Gene Transfer for Herbicide Resistance

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ABSTRACT

Since 1990 Maribo Seed has conducted field trials with transgenic sugarbeet. Glyphosate tolerance has been the main objective. Trials are performed in Denmark, France, England, and Belgium, and in 1993 for the first time in USA. As sugarbeet varieties are hybrids, the transformation can be made on either multigerm, diploid fatherlines or monogerm, diploid motherlines (CMS-lines). Our development work has involved a range of different genetical constructs from Monsanto. The *Agrobacterium* vector transfers the insert into random chromosomal positions in single or multiple copies. Transformed plants are cloned to about 10 copies each and tested. If the GUS-gene is present, GUS analysis on pollen can distinguish between plants which are homozygous and heterozygous for the introduced traits. Transgenic beets, but also progenies of transgenic beets, show considerable variation in the expression of the genes inserted (position effects). We have seen indications of interactions between transgenes and native genes. In most cases transgenes segregate according to Mendel's laws. The occurrence of meiotic irregularities, chimerics and multi-copy inserts can add to the complexity of developing transgenic lines, but classical breeding techniques are able to select lines which are identical to the parental line except for the introduced trait.

Additional Key Words: Sugarbeet, *Beta vulgaris* L., glyphosate-tolerance, transformation, positype, position effect.

Being perennial, sugarbeet *(Beta vulgaris* L.) is time consuming to backcross, especially if the gene source is a wild relative. The idea of inserting nothing but the new trait is therefore fascinating. Genetic engineering and other biotechnological techniques provide a range of possibilities to speed up breeding programs. In 1987 Maribo Seed and Monsanto started a joint research program to introduce transgenes by means of biotechnology. This collaboration includes herbicide-tolerance as well as tolerance against diseases like Rhizomania (Beet necrotic yellow vein virus). In 1990 the first sugarbeets transformed with a glyphosate-tolerance gene were field tested in Denmark, France, England and Belgium. In 1993, we have extended our activities to include USA, where field trials are performed in collaboration with American Crystal Sugar Company (ACS).

MATERIALS AND METHODS

Since sugarbeet varieties are hybrids one can choose to introduce the gene either into multigerm, diploid fatherlines or monogerm, diploid motherlines. The latter strategy is more difficult as it involves both a cytoplasmatic male-sterile line and a maintainer line. On the other hand such a strategy opens the possibility of creating both 2n and 3n varieties (Figure 1).

Sugarbeet is amenable to genetic transformation using the *Agrobacterium* T-DNA technology (Lindsay and Gallois, 1990; Fry et al., 1990; D'Halluin et al., 1992). We have employed this technology to insert a number of gene constructs harboring different promoters, marker genes, and glyphosate-tolerance genes (all provided by Monsanto Co., St. Louis MO 63617, USA) into cotyledon and other types of beet tissue explants. Because transgenes are inserted into different positions on the plant chromosomes, each transformation event is recorded separately. We talk about position types or "positypes" for short, and we keep track of the different positypes wherever they are used: in clones, selfings and crossings. The initial transformed shoots are cloned to about 10 copies. The plants are sprayed with Roundup® (glyphosate; Monsanto Co., St. Louis MO) in a greenhouse to identify clones which have the best expression of glyphosate-tolerance. The best clones are analyzed for the number of insertions and are then vernalized. During the winter we make both selfed seed and hybrid seed by crossing the transformed clones to non-transformed CMS plants. The following year progenies are tested in the greenhouse and in the field. Each progeny is analyzed for morphology, yield components, segregation of the introduced gene, glyphosate-tolerance and reporter genes like the ß-glucuronidase gene

(GUS) (Jefferson et al., 1987). GUS analysis is carried out at a pH of 7.0. Both Southern and PCR analysis are routinely used to check for the presence of the glyphosate tolerant genes and the number of copies inserted. These analyses, however, are not made on pollen but on tissue from leaves and roots.

RESULTS AND DISCUSSION

In almost all cases the transgenic positypes segregate according to Mendel's laws. Thus, the introduction of foreign genes into breeding lines is straightforward. Few positypes, however, do not follow the expected classical segregation or differ in other respects. In the 1990 and 1991 field trials, the tested positypes containing construct 1 or 2 showed a relatively good tolerance to glyphosate but were arrested in growth for a period of time after spraying. As a consequence, the yield loss was too high and the positypes considered to be of no value for commercial use.

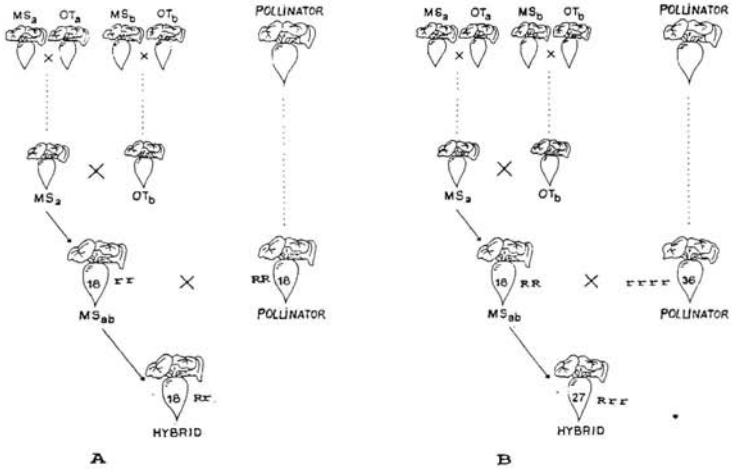

Figure 1. Breeding strategies for the introduction of a herbicide-tolerance gene into hybrid varieties of sugarbeet. **A:** If the gene is introduced as a fixed trait into the diploid pollinator, diploid varieties can be created. **B:** If the gene is inserted into the diploid mother line, both the inbred OT and the inbred MS must be homozygous regarding the tolerance-gene, and the gene must be in the same position in both components. Using the latter strategy, both diploid and triploid varieties can be created.

R = herbicide-tolerance.

Field tests in 1992 revealed good tolerance in progenies from positypes transformed with construct 4 and as of this date, the leaf phenotype observations of the 1993 field trials are positive. Several positypes with construct 3 show a very good tolerance to glyphosate at low rates. Under higher rates these plants are somewhat delayed, but resume normal development 2-3 weeks after the last application. A few positypes containing construct 4 are tolerant although they do show some transitory effects (e.g., chlorosis) shortly after spraying. Among positypes transformed with construct 6 we have found several which are extremely tolerant to glyphosate. Both top and root appear unaffected by spraying with Roundup®. After harvest in the autumn these plants will be analyzed for sugar content, juice purity and other substances.

Considering the number of positypes we have analyzed, introduced changes in morphological and physiological characters, other than those which could be explained by the natural variation, are rare but interesting for genetic studies. The GUS gene appeared not to be linked to the glyphosate tolerance gene. Table 1 shows results from plants transformed with construct 2.

The segregation in the unsprayed plots is close to the expected 3:1 and 1:1 in the OT and the hybrid respectively. Segregation in sprayed plots, however, was also 3:1 and 1:1, suggesting independent transmission of GUS and glyphosate-tolerance in this transformant.

In one of the construct 4 positypes tested in 1992 a possible interaction between the transgenes and the native genes seems to have occurred. Both the S1 and the hybrid plants segregate into plants with normal looking roots and roots with altered morphology.

Table 1. GUS analyses on seedlings from a selfed transgenic OT and its hybrid unsprayed and sprayed with Roundup®.

Progeny	GUS+	GUS−	Total	GUS+	Expected
	Plants	Plants	Plants	%	%
OT:					
Sprayed	40	14	54	74.1	100
Unsprayed	36	12	48	75.0	75
Hybrid:					
Sprayed	35	29	64	54.7	100
Unsprayed	30	28	58	51.7	50

In the sprayed plots only plants with modified roots were found. The segregation in tolerant and non-tolerant beets was 3:1 in the OT and 1:1 in the hybrid and the surviving plants were all GUS-positive. Whether the root modifications seen in this positype are caused by an interaction between one of the introduced genes and endogenous genes (Jorgensen, 1990), position effect, or is an example of tissue culture induced somaclonal variation (Steen et al., 1986; Karp, 1993) will be explored further.

Independently of the construct used, we have occasionally found biased segregations in tolerant and non-tolerant plants. Too few surviving plants indicates loss of tolerance genes during meiosis, silencing of genes or presence of chimeric seed plants. Too many surviving plants indicates multi-copy inserts, which can be confirmed by Southern analyses. Although multiple linked insertions (tandems, etc.) normally segregate as one gene, the expression in such positypes can be rather complex to analyze.

Further studies on the transgenic material have demonstrated interesting perspectives. In 1992 we used an O-Type (maintainer) containing construct 2 as pollen source. One transgenic OT and two non transgenic CMS plants were planted in each isolation tent. The O-Type plants were selected from a plot sprayed with Roundup®. Therefore they were either homozygous or heterozygous regarding the insert, which was in the same chromosome position in all plants. While the plants were still in the bud stage we analyzed the pollen for GUS reaction (Pedersen and Steen, personal communication). These findings were compared to GUS analyses on seed harvested on the OT's (selfings) and on the CMS's (hybrids). The results are shown in Table 2 and Figure 2.

This investigation shows that with GUS as reporter gene it is possible in a population to identify homozygous resistant plants before flowering, provided that GUS is expressed in pollen and is still linked to the tolerance gene. This technique can help speed up the process of fixing inserted traits.

Table 2. GUS analyses on leaves and pollen from transgenic OT's plants and on seedlings from their progenies.

OT No.	GUS+ Leaves	GUS+ Pollen	Allelic Status	GUS+ on seedlings from Selfing	Hybrid
4	Yes	96.4%	Homozygous	98%	–
5	No	0.0%	Control	–	0%
6	Yes	48.7%	Heterozygous	73%	54%
7	Yes	93.0%	Homozygous	100%	100%
9	Yes	50.5%	Heterozygous	80%	45%

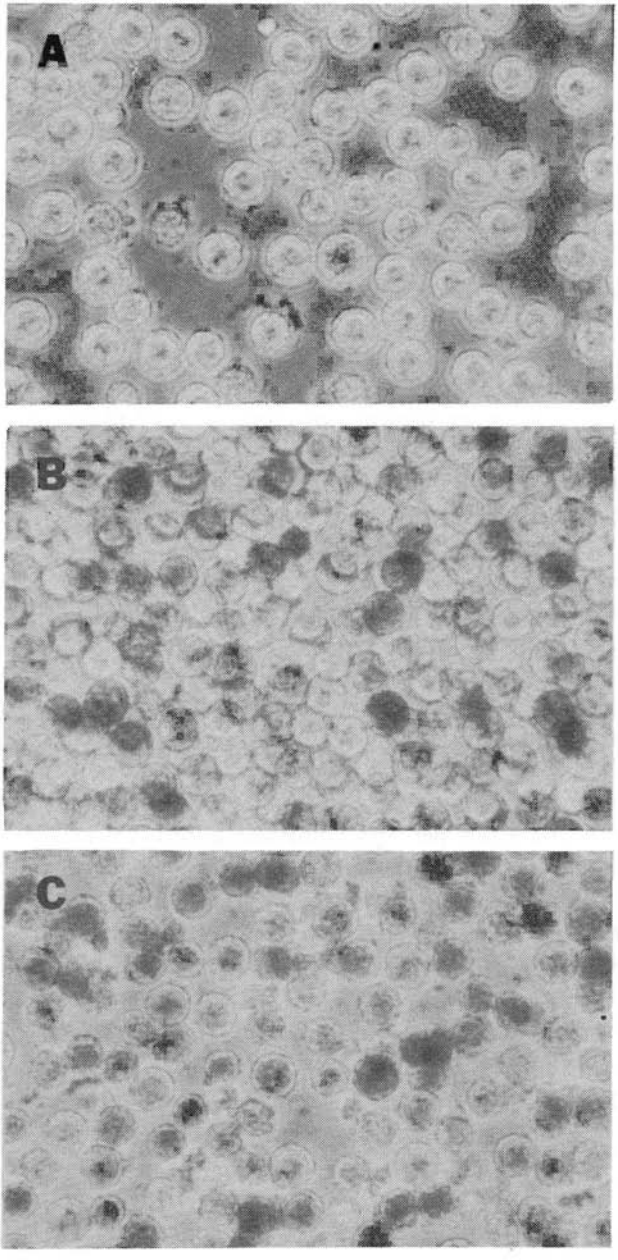

Figure 2. GUS test on pollen from S1 plants originating from a transgenic O-type heterozygous for the inserted genes. **A:** 100% GUS negative pollen shows that the insert has been lost due to segregation. **B:** 50% GUS positive pollen on a heterozygote. **C:** 100% GUS positive pollen on a homozygote.

CONCLUSIONS

When the new techniques of biotechnology made their entry, it was suggested that in the future breeders would not be needed any more, because their work would be done in laboratories. Our experience is that with transformation the possibilities for introducing new, interesting genes are unlimited. These genes are, however, placed randomly among native genes. The position of the transgenes, their stability and expression and their possible interaction with other genes creates a huge number of combinations. Good combinations very seldom occur by themselves. They have to be created and identified by individuals, who know their material, who have an overview of gene sources, who can combine new and traditional breeding techniques and who have the vision—in other words, the breeders. As a tool genetic engineering seems excellent. Many questions about genetics may be answered, adding to the breeders' knowledge about their material, and new questions will arise. We therefore believe that the future will see a close and fruitful collaboration between breeders, molecular biologists, and other scientists in creating new varieties to benefit the environment and agriculture.

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