using an identical *gox*-specific hybridization probe, a unique band (greater than 3.7 kb) should be visible for each *gox* insert. The data from the Southern blot indicate one band of approximately 6.8 kb, supporting the *HindIII* restriction data that indicates a single insert of this portion of the T-DNA. There was no hybridization of the gox probe to the DNA digested with *BamHI*.

The observation that the *gox*-homologous DNA present in the genome of GTSB77 is smaller than expected suggested that the left portion of T-DNA might not have integrated as a complete entity. In order to elucidate the exact nature of the inserted DNA, the nucleotide sequence of the flanking DNA sequences to the inserted DNA was determined.

A Lambda FIXII phage library of GTSB77 genomic DNA was probed with both *cp4/epsps* and *gox* probes. Of 25 of the initial plaques pulled from the library, two hybridized to both the *cp4/epsps* and the *gox* probe. The DNA from one of these was recloned and the nucleotide sequence of the adjacent sugarbeet DNA determined, revealing the junction sites of the sugarbeet genome and the integrated DNA. The right border junction of the integrated DNA was at bp 15116 (Figure 3.1), between the 25 bp right border and the FMV promoter. The left border junction of the integrated DNA was at bp 7372, *within* the coding region of the *gox* gene, 897 basepairs downstream of the *gox* gene start codon (Figures 3.1, 3.5). Downstream (3-prime) of the *gox* gene fragment (within the sugarbeet genomic DNA), two translational stop codons located 130 and 234 bp from the junction were identified. In addition, a *HindIII* site was found 231 bp downstream from the junction site (~2.0 kb from the FMV promoter *HindIII* site) as well as a transcription termination signal (AATAAA) 650 bp from the junction point. Based upon these data, it is apparent that the complete DNA insert within the left and right T-DNA borders present in the transformation vector PV-BVGT03 is not present in the genome of GTSB77. This resulted in a truncated form of the *gox* gene, which is fused to sugarbeet genomic DNA.

4. Other PV-BVGT03 sequences

To determine whether sequences outside of the T-DNA border region of PV-BVGT03 had been transferred into the genome of GTSB77, total DNA was digested with appropriate restriction enzymes, and subject to Southern hybridization using either a PCR-generated DNA probe homologous to the entire *oriV* sequence present in plasmid PV-BVGT03 (nucleotides 9906-11912; Figure 3.1), or a probe homologous to the entire *ori322/aad* sequence present in plasmid PV-BVGT03 (nucleotides 12571-14980; Figure 3.1). There was no hybridization between either of these probes and GTSB77 genomic DNA, indicating that these sequences were not transferred (or stably integrated) into the sugarbeet genome (Figures 3.6, 3.7).

D. Mendelian Inheritance

Glyphosate tolerance in other commercial crops (e.g., soybean, canola, and cotton) transformed with a similar *cp4 epsps* gene is inherited as a dominant trait; a single copy (allele) of the introduced *cp4 epsps* confers whole plant tolerance to glyphosate. Novartis Seeds' plant breeders have conducted numerous backcrosses and selfing (utilizing conventional breeding techniques) with the original GTSB77 line. The inheritance of the introduced DNA in the progeny from these crosses is monitored phenotypically at the whole plant level by application of glyphosate herbicide and/or performing *in vitro* β-D-glucuronidase (GUS) assays. Data from these analyses provide further evidence of the number of loci as well as the stability of the introduced DNA. The results from a typical analysis are presented in Table 3.2. The number of GUS-positive plants (222) in the F2 generation is very close to the expected value (213) for a single locus (as predicted by the Southern analysis) acting in a dominant fashion. Further, the number of glyphosate-tolerant plants in the F3-generation (derived from selfed-F2) were also as predicted for a single-dominant locus. These results prove that the single T-DNA insert present in GTSB77 is inherited as a single locus in a stable manner.

Table 3.1. Summary of the Genetic Elements in PV-BVGT03

Genetic Element	Size (Kb)	Function
Right Border	0.03	A 25 nucleotide sequence that acts as the initial point of DNA transfer into plant cells originally isolated frompTiT37 (Depicker et al., 1982).
P-FMV	0.67	The 35S promoter from a modified figwort mosaic virus used to drive expression of CP4 EPSPS and *gox* genes (Shepard *et al.*, 1987; Richins *et al.*, 1987; Gowda *et al.*, 1989).
AEPSPS/CTP2	0.31	The N-terminal chloroplast transit peptide sequence from the *Arabidopsis thaliana* EPSPS gene (Richins *et al.*, 1987; Gowda *et al.*, 1989; Sanger *et al.*, 1993).
CP4syn	1.36	The C-terminal 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) gene from *Agrobacterium* sp. strain CP4 (Padgette *et al.*, 1993a).
E9 3'	0.63	The 3' end of the pea rbcS E9 gene which provides the polyadenylation sites for the CP4 EPSPS and GUS genes (Coruzzi *et al.*, 1984; Morelli *et al.*, 1985).
P-35S	0.62	The cauliflower mosaic virus (CaMV) promoter (Odell *et al.*, 1985) with the duplicated enhancer region (Kay *et al.*, 1985) used to drive expression of the GUS and *nptII* genes.
GUS:1	1.81	The *uidA* gene from *E. coli* encoding a β-D-glucuronidase or GUS protein (Jefferson *et al.*, 1986).
E9 3'	0.63	The 3' end of the pea rbcS E9 gene which provides the polyadenylation sites for the CP4 EPSPS and GUS genes (Coruzzi *et al.*, 1984; Morelli *et al.*, 1985).
P-FMV	0.67	The 35S promoter from a modified figwort mosaic virus used to drive expression of CP4 EPSPS and *gox* genes (Shepard *et al.*, 1987; Richins *et al.*, 1987; Gowda *et al.*, 1989).
CTP1	0.17	The N-terminal chloroplast transit peptide sequence from the small subunit 1A of rubisco from *A. thaliana* (Timko *et al.*, 1988).
GOXsyn	1.30	The glyphosate oxidoreductase (*gox*) gene isolated from *Achromobacter* sp. strain LBAA (Barry *et al.*, 1994).
NOS 3'	0.26	The 3' nontranslated region of the nopaline synthase gene from *Agrobacterium* which terminates transcription and directs polyadenylation (Fraley *et al.*, 1983).
P-35S	0.62	The cauliflower mosaic virus (CaMV) promoter (Odell *et al.*, 1985) with the duplicated enhancer region (Kay *et al.*, 1985) used to drive expression of the GUS and *nptII* genes.
KAN	0.80	The neomycin phosphotransferase II gene from Tn5. This enzyme confers resistance to aminoglycoside antibiotics and used as a plant selectable marker (Beck *et al.*, 1982).
NOS 3'	0.26	The 3' nontranslated region of the nopaline synthase gene from *Agrobacterium* which terminates transcription and directs polyadenylation (Fraley *et al.*, 1983).
Left Border	0.03	A 25 nucleotide sequence that delimits the T-DNA transfer and acts as the endpoint of DNA transfer into plant cells. It was originally isolated frompTiA6 (Barker et al., 1983).
ori-V	0.39	origin of DNA replication, originally isolated from plasmid RK2; permits plasmid replication in *Agrobacterium*. (Rogers *et al.*, 1987).
ori-322	0.63	Origin of replication isolated from the plasmid pBR322; permits plasmid replication in *E. coli* (Sutcliffe, 1979).
Spc/Str	0.79	The bacterial gene encoding the Tn7 AAD 3" adenyltransferase conferring spectinomycin and streptomycin resistance to bacterial cells that carry the plant vector (Fling *et al.*, 1985).

Table 3.2. Segregation of glyphosate-tolerant sugarbeet obtained from GTSB77

Generation	Number of glyphosate-tolerant plants (*RR* or *Rr*)	Number of non-tolerant plants (*rr*)
BC_1, F2 [1] -actual results: -expected results:	 222 [3] 213	 62 71
BC_1, F3 [2] -actual results: -expected results:	 25 [3] 25.5	 9 8.5
Expected proportions (Mendelian inheritance)	75 %	25 %

[1] Selection of plants made with the GUS test.

[2] Selection of plants made with an application of Roundup herbicide, applied at the anticipated label rate (1 liter/acre)

[3] Chi-square probability: 21 %.

V. AGRONOMIC PERFORMANCE OF GTSB77

Summary
The agronomic performance of GTSB77 was compared to non-transgenic sugarbeet in order to ascertain whether any unintended changes had occurred as a result of the transformation process or the expression of novel proteins. Evaluations included both laboratory studies as well as numerous field trials under a diverse set of geographical and environmental conditions. Parameters evaluated include disease susceptibility, sensitivity to fungicides, insecticides, and herbicides, plant morphology, and vigor. The results from these studies indicate that except for tolerance to glyphosate, GTSB77 is indistinguishable from non-transgenic sugarbeet.

A. Germination rate (emergence), seed dormancy, and invasiveness

Results from field trials indicate no differences in germination and emergence of GTSB77 compared to non-transgenic control (Table 5.1).

Overwintering capacity (frost /cold tolerance) of GTSB77 sugarbeet seed and plant tissue has been evaluated. Observations of fields (following harvest) in which GTSB77 had been cultivated indicate no germination of sugarbeet seed. Results from whole plant studies also indicate that the overwintering capacity nor competitiveness (invasiveness) of GTSB77 has not been altered relative to non-transgenic sugarbeet (Appendices 2 - 5).

B. Vegetative vigor

As a hybrid crop, the vegetative vigor of sugarbeet is dependent on the genetic composition of the parental lines. Commercial varieties are produced by crossing a monogerm cytoplasmic male sterile line (CMS) and a multigerm pollinator (the glyphosate-tolerant trait can be introduced from either parent in the cross). Field trials have been conducted with populations segregating for the glyphosate-tolerant trait. Plants exhibiting glyphosate tolerance are indistinguishable from the controls (glyphosate-sensitive) in terms of growth rate, general appearance, and final yield. In addition, observations by plant breeders during European and North American field trials indicate that GTSB77 is indistinguishable from non-transgenic sugarbeet with regards to susceptibility to predation by insects, as well as to diseases and abiotic factors (Table 5.1; Appendix VIII).

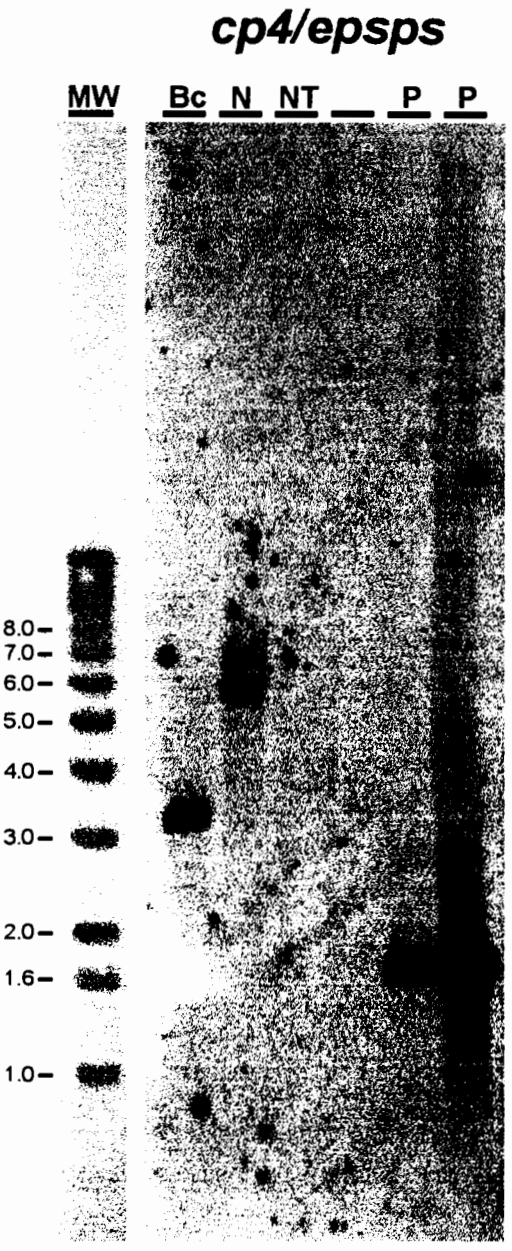
Table 6.2. Proximate Analyses of Top Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature range [2]
	Mean [3]	Range [3]	Mean [3]	Range [3]	
Crude Ash [4]	21.99	18.70-24.79	22.51	18.17-26.84	11.5-34.4
Crude Fibre [5]	9.18	8.46-9.84	9.34	7.83-10.14	5.9-15.9
Crude Protein [6]	13.00	9.45-16.24	13.24	9.73-16.25	8.4-23.2
Crude Fat [7]	2.56	2.06-3.26	2.51	2.06-3.08	0-4.7
Dry Matter [8]	14.79	11.93-17.41	14.89	11.99-17.25	16.0-20.0
Soluble Carbohydrates [9]	53.27	49.78-55.13	52.39	48.92-55.03	38.3-64.5

1 Tissue samples were collected from field studies conducted at various locations in Europe in 1996.
2 See reference DLG, 1991.
3 n=6, all analyses were conducted in triplicate, and all values are given on a dry matter basis except dry matter.
4 Crude ash was determined using an oven method # EF L 155/13 p.430 12/7-71 modified.
5 Crude fibre was determined using the Weende method # EF L 344/36-3726/11-92.
6 Crude protein was determined using a total nitrogen value determined using a Kjeldahl method (# EF L 179/9-10 22/7-93 modified) multiplied by 6.25.
7 Crude fat was determined using a soxhlet method # EF L 15/29-30 18/1-84 modified.
8 Dry matter was determined using an oven method # EF 71/393/EOF; L 279/7 p.858-61 20/12-71.
9 Carbohydrate Calculation was based on Plantedirecktoratet bek. #19 13/1-92.

Figure 3.2

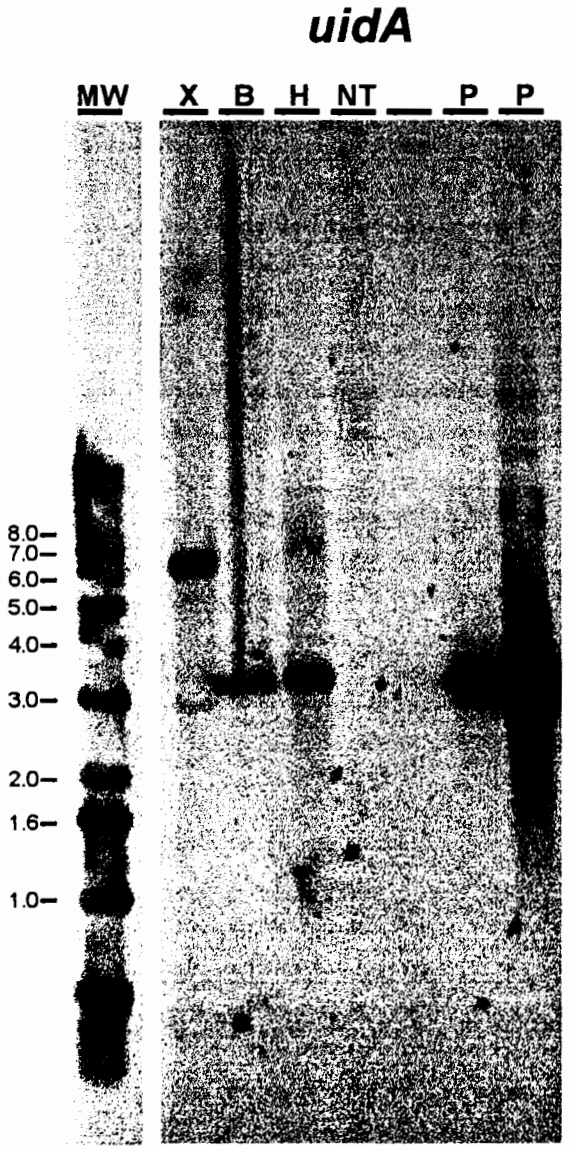
Southern blot analysis of GTSB77 using *cp4/epsps* sequence as probe



Probe: *cp4/epsps* sequence from vector PV-BVGT03(nucleotides 356-1147, Figure 3.1).
Lane Bc: GTSB77 DNA (10 ug) digested with restriction enzyme *Bcl* I.
Lane N: GTSB77 DNA (10ug) digested with restriction enzyme *Nco* I.
Lane NT: Non-transformed control sugarbeet DNA digested with restriction enzyme *Nco*I.
Lane P: Plasmid PV-BVGT03 digested with restriction enzyme *Eco*RI.
MW: 1 Kb molecular weight standard.

Figure 3.3

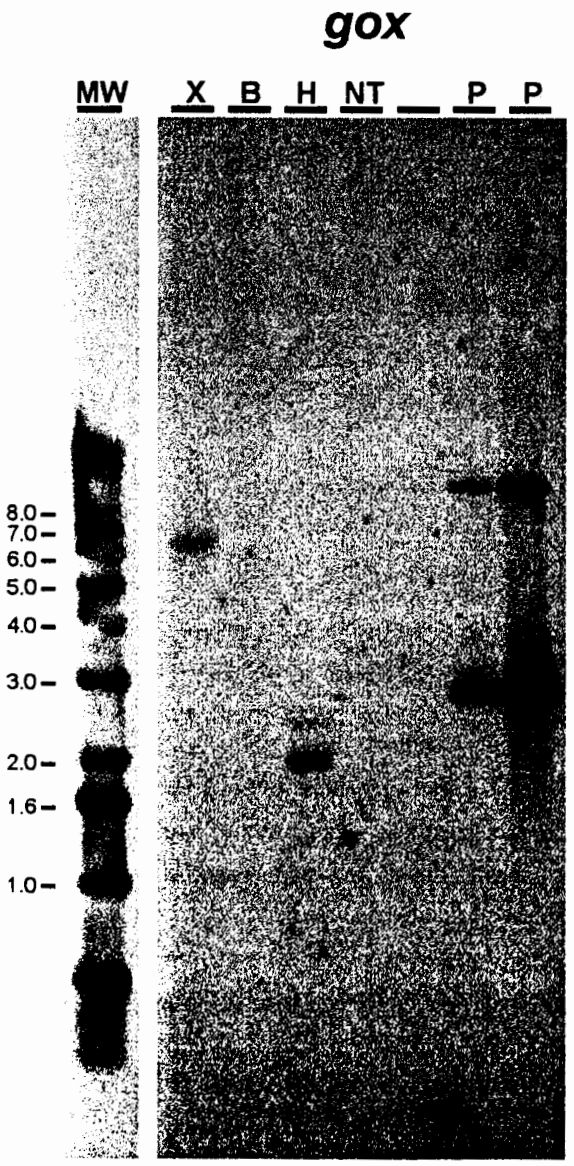
Southern analysis of GTSB77 using *uid*A (*gus*) sequence as probe



Probe: *uid*A (*gus*) sequence from vector PV-BVGT03(nucleotides 3177-4218, Figure 3.1).
Lane X: GTSB77 DNA (10 ug) digested with restriction enzyme *Xba* I.
Lane B: GTSB77 DNA (10 ug) digested with restriction enzyme *Bam*HI.
Lane H: GTSB77 DNA (10ug) digested with restriction enzyme *Hin*d III.
Lane NT: Non-transformed control sugarbeet DNA digested with restriction enzyme *Bam*HI.
Lane P: Plasmid PV-BVGT03 digested with restriction enzyme *Bam*HI.
MW: 1 Kb molecular weight standard.

Figure 3.4

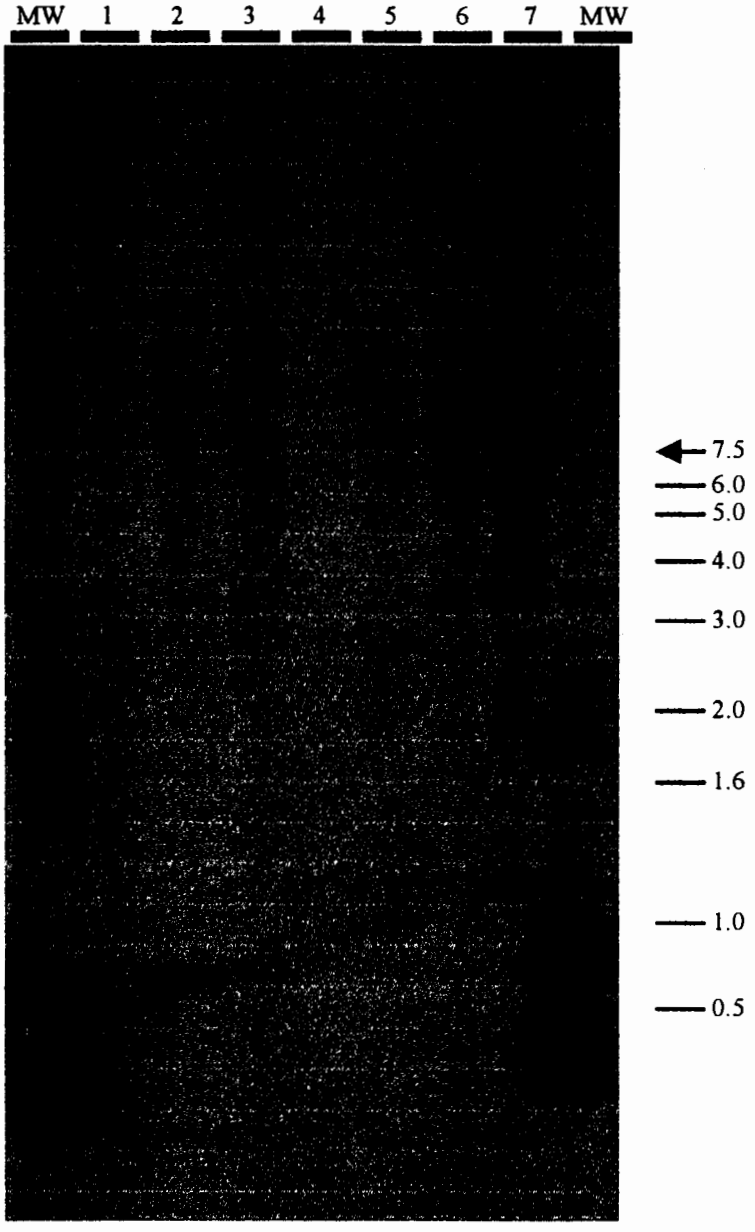
Southern blot analysis of GTSB77 using *gox* sequence as probe



Probe: *gox* sequence from vector PV-BVGT03 (nucleotides 6489-6916, Figure 3.1).
Lane X: GTSB77 DNA (10ug) digested with restriction enzyme *Xba* I.
Lane B: GTSB77 DNA (10 ug) digested with restriction *Bam*HI.
Lane H: GTSB77 DNA (10 ug) digested with restriction enzyme *Hin*dIII.
Lane NT: Non-transformed control sugarbeet DNA digested with restriction enzyme *Bam*HI.
Lane P: Plasmid PV-BVGT03 digested with restriction enzyme *Bam*HI.
MW: 1 Kb molecular weight standard

Figure 3.6

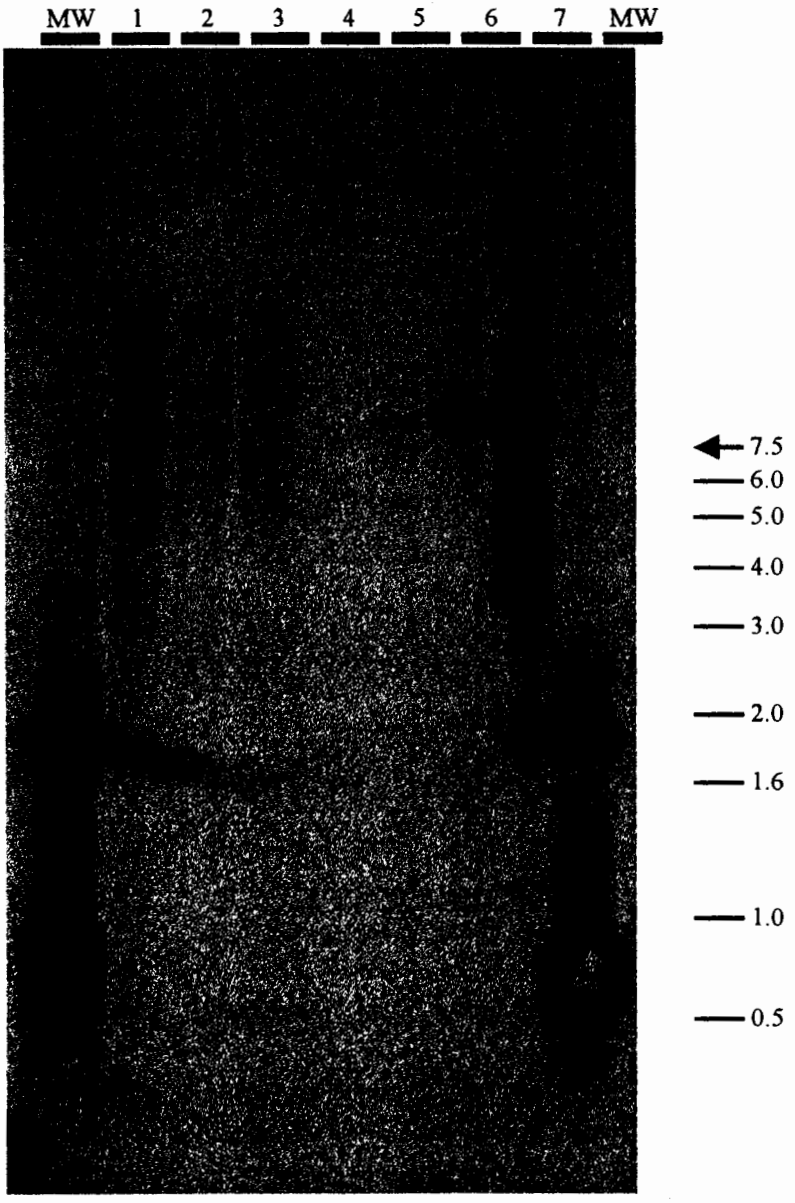
Southern analysis of GTSB77 using *ori-322* sequence as probe



Probe: *ori-322* sequence from vector PV-BVGT03 (nucleotides 12571-14980, Figure 3.1).
Lane 1: GTSB77 DNA (10 ug) digested with restriction enzyme *EcoRI*.
Lane 2: GTSB77 DNA (10 ug) digested with restriction enzyme *HindIII*.
Lane 3: Non-transformed control sugarbeet DNA digested with restriction enzyme *EcoRI*.
Lane 4: Empty lane.
Lanes 5-7: Increasing amounts of PV-BVGT03 DNA digested with *EcoRI*.
MW: 1Kb molecular weight standard.

Figure 3.7

Southern analysis of GTSB77 using *ori-v* sequence as probe



Probe: *ori-v* sequence from vector PV-BVGT03 (nucleotides 9906-11912, Figure 3.1).
Lane 1: GTSB77 DNA (10 ug) digested with restriction enzyme *EcoRI*.
Lane 2: GTSB77 DNA (10 ug) digested with restriction enzyme *HindIII*.
Lane 3: Non-transformed control sugarbeet DNA digested with restriction enzyme *EcoRI*.
Lane 4: Empty lane.
Lanes 5-7: Increasing amounts of PV-BVGT03 DNA digested with restriction enzyme *EcoRI*.
MW: 1 Kb molecular weight standard.

Table 6.1. Proximate Analyses of Top Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature range [2]
	Mean [3]	Range [3]	Mean [3]	Range [3]	
Crude Ash [4]	21.69	14.10-25.78	20.56	15.82-25.87	11.5-34.4
Crude Fibre [5]	10.52	9.59-11.70	10.64	9.03-12.40	5.9-15.9
Crude Protein [6]	15.56	12.88-16.88	16.13	13.69-17.81	8.4-23.2
Crude Fat [7]	2.22	1.47-3.17	2.19	1.43-3.07	0-4.7
Dry Matter [8]	14.37	12.95-16.43	13.99	12.76-16.50	16.0-20.0
Soluble Carbohydrates [9]	49.98	45.03-61.41	50.52	46.06-57.94	38.3-64.5

1 Tissue samples were collected from field studies conducted at various locations in Europe in 1995.
2 See reference DLG, 1991.
3 n=6, all analyses were conducted in triplicate, and all values are given on a dry matter basis except dry matter.
4 Crude ash was determined using an oven method # EF L 155/13 p.430 12/7-71 modified.
5 Crude fibre was determined using the Weende method # EF L 344/36-3726/11-92.
6 Crude protein was determined using a total nitrogen value determined using a Kjeldahl method (# EF L 179/9-10 22/7-93 modified) multiplied by 6.25.
7 Crude fat was determined using a soxhlet method # EF L 15/29-30 18/1-84 modified.
8 Dry matter was determined using an oven method # EF 71/393/EOF; L 279/7 p.858-61 20/12-71.
9 Carbohydrate Calculation was based on Plantedirecktoratet bek. #19 13/1-92.

Table 6.3. Proximate Analyses of Top Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature
	Mean [3]	Range [3]	Mean [3]	Range [3]	range [2]
Crude Ash [4]	20.60	18.3-24.3	21.6	16.2-28.2	11.5-34.4
Crude Fibre [5]	8.46	6.11-10.4	8.76	6.56-10.7	5.9-15.9
Crude Protein [6]	16.1	10.5-18.4	14.7	10.0-18.3	8.4-23.2
Crude Fat [7]	0.79	0.73-1.03	0.92	0.76-2.16	0-4.7
Dry Matter [8]	15.3	13.9-16.5	16.3	14.9-19.6	16.0-20.0
Soluble Carbohydrates[9]	54	47.0-62.3	53.1	45.0-61.4	38.3-64.5

1 Tissue samples were collected from field studies conducted at various locations in the USA in 1996.
2 See reference DLG, 1991.
3 Values are taken from analyses of samples from 5 sites (n=5) for line #77 and for control, with the exception of the ash analyses conducted in duplicate for line #77 (n=10). All values are given on a dry matter basis except dry matter.
4 Crude ash was determined using method AOAC Official Ash Method 923.03, 1990, modified.
5 Crude fibre was determined using AOAC method 962.09, 1990, modified.
6 Crude protein was determined via total nitrogen determination. (AOAC Official Methods 992.03 and 990.03, 1995, modified.
7 Crude fat was determined using AOAC Official Method 960.39, 1990, modified.
Means include all available data some of which are below the limit of detection of the assay. The range highlights the lowest detectable value.
8 Dry matter was determined using an oven method. (AOAC Official Method 925.45, 1990).
9 Carbohydrates were calculated by difference using the fresh weight-derived data.

Table 6.4. Proximate Analyses of Root Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature range [2]
	Mean [3]	Range [3]	Mean [3]	Range [3]	
Crude Ash [4]	5.47	4.58-6.26	6.62	4.76-9.02	3.3-17.7
Crude Fibre [5]	4.10	2.76-5.01	3.96	3.28-4.72	3.4-7.4
Crude Protein [6]	6.28	3.41-9.54	5.60	2.43-8.04	1.2-12.4
Dry Matter [7]	19.40	17.8-22.6	21.10	19.4-22.6	23.00
Soluble Carbohydrates [8]	84.1	80.3-87.2	84.1	79.0-88.1	67.3-90.9

1 Tissue samples were collected from field studies conducted at various locations in the USA in 1996.
2 See reference DLG, 1991.
3 Values are taken from the analyses of samples from 5 sites (n=5) for line #77 and for the control, with the exception of the ash analyses conducted in duplicate for 2 of the 5 sites for line #77 (n=7). All values are given on a dry matter basis except dry matter.
4 Crude ash was determined using method AOAC Official Ash Method 923.03, 1990, modified.
5 Crude fibre was determined using AOAC method 962.09, 1990, modified.
6 Crude protein was determined via total nitrogen determination. (AOAC Official Methods 992.03 and 990.03, 1995, modified.
7 Dry matter was determined using an oven method. (AOAC Official Method 925.45, 1990).
8 Carbohydrates were calculated by difference using the fresh weight-derived data.

Table 6.5. Proximate Analyses of Root Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature range [2]
	Mean [3]	Range [3]	Mean [3]	Range [3]	
Crude Ash [4]	3.42	2.71-4.94	3.40	2.66-5.08	3.3-17.7
Crude Fibre [5]	4.10	3.47-5.22	3.97	3.09-5.33	3.4-7.4
Crude Protein [6]	6.25	4.81-8.19	6.25	4.94-7.88	1.2-12.4
Dry Matter [7]	20.46	14.05-23.48	20.45	13.57-23.12	23.00
Soluble Carbohydrates [8]	86.25	81.65-88.89	86.34	81.69-88.72	67.3-90.9

1 Tissue samples were collected from field studies conducted at various locations in Europe in 1995.
2 See reference DLG, 1991.
3 n=6, all analyses were conducted in triplicate, and all values are given on a dry matter basis except dry matter.
4 Crude ash was determined using an oven method # EF L 155/13 p.430 12/7-71 modified.
5 Crude fibre was determined using the Weende method # EF L 344/36-3726/11-92.
6 Crude protein was determined using a total nitrogen value determined using a Kjeldahl method (# EF L 179/9-10 22/7-93 modified) multiplied by 6.25.
7 Dry matter was determined using an oven method # EF 71/393/EOF; L 279/7 p.858-61 20/12-71.
8 Carbohydrate Calculation was based on Plantedirecktoratet bek. #19 13/1-92

Table 6.6. Proximate Analyses of Root Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature range [2]
	Mean [3]	Range [3]	Mean [3]	Range [3]	
Crude Ash [44]	2.53	1.95-3.22	2.51	2.09-3.35	3.3-17.7
Crude Fibre [5]	4.19	3.87-4.60	4.15	3.88-4.62	3.4-7.4
Crude Protein [6]	4.26	3.02-5.44	4.30	3.02-5.18	1.2-12.4
Dry Matter [7]	23.88	19.18-26.37	23.93	19.53-26.22	23.00
Soluble Carbohydrates [8]	89.01	87.12-91.06	89.03	87.59-90.87	67.3-90.9

1 Tissue samples collected from field studies conducted at various locations in Europe in 1996.
2 See reference DLG, 1991.
3 n=6, all analyses were conducted in triplicate, and all values are given on a dry matter basis except dry matter.
4 Crude ash was determined using an oven method # EF L 155/13 p.430 12/7-71 modified.
5 Crude fibre was determined using the Weende method # EF L 344/36-3726/11-92.
6 Crude protein was determined using a total nitrogen value determined using a Kjeldahl method (# EF L 179/9-10 22/7-93 modified) multiplied by 6.25.
7 Dry matter was determined using an oven method # EF 71/393/EOF; L 279/7 p.858-61 20/12-71.
8 Carbohydrate Calculation was based on Plantedirecktoratet bek. #19 13/1-92.

Table 6.7. Quality Analyses of Root Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature range [2]
	Mean [3]	Range [3]	Mean [2]	Range [3]	
Polarization[4]	14.36	8.40-17.43	14.48	7.89-17.18	10.8-20.7
Sodium[5]	1.68	0.50-3.08	1.77	0.40-3.50	0.35-5.48
Potassium[6]	5.28	4.55-5.87	5.29	4.22-5.95	4.19-10.2
Invert Sugar[7]	1.66	0.32-3.69	1.76	0.35-4.24	0.3-2.7
Amino Nitrogen[8]	2.84	2.01-4.00	2.88	1.98-3.93	0.93-5.14

1 Tissue samples collected from field studies conducted at various locations in Europe in 1995.
2 See reference Märländer *et al.*, 1996 and Smed *et al.*, 1996.
3 n=6, all analyses were conducted in triplicate.
4 Polarization is reported as g/100g root fresh weight, and was determined using a polarimeter, ICUMSA method Sugar Analysis 1979, Proc. 1990.
5 Sodium is reported as mmol/ 100g root fresh weight, and was determined using an SMA method Technicon, technical publication THO-0160-10.
6 Potassium is reported as mmol/100g root fresh weight, and was determined using an SMA method Technicon, technical publication THO-0160-10.
7 Invert Sugar is reported as mmol/100g root fresh weight, and was determined using an SMA method Technicon, technical publication THO-0160-10.
8 Amino nitrogen is reported as mmol/100g root fresh weight, and was determined using ICUMSA method Sugar Analysis 1979 modified.

Table 6.8. Quality Analyses of Root Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature range [2]
	Mean [3]	Range [3]	Mean [3]	Range [3]	
Polarization[4]	17.26	13.79-19.37	17.33	14.12-19.41	10.8-20.7
Sodium [5]	0.46	0.26-0.82	0.54	0.20-0.82	0.35-5.48
Potassium [6]	4.89	4.12-6.01	5.03	3.97-6.38	4.19-10.2
Invert Sugar [7]	0.40	0.29-0.54	0.39	0.28-0.53	0.3-2.7
Amino Nitrogen[8]	1.60	0.67-2.84	1.63	0.76-2.48	0.93-5.14

1 Tissue samples collected from field studies conducted at various locations in Europe in 1996.
2 See reference Märländer et Al., 1996 and Smed et al., 1996.
3 n=6, all analyses were conducted in triplicate.
4 Polarization is reported as g/100g root fresh weight, and was determined using a polarimeter, ICUMSA method Sugar Analysis 1979 Proc.1990.
5 Sodium is reported as mmol/ 100 g root fresh weight and was determined using an SMA method Technicon, Technical publication THO-0160-10.
6 Potassium is reported as mmol/ 100 g root fresh weight and was determined using an SMA method Technicon, technical publication THO-0160.10.
7 Invert Sugar is reported as mmol/ 100 g root fresh weight and was determined using an SMA method Technicon, technical publication THO-0160-10.
8 Amino nitrogen is reported as mmol/100 g root fresh weight, and was determined using ICUMSA method Sugar Analysis 1979, modified.

Table 6.9. Quality Analyses of Root Tissue from GTSB77[1]

Analysis	Control Sample		GTSB77		Literature
	Mean [3]	Range [3]	Mean [3]	Range [3]	range [2]
Polarization[4]	14.80	12.9-17.1	14.6	12.7-16.2	10.8-20.7
Sodium[5]	1.53	0.96-2.28	1.54	1.26-1.92	0.35-5.48
Potassium[6]	8.17	6.79-11.7	8.02	6.73-11.5	4.19-10.2
Amino Nitrogen[7]	5.56	2.66-7.62	5.67	3.37-7.19	0.93-5.14

1 Tissue samples collected from field studies conducted at various locations in the USA in 1996.
2 See reference Märländer *et al.*, 1996 and Smed *et al.*, 1996.
3 n=5, all analyses were conducted in triplicate.
4 Polarization is % sucrose and was determined using a "Pro-Pol" short-path polarimeter.
5 Sodium is reported as mmol/ 100g root fresh weight, and was determined spectroscopically using a Model FP-2 flame photometer.
6 Potassium is reported as mmol/100g root fresh weight, and was determined spectroscopically using a Model FP-2 flame photometer.
7 Amino nitrogen is reported as mmol/100g root fresh weight, and was determined by fluorescence after derivitization of the filtrate with orthophthalic dicarboxaldehyde.

Table 6.10. Saponin Analyses of Root and Top Tissue from GTSB77[1]

Tissue	Control Sample		GTSB77		Literature
	Mean [3,4]	Range [3]	Mean [3,4]	Range [3]	range [2]
Roots	215	111-304	208	128-260	75-965
Tops	175	125-242	215	98-358	50-600

1 Tissue samples collected from field studies conducted at various locations in the USA in 1996.
2 See reference Lüdecke *et al.*, 1958.
3 Values are taken from analyses of samples from 5 sites (n=5) for line #77 and for control. Values are given on a mg/kg fresh weight basis.
4 Saponin method was based on a published method. (J. Agric. Food Chem. 1994, 42, 279-282 (Ridout, et al., 1994)).

Table 6.11. Saponin Analyses of Root and Top Tissue from GTSB77[1]

	Control Sample		GTSB77		Literature
Analysis	Mean [3,4]	Range [3]	Mean [3,4]	Range [3]	range [2]
Roots 1995	151	72-233	137	60-261	75-965
Tops 1995	116	52-193	103	51-165	50-600
Roots 1996	529	304-999	484	293-846	75-965
Tops 1996	478	115-727	353	139-564	50-600

1 Tissue samples were collected from field studies conducted at various locations in Europe in 1995-1996.
2 See reference Lüdecke et al., 1958.
3 n=5, all analyses were conducted, and values are given on a mg/kg fresh weight basis.
4 Saponin method was based on a published method. (J. Agric. Food Chem. 1994, 42, 279-282 (Ridout, et al., 1994)).