Chapter 15

Transgenic Herbicide-Resistant Turfgrasses

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Additional information is available at the end of the chapter

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1. Introduction

Turfgrasses grow in different habitats for numerous purposes worldwide. They are cultivated for their agronomical, environmental, ornamental, recreational and stock feeding values [1, 2]. Various turfgrasses are used for environmental beautification and for the protection of resources such as land, soil and water. Many varieties of turfgrasses cover home yards, golf courses, parks, soccer fields, and roadsides, etc. To cite a few examples of renewed interest in turfgrasses, they play a significant environmental role in photosynthetically fixing carbon dioxide to evolve oxygen into the atmosphere. In addition to their vast acreage of widespread forage, planting of the grasses in urban areas such as rooftops, parks and, more recently automobile parking lots, contributes to the suppression of urban heat island phenomena [3]. Various causes of soil erosion and losses due to flood washout and landslide can also be circumvented and managed, as the damages are greatly reduced and the conservation of soil moisture and underground water is effectively sustained by the planting of turfgrass varieties. Recreational and sporting activities on the natural turfgrass field, compared to an artificial turf, greatly reduce the risk of personal injuries, thus contributing to the wellbeing of people in general.

Not surprisingly, the worldwide turfgrass market and its associated herbicide sales are substantial; in the United States alone, turfgrass is one of the four major staple crops, second only to corn [4, 5]. In facing the challenge of global warming, turfgrasses are gaining attention of both environmentalists and agronomists for their role in the certified emission reductions. Relatively high production costs of cultivating and maintaining turfgrasses concerns them, however. Healthy swarth growth and well-maintained turf habitats entail herbicide spraying because otherwise dominant weed varieties easily overtake the sward. Annually, their maintenance costs alone run around 4.5 billion dollars in the United States [4, 6]. One of the major costs is certainly herbicidal requirement.

Herbicidal agrochemicals are classified into two categories, selective and non-selective herbicides. The latter kills all plant species, whereas the former is targeted at specific plant(s)/ weed(s) for herbicidal action. The biochemical mechanisms of herbicides include the disruptions of (i) the photosynthesis by blocking the photosynthetic reaction centers, electron transport system or photo-oxidative membrane damages, (ii) cell division and root development, (iii) energy transduction and metabolism, (iv) plant growth hormones, (v) biosynthesis of amino acids/proteins and (vi) disruption of other physiologically significant molecules such as chlorophylls and carotenoids, as discussed elsewhere in this volume.

Frequent herbicide applications also pose serious environmental and health concerns, for example, to the authors' residential island of Jeju where there are 30 golf courses open for business. In spite of the current difficulties arising from the public objections, genetically modified turfgrasses with a herbicide-resistant gene provide an effective alternative to the wide applications of agrochemical herbicides. Since the development and ecological impact studies of transgenic herbicide-resistant creeping bentgrass [7, 8] and zoysiagrass [9, 10], several GM varieties of turfgrasses including those of herbicide-resistant cultivars have been developed (see Table 1). Most recently, in reference [11] bentgrass ASR-368 has been patented for its commercial rights. With an increasing number of reports on transgenic herbicide-resistant turfgrasses, it is appropriate to review the subject at this time. Discussion in this chapter focuses on the transgenic herbicide-resistant turfgrasses developed primarily in our laboratory here in Jeju and Gwangju, Korea. For a review of other transgenic grasses with herbicide-resistance traits, see Table 1 and references therein.

Plant species	**Cultivar**	**Method**	**Marker gene**	**Target gene**	**Target trait**	**References**
Agrostis stolonifera (creeping bentgrass)	Crenshaw	*Agrobacterium*	*bar*	*bar*/Rice *tlpd34*	Disease resistance	[16]
	Crenshaw	*Agrobacterium*	*bar*	*bar*/Barley *hva1*	Drought tolerance	[33]
	Crenshaw	*Agrobacterium*	*bar/gus*	*bar/PepEST*	Herbicide resistance/ Disease resistance	[34]
	Crenshaw	*Agrobacterium*	*bar/gus*	*bar*/Maize *Lc+Pl*	Purple-color	[35]
	Crenshaw	*Agrobacterium*	*bar/gus*	*bar/AtBG1*	Herbicide resistance/ Drought tolerance/ dwarf	[36]
	Crenshaw, Penncross	*Agrobacterium*	*bar/gus*	bar	Herbicide resistance	[37]
	Penncross	Electroporation	*bar*	*bar*	Herbicide resistance	[38]
	Penncross	Electroporation	*bar/gus*	*bar*	Herbicide resistance	[39]

Plant species	Cultivar	Method	Marker gene	Target gene	Target trait	References
	Penncross	*Agrobacterium*	*bar*	*bar*/Cowpea *VuNCED*1	Drought/salt tolerance	[40]
	Penncross	*Agrobacterium*	*bar/CP4-EPSPS*	*bar/CP4-EPSPS*	Herbicide resistance	[22]
	Penncross	*Agrobacterium*	*bar*	*bar/ZjLsL*	Herbicide resistance/ dwarf	[41]
	Province Penn-A-4	Biolistics	*bar/gus*	*bar/chitinase +glucanase*	Herbicide resistance/ Disease resistance	[42]
	Penn-A-4	*Agrobacterium*	*hph/gus, bar*	bar	Herbicide resistance	[43]
	Penn-A-4	*Agrobacterium*	*bar*	*bar/Pen4-1*	Herbicide resistance/ Disease resistance	[44]
	Penn-A-4	*Agrobacterium*	*bar*	*bar/AVP1*	Herbicide resistance/ Salt tolerance	[45]
Agrostis palustris (creeping bentgrass)	Suthshore Emerald	Biolistics	*bar/gus*	*bar*	Herbicide resistance	[46]
	Regent Tiger	*Agrobacterium*	*bar/gfp*	*bar*	Herbicide resistance	[47]
	Cobra	Electroporation	*bar*	*bar*	Herbicide resistance	[48]
		Biolistics	*bar*	*bar/hs2*	Herbicide resistance	[49]
Cynodon spp. (bermudagrass)	TifEagle	Biolistics	*bar*	*bar*	Herbicide resistance	[50]
	TifEagle	*Agrobacterium*	*bar/gus*	*bar*	Herbicide resistance	[51]
Dactylis glomerata (orchardgrass)	Embryogen-P	Biolistics	*bar/gus*	*bar*	Herbicide resistance	[52]
	Rapido	Biolistics	*bar/hph/gus*	*bar*	Herbicide resistance	[53]
Festuca arundinacea (tall fescue)		Protoplasts	*bar/hph*	*bar*	Herbicide resistance	[54]
	Alley	Biolistics	*bar*	*bar/Ipt*	Herbicide resistance/ Cole tolerance	[55]
Festuca rubra (red fescue)		Protoplasts	*bar*	*bar*	Herbicide resistance	[56]
Lolium perenne (perennial ryegrass)	Riikka	Biolistics	*bar*	*bar/wft1/wft2*	Herbicide resistance/ Freezing tolerance	[57]
	TopGun	*Agrobacterium*	*bar*	*bar/OsNHX1*	Herbicide resistance/ Salt tolerance	[58]
Panicum virgatum (switchgrass)	Alamo	Biolistics	*bar/gfp*	*bar*	Herbicide resistance	[59]

Plant species	Cultivar	Method	Marker gene	Target gene	Target trait	References
	Alamo	*Agrobacterium*	*bar/gus*	*bar*	Herbicide resistance	[60]
Paspalum notatum (bahiagrass)	Tifton-7	Biolistics	*bar*	*bar*	Herbicide resistance	[61]
	Pensacola	Biolistics	*bar/gus*	*bar*	Herbicide resistance	[62]
Paspalum vaginatum Swartz (Seashore Paspalum)		*Agrobacterium*	*bar/gus*	*bar*	Herbicide resistance	[63]
Zoysia japonica (zoysiagrass)		*Agrobacterium*	*bar/gus*	*bar*	Herbicide resistance	[15]
	Zenith	Biolistics	*bar/hpt*	*bar*	Herbicide resistance	[64]
		Agrobacterium	*bar*	*bar/phyA*	Herbicide resistance/ Shade tolerance	[10]
Zoysia sinica (Chinese lawngrass)		*Agrobacterium*	*bar*	*bar/CBF1*	Herbicide resistance/ Chilling tolerance	[65]

bar: bialaphos resistance gene, gus: β-glucuronidase, hph: hygromycin phosphotransferase. gfp: green fluorescent protein

Table 1. Transgenic herbicide-resistant turfgrasses

2. Turfgrass species

There are some 7,500 turfgrass species of more than 600 genera distributed worldwide. Of these, 30~40 species are cultivated as agronomic plants [1]. Turfgrasses are generally classified into two major species, warm and cold season grasses. The plants are also divided into two groups based on their mechanism of photosynthetic carbon dioxide fixation, C3 and C4 plants. As representative C4 warm season turfgrasses with optimal growth temperatures of 27~35°C, zoysiagrass and Bermuda grass species are widely used for sports fields because of their strong traits such as swarth growth, vegetative propagation and drought tolerance as they are cultivated widely, especially in China, Japan and Korea. However, they tend to grow relatively slowly and particularly with zoysiagrasses prematurely lose their greenness by late autumn. Typical C3 cold season turfgrasses with optimal temperatures in the 15~25°C range include blue grass and bentgrass varieties. The latter is particularly advantageous for the putting greens [1, 4, 5, 12]. In this chapter, the review will be concerned with two main varieties, zoysiagrass (*Zoysia japonica* Steud.) and bentgrass (*Agrostis palustris* L., Crenshaw and Penn-cross varieties), focusing on their herbicide resistant transgenic cultivars.

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3. Transgenes and mechanisms of herbicidal action

Turfgrass has been a subject of classical breeding for trait improvement over decades, especially in Japan and United States. However, conventional breeding suffers from such drawbacks as low efficiency, time consuming and labor intensiveness. With an increasing trend in turfgrass cultivation worldwide, excessive applications of herbicides and other agrochemicals over the grass habitats adversely impact the environment, biodiversity and human health [13, 14]. Several attempts to develop GM turfgrass lines with improved traits have been reported; for example, herbicide-resistant turfgrass varieties in references [15], [16], 17] and [10] and insect-resistant turfgrass in reference [18]. A number of laboratories are developing herbicide-resistant and other transgenic turfgrasses with biotic and abiotic stress tolerances (Table 1).

So far, several genes including the two widely adopted ones, *CP4 EPSPS* encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs) and *BAR or PAT* encoding a phosphinothricin acetyl transferase (PAT), have been introduced to generate herbicide-resistant turfgrasses. Other target genes for herbicide resistance include *BXN* (bromoxylnil nitrilase gene), *DHPS* (dihydropteroate synthase gene), *ALS* (acetolactate synthase gene) and others (Table 1). Transgenic bentgrass and zoysiagrass stacked with *BAR* and *PHYA* (phytochrome A) genes conferring herbicide- and shade-resistance traits, respectively, have also been developed [10] and will be reviewed in this chapter.

The widely used herbicide, bialaphos (also phosphinothricin-alanyl-alanine tripeptide, PTT), is an antibiotic produced by certain *Streptomyces* genera and used as an agrochemical, which has been commercialized under the trade name Basta by Bayer Crop Science. It kills plants non-selectively. Bialaphos itself is an inactive compound as a herbicide, but it is cleaved by intracellular peptidases to phosphinothricin (L-PPT), Phosphinothricin (glufosinate) so produced *in situ* binds glutamine synthetase (GS), the key enzyme in the nitrogen fixation in plants, inhibiting its catalytic activity to fix the ammonium with L-glutamate to form glutamine [19] (See Figure 1).

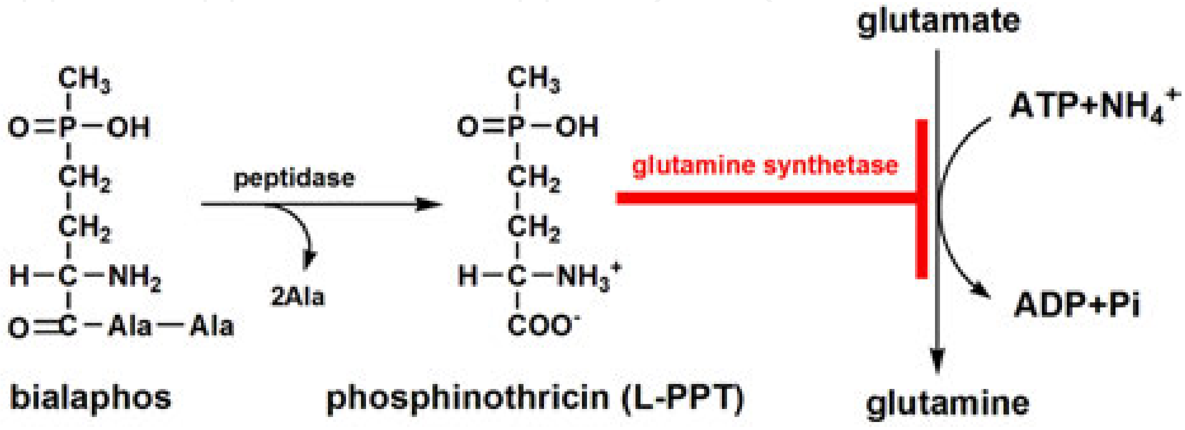

Figure 1. Biochemical mechanism for the herbicidal action of glufosinate through the inhibition of glutamine synthetase by the herbicide.

The glufosinate herbicide causes accumulation of lethal levels of ammonia in both soil bacteria and plant cells. The GS inhibiting activity of glufosinate is lost when its amino group is acetylated by a phosphinothricin acetyl transferase (PAT encoded by *PAT;* also known as *bar or BAR* for bialaphos resistance) (Figure. 2).

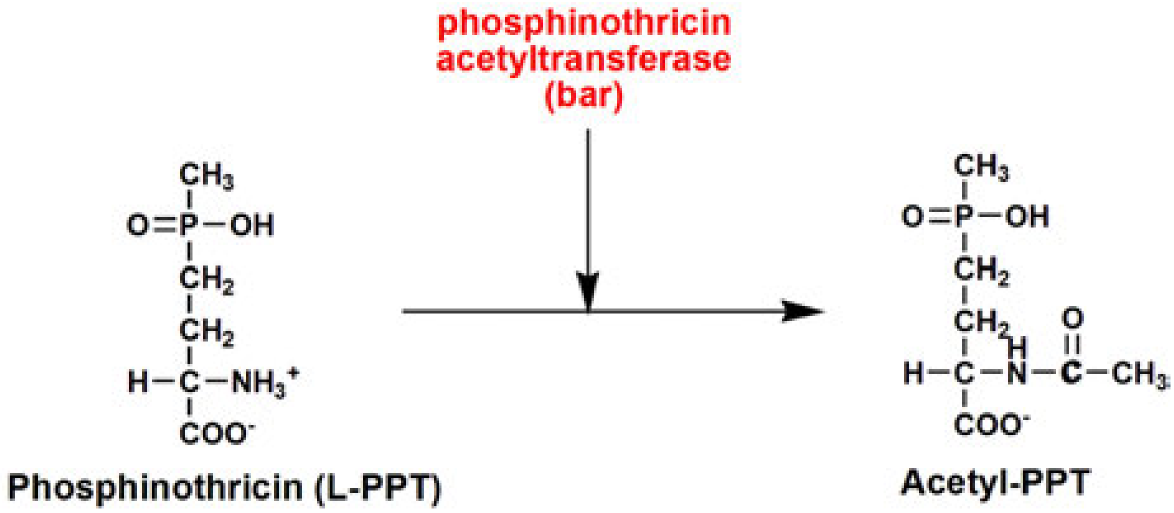

Figure 2. Detoxication of glufosinate by phosphinothricin acetyl transferase (*BAR* or *PAT*).

Thus, a transgenic turfgrass transformed with *BAR* gene becomes resistant to the Basta spray, as glufosinate from the Basta is effectively detoxicated in the plant. The transgenic zoysiagrass and bentgrass developed in our laboratories carry the *BAR* gene isolated from *Streptomyces hygroscopicus* in the soil [10].

Glyphosate is a non-selective herbicidal agent commercialized under the trade name "Round-up" by Monsanto. It exerts its herbicidal action by competitively inhibiting the 5-enolpyru-vylshikimate-3-phosphate synthase (EPSPs) centrally involved in the biosynthesis of aromatic amino acids (phenylalanine, tryptophan and tyrosine). Plants treated with glyphosate are killed for the lack of these amino acids in protein biosynthesis. Accumulation of shikimate also leads to cell death, thus contributing to the herbicidal action of glyphosate [20] (Figure. 3).

A transgenic bentgrass carrying the EPSPS gene ("Roundup Ready") then develops resistance to Roundup [7, 21].

Although both *BAR*- and *EPSPS*-.transgenic turfgrasses are yet to be released for agronomic cultivations, second and third generation GM crops including turfgrasses are forthcoming to deal with the intolerance and tolerance being developed to the non-specific herbicides in the transgenic herbicide-resistant turfgrasses and weed plants, respectively. Such next generation crops are also being developed with the hope of leading consumer acceptance. In reference [22] the authors stacked both *BAR* and *CP4 EPSPS* genes in creeping bentgrass to generate dual (glufosinate and glyphosate) herbicide-resistant turfgrasses, hoping that less amounts of two herbicides together are required for weed necrosis than with the greater amount needed with one herbicide alone. The bentgrass species so developed showed an expected degree of

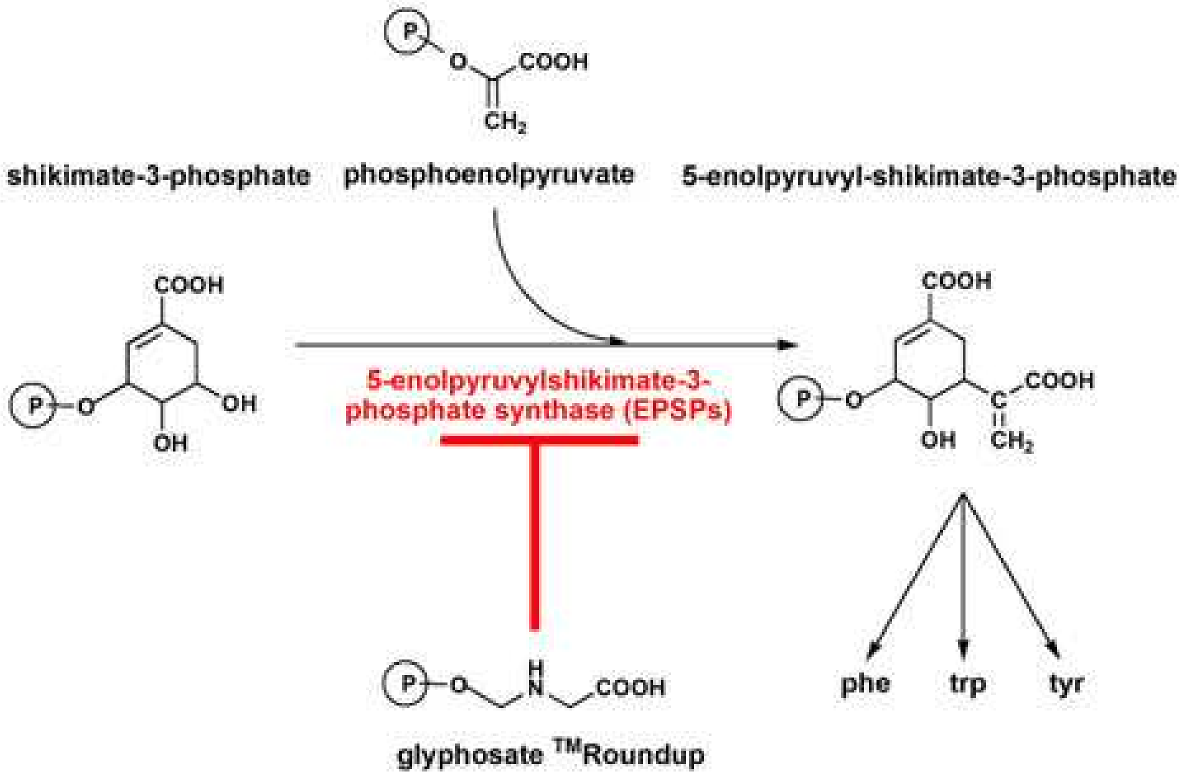

Figure 3. The reaction catalyzed by 5-enolpyruvylshikimate 3-phosphate synthase(EPSPS) (Modified from reference [32])

tolerance to both Basta and Roundup, respectively. While such dual transgene herbicide resistance may counter for a single-transgene plant to lose tolerance to the herbicide and/or for the weeds to develop tolerance to the herbicide, it remains to be seen if this expectation is borne out in natural habitats.

One of the most promising herbicide-resistant traits can be conferred by dicamba monooxygenase gene (*DMO*). Dicamba (3, 6-dichloro-2-methoxybenzoic acid) is an active auxin analog and its presence in the plant cells exaggerate the hormonal effects that lead to the cell and plant death. It is widely used in the Unites States for over four decades. It is a relatively non-toxic and environment-friendly herbicide. Its herbicidal activity is lost in a *DMO*-transgenic crop as dicamba is detoxified to its inactive 3, 6-DCSA (3, 6-dichlorosalicylic acid) [23]. Attempts are being made to generate DMO-transgenic turfgrass plants in several laboratories.

4. Herbicide-resistant zoysiagrass and bentgrass

In a previous report, we discussed the development of the *BAR*-transgenic *Zoysia japonica* Steud., currently undergoing a regulatory approval process under the cultivar name "Jeju Green 21" and compared its phenotypic traits with those of non-transgenic control [9]. Figure 4 (A, B) illustrates the effect of spraying Basta on the test plot containing both control and herbicide-resistant zoysiagrasses. In Figure 4(A), the herbicide-resistant runners were planted in the GMO-spelled area, which continued to grow healthily after Basta spray, showing "Jeju

Green 21" plants growing in "GMO" spell pattern before and after the herbicide treatment at a concentration of 0.1% (w/v) glufosinate. Figure 4(B) shows the mixed turfgrass/weed habitat treated with a 0.5% Basta spray, showing an effective herbicidal killing of the weeds. Non-transgenic grasses are effectively wilted out, whereas the resistant plants remain healthy and indistinguishable from their non-transgenic counterparts physiologically and phenotypically [9]. Figure 5 displays the herbicidal performance of *BAR*-transgenic creeping bentgrass in which a wild type or mutant *PHYA* (*Ser599Ala PHYA*) gene is stacked with the *BAR* gene, *vide infra*. The results show that the gene stacking has not compromised the herbicide-resistance function conferred by the *BAR* gene. Qualitatively, both *BAR*- and *EPSPS*-transgenic bent-grasses effectively tolerate the herbicides, Basta and Roundup, respectively, but quantitative comparisons of the herbicide resistances exhibited by different transgenic zoysiagrass and bentgrass varieties entail further study.

Figure 4. Herbicide resistance assay of putative transgenic zoysiagrass plants. A. 0.8% BASTA® was sprayed onto non-transgenic plants (NT) and bialaphos-resistant zoysiagrass, "GMO" was spelled by removing the plants; GM grass was then planted into the letters, B. 0.5% BASTA® was sprayed onto the weed and bialaphos-resistance zoysiagrass plants.

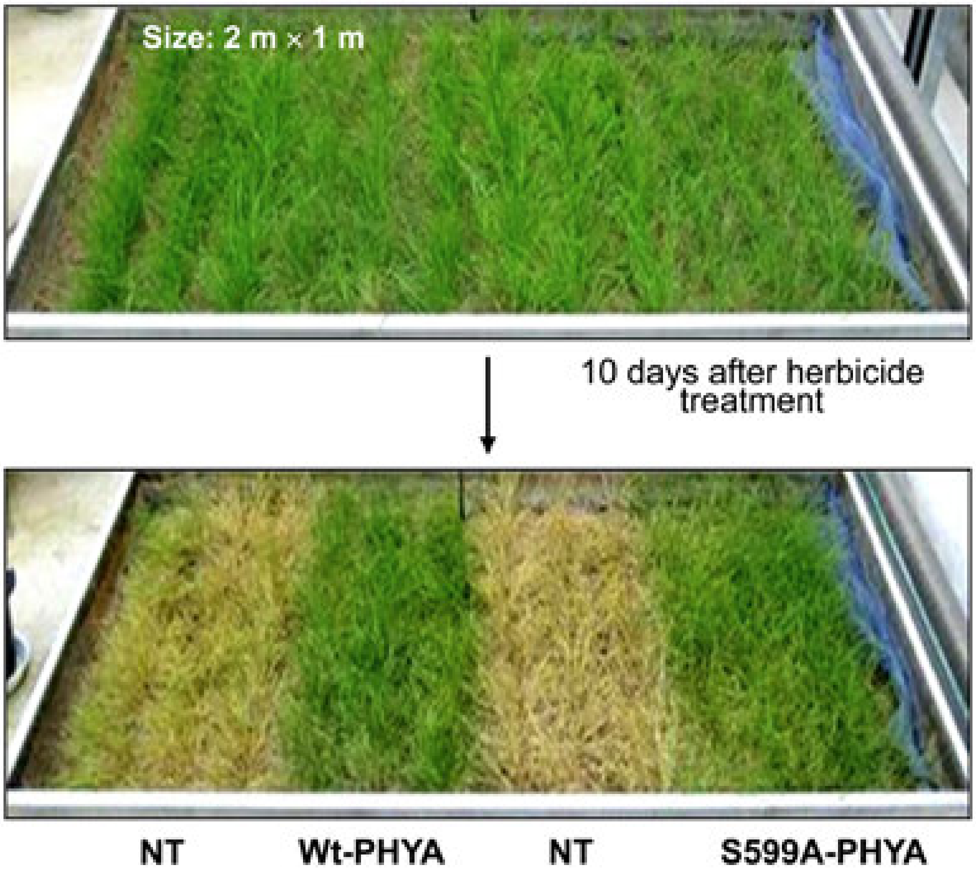

Figure 5. Herbicide resistance assay of putative transgenic creeping bentgrass plants. 0.8% BASTA® was sprayed onto non-transgenic plants (NT) and transgenic plants over-expressing *Wt-PHYA* or *Ser599Ala-PHYA*, and the herbicide resistance of the plants was determined 10 days after the spraying. *Wt-PHYA*, transgenic bentgrass plants with wild-type *PHYA* gene; *Ser599Ala-PHYA*, transgenic bentgrass plants with *Ser599Ala-PHYA* mutant.

When zoysiagrass and possibly other turfgrass species are left unmanaged under natural habitats, their populations and swarth growth are easily overtaken by the dominant weed plants. Figure 6 shows our own observations of herbicide-resistant zoysiagrass plants growing in natural habitats during the four consecutive years (2006~2009). In four years, the ground coverage of zoysiagrass was dominated by the weeds when the grass plot was left unmanaged. On the other hand, the herbicide-resistant plants continued healthy population and swarth growths under managed conditions involving fertilizer applications, herbicide sprays and timely mowings.

Recently, we reported the development and morphological characterization of transgenic *Zoysia japonica* and *Agrostis stolonifera* plants transformed with both *BAR* and *PHYA* genes [1]. The two transgenes confer herbicide resistance and shade tolerance to the grass, respectively. We developed these turfgrass plants by harboring wild-type *Avena PHYA* or *Ser599Ala PHYA* mutant (*S599A-phytochrome A hyperactive mutant* gene [24]) on the *BAR*-decked *pCAMBIA3301* vector in order to confer both herbicide and shade tolerant phenotypes to them. The transgenic plants with *Ser599Ala-PHYA* and *Wt-PHYA* also displayed the shorter phenotypes desired, in addition to their herbicide resistance trait (Figure 7).

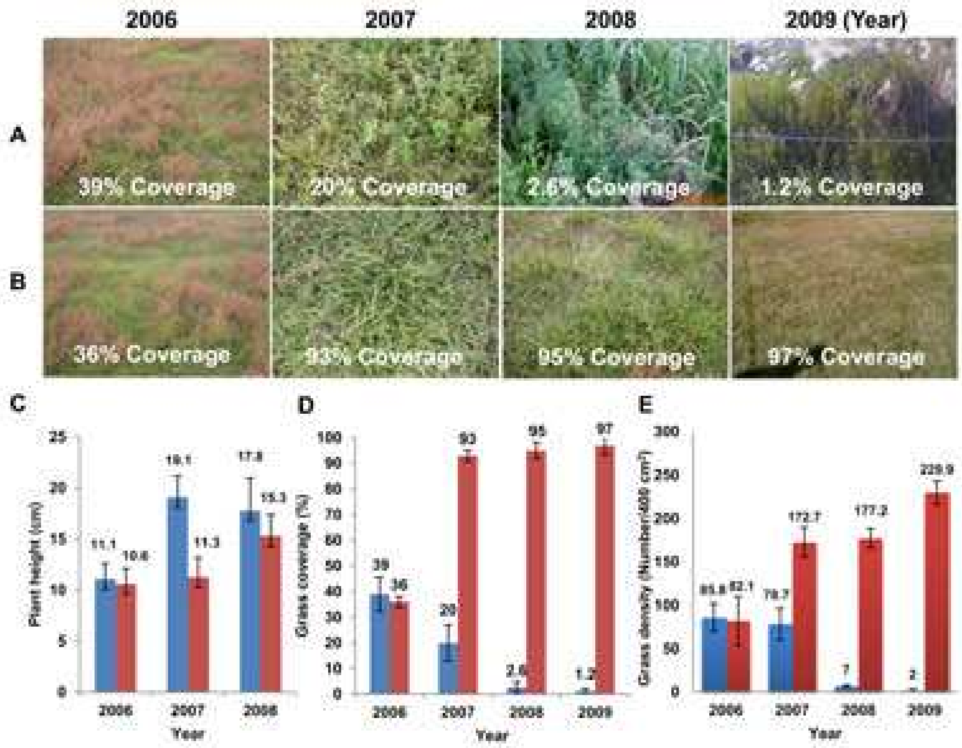

Figure 6. Survival of the transgenic herbicide-resistant zoysiagrass during 4 years (2006-2009) in natural habitats. A. Natural habitats during 4 years, B. Managed field, C. Plant height of zoysiagrass, D. Grass coverage of zoysiagrass, E. Grass density of zoysiagrass. Blue bar, natural habitat; red bar, managed field.

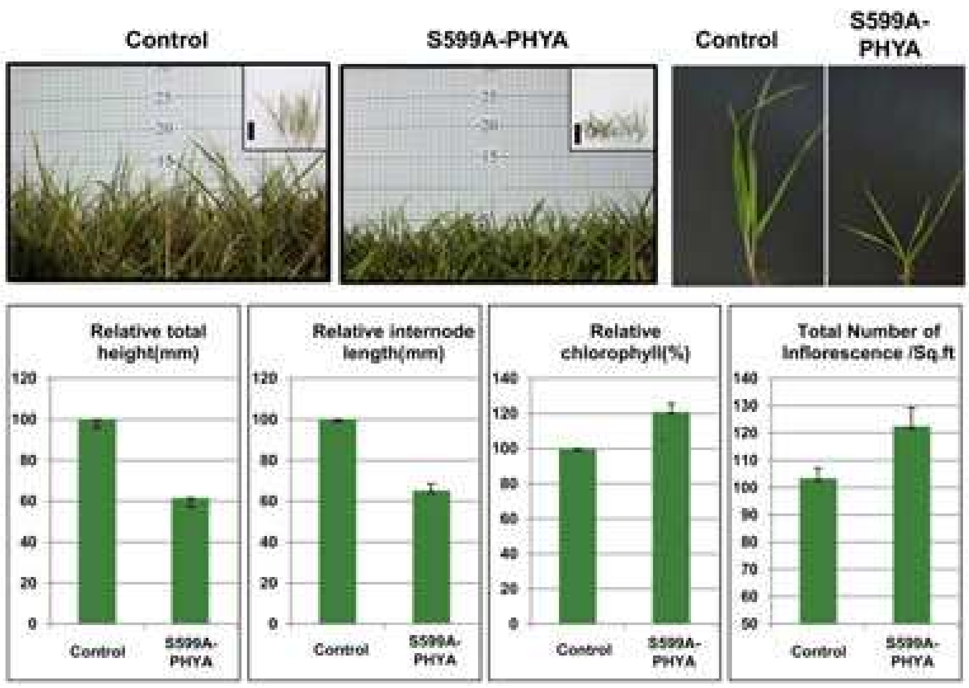

Figure 7. Growth performance of transgenic zoysiagrass plants over-expressing *Ser599Ala-PHYA* showed short phenotypes compared with control plants *(BAR* gene) under field conditions. Bar in insert 1 cm.

We observed a delay in necrosis (senescence) of *Ser599Ala-PHYA* leaves under outdoor conditions in early winter (Figure 8). During the rejuvenation of zoysiagrass after the winter season, various weeds began to dominate over the transgenic turfgrass habitats. However, zoysiagrass plants expressing both *BAR* and *Ser599Ala-PHYA* genes exhibited a significant increase in tiller number and runner length relative to the non-transgenic controls [10]. These traits will be helpful for the zoysiagrass plants to compete effectively with the weeds, especially in disrupting the germination of unwanted weeds.

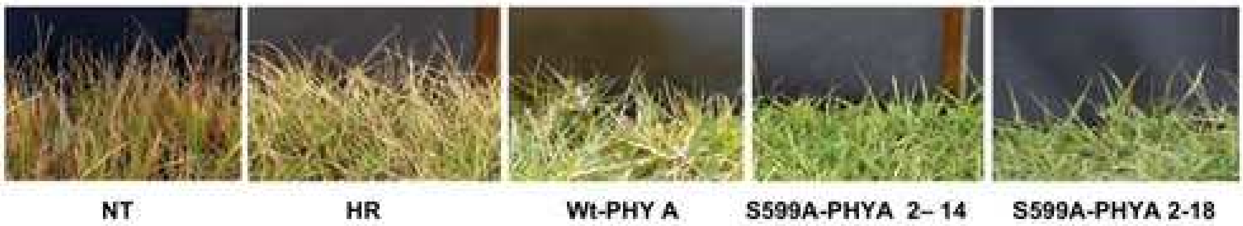

Figure 8. Photographic view of browning (necrosis) in zoysiagrass transformant lines in early winter. NT, non-transgenic zoysiagrass plants; HR, herbicide-resistant zoysiagrass plants with *BAR* gene; *Wt-PHYA*, transgenic zoysiagrass plants with wild-type *PHYA* gene; *Ser599Ala-PHYA* 2-14 & 2-18 transformant lines, transgenic zoysiagrass plants with *Ser599Ala-PHYA* mutant gene.

5. Environmental risk assessment

To commercialize any of the transgenic turfgrass varieties listed in Table 1, their environmental risks must be assessed under their natural habitats [7, 8, 9, 25]. This chapter briefly reviews our own studies and discusses attempts to block or minimize the risks of gene flow from the transgenic turfgrass habitats to the plants at neighboring and remote sites. For example, in reference [26] and [27] the workers introduced a male-sterility gene into GM crops to block the escape of a transgene from the latter, and this strategy may be applied to turfgrasses. We developed a sterile herbicide-resistant zoysiagrass through γ-radiation mutation, making the latter unbolting and deficient in fertile pollens [28, 29]. The γ-radiation generated herbicide-resistant zoysiagrass can be cultivated in agronomic habitats for eventual commercialization [25].

A preliminary study showed that the transgene (*BAR*) of herbicide-resistant *Zoysia japonica* unintentionally escaped from the test plants to the close neighbored non-transgenic zoysiagrass species [9]. However, the introgression is likely to be suppressed under natural conditions (see Figure. 6) and can be easily terminated by applying non-specific herbicides such as glyphosate and paraquat [25].

According to the “Weed risk assessments for Hawaii and Pacific Islands” database (http://www.botany.hawaii.edu/faculty/daehler/wra/default.htm), transgenic *Zoysia japonica and Zoysia tenuifolia* are classified as being L grade, i.e. not currently recognized as invasive in

Hawaii, and not likely to have major ecological or economic impacts on other Pacific Islands based on the HP-WRA screening process. On the other hand, bentgrass (*Agrostis stolonifera*) belongs to an H grade group of plants, suggesting that transgenic herbicide-resistant bentgrass is a higher risk turfgrass than the zoysiagrass; according to the Hawaii database, *Agrostis stolonifera* is likely to be invasive in Hawaii and on other Pacific Islands as determined by the HP-WRA screening process. In fact, the transgene of the Roundup Ready creeping bentgrass introgressed other recipient plant species 3.8 km away from the test plot [8]. In conclusion, the herbicide-resistant zoysiagrass developed in our laboratory poses substantially less risk of transgene flow than the bentgrass (Figure. 5).

Although the risk of transgene escape and flow from the genetically modified zoysiagrass is low, pollen-induced gene flow cannot be completely discounted. In reference [30] we examined the pollen releases from the defined boundary of *BAR* –transgenic *Zoysia japonica* habitats as a function of physical variables including the boundary, temperature, atmospheric humidity, and lighting condition/duration. Results suggest that zoysiagrass' pollen escape is essentially limited to the close neighborhood, in contrast to bentgrass pollens.

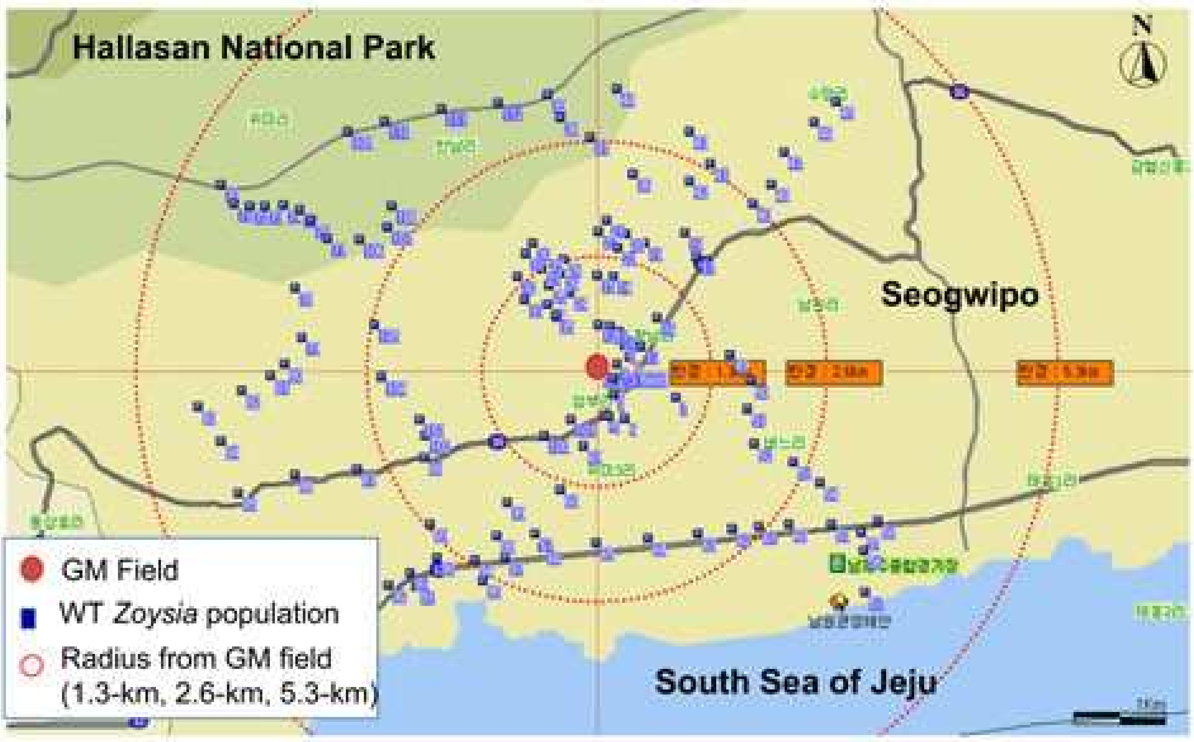

Figure 9. Monitoring for the potential gene flow from the genetically modified zoysiagrass to wild-type zoysiagrass plants within a 5-km radius in natural habitat. Samples were taken from 112 zones (448 sites): *Zoysia japonica* 96 zones (384 sites) and *Zoysia matrella* 16 zones (64 sites).

Figure 9 shows the sites in Jeju Island monitored for the potential gene flow from the herbicide-resistant *Zoysia japonica* to wild-type zoysiagrass within a 5-km radius in natural habitat. No introgression was observed at these sites as of this writing.

6. Commercial potentials and outlook

Turfgrass is a highly value-added crop in terms of commercial profits per land acreage, when compared to other crops. Turfgrasses sward vigorously through vegetative propagation and swarth growth. According to TPI data (Turfgrass Producers International), the turfgrass market size increased by 35% during the five year (2002-2007) period [31]. Based on the data available, transgenic zoysiagrasses pose considerably less risk of transgene escape than does bentgrass. Furthermore, the former can be effectively propagated vegetatively, and sterile herbicide-resistant zoysiagrass (and bentgrass) can be developed through γ-radiation treatment [30]. This will circumvent to a large extent the public's objections to genetically modified plants and their unintended escapes.

7. Conclusion

We compiled a table of transgenic herbicide-resistant turfgrass varieties in various stages of development and eventual agronomic cultivations. As can be seen in Table 1 of this chapter, several transgenes have been introduced into zoysiagrass, bentgrass and other lawn grass species primarily through Agrobacterium-mediated transformation and biolistic transfection. These grasses all exhibit resistance to their intended herbicides such as Basta, Roundup and others, but how well each of the transgenics developed performs in test plots and natural habitats cannot be assessed at this point largely because quantitative data such as the dose-response curves and the outdoor performances are lacking in most cases. In this chapter, we focused our discussion to the *BAR* transgenic *Zoysia japonica* and *Agrostis stolonifera* species. We conclude that these cultivars offer promising potentials as environmentally friendly and economically beneficial turfgrass varieties, especially the former, for Jeju Island and elsewhere.

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