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Breeding Barley Ornamented with the Novel Agronomical Attributes

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Under the auspice of the project WNP00251, entitled "Breeding Barley for Imidazolinone Resistance and High Grain Lysine Content" we are committed to develop nutritionally enhanced and ecologically adapted high yielding barley cultivars for the State of Washington. Spring barley, which is one of the preferred rotational crops after winter wheat, has lost acreage throughout the US [1]. Specifically, in the State of Washington observed decline in acreage was quite stark, from 500,000 acres planted in 1999 to 115,000 acres in 2014. A combination of biological, edaphoclimatic and agronomical factors are responsible for this decline, in particular susceptibility of barley to commonly used herbicides and prevailing races of foliar as well as rhizopathogens contribute to it [2]. No natural resistance to imidazolinone (IMI) herbicides and major rhizopathogens of the dryland barley production exist in the extant germplasm [2]. In addition, since, feed barley is the preferentially cultivated class of barley in the State of Washington we focused our efforts both on enhancement of grain feed quality and sustainability of barley production by respectively improving grain lysine content, and developing resistance to major rhizopathogens and tolerance to IMI herbicides. The progress made in this direction is discussed below:

i) Breeding for imidazolinone tolerance: The decline in barley acreage can be in part explained by the large-scale application of IMI herbicides and adaptation of IMI-resistant crops. Making transfer of IMI-resistance, characterized by us in the feed barley cultivar Bob to other relevant food, feed and malting barleys a primary breeding objective [3]. In this connection the seed grant received from the Wash. Grain Commission, allowed us to transfer IMI resistance to six barley cultivars two each belonging to three market classes of barley. The transfer of resistance was primarily confirmed by herbicide spray at a 2× (8 oz/acre) field recommended dose (for winter wheat) followed by determination of plant vigor a month after herbicide spray. Based on the phenotypic screen 6-8 most vigorous F_2 plants per cross-combination were tested for mutant allele at the *AHAS* (acetohydroxy acid synthase) locus by DNA sequencing, and the recovery of recipient parent genome by genotyping with the carrier chromosome 6H specific DNA markers. Results of this pilot study revealed a range of 20-90% recovery of the recipient parent genome for the carrier chromosome in different cross combinations. Collectively, this study unambiguously showed that it is possible to identify plants with good recipient parent genome recovery without involving the laborious backcrossing steps [4]. In view of these results we are now screening 192 F_2 individuals per cross combination for the recovery of the recipient parent genome using a three step marker-assisted selection approach. This approach involves a foreground selection step followed by two background selection steps, which include sequential screening with carrier chromosome specific and genome-wide DNA markers. The plants showing good recovery of the recipient parent genome will be evaluated for their performance in field on herbicide residue and under spray trials.

ii) Pre-breeding for the high grain lysine content: All cultivated barleys are lysine deficient, and require lysine fortification for feeding livestock. Thus, improving lysine content has always remained a primary breeding target. To cope with this problem the breeders have produced high-lysine barley mutants, but despite of several attempts, were unsuccessful in dissecting yield penalty associated with the only agronomically relevant high-lysine barley mutant Risø 1508 (*Lys3a*) with 44% more lysine than wild type. The possible reasons behind the unsuccessful attempts may be either the size of the primary mutation or its tight association with other undesirable background mutations. Moreover the miss-localization of the *Lys3* locus on barley chromosome 5H made its cloning virtually impossible. With the help of our collaborators in Germany we determined that ~10 Mb DNA stretch from Risø 1508 on the short arm of chromosome 1H carries *Lys3* gene. It is a big chromosome segment harboring several hundred genes [5]. Thus, in order to narrow down the effect to a smaller chromosomal region with fewer genes, crosses were made between a near iso-genic line dubbed BW496 carrying the *Lys3* mutant allele in the Bowman background with Golden Promise, and >2000 F_2 grains were obtained. DNA markers flanking the region of interest will be used to evaluate these F_2 plants to identify genotypes carrying recombinations within this region. Identified recombinants will be genotypes with additional DNA markers mapping within the region [5]. If required more DNA markers will be developed and more crosses will be made, and the process will be repeated until a limited number of candidate genes will be identified whose effect on the lysine content will be confirmed by gene silencing.

iii) Breeding for root and crown rot resistance: The aim of this research is to address a gap in genetic resources available to barley breeders to combat the most devastating root pathogens of the dryland barley production. Direct seeding or conservation tillage are the preferred management practice in the low rainfall areas, where crop residue left on the soil surface provides an ideal environment for the proliferation of root and crown rot pathogens specifically *Rhizoctonia solani*, *R. oryzae*, *Fusarium culmorum* and *F. pseudograminearum* [2]. Using biotechnology, an endochitinase (*ThEn42*) gene from a mycotrophic fungus *Trichoderma harzianum* was introduced in barley genomes, and expressed ectopically under the control of *35S* promoter [6,7]. Production of enzyme in barley transformants was determined with the help of a fluorometric assay [7]. The initial

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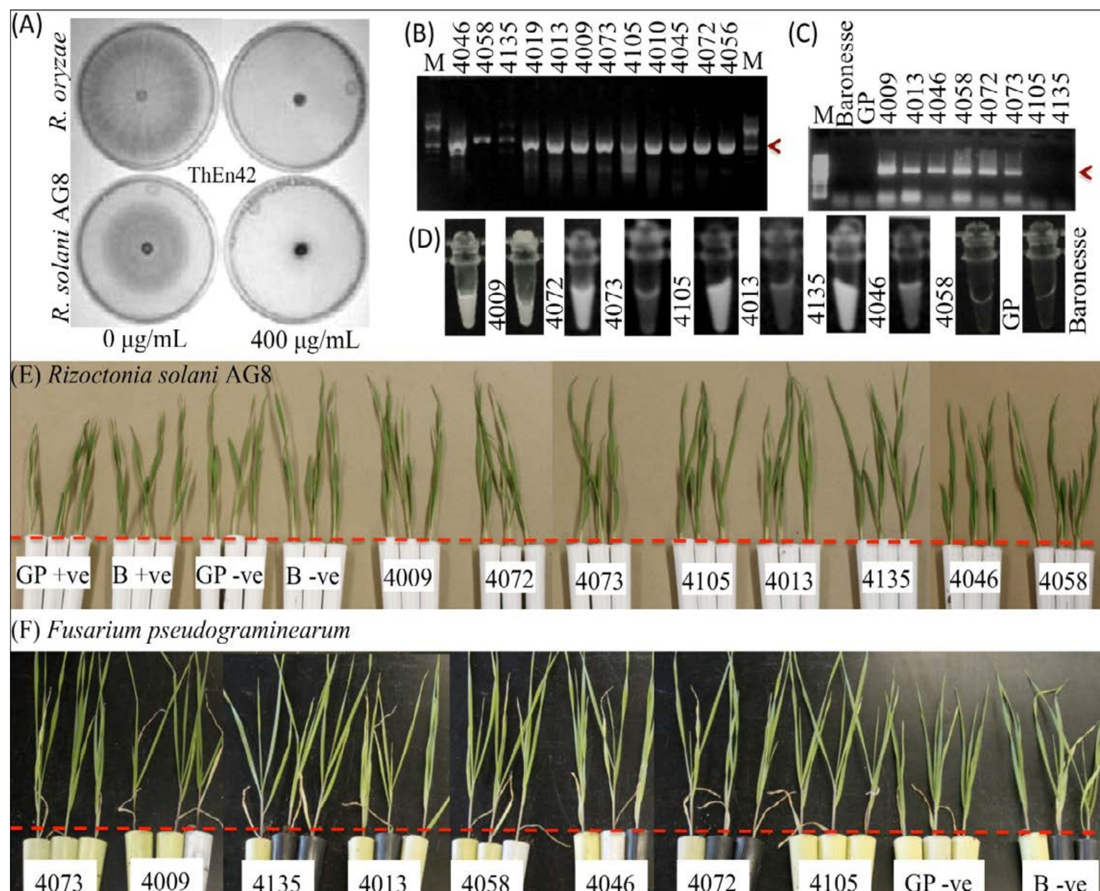

Figure 1: (a) Expression of codon-optimized *ThEn42* gene in methylotrophic yeast (*Pichia pastoris*). The enzyme extracted from yeast cells showed effective inhibition of *R. solani* and *R. oryzae* at a concentration of 400µg/ml. (b) Transfer of transgene *ThEn42* to barley cv. Baronesse confirmed using gene-specific primers. (c) Confirmation of transgene expression by RT-PCR on RNA extracted from the seedling roots (germinated on Petri plate). Six lines showed high level *ThEn42* expression (more than *Actin*), whereas the other two lines showed mild expression (close to *Actin*) at the 25th PCR cycle. (d) Biochemical enzyme activity assay performed using fluorogenic substrate methylumbelliferyl-chitotrioside and root extracts showed variable activity in different transformants. *In planta* pathogen challenge assay performed using eight selected transformants infested with *R. solani* AG8 (e), and *F. pseudograminearum* (f). The *Rhizoctonia* infested control plants (+ve) showed reduced vigor whereas the *Fusarium* infected control plants died. In contrast the transformants showed variable responses to both pathogens. Interestingly the *Fusarium* infested transformants showed higher biomass than the negative (-ve) controls, which is likely a result of in general high resistance in these plants against several other pathogens. The pictures were taken two week after co-cultivation with pathogens. B: Baronesse and GP: Golden Promise.

transformants identified in Golden Promise background were used to transfer transgene to barley cultivar Baronesse. Eight transformants in Baronesse background that showed high expression levels of *ThEn42* gene in roots were identified, and recently challenged with root pathogenic fungi in greenhouse (Figure 1).

iv) Breeding for proanthocyanidin-free grains: In view of reducing the cost of the brewing process and to increase the industrial value of food barley a proanthocyanidin-deficient barley mutant *ant-499* in Apex background was deployed to develop a proanthocyanidin-free barley genotype adapted to the US Pacific Northwest (PNW). This characteristic provides barley with an added advantage of being free of proanthocyanidins (condensed tannins) in the seed coat that cause haze formation in beer after refrigeration (by binding to the protein), and give barley porridge a characteristic gray color that makes it unsuitable as baby food and breakfast cereal [8]. Since, feed barley is the preferentially cultivated class of barley in the US PNW a proanthocyanidin-free genotype, 2004NZ151 with high protein content was selected. Results of the multi-location yield trails in the Western Washington showed that 2004NZ151 yields at least 1000 lb/a more than the large acreage varieties (i.e., Bob, Baronesse, and Champion), and exhibits resistance to lodging as well as prevalent races of stripe rust, leaf rust and powdery mildew [9]. In view of its outstanding agronomical performance 2004NZ151 was approved for release under the name Richard for cultivation in the Western Washington. Since, Richard breeds true for *ant-499* mutation it will serve as an excellent material to breed for the proanthocyanidin-free malting and food barley cultivars adapted to the US PNW or elsewhere.

v) Breeding for increased bioavailability of β-glucans: The low nutritional value of barley for poultry is due to the absence of an intestinal enzyme for efficient depolymerization of (1,3;1,4)-β-d-glucan, the major polysaccharide of the endosperm cell walls. Incomplete digestion of β-glucans leads to high viscosity in the intestine, limited nutrient uptake, decreased growth rate, and unhygienic sticky droppings that adhere to chickens and floors of the production facility. Consequently, the 8 billion broiler chickens produced annually in the United States are primarily raised on corn-soybean diet. If barley is used as feed the diet is supplied with enzymes from *Trichoderma*. Thus, in order to introduce barley as preferred feed choice for

poultry animals, we transformed barley cultivar Golden Promise with the protein-engineered thermostable (1,3;1,4)-β-glucanase from *Bacillus* to be expressed in endosperm. The feeding experiments with broiler chickens suggested that it is sufficient to add 0.2 g (0.02%) transgenic grains in a kilogram of barley-soybean diet containing 620 g nontransgenic barley, to achieve the nutritive value of the corn-soybean diet [8]. These encouraging results promoted us to breed in the transgene from Golden Promise to the widely cultivated spring feed barley cultivar Baronesse. Successful transfer of the β-glucanase gene from Golden Promise to Baronesse was confirmed using the construct-specific primers [7], and enzyme activity in grains of selected genotypes will be determined using the Megazyme biochemical assay.

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