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DEVELOPMENT OF IMPROVED TURFGRASS WITH HERBICIDE RESISTANCE AND ENHANCED DISEASE RESISTANCE THROUGH TRANSFORMATION

Executive Summary

This program aims to establish a transformation system for creeping bentgrass (*Agrostis palustris*) which will enable us to improve the utility of this important recreational turfgrass by incorporating genes to confer herbicide resistance or enhanced resistance to fungal pathogens. The improvements will help conserve natural resources by reducing chemical treatments against major fungal pests while providing high quality playing surfaces for golf through the ability to remove weeds and competing species using available, broad-spectrum and safe herbicides.

Milestones for successful turfgrass transformation are: (1) establishment of a plant regeneration system to produce regenerants at high efficiency, (2) development of a high frequency transformation system to obtain large numbers of transgenic plants. Using surface sterilized mature seeds as explants for callus initiation, we developed a turfgrass tissue culture and regeneration system. Embryogenic callus lines with high regeneration potentials were established from eight commercially important creeping bentgrass varieties. Suspension cultures were initiated from embryogenic callus lines. Both embryogenic callus cultures and suspension cells were used as targets for stable transformation using a gene gun. The *E.coli* ß-glucuronidase (GUS) gene was used as a scorable maker and the *bar* gene, which confers resistance to the herbicide bialaphos (*Basta*™), was used as a selectable marker. We optimized various parameters to improve transient expression of GUS in cultured bentgrass to high levels, and we have developed a turfgrass transformation system with particle bombardment. Transformants were obtained from Emerald, Putter, and Southshore creeping bentgrass. Experiments incorporating several herbicide resistance genes in other cultivars are in various stages of completion. Both plate and liquid selection were successful in obtaining herbicide resistant bentgrass. Transgenics have been confirmed by herbicide tests, and by polymerase chain reaction (PCR) assay and southern blot hybridization to show the presence of the transgenes. We are also developing a protoplast transformation system. Regenerants were obtained from protoplasts through direct DNA uptake with polyethylene glycol (PEG) or electroporation. These plants will be tested for herbicide sensitivity by spraying in the greenhouse.

Introduction

This project seeks to improve creeping bentgrass through transformation to provide golf course managers with more effective and selective weed control with herbicides and more environmentally sound and cost-effective control of plant diseases with reduced use of fungicides. Milestones for successful turfgrass transformation are: (1) establishment of a plant regeneration system to produce regenerants at high efficiency, (2) development of a high frequency transformation system to obtain large numbers of transgenic plants. We have reached both milestones as we have established tissue culture and regeneration systems and now have recovered several varieties of creeping bentgrass with herbicide resistance.

Our objective was to incorporate single gene traits for herbicide resistance in turfgrass and enhanced disease resistance in turfgrass. Results expected by the end of year 1 are: (1) to obtain embryogenic callus lines from several creeping bentgrass varieties, (2) to establish reasonably high efficiency regeneration systems from these creeping bentgrass varieties, (3) with the particle bombardment approach, to use our Putter bentgrass transformation protocol, optimize transformation systems for these varieties using the GUS reporter gene, (4) to tailor gene constructions for monocots, (5) to start transformation experiments with embryogenic calli and useful gene traits. We are making good progress.

I. Initiation of embryogenic callus cultures and regeneration

Embryogenic callus cultures were started from surface sterilized seeds of eight commercially important creeping bentgrass cultivars: Cobra, Emerald, Penncross, Pennlinks, Providence, Putter, Southshore, and SR1020. The cultures of different ages are listed in Table 1. The number of embryogenic callus lines normally decreases over time, so it is critical to establish embryogenic callus cultures periodically to maintain sufficient materials.

Mature seeds of all eight cultivars are satisfactory sources of explants for the establishment of embryogenic callus cultures. Callus initiation media included MS (Murashige and Skoog) (ref. 1) basal medium, MS vitamins, 100 mg/L myo-inositol, 3% sucrose with MSA2D (150 mg/L asparagine and 2 mg/L 2,4-D) (ref. 2) or MMS (500 mg/L casein hydrolysate, 6.6 mg/L dicamba, and 0.5 mg/L 6-BA (6-benzyladenine). (ref. 3).

A high efficiency regeneration system was established for Putter bentgrass. Embryogenic callus cultures of this cultivar have many embryoids, which on transfer readily give rise to shoots. The regeneration system was optimized to obtain high rates of regeneration for all eight creeping bentgrass cultivars. Table 2 shows that even 2-year-old embryogenic callus cultures are as regenerable as younger cultures. Around 200 - 400 plants can be obtained from 1 gram fresh weight of callus.

Embryogenic suspensions were established from embryogenic callus cultures by bulking up sufficient materials (about 2g) and growing suspensions in 250ml flasks with 50ml liquid media in the dark at 25°C and subcultured biweekly. Embryogenic suspensions are as regenerable as callus cultures in the first 2 - 3 months after establishment, but became less regenerable with time. Periodic initiation of suspensions is important to have sufficient materials for experiments.

Embryogenic callus cultures and suspension cells of different ages were used for transformation experiments.

II. Particle Bombardment

Using the *E. coli* ß-glucuronidase (GUS) gene as a scorable marker and the *bar* gene, which confers resistance to the herbicide bialaphos, as a selectable marker, we have optimized a particle bombardment protocol for stable transformation. The flow chart in figure 1 shows the procedure and time table. The establishment of embryogenic callus cultures and suspensions take 3 - 4 months. Bombardment and selection take 3 - 4 months. If regeneration goes well, many plants are ready to be transferred to soil in the greenhouse in another 2 months.

We have successfully obtained turfgrass transformants by using embryogenic suspensions as target tissues. Several experiments are in progress using embryogenic callus directly as the target. Successful bombardment of callus tissues will avoid the task of maintaining suspension cultures and shorten the time needed for obtaining transgenics.

Comparison of transient expression of GUS gene constructs with different promoters in creeping bentgrass suspension cells showed that the rice actin promoter was much stronger than the cauliflower mosaic virus 35S and the maize alcohol dehydrogenase (Adh) promoters (Table 3). We plan to tailor gene constructs using the actin promoter to enhance their expression. We are in the process of constructing a maize chitinase A cDNA clone in a plant gene expression vector. We have also obtained a bean chitinase gene construct (from Dr. Richard Broglie, DuPont, DE). These chitinase constructs will be used to enhance resistance to fungal diseases turfgrass.

Table 4 summarizes one transformation experiment that was completed with herbicide spraying. Suspension cultures were bombarded with a *bar* gene construct and transferred to medium with 2 mg/L bialaphos. One resistant colony was recovered from each of two treatments of Southshore creeping bentgrass. All regenerants obtained from both colonies survived spraying with the herbicide Herbiace (active principle bialaphos). From this experiment, we obtained a total of 126 plants. Some of the transgenics had mixed yellow and green tissues while others were relatively dark green in color. These transformants will be tested to confirm the insertion of transgenes in their genomes. DNA was recently isolated from some of these transformants. Table 5 lists the yields of genomic DNA from transgenics following a CTAB (hexadecyltrimethylammonium) extraction method (ref. 4).

To evaluate transformants further, we are testing the growth chamber conditions required for flowering, and are also preparing an application for field testing from USDA-APHIS.

Table 6 summarizes other particle bombardment experiments in progress: 3 have produced plants in soil, 2 are still in regeneration media and the rest are in various stages of selection with several different herbicides. Seven cultivars of creeping bentgrass were bombarded. Both embryogenic callus and suspension cells were used as targets. Cultures were selected on solid and liquid media. Plants in soil will be sprayed as soon as they are ready.

III. Direct DNA uptake by protoplasts

We are also investigating protoplast regeneration and transformation. Figure 2 shows the scheme. We are using a feeder layer system to support regeneration of callus from protoplasts isolated from embryogenic suspension cells. Plantlets were obtained from the regenerated callus tissues. Both polyethyleneglycol (PEG) and electroporation are being tested to enhance DNA uptake by protoplasts. Parameters such as PEG concentrations, voltage used in electroporation, and the selection pressures are being optimized to establish protoplast transformation. Although still in an early stage of development, putative transformed plants have already been transferred to soil for testing with herbicides.

IV. Related Project

A. Endophytes of turfgrasses: new tools and approaches (USGA 1990-Feb. 1993)

The Emerald bentgrass clone obtained from AS4 endophyte inoculation was multiplied and, with Dr. Funk's help, twelve plants were sent to Dr. William A. Meyer of Pure-Seed Testing for field planting. Drs. Funk and Huff of the Dept. of Plant Science are also planting this clone in their N.J. nurseries. The clone was tested for insect resistance. Detached leaves fed to third instar fall armyworm (*Spodoptera fruigiperda* J.E. Smith) delayed pupation, reduced the number of surviving pupae, and increased larval mortality (Figure 3).

V. Future Directions

We will continue to work on (1) transformation by particle bombardment of turfgrass with agronomically important genes, (2) analysis of putative transgenics by herbicide tests, polymerase chain reaction (PCR), and southern blot hybridization, (3) evaluation of transformants further by preparing applications for field testing to USDA-APHIS.

VI. Manuscripts and presentations

1. Lee, L., N. Tumer, and P. Day. 1993. Infection of creeping bentgrass with *Acremonium* endophytes. (in preparation)

2. Hartman, C., L. Lee, P.R. Day, and N. Tumer. 1993. Creeping bentgrass transformation with particle bombardment. (in preparation)

3. Presented posters at "Molecular Genetics of Plant-Microbe Interactions" Symposium, April 21-25, 1993, East Brunswick, NJ.

4. Dr. Hartman attended 1993 Congress on Cell and Tissue Culture, June 5-9, 1993, San Diego, California.

VII. References

1. Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

2. Lee, L., N. Tumer, and P. Day. 1993. Infection of creeping bentgrass with Acremonium endophytes. (in preparation)

3. Zhong, H., C. Srinivasan, and M. B. Sticklen. 1991. Plant regeneration via somatic embryogenesis in creeping bentgrass (*Agrostis palustris* Huds.) Plant Cell Reports 10: 453-456.

4. Saghai-Maroof, M. A., et al. 1984. Ribosomal DNA spacer length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci., 81: 8014-8018.

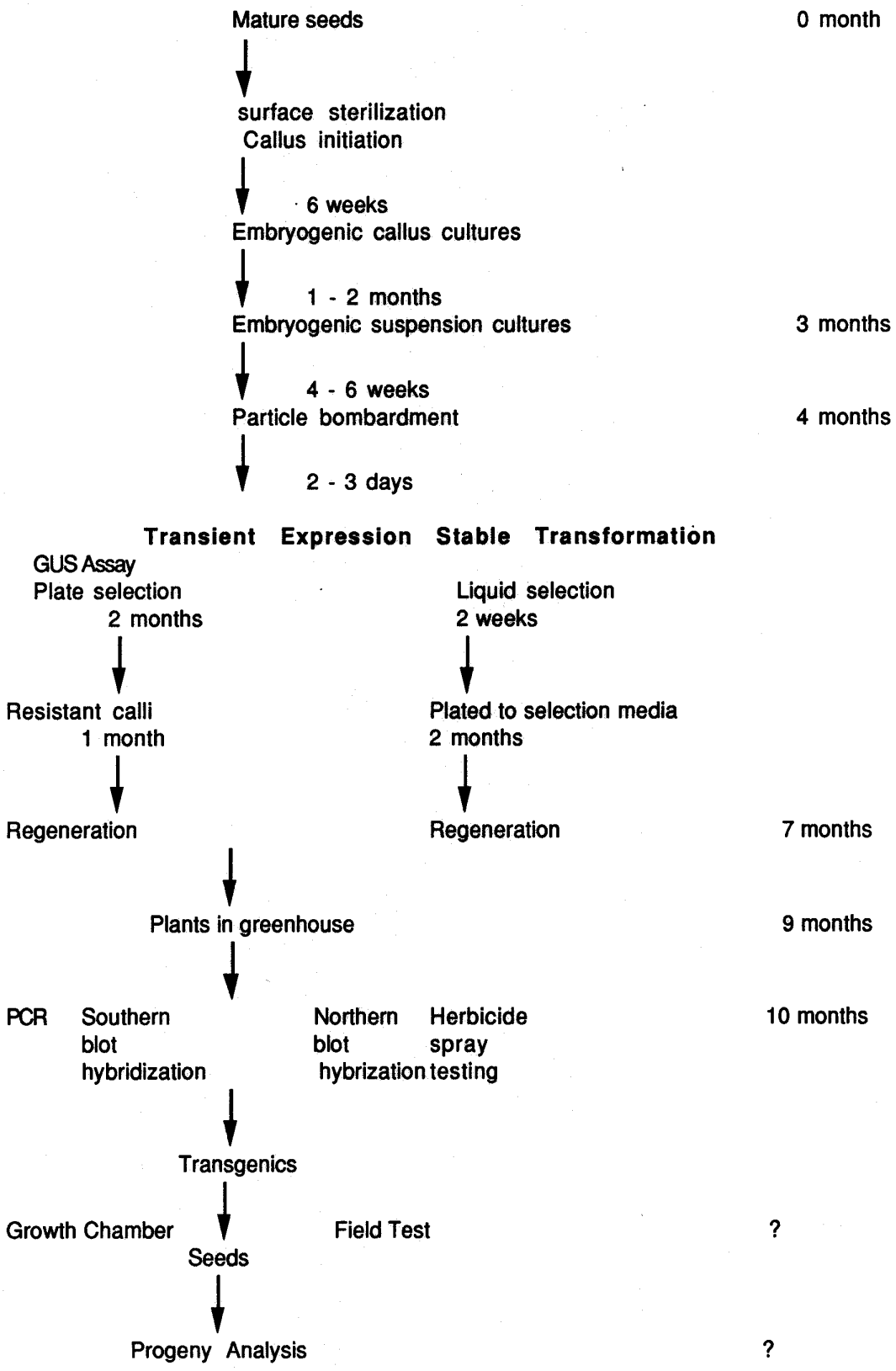


Fig. 1 Flow chart of biolistic particle bombardment procedure for turfgrass transformation

Fig. 2 Protoplast Regeneration and Transformation Scheme

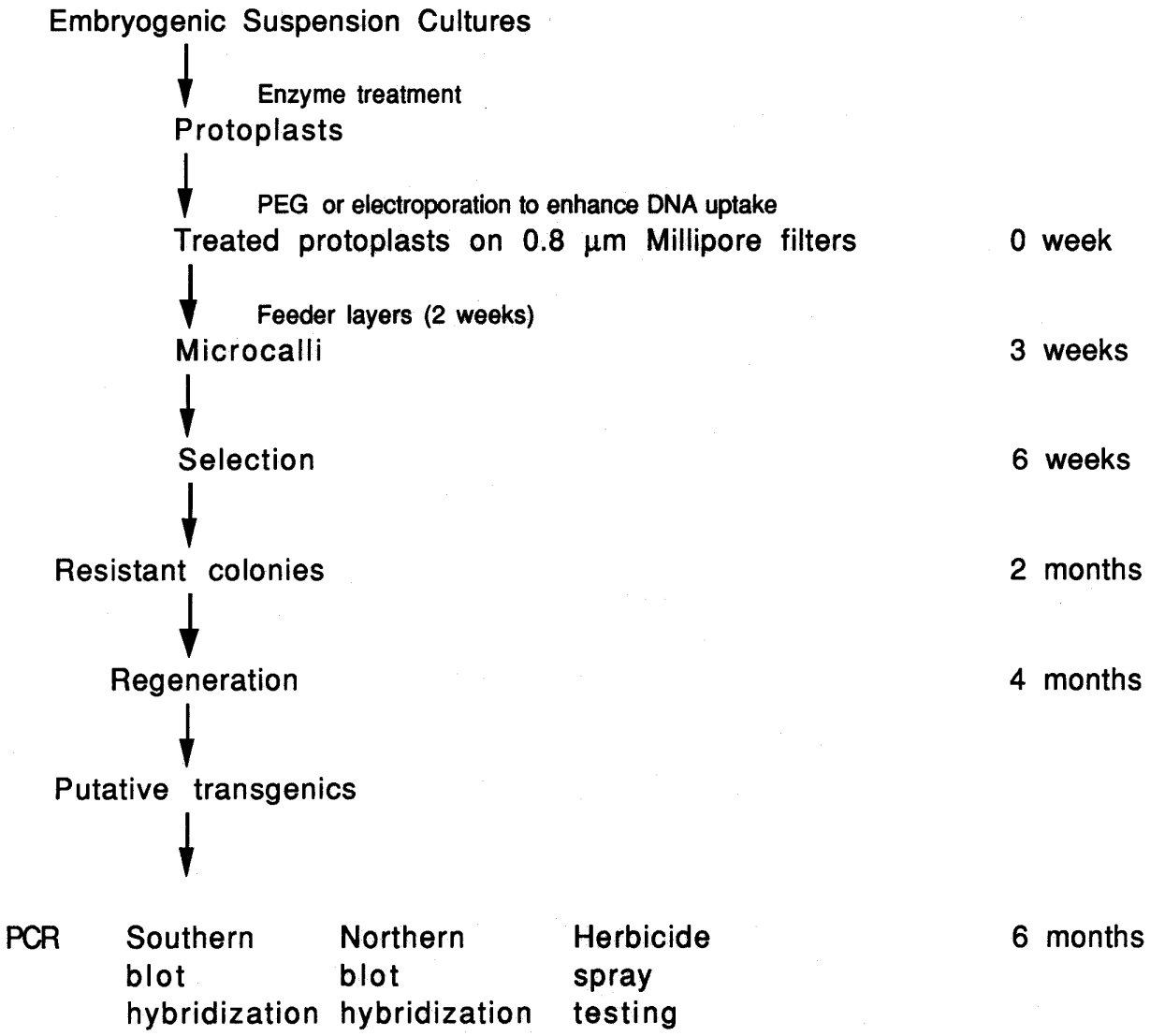
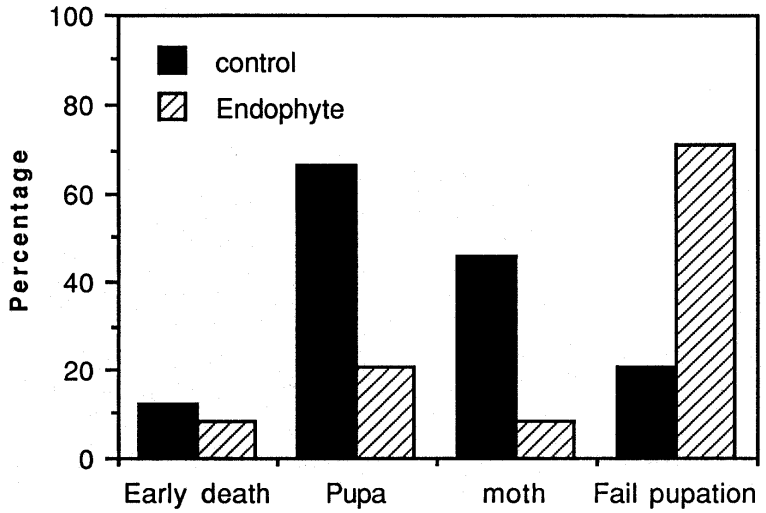


Fig. 3 Fall armyworm test of E- and E+ tillers.



Twenty-four larvae were used in each treatment.

Table 1. Creeping bentgrasses used to initiate embryogenic callus cultures

Variety	Embryogenic callus culture age (months)	Number of embryogenic callus lines
Putter	29	3
Putter	15	20
Emerald	25	4
Emerald	15	20
Emerald	6	65
Penncross	15	10
Southshore	17	1
Southshore	12	20
Providence	15	1
Providence	12	5
SR1020	15	5
SR1020	6 1/2	44
Pennlinks	7	57
Pennlinks	6 1/2	42
Cobra	6	51

Table 2. Regeneration of embryogenic callus cultures of Putter, Emerald, and Southore creeping bentgrasses

Varieties	Culture age (months)	No. of Calli regenerated	Regeneration (%)	No. of shoots per gm FW callus
Putter	24	20	95	384
Emerald	20	20	75	324
Southshore	10	20	100	183

Table 3. Transient expression of GUS gene constructs with different promoters in cultured creeping bentgrass cells. GUS activity was assessed histochemically by counting visible blue spots, using 5-bromo-4-chloro-3-indolyl glucuronide.

Experiment Number	Variety Bombarded	35S-GUS (pPHI460)	Adh-GUS (pBARGUS)	Act-GUS (pACTID)
		Blue spots/filter disc		
22	Emerald	1	1	548
		1	8	- -
	Providence	1	4	147
		2	6	130
	SR1020	1	2	245
		-	0	97
23	Providence	-	2	98
	SR1020	-	1	145
24	Emerald	-	-	30
		-	-	13
	Penncross	17	35	87
	SR1020	9	11	56
		5	14	165

Table 4. Summary of transformation experiment 19. Turfgrass suspension cultures were bombarded with *bar* gene construct and selected with 2 μg/ml bialaphos.

Variety	Filters bombarded	Filters with resistant calli	Calli that produced regenerants	Plants survived herbicide spray
Emerald	2	1	0	0
Putter	2	0	0	0
Southshore	2	2	2	72 51
BMS	2	2	NA	NA

Table 5. DNA yields of Southshore transgenics of HB019

Plant	FW(g)	Yield (ug/g)
Control	1.7	258
803	1.1	321
885	1.4	253
929	1.3	235
949	2.0	256
955	2.5	229

Table 6. Summary of particle bombardment transformation experiments in progress.

Experiment	Variety	Filters bombarded	Selection	Filters produced regenerants		Status
23	Providence	3	Liquid	1	{23-16	in soil
	SR1020	4	Liquid	0		
26	Providence	1 (3)	Liquid	0		
	SR1020	1 (3)	"	0		
	Southshore	2 (3)	"	2	{26-4	in regeneration
					{26-11	in soil
27	Emerald callus	3	plate	2	{27-11	in regeneration
					{27-15	
	Cobra callus	3	"	1	{27-12	"
28	SR1020	8	plate	-		in selection
	Pennlinks	4	"	-		"
30	Emerald callus	5	plate	-		in selection
	Southshore callus	5	"	-		"
31	SR1020 callus	5	plate	-		in selection
	Pennlinks	5	"	-		"
32	Southshore	3	Liquid	-		in selection
	SR1020	3	"	-		"
	Pennlinks	3	"	-		"
33	Southshore	9	plate	-		in selection
	SR1020	9	"	-		"