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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 in the management of creeping bentgrass turf include several pathogenic disorders and certain environmental extremes such as drought, heat, and cold stress. In addition, 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 stress in plants has been reported to be associated with relatively complex genetic factors. Most biotechnological approaches of the last two decades, specially 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, however, this approach may result in more serious problems over longer periods as populations of biotic agents develop resistance to the single gene increase. 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 (1) the glufosinate ammonia (Liberty) resistant herbicide [1], a chitinae gene [2], a proteinase inhibitor gene [3], and a drought and salt tolerance mannitol dehydrogenase (mt1D) gene [2]. So far, our research team confirmed that glofosinate ammonia has fungicidal as well as herbicidal properties. Therefore, we have been able to simultaneously control weeds as well as turfgrass pathogens (mainly *Sclerotinia ulnocarpal* and *Rhizoctonia solani*) by spaying this herbicide on transgenic creeping bentgrass expressing this gene under greenhouse conditions [4].

Studies have shown that the chitinase genes can make transgenic plants resistant to pathogenic fungi such as *R. solani*, etc. [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 of plants to R. *solani* under controlled environmental conditions. Recently, Dr. Sticklen laboratory has constructed a plasmid containing the elm chitinase gene controlled by rice actin promoter (shown to provide greater gene expression in grass family than the 35S promoter) and transformed creeping bentgrass with this construct. Theoretically, using this grass-specific promoter, we should improve the level of expression of the chitinase gene, and the degree of resistance to *R. solani* in transgenic creeping bentgrass plants.

The mannitol 1-phosphate dehydrogenase (mt1D:known for its drought tolerance) gene that we have used to transform creeping bentgrass [6; 2] is also associated with salt tolerance [7], A preliminary experiment performed by Dr. Baird laboratory has not shown any drought tolerance of transgenic plants. More studies are needed to confirm whether these plants have any tolerance to drought and/or salt.

II. List of Graduate Students Working on the Project

1. Benli Chai	Ph. D. Student in Dr. Sticklen laboratory
2. Susan Redwine	MS student in Dr. Baird laboratory
3. Dr. David Green	Postdoctoral Associate 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 of pathogens causing disease in turfgrass contain chitin in their cell walls. Therefore, expression of 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 which 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 collaboration of Dr. Vargas's research team, she confirmed 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 under controlled and natural environmental conditions.

B. A higher expression of chitinase gene in transgenic plants

1. Plasmid construction:

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

2. Plant regeneration and confirmation of gene integration and expression:

Regeneration of the 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.

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

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

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

Eleven clones of independent transgenic creeping bentgrass containing the 35S-elm chitinase construct were screened 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 are being propagated in the greenhouse and will be screened under controlled environmental conditions for resistance to the dollar spot pathogen *S. homoeocarpa.*

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

Clones of herbicide resistant creeping bentgrass clones from the the Act1-chitinase construct are in 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 with and will be screened for resistance to both brown patch pathogen (*R. solani*) and dollar spot (*S. homoeocarpa)* pathogens.

D. Testing drought tolerance of transgenic creeping bentgrass

Many independent transgenic creeping bentgrass 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 osmatic adjustment, which is the accumulation of solutes in plant tissue in response to dehydration. Osmatic adjustment can result in turgor maintenance, thereby sustaining cell elongation and leaf expansion as water deficit develop [13].

So far, Dr. Baird laboratory conducted a preliminary study using seven turfgrass clones containing the mtlD gene to test for drought tolerance imparted by this gene. In this preliminary experiment, we found no obvious significant trends for drought tolerance among transgenic clones tested. In our studies, the drought stress was incurred by withholding water from the plants for the whole duration of the experiment. All stressed plants decreased leaf expansion over period of the experiment, until the plants were visibly desiccated. Every clone decreased leaf extension dramatically with none displaying obvious retardation of water stress. Evapotranspiration rates were very similar among the clones. Therefore, this 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 thought the experiment. Clones had to grow for a long enough period of time so that the mass of clipping yields were large enough to be weighed. Because the area of each container is 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 (data not shown). The second set of clippings revealed no significant differences and only included the non-stressed plants, as the stressed plants were desiccated by this time.

We believe that the amount of drought stress given in our preliminary studies was too sudden and sever for mtlD gene activity to compensate for. Further experiments testing drought tolerance of transgenics will involve maintaining a longer period of sublethal stress. This includes using a gravimetric method to evaluate the containers, and then supplementing transpired water to maintain a desired level of stress. The level of water stress which the plants will be subjected to will be based on data from preliminary experiments. Evapotranspiration rates between six and ten mm per day $^{-1}$, which was found between four and six days after watering appear to be critical.

Literature [7] only alludes to possible drought tolerance resulting from osmatic adjustment within plants. Published research on the effects of the mtlD gene in transgenic tobacco almost exclusively involves investigating its role in salinity tolerance. Our strategy is to conduct salinity experiments using our preliminary research as a guideline in addition to pursuing the evaporation of drought stress with our revised experimental plan.

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

Michigan State University has signed a contract with the Pure Seed Company so that this Company cross breeds our transgenic plants. As per Mr. Bill Rose report, the company has performed cross breeding of our herbicide resistant plants with their desirable genotypes. Michigan State University is also in process of recruiting a turfgrass breeder to collaborate with our team in this regard.