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Generation of transgenic asulam-resistant potatoes to facilitate eradication of parasitic broomrapes (*Orobanche* spp.), with the *sul* gene as the selectable marker

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Abstract Root-attaching parasitic flowering broomrapes (*Orobanche* spp.) are major constraints to vegetable, legume and sunflower production around the Mediterranean and elsewhere, with banned methylbromide fumigation or land abandonment of these affected crops as the major "solutions" to the problem. We report the specific generation of transgenic asulam-resistant potatoes as a way to eradicate this pest. The target-site resistance in the crop allows the herbicide to be translocated from treated leaves to the parasite via crop roots. This inhibitor of dihydropteroate synthase lethally then prevents folic acid biosynthesis in the parasite. Additionally, we demonstrate that asulam can be used directly in selecting resistant transformants, without the need for another selectable marker.

Key words Asulam · Herbicide resistance · *Orobanche* · Potato · Selectable marker

Introduction

The parasitic broomrapes (*Orobanche* spp.) are pernicious weeds throughout the Mediterranean region that attack almost all vegetable and legume crops as well as sunflowers. These achlorophyllous holoparasites attach to compatible crop roots, forming tubercles that store much of the crops' photosynthate, thereby debilitating crop growth and yield. Tens of such attachments can occur on each host plant. The parasites later send up flower stalks, each bearing tens of thousands of seeds. The seeds remain dormant until a host root passes nearby and chemically stimulates their germination. The only mechanical eradication possible is the manual removal of broomrape flower stalks before the seeds are set, but damage at this point has been done to the current crop. No selective herbicides are available that effectively deal with these parasites on most crops without damaging the crop (Foy et al. 1989). In Israel (Jacobsohn 1994) and throughout the Middle East much of the noxious methyl bromide used to fumigate vegetable fields is aimed at destroying dormant broomrape seed in the soil. The use of methyl bromide is being phased out due to international accords. There are few alternatives to deal with the parasitic weeds other than suffering severe yield reduction, or land abandonment; i.e. no longer cultivating affected high-value crops.

In most situations where there are herbicides that selectively kill weeds in crops, the crops are resistant due to the crops' abilities to metabolize the herbicides to non-toxic products. Such metabolic selectivity would be ineffective in controlling broomrapes with systemic herbicides as the parasite would receive inactive herbicide metabolites from the crop's vascular system. It has been hypothesized that transgenic crops carrying resistance due to a modification of the target site of the herbicide would allow the systemic translocation of unmetabolized herbicide to the attached parasite (Gressel 1992). This hypothesis was borne out using model transgenic crops bearing genes for resistance to three different herbicides (Joel et al. 1995a). The herbicide must be of a type that is toxic to the achlorphyllous parasite, thereby precluding the many herbicides that affect photosynthesis. Thus, broomrape would not be affected by triazines used on the previously generated target-site triazine-resistant potatoes (Gressel et al. 1989; Smeda et al. 1993) as this herbicide group affects only photosynthesis.

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We report here our results with potatoes, the first crop specifically engineered with a gene for herbicide resistance to allow the eradication of parasitic weeds. The broomrapes *Orobanche aegyptiaca*, *O. ramosa* or *O. cernua* cause heavy damage to potatoes (Jacobsohn 1994). Yields can be reduced by more than 50%, although this is not common; farmers either fumigate with methyl bromide or avoid growing potatoes in infested fields, consequently decreasing the crop diversity available to the grower.

This is the first report of asulam being used as the sole selection compound in the transformation procedures. Many descriptions of transgenic, herbicide-resistant plants suffer from the problem that there are two variables engineered into the crop, the purported resistance gene and the selectable marker. The selectable marker may have a broader substrate specificity than assumed. Indeed, it was recently claimed that the gene coding for hygromycin resistance, often used as a selectable marker, also confers resistance to the herbicide glyphosate (Peñaloza-Vásquez et al. 1995).

The herbicide resistance gene chosen was *sul*, which codes for a modified dihydropteroate synthase (DHPS), the target of the herbicide asulam and other sulfonamide-type antimetabolites (Guerineau et al. 1990). DHPS is the second enzyme before folic acid, and the herbicide prevents the synthesis of this vitamin unless the modified gene is present. Because asulam kills the parasite (Joel et al. 1995a), we may now assume that the parasite does not receive folic acid from the host-crop and that it must synthesize this vitamin, contrary to general opinion about the wide-ranging nutrition of these parasites from their hosts (Press and Graves 1995). Asulam is not widely used, as too few crops are resistant to it. Thus, the potential increase in its use could add to the diversity of herbicides used, relieving the pressure on heavily used herbicides that often leads to resistance problems in weeds.

Materials and methods

Plasmids and constructs

Plasmid pJIT118 containing the bacterial *sul* gene, which encodes a modified dihydropteroate synthase (DHPS) insensitive to inhibition by asulam, was kindly provided by Drs. G. Freyssinet and R. deRose of Rhone-Poulenc. pJIT118 contains a *Kpn*I fragment that is composed of the *sul* gene preceded by a chloroplast transit peptide and a double 35S promoter and followed by a CaMV poly A site (Guerineau et al. 1990). The *Kpn*I fragment was transferred into pGA492 and then introduced into *Agrobacterium tumefaciens* strain EHA101 by electroporation according to An (1987) (Fig. 1).

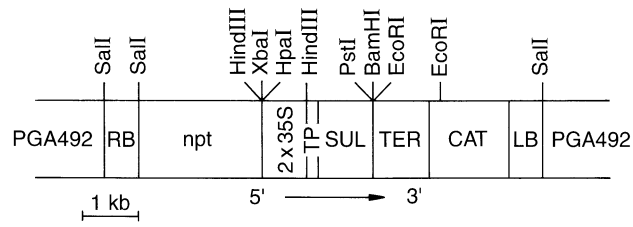

Fig. 1. Schematic representation of the sulfonamide resistance gene constructed into the binary vector pGA492. The *Kpn*I fragment that contains a double CaMV 35S promoter, Rubisco small subunit transit peptide (*TP*), the sulfonamide resistance gene (*sul*) and the CaMV poly A as a terminator (*TER*) was excised from the pJIT118 plasmid and ligated to a linearized binary vector PGA492 at the *Kpn*I site. *RB* and *LB* are the T-DNA, the *right* and the *left* borders, respectively, *npt* neomycin phosphotransferase gene, and *cat* chloramphenicol acetyltransferase genes. Important restriction endonuclease sites are indicated

Potato transformation with EHA 101-sul

Transformation of potato was essentially as described by Perl et al. (1993). Potato tubers (*Solanum tuberosum* cv 'Desireé') were disinfected, cut into thin slices and incubated in *Agrobacterium tumefaciens* EHA101-*sul* for 30 min. The slices were then dried on filter paper and transferred for co-cultivation onto regeneration plates containing MS medium (Murashige and Skoog 1962) with 9.8 μ*M* zeatin riboside and 3.4 μ*M* indole-3-acetyl-L-aspartic acid (Sigma). The tubers were washed after 48 h, dried and transferred onto selection agar plates containing regeneration medium plus 948 μ*M* carbenicillin and a sulfonamide; either 184 μ*M* sulfadiazine (Sigma) or 217 μ*M* asulam (Riedel-de Haen). Two to four weeks later regenerated shoots were transferred for rooting on Nit (Nitsch 1969) agar plates containing 237 μ*M* carbenicillin and 435 μ*M* asulam.

Rooting in asulam

Tubers of putative transformant lines were germinated in pots in the greenhouse. When the shoots reached 15–20 cm they were cut and transferred to flasks containing an aqueous dilution of asulam (formulated as Asulox, Rhone-Poulenc) to a final concentration of 652 μ*M* indolebutyric acid. The flasks were kept in the greenhouse for 2–3 asulam plus 5 μ*M* weeks.

Plant DNA isolation and Southern analysis

Genomic DNA was isolated from potato-leaf tissue using a Nucleon Phytopure kit (Ornat). Samples of 10 μg DNA were digested with *Asp*718 restriction endonuclease and electrophoretically fractionated on 0.8% agarose gels. Southern blots were performed using a radiolabelled Random Primer Labelling System (Amersham-PRN 1633) with a 790-bp fragment from the *npt* gene as a probe.

Greenhouse experiments

Young asulam-resistant potato plants of eight different clones that emerged from tubers were transplanted into 10-l pails filled with soil that was artificially inoculated with 40 mg *Orobanche aegyptiaca* Pers. seeds per kilogram soil (approx. 2,500 seeds·kg^{-1}). Altogether we used 350 transgenic potato plants in three consecutive experiments. The pots were placed in a greenhouse in random blocks for asulam treatments and for genetic clones.

Different doses of asulam (0, 350, 500, 600 m*M*) were sprayed using an air-pressurized sprayer at 200 l·ha^{-1} on the plant foliage 6,

15, 24, 36, and 50 days after transplanting. These rates are the equivalent of 0, 16, 24 and 32 $kg \cdot ha^{-1}$, respectively. Potato shoot survival was recorded 85 days after transplanting. The root systems of the potato plants were washed free of soil and checked for young broomrape infections 2 weeks later.

Results

Potato transformation

The first step in the strategy of combating broomrape was to produce asulam-resistant potato plants. We infected tuber slices with *Agrobacterium tumefaciens* strain EHA101-*sul* (Fig. 1), which contains the bacterial *sul* gene under a 35S CaMV promoter (Guerineau et al. 1990). Tobacco seedlings transformed with that same construct, but selected on kanamycin, were later found to be co-resistant to either sulfadiazine or asulam, which both inhibit DHPS (Guerineau et al. 1990). We directly selected potato transformants harboring the *sul* gene on either 0.15–0.18 m*M* sulfadiazine or 0.20–0.26 m*M* asulam in different experiments, without an additional selector. Indeed, some tens of shoots were regenerated under these selection conditions in three independent transformation experiments (Fig. 2A). Each shoot was transferred onto Nit agar medium containing 435 μ*M* asulam. Non-transformed potato shoots did not root under these conditions, while those of transformed lines did (Fig. 2B). Isolated shoots from another 30 independent lines also rooted very strongly in this test (data not shown). All viable rooted shoots were transferred to the greenhouse for further analysis and for obtaining tubers.

Verification of transformation

We employed several tests to verify the transformed nature of the putative transformants:

(1) Total DNA was isolated from 4 plants, digested with *Asp*718, run on a gel and blotted. The blots were hybridized with the *npt* fragment. In all 4 plants, the 2.8-kb bands were hybridized and visible as shown in Fig. 3 for transformant 4–1. No such band was observed in the control, non-transformed line (Fig. 3). These results indicate that the *sul* gene was integrated into the potato genome.

(2) Potato plants grown in Rehovot rarely bear fruits that set seeds, but 2 of the putative transformants did set seeds. The seeds were germinated on Nit agar plates containing 435 μ*M* asulam. While seeds of control non-transformed cv 'Desireé' developed very short, inhibited roots on 217 μ*M* asulam; the germinated seeds of transformants 9-3 and 4-1 developed nice long roots on 435 μ*M* asulam (Fig. 4). This indicates that the trait is dominant, integrated and is transmitted through a meiotic cycle.

(3) Shoots appearing on tubers from all other lines that were not analyzed by either Southern hybridization or by the seed-germination test were subjected to a rooting test. Shoots were excised from the tubers when they reached about 15–20 cm and incubated in 652 μ*M* asulam with 5 μ*M* indolebutyric acid (a root-inducing hormone). Non-transformed cv 'Desireé' did not develop any roots in the presence of asulam while they rooted normally in its absence (Fig. 5). All of the putatively transformed lines tested formed roots in the presence of 652 μ*M* asulam (data not shown).

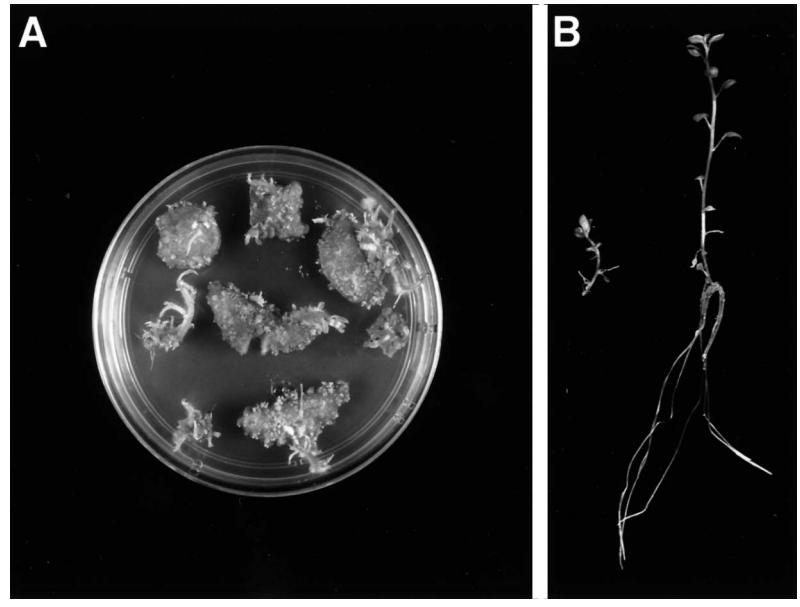

Fig. 2A, B Shoot regeneration of potato tuber slices and rooting of EHA101-*sul* transformants. **A** Regeneration of potato tuber slices in the presence of 150 μM sulfadiazine, a dihydropteroate synthase inhibitor, 1 month after transformation with EHA101-*sul*. **B** Rooting of a shoot of putative *sul* transformant 8-1 (*right*) on medium containing 375 μ*M* sulfadiazine. A non-transformed control shoot of the same original size is on the *left*

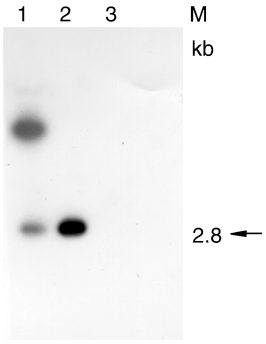

Fig. 3 Integration of the *sul* gene coding for asulam resistance in transformed potato. Genomic Southern hybridization: the *npt* fragment (790 bp) was hybridized with genomic DNA from putatively transformed potato plants cut with the *Asp*718 restriction enzyme. *Lanes 1–3* contain *1* the positive control plasmid (PGA-*sul*), *2* restricted DNA from transformant 4-1, *3* DNA from a non-transformed plant

Fig. 4 Transmission of asulam resistance through true seeds. True seeds were germinated on 200 μ*M* asulam. Seedlings on the *right* are from *sul* transformant 9-3, and seedlings from non-transformed seeds are on the *left*

Efficacy in eradicating broomrape

Transplants of asulam-resistant transformants were grown in *Orobanche*-inoculated soil in pails, and the resulting plants were sprayed with various concentrations of asulam at different times after shoot emergence. Broomrape attachments to potato roots were counted 3 months after planting; any broomrape attaching after this time would have little influence on crop yield. By this time many untreated potato plants had dead shoots; the flower stalks of broomrape constituted the only living material above ground (Fig. 6).

Broomrape was completely suppressed on the best potato transformant (4-1), while potato viability was preserved (Fig. 7). The early treatment at 15 days after transplanting (near the time broomrape seedlings began to attach to potato roots) did not give the best eradication of broomrape. Later treatments with asulam provided far better protection, as seen in Fig. 7, and in other experiments. Neither broomrape flower stalks nor live underground attachments were seen after the later application times. The rates of asulam used (16–32 $kg \cdot ha^{-1}$) are much higher than those typically used in agriculture (1–7.5 $kg \cdot ha^{-1}$), indicating the robustness of the transformants in coping with high rates of asulam. This is very important for any transgenic; they must be resistant to at least 4 times the field use rate. We must now ascertain the lowest effective concentrations for broomrape eradication in the field.

Discussion

There is an increasing number of reports demonstrating that genes conferring herbicide resistance can be transferred into numerous plant species. In the majority of such transformation protocols, a selectable marker such as the *npt*II (neomycin phosphotransferase) gene or *hpt* (coding for hygromycin phosphotransferase) is included in the DNA constructs together

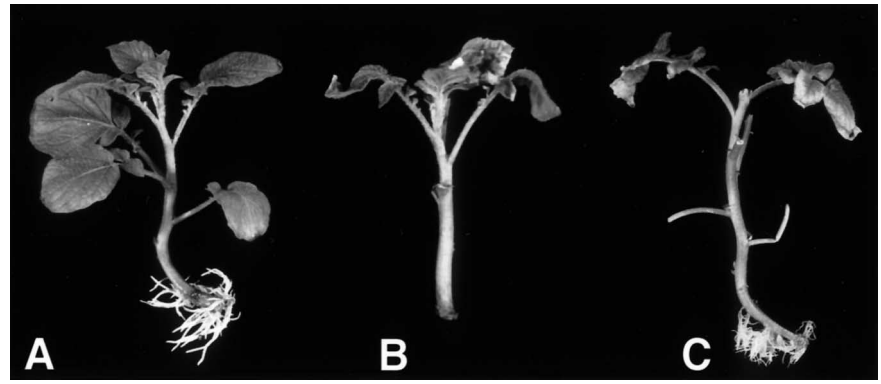

Fig. 5A, B, C Transmission of asulam resistance through tubers; a rooting test of tuber-derived shoots. **A, B** Control, non-transformed shoot in water (**A**) and 600 μ*M* asulam (**B**). **C** *sul* transformant 9-3 in 600 μ*M* asulam

Fig. 6 Eradication of broomrape on transgenic potato. Transgenic potatoes were planted on broomrape-inoculated soil. The plant on the *right* was sprayed with 500 μM asulam 40 days after planting, and the plant on the left was not sprayed. No broomrape infection developed on the transgenic, treated plant, while the shoot of the untreated potato died and a parasite flower stalk developed

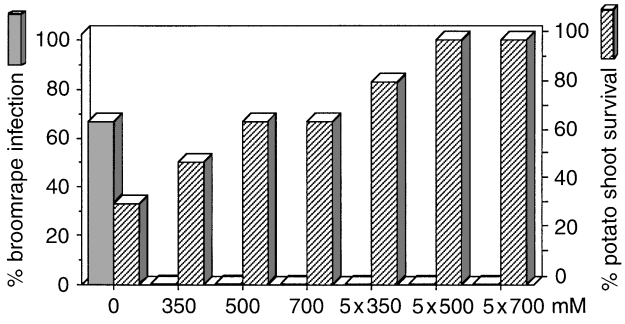

Fig. 7 Enhanced potato survival and lack of broomrape on asulam-treated transgenic plants. In this experiment with *sul* transformant 4-1, plants were either sprayed once at 15 days after transplanting or at 6, 15, 24, 36 and again at 50 days after transplanting at the rates indicated

with the gene of interest. The relevant antibiotic is then added to the transformation selection media. Thus, antibiotic-resistant transformants are isolated in the first step, and only later is there a test for the presence of the gene of interest.

As most of the herbicide resistance genes are expressed in the early stages of the transformation process and most herbicides are inhibitory under tissue-culture conditions (Gressel 1984), the former can be used directly as selectable markers. Glufosinate was among the first herbicides to be used in this way, in combination with the *bar* gene that confers resistance to it (e.g. DeBlock et al. 1989), and later the mutated acetolactate synthase gene was used that confers resistance to the sulfonylurea herbicides (e.g. Li et al. 1992). While this manuscript was in preparation, a paper appeared describing the use of the *sul* gene as a selectable marker in 'Russet Burbank' potatoes, with sulfa drugs as the selector (Wallis et al. 1996). The researchers did not present direct evidence for the stable integration of the gene, just root and shoot formation. To the best of our knowledge we are the first to demonstrate that asulam itself can be used directly for the selection of *sul* transformants.

Our results indicate that broomrape can be selectively suppressed on transgenic potatoes by treatment with asulam, a systemically translocated herbicide. Such a treatment allows potatoes to be grown in broomrape-infested soils without the need of fumigation. This complete eradication of broomrape by asulam in potato is superior to our previous results with tobacco, where only partial eradication was achieved (Joel et al. 1995a). Weeds have not evolved resistance to asulam, but this herbicide was not widely used in repeated monoculture, the typical condition leading to resistance. The *sul* gene used is a mutant easily selected for in bacteria, but the mode of selection (Guerineau et al. 1990) precludes knowing the mutation frequency in the bacteria, so one cannot easily extrapolate to plants. This does not allow modelling the rate at which the evolution of resistance will occur, as has been done with another parasitic weed with herbicide-treated maize (Gressel et al. 1996). Still, the existence of such target site mutants in DHPS suggests that such a resistance might eventually evolve in broomrapes, especially as these weeds have a very large seed output, often 250,000 per broomrape (Joel et al. 1995b), providing large populations to choose from. It would be wise to follow the precepts of the above models and scout fields for the rare, early-appearing, single stalks of broomrape, which might be resistant mutants, and remove them from fields by hand before they set seed. Despite this worry about resistance, the effective eradication of broomrape, for whatever duration, will be far superior to the situation at present. For this reason we are continuing to transform crops attacked by broomrape with the *sul* gene and with other genes conferring crop resistances to other broomrape-toxic herbicides. We believe that such crops will be a needed addition to the many other herbicide resistant crops that have been developed (Duke 1996).

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