Theor Appl Genet (2009) 119:293–303
DOI 10.1007/s00122-009-1038-x

ORIGINAL PAPER

Scarlet-Rz1, an EMS-generated hexaploid wheat with tolerance to the soilborne necrotrophic pathogens *Rhizoctonia solani* AG-8 and *R. oryzae*

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Received: 14 November 2008 / Accepted: 8 April 2009 / Published online: 1 May 2009

Abstract The necrotrophic root pathogens *Rhizoctonia solani* AG-8 and *R. oryzae* cause Rhizoctonia root rot and damping-off, yield-limiting diseases that pose barriers to the adoption of conservation tillage in wheat production systems. Existing control practices are only partially effective, and natural genetic resistance to *Rhizoctonia* has not been identified in wheat or its close relatives. We report the first genetic resistance/tolerance to *R. solani* AG-8 and *R. oryzae* in wheat (*Triticum aestivum* L. em Thell) germplasm 'Scarlet-Rz1'. Scarlet-Rz1 was derived from the allohexaploid spring wheat cultivar Scarlet using EMS mutagenesis. Tolerant seedlings displayed substantial root and shoot growth after 14 days in the presence of 100–400 propagules per gram soil of *R. solani* AG-8 and *R. oryzae* in greenhouse assays. BC_2F_4 individuals of Scarlet-Rz1 showed a high and consistent degree of tolerance. Seedling tolerance was transmissible and appeared to be dominant or co-dominant. Scarlet-Rz1 is a promising genetic resource for developing *Rhizoctonia*-tolerant wheat cultivars because the tolerance trait immediately can be deployed into wheat breeding germplasm through cross-hybridization, thereby avoiding difficulties with transfer from secondary or tertiary relatives as well as constraints associated with genetically modified plants. Our findings also demonstrate the utility of chemical mutagenesis for generating tolerance to necrotrophic pathogens in allohexaploid wheat.

P. A. Okubara and C. M. Steber contributed equally to this work.

Communicated by R. Waugh.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-009-1038-x) contains supplementary material, which is available to authorized users.

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Introduction

The necrotrophic basidiomycete fungi *Rhizoctonia solani* Kühn AG-8 and *R. oryzae* Ryker and Gooch cause Rhizoctonia root rot and pre-emergence damping-off, two major yield-limiting diseases in cereal production regions worldwide (MacNish and Neate 1996; Demirci 1998) and especially in conservation tillage systems of the Pacific Northwest USA (Cook et al. 2002; Paulitz et al. 2002). Both are highly pathogenic to wheat and barley seedlings (Weller et al. 1986), attacking young seminal and crown roots, killing root tips and causing root rot that prevents further root growth. *R. solani* AG-8 can cause stunting of plants in the field, often in patches several meters in diameter, which reduce grain yields by 30% or more (Cook et al. 2002). The bare patch phenomenon is particularly severe in fields that are under transition from conventional tillage to direct seeding (no-till) (Cook et al. 2002). *R. oryzae* was first identified as a foliar pathogen of rice, and has emerged as a major root pathogen of direct-seeded spring wheat in the Pacific Northwest (Mazzola et al. 1996; Paulitz et al. 2003). The pathogen causes stunting and

uneven stand height, and, in severe infections, necrosis of emerging roots and death of germinating seedlings (Paulitz et al. 2002).

Lack of control of *Rhizoctonia* poses a major barrier to the widespread adoption of direct seeding in the Pacific Northwest (Schroeder and Paulitz 2006). Rotation, tillage, fungicides, and the control of volunteers and weed hosts between plantings by the herbicide glyphosate are currently used to manage root diseases such as Rhizoctonia root rot and damping-off (Bockus and Shroyer 1998; Paulitz et al. 2002; Rainbolt et al. 2004). However, no single practice or combination of practices is effective. *Rhizoctonia*-tolerant or -resistant wheat could improve the economic viability of cereal-based production systems because of its potential to complement conservation tillage, reduce agricultural inputs, and reduce the risk of developing glyphosate-resistant weed populations.

All known cultivars of wheat and barley, including those having resistance to rusts and other pathogens, are susceptible to the necrotrophic root pathogens *Rhizoctonia solani* and *R. oryzae* Ryker and Gooch (teleomorph *Waitea circinata* Warcup & Talbot) (Mazzola et al. 1996). Extensive screening of adapted wheat germplasm and wheat relatives has demonstrated the paucity of effective genetic resistance to *R. solani* AG-8 (Smith et al. 2003) and to *Pythium ultimum* or *P. irregulare* group IV (Higginbotham et al. 2004). The most effective resistance to *Rhizoctonia* was found in *Dasypyrum villosum* (L.) P. Candargy (Smith et al. 2003); however, traits in this wild Triticeae relative are not readily transferable to cultivated wheat germplasm. In contrast, resistance to *R. solani* has been characterized in several crop species, including soybean (Bradley et al. 2005), crucifers (Keinath and Farnham 1997), sugar beet (Scholten et al. 2001), tall fescue (Green et al. 1999) and rice (Li et al. 1995; Pinson et al. 2005).

Chemical mutagenesis provides an alternative approach to traditional germplasm screening for developing *Rhizoctonia*-resistant wheat. Chemical- or radiation-induced mutagenesis has been used to increase genetic diversity in wheat and other crop plants since 1950s (Brock 1971; reviewed in Strader et al. 2004). Chemical mutagenesis was used to produce Clearfield™ crops that harbor induced tolerance to imidazoline herbicides (Tan et al. 2005). Mutagenesis also has been used to generate wheat plants with resistance to powdery mildew (*Erisyphe graminis*) (Kinane and Jones 2001), leaf rust and stem rust (*Puccinia recondita* and *P. graminis*, respectively) (Kerber 1991; Williams et al. 1992; Boyd et al. 2002, 2006).

Mutagenesis using the alkylating agent ethyl methane sulfonate (EMS) was chosen for this project because EMS generates C–T transitions or small deletions; these changes are more likely to alter rather than ablate gene function, in contrast to ionizing radiation, which causes large deletions and chromosomal rearrangements. Gain-of-function disease resistance could result from altered gene function or from ablation of a dominant suppressor of resistance. The hard red spring cultivar Scarlet was used for mutagenesis because spring wheat cultivars are more susceptible to damage by necrotrophic root pathogens in the Pacific Northwest, and Scarlet is a widely grown, adapted hard red spring cultivar with excellent agronomic and end-use (milling and baking quality) traits (Kidwell et al. 1999).

This study describes the identification of a *Rhizoctonia*-tolerant adapted wheat germplasm or genetic stock called 'Scarlet-Rz1,' and inheritance of the tolerance trait in BC_1 and BC_2 populations. At present, we prefer the term "tolerance" rather than "resistance" because the mechanism of protection is unknown. Protection against both *Rhizoctonia solani* AG-8 and *R. oryzae* are reported.

Materials and methods

Fungal isolates and inoculum preparation

R. solani AG-8 isolates C1 (Weller et al. 1986), 050539, 070308 and 1202262 (Okubara et al. 2008) and *R. oryzae* isolates 0801387 (Paulitz et al. 2003), 030111, 0801387 and 1202119 (Okubara et al. 2008) were maintained on potato dextrose agar (PDA, Difco Laboratories, Sparks, NV, USA) and used to inoculate autoclaved whole oats. Oat inocula were incubated at 23°C in darkness for 3–4 weeks; fresh inocula were prepared every 3 weeks. Inocula were homogenized using a coffee grinder and passed through 1- and 0.25-mm opening sieves to obtain particle sizes of 250–1,000 μm. Inocula were enumerated from a suspension of 100 mg of homogenized material in 5 mL water and from a 10-fold dilution of the suspension. Triplicate 0.2-mL aliquots of both the original and diluted suspensions were spread onto water agar containing 100 μg/mL chloramphenicol, a broad-spectrum antibacterial agent, and 1 μg/mL a.i. benomyl (Benlate®, DuPont, Wilmington, DE, USA), a fungicide with activity against Ascomycetes. Plates were incubated at 24°C in darkness for 3 days. Inoculum titer was calculated as the mean colony density of both undiluted and 10-fold dilutions. Homogenized inoculum was stored up to 3 weeks at 8°C. Soil infestation was carried out by thoroughly mixing the inoculum into Palouse silt loam soil from the Spillman Agronomy Farm, Pullman, WA, USA. Soil was pasteurized at 60°C moist heat for 30 min before infestation.

Plant material and mutagenesis

Approximately 5,000 seeds of cultivar Scarlet (Kidwell et al. 1999) were soaked in 200 mL 50 mM sodium

phosphate buffer (pH 7.0) for 5 h, transferred to 200 mL of 0.3% EMS (Sigma Chemicals, St. Louis, MO, USA) solution in phosphate buffer in a sealed 2 L flask, and incubated with shaking for 16 h at 22°C. EMS was neutralized by adding an equal volume of 10% sodium thiosulfate (w/v). Seeds were allowed to stand for 5 min in sodium thiosulfate solution before washing ten times with water, with 30-min intervals of soaking in water between washes.

EMS-treated seeds (M_1) were grown in a greenhouse under 16 h day^{-1} supplementary sodium lighting (300 mmol m^{-2} s^{-1}) at a constant temperature of 16°C. Seeds from M_1 plants were considered to be M_2.

Mutant screen, M_2 and M_3 plants

The first spike from each of 1995 M_1 plants was harvested by hand and comprised an M_2 family for screening. To avoid resampling, six grains from each M_1 head (total of 11,970 M_2 seedlings) were grown in 8-in × 1.5-in plastic cones (Stuewe & Sons, Corvallis, OR, USA). Before planting, each cone was disinfected in 10% (v/v) bleach, plugged with a cotton ball, and filled with approximately 115 g of Spillman soil infested with 1.5 g *R. solani* AG-8 inoculum per 1,000 g soil (Smith et al. 2003), or about 80 propagules per gram of soil (ppg). Soil-filled cones were watered to near-saturation and incubated 1 week at 16°C to allow fungal mycelium to colonize the soil prior to planting M_2 seed. Seedlings were maintained in a Conviron™ (Pembina, ND, USA) growth chamber for 3 weeks under 95% humidity, 14 h day^{-1} supplemental lighting, with day and night temperatures of 23–26°C and 15–18°C, respectively (Smith et al. 2003). These conditions limited soil water loss by evaporation and plant transpiration, and allowed development of Rhizoctonia root rot. Disease tolerance was assessed relative to wild-type Scarlet. M_2 seedlings displaying greater plant height (mm) were transferred to sterile, non-infested soil to advance candidates to M_3.

Twenty-eight M_3 seeds from each of three putative tolerant M_2 plants were treated and grown as described for the M_2. M_3 plants were evaluated on the basis of plant height, and number of infected seminal and crown roots; total root length (cm) and number of root tips were obtained using digital images of roots (HP ScanJet 5370C, Hewlett Packard, Palo Alto, CA, USA) and the pixel-counting software WinRHIZO 5.0 (Regent Instruments, Inc., Quebec, Canada).

BC and F generations

For all generations, plants showing disease severity ratings of 0 (or 1, in one instance) were rescued and advanced in the greenhouse. Tolerance assays are described in the following section. A diagram of crosses and families is shown in Suppl. Fig. 1. Two M_3 individuals, derived from a single tolerant M_2 plant and displaying reduced disease symptoms, were crossed to wild-type Scarlet to obtain two independent BC_1F_1 plants derived from the same cross, named "1" and "2." Eight tolerant progeny of plants 1 and 2 (BC_1F_2 individuals) were self-pollinated to generate BC_1F_3 families; four of these (1–1 to 1–4) were derived from plant "1," and four (2–1 to 2–4) were derived from plant "2." BC_2 progeny were derived from four tolerant BC_1F_3 individuals from plant "1" and two from plant "2" as pollen donors in crosses to wild-type Scarlet. The six BC_2F_1 families, four from plant "1" (1–5 to 1–8) and two from plant "2" (2–5 and 2–6), were used to generate BC_2F_2 and BC_2F_3 progeny through self-pollination. Individuals of BC_2F_3 family 1–5 that showed the highest overall tolerance were advanced to the F_4 generation.

BC_1 and BC_2 seedling tolerance assays

A total of four BC_1F_2, eight BC_1F_3, six BC_2F_2 and six BC_2F_3 families were tested for tolerance to *Rhizoctonia* species. For seedling tolerance assays, seeds of each BC_1 and BC_2 family were germinated on Whatman No. 1 filter paper in Petri plates at 23°C for 3 days in darkness prior to planting. 16–24 individuals of each family were tested to ensure a >95% and >99% probability of obtaining at least one homozygote, respectively, assuming a single-gene mutation. Seedlings were sown individually in 6-inch plastic cones (Stuewe & Sons) containing 70 g pasteurized Spillman soil infested with a mixture of *R. solani* AG-8 isolate C1 and *R. oryzae* isolate 0801387 at 200, 250 or 400 ppg of each isolate for BC_1F_2, BC_1F_3, BC_2F_2 and BC_2F_3, respectively. Inoculum levels were incrementally increased from 200–400 ppg per pathogen for each subsequent generation, so that tolerance and susceptibility were more clearly distinguished, and false positives or "escapes" were minimized. At these inoculum levels, infection was moderate enough for survival of tolerant plants. BC_2F_4 progeny of family 1–5 were sown in soil infested with 20, 100 or 400 ppg of either *R. solani* or *R. oryzae*. The soil in each cone was drenched with 50 mL of metalaxyl (75 mg/L a.i., Novartis, Greensboro, NC, USA) to control *Pythium* and other Oomycetes (Okubara et al. 2004). Plants were maintained at 15 ± 1°C, with 12 h daily supplemental lighting (66–90 μmol m^{-2} s^{-1}) for 14 days (Okubara et al. 2004). Controls were wild-type Scarlet grown in infested and non-infested soil.

Washed roots were rated for disease symptoms on a scale of 0–8, where 0 indicated no symptoms, 5 indicated significant root stunting and more than one lesion in all

seminal roots, and 8 indicated lesions and severe stunting in all seminal roots (Kim et al. 1997). Tolerance also was assessed using length between the crown and tip of the longest leaf (shoot length), percentage of infected seminal roots, root or whole seedling fresh weight and total root length relative to wild-type Scarlet, as described in 'Mutant screen, M_2 and M_3 plants.' Seedling weights rather than root weights were measured in cases where tolerant plants were rescued for advance to the next generation. Ratings of "6," rarely obtained for inoculated wild-type at the inoculum density used, were pooled with the "5"s.

To evaluate pre-emergence damping-off in BC_2F_4 family 1–5, 24 seeds were sown in pasteurized Spillman soil containing 400 or 800 ppg of *R. oryzae* isolate 0801387 as described for seedling tolerance assays. The number of emerged seedlings and root or whole-seedling weights were measured after 8 days. Total root length and number of root tips were analyzed from digital scan images using WinRHIZO 5.0 (Regent Instruments, Inc.) Controls consisted of 12 wild type Scarlet grown in infested and non-infested soil.

Tolerance was assessed in 11 F_1 progeny of a cross of Scarlet-Rz1 (BC_2F_4 family 1-5) and a susceptible BC_1F_3 individual.

Tolerance to multiple isolates of *R. solani* AG-8 and *R. oryzae* was evaluated in Scarlet-Rz1 BC_2F_5 plants derived from BC_2F_4 family 1–5. Twelve seeds of either Scarlet-Rz1 or wild type were grown in pasteurized Spillman soil containing 400 ppg of *R. solani* isolate C1, 050539, 070308 or 1202262, or 400 ppg of *R. oryzae* isolate 030111, 0801387 or 1202119 for 14 days. Wild-type Scarlet and Scarlet-Rz1 without pathogen were included. Disease severity ratings and root fresh weight data were collected.

Statistical analyses

Comparisons of mean disease severity ratings and percentages of infected seminal roots among samples were conducted using Kruskal–Wallis one-way ANOVA (Statistix vers. 8.1, Analytical Software, Tallahassee, FL, USA), where family (genotype) and pathogen treatment were combined to produce one dependent variable. Comparisons of mean leaf length, root or seedling fresh weight and total root length among treatments were done using general analysis of variance, and mean separations were performed using Fisher's protected least significant difference test at $P < 0.05$ (Statistix vers. 8.1). Inoculum by genotype interactions for BC_2F_4 assays were analyzed using JMP 4.0 (SAS Institute Inc, Cary, NC) and significant differences among means were determined using LSD. BC_2F_2 and BC_2F_3 assays each were conducted in two to three separate experiments. Data from each BC_2F_2 or BC_2F_3 experiment was analyzed independently to determine if tolerance phenotypes were reproducible, and Bartlett's t test for equal variances was applied to determine whether data from separate assays could be combined.

Chi-square analysis

To test the prediction that tolerance was conferred by a single dominant or co-dominant gene, root-length values of 61 BC_2F_2 individuals of families that showed an intermediate level of tolerance (Suppl. Table 1, hatched bars in Fig. 1) were sorted into three length classes, 'long' = 61.8–82.8 cm, 'intermediate' = 41.0–61.7 cm and 'short' = 19.9–40.9 cm, representing resistant, intermediate and susceptible phenotypes. These classes were established by dividing the difference between the highest and lowest lengths into three equal intervals. To test predictions for inheritance of two dominant genes of unequal tolerance activity, root-length values of the 61 BC_2F_2 individuals were divided into four equal length intervals: 67.3–82.8 cm (long or resistant), 51.5–67.2 cm (intermediate-long or moderately resistant), 35.7–51.4 cm (intermediate-short or moderately susceptible) and 19.9–35.6 cm (short or susceptible). Chi-square values were calculated using the equation $X^2 = \Sigma(o_i - e_i)^2/e_i$ (Excel, Microsoft Corp., vers. 2002 SP3).

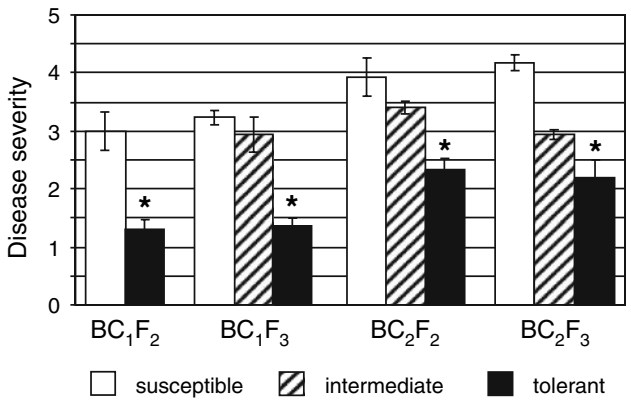

Fig. 1 Mean disease severity scores for three tolerance classes of BC_1F_2, BC_1F_3, BC_2F_2 and BC_2F_3 progeny. Individual plants were rated on a scale of 0 (no symptoms) to 8 (lesions and severe stunting in all seminal roots) after 14 days of pathogen challenge. Families were sorted into phenotypic groups "susceptible", "intermediate" or "tolerant" according to the lowest, intermediate or highest proportion of individuals with ratings of 0–1. Sample sizes were 16 individuals for each BC_1F_2, BC_1F_3 and BC_2F_2 family, and 24 individuals for BC_2F_3. *Asterisks* indicate significant ($P < 0.05$) differences between means of the "tolerant" group compared with the other two phenotypic groups within each generation

Results

Progeny of six generations (M_3, BC_1F_2, BC_1F_3, BC_2F_2, BC_2F_3 and BC_2F_4) derived from a tolerant M_2 plant of allohexaploid wheat cv. Scarlet showed improved foliar and root growth and reduced root infection in the presence of *Rhizoctonia solani* AG-8 and *R. oryzae* compared with wild-type Scarlet. The original tolerant plant and its progeny were designated "Scarlet-Rz1." Family 1–5 showed consistent tolerance, but the degree of tolerance in each generation depended on whether the trait was segregating, as in the BC_2F_2.

Criteria for evaluating *Rhizoctonia* tolerance

Tolerance was evaluated on the basis of disease severity ratings, percentage of infected seminal roots (Table 1; Fig. 1), root or seedling fresh weight, shoot length and total root length (Table 2). *R. solani* AG-8 was used for the initial mutant screen at approximately 80 ppg. Because *R. solani* AG-8 and *R. oryzae* often occur together in agricultural soils and are the major causal agents of Rhizoctonia root rot, both pathogens were combined in equal amounts (total of 200–800 ppg) and used to monitor transmission of tolerance in the BC_1 and BC_2 generations of mutagenized Scarlet. At 14 days post-inoculation, disease symptoms were readily visible, and roots were small enough to scan on a flatbed scanner for root-length analysis.

Disease severity ratings generally concurred with proportion of infected seminal roots; however, the latter was less informative than the former because each seminal root often sustained more than one lesion. Unless infection was severe, gross measurements of root or seedling fresh weight and shoot length were not consistent indicators of tolerance or susceptibility, especially if pathogen-damaged roots became stunted and thickened with only minor loss of root mass. In such cases, lateral roots growth was severely reduced, but crown roots often formed in response to the loss of seminal and lateral roots. The most reliable combination of variables for tolerance was deemed to be the disease severity rating and total root length, the latter including seminal, lateral and crown roots (Table 1).

Table 2 Mean total root lengths of wild-type and EMS mutagenized Scarlet after challenge with *Rhizoctonia solani* AG-8 plus *R. oryzae*

Plant/family	Mean total root length (cm)[a]		
	BC_1F_2	BC_2F_2	BC_2F_3
Wild-type control[b]	124.5 b	81.2 a	136.7 a
Wild type	72.7 c	30.6 c	89.5 c
Family 1	148.0 ab	–	–
Family 1–5	–	60.0 b	132.1 ab
Family 2	159.2 a	–	–
Family 2–5	–	49.7 b	115.6 b

[a] Mean of 16–24 individuals, except for eight individuals of wild-type Scarlet in BC_1F_2 experiments. Letters indicate significance ($P < 0.05$) classes among plants and treatments within a generation

[b] Control plants were not inoculated; all others were grown for 14 days in soil infested with equal amounts of *R. solani* AG-8 and *R. oryzae*, as listed in Table 1

Transmission of *Rhizoctonia* tolerance based on disease severity ratings

Three mutant candidates for *Rhizoctonia* tolerance were identified among 11,970 M_2 seedlings derived from 1995 M_1 spikes. Of these candidates, only one showed a reproducible reduction in the number of infected seminal roots in tests of M_3 progeny (Table 1). This individual was used to generate the first backcross (BC_1) to cv. Scarlet. The other two candidates displayed disease severity ratings of '0' in the M_2 screen but were not found to be tolerant in the M_3 test.

Table 1 *Rhizoctonia* disease severity in wild-type and EMS-mutagenized Scarlet

Experiment	Inoculum[a]	Infected seminal roots		Disease severity	
		Wild type[b]	EMS Scarlet[c]	Wild type[b]	EMS Scarlet[c]
M_3	~80	88.1 a	65.5 b	n.a.	n.a.
BC_1F_2	400	75.0 a	42.3 b	3.0 a	1.3 b
BC_1F_3	500	88.9 a	34.0 b	3.3 a	1.4 b
BC_2F_2	500	75.1 a	48.3 b	3.9 a	2.3 b
BC_2F_3	800	83.5 a	51.9 b	4.1 a	2.2 b

[a] Total inoculum in propagules per gram soil, consisting of equal amounts of *R. solani* AG-8 C1 and *R. oryzae* 0801387

[b] Mean percent values derived from 16 to 24 individuals in each experiment, except for eight individuals in BC_1F_2. Letters indicate significance ($P < 0.05$) classes among wild-type and EMS-mutagenized Scarlet within an experiment

[c] Mean values for individuals of families displaying tolerance (Fig. 1) in each experiment: for BC_1F_2, Plant 1 and Plant 2 ($n = 32$); for BC_1F_3, 1–1, 1–2, 1–3, 2–1, 2–2 ($n = 76$); for BC_2F_2, 1–5 ($n = 15$); for BC_2F_3, 1–5 ($n = 24$)

Rhizoctonia tolerance was evaluated in a total of six generations of backcrossed material derived from the tolerant M_3 plant. All non-inoculated plants had ratings of "0" (data not shown). Tolerant BC_1F_2 families 1 and 2 displayed a combined mean disease severity score of 1.3 when inoculated at 400 ppg. This result suggests that these populations are segregating for tolerance since this mean disease severity is significantly ($P < 0.05$) lower than that of 3.6 for wild-type Scarlet (Fig. 1). Eight BC_1F_3 populations derived from BC_1F_2 individuals having a '0' disease severity rating were placed into one of three phenotypic classes, "susceptible" (two families) "intermediate" (one family) or "tolerant" (five families) based on the proportion of individuals with disease severity ratings of 0–1 when inoculated at 500 ppg (Fig. 1; Suppl. Table 1). The combined mean disease severity scores were 1.3 for the tolerant, 2.9 for the intermediate, and 3.2 for the susceptible BC_1F_3 families (wild-type Scarlet included), respectively. The mean of the tolerant families was significantly ($P < 0.05$) different from those of the intermediate and susceptible groups. These data suggested that BC_1F_3 populations harbored the mutation conferring the tolerance phenotype.

Among 32 BC_1F_2 individuals, seven were assigned disease severity ratings of 0 and were considered tolerant, 15 had a rating of 1 and 10 were rated susceptible at 2 to 3 (Suppl. Table 1). Thus, 68.8% of the BC_1F_2 had a disease severity rating between 0 and 1. In contrast, three (37.5%) of the eight wild-type Scarlet plants were rated at 2, and none between 0 and 1 (Suppl. Table 1). The high percentage of tolerant BC_1F_2 individuals relative to Scarlet suggested that the phenotype was dominant or co-dominant. Individuals showing disease severity ratings of 0 were observed in every generation, indicating that tolerance was genetically transmissible.

Tolerance also appeared to be segregating within the BC_2F_2 and BC_2F_3 generations (Fig. 1). Of six BC_2F_2 families showing tolerance when inoculated at 500 ppg, family 1–5 was considered to be the most tolerant, displaying a mean disease severity score of 2.3. The five remaining BC_2F_2 families displayed intermediate levels of tolerance and mean ratings ranging from 3.1 to 3.5, whereas the susceptible wild-type Scarlet had a mean score of 3.9 (Fig. 1). BC_2F_3 families were derived from single tolerant BC_2F_2 individuals and evaluated at 800 ppg of inoculum in order to better differentiate between tolerant and susceptible responses. Although all seven BC_2F_3 families showed greater tolerance than wild-type Scarlet, the BC_2F_3 family 1–5 showed the highest degree of tolerance; its mean disease severity rating of 2.2 was significantly ($P < 0.05$) different from those of the remaining intermediate families (Fig. 1, Suppl. Table 1). Wild-type Scarlet had a mean disease severity rating of 4.1.

Transmission of *Rhizoctonia* tolerance based on root length

Root-length data (Table 2) was examined for consistency with disease severity ratings. BC_1F_2 family 2 challenged with the *Rhizoctonia* mixture fell into a root length class distinct ($P < 0.05$) from wild-type Scarlet (Table 2). This trend was repeated for families 1–5 and 2–5 in the BC_2F_2 and BC_2F_3 generations (Table 2). Over multiple generations, root-length values were inversely proportional to disease ratings, indicating that these parameters are consistent in monitoring *Rhizoctonia* tolerance or damage. We also noted that pathogen challenge resulted in increased mean root length of tolerant BC_1F_2 progeny relative to non-inoculated wild-type (Table 2). This phenomenon sometimes was seen in other greenhouse experiments (P. Okubara, unpublished data).

BC_2F_3 individuals of family 1–5, which gave consistently lower disease ratings than other genetic lines, showed a mean shoot length of 22.1 cm that was significantly ($P < 0.05$) different from 19.4 cm obtained for 'intermediate' families, and for 16.9 cm obtained for wild-type Scarlet (Fig. 2a). Family 1–5 showed significantly ($P < 0.05$) longer mean total root lengths after pathogen challenge compared with family 2–5 (Table 2), and three other families, 1–6, 1–7 and 1–8, that were derived from the same parent (Plant 1) (data not shown). Roots of family 1–5 more closely resembled non-inoculated wild type (Fig. 2b–d). Mean seedling weight values of BC_2F_3 family 1–5 also were significantly ($P < 0.05$) greater than those of inoculated wild-type Scarlet and BC_2F_3 families 2–5 and 2–6 (Fig. 2e). Mean seedling weights were 0.51, 0.28 and 0.29–0.40 g for family 1–5, wild type and 'intermediate' families 2–5 and 2–6, respectively. BC_2F_3 plants of 1–5 were selected for advance to the F_4 and F_5 generations and a seed increase was carried out using the BC_2F_4 family.

Tolerance to *R. solani* AG-8 and *R. oryzae* in Scarlet-Rz1

To examine tolerance to each pathogen species separately, we challenged BC_2F_4 seedlings of family 1–5 with 20, 100 and 400 ppg of either *R. solani* AG-8 or *R. oryzae* instead of a mixture of isolates. Significant inoculum by genotype interactions were observed. Scarlet-Rz1 displayed disease severity ratings of 1.6–2.8 after challenge with *R. solani* AG-8, and ratings of 1.7–4.9 for *R. oryzae* (Table 3). These values were lower than those for wild-type Scarlet, and the differences were significant ($P < 0.05$) at 20, 100 and 400 ppg of *R. solani* AG-8, and at 100 and 400 ppg of *R. oryzae*. Mean total root length of Scarlet Rz-1 was significantly ($P < 0.05$) greater than that of wild-type Scarlet at 100 and 400 ppg of *R. solani* AG-8 and at

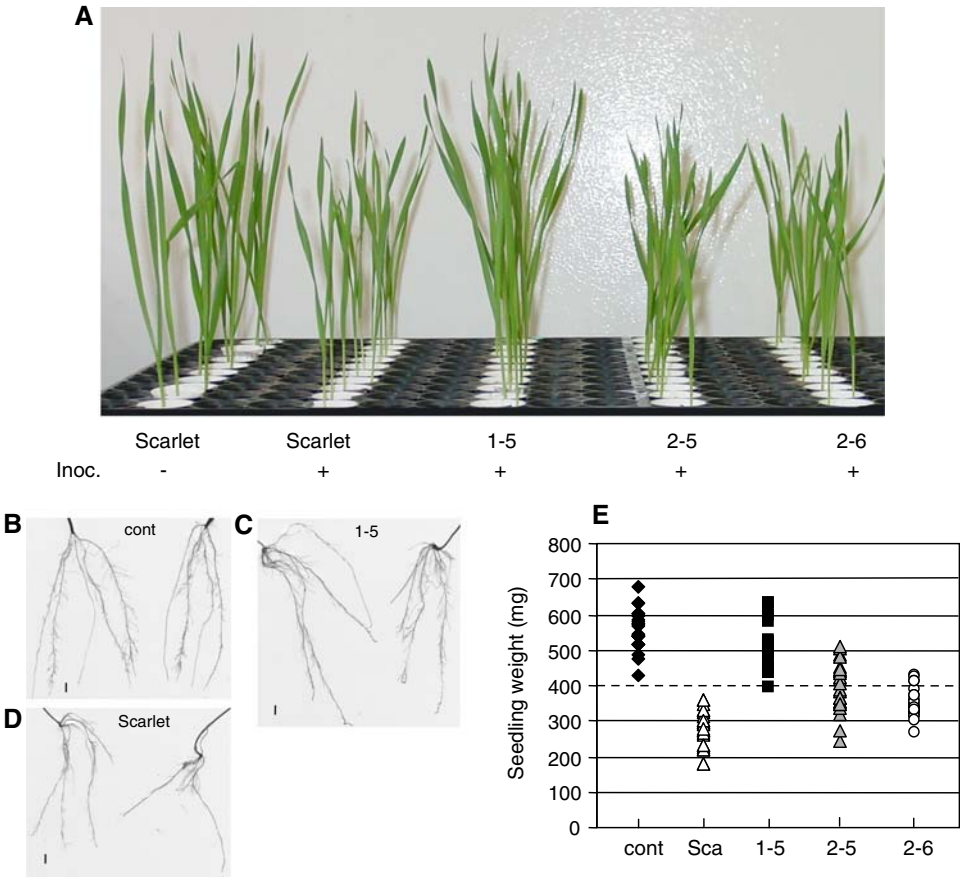

Fig. 2 Seedling tolerance to *Rhizoctonia* displayed in BC_2F_3. Plants were challenged with 400 ppg each of *Rhizoctonia solani* AG-8 and *R. oryzae* (800 ppg total) for 14 days. **a** Comparison of shoot heights in a sampling of inoculated plants of families 1–5, 2–5 and 2–6, and Scarlet wild-type plants without (−) and with (+) inoculation. Digital images of roots of typical plants. *Bars* represent 1 cm. Scarlet control (**b**) and 1–5 (**c**) roots are longer and contain more lateral roots than inoculated Scarlet (**d**). **e** Distributions of seedling weights of 24 individuals from families 1–5, 2–5 and 2–6, wild-type Scarlet with (*Sca*) and without (*cont*) inoculation. The *dashed line* represents the mean seedling weight of all individuals in the *graph*

Table 3 Seedling tolerance to *Rhizoctonia solani* AG-8 and *R. oryzae* in Scarlet-Rz1 BC_2F_4 family 1-5

Pathogen treatment	Disease severity[a]		Root length (cm)[a]	
	Scarlet-Rz1	Wild type	Scarlet-Rz1	Wild type
R. solani AG-8 (ppg)				
0	0	0	83.1 a	61.2 b
20	1.6 a	2.8 b	62.3 b	49.4 b
100	2.4 a	4.5 b	76.8 a	32.6 b
400	2.8 a	5.3 b	65.7 a	44.8 b
R. oryzae				
0	0	0	63.5 a	66.8 a
20	1.7 a	2.2 a	62.2 a	55.0 a
100	2.8 b	4.1 a	55.8 a	43.8 a
400	4.9 b	6.6 a	38.0 a	20.1 b

[a] Means and significance ($P < 0.05$) classes between genotypes for each pathogen treatment, $n = 24$

Table 4 Seedling tolerance to different isolates of *R. solani* AG-8 and *R. oryzae*

Isolate	Disease severity[a]		Root weight[a] (mg)	
	Scarlet-Rz1	Wild type	Scarlet-Rz1	Wild type
R. solani AG-8[b]				
C1	1.5 c	4.0 a	300 a	162 bc
1202262	1.7 c	3.2 b	303 a	199 b
070308	1.6 c	3.6 ab	310 a	156 c
050539	1.2 c	3.5 ab	317 a	170 bc
R. oryzae[b]				
1202119	3.5 b	4.2 a	170 a	126 b
0801387	3.2 b	4.3 a	167 a	125 b
030111	3.4 b	4.6 a	171 a	109 b

[a] Means and significance ($P < 0.05$) classes of 12 individuals per isolate

[b] At rate of 400 ppg for each isolate

400 ppg of *R. oryzae* (Table 3). In addition, Scarlet-Rz1 (BC_2F_5 seedlings of family 1–5) displayed tolerance to multiple isolates of *R. solani* AG-8 and *R. oryzae* (Table 4). Our findings show that Scarlet-Rz1 was somewhat more tolerant to *R. solani* AG-8 than to *R. oryzae*, and that tolerance was not isolate-specific.

Inherent differences in root development were observed between wild type and Scarlet-Rz1 (Fig. 3a, b; Tables 3, 5). Roots of non-inoculated Scarlet-Rz1 were both longer and more massive ($P < 0.05$) compared with those of wild type in four of five experiments in which comparisons were made.

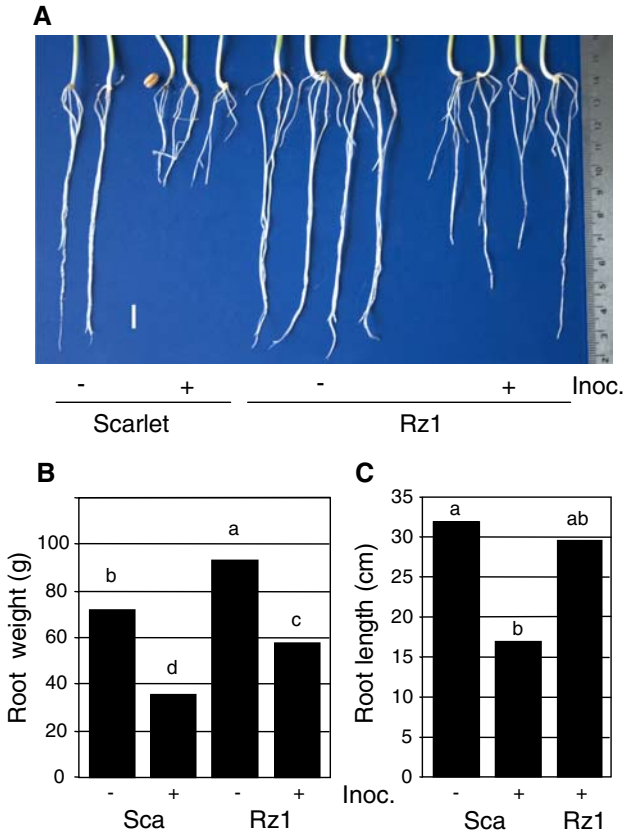

Fig. 3 Damping-off tolerance to *R. oryzae* in Scarlet-Rz1. Seeds of BC_2F_4 family 1–5 (Rz1) were sown in soil infested with 400 ppg *R. oryzae* and evaluated for root morphology at 8-day post-inoculation. **a** Examples of typical root growth of wild-type Scarlet and Rz1 plants with (+) and without (−) inoculation. The *bar* and each major division on the ruler is 1 cm. **b** Mean root fresh weights and **c** total root lengths of wild-type Scarlet (*Sca*) and Scarlet-Rz1. *Letters* indicate significant ($P < 0.05$) differences among the means

Damping-off tolerance

Scarlet-Rz1 displayed tolerance to *R. oryzae* in damping-off assays, in which seeds rather than 3-day-old seedlings were sown in infested soil (Fig. 3a). Seedling emergence, evaluated at 8 days in six plants as part of a larger screen, was 67% for wild-type Scarlet sown in soil containing 800 ppg of *R. oryzae*, but was unaffected (100%) in Scarlet-Rz1. At 400 ppg *R. oryzae*, emergence was 94.4% for wild-type and 100% for Scarlet-Rz1 (24 plants of each genotype; data not shown). Furthermore, Scarlet-Rz1 sustained significantly ($P < 0.05$) less damage on a root-weight- and root-length basis than wild-type Scarlet at 400 ppg of *R. oryzae* (Fig. 3b, c). The data indicated that the mutation conferred tolerance to *R. oryzae* at a very early stage of emergence. This was not tested for *R. solani* AG-8.

To determine whether 8-day seed-based emergence assays could substitute for the more time-consuming seedling tolerance assays in future mutant screens, we tested previous generations of mutagenized Scarlet. One BC_1F_3 family, 1–3, displayed significant ($P < 0.05$) enhancement of root fresh weight and root length compared with wild-type Scarlet, and enhanced growth also was observed in the BC_2F_3 generation of Scarlet-Rz1 family 1–5 (data not shown). However, individuals of the BC_1F_2 and BC_2F_2 generations showed little or no early tolerance to *R. oryzae* (data not shown). This suggested that the damping-off assay was not sufficiently sensitive or reproducible for mutant screens and not suitable for early generation genetic analysis.

Table 5 Comparison of mean root weight and mean total root length for non-inoculated wild-type Scarlet and tolerant Scarlet genotypes in four experiments

Experiment[a]	Mean root weight (g)	Mean total root length (cm)
BC_2F_2 #1		
Wild-type	0.0945 b	81.234 b
Tolerant	0.1498 a	103.19 a
BC_2F_2 #2		
Wild-type	0.0866 b	82.86 b
Tolerant	0.1132 a	97.52 a
BC_2F_4 #1		
Wild-type	0.0841 b	61.174 b
Tolerant[b]	0.1145 a	83.071 a
BC_2F_4 #2		
Wild-type	0.1003 a	66.799 a
Tolerant[b]	0.1163 a	63.512 a

[a] Means and significance ($P < 0.05$) classes determined for eight individuals per treatment, except for 16 individuals of BC_2F_2 wild-type

[b] Scarlet-Rz1 (family 1–5)

Segregation analysis

We performed Chi-square tests for a single-locus or two-locus mode of inheritance of *Rhizoctonia* tolerance using root-length data from individuals of four BC_2F_2 families. These families were derived from the same parent (Plant 1) and were deemed to be segregating because they showed "intermediate" tolerance (Fig. 1, Suppl. Table 1). When root-length values were sorted into three length classes representing three phenotypes expected for single-locus co-dominant inheritance, the observed numbers of individuals in each length class were 17 'long' (15.25 expected for a single co-dominant gene), 26 'intermediate' (30.5 expected), and 18 'short' (15.25 expected) for 61 BC_2F_2 individuals, where $\chi^2 = 1.361$ ($P = 0.51$). A χ^2 value of 3.84 or lower indicates a 95% probability that the model hypothesis, in this case, a single co-dominant gene, is correct. For

inheritance of a single dominant locus, 43 individuals were 'long' (45.75 expected) and 18 were 'short' (15.25 expected), resulting in $\chi^2 = 0.661$ ($P = 0.42$), suggesting that there is a high likelihood that the single dominant locus hypothesis is true. Therefore, the data support either a single dominant or co-dominant gene inheritance. Chi-square values for two additive or two epistatic dominant loci were 45.41 ($P < 0.001$) and 44.9 ($P < 0.001$), respectively, indicating that these hypotheses are not likely to be true. Thus, the most likely hypotheses are that the tolerance in Scarlet-Rz1 segregates as a single dominant or co-dominant gene.

If the tolerance phenotype is conferred by a single dominant locus, then Scarlet-Rz1 heterozygous plants would be expected to display tolerance similar to Scarlet-Rz1 homozygous plants, whereas if tolerance was co-dominant, then heterozygotes would display an intermediate degree of tolerance. To evaluate tolerance in heterozygous individuals, we used available F_1 progeny of a cross of Scarlet-Rz1 (BC_2F_4 family 1–5) and a susceptible BC_1F_3 individual. Wild-type Scarlet was substituted for the BC_1F_3 parent because seed of the latter was limited, and both displayed very similar responses to pathogen challenge in previous experiments (data not shown). Statistical analysis showed that the F_1 progeny did not differ significantly from Scarlet-Rz1 in root length or disease severity rating, and were not significantly different from wild type in shoot length, height and root fresh weight. Although the F_1 disease severity score did not differ significantly from Scarlet-Rz1 ($P > 0.05$), the F_1 mean disease severity rating of 3.1 was intermediate relative to the scores of 4.5 and 2.4 for the susceptible wild type and Scarlet-Rz1, respectively (Suppl. Table 2). Taken together, our findings suggest that the *Rhizoctonia* tolerance in Scarlet-Rz1 is conferred by a single co-dominant locus showing strong but incomplete dominance.

Discussion

Scarlet-Rz1 is the first wheat germplasm to be identified with seedling tolerance to *R. solani* AG-8 and *R. oryzae*. Protection against both pathogen species confers a significant advantage over protection against either pathogen alone because they often are found together in commercial production fields (Paulitz and Schroeder 2005). Furthermore, tolerance against multiple isolates of each pathogen species indicates a broad activity that is not limited to specific isolates. The tolerance limits of >100 ppg that were observed in our greenhouse studies exceed the 20–85 ppg that are associated with Rhizoctonia root diseases of cereals in the field (Paulitz and Schroeder 2005). Assessing the performance of Scarlet-Rz1 in the field, under natural growth conditions and environmental influences, is a priority; field studies are in progress to determine how the significant tolerance observed in the greenhouse will provide protection.

Evaluating disease tolerance was complicated by plant-to-plant variation in reaction to *Rhizoctonia* spp. We have applied carefully controlled experimental conditions, including standardized inoculum preparation, soil infestation and planting protocols, to reduce such variation within and among experiments. Despite these measures, it was still difficult to assess populations of mutagenized Scarlet that were segregating for the tolerance phenotype. Susceptible sister lines of Scarlet-Rz1 and previous generations were not selected beyond BC_1F_3; these would have provided more suitable controls for susceptibility than wild-type Scarlet. However, comparisons of the families derived from the EMS mutant relative to wild-type Scarlet provided evidence for stable tolerance in two different lineages of the original mutant over a total of six generations. *Rhizoctonia* tolerance was readily detected in multiple families per generation, and was not attenuated after two backcrosses.

Chi-square analysis indicated that tolerance was not recessive and suggested that it segregated as a single dominant or co-dominant locus, which we provisionally designate *Rot1* for *Rhizoctonia tolerance1*. Although it is more likely that the tolerance phenotype resulted from a single EMS-induced point mutation or small deletion (Pastink et al. 1991; Strader et al. 2004), the possibility that the phenotype results from two independent, closely linked mutations cannot be ruled out at this time. Alternatively, a second mutation(s) might condition the expression of the tolerance locus, resulting in the differences seen in the degree of tolerance in the BC_2F_2 families. The disease symptoms displayed in F_1 progeny of a cross between tolerant Scarlet-Rz1 and a susceptible BC_1F_3 generally supported the single-gene model. Plant-to-plant variation in pathogen response was more obvious among the small number of available F_1 progeny of Scarlet-Rz1, so that means of genotypes were not significantly ($P > 0.05$) different. Clearly, larger numbers of segregating and F_1 progeny are needed to determine mode of inheritance of *Rot1*. Eventually, tolerance in Scarlet-Rz1 will be mapped using molecular markers; a linked marker will greatly facilitate deployment of the *Rot1* locus.

The molecular basis for a dominant or co-dominant phenotype arising from a single-nucleotide change might involve an altered protein that confers constitutive tolerance to *Rhizoctonia*. Such mutations have been described for the salicylate-mediated defense pathway in *Arabidopsis* (Rate et al. 1999; Grant et al. 2003; Zhou et al. 2008) and for jasmonate-mediated defense against *Botrytis cinerea* (Bonaventure et al. 2007). Mutations in a regulatory

element that result in loss of negative regulation of a defense component, or in constitutive activation of a positive regulator, are possible.

We noted that roots of challenged Scarlet-Rz1 were slightly, but significantly ($P < 0.05$) larger than those of wild-type Scarlet in four experiments, indicating an inherent difference in root mass between the two genetic lines. The stimulation of crown root development at moderate inoculum densities of *Rhizoctonia* occasionally has been observed in greenhouse assays (Schroeder and Paulitz 2008); the physiological basis underlying this response is not yet known. In the presence of severe pathogen challenge, seedlings of Scarlet-Rz1 displayed more robust root growth than wild type, which likely accounts for its better shoot growth. The role of phytohormones (Adie et al. 2007; Vijayan et al. 1998) in this characteristic remains to be explored.

Genetic resistance to *R. solani* in dicots (Keinath and Farnham 1997; Scholten et al. 2001; Bradley et al. 2005) and monocots (Green et al. 1999; Li et al. 1995; Pinson et al. 2005; Sharma et al. 2009) is quantitative. Rice varieties having resistance to *R. solani*, the causal agent of sheath blight of rice, displayed improved plant height and heading date (Sharma et al. 2009), and yield (Tang et al. 2007). Rice cultivars displaying insensitivity to pathogen-derived tox-S were less susceptible to *R. solani* (Brooks 2007), indicating that toxins can have a role in host–*Rhizoctonia* interactions. The production of toxins by the isolates used in the study has not been determined. In specific cases, transgenes conferred protection against *R. solani* (e.g., Lorito et al. 1998; Punja 2001; Almasia et al. 2008). Although transgenes can be effective in reducing disease symptoms and seedling mortality, their impact on yield generally has not been determined, and lack of consumer acceptance of "genetically modified" products is a major economic barrier to commercial development of transgenic crops at this time.

Rhizoctonia tolerance in Scarlet-Rz1 was quantitative rather than qualitative, but in advanced generations, disease symptoms, including reductions in total root length, numbers of root tips (data not shown) and root or seedling weight, were almost completely alleviated. Real-time PCR quantification of pathogens and microscopy-based studies will be needed to determine whether pathogen ingress and spread is altered in Scarlet-Rz1. Tolerance appeared to be localized to roots, as Scarlet-Rz1 did not exhibit tolerance to stripe rust (X. Chen, unpublished data). Experiments are in progress to determine whether Scarlet-Rz1 is tolerant to *Pythium*, *Gaeumannomyces graminis* var. *tritici, Fusarium culmorum* and other necrotrophic soilborne pathogens, and whether greenhouse tolerance is correlated to improved seedling survival and yield.

Protection against *Rhizoctonia solani* or *R. oryzae* was not available to wheat breeders prior to the generation of Scarlet-Rz1. Genetic variants, such as Scarlet-Rz1, that are produced by mutagens offer acceptable alternatives to transgenic plants; their planting, distribution and market are not subjected to regulatory or societal constraints. Gene silencing observed with high levels of transgene expression in plants, including wheat (Alvarez et al. 2000), is not expected to occur with EMS-induced mutations. As Scarlet-Rz1 plants appear to be indistinguishable from wild type in the greenhouse (unpublished data), transmission of visible undesirable traits is not anticipated. The performance of the trait in other genetic backgrounds currently is being examined. Availability of non-recessive, non-transgene tolerance in an adapted wheat variety will make the trait readily deployable in wheat breeding programs.

Acknowledgments We are grateful to Jamie Baley, Kurtis Schroeder, Lucia Strader and Xianming Chen for expert assistance, and to Kim Garland Campbell and Scot Hulbert for critical reading of the manuscript. This research was supported by Washington Wheat Commission Project Numbers 3019-5850 (C.S., K.K., P.O.) and 3061-3564 (P.O., T.P.) and by USDA-ARS Project Numbers 5248-22000-012-00D (P.O., T.P.) and 5348-21220-003-00D (C·S.). References to a company and/or product by the USDA are only for the purposes of information and do not imply approval or recommendation of the product to the exclusion of others that may also be suitable.

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