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A Multigene-Transfer Strategy to Control Pathogens and Enhance Environmental Stress Tolerance in Creeping Bentgrass

from

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I. Executive Summary

Major biotic and abiotic problems associated with the management of creeping bentgrass turf include: several pathogenic disorders and certain environmental extremes such as drought, heat, and cold stress. Environmental extremes such as drought can influence the health of the plant and its ability to resist infection by biotic agents.

Resistance to biotic and abiotic stresses in plants has been reported to be associated with relatively complex genetic factors. Most biotechnological approaches of the last two decades, especially those related to the control of insects and diseases, have concentrated on transferring a single gene to plants. The single gene approach may sound attractive over a short period of time, however, this approach may result in more serious problems over longer periods of time as populations of biotic agents develop resistance to the single gene. Our long term goals include development of transgenic turfgrasses with improved resistance to pathogens and drought tolerance.

Previously, Sticklen's research team developed creeping bentgrass clones that contain: a glufosinate ammonium (Liberty) herbicide resistance gene [1]; a chitinase gene [2]; a proteinase inhibitor gene [3]; and a mannitol dehydrogenase (*mtlD*) gene for drought and salt tolerance [2]. So far, our research team confirmed that glofosinate ammonium has fungicidal as well as herbicidal properties. Therefore, we have been able to simultaneously control turfgrass pathogens (mainly *Sclerotinia ulnocarpal* and *Rhizoctonia solani*) as well as weeds by spraying this herbicide on transgenic creeping bentgrass expressing the herbicide resistance gene under greenhouse conditions [4].

Studies have shown that chitinase genes can make transgenic plants resistant to pathogenic fungi such as *R. solani*. [5]. Research by Dr. Vargas' laboratory has shown that our transgenic creeping bentgrass clone 711, transcribing the elm chitinase gene controlled by the cauliflower mosaic virus 35S promoter, has improved resistance to *R. solani* under controlled environmental conditions. Dr. Sticklen's laboratory has constructed a plasmid containing the elm chitinase gene controlled by a rice actin promoter and transformed creeping bentgrass with this construct. Molecular analysis is in progress to test the expression of the chitinase gene regulated by this grass-specific promoter in creeping bentgrass.

The mannitol 1-phosphate dehydrogenase gene for drought tolerance (*mtlD*) that we have used to transform creeping bentgrass [6; 2] is also associated with salt tolerance [7]. Experiments performed by Dr. Baird's laboratory have not shown any drought tolerance in transgenic plants, nor have they shown accumulation of mannitol in transgenic plants. Antibodies were made against the enzyme, mannitol dehydrogenase, using synthetic peptides. Western blotting will be performed to test the level of expression of this gene in different transgenic lines.

II. List of Graduate Students and Postdoctoral Research Associates Working on the Project

1. Dr. Chien-An Liu	Graduated with Ph.D. from Dr. Sticklen's laboratory
2. Dr. Donald Warkentin	Graduated with Ph.D. from Dr. Sticklen's laboratory
3. Dr. Benli Chai	Graduated with Ph.D. from Dr. Sticklen's laboratory
4. Susan Redwine	M.S. student in Dr. Baird's laboratory
5. Dr. David Green	Completed Postdoctoral Research Associateship in Dr. Vargas' laboratory

III. Description of the Report

A. Introduction

Some of the major diseases of turfgrass include: dollar spot caused by *Sclerotinia homoeocarpa*; brown patch caused by *Rhizoctonia solani*; and pythium blight caused by *Pythium aphanidermatum*. Many pathogens causing diseases in turfgrass contain chitin in their cell walls. Therefore, expression of a chitinase gene in creeping bentgrass plants is expected to provide control of these pathogens by degradation of chitin in their hyphae. Sticklen's laboratory team has cloned and characterized a full length chitinase gene that contains the necessary chitin-binding domain [8; 9; 10] (Gene Bank Number L22032). Also, her laboratory initially developed a system for genetic engineering of creeping bentgrass using a reporter (*gus*) blue gene [11; 12], and transferred the elm chitinase gene [2], and the biolophos resistance gene into creeping bentgrass. With the collaboration of Dr. Vargas's research team, they observed the simultaneous control of weeds, dollar spot, and brown patch diseases in transgenic plants expressing the bialaphos resistance gene[4].

This report covers construction of a new plasmid to increase levels of expression of a chitinase gene in plants, genetic engineering of creeping bentgrass with the improved construct of this chitinase gene and a second drought tolerance gene, and testing transgenic plants for drought tolerance and pathogen resistance [13] under controlled and natural environmental conditions.

B. A higher level of expression of a chitinase gene in transgenic plants

1. Plant regeneration and confirmation of gene integration and expression

Regeneration of putatively transformed plants was performed by placing the cultures in selection media (10 mg/l of bialaphos) under light. Many putatively transgenic plantlets have been developed. We have confirmed herbicide resistance of several clones from this experiment. Work is in progress to test the integration and expression of the elm chitinase gene from this construct (controlled by a grass-specific promoter) in creeping bentgrass.

2. Plasmid construction

A protocol developed for regeneration of creeping bentgrass [11] was used to produce embryogenic callus from caryopses of creeping bentgrass. A construct containing an elm chitinase gene controlled by a grass-specific promoter (rice actin 1) was developed for a higher level of expression of chitinase gene in transgenic creeping bentgrass. In this construct, the chitinase gene cassette was linked to a cassette containing the bar herbicide resistance gene controlled by the 35S promoter. Therefore, herbicide resistant transgenic plants are highly likely to contain the chitinase gene as well as the herbicide resistance gene.

C. Comparisons of disease resistance in transgenic creeping bentgrass transcribing the chitinase gene controlled by the 35S promoter versus those transcribing the elm chitinase gene controlled by the rice actin promoter

So far, inoculations of transgenic creeping bentgrass expressing the elm chitinase gene controlled by the 35S promoter have been performed. The results are presented below.

1. Resistance to *R. solani* in transgenic creeping bentgrass transcribing elm chitinase controlled by the 35S promoter:

Eleven clones of independent transgenic creeping bentgrass containing the 35S-elm chitinase construct were screened [13] for resistance to the brown patch pathogen *R. solani* under controlled environmental conditions. Comparisons between creeping bentgrass cultivars Pencross and Putter versus the transgenic creeping bentgrass clones found that clones 711 and 9603 had 3-fold and 1-fold improved levels of resistance, respectively, to an isolate of *R. solani* AG 1-1a obtained from symptomatic creeping bentgrass in Pennsylvania. Current studies are being conducted to compare the levels of resistance in clones 711 and 9603 to other isolates of *R. solani* obtained from across the United States and to newer creeping bentgrass cultivars L93, G2, and A4.

2. Resistance to *S. homoeocarpa* in transgenic creeping bentgrass transcribing the elm chitinase gene controlled by the 35S promoter:

Clones of transgenic creeping bentgrass containing the 35S-chitinase construct were propagated in the greenhouse and were screened under controlled environmental conditions for resistance to the dollar spot pathogen *S. homoeocarpa.* There was no significant resistance in transgenic lines against this pathogen.

3. Resistance to turfgrass diseases in creeping bentgrass transcribing elm chitinase gene controlled by rice actin1 promoter:

Clones of herbicide resistant creeping bentgrass clones from the the Act1-chitinase construct are in the process of molecular studies and propagation in the greenhouse. After integration and expression of chitinase gene is confirmed in these clones, they will be screened for resistance to both brown patch (*R. solani*) and dollar spot (*S. homoeocarpa)* pathogens.

D. Testing drought tolerance of transgenic creeping bentgrass

Many independent transgenic creeping bentgrass lines containing the mannitol 1-phosphate dehydrogenase gene [7] have been produced in Sticklen's laboratory [2].

The mannitol 1-phosphate dehydrogenase gene has been documented to provide stress tolerance under high salinity conditions [7]. The gene is thought to aid in stress tolerance through osmotic adjustment, which is the accumulation of solutes in plant tissue in response to dehydration. Osmotic adjustment can result in turgor maintenance, thereby sustaining cell elongation and leaf expansion as water deficit develops [14].

So far, Dr. Baird's laboratory conducted studies using several turfgrass clones containing the *mtlD* gene to test for drought tolerance imparted by this gene. In this experiment, we found no obvious significant trends for drought tolerance among transgenic clones tested. In our studies, the drought stress was obtained by withholding water from the plants for the whole duration of the experiment. All stressed plants exhibited decreased leaf expansion over the duration of the experiment, until the plants were visibly desiccated. Every clone exhibited decreased leaf extension dramatically, with none displaying obvious retardation of water stress. Evapotranspiration rates were very similar among the clones. Therefore, these very preliminary studies show that the differences in response to water stress among clones do not appear to be caused by differences in evapotranspiration rates. Clippings were taken twice during the experiment. Clones had to grow for a time period long enough to provide clipping yields adequate for accurate weighing. Because the area of each container was so small, this time period was too long to take several sets of observations. Comparison of the first set of clippings did not reveal a significant trend among the clones. The second set of clippings revealed no significant differences and only included the non-stressed plants, as the stressed plants were desiccated by that time.

Since experiments did not show any drought tolerance of transgenic plants, mannitol accumulation in tissues of the transformants was evaluated. Regenerated clones were established in containers using a sand-peat medium. Plants were exposed to salt stress for 4 days by irrigation with 100mM NaCl, followed by irrigation with tap water for 3 days. Carbohydrates were extracted three times by heating samples in 80% ethanol for 30 minutes. Extracts were evaporated to dryness then rehydrated using millepore water. Mannitol content was measured using normal phase high-performance liquid chromatography (HPLC) with an Alltech 700CH carbohydrate column combined with a differential refractometer detector. Celery extracts containing a known amount of mannitol were measured as comparison. The experiment was repeated for plants which had been exposed to 17 days of irrigation with 300 mM NaCl. These were allowed to recover from stress for 4 days prior to extraction and mannitol content determination. Results of all experiments showed no detection of mannitol in any of the transformed plants.

Because there has been no evidence of mannitol accumulation or stress tolerance of the plants, gene expression needs to be assessed. Antibodies were made against the mtlD enzyme using synthetic peptides. These are being tested before performing western blotting to test the level of expression of this gene in different transgenic

lines.

E. Cross breeding of transgenic creeping bentgrass expressing disease resistance, drought tolerance, and biolophos herbicide resistance genes

Michigan State University has employed a plant breeder, Dr. Suleiman Bughrara. Dr. Bughrara will cross breed pathogen resistant and/or drought tolerant transgenic lines in the near future.

Also, Pure Seed Company has cross bred our transgenic plants containing the herbicide resistance gene, *bar*. This company also conducted extensive studies on flow of transgenic pollen grains in the field. Results of their studies will be reported prior to the completion of this USGA contract.

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