Evaluation of Progeny of Genetically Engineered Barley Plants for Resistance to *Rhizoctonia oryzae* and *Rhizoctonia solani* AG-8

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Table of Contents: Figures

PRECIS

In recent years, farmers in the Pacific Northwest (PNW) have been adopting agricultural practices known as direct-seed, or no-till farming, for both economic and conservation benefits. Unfortunately, Rhizoctonia root rot caused by *Rhizoctonia solani* AG-8 and *Rhizoctonia oryzae*, has become a major limiting factor to crop yields in these systems. This situation has developed in part because of an increase in cropping intensity made possible by the additional water captured and saved for crop growth in direct-seed systems, and in part because of apparently higher inoculum potentials for the pathogens when infested host remains are left undisturbed in and on the soil. If direct-seeding is to move forward and succeed in the PNW, cereal genotypes must be identified that have resistance or tolerance to Rhizoctonia root rot.

Six barley plants were each genetically engineered (transformed) by Ph.D candidate Yongchun Wu in the Plant Pathology Department at Washington State University to express a gene from the soil fungus *Trichoderma harzianum.* The purpose of this work is to test progeny of the six transformed barley plants for resistance to *Rhizoctonia oryzae* and *Rhizoctonia solani* AG-8 by exposing seedlings to the pathogens in growth chamber trials, and thereby determine whether the *ThEn42* gene will confer some level of resistance to these pathogens relative to the non-transformed control seedlings. It was hypothesized that there would be a difference in level of resistance between the seedlings of the transformed plants and the seedlings representing the non-transformed control parent variety.

The hypothesis was tested by exposing progeny of all six transformed barley plants and non-transformed control plants to each pathogen as well as no pathogen

(healthy control), and then comparing the amount of root disease and size of seedlings for each progeny x pathogen treatment. With the objective of obtaining a complete data set, I tested progeny from the six plants and one non-transformed (control) plant by planting seeds in soil with a low level of *Rhizoctonia oryzae*, a low level of *Rhizoctonia solani* AG-8, a high level of *Rhizoctonia oryzae*, a high level of *Rhizoctonia solani* AG-8, or in pathogen-free soil.

The soil was infested with the two respective *Rhizoctonia* species at the respective "low" and "high" levels by mixing an artificial inoculum source into the pasteurized soil. Seeds of the six transformed plants as well as one non-transformed (control) plant were planted in small, plastic cone-shaped containers filled with respective infested and pathogen-free soil. The 245 planted containers were completely randomized, placed in trays and allowed to grow in a controlled climate growth chamber at 15° C and 12 hour day/night cycles for 20 days.

Data collection commenced during and immediately upon conclusion of the experiment. The emergence date of each plant and the plant height after the 20-day period were recorded as both reflect symptoms of Rhizoctonia root rot. These data were compared with the emergence date and plant height of unmodified plants in the same pathogen conditions. I found at least one progeny from each genetic transformation to be resistant to both species causing Rhizoctonia root rot. The plants that have shown resistance have been grown to maturity for seed and may be used as parents for making crosses in barley breeding programs where resistance to Rhizoctonia root rot is needed in order for direct-seed cropping systems to succeed.

This thesis is dedicated to my father, Dan, for teaching me
the joys and challenges of the agricultural life.

Evaluation of Progeny of Genetically Engineered Barley Plants for Resistance to *Rhizoctonia oryzae* and *Rhizoctonia solani* AG-8

Introduction

The dryland Pacific Northwest (PNW) region of the United States, which includes eastern Washington, northern Idaho, and northeastern Oregon, is one of the most productive dryland cereal-producing areas in the world. The cropland is endowed with deep, rich mollisol soils and relatively high organic matter. Unfortunately, these loess soils lie on steeply rolling hills and are prone to soil erosion under conventional farming systems that rely heavily on tillage. Standard conventional practices may include 8 to 10 passes of tillage and cultivation to bury previous crop residue, prepare a seedbed, fertilize and seed.

In order to conserve soil and water resources, many PNW growers are adopting new conservation practices known as direct-seeding. With direct-seeding, also known as minimum-till or no-till, all residue from the previous crop is left on the soil surface and the next crop is seeded directly through this residue. No preplant tillage to mix or stir the soil and bury crop residue prior to planting occurs in direct-seed systems. In the PNW, direct-seeding has been defined as "any method of planting and fertilizing done with no prior tillage to prepare the soil (Anon., 2001). The Pacific Northwest Direct Seed Association, a grower organization, has as one of its goals that 2 million acres of farmland in the PNW will be direct-seeded by the year 2005. (www.directseed.org).

Although direct-seeding conserves natural resources and improves environmental quality, Rhizoctonia root rot caused by *Rhizoctonia solani* AG-8 and *Rhizoctonia oryzae*, has become a major limiting factor to crop yields in these systems (Cook, 2001; Paulitz *et al.,* 2002). This situation has developed in part because of an increase in cropping

intensity made possible by the additional water captured and saved for crop growth in direct-seed systems, and in part because of apparently higher inoculum potentials for the pathogens when infested host remains are left undisturbed in and on the soil (Paulitz *et al.,* 2002). *R. solani* AG-8 has become particularly important in direct-seed systems (Weller *et al*., 1986). In particular because this pathogen affects all crops grown in the region, making crop rotation ineffective as a means of controlling the disease. As direct-seed systems become more widespread, Rhizoctonia root rot has rapidly become the single most important root disease of cereal crops in the PNW. A chronic form of the disease limits plant vigor without causing visual symptoms in the crop, although yields are lower by 10% or more (Wesselius *et al.,* 2002). The acute form of this disease causes plant stunting in distinct patches in fields ranging from several centimeters to several meters in diameter (MacNish and Neate, 1996; Cook *et al*., 2002). Infected roots have brown sunken lesions that girdle and often sever the roots, leaving the roots as characteristic "spear points or "pinched off" pointed brown tips. The development of patches of stunted plants is often associated with the presence of volunteer plants and weeds, also known as the "green bridge," when allowed to thrive in the field between harvest of one crop and planting of the next (Smiley *et al*., 1992, Cook, 2001). If direct-seeding is to move forward in the PNW, methods of controlling Rhizoctonia root rot must be identified and implemented.

In recent years, many attempts have been made to control Rhizoctonia root rot. Unfortunately, the methods have had negative impacts on the environment, the economy and even on society. Moreover, none of these methods, alone or combined, have provided better than 85% control of Rhizoctonia root rot. One of the earliest

management practices used to control Rhizoctonia root rot was the use of intensive tillage in the fields of the Palouse. This treatment of the soil promotes erosion, an environmental hazard, and reduces soil water-holding capacity, which reduces the potential revenues from the next crop. Another method often used in conjunction with intensive tillage is leaving a field fallow, i.e. without crop, for an entire year. While this starves the pathogen, no crop and therefore no income for the grower from his land for a full year. Another venue, waiting 2-3 weeks or longer between application of a burn-down herbicide and seeding the next crop, can lower the incidence of Rhizoctonia root rot, but will not prevent the disease altogether (Smiley *et al*., 1992). Placement of fertilizer directly beneath each seed row makes nutrients more accessible to diseased roots, but this practice can only elevate grain yields to 80-85% of potential yield without root disease (Cook *et al*., 2002). Placement of fungicides in the seed row and fumigation of the soil have also been explored, but have been determined to be cost-prohibitive.

In a less traditional attempt, scientists in the 1960's tried to enhance chitinase production by soil microbes by adding crab shells to the soil. Crab shells are composed mostly of chitin and it was thought that soil microbes would increase production of chitinase to digest the crab shells and also digest the inoculum of pathogens such as *R. solani* AG-8 and *R. oryzae.* The cell walls of the *Rhizoctonia* species are composed mostly of chitin, and therefore it was thought that by increasing the activity of chitinase in the soil, this would lead to elimination of pathogens with chitin in their cell walls. This method was found to be cost-prohibitive, as well as generally ineffective. As past management practices have been determined unacceptable economically, for the environment or to society, research in recent years has started in a new direction: use of

biotechnology to provide a safe and effective means of controlling *R. solani* AG-8 and *R. oryzae.*

McDonald and Rovira (1985) were the first to screen cereal varieties for genetic resistance to *R. solani* AG-8. They found some cultivars of barley and oats to be more resistant to or tolerant of the pathogen than others, but none were highly resistant. Neate (1989) evaluated the same cultivars as well as additional wheat, barley, oat, rye and triticale cultivars for resistance to *R. solani* AG-8 under both controlled and field conditions. He was unable to confirm the disease resistance reported by McDonald and Rovira (1985). Jitkov (1997) evaluated 1, 214 cultivars from the United States Department of Agriculture National Small Grains barley collection for resistance to *R. solani* AG-8 in growth chamber analyses and detected varying levels of susceptibility, including one cultivar with apparent resistance to this pathogen. However, subsequent testing of this line in the field and in controlled conditions could not confirm this resistance (Wesselius *et al*., 2002). Smith et al. (2002) evaluated members of the primary, secondary and tertiary gene pools of wheat for genetic resistance to Rhizoctonia root rot for use in spring wheat cultivar improvement. One variety, a Mediterranean annual grass, *Dasapyrum villosum,* expressed a significant level of resistance, indicating that it may be useful as a gene donor to wheat for resistance to *R. solani* AG-8 (Wesselius *et al*., 2002). In 2000 and 2001, Wesselius et al. (2002) continued to search for a Rhizoctonia root rot resistant gene donor for barley, the crop most affected by the Rhizoctonia root rot. While no resistant cultivars were detected, two varieties were identified as partially resistant/less susceptible (Wesselius *et al*., 2002). However,

subsequent greenhouse and field tests with these varieties failed to confirm any level of resistance or tolerance (R. J. Cook, unpublished).

Identifying cereal gene donors to PNW grains for Rhizoctonia root rot resistance has been a long and inconclusive process. Due to its wide host range, which includes peas, lentils, canola, and chickpeas as well as cereal grains (Cook *et al.,* 2002), and lack of resistance or tolerance in small-grain cereals, Rhizoctonia root rot caused by *R. solani* AG-8 and *R. oryzae* can be considered a logical candidate for control by transgenic resistance (Wu, 2003), i.e, by the use of one or more genes from outside the normal gene pool of cereals, including from outside the plant kingdom, introduced into the crop plant by genetic engineering.

Trichoderma harzianum is one of the most widely recognized and accepted biocontrol agents of fungal pathogens in the soil (Wu, 2003). This fungus has been found particularly effective at biologically controlling diseases caused by *R. solani* (Lewis and Papavizas, 1991). The mechanism by which *T. harzianum* provides biocontrol involves a combination of antibiotic and enzymatic activity that act synergistically to suppress infection (Wu, 2003). Hyphae of the *Trichoderma* fungus attach to the to the hyphae of the plant-pathogenic fungus and secrete enzymes that degrade the hyphae of the fungal pathogen. (Benhamnou and Chet, 1993). The enzymes secreted by *T. harzianum* are chitinases, cellulases, glucanases and proteinases. Among these, chitinases are thought to be the most important and have been the focus of most studies (Elad and Kapat 1999).

Studies of pathogen-host plant interactions determined that upon infection by plant pathogens, plants express a number of proteins known as pathogenesis-related (PR) proteins, many of which are chitinases (Punja and Zhang, 1993). Since chitinases have

been identified as an important part of plant defense systems against pathogens, efforts are being made to enhance the plants' natural defense mechanisms by introducing foreign genes that code genetically for chitinase synthesis. In recent years there have been numerous reports of improved resistance to fungal pathogens, specifically *R. solani*, by plants transformed to express foreign chitinase genes (Wu, 2003).

Several chitinases with anti-fungal activity have been found in barley, a PNW crop that is highly susceptible to infection by Rhizoctonia root rot. Since the crop is apparently the most susceptible of all crop plants to the disease (Cook *et al.,* 2002), it seems that the chitinases produced in barley are not adequate in activity, concentration or location in the plant to provide defense against Rhizoctonia root rot (Wu, 2003). Due to these circumstances, transfer of other chitinase genes into barley and other fungal disease-prone plants has become the focus of much research in genetics, mycology and plant pathology.

In an effort to address the substantial problem of Rhizoctonia root rot of barley grown in the PNW, a study was initiated transforming barley by introducing the *ThEn42* gene, a chitinase-producing gene, from *Trichoderma harzianum* into the genome of the variety 'Golden Promise.' This variety was selected because of its prior use at Washington State University as kind of a barley "lab rat" that can be genetically engineered with relative ease. The *ThEn42* gene was cloned from *T. harzianum*, and incorporated into the barley genome via *Agrobacterium tumefaciens,* another soil microbe with the unique ability to introduce its own genome into plants. Plasmids of *Agrobacterium* were constructed in a lab to contain the *ThEn42* gene as well as various combinations of gene-promoters and signal peptides that were expected to drive the

expression of the *ThEn42* gene in the barley plants and assure that the chitinase enzyme once produced in barley cells would be exported out of those cells and into contact with the infection hyphae of *Rhizoctonia*. The *Agrobacterium tumafaciens* containing the various plasmids with differing promoters and signal peptides were then used to infect barley callus tissue. Upon infection, *Agrobacterium* transfers its plasmid to the host plant cells and the plasmid is incorporated into one of the host-plant's chromosomes. In this way, the barley callus tissue was genetically transformed to contain the *ThEn42* gene for endochitinase production (Wu, 2003).

Following the genetic transformations of the barley, hormones were added to the barley callus tissues to promote shoot and root production. The developing plants that tested positive for the *ThEn42* gene in a Western Blot lab test were transferred to a potting soil and grown to maturity for seed. The seed from these transformed plants was then used to test whether the transformed plants had useful resistance to Rhizoctonia root rot.

The overall objectives of Wu's study (2003) were to: a) assemble the *ThEn42* gene optimized for expression in barley, b) assure that the expression of the gene produced the correct endochitinase enzyme, and c) determine that the enzyme has activity against both *Rhizoctonia* species responsible for the disease. My study focused on the third goal of Wu's research: evaluating the effectiveness of the gene against *Rhizoctonia* species by growing the progeny of the transformed plants in soil infested with the *Rhizoctonia* species and taking pathogen reaction data specific to the different genetic modifications made. It is hypothesized that there will be a difference in level of

resistance between the seedlings representing the genetically transformed plants and those representing the non-transformed control parent variety.

Materials and Methods

The hypothesis was tested by exposing progeny of six transformed barley plants and one non-transformed (control) plant to each of the two pathogens, as well as no pathogen (healthy control), and then comparing the emergence date and size of seedlings for each progeny x pathogen treatment. In order to conduct the experiment, a specific protocol designed by Ph.D candidate Kurt Schroeder in the Department of Plant Pathology at Washington State University was followed.

With the objective of obtaining a complete data set, I tested progeny from the six transformed plants and one non-transformed (control) plant by planting seeds in soil with a low level of *Rhizoctonia oryzae*, a low level of *Rhizoctonia solani* AG-8, a high level *Rhizoctonia oryzae*, a high level of *Rhizoctonia solani* AG-8, or in pathogen-free soil. Seven replications of each of the six transformed plants and one non-transformed (control) plant were planted in each of the four pathogen treatments and pathogen-free control soil. Soil was collected from winter wheat ground at the Washington State University Palouse Conservation Field Station, near Pullman, air-dried, sieved to remove debris, and then pasteurized in an aerated-steam pasteurizing unit. The pasteurizing unit eliminated all pathogens in the soil by mild moist-heat (60°C/ 30 min.) treatment. Some pasteurized soil was set aside and used for the pathogen-free control.

The soil was then infested with each of the respective *Rhizoctonia* species. Inoculum was created by filling a 500 mL flask with 250 mL of whole oat grains and 250

mL of water. This mixture was then autoclaved at 121°C for 90 minutes. The process eliminated most microbes, but stimulated germination of some microbes, e.g. those with heat-resistant spores, that survived the first autoclaving in the oat grains as spores. One day later, the oat grain and water mixture was autoclaved again for 90 minutes to eliminate any spores that had germinated in response to the first autoclave. *R. oryzae* and *R. solani* AG-8 were each cultured on a nutrient agar medium in Petri plates. The sterile oat grains were then inoculated with each of the respective pathogens by adding eight to ten, 1-cm^2 segments of the fresh agar culture. The flasks were stored for four weeks at room temperature and shaken once weekly to encourage complete colonization of the oat grains by the respective *Rhizoctonia* species.

At the conclusion of four weeks, the colonized oat grains were laid out on a sterile surface to dry under a fume hood. They were allowed to air-dry for two days. Once dry, the *Rhizoctonia*-colonized oat grains were ground slightly in a coffee grinder. The ground oats were sieved through a #18 (1 mm) sieve and collected on a #60 sieve (0.25 mm). Pasteurized soil was measured into 840 g portions for infestation. Precise weights of the ground oat inoculum were added to the portions of pasteurized soil to simulate low and high levels of infestation of the respective *Rhizoctonia* species. The necessary weights of oat grain inoculum for each species had been determined previously by dilution plating studies conducted by Schroeder. *Rhizoctonia solani* AG-8 inoculum was added to pasteurized soil at a rate of 2.352 g/ 840 g of soil for the low infestation rate and at a rate of 4.704 g/ 840 g of soil for the high infestation rate. *Rhizoctonia oryzae* inoculum was added to pasteurized soil at a rate of 1.354 g/ 840 g of soil for the low infestation rate and at a rate of 2.716 g/ 840 g of soil for the high infestation rate.

Soil was infested in small (840 g) portions to ensure uniform distribution of the pathogen inoculum throughout the soil. Each 840 g lot of soil was spread evenly and shallow and the ground oat inoculum was spread in an even layer on the surface of the soil. For the low rates of infestation, the inoculum was suspended in 45 mL of water then spread on the soil surface to promote more even distribution. The samples were then mixed thoroughly in a clean plastic bag inflated with air so as to make it into a container that could be shaken vigorously. Each 840 g sample of soil with added inoculum was then distributed evenly among 7 cone-shaped containers that represented the seven repetitions for a given plant x pathogen treatment. Thirty milliliters of water were added to each container and a single barley seed placed on top of the infested and watered soil. Each barley seed was covered with approximately 15 g of pasteurized (non-infested) soil. The 245 planted containers were completely randomized, placed in trays to support their vertical position, and incubated in a controlled climate growth chamber at 15° C with 12 hour day/night cycles for 20 days.

Data collection commenced during and immediately upon completion of the experiment. Data were collected to reflect symptoms of Rhizoctonia root rot, which include delayed emergence, stunted plants and reduced root systems. The date of the each seedling's emergence was recorded during the 20-day period in the growth chamber. At the conclusion of the 20 days, plant height was measured. Roots were then washed free of all soil and photographed for comparison. These data have been used to determine that there is a marked difference in resistance to Rhizoctonia root rot among the progeny of the six transformed plants, compared to the non-transformed control.

Results

The variety ‘Golden Promise’ was successfully genetically transformed to confer resistance to both species causing Rhizoctonia root rot. Complete data on time to emergence and plant height were collected on the unmodified control plants in each of the pathogen treatments and no pathogen treatment. These data have been used for comparison to the six genetically modified plants in each respective treatment.

Figure 1: Validation of the Seedling Assay: Response of the Unmodified Golden Promise to the Rhizoctonia Species

Soil Infestations	Rep	Emergence (days)	Height (mm)
Pasteurized soil	1	5.00	186.00
	2	5.00	165.00
	3	6.00	176.00
	4	5.00	157.00
	5	6.00	148.00
	6	6.00	156.00
	7	5.00	170.00
	Avg	5.43	165.43
R. solani low	1	5.00	166.00
	2	5.00	127.00
	3	6.00	118.00
	4	18.00	30.00
	5	13.00	66.00
	6	15.00	56.00
	7	6.00	117.00
	Avg	9.71	97.14
R. oryzae low	1	13.00	82.00
	2	13.00	62.00
	3	17.00	7.00
	4	17.00	33.00
	5	14.00	82.00
	6	18.00	47.00
	7	15.00	80.00
	Avg	15.29	56.14
R. solani high	1	6.00	88.00
	2	17.00	15.00
	3		
	4	17.00	4.00
	5		
	6	13.00	21.00
	7	16.00	21.00
	Avg	13.80	29.80
R. oryzae high	1		
	2	16.00	12.00
	3	7.00	44.00
	4	16.00	24.00
	5	17.00	16.00
	6	16.00	21.00
	7	17.00	12.00
	Avg	14.83	21.50 [1]

[1] Averages calculated on the basis of plants emerged.

The unmodified (control) plants responded as expected when subjected to the five respective treatments. The unmodified (control) plants grown in pasteurized soil all emerged on schedule (5 days) and were uniform in height. Average time to emergence for unmodified plants in pasteurized soil was 5.43 days. Average height for the unmodified plants in pasteurized soil was 165.43 mm. Some unmodified plants in the low level treatments of *R. solani* and *R. oryzae* showed symptoms of delayed emergence and stunting. At least one plant in each of the low level treatments was retarded in development. The average time to emergence for unmodified plants in *R. solani* low was 9.71 days and the average plant height was 97.14 mm. The average time to emergence for unmodified plants in *R. oryzae* low was 15.29 days and the average plant height was only 56.14 mm.

Results acquired from growing unmodified plants in high level infestations of *Rhizoctonia* species were most useful. The symptoms of Rhizoctonia root rot in the high level pathogen treatments were severe. In the *R. solani* high treatment average time to emergence was 13.80 days and the average plant height a mere 29.80 mm. A high level of *R. oryzae* caused an average time to emergence of 14.83 days and an average plant height of only 21.50 mm on unmodified Golden Promise plants.

The difference in Rhizoctonia root rot symptoms of the unmodified plants between pasteurized soil and the low level pathogen treatments in considered minor, while the difference between the pasteurized soil and the high level pathogen treatment is significant. For this reason only the pasteurized treatment, the *R. solani* high treatment and the *R. oryzae* high treatment were used for comparison of the genetically modified progeny with the unmodified check. Moreover, the values for time to emergence and

plant height of the genetically modified plants have been expressed as both absolute values and as a percentage of the average of the unmodified plants in each respective treatment.

Figure 2-1: Response of Progeny of Transformed Plant #1 to Rhizoctonia Species

Soil Infestations	Plant	Emergence (days)	Height (mm)	% of Control
Pasteurized Soil	1			
	2	7.00	176.00	**106.39**
	3	5.00	160.00	**96.72**
	4	5.00	148.00	**89.46**
	5	6.00	147.00	**88.86**
	6	6.00	164.00	**99.14**
	7	6.00	158.00	**95.51**
R. solani high	1	6.00	85.00	**285.23**
	2	11.00	32.00	**107.38**
	3	16.00	47.00	**157.72**
	4	6.00	52.00	**174.50**
	5	6.00	36.00	**120.81**
	6			
	7			
R. oryzae high	1	6.00	85.00	**395.35**
	2	11.00	32.00	**148.84**
	3	16.00	47.00	**218.60**
	4	6.00	52.00	**241.86**
	5	6.00	36.00	**167.44**
	6			
	7			

[2]

Progeny of the first transformed plant responded as expected. The primary transformants all emerged on schedule (about 5 days) and were within normal variation (85%-115%) of the unmodified plants when grown in pasteurized soil. This indicates that transformation itself did not add to retardation in emergence or abnormal plant growth. The primary transformants from plant #1 generally performed better than the unmodified plants in the presence of a high level of *R. solani*. Four of the seven progeny

[2] Averages calculated on basis of plants emerged.

surpassed performance of the control plants in the *R. solani* high treatment. One plant was even 285% of the average of the unmodified plants in the same treatment. In the *R. solani* high treatment one plant was the same as the unmodified plants and two did not emerge at all. In the *R. oryzae* high treatment, while two plants did not emerge at all, the other five not only emerged, but surpassed the performance of the unmodified plants in the same treatment. Responses range from 148% to 395% of the unmodified plants in the *R. oryzae* high treatment.

Figure 2-2: Response of Progeny of Transformed Plant #2 to Rhizoctonia Species

Soil Infestations	Plant	Emergence (days)	Height (mm)	% of Control
Pasteurized Soil	1			
	2	7.00	176.00	**106.39**
	3	5.00	160.00	**96.72**
	4	5.00	148.00	**89.46**
	5	6.00	147.00	**88.86**
	6	6.00	164.00	**99.14**
	7	6.00	158.00	**95.51**
R. solani high	1	6.00	85.00	**285.23**
	2	11.00	32.00	**107.38**
	3	16.00	47.00	**157.72**
	4	6.00	52.00	**174.50**
	5	6.00	36.00	**120.81**
	6			
	7			
R. oryzae high	1	6.00	85.00	**395.35**
	2	11.00	32.00	**148.84**
	3	16.00	47.00	**218.60**
	4	6.00	52.00	**241.86**
	5	6.00	36.00	**167.44**
	6			
	7			

[3]

Progeny of the second transformed plant responded much like the progeny from the first transformation. Six of the seven primary transformants emerged on schedule and

[3] Averages calculated on basis of plants emerged.

were in the normal variation of plant height in the pasteurized soil treatment. In the *R. solani* high treatment, five out of seven plants emerged, a bit delayed and a bit shorter than the plants in pasteurized soil, but better than the unmodified plants in the *R. solani* high treatment. Of the five primary transformants that emerged in the *R. solani* high treatment, four surpassed the performance of the unmodified plants in the same treatment. Primary transformants ranged from 120% to 285% taller than the unmodified control plants in the same treatment. In the *R. oryzae* high treatment, again five out of seven plants emerged. Of these five, all significantly outperformed the unmodified plants in the same treatment, ranging from 148% to 395% the height of the unmodified plants in the *R. oryzae* high treatment.

Figure 2-3: Response of Progeny of Transformed Plant #3 to Rhizoctonia Species

Soil Infestations	Plant	Emergence (days)	Height (mm)	% of Control
Pasteurized Soil	1	6.00	144.00	**87.05**
	2	8.00	165.00	**99.74**
	3	6.00	156.00	**94.30**
	4			
	5	8.00	139.00	**84.02**
	6	6.00	150.00	**90.67**
	7	6.00	163.00	**98.53**
R. solani high	1	7.00	39.00	**130.87**
	2	15.00	51.00	**171.14**
	3			
	4	17.00	6.00	**20.13**
	5	6.00	24.00	**80.54**
	6	6.00	31.00	**104.03**
	7	6.00	65.00	**218.12**
R. oryzae high	1			
	2	17.00	44.00	**204.65**
	3	17.00	3.00	**13.95**
	4	7.00	56.00	**260.47**
	5			
	6	17.00	22.00	**102.33**
	7	17.00	24.00	**111.63**[4]

Progeny of the third genetically transformed plant responded similarly to the progeny of the first two transformations. In pasteurized soil, six of the seven primary

[4] Averages calculated on basis of plants emerged.

transformants emerged on schedule and were of uniform height. One transformed plant did not emerge in pasteurized soil. In the *R. solani* high treatment, only six plants emerged and only three of these performed better than the unmodified control plants. These, again, were stunted compared to the same progeny in pasteurized soil, but much taller than unmodified plants exposed to the same pathogen conditions. In the *R. oryzae* high treatment two plants did not emerge, three emerged but performed the same or worse than the unmodified plants. Only two plants in the *R. oryzae* high treatment emerged and outperformed unmodified plants in the same disease conditions. One plant was 204% the height of unmodified plants and one was 260% the height of the unmodified plants in *R. oryzae* high.

Figure 2-4: Response of Progeny of Transformed Plant #4 to Rhizoctonia Species

Soil Infestations	Plant	Emergence (days)	Height (mm)	% of Control
Pasteurized Soil	1	7.00	156.00	**94.30**
	2	5.00	131.00	**79.19**
	3	5.00	162.00	**97.93**
	4	5.00	169.00	**102.16**
	5	5.00	190.00	**114.85**
	6	5.00	153.00	**92.49**
	7	7.00	100.00	**60.45**
R. solani high	1			
	2	5.00	63.00	**211.41**
	3	15.00	16.00	**53.69**
	4	5.00	22.00	**73.83**
	5	13.00	10.00	**33.56**
	6			
	7	5.00	34.00	**114.09**
R. oryzae high	1	5.00	63.00	**293.02**
	2	5.00	64.00	**297.67**
	3	5.00	34.00	**158.14**
	4	13.00	20.00	**93.02**
	5			
	6			
	7			[5]

Progeny of transformed plant #4 responded as expected and similar to the other three transformations. Primary transformants in pasteurized soil all emerged on schedule and were of uniform height. It would seem that transformation was not particularly successful or there is an abnormally high rate of segregation among these primary transformants due to a lower performance than expected in the presence of high levels of *R. solani* and *R. oryzae*. In the *R. solani* high treatment only one plant outperformed unmodified plants at 211% the height of the average of unmodified plants in the *R. solani* high treatment. Performance results were better in the *R. oryzae* high treatment. Though three plants did not emerge, three of the four that emerged outperformed control plants,

[5] Averages calculated on basis of plants emerged.

ranging from 158% to 297% the height of the unmodified plants in the same pathogen conditions.

Figure 2-5: Response of Progeny of Transformed Plant #5 to Rhizoctonia Species

Soil Infestations	Plant	Emergence (days)	Height (mm)	% of Control
Pasteurized Soil	1	7.00	144.00	**87.05**
	2	6.00	149.00	**90.07**
	3	6.00	160.00	**96.72**
	4	6.00	168.00	**101.55**
	5			
	6	5.00	178.00	**107.60**
	7	5.00	135.00	**81.61**
R. solani high	1			
	2	19.00	3.00	**10.07**
	3	5.00	28.00	**93.96**
	4			
	5	17.00	4.00	**13.42**
	6			
	7			
R. oryzae high	1	18.00	13.00	**60.47**
	2			
	3	16.00	14.00	**65.12**
	4	15.00	49.00	**227.91**
	5	16.00	21.00	**97.67**
	6	18.00	4.00	**18.60**
	7	16.00	20.00	**93.02** [6]

The poorest performers of the group, progeny of plant transformation #5 did not gain the enhanced performance afforded progeny of other transformations. Though six of the seven primary transformants emerged on schedule and were uniform height in pasteurized soil, only one plant between the two respective pathogen treatments outperformed control plants. One plant in the *R. oryzae* high treatment was measured to be 227% the height of the control plants. All other plants either did not emerge (5) or performed the same or worse as control plants (8).

[6] Averages calculated on basis of plants emerged.

Figure 2-6: Response of Progeny of Transformed Plant #6 to Rhizoctonia Species

Soil Infestations	Plant	Emergence (days)	Height (mm)	% of Control
Pasteurized Soil	1			
	2	5.00	150.00	**90.67**
	3	6.00	140.00	**84.63**
	4	6.00	161.00	**97.32**
	5	6.00	167.00	**100.95**
	6	5.00	165.00	**99.74**
	7	6.00	152.00	**91.88**
R. solani high	1	5.00	72.00	**241.61**
	2	5.00	42.00	**140.94**
	3	6.00	74.00	**248.32**
	4	5.00	75.00	**251.68**
	5	5.00	85.00	**285.23**
	6	6.00	81.00	**271.81**
	7	5.00	70.00	**234.90**
R. oryzae high	1	5.00	93.00	**432.56**
	2	6.00	75.00	**348.84**
	3	5.00	105.00	**488.37**
	4	5.00	84.00	**390.70**
	5			
	6	17.00	10.00	**46.51**
	7	5.00	95.00	**441.86**

The progeny of the sixth and final transformation were some of the better and more consistent performers of the entire group of progeny. Again, six of the seven primary transformants emerged on schedule and grew to uniform height in the pasteurized soil. All seven primary transformants emerged *and* outperformed the unmodified plants in the *R. solani* high treatment with performances ranging from 140% to 285% of the control plants. Although only six of the seven plants emerged in the *R. oryzae* high treatment, five of the six emerged plants outperformed control plants by an impressive margin ranging from 348% to 488%, four to five times the performance of the unmodified plants in the same pathogen conditions.

Dicussion

These results provide clear and conclusive support for the hypothesis that resistance to Rhizoctonia root rot is greater among seedlings representing plants genetically transformed with the *ThEn42* gene than in seedlings representing the non-transformed (control) parent variety. Seedlings representing the six transformed progenies showed more timely emergence and more uniform plant height compared to the non-transformed Golden Promise control when grown in soil infested with either *R. solani* AG-8 or *R. oryzae.* As predicted by Wu's study (2003), the endochitinase enzyme produced in transformed barley is effective against both species of *Rhizoctonia*. Resistance to both species responsible for this disease is critical since both occur as a mixture in PNW direct-seed cropping systems. However, it is not possible to determine with the seedling assays conducted in this experiment whether the resistance expressed by transformed barley is more effective against one species than the other.

Each group of progeny tested had at least one plant that excelled allowing us to conclude that each transformed parent plant definitely had the *ThEn42* gene. Many plants tested did not perform acceptably in the presence of the *Rhizoctonia* species due to segregation of the gene in this T_1 generation. Though all parent plants had the *ThEn42* gene, all were heterozygous for the gene as the transformation process via *Agrobacterium tumafaciens* transforms only one chromosome in the affected pair of the host plant. These chromosomes segregate as gametes are formed causing some progeny to receive the chromosome while others do not. Since the *ThEn42* gene is dominant, I expected to see the progeny segregate between having the gene or not at a ratio of 3:1. This ratio was not observed in all treatments due to relatively few seedlings tested compared to the

number of seed produced by each transformed parent plant. If all primary transformant seed had been assayed I would have expected to observe a more consistent 3:1 ratio for resistance to Rhizoctonia root rot. Moreover, some retarded emergence or failure to emerge can be attributed to normal variation in seed viability.

Conclusion

It must be emphasized that even those plants with apparent resistance to Rhizoctonia root rot are not completely immune to the disease. The resistant plants significantly outperform the unmodified plants placed in similar pathogen conditions, but do not outperform plants grown in complete absence of the pathogen (pasteurized soil). For this reason, genetic modification is not the ultimate remedy for control of Rhizoctonia root rot, but rather must be used in conjunction with previously established best management practices for Rhizoctonia root rot including: green bridge management and placement of starter fertilizer near the seed.

While the barley variety Golden Promise has been successfully transformed to confer resistance to both species of *Rhizoctonia* responsible for causing Rhizoctonia root rot, Golden Promise is not a variety that could be successfully grown in the PNW. Golden Promise was chosen for the transformation because of its ability to be transformed with relative ease. Although PNW growers will not use this transformed Golden Promise variety in their cropping systems, the variety can be used as a parent plant in barley breeding programs. In this way, plant breeders can use one or more of these transformed plants to cross with barley varieties commonly produced in the PNW in an effort to incorporate resistance to Rhizoctonia root rot for practical purposes.

In this study has shown very promising results from genetic modification for the future of direct-seed cropping systems in the PNW. While this is not the first instance of genetic modification to enhance agricultural production it is the first example of genetic engineering to control a root disease on any crop. The seedlings conferring genetic resistance to the crop have been grown to maturity to produce seed so that this research may continue and breeding lines of barley with genetic resistance to Rhizoctonia root rot may begin development. On the horizon is a means of finally controlling one of the most limiting factors to production in PNW agriculture in direct-seed systems.

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