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T. R. Wright · D. Penner

Cell selection and inheritance of imidazolinone resistance in sugarbeet (*Beta vulgaris*)

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Abstract Sugarbeets are sensitive to imidazolinone herbicide residues applied to rotational crops. Two imidazolinone-resistance (IMI-R) sugarbeet traits were developed by somatic cell selection to overcome rotation restrictions for sugarbeets where imidazolinones have been applied. Sir-13 is an IMI-R/SU-S (sulfonylurea-sensitive) variant selected from an imidazolinone-sensitive (IMI-S) sugarbeet clone, REL-1. A second variant, 93R30B, resistant to imidazolinone as well as to sulfonylurea herbicides (IMI-R/SU-R), was selected from a plant homozygous for a previously described sulfonylurea-specific resistance trait, *Sur* (IMI-S/SU-R). The IMI-R alleles (*Sir-13* and *93R30B*) were found to be corresponding allelic variants at the same ALS locus and both were tightly associated with the *Sur* allele. Each resistant allele is dominant to the sensitive wild-type allele; however, incomplete dominance is shown among resistance alleles. Diploid sugarbeet contains a single ALS gene copy, limiting the ability to stack these resistance traits in the same plant by traditional breeding.

Key words Chlorsulfuron, 2-chloro-*N*-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide · imazethapyr, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-5-ethyl-3-pyridinecarboxylic acid · Sugarbeet, *Beta vulgaris* L. 'SR93' · 'L03' · REL-1 · Acetolactate synthase, ALS · Acetohydroxyacid synthase, AHAS · *Sur* · *Sir-13* · *93R30B* · Sulfonylurea resistance · Somatic cell selection

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T. R. Wright · D. Penner (✉)
Department of Crop and Soil Sciences, Plant and Soil Science Buildings, Michigan States University, East Lansing, MI 48824-1325, USA
Voice; U.S. eastern time zone: 517-353-8853
Fax: 517-353-5174

Introduction

Imidazolinone herbicides effectively control a broad spectrum of weeds in leguminous and small-grain crops. This herbicide class provides season-long weed control afforded by its persistence in the soil. Unfortunately, the long residual activity of these herbicides can also injure susceptible rotational crops (Renner and Powell 1991; Johnson et al. 1993; Walsh et al. 1993; Krausz et al. 1994) . Sugarbeets are more sensitive to imidazolinone and other acetolactate synthase [ALS (EC. 4.1.3.18, AHAS, acetohydroxyacid synthase)]-inhibiting herbicide residues than most other crops (Moyer et al. 1990). Imazethapyr can control many of the problem weeds faced in soybean and dry bean production. These crops are commonly grown in rotation with sugarbeets; however, the 40 month rotation restriction (Pursuit Herbicide label, 1996, American Cyanamid Company, Wayne, New Jersey) between imazethapyr application and subsequent sugarbeet planting can preclude the use of imazethapyr for weed control in these areas. Additionally, imidazolinone and sulfonylurea herbicide persistence is significantly affected by soil and environmental factors, making the risk of herbicide carry over injury more unpredictable (Frederickson and Shea 1986; Stougaard et al. 1990). The development of sugarbeets resistant to ALS-inhibiting herbicides could minimize the potential for herbicide carry over injury, shorten rotation restrictions for sugarbeets, increase flexibility in crop rotations, and potentially provide new, effective chemical weed-control options for sugarbeets with currently registered products.

An abundance of herbicide-resistant crops have been reported. Herbicide-resistant plants have been derived by genetic engineering (Haughn et al. 1988; McHughen 1989; D'Halluin et al. 1992), mutation breeding (Sebastian et al. 1989; Newhouse et al. 1992), somatic cell selection (Newhouse et al. 1991; Heering et al. 1992;

Saunders et al. 1992; Iler et al. 1993), somatic hybridization (Harms et al. 1982), and interspecific crosses (Beversdorf et al. 1988; Mallory-Smith et al. 1990). In sugarbeets, sulfonylurea-resistant sugarbeet was developed by tissue-culture somatic cell selection from a herbicide-sensitive, highly regenerable clone (REL-1, Regenator East Lansing-1; Saunders et al. 1992). An altered ALS sensitivity provided resistance to specific sulfonylurea herbicides but no cross resistance to imidazolinone herbicides (Hart et al. 1992). This sulfonylurea resistance, *Sur* was inherited in sugarbeets as a semi-dominant monogenic trait (Hart et al. 1993). All other altered ALS-based herbicide resistances reported have been inherited as dominant or semi-dominant traits (Chaleff and Ray 1984; Anderson and Georgeson 1989; Sebastian et al. 1989; Haughn and Sommerville 1990). Resistance to ALS-inhibiting herbicides in weeds and crops can be specific to sulfonylureas (Haughn and Sommerville 1986; Mallory-Smith et al. 1990; Hart et al. 1992), specific to imidazolinones (Anderson and Georgeson 1989; Newhouse et al. 1992), or cross resistant to all classes of ALS-inhibitors, including the triazolopyrimidine sulfonanilide and pyrimidinylthiobenzoate herbicides (Lee et al. 1988; Anderson and Georgeson 1989; Bernasconi et al. 1995). No yield or fitness penalty is apparent in progeny containing an altered ALS enzyme (McHughen and Holm 1991; Newhouse et al. 1991, 1992), unlike the reduced fitness of triazine-resistant plants (Conard and Radosevich 1979; Ort et al. 1983).

In this paper we describe the development by somatic cell selection of two imidazolinone-resistant sugarbeet lines which differ in their resistance to the sulfonylurea class of herbicides. The mode of inheritance and the allelic characterization of the different resistance traits are also reported.

Materials and methods

Somatic cell selection

Tissue culture media

Sugarbeet tissue culture utilized the growth media described by Saunders et al. (1992). Ingredients common to all media were: MS mineral salts (Murashige and Skoog 1962), 30 $g l^{-1}$ sucrose, 100 mg l^{-1} *myo*-inositol, 0.5 $mg l^{-1}$ nicotinic acid, 0.5 $mg l^{-1}$ pyridoxine·HCl, and 1.0 $mg l^{-1}$ thiamine·HCl. Three functionally different media were used that differed only in the growth regulator added to the base medium above: callus induction and later shoot induction (B1), 1.0 $mg l^{-1}$ benzyladenine; shoot multiplicaton (M20), 0.25 $mg l^{-1}$ benzyladenine; and root induction (N3), 3.0 $mg l^{-1}$ a-naphthaleneacetic acid. Media pH was adjusted to 5.95 before autoclaving. Solid media were prepared as above except that 9 $g l^{-1}$ of agar (Sigma A-1296; Sigma Chemical Company, PO Box 14508, St. Louis, MO 63178) was added before autoclaving. When modified with herbicides, filter-sterilized stock herbicide solutions were added during cooling after autoclaving. Solid B1 and M20 media were poured as 30 ml and 40 ml aliquots into 15×100 mm and 20×100 mm Petri dishes, respectively. Liquid B1 and solid N3 media were poured as 40 ml aliquots into 125 ml Erlenmeyer flasks and sealed with foil-covered foam plugs.

Imidazolinone resistance

The methods of Saunders et al. (1992) were modified to select for imidazolinone resistance from the herbicide-sensitive REL-1 clone. REL-1 was chosen as a starting germplasm because of its ability to regenerate plants from leaf callus tissue (Saunders et al. 1992) together with its monogenic annualism and faster genetic analysis. Partially expanded leaves were removed from greenhouse-grown vegetative REL-1 plants and surface-sterilized by two successive 20-min washes with 15% (v/v) commercial bleach (5.25% w/v NaOCl) plus 0.025% (v/v) Triton X-100. Leaves were rinsed twice with sterile de-ionized water. Leaf disks were excised using a 5-mm-diameter cork borer. White, friable callus tissue proliferated from leaf disks (2–5 per plate) placed aseptically onto solid B1 callus-induction media and incubated in the dark at 30°C for 4–8 weeks. Suspension cultures were initiated by adding approximately 2 g of mechanically dissociated callus tissue to 125-ml Erlenmeyer flasks containing 40 ml of liquid B1 media. Suspension cultures were maintained on a gyratory shaker (150 Hz) under continuous low-intensity fluorescent lighting (30 $\mu Em^{-2}s^{-1}$) at room temperature. Suspensions were sub-cultured after 1 week by diluting 1:1 with fresh B1 liquid media.

Two weeks after suspension-culture initiation, cell clumps were separated using a cell-dissociation sieve (Sigma, CD-1) with a 60-mesh screen. Approximately two-thirds volume of the liquid media was removed by aspiration after cell sedimentation. Cells re-suspended in the remaining liquid were spread evenly over B1 medium supplemented with 50 nM imazethapyr in 15×100 mm disposable Petri dishes (approximately 300 viable cell clusters per plate). Plates were incubated under dim fluorescent light (5–10 $\mu Em^{-2}s^{-1}$) for 8–12 weeks. At 50-nM imazethapyr concentration all sensitive cells died. Cell clumps that survived and grew at this concentration were identified as possible resistant variants. Putatively resistant cell clumps (1–3 mm diam) were transferred to fresh B1 solid media without herbicide and allowed to grow under low light (10–20 $\mu Em^{-2}s^{-1}$). Each putatively resistant variant was individually identified and separately maintained.

Healthy callus was subdivided every 4 to 6 weeks to fresh B1 solid media until individual shoots regenerated from the callus. Shoots were maintained on M20 media under continuous low-intensity fluorescent lighting (30 $\mu Em^{-2}s^{-1}$) and multiplied by subdivision on M20 every 2–8 weeks. Imidazolinone resistance in the regenerated shoots was re-assessed by placing shoot cultures onto M20 solid agar modified with 50 nM imazethapyr. Herbicide-resistant whole plants were generated by placing shoots into 125 ml Erlenmeyer flasks containing 40 ml of solid N3 rooting media. Cultures were maintained under moderate-intensity fluorescent lighting (40–60 $\mu Em^{-2}s^{-1}$) at 25°C until sufficient root mass had formed to support ex vitro survival (generally 4–8 weeks). Plantlets of the selected isolate (R_0 generation) were transferred to potting substrate (BACCTO potting mix, Michigan Peat Company, PO Box 980129, Houston, TX 77098) in the greenhouse. This isolate was known as Sir-13 and the corresponding resistance allele as *Sir-13*.

Imidazolinone and sulfonylurea resistance

A second selection effort seeking imidazolinone resistance utilized an annual sugarbeet plant exhibiting good shoot regeneration from callus, as well as a homozygous sulfonylurea resistance (*Sur*) trait (Hart et al. 1992, 1993; Saunders et al. 1992) as the starting material. This plant was identified as 93R30. Leaf-disk explants from 93R30 generated callus, and imazethapyr selection was accomplished on B1 media supplemented with 50 nM imazethapyr as previously

described. A callus clump surviving this selection procedure ultimately regenerated into shoots, whole plants were obtained, and transferred to the greenhouse. This isolate was known as 93R30B and the corresponding resistance allele as *93R30B*.

Genetic analysis

Imidazolinone resistance

A Sir-13 R_0 plant was outcrossed with plants from the herbicide-sensitive smooth-root sugarbeet line, 'SR93 (Acquired from J. W. Saunders, USDA-ARS, East Lansing, Michigan). F_1 progeny were screened for imazethapyr resistance by applying 25% of the field rate POST [18 g ai ha^{-1} plus 2.4 l ha^{-1} (SunIt-II, AGSCO, PO Box 458, Grand Forks, N.D. 58206) plus 2.4 l ha^{-1} 28% nitrogen as urea ammonium nitrate solution applied in 239 l ha^{-1} spray volume] and were maintained in the greenhouse (25° ± 3C°) under supplemental lighting from high-pressure sodium lamps for 2 weeks. An F_1 plant surviving the POST imazethapyr screen was self-pollinated to yield F_2 seed. Progeny of F_2 plant self-pollinations were screened as above for resistance to POST imazethapyr. The percent progeny survival identified each F_2 individual as homozygous resistant (100% survival), heterozygous resistant (approximately 75% survival), or homozygous sensitive (0% survival). Genotype distribution was tested for "goodness of fit" with a monogenic dominant trait by chi square analysis at the 5% level.

Imidazolinone and sulfonylurea resistance

93R30B was outcrossed with plants of the sensitive sugarbeet line, 'SR93'. 93R30B was unable to self-pollinate or successfully pollinate a cytoplasmically male-sterile (CMS) line (L03), acquired from J. W. Saunders, USDA-ARS, East Lansing); however, pollen from SR93 successfully produced seed on the 93R30B R_0 plants. Subsequent generations were both male- and female-fertile. With the addition of imidazolinone resistance (IMI-R) in *93R30B* to sulfonylurea resistance (SU-R) in *Sur*, the leaf-disk assay of Saunders et al. (1992) was modified to non-destructively and simultaneously assay for the presence of herbicide class resistance (i.e., IMI or SU) independently. The resistance of F_1 plants to IMI, SU, or both classes, were determined by placing two to three sterilized leaf-disk explants (described above) from each individual onto B1 media with no herbicide, or modified with 50 nM imazethapyr or 50 nM chlorsulfuron. Leaf-disk tests were maintained at 25°C under continuous low-intensity fluorescent light (30 $\mu Em^{-2}s^{-1}$) for 1–2 weeks. Herbicide resistance for a given individual was determined by the presence of green, expanding leaf disks on the herbicide tested. Herbicide sensitivity was manifested by non-expanding, brown, dead or dying leaf disks.

Individual (93R30B × SR93) F_1 plants segregated strictly as IMI-R plus SU-R (IMI-R/SU-R) or as IMI-S/SU-R in approximately a 1:1 ratio. Most prior tissue-culture selections for ALS-inhibitor resistance have resulted in a (semi-)dominant trait with an enzyme altered in herbicide sensitivity. Diploid sugarbeet was reported to have one ALS gene copy (Hartnett et al. 1991); consequently, we hypothesized that *Sur* had attained a separate, genetically-linked IMI-R characteristic which was most likely a second mutation to the ALS gene (the SU-R of *Sur* representing the first mutation). Accordingly, for further genetic analysis, the phenotypes listed in Table 1 were assumed for each individually selected resistance allele.

An IMI-R/SU-R F_1 plant (presumed genotype 93*R*30*B*/*wt*) was self pollinated and progeny were tested for IMI and SU resistance segregation in the F_2 generation by the leaf-disk assay described above. Data were tested against the hypothesis of a single dominant nuclear trait. An alternative, unlinked two-locus hypothesis was also investigated. Data were tested for goodness of fit with the models by chi-square analysis at the 5% level.

Table 1 Phenotype of sugarbeet ALS alleles

Allele identification	Phenotype
wt[a]	IMI-S/SU-S
Sur	IMI-S/SU-R
Sir-13	IMI-R/SU-S
93R30B	IMI-R/SU-R

[a] *wt* represents the wild-type, herbicide-sensitive allele

Allelism of herbicide resistance traits

Genetic evidence

The male sterile phenotype of 93R30B R_0 plants allowed opportunistic, unidirectional crosses with various pollinator genotypes to test for allelism of the various herbicide resistance traits. An IMI-R/SU-S (genotype *Sir-13*/*wt*) individual pollinated a 93R30B R_0 plant (genotype *93R30B*/*Sur*) in an individual cross. Seed set was poor, but 12 F_1 plants from this cross were analyzed for IMI-R and SU-R by leaf-disk assay. *Sur* and *93R30B* were shown to be allelic (above). Assuming a single ALS locus for diploid sugarbeet and previously described phenotypes for each resistance trait, the expected F_1 genotype and phenotype distribution was determined based on a single dominant nuclear trait (Fig. 1 A). Only IMI-R/SU-R F_1 plants were used for further genetic analysis. Given the previously stated assumptions, only three genotypes would account for this phenotype in F_1 plants (Fig. 1 A, shaded box). *Sir-13* IMI-R allelism with one or both of the other herbicide resistance traits was determined by F_1 testcross or progeny test resistance phenotype-segregation data.

Nine IMI-R/SU-R F_1 plants were simultaneously testcrossed and self-pollinated by bagging with a cytoplasmically male-sterile line (L03). Seed set was successful for one testcross and two self-pollinations. Three herbicide class resistance phenotype-segregation patterns would be expected based on the genotypes listed in Fig. 1 A for IMI-R/SU-R F_1 plants. Expected testcross and self-pollination phenotype distributions for a one-locus model (i.e., all resistance traits allelic) were calculated (Fig. 1 B) and observed distributions determined by leaf-disk assay (Fig. 1 C). Chi-square analysis was used to test data for goodness of fit with the single-locus assumption at the 5% level.

Southern-blot analysis

Molecular evidence for ALS locus number in diploid sugarbeet was obtained by DNA-blot analysis as described by Southern (1975). Genomic DNA was isolated from F_3 plants descended from the original Sir-13 variant (Ausubel et al. 1993). Fifty micrograms of genomic DNA were digested overnight at 37°C with restriction endonucleases (Gibco BRL, 8400 Helgerman Court, Gaithersberg, MD 20887) (1000 U) in a 200-µl total volume of manufacturer's buffer solutions. Restriction enzymes were used that cut at zero internal sites (*Bam*HI and *Hin*dIII), one internal site (*Xba*I), and two internal sites (*Eco*RI) according to a wild-type (wt) sugarbeet ALS gene sequence (Fig. 2 B; B. Mazur, DuPont Agricultural Products, Wilmington, Del., personal communication). Digested DNA was re-precipitated, re-suspended, and DNA fragments separated electophoretically in a 0.7% agarose gel. DNA was de-purinated, denatured, and vacuum-blotted (VacuGene XL, Pharmacia, S-752 82, Uppsala, Sweden) to a nylon membrane according to the protocol supplied by the manuacturer.

A 1.7-kilobase pair (kb) homologous-ALS DNA fragment was generated by polymerase chain reaction (PCR) amplification using primers designed from the wild-type ALS gene sequence. This fragment was fluorescein-labeled (Prime It Fluor fluorescein labeling kit,

Stratagene Inc., 11099 North Torrey Pines Road, La Jolla, CA 92037) and used as a non-radioactive probe for the detection of homologous DNA fragments on the DNA blot. Probe hybridization followed the manufacturer's instructions (QuikHyb hybridization solution, Stratagene Inc., 11099 North Torrey Pines Road, La Jolla, CA 92037) hybridizing at 68°C for 2 h. The blot was rinsed and signal-amplified by the chemifluorescence reaction (Signal amplification module for the FluorImager, Amersham Life Sciences, Amersham Place, Little Chalfont, Buckinghamshire, HP79NA England) and then detected by a FluorImager scanner (FluorImager SI scanner, Molecular Dynamics, 928 East Arques Avenue, Sunnyvale, CA 94086).

Results and discussion

Somatic cell selection

Imidazolinone resistance

Challenging cells of REL-1 with a lethal dose of imazethapyr resulted in three putatively resistant cell isolates growing on 50 nM imazethapyr 8 to 10 weeks after plating onto selective media. Calli of these isolates were propagated both in the presence and absence of 50 nM imazethapyr on B1 solid media. Shoots were successfully regenerated from three isolates on B1 without herbicide. One isolate was identified as an escape when shoots were placed on M20 modified with 50 nM imazethapyr; only isolates identified as Sir-13 and Sir-30 survived. Sir-13 shoots successfully generated roots 6–8 weeks after transfer to N3 rooting media without herbicide. Sir-13 R_0 plants transplanted to soil and grown in the greenhouse were successfully outcrossed to SR93 plants. Sir-30 displayed abnormal morphology both in vitro and in the greenhouse, and attempts to cross plants in the greenhouse failed. Abnormal plant morphology, chimeras, and other deleterious somaclonal variations commonly occur from the tissue-culture process and can affect the ability to regenerate viable, fertile plants (Saunders et al. 1992).

Imidazolinone and sulfonylurea resistance

A single callus derived from cultures initiated from the SU-R plant 93R30 (*Sur*/*Sur*) grew on a background of dead cells 8 weeks after plating onto 50 nM imazethapyr. Callus propagated from these cells in the absence of herbicide regenerated shoots from separate callus clumps over several weeks. Regenerated shoots were individually identified and were maintained and propagated on M20 media without herbicide. Re-testing shoots for IMI-R showed no differences among the individual regenerates indicating that the shoots were most likely genetically identical for IMI-R although further somaclonal variation can continue to be generated after herbicide resistance mutation (Harms et al. 1992; Saunders et al. 1992). Whole plants were generated from one isolate and labeled as 93R30B. Clonal copies of 93R30B (the R_0 generation) were transferred to the greenhouse, and self-pollination and outcrossing attempted.

Genetic analysis

Imidazolinone resistance

An F_1 (*Sir-13*/*wt*) plant surviving 25% of a POST field-application rate of imazethapyr was self-pollinated to produce F_2 seed. Most previous examples of ALS-inhibitor resistance in other species have resulted from point mutations in the ALS gene and were inherited as (semi-)dominant traits (Chaleff and Ray 1984; Haughn and Sommerville 1986; Sebastian et al. 1989; Hart et al. 1993). Identification of F_2 segregates as homozygous sensitive, heterozygous resistant, or homozygous resistant was determined by F_3 progeny testing with POST-applied imazethapyr (Table 2). IMI-R segregation in the Sir-13 F_2 population was tested for "goodness of fit" with a 1:2:1 Mendelian inheritance and *Sir-13* was determined to be inherited as a monogenic dominant trait. Within segregating F_2 progeny families, imazethapyr injury and stunting was observed on approximately two-thirds of the screen survivors. The heterogeneous response to imazethapyr was presumably due to a mixture of homozygous and heterozygous *Sir-13* individuals in these populations. This observation implies that *Sir-13* is most likely a semi-dominant trait, providing greater resistance in the

Table 2 IMI-R genotype distribution for the *Sir-13* F_2 progeny test. The chi-square analysis tested the IMI-R segregation data goodness of fit with a single dominant nuclear gene model

Genotype	Theoretical distribution	Observed frequency	Expected frequency	$\frac{(o-e)^2}{e}$
Homozygous susceptible (0% progeny survival)	1	12	15.5	0.8
Heterozygous resistant (75% progeny survival)	2	35	31	0.5
Homozygous resistant (100% progeny survival)	1	15	15.5	0.0
				$\chi^2 = 1.3$ $0.9 < P < 0.7$[a] $df = 2$, ns

[a] Probability of exceeding chi-square when hypothesis is true. Accept proposed mode of inheritance for $P > 0.05$

homozygous condition. Chlorsulfuron resistance (*Sur*) in sugarbeets, likewise, was inherited as a semi-dominant trait (Hart et al. 1993) and segregating plants could be accurately grouped as heterozygous or homozygous based on the level of injury caused by a post-emergence sulfonylurea herbicide application. Most other plant ALS-herbicide site-of-action resistances also exhibit a semi-dominant resistance (Chaleff et al. 1984; Anderson and Georgeson 1989; Sebastian et al. 1989; Haughn and Sommerville 1990; Mallory-Smith 1990).

Imidazolinone and sulfonylurea resistance

93R30B R_0 plants did not produce seed when bagged for self-pollination or crossed to CMS plants. Decreased fertility has been seen previously in some plants regenerated from callus (Saunders et al. 1992). Female fertility, however, was maintained and seed was produced when 93R30B R_0 plants were pollinated by 'SR93' sugarbeets. F_1 plants were tested simultaneously for IMI-R and SU-R by the leaf-disk assay. Two phenotypes were observed: (1) resistance to both IMI and SU herbicides (IMI-R/SU-R) and (2) sensitive to IMI herbicide but resistant to SU herbicide (IMI-S/SU-R). IMI-R/SU-R and IMI-S/SU-R segregated approximately 1:1 in the F_1 generation (data not shown), suggesting that the newly acquired IMI-R was dominant. Outcrossing with SR93 restored male and self-fertility and IMI-R/SU-R (determined by leaf-disk assay) F_1 plants were self-pollinated to produce an F_2 population. Segregation of IMI-R and SU-R was examined in the F_2 population by the leaf-disk assay. IMI-R and SU-R strictly co-segregated, indicating that these traits were tightly associated. Segregation of the IMI-R/SU-R trait in the F_2 population was consistent with a single dominant nuclear trait (Table 3). We have labeled this allele *93R30B*.

Based on the 1:1 F_1 segregation of IMI-R/SU-R and IMI-S/SU-R, as well as strict co-segregation of IMI-R and SU-R in the F_2 generation, we hypothesized that IMI-R of *93R30B* resulted from a genetic change in the *Sur* allele which previously provided no resistance to IMI herbicides. Alternatively, a second, unlinked locus hypothesis was examined with the following

Table 3 IMI-R/SU-R phenotype distribution for the *93R30R* F_2 herbicide resistance screen. The model tested assumes that the *93R30B* trait (IMI-R/SU-R) is a single nuclear dominant trait

Genotype	Theoretical distribution	Observed frequency	Expected frequency	$\frac{(o-e)^2}{e}$
Susceptible (IMI-S/SU-S)	1	53	42.5	2.6
Resistant (IMI-R/SU-R)	3	117	127.5	0.9
(homozygous + heterozygous)				$\chi^2 = 3.5$ $0.1 < P < 0.05$[a] $df = 1$, ns

[a] Probability of exceeding chi-square when hypothesis is true. Accept proposed mode of inheritance for $P > 0.05$

Table 4 Chi-square analysis of the observed *93R30B* F_2 progeny test phenotype distribution versus proposed one- or two-ALS locus models. Assumptions made: if one-locus, *93R30B* had a IMI-R/SU-R phenotype; if two-locus, *93R30B* could be either IMI-R/SU-R or IMI-R/SU-S

Phenotype	Observed frequency	Assumed F_1 genotype					
		One-locus		Two-locus			
		(*93R30B*/*wt*)		(*93R30B*/*wt*, *Sur*/*wt*)[a]		(*93R30B*/*wt*, *Sur*/*wt*)[b]	
		Theoretical distribution	Expected Frequency	Theoretical distribution	Expected frequency	Theoretical distribution	Expected frequency
IMI-R/SU-R	117	3	127.5	12	127.5	9	95.6
IMI-R/SU-S	0	0	0	0	0	3	31.8
IMI-S/SU-R	0	0	0	3	42.5	3	31.8
IMI-S/SU-S	53	1	42.5	1	10.6	1	10.6
		$\chi^2 = 3.5$ $0.1 > P > 0.05$[c] $df = 1$		$\chi^2 = 213.0$ $0.001 < P$[d] $df = 2$		$\chi^2 = 238.0$ $0.001 < P$[d] $df = 3$	

[a] *93R30B* allele phenotype assumed = IMI-R/SU-R
[b] *93R30B* allele phenotype assumed = IMI-R/SU-S
[c] Probability of exceeding chi-square when hypothesis is true. Accept proposed mode of inheritance for $P > 0.005$
[d] Probability of exceeding chi-square when hypothesis is true. Reject proposed mode of inheritance for $P < 0.05$

constraints: the *93R30B* allele was dominant (or semi-dominant) and imparted a phenotype of IMI-R/SU-R or IMI-R/SU-S. An F_1 resistance segregation of 1 : 1 (IMI-R/SU-R : IMI-S/SU-R) would be expected for either the one- or two-locus models; however, because IMI-R and SU-R strictly co-segregated in the F_2 generation, the two-locus hypothesis was rejected (Table 4). If two unlinked loci were involved, IMI-specific or SU-specific resistance would have been observed. These data indicated that IMI-R of *93R30B* and SU-R of *Sur* were tightly associated and most likely represented two independent changes to the same allele. 93R30B ALS enzyme assays demonstrated a more than a 1000-fold decrease in enzyme sensitivity to representative IMI and SU herbicides, consistent with whole-plant herbicide resistance (data not shown). *Sur* and *93R30B* , therefore, represent different semi-dominant alleles of the sugarbeet ALS locus with phenotypes of IMI-S/SU-R and IMI-R/SU-R, respectively, obtained by a two-step selection process. Creason and Chaleff (1988) described a two-step selection in tobacco where the second selection attempted to improve the level of SU-R obtained in the first selection. They selected for increased SU-R in vitro from a plant already homozygous for a SU-R trait (*S4*/*S4*). The increased resistance allele (*S4-Hra*) was also linked to the initial SU-R trait derived by a previous round of somatic cell selection. Lee et al. (1988) reported that *S4* had a single amino-acid change within the deduced ALS amino-acid sequence and that *S4-Hra* had acquired a second mutation within the same deduced peptide sequence.

Fig. 1A–C Phenotype evaluation to determine herbicide resistance allelism. **A** Expected F_1 genotype and phenotype segregation ratio, and observed F_1 phenotype distribution from a four-allele cross. **B** Possible testcross and progeny test herbicide class resistance segregation ratios for three IMI-R/SU-R F_1 genotypes (**A**, *shaded box*). **C** Observed testcross and progeny test herbicide class resistance segregation ratios for three IMI-R/SU-R F_1 individuals

93R30B/Sur x Sir-13/wt

A

	Genotype	Phenotype	Expected	Observed
A	*93R30B/Sir-13*	IMI-R/SU-R		
B	*93R30B/wt*	IMI-R/SU-R	9	9
C	*Sur/Sir-13*	IMI-R/SU-R		
D	*Sur/wt*	IMI-S/SU-R	3	3
				$x^2 = 0$
				$P = 1.0^a$, $df = 1$

Select IMI-R/SU-R F_1 phenotypes only

Testcross (*wt/wt* x F_1) or Self pollinate

B

	Expected testcross class-resistance distribution				Expected testcross class-resistance distribution			
	IMI/SU	IMI	SU	wt	IMI/SU	IMI	SU	wt
A	1	1	0	0	3	1	0	0
B	1	0	0	1	3	0	0	1
C	0	1	1	0	2	1	1	0

C

	Observed testcross class-resistance distribution						Observed testcross class-resistance distribution					
	IMI/SU	IMI	SU	wt	x^2	*df*	IMI/SU	IMI	SU	wt	x^2	*df*
A	-	-	-	-			-	-	-	-		
B	-	-	-	-			96	0	0	22	2.5	$1^{a,c}$
C	0	27	22	0	$0.5^{a,b}$	1	13	4	5	0	0.8	$2^{a,d}$

[a] Probability of exceeding chi-square when hypothesis is true. Accept proposed mode of inheritance for $P > 0.05$

[b] $(0.5 > P > 0.3)$

[c] $(0.2 > P > 0.1)$

[d] $(0.7 > P > 0.5)$

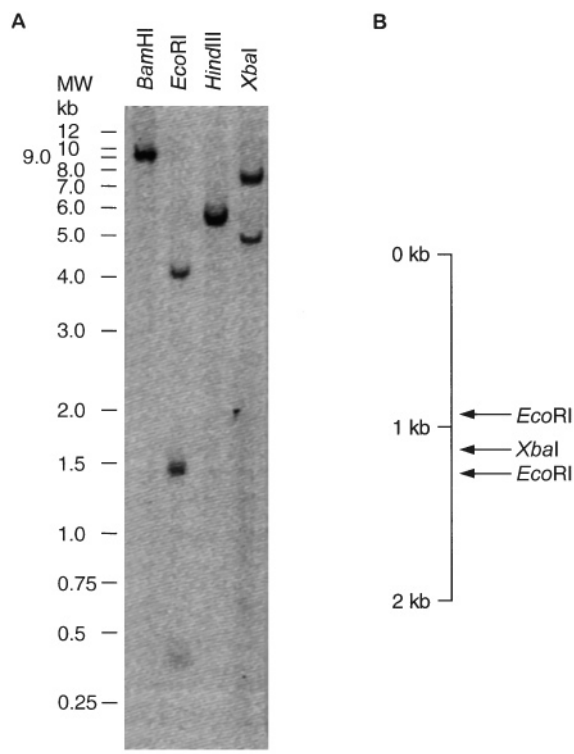
Allelism of herbicide resistance traits

Genetic evidence

Twelve individual F_1 plants from the *93R30B*/*Sur* × *Sir*-13/*wt* cross were tested by leaf-disk assay for IMI-R and SU-R. Two phenotypes were expected in a 3 : 1 ratio (IMI-R/SU-R : IMI-S/SU-R) whether *Sir-13* was allelic (i.e., another variant at the same ALS locus) or present at a different locus. The observed phenotype segregation agreed perfectly with expected; however, the low number of individuals tested warrants caution in any conclusions from these data (Fig. 1 A). Subsequent testcrossing and self-pollination resulted in seed production in only three plants (one testcross and two self-pollinations). The expected class resistance segregation ratios were calculated for each possible F_1 IMI-R/SU-R genotype (Fig. 1 A, shaded box) for each possible testcross and self-pollination (Fig. 1 B). Herbicide class resistance segregation for each family was adequately described by the predicted segregation for the single-locus model (Fig. 1 C). Phenotype distributions were not adequately described by an alternative, two-locus model with *Sir-13* at a separate locus from *Sur* and *93R30B* . The two-locus model is constrained by co-dominance phenotype distributions which could not be reconciled with the observed phenotypic ratios. For example, under the two-locus hypothesis, the expected phenotype segregation for the testcross population in line C (Fig. 1 C) (unliked two-locus genotype = *Sur*/*wt*, *Sir*-13/*wt* × *wt*/*wt*, *wt*/*wt*) would be 1 : 1 : 1 : 1 for IMI-R/SU-R:IMI-R/SU-S : IMI-S/SU-R : IMI-S/SU-S. The observed 0 : 1 : 1 : 0 segregation is well described by the single-locus model but does not fit any expected segregation distribution for the two-locus model. Two F_1 plants were identified as having a *Sur*/*Sir-13* genotype, indicating these two traits were indeed allelic. *Sur* and *93R30B* were previously described as allelic. These crosses did not directly demonstrate that *93R30B* and *Sir-13* were allelic; however, since both traits are allelic with *Sur* it can be surmised that both IMI-R traits are also allelic. As with *Sur* and *Sir-13*, two herbicide class-specific resistance ALS alleles located at the *Csr1* locus (*Csr1-1* = IMI-S/SU-R and *Csr1-2* = IMI-R/SU-S) were selected from mutagenized *Arabidopsis thaliana* (L.) Heyhn. seed (Haughn and Sommerville 1986, 1990). A third allele located at the *Csr1* locus (*Csr1-4*) was derived by intragenic recombination between the *Csr1-1* and *Csr1-2* alleles resulting in cross resistance to both classes of herbicides (Mourad et al. 1994, 1995). It is very probable that more than three herbicide resistance alleles can exist for a single higher-plant ALS locus.

Southern blot analysis

The evidence for allelism of the three herbicide resistance traits was strengthened by Southern-blot analysis data displaying for the first time that a single ALS gene-copy exists in diploid sugarbeet. The observed Southern-blot banding pattern exactly matched the expected band number and sizes (Fig. 2 C) predicted for a single copy given the restriction map for endonucleases used in this experiment (Fig. 2 B). Additional, anomalous bands would be expected if more than one ALS gene copy were present. Sugarbeet was previously stated to have a single ALS gene copy (Hartnett et al. 1991); however, no evidence to this effect has ever been published. A single ALS locus and allelism of the *Sur* and *Sir-13* resistance traits would limit the ability to stack these resistances in a homozygous condition in order to achieve even greater levels of resistance. The homozygous state for each allele would be desired as each is semi-dominant and a greater level of herbicide resistance is expected for a plant homozygous for a semi-dominant trait. The combination of IMI-R and



C

Restriction enzyme	Expected bands # of bands	Band size (s) (kb)	Observed bands # of bands	Band size (s) (kb)
*Bam*HI	1	>2.0	1	9.1
*Eco*RI	3	0.35, >0.7, >0.9	3	0.35,1.5, 4.0
*Hind*III	1	>2.0	1	5.5
*Xba*I	2	>0.7, >1.3	2	5.0, 7.5

Fig. 2A–C Sugarbeet Southern-blot analysis for ALS gene copy determination. **A** Southern blot. **B** Restriction map for the 1998-bp sugarbeet ALS gene: no internal *Bam*HI or *Hin*dIII sites exist. **C** Expected and observed band number and sizes for a single ALS gene copy number

SU-R in the *93R30B* trait may circumvent the limitation of trying to combine the SU-R of *Sur* and IMI-R of *Sir-13* and so present the possibility to produce cross-resistant sugarbeets in the homozygous condition.

In this manuscript we have described the selection of two unique imidazolinone resistance alleles (*Sir-13* and *93R30B*). These resistances were allelic to a previously-described sulfonylurea resistance factor, *Sur*. Herbicide class-specific resistances for *Sir-13* (IMI-R) and *Sur* (SU-R) were combined in a single allele (*93R30B*), apparently an ALS double mutant, by a two-step selection process. ALS herbicide resistance expressed by whole sugarbeet plants was affected by the number of semi-dominant alleles present. Since all three sugarbeet resistance traits are allelic, stacking of these traits can only be accomplished in a mutually heterozygous condition in diploid sugarbeets. The cross-resistant phenotype of *93R30B* , however, will allow a IMI-R/SU-R phenotype in a homozygous state. The prospects for using these traits for overcoming ALS-herbicide carry over injury to sugarbeets or for weed control in sugarbeets appear promising.

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