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Establishment of transgenic herbicide-resistant creeping bentgrass (*Agrostis stolonifera* L.) in nonagronomic habitats

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Abstract

Concerns about genetically modified (GM) crops include transgene flow to compatible wild species and unintended ecological consequences of potential transgene introgression. However, there has been little empirical documentation of establishment and distribution of transgenic plants in wild populations. We present herein the first evidence for escape of transgenes into wild plant populations within the USA; glyphosate-resistant creeping bentgrass (*Agrostis stolonifera* L.) plants expressing *CP4 EPSPS* transgenes were found outside of cultivation area in central Oregon. Resident populations of three compatible *Agrostis* species were sampled in nonagronomic habitats outside the Oregon Department of Agriculture control area designated for test production of glyphosate-resistant creeping bentgrass. *CP4 EPSPS* protein and the corresponding transgene were found in nine *A. stolonifera* plants screened from 20 400 samples (0.04 ± 0.01% SE). *CP4 EPSPS*-positive plants were located predominantly in mesic habitats downwind and up to 3.8 km beyond the control area perimeter; two plants were found within the USDA Crooked River National Grassland. Spatial distribution and parentage of transgenic plants (as confirmed by analyses of nuclear ITS and chloroplast *matK* gene trees) suggest that establishment resulted from both pollen-mediated intraspecific hybridizations and from crop seed dispersal. These results demonstrate that transgene flow from short-term production can result in establishment of transgenic plants at multi-kilometre distances from GM source fields or plants. Selective pressure from direct application or drift of glyphosate herbicide could enhance introgression of *CP4 EPSPS* transgenes and additional establishment. Obligatory outcrossing and vegetative spread could further contribute to persistence of *CP4 EPSPS* transgenes in wild *Agrostis* populations, both in the presence or absence of herbicide selection.

Keywords: *Agrostis stolonifera*, *CP4 EPSPS*, creeping bentgrass, transgene escape

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Introduction

The potential for unintended ecological impacts of transgene introgression from genetically modified (GM) crops into wild populations has been extensively examined in recent years (reviewed in Snow 2002; Ellstrand 2003; Jenczewski *et al*. 2003; Stewart *et al*. 2003; Pilson & Prendeville 2004; Marvier & Van Acker 2005). To assess changes in populations associated with transgene introgression there is a need to identify wild (unmanaged native, naturalized or weedy) plant populations that contain established transgenic individuals. However, there are very limited published empirical data that document the relative frequency of establishment and distribution of transgenic plants in wild populations (i.e. data are available primarily for agricultural fields and nearby bordering areas). Thus, the first global evidence of transgene escape into natural weedy populations was only recently reported for herbicide (glyphosate) resistant *Brassica* (Warwick *et al*. 2003, 2004).

It could be argued that the lack of numerous documented cases of transgene escape indicates that such events are rare, and are therefore of minimal concern. This hypothesis

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may prove to be generally true; however, it has remained largely untested. On the other hand, it is reasonable to expect that additional transgenic hybrid plants have or will soon become established within noncultivated populations located near other GM crops. Many crops, including some for which GM cultivars have been developed, are sympatric with sexually compatible wild relatives. There are numerous examples of conventional gene flow (not involving transgenes) and hybridization between cultivated and noncultivated populations (*Cucurbita*, Kirkpatrick & Wilson 1988; *Raphanus*, Klinger *et al*. 1992; *Helianthus*, Arias & Rieseberg 1994; *Sorghum*, Arriola & Ellstrand 1996; *Beta*, Bartsch *et al*. 1999; *Brassica*, Rieger *et al*. 2002) that suggest the potential for transgene escape from GM cultivars via hybridization. Research using non-GM crop alleles (Linder *et al*. 1998) and modelling studies (Meagher *et al*. 2003; Thompson *et al*. 2003) also suggest that under certain conditions, transgene introgression and persistence are likely in wild populations. The likelihood of transgene introgression depends on many factors including the fertility of F_1 progeny and subsequent backcrossed hybrids plus the effects that particular engineered traits have on the fitness of hybrids (cf. Bartsch *et al*. 2001; Spencer & Snow 2001; Burke & Rieseberg 2003; Snow *et al*. 2003; Halfhill *et al*. 2005). Transgene flow to wild populations and opportunities to form fertile hybrids can also be expanded spatially and temporally by dissemination of GM crop seeds that result in the establishment of feral transgenic plants. Nontransgenic feral *Brassica rapa* has been shown to persist outside of cultivated fields in France for at least 8 years (Pessel *et al*. 2001). As with transgenic crop × wild hybrids, engineered traits effecting fitness are predicted to affect the persistence of feral transgenic *B. rapa* as well (Claessen *et al*. 2005).

In 2003, under USDA Animal and Plant Health Inspectional Service (APHIS) permit, flowering of approximately 162 ha of glyphosate-tolerant GM creeping bentgrass (event ASR368 by Scotts Company and Monsanto) occurred for the first time in Jefferson County Oregon, USA, within a 4453-ha agricultural bentgrass control area established by the Oregon Department of Agriculture (Oregon Administrative Rules 2002). Following that initial flowering event, we documented transgenic pollen-mediated hybrid seed formation in resident *Agrostis* populations on a landscape level, based on greenhouse and laboratory analyses of seeds from panicles collected in the field (Watrud *et al*. 2004). The current investigation builds upon our previous work and was driven by our interest in determining whether or not transgenic plants could become established in the environment in nonagronomic habitats.

Our interest in GM glyphosate-resistant creeping bentgrass as an experimental study system is based on its life history characteristics, its geographical distribution in diverse habitats and the availability of *CP4 EPSPS* as a selectable marker for tracking gene flow from GM cultivars and its potential introgression into wild compatible populations. *Agrostis stolonifera* transformed with a *CP4 EPSPS* construct (5-enolpyruvylshikimate-3-phosphate synthase gene from *Agrobacterium* spp. strain CP4) is one of the first transgenic, perennial, wind-pollinated crops with sexual and asexual modes of reproduction that is intended to be grown outside of agricultural fields (i.e. on golf courses). *A. stolonifera* belongs to a cosmopolitan genus that includes approximately 200 species worldwide (Sell & Murrell 1996; Kartesz 2003; Soreng *et al*. 2003). There are approximately 34 North American species of *Agrostis*, 26 of which are native. Fourteen native and naturalized species are found in Oregon. North American taxa include diploid, tetraploid, hexaploid and possibly octaploid species (primarily allopolyploids) plus various aneuploid specimens. *A. stolonifera* is an obligate outcrossing species and member of a hybridizing network of at least 12 other grass species from *Agrostis* and *Polypogon*. Of the four species from this complex that grow wild in central Oregon, *Agrostis exarata* Trin. (spike bentgrass) is native, while *Agrostis gigantea* Roth,(redtop), *A. stolonifera* and *Polypogon monspeliensis* (L.) Desf. (annual rabbit's-foot bentgrass) are naturalized (reviewed by MacBryde 2005). Using Grant's (1981) system of classification, the network has characteristics of both polyploid and clonal hybrid complexes. Naturally occurring interspecific hybrids of *Agrostis* tend to be sterile or produce low numbers of viable seeds. Under field conditions, few viable transgenic F_1 *A. stolonifera* × *A. capillaris* L. (colonial bentgrass) and *A. stolonifera* × *A. castellana* Boiss & Reut. (dryland bentgrass) hybrids were recovered by Belanger *et al*. (2003a). However, under optimum conditions, there was notable fertility of transgenic *A. stolonifera* × *A. canina* L. (velvet bentgrass), *A. stolonifera* × *A. capillaris*, *A. stolonifera* × *A. castellana* and *A. stolonifera* × *A. gigantea* among viable hybrid progeny detected through backcrosses (Belanger *et al*. 2003b). Backcrossing of hybrid plants may restore full fertility. Likewise, fertility of F_1 *A. gigantea* × *A. capillaris* and *A. stolonifera* × *A. capillaris* hybrids has been observed in self crosses that occasionally produce viable F_2 progeny (Bradshaw 1975). Even though individual *A. stolonifera* interspecific F_1 hybrids may have low fertility, they can still undergo extensive clonal propagation via spread of stolons and/or rhizomes (Edgar & Connor 2000). Furthermore, there is evidence for persistence and adaptability of naturally occurring *Agrostis* hybrids (*A. gigantea* × *A. capillaris*) in hybrid swarms (Stuckey & Banfield 1946; Meerts & Lefèbvre 1989). *Agrostis* species are found in a remarkable variety of agronomic and nonagronomic habitats (Grime *et al*. 1988; Kik *et al*. 1991). Species like *A. stolonifera* also spread by movement of their small seeds (approximately 2 mm × 0.5 mm; 80 μg/seed for *A. stolonifera*) that are readily dispersed by wind, water, and animals (Hunt *et al*. 1987; Grime *et al*. 1988). *A. stolonifera*, which was regionally introduced to the USA from Europe, is sometimes considered to

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be a weed in crop fields as well as a colonizer of nonagricultural habitats. At least eight other countries also consider it to be weedy (MacBryde 2005).

A key factor affecting the potential for transgene escape from GM *A. stolonifera* is the long distance over which viable pollen may be transported to compatible recipient plants via wind-mediated transport. Previously, we documented hybridization of *Agrostis* plants by viable transgenic pollen as far as 21 km beyond the perimeter of the bentgrass control area in central Oregon in 2003 (Watrud *et al.* 2004). In that study, seeds were collected from panicles of sentinel and resident plants that had been placed or were naturally growing outside of the GM bentgrass control area; seeds were germinated in a greenhouse and were sprayed with the herbicide glyphosate, the active ingredient in RoundUp (Monsanto). Survivors of herbicide treatment were tested for expression of *CP4 EPSPS* protein, and additional molecular tests [polymerase chain reaction (PCR) and sequencing] confirmed presence of the transgene. Field surveys for establishment of wild transgenic *Agrostis* pollen-mediated hybrids were not conducted during that study. Transgene flow by pollen transport represents one avenue of successful escape of transgenes into wild populations; dispersal of transgenic crop seeds is another. In 2004–2005, a separate survey conducted by Mallory-Smith *et al.* (2005) found numerous *CP4 EPSPS*-positive *Agrostis stolonifera* plants in agronomic settings inside the bentgrass control area at locations where either no *Agrostis* plants were detected in the previous year, or where they had been removed. The volunteers found were presumed to be GM seed progeny because of their growth in plowed fields of other crops, in open disturbed spaces or along irrigation canals near ASR368 fields. In contrast, it is more logistically challenging to detect diffuse sites of successful establishment and persistence of transgenic *Agrostis* plants in populations at multi-kilometre distances outside of the control area.

This study thus focuses on locating and phylogenetically identifying *CP4 EPSPS*-positive *Agrostis* plants that became established in nonagronomic habitats following the initial 2003 test production of GM creeping bentgrass. We also discuss factors affecting establishment and persistence of *CP4 EPSPS* in wild *Agrostis* populations.

Materials and methods

Experimental overview

The objective of our field studies was to locate wild (native and naturalized) *Agrostis* populations outside the control area containing transgenic plants established by either crop × wild hybridization or by crop seed dispersal. Leaf samples from 50 or more plants at a location were combined and tested for presence of *CP4 EPSPS* protein using TraitChek tests (Strategic Diagnostics). Bulk sample

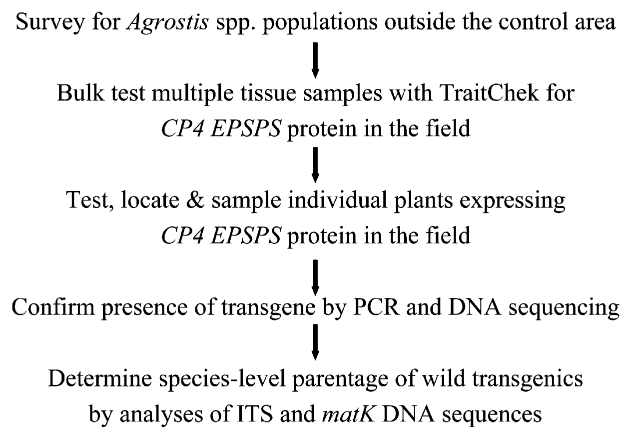

Fig. 1 Flow chart of the field and molecular experiments used to locate and identify wild transgenic plants outside of the bentgrass control district.

that tested positive were subsampled in the field to identify specific *CP4 EPSPS*-positive plants. In the laboratory, we utilized PCR- and sequencing-based approaches to confirm presence of the transgene in individual samples. We also tested a procedure for detection of F_1 interspecific hybrids among wild transgenics by determining their parentage with nuclear ribosomal ITS1-5.8S-ITS2 (ITS) and maternally inherited chloroplast *trnK* intron maturase (*matK*) DNA (see Fig. 1).

Field surveys and sampling of nonagronomic populations for transgenic Agrostis *plants*

Our searches for newly established transgenic plants in nonagronomic areas were guided by field surveys that we conducted in conjunction with our previous study (Watrud *et al.* 2004) and the literature on *Agrostis* (Hitchcock 1950; Frenkel *et al.* 1978; Esser 1994; Crowe & Clausnitzer 1997; Crawford 2003). These sources of information suggested that wild *Agrostis* populations within the arid, high desert plateau of central Oregon including the control area would be more restricted to mesic habitats. We also took into account our observations that the highest frequencies of viable transgenic seeds (assayed through greenhouse and laboratory experiments) came from *Agrostis* plants that were within 4.8 km outside of the control area (Watrud *et al.* 2004). Consequently, we used a sampling design that focused on nonagronomic mesic habitats within 4.8 km outside of the control area rather than a spatially uniform sampling design in light of the a priori information on habitat preference. Due to property ownership constraints, we concentrated our 2004–2005 *Agrostis* population surveys on publicly accessible regions along the Deschutes River, Willow Creek and in the Crooked River National Grassland as well as canals, roadside ditches, natural drainages and

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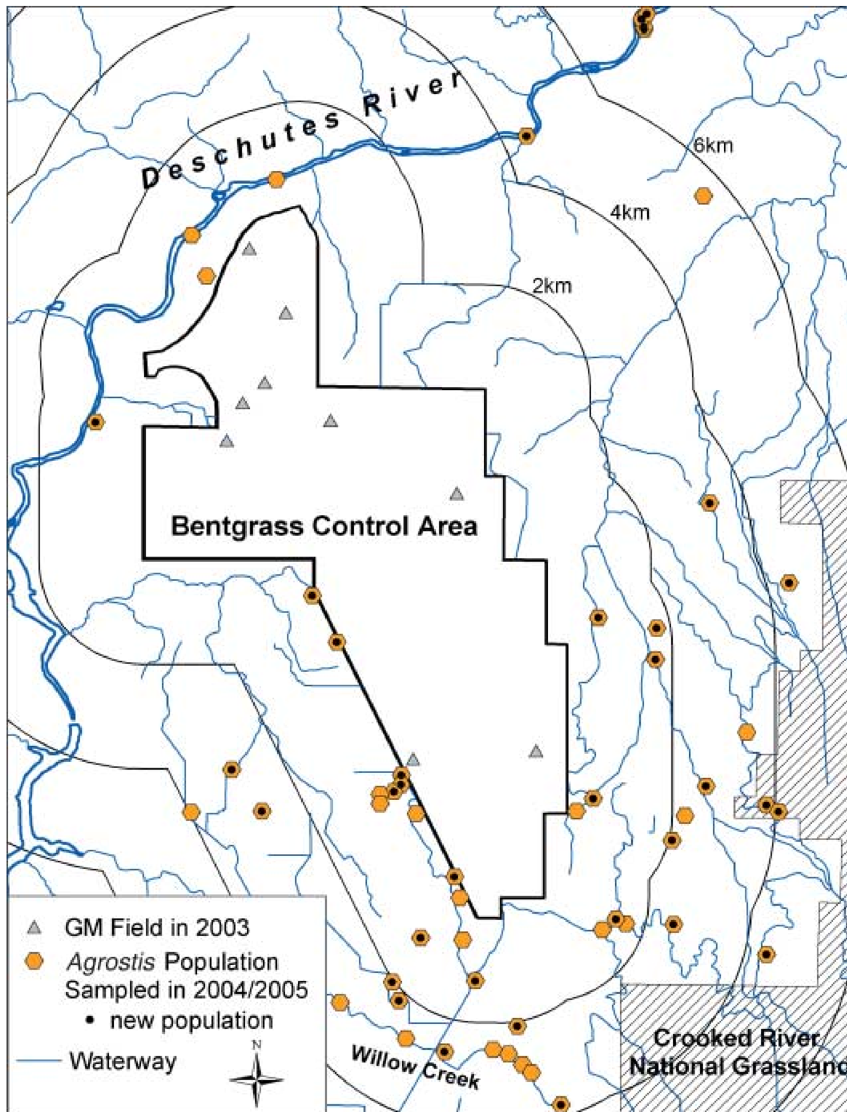

Fig. 2 Map of the *Agrostis* populations located and sampled for transgenic plants outside the GM creeping bentgrass control area Oregon, USA. The 34 new sites here were those not previously sampled by Watrud *et al*. (2004).

ponds out to 4.8 km beyond control area. In addition, we performed extended sampling 6.9 km downstream along the Deschutes River to the northeast of the control area because this is the major natural waterway that passes near the test production area (Fig. 2). While *Agrostis* populations sampled within this study partially overlapped with resident sites where seeds were collected for testing by Watrud *et al*. (2004), additional sites identified after the 2003 growing season are included in the current study.

As putative *Agrostis* populations were located, morphological identification of *Agrostis* and other species present was based on the taxonomy prescribed by the PLANTS database (US Department of Agriculture, National Resources Conservation Service 2004), and both regional and national floras were used for plant identification (Hitchcock 1950; Hitchcock *et al*. 1969; Hickman 1993). At each site, the search was conducted in exposed soil microsites that were considered to be most conducive to seedling establishment due to soil disturbance or reduced competition from established plants. Sampling procedures were conducted as follows: On canal banks where *Agrostis* and likely establishment sites were in a relatively continuous and narrow band along the water's edge, samples were taken along linear transects of variable length that depended on the density of seedlings and patch length (typically 2 m). Where *Agrostis* plants were more dispersed, samples were collected within the 1-m radius of a centrally placed reference stake. At more discrete *Agrostis* locations, individual plants were sampled within a marked area encompassing the observed *Agrostis* plants.

Collection of leaf samples focused on juvenile individuals because these would be the most recently established plants within disturbed sites. Young plants sampled were not in flower, so *Agrostis* species were differentiated in the

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field from other grasses using vegetative characteristics including leaf colour, size, shape, and texture; presence of stolons or rhizomes; ligule characteristics; and overall plant morphology. To the extent possible, a single leaf was sampled from each individual plant. Initial leaf samples were pooled by site to allow for rapid field screening of multiple samples with TraitChek tests for *CP4 EPSPS* protein. Previous pilot laboratory studies with homogenates of mixtures of one known positive *CP4 EPSPS* leaf tissue disc sample with dilutions (e.g. 1 : 4–99) of non-GM leaf tissue discs of equal size (8 mm^2) obtained by using a one-hole paper punch, suggested that under field conditions collections of leaf samples could initially be tested in larger, pooled batches. In the field, leaves were counted and grouped into bundles of up to 40–50 leaves, and then 1–2 cm sections of each leaf were cut and placed in vials. Following the addition of approximately 0.5 mL of water to a 1.7 mL microfuge tube, the pooled sample was homogenized with a disposable plastic pestle and tested with a TraitChek test strip. If the larger pooled sample homogenate tested positive for the protein, successively smaller sets of leaf samples were similarly tested until the individual *CP4 EPSPS*-positive plant(s) could be identified and labelled *in situ*. Leaf samples also were collected from the closest mature *Agrostis* species neighbours to established transgenic plants for later molecular characterization as potential paternal parents of the *CP4 EPSPS* positive plants.

Molecular tests for transgenes and for determination of parentage of wild transgenic Agrostis *plants*

We wanted to ensure that field sampling procedures had correctly identified individual transgenic plants within *Agrostis* populations found in the field survey. To do this, we first extracted genomic DNA from TraitChek positive plant leaf tissue, then used the same PCR methods, primers and reagents reported in Watrud *et al*. (2004) designed to amplify 1 kb segments of the *CP4 EPSPS* coding regions. Positive, negative and no template controls were included in all amplifications. Amplified fragments were then sequenced as before for comparison with known *CP4 EPSPS* coding sequence.

Our next goal was to determine the parentage of field-positive plants in order to identify interspecific hybrids that may have formed. Laboratory detection of first- and second-generation plant hybrids is often done using combinations of chromosome counts, ploidy determination and various population-level molecular markers (cf. Hall *et al*. 2000; Warwick *et al*. 2003; Halfhill *et al*. 2005). Unfortunately, proprietary constraints by Scotts and Monsanto currently limit our access to ASR368 plants or their potential hybrid progeny. We thus used molecular systematic methods to characterize and identify *Agrostis* hybrids. Species-level molecular phylogenetic analyses based on nuclear and/or plastid DNA sequence data have successfully been used to identify interspecific hybrids in several different plant lineages including those with a history of reticulation (Rieseberg 1995; Sang *et al*. 1995; Rauscher *et al*. 2002; Koch *et al*. 2003; Dobes *et al*. 2004; Weeks & Simpson 2004; Ellison *et al*. in press). Hybridization events are frequently discovered through incongruity between sequence placement in gene phylogenies derived from nuclear and plastid loci. For angiosperms, disagreements can reflect the biparental inheritance of nuclear alleles vs. the maternal inheritance of plastid alleles (Weeks & Simpson 2004 and references therein). We chose to separately analyse ITS and *matK* gene trees in this project, to check for incongruities in sequences from transgenics that could indicate recent interspecific hybrid origin.

Reference taxa used here for species-level parentage determination of field positives included all species from the temperate North American *Agrostis* hybridizing network summarized by MacBryde (2005) except *Polypogon fugax* (Table 1). Each taxon was represented by an accession from herbaria or a germplasm repository. Additional samples were included from *Agrostis exarata*, *Agrostis gigantea*, *Agrostis stolonifera* and *Polypogon monspeliensis* resident plants from our central Oregon study sites, an ASR368 plant, a volunteer from presumptive ASR368 seed dispersal and a known transgenic hybrid of resident *A. gigantea* pollinated by ASR368 during 2003 (interspecific hybrid control). DNA extraction from reference leaf tissue was conducted with the same procedures used for field positive plants.

Primers for the ITS were ITS5 (White *et al*. 1990) and new plant-specific P216R CGTCGTGCGCACCGTTCAWAGGG. Primers for *matK* amplification and outermost sequencing were *trnK*F and *trnK*R (Cronn *et al*. 2002). Additional new midstream *matK* sequencing primers were P211R CAGTAGCGGGAACTATGGTATCG, P221F TAATTGGG-TAGAAAAGGAACG, P226R AAGCATTTCTCGGTT-TATCG, P227R GCATTTTTCATTGCACACGAC and P228F GAGCAACAAATTCGTCCAGA. Novel primers were designed with PrimerSelect (DNAStar) and were synthesized by QIAGEN. Amplification of ITS regions was done with standard reagents and concentrations (Roche Diagnostics Corporation). The ITS thermal profile was 40 cycles of 95 °C for 30 s, 56 °C for 15 s and 72 °C for 50 s. For amplification of *matK*, the final $MgCl_2$ concentration was raised to 2 mM and was conducted with 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min 30 s. PCR products were purified with QIAquick Gel Extraction kits. Purified ITS amplicons were cloned with TA Cloning Kits (Invitrogen). Cloning was conducted to test the variability of ITS sequences within a given tissue sample. Ten random clones were screened by PCR and products were sequenced. Cloning of *mat*K fragments proved unnecessary due to minimal variation between clones from an individual. Thus, *matK* fragments were sequenced directly. Cycle-sequencing

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Table 1 *Agrostis* and *Polypogon* taxa included for molecular phylogenetic identification of transgenic plants found in the wild

Classification	Internal designation	Source/voucher information
Poaceae; Pooideae; Aveneae; *Agrostis*		
A. canina L.	*A. canina* USU4	Wales, Great Britain; Utah State University Intermountain Herbarium; USU124245
A. capillaris L.	*A. capillaris* OSU5	Yamhill County, Oregon; Oregon State University Herbarium; OSC84615
A. castellana Boiss & Reut.	*A. castellana* USU6	Yolo County, California; Utah State University Intermountain Herbarium; USU234649
A. exarata Trin.	*A. exarata* USU43	Malheur County, Oregon; Utah State University Intermountain Herbarium; USU154401
A. exarata	*A. exarata* NGC2	Jefferson County, Oregon; US EPA Western Ecology Division
A. gigantea Roth	*A. gigantea* USHN32	Roth, Kalamazoo, Michigan; US National Herbarium; USNH3110826
A. gigantea	*A. gigantea* EPR12	Jefferson County, Oregon; US EPA Western Ecology Division
A. idahoensis Nash	*A. idahoensis* USU13	Uinta Mountains, Utah; Utah State University Intermountain Herbarium; USU2708
A. mertensii Trin.	*A. mertensii* USU20	Horton Mackie Lake, Canada; Utah State University Intermountain Herbarium; USU204867
A. pallens Trin.	*A. pallens* OSU32	Lane County, Oregon; Oregon State University Herbarium; ORE8264
A. scabra Willd.	*A. scabra* OSU40	Crook County, Oregon; Oregon State University Herbarium; ORE8317
A. stolonifera L.	*A. stolonifera* OSU2	Lincoln County, Oregon; Oregon State University Herbarium; OSC61973
A. stolonifera	*A. stolonifera* GP1	Jefferson County, Oregon; US EPA Western Ecology Division
A. stolonifera	*A. stolonifera* NGC2	Jefferson County, Oregon; US EPA Western Ecology Division
A. stolonifera cultivar ASR368	*A. stolonifera* ASR368	Scotts Company, Marysville, Ohio
A. stolonifera cultivar ASR368 SS	*A. stolonifera* ASR368 SS	Jefferson County, Oregon; Oregon State University; seed scatter within Control District
A. vinealis Schreb.	*A. vinealis* GRIN3	USDA ARS National Plant Germplasm System; Pullman, Washington; PI440110
A. gigantea × *A. stolonifera* hybrid	*A. gig.*♀ × *A. stol.*♂ OSU4911	Jefferson County, Oregon; Oregon State University; transgenic hybrid progeny of resident *A. gigantea* pollinated by *A. stolonifera* ASR368 during 2003
Poaceae; Pooideae; Aveneae; *Polypogon*		
P. monspeliensis (L.) Desf.	*P. monspeliensis* USNH24	Malheur Forest, Grant County Oregon; US National Herbarium; USNH305585
P. monspeliensis	*P. monspeliensis* MAD1	Jefferson County, Oregon; US EPA Western Ecology Division
P. viridis (Gouan) Breistr.	*P. viridis* USNH11	Jalisco, Mexico; US National Herbarium; USNH2432658

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reactions used BigDye v3.1 chemistry and standard thermal profiles suggested by the manufacturer (Applied Biosystems). Labeled fragments were purified with CleanSeq kits (AgenCourt Bioscience). Sequence data were collected on a PRISM 3100 Genetic Analyser (Applied Biosystems).

Contigs for both regions were assembled with SEQMAN 5.05 (DNAStar). All ITS sequences were screened against GenBank accessions by using BLASTN searches to insure that sequences from fungal endophytes were not included in further analyses. ITS and *mat*K sequences were deposited in GenBank under accession numbers (DQ146766–DQ146826). Sequences were aligned using MEGALIGN 5.05 (DNAStar) and edited in MACCLADE 4.06 (Maddison & Maddison 2003). Heuristic parsimony tree searches and bootstrap support analyses for the ITS and *mat*K data matrices were executed in PAUP*4.03b10 (Swofford 2003). Unique ITS sequences from each taxon were evaluated for their effect on tree topology. Monophyly of ITS sequences from individual reference taxa was evaluated by similar searches. Additional details about the PAUP settings are available upon request.

Results

Locations and habitats of transgenic plants

Field surveys located 55 *Agrostis* spp. populations on publicly accessible lands in the study area. Sixty-two per cent of these sites (34/55) were newly located in this study (Fig. 2). The species that were sexually compatible with the glyphosate-resistant creeping bentgrass crop and that were present in the sampled populations included *Agrostis stolonifera*, *Agrostis gigantea* and *Agrostis exarata* but not *Polypogon monspeliensis*. A total of 20 400 plant tissue samples were collected from the three species present for analysis with TraitChek kits for the *CP4 EPSPS* protein. Approximately 0.04% (9/20 400) of plant tissue samples tested positive for the protein. Nine positive plants distributed between six of the surveyed populations were identified as *A. stolonifera* based on morphology (Fig. 3). These plants were 4–10 cm tall and 8–36 cm wide, and had not yet flowered. Eight of nine plants had produced stolons. All but one (EPR12 #1) of the *CP4 EPSPS*-positive plants were found among the 34 newly located sites, and where there were no viable transgenic seeds previously recovered from the EPR12 site by Watrud *et al*. (2004).

Spatial distribution of positive plants was consistent with wind movement as the primary physical mechanism for transport of both pollen and seeds from the ASR368 crop fields to resident populations (Fig. 3). Detailed information about the distribution of the positive plants, habitats in which they were located and plant taxa nearby these plants is presented in Table 2. It was not possible to determine which particular GM crop field was the source for any of the nine plants, so distances from potential sources are given as ranges. However, for consistency with our previous work (Watrud *et al*. 2004), emphasis is placed on reporting direction and distances of establishment beyond the border of the agricultural bentgrass control area. Seven *CP4 EPSPS*-positive plants were distributed south and southeast of the control area in the direction of the prevailing

Table 2 Distribution, habitat and sympatric species for the transgenic *Agrostis stolonifera* plants outside of the control area

Transgenic *A. stolonifera* plant ID numbers	Distance and direction from perimeter of control area (km)	Minimum–maximum distance (km) from ASR368 GM crop potential source fields	Habitat	Resident sympatric non-transgenic *Agrostis* species	Other sympatric species
EPR12 #1	1.2, SE	3.6–14.6	Main canal	*A. gigantea* and *A. stolonifera*	*Eleocharis palustris*, *Phalaris arundinacea*, *Melilotus officinalis*
ESL1 #1	0.4, E, SE	1.4–12.4	Irrigation canal	*A. gigantea*	*Lolium arundinaceum* and *Melilotus officinalis*
GP1 #1, #2 and #3	2.1, S	5.2–15.7	Artificial pond	*A. stolonifera*	*Juncus balticus and Poa* sp.
NGC1 #1	3.7, E	4.4–14.4	Main canal	*A. exarata*, *A. gigantea* and *A. stolonifera*	*Holcus lanatus*, *Lolium arundinaceum*, *Verbascum thapsus*, *Bromus tectorum* and *Panicum capillare*
NGC2 #1	3.8, E	4.6–14.6	Main canal	*A. exarata*, *A. gigantea* and *A. stolonifera*	*Holcus lanatus*, *Lolium arundinaceum*, *Verbascum thapsus*, *Bromus tectorum* and *Panicum capillare*
WPR08 #1 and #2	0.2, W	5–10.6	Roadside	*A. gigantea*	*Elymus repens*

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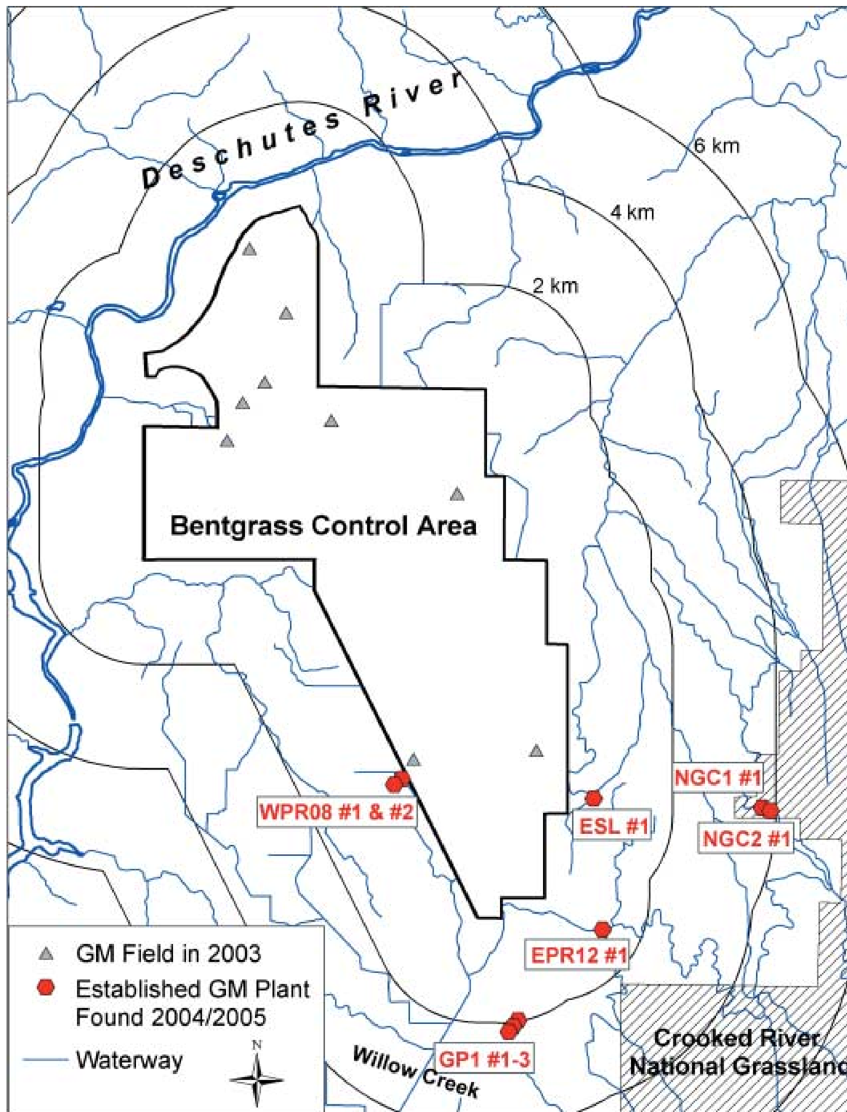

Fig. 3 Map of the wild transgenic *Agrostis stolonifera* plants located within *Agrotis* populations outside the GM bentgrass control area perimeter during 2004–2005. Transgenic plants were found 0.2–3.8 km beyond the boundary of the bentgrass control area. The two *CP4 EPSPS*-positive plants that were found furthest from the control area were located within the USDA Crooked River National Grassland.

winds, while two were established 0.2 km from the southwestern border (Fig. 3). All seven plants to the south and southeast were found growing in mesic habitats specifically preferred by *A. stolonifera*. Three of these plants were located along banks of main irrigation canals, three were at a pond and one was found along the bank of a small irrigation canal. The two plants found at the greatest distances outside the control area (3.7 km and 3.8 km to the southeast, respectively) were located along canal in the Crooked River National Grasslands. The two transgenic plants to the southwest were not associated with a waterway, but were located on a roadside. Resident *A. stolonifera* was present within all populations where *CP4 EPSPS*-positive plants were found except the two in closest proximity to the control district (ESL-1 and WPR08). The only *Agrostis* species located at these two sites was *A. gigantea*. No plants expressing *CP4 EPSPS* were found along the Deschutes River.

Molecular transgene detection and species-level parentage determination

For all nine plants that tested positive for *CP4 EPSPS* protein in the field, the presence of the engineered construct encoding the protein was confirmed in the laboratory by PCR and DNA sequencing. In PCRs for each plant, a fragment of the expected 1 kb size was amplified. Direct sequencing showed the 1 kb product from each filed positive had 100% identity with that from the ASR368 positive control and with the same region of GenBank accessions AF464188.1, *Glycine max CP4 EPSPS* (data not shown).

Analyses of ITS and *matK* gene trees (Fig. 4) indicated that both the paternal and maternal parents of the wild transgenic plants were *A. stolonifera*. Several key results supported these identifications. A single most parsimonious tree (MPT) island was found for each data matrix. While there were 240 ITS MPTs and 6 *matK* MPTs resulting

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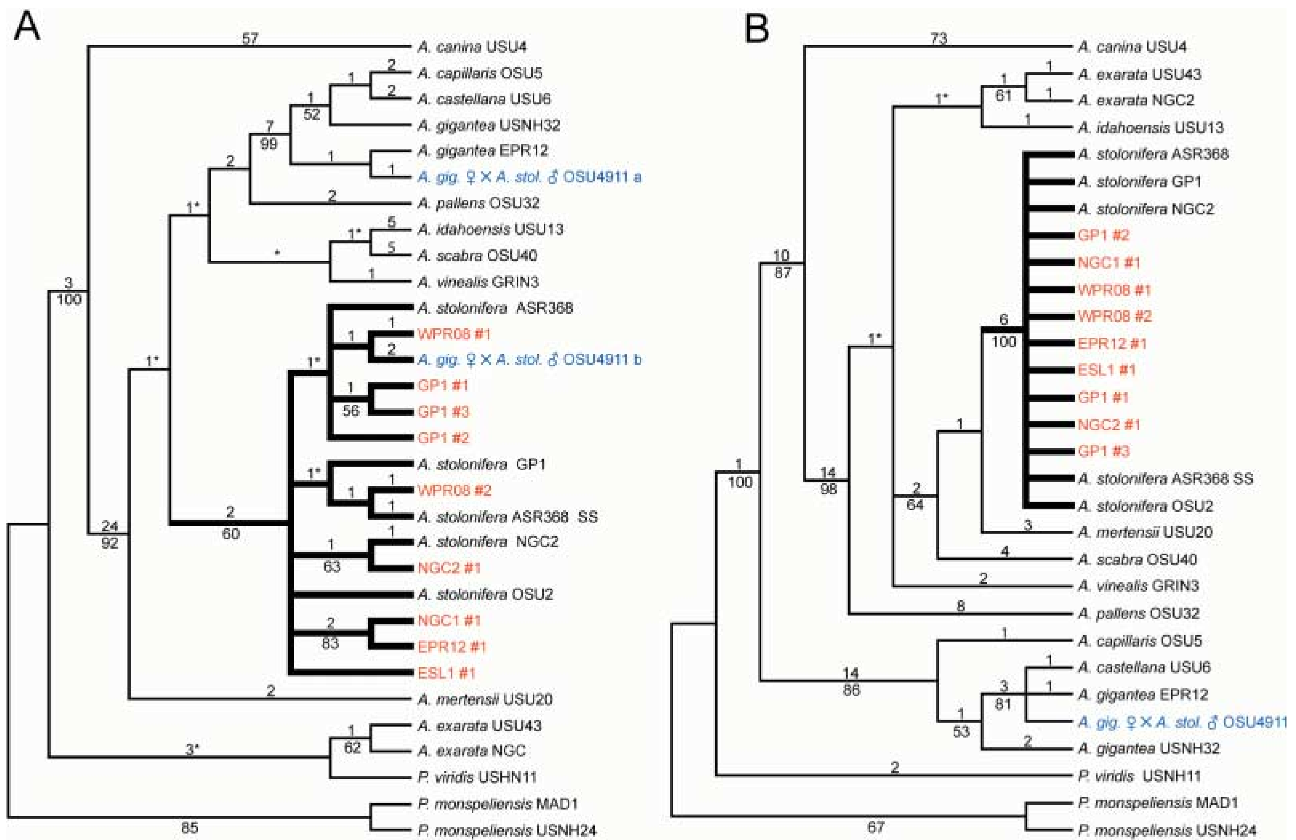

Fig. 4 Molecular identification of wild *CP4 EPSPS*-positive plants established outside the GM bentgrass control area: Randomly chosen most parsimonious trees (MPT) are shown for both the ITS and *matK* data matrices. In the analyses, gaps were treated as missing data. Simple gap coding was used to account for the presence/absence of insertions and deletions (Simmons & Ochoterena 2000). *Polypogon monspeliensis* sequences were assigned to the outgroup. (A) All ITS MPTs were based on 45 informative characters and had Tree Length = 122, Consistency Index (CI) = 0.836, Homoplasy Index (HI) = 0.164, Retention Index (RI) = 0.902, Rescaled Consistency Index (RC) = 0.754. (B) Likewise, all *matK* MPTs were based on 42 informative characters and had Tree length = 139, CI = 0.885, HI = 0.115, RI = 0.939, RC = 0.830. Branch lengths greater that one are given above branches, and those marked with an '*' collapse within the strict consensus of MPTs. Bootstrap values = 50% are shown below the branches. Relative positions of control sequences from a known transgenic hybrid offspring of resident *Agrostis gigantea* pollinated by *Agrostis stolonifera* ASR368 during 2003 are indicated in blue. Wild transgenic plants collected outside the control area in 2004–2005 are indicated in red. Each phylogeny places these wild *CP4 EPSPS* positive plants strictly within the *A. stolonifera* clade (bold branches).

from branches that collapse in the strict consensus, with both loci a single clade containing all *A. stolonifera* accessions plus the wild transgenic plants was obtained. Bootstrap support for the *A. stolonifera* clade within *matK* tree was 100%. Although bootstrap support for the ITS *A. stolonifera* clade was low (60%), there was no support for any *A. stolonifera* accession placement outside of this clade. A separate parsimony analysis confirmed the monophyletic relationship of each group of ITS sequences from the *A. exarata*, *A. gigantea* and *A. stolonifera* accessions used here (data not shown). As expected, sequence variation between ITS clones produced incongruity in the placement of F_1 transgenic *A. gigantea* × *A. stolonifera* hybrid, with alternate placements reflecting the species of both parents. And, the *matK* tree correctly indicated that the recipient plant of the transgenic pollen had been *A. gigantea*. There also was agreement on placement of nontransgenic *A. exarata*, *A. gigantea*, *A. stolonifera* and *P. monspeliensis* samples collected near the control area with herbarium specimens from the same species. These molecular results are consistent with morphologically based taxonomic identifications made in the field and with herbarium reference samples. Furthermore, while our analyses of ITS and *matK* DNA sequence data have the capacity to identify recently formed interspecific *Agrostis* hybrids, none were present in the nine wild transgenic plants that were found established in nonagronomic habitats.

Discussion

Ecological effects of transgene flow from GM crops into wild populations can be studied through controlled field and greenhouse experiments and also by modelling

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investigations. However, ultimately the documentation of ecological effects requires identification and monitoring of wild populations into which transgenes have escaped. To that end, *CP4 EPSPS*-positive *Agrostis stolonifera* plants identified here provide the first evidence for escape of transgenes into native and naturalized plant populations outside of cultivated habitats within the USA. Based on recent results from Watrud *et al*. (2004) and Mallory-Smith *et al*. (2005), we anticipated that transgenic *Agrostis* plants that formed through both crop × noncrop hybridization and through dispersal of GM crop seeds would be found downwind beyond the bentgrass control area. Potential for transgene escape is not the same for all GM crops; however, for *Agrostis*, numerous factors suggested the likelihood of escape from transgenic *A. stolonifera* source fields into nearby wild sympatric populations. The combination of pollen size, density and viability, wind speed and direction, proximity to wild sexually compatible species, floral synchrony between GM crops and wild relatives, relatively large area of source fields compared to individual patches of compatible wild species, all enhanced the potential for formation of viable transgenic seeds on wild plants. In addition, the extremely small size of *A. stolonifera* seeds allows them to easily become air-borne, making their full containment within production fields highly unlikely.

Locating *Agrostis* populations containing transgenic individuals in nonagronomic habitats required extensive field work over a large geographical area (310 km^2) for almost 2 years. Established transgenic plants were spread over an appreciable distance beyond the border of the control area (0.2–3.8 km). Mesic habitats preferred by *A. stolonifera* were also diffuse and many were seasonally transient in surrounding nonagronomic regions. Where such habitats did exist, dense stands of perennial *Agrostis* species were not optimal sites for establishment of new seedlings due to resource limitations on open soil, nutrients and light. Thus, new seedlings most frequently grew in the margins of these habitats or where disturbance of existing plants had occurred. Bulk field testing of seedlings in these areas did prove effective as a quick screen for transgenic individuals within these populations, as born out by follow-up individually based field testing and later molecular transgene confirmations. One limitation of this approach is that bulk plant tissue sampling may underestimate the number of sites where transgenic plants established in the wild through false negatives. All nine wild transgenic *A. stolonifera* plants reported here were found by surveys that were restricted to publicly accessible areas comprising = 10% of total estimated *Agrostis* habitat in the study region. These factors suggest that the extent of establishment of wild transgenics may be substantially more than what is documented in this report, if one were to consider total likely habitat available for establishment of *Agrostis*.

The distribution and parentage of the wild transgenic plants suggest that six of the established plants resulted from pollen-mediated gene flow to wild *A. stolonifera* plants and that three came from dispersed GM seeds. As expected, transgenics were generally found at sites in the direction of prevailing winds. Evidence for feral ASR368 comes from three plants established at two sites (WPR08 and ESL-1) nearest the control area and where there was an absence of sympatric noncultivated *A. stolonifera* plants. These three plants may have resulted from crop seeds that were dispersed by various mechanisms: wind, water, wildlife and/or mechanical means (e.g. vehicles). Association between most of the wild transgenic *A. stolonifera* plants with mesic habitats initially points to waterways themselves as conduits for establishment. While it is possible that water could have transported GM crop seeds (or vegetative propagules) to some of the distant sites on canal banks, this appears less likely than wind transport of either pollen or seeds. For instance, the GP-1 mesic habitat was formed by localized collection of seasonal rainwater and was not connected to a flowing waterway. Also, no transgenic plants were found in any *Agrostis* populations upwind of the control area and downstream along the Deschutes River that does receive runoff from the plateau where the control area sits. Assuming transport by wind, seeds would travel shorter distances than lighter, smaller pollen particles because the settling velocity of a sphere through air is positively related to the size and density of the particle according to Stokes' law (Nathan *et al*. 2001). In addition, we demonstrated that our ITS and *matK* comparisons can detect recently formed interspecific *Agrostis* hybrids, and these methods confirmed that all transgenic plants that we found had paternal and maternal *A. stolonifera* parents. Establishment of three transgenic *A. stolonifera* plants within *A. gigantea* WPR08 and ESL-1 populations further suggests that these three were most likely to have established from seed scatter. The other six, all of which were found further out from the control area (1.2–3.8 km) within *A. stolonifera* populations, are most likely to be intraspecific hybrids. The lack of detection of wild interspecific hybrids among any sites could simply be due to insufficient sampling, but it may also reflect viability of fewer interspecific hybrid seeds that could have formed. Population-level *Agrostis* markers (Casler *et al*. 2003; Vergara & Bughrara 2003) will be needed for particular GM *Agrostis* cultivars to distinguish transgenic intraspecific hybrids from feral crop plants found growing in the wild and to identify *Agrostis* populations undergoing transgene introgression. Given the likelihood that wind movement of both GM pollen and crop seeds contributed to the establishment of this cohort of plants, it is perhaps only coincidental that the incidence of positives reported here (0.04 ± 0.01% SE) closely matched that for viable transgenic hybrid seeds recovered from resident plants in the previous season (Watrud *et al*. 2004). On the other hand, both samples measured the probability of such a rare event

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(i.e. incidence of positives) with accuracy and precision given the large sample sizes and the small standard errors associated with a binomial distribution. Furthermore, our sample had few instances of transgenic plants via seed scatter which resulted in negligible bias in reported incidences of positives from transgene flow due to pollen movement. If similar sampling strategies are applied, these percentages can serve as a useful point of reference by which to compare numbers of transgenic *Agrostis* plants discovered from future samplings in this region or in other regions where GM *Agrostis* has been grown, allowed to flower and to set seed.

Our results clearly demonstrate that transgene flow from even short-term production can result in transgene establishment within wild populations at multiple locations that provide suitable habitat. When such establishment involves or leads to the formation of hybrids with either full or partial fertility, then transgene introgression into wild populations through backcrossing becomes possible. Several recent reports have discussed movement of transgenes from *A. stolonifera* to other *Agrostis* species (Wipff & Fricker 2001; Christoffer 2003; Watrud *et al*. 2004), the dynamics of transgene introgression through hybridization (Conner *et al*. 2003), and likely impacts of particular transgenes that become established in resident populations and non-GM crops (Faure *et al*. 2002; Rieger *et al*. 2002; Lu *et al*. 2003; Snow *et al*. 2003; Lu & Snow 2005). Where compatible species are sympatric in the environment as in the current example, establishment of both wild hybrids and feral transgenic plants facilitate introgression of transgenes (and other crop-specific alleles) into previously nontransgenic populations. Transgene flow from GM crop fields to resident populations through pollen-mediated hybridization (especially with wild plants of the same species as the crop) may, at least initially, have greater introgressive potential than seed loss from engineered fields. Formation of fertile hybrids is the first critical step towards future introgression through backcrossing. However, once established, feral GM plants may also expand the exposure of compatible wild relatives to transgenic pollen and increase opportunities for hybridization. Introgression is typically considered most likely when engineered traits may give selective advantages to fertile hybrids that backcross to wild parental species. However, it may also occur if the novel traits are not associated with fitness costs in hybrids, where the frequency of the transgene within a wild population is governed by genetic drift. If hybrids and/or their progeny have increased fitness characteristics relative to other plants with which they compete, or if there is a loss of genetic diversity in introgressed populations, then changes in community structure and ecological transitions also become possible (Thompson *et al*. 2003; Rieseberg *et al*. 2003).

While herbicide resistance is generally not expected to provide a direct selective advantage to plants outside of cultivation, there are at least three scenarios in which *CP4 EPSPS* transgenes may yet introgress and persist in wild *Agrostis* populations. First, glyphosate is one of the only herbicides approved for use near waterways; thus direct or indirect (e.g. herbicide drift) application of glyphosate for weed control in mesic habitats where *CP4 EPSPS*-positive *Agrostis* plants are established could provide selective pressure to enhance transgene introgression and foster establishment of further glyphosate-resistant plants. Second, obligatory outcrossing, formation of fertile hybrids and/or vegetative spread could also contribute to spread of the *CP4 EPSPS* transgenes in wild *Agrostis* populations especially if transgene frequency is not being reduced through selection. And third, there would be considerable potential for unintended transgene stacking between different transgenic *Agrostis* crops if commercialized. Just as gene flow between different cultivars of *Brassica* resulted in the hybrid volunteers within Canadian crop fields with triple stacked resistance to herbicides (Hall *et al*. 2000), a similar situation could easily occur if fully fertile cultivars of GM *A. stolonifera* engineered with different traits (e.g. resistance to additional herbicides, drought resistance, plant incorporated protectants) were grown for seed production in the same geographical region. *Agrostis* hybrids and/or feral plants with stacked traits that more distinctly increase their fitness also could become established in the wild. It should be noted that biotechnological developments aimed at transgene containment, such as constructs that produce male sterility, may reduce but not eliminate concerns about hybridizations due to the possibility of wild pollen movement into crop fields and subsequent seed scatter into wild populations.

The long-term fate and ecological impacts of *CP4 EPSPS* transgenes within wild *Agrostis* populations in central Oregon remain to be determined. Only one (approximately 2.5 ha) GM bentgrass field remained in production and flowered in the control area during the 2004 growing season; thus, *in situ* monitoring of the central Oregon populations continues. There may be other regions where transgenic *Agrostis* has become or will become established in the wild. Additional experimental work under greenhouse conditions is recommended to identify and measure factors that may mitigate or enhance the establishment of wild transgenic plants, transgene introgression, persistence and their potential ecological effects on plant communities.

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The authors are molecular ecologist and plant physiologists that are interested in developing methods to determine the potential unintended ecological effects of gene flow from GM crops to compatible wild relatives.

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