Tissue Culture and Genetic Transformation of Hexaploid Wheat for Stripe Rust Resistance

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Authorization to Submit Thesis

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Abstract

Wheat stripe rust, caused by *Puccinia striiformis* f.sp *tritici* (*Pst*), can be a highly destructive disease, resulting in significant yield losses in the Pacific Northwest in epidemic years. Brundage, a top-quality, high-yielding, soft white winter wheat variety has been superseded in the last five years due to its susceptibility to the disease. Because of climate conditions in this region, infection by *Pst* is common. Therefore, introgression of one or more *Pst* resistance genes into regionally adapted genetic backgrounds is highly desirable. The goals of the present study were, firstly, to establish a tissue culture and genetic transformation system for two regionally adapted varieties: Brundage and UI Platinum. Secondly, to use genetic transformation to improve stripe rust resistance in Brundage. Six wheat varieties were initially tested for the tissue culture response of immature embryos in order to determine their regenerability into whole plants. The results indicated that there were two related groups in regard to regeneration; Fielder, Florida 303, and UI Platinum had similar but higher regeneration capacities than Florida 301, Florida 302, and Brundage. Fielder and UI Platinum are spring varieties, Florida 301 and 303 are facultative, and Florida 302 and Brundage are winter types. The shoot regeneration rate mean was highest for Fielder at 51.72% and lowest for Brundage at 9.90%. Five of aforementioned genotypes, excepting UI Platinum, were used in biolistic bombardment with the *Yr28* gene, which is derived from *Aegilops tauschii* and confers all-stage resistance to stripe rust. There was no significant difference in the transformation rate of each genotype. The Fielder genotype had the highest mean transformation rate at 0.43% with a total of four transformed plants produced from 1,009 embryogenic calli, and Florida 301 had the lowest mean transformation rate at 0.13%, with one transformed plant produced from 952 embryogenic calli. However, the most successful replicate, KJ3, was completed with the Brundage genotype and had a transformation rate of 1.54% with three transformed plants produced from 195 embryogenic calli. Fifteen of the 33 replicates completed produced no plants whatsoever, and eight replicates produced plants, but those plants did not test positive for the for the *Yr28* transgene. In total, 24 transformed plants were produced, 11 of which were from the Brundage genetic background, four from Fielder and Florida 302 respectively, three from Florida 301, and one from Florida 303. Ten of the T_0 transformed plants showed distinctively resistant phenotypes, six of which were from the Brundage background. Lines carried to the T_1 generation displayed genetic segregation for the *Yr28* transgene. Some T_1 plants were associated with stripe rust resistance. All wheat genotypes selected for transformation were confirmed to be susceptible to the currently prevalent *Pst* races. The insertion of *Yr28,* or other *Pst*-resistance genes, is highly desirable to impart disease resistance in wheat that is otherwise limited in this regard. The current study illustrated that unexploited genes from wild wheat relatives can be harnessed for wheat improvement.

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Dedication

This work is dedicated to my parents and my dear friends for supporting me while I have pursued a number of life paths.

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Chapter 1: Wheat Tissue Culture and Genetic Transformation

Importance of Wheat

Wheat, due to its richness in carbohydrates and proteins, is an important source of nutrition for humankind. Wheat is adaptable to various moderate climates and is therefore grown nearly globally. For reasons of food security, cereal production must increase concomitant with growing populations, which includes an increase in wheat production (Eissa et al., 2017). In regard to land mass, wheat production has fluctuated, but with regard to tonnage it is projected to reach 860 million metric tons by 2030 (Sparks & Jones, 2004).

Bread wheat (*Triticum aestivum*) resulted from the crossing of the cultivated emmer (*Triticum turgidum* spp. *dicoccum)* with *Aegilops tauschii* in the Caspian plains region approximately 8,000 years ago. Human cultivation and selection gave rise to the predecessors of our elite varieties. In the past century, scientific practices in breeding have significantly improved wheat growth and yield in the field. Selective breeding based on Mendel's genetic models farther helped to improve productivity and consistency – crucial factors in wheat development. The "green revolution" of the 1960's aided in refining wheat with the introgression of dwarfing alleles and other desirable alleles. Mendel's principles are now giving rise to molecular breeding based on the ability to distinguish and interpret DNA. From these understandings, the ability to transform plants with foreign genes has become a reality (Sparks & Jones, 2004). Since 2000, tissue culture and gene manipulation have been powerful tools in advancing wheat genetics and breeding.

Tissue Culture of Wheat

Plant tissue culture is based on cellular totipotency which describes the process of callus induction, and subsequent shoot and root regeneration, and ultimately the genesis of fertile plants, from an isolated tissue explant of a donor plant. Plant tissue culture is critical for success of genetic transformation and other crop improvement techniques including interspecies crosses and induced mutation breeding (Kumar et al., 2017). Some plant species or genotypes are tissue culture-prone, which means it is easy to recover regenerated plants from them, but some plant species or genotypes are recalcitrant to tissue culture (Sears & Deckard, 1982). Wheat has historically been difficult to establish and maintain in tissue culture. This difficulty had made it one of the later crops to be transformed (Vasil et al. 1992; Sparks & Jones, 2004).

Explant Types

Wheat as an explant is limited, in that the tissue for transformation, particularly particle bombardment, is generally limited to immature embryos and the resultant embryogenic calli from

them. These tissues have limited viability of just a few days. Additionally, variability exists in the tissue culture response of many wheat genotypes, and thus responsive varieties such as Bobwhite are typically used. Many of the model wheats used for transformation are agronomically unacceptable, which is a drawback to creating valuable varieties. The alternative techniques in traditional breeding for gene-transfer can take many generations (Sparks & Jones, 2004). Calli can be formed from protoplasts or scutella of immature embryos (Chowdhury et al., 1994) or other explants such as leaf segments and immature inflorescences (Khan et al., 2015). Mature embryos from dry seeds have also been used but without considerable success, although mature embryo culture has been explored and may become a more viable option with appropriate techniques (Alikina et al., 2016; Khan et al., 2015; Kumar et al., 2017). Other marginally successful options for callus cultures include anthers, leaf segments, immature inflorescence, coleoptiles, shoot tips and microspores (Khan et al., 2015; Kumar et al., 2017).

General Process for Tissue Culture of Wheat

Wheat tissue culture with immature embryos is often conducted with a fairly basic two-step protocol that includes four-week cultivation of explants in the dark on media containing the growth regulator 2,4-D or another auxin source, followed by plant differentiation in the light on media either void of growth regulators or with different concentrations of cytokinin and auxin. Kumar et al. (2017) posits that a third root-inducing media composition is more successful at regenerating whole plants. Similar to preparation of embryos for bombardment, the immature seeds are surface sterilized. The embryos are then removed in an aseptic environment and placed on a callus induction medium with scutella up. Results indicate that variation of media compositions can promote better responses based on genotype or can be optimized to suit a variety of genotypes (Alikina et al., 2016; Kumar et al., 2017)

Reportedly successful media compositions varied greatly. Generally, a callus induction medium contained an auxin source combined with essential vitamins and minerals. Regeneration media compositions vary more widely than those for callus induction, but in general featured an auxin source at a lower level than that of the callus induction media, a cytokinin source, and often an inducing agent such as a sulfate. Auxins are known to promote cell division and growth with 2,4-D or Dicambia being common choices, whereas cytokinins enhance cell division and include zeatin and benzyl aminopurine (Khan et al., 2015). A root-promoting medium was generally lacking any plant growth regulators. Kumar et al. (2017) reported their best success with callus induction media using picloram as the auxin, though they noted that fewer precocious sprouts occurred with 2,4-D, and as a chemical was a highly stable choice. Their most successful regeneration medium consisted of low levels of 2,4-D in combination with zeatin as the cytokinin source and $CuSO_4$ which acted as a stress-

inducing agent known to promote shoots. Finally, a rooting media consisting of half the level of MS salts but void of any growth regulators was used to develop roots of regenerated plantlets (Kumar et al., 2017).

Data regarding tissue culture was generally gathered in a two-step manner like the steps of media compositions. Data usually consisted of callus and differential measurements. First the callus induction score was composed of the number of embryos forming embryogenic calluses divided by the number of embryos plated prior to transfer to regeneration medium. Secondly, evaluation of regeneration capacity was calculated as the number of plants regenerated over the number of calluses plated (Zhang et al., 2015). Another measure of calli sometimes employed was that of growth rate via embryo weight at 12 to 30-day intervals. The formation of calli does not necessarily equate to regeneration. Calli need to develop into somatic embryogenic states characterized by green regions that then produce leaf-like tissue and shoots. If transfer of these totipotent calli to a media lacking 2,4-D prior to shoot formation was undertaken, root development occurred but plant regeneration was incomplete (Sears & Deckard, 1982).

Other factors play a role in plant regeneration through tissue culture beyond the genotype, which is discussed briefly later. In experimentation carried out by Rasco-Gaunt et al. (2001) 11 culture variables were tested. Five of those variables were shown to be important for somatic embryogenesis: appropriate embryo size, sucrose content of the induction medium, 2,4-D concentration of the induction medium, silver nitrate in callus induction and regeneration media, and a weak connection to the type of sealing film utilized (Rasco-Gaunt et al., 2001). Other testing by Kumar et al. in 2017 considered explant sterilization technique, callus induction media composition, tissue regeneration and subsequent root induction via media compositions, and also considered a hardening process for plants produced from those experiments (Kumar et al., 2017).

The size of the embryos used as the source of scutella has been widely noted as being important for successful tissue culture endeavors. For greatest success, Pellegrineschi et al. (2002) recommended that donor plants should be stress-free and have an embryo length of 1 mm, which corresponded to a maturity of the embryo appropriate for cell culture. This is a fairly typical size, as many other researchers recommend between 0.5 to 1.5 mm in length (Alikina et al., 2016; Khan et al., 2015; Kumar et al., 2017; Sparks & Jones, 2009; Zhang et al., 2015)

Genotype Dependence

Different wheat genotypes have varying ability to regenerate in tissue culture, and this is true across genotypes in all major cereal crops. Chromosomal studies of wheat have drawn links to chromosomes

1A, 2A, 4B, 1D, and 4D in wheat in governing callus induction and regeneration. These myriad links point to the complexity of the tissue culture response (Khan et al., 2015).

Zhang et al. (2015) have screened an array of Chinese elite wheat varieties for tissue-culture-responsive genotypes and subsequently adjusted transformation factors in order to increase efficiencies. Culture response was measured in callus induction and subsequent plant regeneration averages. Callus induction ranged from 10% to 100% across genotypes tested with an average of 73.5%. Plant regeneration varied from 3.5% to 31% with a mean of 14.5% (Zhang et al., 2015). Kumar et al.'s most successful combination of media is purported to be genotype independent after testing with six different Indian elite varieties (Kumar et al., 2017).

As Bobwhite is a model genotype, Pellegrineschi et al. (2002) reported that of the 129 Bobwhite sister lines tested for tissue culture response, nearly all the genotypes produced somatic embryos, with only 18% failing to form embryogenic calli. Several of the genotypes had nearly 100% of embryos producing embryogenic calli. They note the necessity of identification of responsive genotypes due to their ability to enhance transformation efficiency and potentially being amenable to other protocols (Pellegrineschi et al., 2002). Other researchers have studied the relationships between agronomic traits of a wide range of spring and winter wheats to their success in tissue culture, and therefore their transformability (Dodig et al., 2008; Li et al., 2012; Varshney & Altpeter, 2002).

DNA fidelity during tissue culture is among researchers' concerns. Variation *in vitro* may arise from chemicals used, genotypes, types of explant, pathways of regeneration and methods for evaluation such as morphology, cytology and molecular studies. Chowdhury et al. used restriction fragment length polymorphism (RFLP) investigation with a variety of 15 DNA probes in regenerants versus the parental plants. Their results indicated no major variation in DNA during short-term culture. The *nor* locus probe showed some variation among cultured regenerants versus their parents of the Pavon and RH770019 varieties used, but none detected from Florida 302 and Chris. These results indicated to those researchers that regenerant variation was likely genotype dependent (Chowdhury et al., 1994).

Wheat Genetic Transformation

Traditional wheat breeding has been relatively successful for meeting the needs of a growing population. However, genes from sexually non-compatible species fail to be introduced via breeding, and genes are unable to be introduced in such a way as to express only in particular tissues. Wheat transformation can provide methods to manipulate even native genes, or those that are possible to introduce through breeding, in order to vary expression levels or even to silence them - this allows for determination of function. Transformation also has the advantage of generational speed, as the need

for backcrossing as is required in traditional breeding is unnecessary. Many factors contributed to wheat being one of the last major cereals to be transformed - early initial efforts of wheat transformation took one year or longer, largely due to poor tissue culture response, to produce fertile plants and had low efficiencies of about 0.2% (Vasil et al., 1992; Sparks & Jones, 2004).

Alteration of plant genomes is a powerful tool for research of functional genomics, ultimately leading to development and improvement of commercial varieties. For wheat, several transformation technologies have been used with varying degrees of success. Among the technologies are methods of direct DNA transfer such as microinjection, liposome mediated protoplast transformation, tissue and protoplast electroporation, and particle bombardment (Fu et al., 2000). Indirect DNA transfer can be done via *Agrobacterium*-mediated transformation (Sparks & Jones, 2009). Particle bombardment was first completed with wheat by Vasil et al. (1992), and *Agrobacterium*-mediated transformation of immature embryos was used by Cheng et al. (1997). Biolistic transformation has historically been used for wheat transformation because the technique is safe, simple, and lacks the need for genetically modified organisms, such as *Agrobacterium*. As of 2006, direct DNA delivery via particle bombardment was the standard technique for genetic modifications (Sparks & Jones, 2009; Vasil et al., 1992). Another advantage of particle bombardment is the potential for delivering multiple agronomically beneficial genes at once. Regardless of the method, obtaining plants without residual vector sequence is highly desirable.

Common practice in animal egg and embryo microinjection is the removal of all vector sequence. Though requiring less work for refinement if removal is unnecessary, the backbone sequences in microprojectile bombardment serve no direct DNA transfer purpose (Fu et al., 2000). Data show that dephosphorylation of the linear genes is also helpful for simple integration of introduced genes, and researchers hypothesized that protection of the 3’ end of the fragment with additional base pairs could further increase this efficiency. This work has the potential to help with the inclusion of genes from other varieties of wheat or related species through transformation of wheat in the future. Additionally, regulatory bodies require gene integration free of vector and backbone sequences for field trials and commercial release (Tassy et al., 2014).

When completing plant transformation, many independently transformed lines must be produced in order to select those for which the gene of interest is highly expressed, or thoroughly silenced, as these are the most useful for breeding purposes. Expression can vary based on several factors including the location in the genome where the foreign gene has integrated. Increasing production can be accomplished by increasing transformation efficiency and/or decreasing the time required for plant production. Increasing transformation efficiencies has been reportedly difficult due to a lack of

understanding of all the factors that contribute to stable transformation (Altpeter et al., 1996). Although a relatively simple process to accomplish, bombardment can result in multiple copies of the introduced gene into the genome at a single locus which has been shown to contribute to gene silencing. Simple integration patterns are crucial to ensure predictable, faithful transmission to future generations and reliable expression. Biolistic transgenic events reportedly often result in five to fifteen copies per plant (Sparks & Jones, 2004). Simple integration can be a difficult thing to accomplish when the efficiencies of biolistic transformation are so low to begin with (Yao et al., 2006). Fu et al. (2000) completed experimentation with backboneless linear constructs with rice. This experimentation was done because of findings of plasmid multimerization and plasmid-plasmid recombination which is hypothesized to increase the complexity of the integration patterns of transgenes. Additionally, people were concerned that new replicons, comprising bacterial origins of replication paired with plant DNA may escape into the environment, or that bacterial antibiotic resistance could be expressed in plants (Fu et al., 2000). Research has shown that integrated vector DNA has been detected in plants produced via *Agrobacterium*-mediated transformation. Whole plasmids are required for *Agrobacterium*-mediated transformation techniques of cointegration, where *vir* genes are linked to the T-DNA. Up to 75% of transformants produced by Kononov et al. (1997) showed vector sequence (Fu et al., 2000). Regardless of the DNA delivery mechanism, vector sequences may exert negative recombinant effects in host genomes. Kohli et al. (1998) proposed that transgene integration from a plasmid occurs as a two-phase action. First, the plasmid is broken in a random location and linearized, then concatemerized with other linear constructs. These concatemers are then integrated into host genomic DNA, sometimes with that host's DNA fragments between. This action results in high copy numbers. Methylation of backbone sequences, due to high frequency of unmethylated CpG dinucleotides common to bacterial sequences, continues downstream into the promotor region of the transgene, ultimately leading to gene silencing (Tassy et al., 2014).

In terms of introducing multiple genes at once via particle bombardment experiments have been completed with simultaneous delivery of different gene constructs. Fu et al. (2000) found that particle bombardment with separate, but co-precipitated DNA, resulted in 100% co-transformation of the genes and 88% co-expression of the two genes. These data indicated that the genes cointegrated at the same locus and are also indicative of the adaptability of particle bombardment for the creation of vector-sequence-lacking plants with more than one introduced gene. The benefits of bombardment include the ability to integrate larger transgenes, multiple constructs at a time and, historically, has been applied to a greater number of genotypes (Tassy et al., 2014).

One of the disadvantages of other direct DNA transformation methods used historically, other than particle bombardment, is the requirement for protoplasts or cell suspension cultures – which are difficult to obtain for some plant species and difficult to regenerate into whole plants for wheat. Other methods suffer from the lack of regeneration ability from other tissues, such as leaf explants. Extended cell-culturing requirements of protoplasts or cell-suspension cultures may also influence the development of additional mutations or allow for greater chances of contamination (Fu et al., 2000; Khan et al., 2015). Indirect DNA transfer is also an imperfect system. Although *Agrobacterium* transformation was believed absent of foreign sequences, many researchers have found vector sequences, random integration of bacterial sequences, and bacterial transposons to be present as recently as 2012 (Tassy et al., 2014). *Agrobacterium*-mediated transformation of wheat via immature embryos, and other explants, of various genotypes has been refined since its first reported use in 1997, however. Different strains of the bacterium have resulted in greater transformation efficiencies, some significantly better than those reported of particle bombardment (Li et al., 2012).

Revising Biolistic Transformation in Wheat

Biolistic bombardment is based on the use of the gene gun, which has changed forms since its advent in the 1980's, but whose principle operation remains the same. In simple terms, DNA-coated microscopic particles are propelled by helium gas within a vacuum chamber to penetrate target cells. Many researchers now use the Bio-Rad PDS-100/HE particle gun due to its relative simplicity of operation. Many factors have the potential to influence the effect of biolistic bombardment.

The process is now largely standardized; however, a range of parameters are considered. The variables to consider are microcarrier type (i.e. gold, silver or tungsten), microcarrier size, the settings of the machine that include rupture-disk pressure (which affects velocity of the microcarriers), target cell distance (which affects microcarriers' penetration of the target cells), vacuum pressure (which reduces air resistance thereby maintaining microcarrier velocity), target cell type (which is plant-dependent based on response to tissue-culture), and microcarrier preparation (i.e., DNA-coating protocols) (Sparks & Jones, 2009).

A protocol developed by researchers Caroline A. Sparks and Huw D. Jones (2009) established an efficient method for transformation that uses the Bio-Rad device to deliver DNA-coated gold micro-particles to bombard freshly isolated immature embryos. High-quality immature embryos and induced calluses were obtained in the following steps. First, spring wheat was regularly sown in greenhouses on a 14-day cycle, and these plants were under regular watering and pest control schedules. Pesticide treatment close to spike harvest was reported to have a negative effect on the recovery of embryos post-bombardment (Altpeter et al., 1996). Appropriate maturity of young embryos is vital to the

success of biolistic transformation. Sparks & Jones (2009) called for stripping donor plants down to the five strongest tillers when they were five to six weeks of age. Spikes from donor plants were collected at 12 to 16 days post anthesis when the immature embryos were translucent and between 0.5 to 1.5 mm in length. Second, seeds that were the source of immature embryos were separated from the spikes, surface sterilized with a 70% ethanol solution and gentle shaking for three to five minutes, and a 10% bleach solution for 10 minutes followed by liberal rinsing and draining with sterile distilled water for several replications and were thereby ready for excision. Third, excision was performed by liberating the embryos from the seeds, and in so doing removing the embryos' axils to prevent precocious germination of meristematic tissue, which precludes the desired callus formation of the scutella of the embryos. Fourth, isolated embryos were placed scutellum-side facing up on callus induction medium, which consisted of MS salts, Agargel, 2,4-D and $AgNO_3$. Thirty embryos were gathered in the center of a 9 cm petri dish so that they were all in the target area for future bombardment. These plates were then cultured for one to two days at 22 to 23°C to initiate callus growth (Sparks & Jones, 2009).

Immature embryos and their induced calluses were then subject to biolistic bombardment, which involved the following key steps. First, prior to bombardment, 0.6 µm gold particles were washed in ethanol, fully suspended in sterile distilled water (20 mg gold in 1 mL H_2O) and divided into 50 µL aliquots. Aliquots were stored at -20°C and defrosted prior to DNA-coating procedures. Each aliquot was purported to be good for approximately 10 to 12 shots but could be scaled based on how many were needed for the number of samples excised. Second, up to 5 µL plasmid DNA (1 µg/µL) was added to the 50 µL gold aliquot and mixed via vortex. Then 50 µL 2.5 M calcium chloride and 20 µL 0.1 M spermidine was mixed then was added to the gold/DNA solution and immediately mixed by vortexing. The researchers then centrifuged the mixture at 12,000 rpm for three to five seconds. After removing the supernatant, the pellet was resuspended with 150 µL 100% ethanol. Vortexing was completed to mix the DNA-coated gold fully in the ethanol, then the tube was centrifuged again, and again the supernatant was discarded. Then the gold/DNA was resuspended again, including vortexing, with a volume of 85 µL of 100% ethanol. If this solution was not used immediately, the tube was sealed and stored on ice. Additional vortexing was done immediately prior to use of the solution by placement on carrier discs. A 5 µL volume of the gold/DNA suspension ware placed on the center of a carrier disc (this resulted in 10 µg gold per shot) and allowed to dry, thus leaving a thin film of gold visible on the disc surface. The carrier discs were used as soon as possible after dry. Third, the PDS-1000/HE device was used to deliver the gold/DNA into the pre-cultured embryos described previously. The device was operated with a 2.5 cm gap between rupture disc and microcarrier disc, a 5.5 cm target distance between the stopping screen and the embryos, and a 0.8 cm distance between

the microcarrier disc and the stopping screen. The chamber was used with 26" Hg vacuum (-8.6 bar), a 5.0 vacuum flow rate and a 4.5 vacuum vent rate. The helium delivery tank was set to ~200 psi higher than the rupture disc specifications. The gene gun components were then assembled, and bombardment was carried out for each prepared plate. Bombarded samples were removed from the apparatus, and the procedure could be repeated for as many samples as were prepared. All components of the device, including discs to be used, were sterilized prior to procedures by dipping in or spraying with 100% ethanol and drying in a horizonal flow hood (Sparks & Jones, 2009).

Following bombardment, embryos were spread evenly, 10 calli per plate, on induction medium and then cultured at 22°C to 23°C for three to four weeks in the dark. Because the plasmid DNA included a selectable marker gene, the treated embryos were placed in media with selectable agents, such as herbicide or antibiotic that can be used for selection of transformed cells during the following tissue culture. Only plantlets surviving selection stages were advanced to soil and farther analysis. Responsive calli were then transferred to regeneration medium, consisting of the same components as callus induction medium with the addition of zeatin and $CuSO_4$, and the subtraction of $AgNO_3$, cultured at the same temperature, but now in the light, for three to four more weeks. After this medium, calli that showed vital shoot development were distributed, to prevent over-crowding, onto selection media that contained the same components as regeneration medium minus $CuSO_4$. Keeping calli in one piece, rather than breaking them up, maintains the clonal identity of those calli. Healthy plantlets were then selected to move to the next round of selection with the same selection media as above, but in larger vessels that allowed for shoot, root, and leaf expansion. Plantlets originating from a single callus were labeled in such a way as to distinguish possible clones. Following the second round of selection in these vessels, selection was apparent due to tissue death and bleaching, and surviving plantlets were transferred to soil following a rinse of root tissues to remove remaining medium. These plants were placed in a contained area for 1 to 2 weeks in order to produce a humid environment in which they could establish more effectively. Plant leaves produced in tissue culture lack a waxy cuticle initially, and therefore a humid environment aids in preventing desiccation while the cuticle forms. Plants having survived to this stage were then sampled for DNA extraction from a leaf, and subsequent PCR testing to determine their genetically modified status – that is, whether they had foreign DNA. Positive transgenic plants were moved into larger containers and moved into an appropriate genetically-modified-organism-suitable containment area. Transformed plants rarely showed significant morphological differences compared to their control counterparts. Wheat transformation using the above protocol took at least 70 days from isolating an immature embryo to obtaining a positive wheat T_0 seedling, and the transformation efficiency was about 5% in the 30 genotypes it was applied to (Sparks & Jones, 2009).

A protocol by Altpeter et al. (1996) was developed to speed transformed plant production to a timeline of 56 to 66 days, which could allow researchers to produce third generation homozygous progeny within a year using Bobwhite as the genotype. Spikes were used fresh or stored at 4°C for up to 5 days prior to seed removal for embryo excision. Seed sterilization and embryo size were similar to the protocol above. Embryos of a length 1.0 to 1.5mm responded best, which is on the larger end of the scale discussed previously. In contrast to Sparks & Jones (2009), 10 µL of DNA was used for gold coating rather than 5 µL as they utilized. Approximately 48 hours after bombardment, transformation was assured. In this experimental data, media differed somewhat to those of the previous protocol. The base MS medium consisted of MS salts, 2,4-D, sucrose and glutamine and Gelrite. The addition of casein hydrolysate resulted in MS+ medium. Osmotic medium was MS+ medium with the addition of mannitol and sorbitol. Shoot-generation medium consisted of MS medium with the addition of zeatin and herbicide selection. Shoot-elongation medium was half-strength MS salts, sucrose and herbicide, but no hormones. Their incubation temperatures during callus formation, osmotic treatment (pre-culturing of excised embryos was optimized for 4 to 6 hours on osmotic medium prior to bombardment and a 16 hour post-bombardment culture on the same medium) and regeneration was conducted at 24°C to 27°C which is all relatively concurrent to the previous protocol. Additionally, embryos were precultured for 5 to 7 days, versus 1 to 2 days by Sparks and Jones (2009), in the dark prior to bombardment, on MS medium. During shoot generation, a period of 8 to 10 days with 16-hour photoperiods was utilized which is markedly shorter than the 14 plus days previously mentioned. That 16-hour photoperiod was also used during shoot elongation for 28 days with a media replacement at 14 days. Altpeter et al.'s (1996) most successful transformations were reported utilizing 30 µg of gold particles per bombardment – differing from 10 µg in the former protocol - though they tested up to 100 µg. The higher concentration of 100 µg resulted in 53% more reporter gene activity, but regeneration was compromised. The optimal length of dark incubation was 20 days, rather than 28 by Sparks & Jones (2009). Other treatments tested, such as selection immediately post-bombardment, low light intensity during callus development, or longer periods of dark treatment, were deemed ineffective. Thirty-two transformed, healthy plants were reported from 2,100 embryos. The reported efficiency was up to 2%. The addition of herbicide in medium, even at low concentrations, reportedly slowed shoot elongation in comparison to control calli. Various herbicides were tested for selection. None of the options tested eliminated escapes (plants that lacked the DNA of interest) but plants were more vigorous when biolaphos was used over other herbicide selection agents (Altpeter et al., 1996).

Other studies have aimed to increase the transformation efficiency of wheat. Tian et al. (2018) presented a protocol that, in their lab, resulted in a reported transformation efficiency of 5% to 10%

across many genotypes. Their protocol differs from others in their use of tungsten rather than gold particles for bombardment, but they use the traditional calcium chloride and spermidine technique for DNA precipitation. As in other publications they induced calli prior to bombardment; the induction incubation used was seven to twelve days which is longer than previous protocols discussed. Post-bombardment they started selection of transformed calli after two to four days of recovery, versus the 16-hour recovery period followed by up to two weeks of unselected callus growth used by other researchers. They found that regardless of co-bombarding with separate constructs on different DNA fragments or having the genes on the same DNA segment, that the integration of the genes often occurred as a single event 90% of the time. This finding is consistent with other publications. Regardless of the integration patterns of the genes, they hoped to produce marker-free plants through segregation in progeny (Tian et al., 2018).

In addition, Ismagul et al. (2018) developed yet another protocol for high-throughput wheat with simple insertion patterns. They note, as others have, that the calcium chloride and spermidine DNA-coating method can be inherently variable and difficult to reproduce. They therefore chose a technique that utilized polyethylene glycol and magnesium chloride to coat gold particles with minimal amounts of cassette DNA – with the intention of achieving single gene insertions. Through this work they reported a single-gene-copy rate of 38.2% of 1,538 transformed plants examined. The overall transformation efficiencies ranged from 3.1% to 20.3% across experiments, with less than 5% only occurring in three of 19 experiments. Transient reporter gene expression with PEG/$MgCl_2$, versus spermidine/$CaCl_2$, was reported to be 1.2x to 1.6x higher with an advantage that the DNA and gold preparations were stable at room temperature up to three hours, and additionally can be stored longer at -20°C. Immature embryos were cultured for seven to 14 days prior to performing bombardment of the calli that formed. Standard set-up for this protocol was a 900 psi rupture pressure, 15 mm flight distance and 60 mm target distance, which differs from that of Sparks & Jones (Ismagul et al., 2018).

To understand transgene multimerization that can lead to high copy numbers Tassy et al. (2014) compared whether simple integration was more likely with either digestion and recovery of the gene cassette or PCR amplification from their construct; they also experimented with dephosphorylating the ends of gene cassettes. This protocol called for 900 psi rupture discs and a target distance of 7 cm. They used a preparation that utilized spermidine and calcium chloride to precipitate DNA on to 0.6 μm gold particles, and then 10 μL DNA/gold suspension per shot, versus the 5 μL of Sparks & Jones' protocol, and DNA concentration of 500 ng/μL for the tested gene of all experiments, whereas Sparks & Jones utilized 1 μg/μL (2x as much). The efficiency of transformation was 2.5%. This figure did

not differ significantly (ranging 2 to 2.7%) based on the construct architecture (that is circular, linear, dephosphorylated, or PCR amplified). Concentration experiments for insertion complexity ranged from 1 ng/µL to 500 ng/µL DNA of either linear cassettes, dephosphorylated or PCR-amplified genes. For PCR-amplified genes there was a significant increase, 46%, in simple insertions with 1 ng/µL DNA versus 8% with 500 ng/µL. Linear cassettes versus those dephosphorylated showed insignificant differences in simple events (50% to 59%, or 56% to 60% respectively). This data was obtained without compromise in average transformation efficiency, indicating much smaller amounts of DNA can be used per shot. In total, 7,687 immature embryos were bombarded to produce 292 T_0 plants. Only samples from embryos bombarded with the whole plasmids showed probe hybridization with backbone sequence, which was to be expected. This research showed that dephosphorylated cassettes produced more simple events with stable transgene expression that was heritable in Mendelian segregation ratios, indicating that concatemer formation did not occur. Also indicative of complex integration is the data that 82% of T_1 plants produced from whole plasmid bombardment presented complete expression loss. Expression for cassettes, either unmodified or dephosphorylated, was significantly higher ($\chi^2 = 4.4$; $\alpha = 0.05$) in the T_1 generation. These modifications resulted in significant improvement in the production of transgenic plants with simple integration patterns and stable expression over at least two observed generations when compared to whole-plasmid bombardment (Tassy et al., 2014).

A protocol by Yao et al. (2006) had some differences to those already reviewed, but like Tassy et al. (2014) used linear constructs. One difference from protocols such as Sparks & Jones' (2009) included culturing excised embryos for one day at 24°C prior to bombardment versus two to seven days. Yao et al. (2006) used two controls: one bombarded and one non-bombarded. For whole-plasmid gold precipitations 5 µL (the same as Sparks & Jones (2009)) was used per carrier disc, and for linear cassettes 3.5 µL was used. Results of their experimentation yielded a 51.2% regeneration of shoots from non-bombarded control cultures. Of the experimentally bombarded explants, the corresponding figure is 56.7% Of 993 scutella bombarded with the linear cassette combination, five independent lines resulted. Whole plasmid bombardment of 995 immature embryos resulted in two plants, which reinforces the hypotheses that linear gene constructs are more efficient. The researchers noted that T_0 plants often produced only one to two tillers, but T_1 plants appeared normally vigorous. The main purpose of bombarding with a gene cassette, rather than whole plasmids, was to achieve more simplified gene integration patterns and, therefore, more consistent expression. Yao et al. (2006) concluded that most integration events of linear gene cassettes involved low copy number insertions. Similar investigation by Fu et al. (2000) recognized that liberated DNA fragments were eroded in 76% of plants transformed. The sequenced individual showed five base removals from the 3' and 5'

ends, respectively. They theorized that other bands of unexpected sizes may have had more extensive erosions. However, their Southern blot analysis concluded that fewer rearrangements from the limited-linear-construct than from the linearized plasmids or whole plasmids that contained backbone sequence (Fu et al., 2000).

Other investigation by Rasco-Gaunt et al. (2001) tested four factors that could influence stable transformation of the elite varieties in their study. Of three bombardment pressures, only 650 and 900 psi produced transgenic plants. Regarding the amount of gold on carrier discs, 60 µg versus 120 µg of gold for bombardment had no statistical difference between them. The media compositions they tested also played a role in transgenic plant generation, with plants only obtained from 9% sucrose in callus induction medium and a level of 0.5 mg/L 2,4-D versus 2.0 mg/L, resulting in better recovery of transformants. These experiments allowed Rasco-Gaunt et al. (2001) to achieve a mean 10-fold increase in transformation over those achieved with the standard protocol for model varieties (Barcelo & Lazzeri, 1995) and gave them the ability to transform two varieties that previously failed to be transformed. From this experimentation, 21 of 32 procedures (66%) resulted in transgenic plants. Osmotic treatment of target tissues a few hours before and after bombardment was noted to prevent extrusion of the protoplasm, which can result in cell death (Rasco-Gaunt et al., 2001).

A report by Li et al. (2012) reviewed the circumstances in which most reviewed biolistic researchers achieved success. Per bombardment, the most effective gold load was 50 to 100 µg coated with 0.5 to 1.0 µg of DNA. Often reported was 1100 psi and a 5 cm target distance, but lower psi and the same target distance as well as greater psi and greater target distance were also used successfully. Selection and media conditions by reviewed researchers varied greatly, from concentration of the selection agent, to when it was applied for greatest reported success, to additional chemical additives to media. Even explant type can be a factor – although immature embryos or embryogenic calli are the general standard, mature embryos have also been tested with limited success (Li et al., 2012).

Genotypes Used Historically for Wheat Transformation

Tassy et al. (2004) used Bobwhite as their experimental genotype, which is by far the most commonly used genotype, reported in over 40% of the publications reviewed by Li et al. (2012). Genotypes noted as being successful with Tian et al.'s (2018) protocol beyond the traditional variety Bobwhite, which is noted as being highly amenable to tissue culture, are other spring wheat varieties: Fielder, and Forefront as well as hard winter varieties: Jagger and Everest. Tian et al. (2018) noted, however, that callus initiation time varies based on the variety. Investigation of 129 Bobwhite sister lines by Pellegrineschi et al. (2002) aimed to study not only embryogenic calli development and regeneration, but transformation efficiency and agronomic characteristics that would contribute to the best choice

for transformation. They noted that unbombarded controls were often more efficient in their formation of somatic embryos than their bombarded counterparts. Their transformation efficiency was reported as the effective number of transgenic plants obtained divided by the number of immature embryos bombarded. The genotype with the highest efficiency reported was at 48% and the lowest was at 19%. Segregation was reported at a 3:1 ratio for 500 of 600 events tested. Plants harboring multiple copies of the gene appeared to co-segregate, suggesting insertion at a single locus (Pellegrineschi et al., 2002).

Sparks & Jones' protocol involved the sowing of the wheat variety Cadenza, although it was noted that many different genotypes, up to 30, had been transformed with the protocol (Sparks & Jones, 2009). In a prior report by Jones (2005), successful varieties included Bobwhite, but also Fielder, Florida and Veery-5. Noted varieties of common use such as Bobwhite and Florida often lack desirable agronomic traits, however, despite being tissue culture responsive. Crossing into elite varieties from these "models" is often associated with linkage drag to undesirable traits (Rasco-Gaunt et al., 2001).

Ismagul et al. (2018) developed a transformation protocol using the spring variety Gladius, due to it being an elite Australian variety where the research took place. However, they noted success with other varieties such as Bobwhite, Akadaruma and others unlisted but that included durum and winter types (Ismagul et al., 2018). Yao et al. (2006) used the variety EM12 due to its reputation for creating flour with poor processing properties and their desire to create a better end-use-quality wheat (Yao et al., 2006).

For Fusarium head blight testing by Shin et al. (2008), the spring varieties Alsen, 2375, Roblin, Sumai 3, Wheaton and Bobwhite were used, with Wheaton and Roblin being hard red varieties, and the others with varying susceptibility to the disease. For their experimentation, Eissa et al. (2017) used the variety Hi-Line for further fungal disease testing with the same gene.

In tissue culture investigations, Chowdhury et al. (1994) utilized several varieties to develop embryogenic calli from immature embryos including Florida 302, Chris, Pavon and RH770019 (Chowdhury et al., 1994). Optimal regeneration ability of individual species and varieties for biolistic transformation needs further investigation (Ismagul et al., 2018).

Major Traits Targeted for Transformation in Wheat

A multitude of traits for which transformation techniques, such as those previously discussed, could be useful include yield increase, pest resistance, tolerance to environmental stressors, herbicide tolerance, plant and/or grain architecture, and nutrient composition. Commercial wheat varieties have

been produced, though not released, for herbicide tolerance via the brand name “Round-Up”. Otherwise, varieties have lack the scrutiny required for public release (Sparks & Jones, 2004).

Selectable Marker and Reporter Genes

In order to save time and money, transformation needs a reliable way to assure that the processes have worked, and that the target cells do, indeed, have foreign DNA integrated in their genome. A way to do this is to introduce a selectable marker or reporter gene that can be easily assayed. In the case of selectable marker genes, selection happens through the regeneration process, as only those cells containing DNA capable of withstanding the selection criteria live. Reporter genes, on the other hand, can be directly observed by the researcher and therefore accomplish a similar confirmation.

The first wheat plants produced via biolistic bombardment by Vasil et al. in 1992 featured insertion of the *bar* gene. Many studies in the four years following this landmark event similarly used either *gus* or *bar* genes, or a combination thereof, to refine the procedure (Li et al., 2012). The *bar* gene encodes for resistance to bialaphos, which is a tripeptide antibiotic that consists of phospinothricin, or glufosinate ammonium, which is the chemically synthesized relative (D’halluin et al., 1995). In their research, Pellegrineschi et al. used the *bar* gene in screening Bobwhite sister lines (Pellegrineschi et al., 2002).

Altpeter et al. used a plasmid that contained the *bar* gene as the selectable marker and the *gus* gene as a reporter (Altpeter et al., 1996). The *gus* gene, or *uidA*, is a reporter, in that it can be directly visualized due to non-native enzyme, ß-glucuronidase, activity that produces blue coloration in tissue where it is active in the presence of the substrate x-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). This reporter gene is remarkably easy to assay, and therefore has been used in thousands of plant experiments (Jefferson, 1989). Another effective reporter is green florescent protein – another visually assayable gene that is non-destructive and permits early detection of transformation. The *gpf* gene is derived from *Aequorea Victoria* and imparts multicellular clusters with emission of green fluorescence under UV light. Green-glowing tissues can be easily seen fourteen days after bombardment, or fewer, thereby streamlining efficient selection of transformed tissues for experimentation. Early detection reduces the number of non-transformed escapes carried to full term and thereby reducing labor and material inputs (Jordan, 2000).

The *hpt* gene is derived from *Streptomyces hygroscopicus* and it prevents death of eukaryotic cells caused by the antibiotic hygromycin B, which acts by inhibiting protein synthesis. Working to understand co-integration with separate constructs bombarded together, Ismagul et al.’s (2018) cassettes contained the *gus* gene (for transient expression experiments only), the *hpt* gene, conferring

hygromycin resistance, and a third gene-of-interest (one of 19 undisclosed wheat genes isolated at the University of Adelaide). The *hpt* and gene-of-interest cassettes were co-bombarded in large-scale experiments (19 total) consisting of 15,496 embryos. Gene-of-interest average transformation efficiency was reported as 7.4%, whereas *hpt* was 9.9%. Single-copy events ranged in efficiency from 16.1% to 73.5%. Co-transformation frequency average was reported as 74.7%. The researchers attributed high levels of co-transformation to impacts of the various genes-of-interest on co-integration and plant regeneration (Ismagul et al., 2018). A few other studies have used marker genes such as *hpt*. Some early experiments claimed transformation efficiencies ranging from 0.2-5.5% (Li et al., 2012). Ortiz et al. (1996) claimed a higher efficiency with *hpt* over *bar* in their experimentation at 5.5% versus 2.6% respectively.

Tassy et al. (2014) used two base constructs in their experimentation: one containing the *bar* gene under the maize ubiquitin promotor, the other with the *pmi* gene under the same control. The *pmi* gene allows transformed plants to use mannose as a carbon source. These constructs were linearized and reduced via enzyme digestion to remove backbone sequences. One experimental set of constructs was dephosphorylated after recovery and in order to test purification of cassettes over PCR amplification, the *pmi* cassette was also amplified for experimental purposes. To study heritability, transformed plants of each experimental construct of the *bar* gene were selected (whole-plasmid, cassette, and dephosphorylated cassette). All plants indicated transmission of the *bar* gene via PCR testing which was indicative of single-locus Mendelian 3:1 segregation. Only 13% of their transgenic plants with this gene did not segregate in this fashion, which is an improvement over other researchers (Tassy et al., 2014).

Because the *pmi* gene allows only transformed cells to live using an alternative carbon source, whereas other selectable agents kill untransformed cells, it is considered a positive selectable marker. Positive selectable markers can be environmentally desirable because they reduce the chance of escape of transformed plants in the environment. Gadaleta et al. (2006) have also used constructs with the *bar* and *pmi* genes, separately, but in durum wheat Svevo rather than bread wheat. They reported a higher transformation efficiency with *pmi* over *bar* at 1.14% versus 0.99% (Gadaletaet al., 2006).

Genes for Trait Improvement

A great deal of research has involved increasing the transformation efficiencies of wheat, most of which have used the marker and reporter genes discussed previously. However, the ultimate goal of transformation is to improve valuable plant traits in such a way that is limited by traditional breeding. To date, there have been a number of efforts to improve quality and endurance characteristics of wheat. One of the most famous is that of “Roundup Ready” wheat which could allow farmers to

apply glyphosate as a broad-spectrum weed control chemical rather than the long-held methods of applying several narrow-spectrum chemicals. The *epsps* gene is responsible for plant tolerance to glyphosate, which has the effect of blocking plant production of aromatic amino acids. The gene used to transform wheat for this tolerance was derived from a strain of *Agrobacterium* and has also been introduced to cotton and soybean. This gene has been introduced via *Agrobacterium*-mediated transformation. One transformant of the cultivar Bobwhite was shown to have a simple, single-gene insertion lacking any extraneous backbone sequence and was thereafter used in back-crossing to introduce the gene to desirable lines (Mergoum et al., 2010; Zhou et al., 2003).

Other genes targeted for trait improvement involve wheat quality, stress tolerance, or disease resistance. Researchers, such as Yao et al., (2006) have done work to eliminate the superfluous backbone sequence of vectors thereby bombarding with "clean" exogenous genes. One of these genes was the *1Ax1* gene which encodes for a high molecular weight glutenin subunit. They thereby introduced a gene that improves the agronomic traits, in this case that of grain quality, in a cereal crop which had been unreported at the time. The experiment used the whole plasmids as a control for the liberated gene cassettes. Their method is based off that of Sparks & Jones (2004). Of 1010 scutella of the *1Ax1-bar* linear cassette bombardment, six independent transgenic lines resulted. The combination of *1Ax1* and *bar* plasmids (co-bombarded) resulted in three lines from 998 scutella (Yao et al., 2006)

Fahim et al. (2012) produced a hairpin RNA construct derived from a viral gene. When this construct was introduced into wheat, it produced microRNAs that conferred immunity to streak mosaic virus. Other researchers report over-expression of specific transcriptional factors can lead to wheat's increased ability to deal with abiotic stressors such as freezing and high salinity (Li et al., 2012). Zhang et al.'s (2015) transformation system has been used to produce transgenic wheat with more than 200 genes of interest as of 2015, including improved quality traits, drought and cold tolerance, and fungal disease resistance.

Fungal Resistance Genes

Fungal diseases of wheat are highly detrimental to crop productivity and require the application of chemical fungicides. Additionally, fungal strains can develop resistances to these chemical inputs. Traditional breeding, often requiring the pyramiding of several resistance genes, can take many generations. Through genetic transformation, resistant varieties can provide an alternative approach in the form of 'integrated pest management' through the integration of wide-range, durable, resistance to fungal pathogens. Plants have fungal defense mechanisms that work through complex networks of pathways that respond and limit the penetration and development of the pathogens. One pathogenesis-

related protein, chitinase, has hydrolytic activity against the fungal cell wall thereby destroying or weakening the fungal cells (Bliffeld et al., 1999).

Some researchers postulate that enforcement and maintenance of the plant response through constitutive expression of anti-microbial genes, such as chitinase, through genetic transformation could be beneficial to wheat's ability to withstand pathogen attack. This approach alone, or in tandem with glucanases and ribosome-inactivating proteins, has historically shown enhanced resistance to particular pathogens (Swords et al., 1997).

Bliffeld et al. (1999) attempted to clarify which chitinases in plant cell walls best protect plants from powdery mildew. In the attempt to protect wheat from powdery mildew caused by *Blumeria graminis* f.sp. *tritici*, a barley-seed class-II chitinase was inserted by the researchers and tested *in vivo* (Bliffeld et al., 1999). Similar proteins had shown promise as a defense against other fungi in prior researchers' experiments. Although wheat has endogenous chitinases, upregulation at the protein or enzymatic level, despite increased transcript levels, during fungal infection had yet to be been demonstrated. They therefore over-expressed the chitinase gene using the maize ubiquitin 1 constitutive promotor, paired with either a ribosome-inactivating protein gene or a glucanase gene. Both two-gene-combination cassettes, protected from cellular degradation and position effects by ~600 bp scaffold attachment region from tobacco, were co-bombarded with a vector containing the *bar* gene. This study produced 15 independent, fertile, transgenic lines from the Bobwhite parent variety in 14 experiments, with an average transformation efficiency of 0.7%. Nearly all transgenic plants were reported to have the chitinase gene. Investigation of the transgene-derived antifungal proteins was conducted via the intercellular wash, and three lines were positive for the presence of the proteins, with variable expression levels between them. Expression levels were, however, reported to be stable within the lines for the four generations tested. The intercellular wash was investigated due to the sequence's N-terminal transport signal which is meant to cause the protein to exit the cell. Disease infection was rated based on the number of *B. graminis* f.sp. *tritici* colonies present on inoculated leaves through several independent experiments. Two of the three transgenic lines tested showed significant reductions in colony formation, at 6% to 17% and 28% to 54% respectively, compared to the non-transformed controls. Additionally, colonies on controls were reported to be both larger and more highly sporulating than those on transgenic lines. Line 6, with more detectable chitinase, was three to six times more resistant compared to other transformed lines. These results indicate that accumulation of transgenic chitinase in the apoplastic region of wheat plants confer greater resistance to powdery mildew without affecting morphology. Resistance to the obligate biotrophic fungi *B. graminis* f.sp. *tritici* through over-expression of a transgene for chitinase has the potential to be an

agronomically important trait, especially considering that many race-specific resistance genes can be overcome by pathogens. Transgenes can be derived from unrelated species, which provides greater diversity of disease resistance than does traditional breeding where only closely related species may be crossed (Bliffeld et al., 1999).

With the same barley class II chitinase, Shin et al. (2008) applied its defense mechanism to Fusarium head blight (FHB). This disease primarily thrives in hot, humid growing regions and is highly detrimental to grain quality due to kernel shriveling and toxin accumulation. Breeding efforts for control of FHB have been stymied by a lack of resistance genes. Transgenes provide an additional defense mechanism. They identified 16 chitinase expressing transgenic plants. These were greenhouse tested for disease resistance using up to T_5 lines. Seven lines showed consistent resistance in two or more independent experiments and were therefore advanced to further testing. T_6 and T_7 generations were tested in field plots in 2005 and 2007. Plants were inoculated with 50 isolates and 41 isolates of Fusarium in the respective years. Inoculation occurred at anthesis and again three days thereafter. Disease severity was evaluated 21 days post-inoculation, with 20 primary tiller spikes selected randomly and rated based on symptomatic spikelets per spike. Visual inspection after harvest for shriveled kernels was completed and toxins were measured. Two of the seven lines showed significant improvement of disease resistance over the Bobwhite control by up to 39% reduction in colony frequency (Shin et al., 2008).

Eissa et al. (2017) took the barley class II chitinase (*chi26*) previously discussed for resistance to powdery mildew and Fusarium head blight and expanded the testing criteria to include both a greater number of fungal diseases and field environmental trials over several generations. Due to the gene's ability to hydrolyze fungal cell walls, this gene's product is a natural choice to test its function on other fungal diseases, such as rusts. Testing included resistance to leaf rust, stem rust, yellow rust and powdery mildew. Following Southern blot testing and enzyme activity assays, four T_0 lines (originally 14 selected out of 72 total transgenics) were advanced to future generations and subsequent testing after transformation with a plasmid carrying *chi26* and *bar*. Field trials were conducted in five growing seasons in a location in Egypt with suitable conditions to access disease response of the four selected lines at the T_4-T_9 generations. These independent lines showed variable transcript levels with two of the lines harboring one transgene copy and the other two carrying two copies. Ultimately various members of the families produced with the four original T_0 lines had the *chi26* gene silenced, with three of the four lines having one member each that was still active in the nine studied generations. They concluded this silencing effect may have been caused by homology-dependent silencing, tandem insertions, or interspersed genomic DNA, rather than tied to any

particular promotor or transformation method. The silencing effect may have been due to the phenomena of promotor methylation – this was supported by fluctuations in the transgene's expression in subsequent generations within families. Unlike other researchers, there seemed to be a lack of correlation with copy number of the transgene and silencing. Despite some advanced generation silencing, the degree of resistance was fairly uniform for each transgenic event, and if not silenced into the T_6 generation was likely to be stable (Eissa et al., 2017). Shin et al. (2008) developed seven transgenic lines with *chi26* demonstrating high Fusarium head blight resistance in the greenhouse; two of those lines exhibited enhanced field resistance also (Eissa et al., 2017; Shin et al., 2008).

Yellow (Stripe) Rust

Stripe rust, also known as yellow rust, is just one of many fungal diseases affecting wheat. It is caused by the pathogen *Puccinia striiformis* f.sp *tritici* (*Pst*). Though generally confined to wheat, the disease may harbor on other cereals and grasses such as barley, triticale, and brome. The disease's development depends on moisture and temperature and as a result infection is often regionalized (Murray et al., 2005). Generally, the disease is confined to cooler climates in which wheat is grown (Johnson, 1988). The disease has been reported in more than 60 countries (Chen, 2005; Liu et al., 2013).

Differentiation from other rusts is based on the color and pattern of sporulation. Stripe rust is most similar to leaf rust in this regard but is more yellow and has distinct banding patterns of sporulation versus the orange-brown and random circular colonies of leaf rust. Control can be achieved, as with other fungal pathogens, with chemical sprays but these are costly and are only cost effective when losses exceed 10%. Chemical inputs are potentially environmentally harmful. Therefore, it is desirable to have agronomically viable resistant varieties. Resistant varieties over long ranges would be especially useful because *Pst* spores can spread great distances with wind assistance. Unlike some other fungal diseases, there is, fortunately, a lack of seed transmission of the disease (Murray et al., 2005).

Host plant resistance occurs in two forms: seedling resistance and adult plant resistance, or APR. Generally, seedling resistance is seen as a gene-for-gene resistance being race specific. While this type of resistance can be pyramided, that is stacking multiple resistance genes into one plant line to increase the number of pathogens a variety can withstand, individual genes are often overcome by the development of new pathogen strains through mutation (Murray et al., 2005). This fungal mutation has historically occurred Britain (Johnson, 1988). APR is usually more durable despite later

development. The benefit of resistant varieties is delaying onset and slowing transmission of the disease epidemics to reduce overall losses rather than eradication of the disease (Murray et al., 2005).

Stripe rust was first detected in the western United States in 1915, but further investigation revealed that the disease had been present at least 23 years prior to this recognition. In the 1950's and 60's, *Pst* became a disease of importance when it caused significant yield losses in California and the Pacific Northwest. Prior to 2000, 59 *Pst* races had been named by the Cereal Disease Laboratory in Pullman, WA, 55 of which had been detected in the Pacific Northwest specifically, where conditions are favorable for the disease nearly every year. In 2000, stripe rust was reported in 25 states, the most widespread distribution up to that year and included 21 pre-existing identified races and 21 new races (Chen et al., 2002).

Various resistance genes have been historically overcome by *Pst* through mutation of the pathogen such that it may still infect a host with one or more of the genes. For example, *Yr5* having been overcome within four years of introgression in Australia, and *Yr10* having been quickly overcome in the USA after introgression into the commercial variety Moro (Johnson, 1988). All-stage resistance of wheat genes *Yr9* and *Yr17* have also been overcome by *Pst* races (Bayles et al., 2000; de Vallavieille-Pope et al., 2011; C. Zhang et al., 2019). Many of these genes originate from closely related genera such as *Triticum* or *Aegilops* (Johnson, 1988). Liu et al. (2013) note that *Aegilops tauschii*, the D-genome progenitor of hexaploid wheat, is a rich resource for wheat improvement (Liu et al., 2013). Other genes seem more durable – being race-nonspecific and possessing slow-rusting mechanisms – such as *Yr18*, which is also known as *Lr34*, that confers leaf rust resistance additionally. *Yr18*/*Lr34* displays a disease-lowering or delaying effect of the disease in a plant's maturity that is typical of adult plant resistance (Singh et al., 2000). Other genes from *Ae. tauschii* have been mapped for stripe rust resistance (Liu et al., 2013; Singh et al., 2000; C. Zhang et al., 2019).

Researchers have mapped gene *Yr28* to *Ae. tauschii* chromosome 4DS, although expression patterns under strict temperature control were yet to be established at the time (Singh et al., 2000). More recently, dominant resistance gene *YrAS2388* was mapped to chromosome 4DS from the *Ae. Tauschii* subspecies *strangulata*. The gene likely predates differentiation of *Ae. tauschii* subspecies, however, because it can also be found in other accessions of both subspecies. All tested accessions that showed resistance to *Pst* were from the Caspian Sea region. Researchers have yet to fully determine the allelic relationships present. Through allelism testing of developed populations this relationship was further elucidated. Liu et al. (2013) went on to hypothesize that these were, in fact, the same gene having been mapped to a highly similar distal region of 4DS. The genetic background of synthetic crosses affected the level of disease resistance expression seen (Liu et al., 2013; Singh et al., 2000).

Only seven of the 80 named stripe rust resistance genes have been cloned to date, and only three of those demonstrate adult plant resistance. Zhang et al. (2019) posit that adult plant resistance and high-temperature adult plant resistance genes are more durable than race-specific ones, less likely to be overcome by specific rust races, and therefore more useful for incorporation into new wheat varieties – particularly when stacked together. They have succeeded in cloning resistance gene *YrAS2388R*, also known as *Yr28*. Although synthetic lines developed with *Ae. tauschii* can be useful breeding resources, many of the biotic and abiotic stress response genes have been shown to be repressed in the hexaploid background. A fosmid library was derived from PI 511383, a subspecies *strangulata* accession, that has shown stripe rust resistance for over 20 years in China. The library has an approximate 8-fold coverage of the genome, with 20 clones encompassing the *YrAS2388* region. Of three active genes in the region, the $NLR_{4DS\text{-}1}$ gene was a classic R (resistance) gene. The gene was validated as the gene of interest, *YrAS2388* (or *Yr28*), through various markers and other validation techniques. This gene has multiple splicing patterns, which appear to confer the ultimate stripe rust resistance of this particular gene. Despite having homologues in Chinese Spring with 86-94% cDNA identity – these lack a distinctive duplicated 3' untranslated region (C. Zhang et al., 2019).

Prospects for Transformation Technologies in the Future of Transgenic Plant Production

The challenge to be met with future plant transformation efforts is the need to produce more food from the same land resources, potentially an even smaller area, in order to meet consumption and nutritional demands of a growing population. A drastic increase in production despite even further land-use restrictions depends, also, on the public acceptance of bioengineering before many of the available and future technologies can be used adequately (Li et al., 2012). The aim is further complicated by more regular occurrences of extreme weather, changing long-term climate patterns, and regional preference shifts. For example, July temperatures in Russia in 2010 were the highest in 130 years thereby reducing wheat yields by 70% compared to the previous year. Innovation must meet these various demands, and Jones (2015) believes commercial cultivation of bioengineered and/or genome-edited wheat will be cultivated by 2025 to help meet demands. Biolistic bombardment can be utilized to facilitate other technologies such as CRISPR/Cas 9 and thereby to develop marker-free genome-edited plants with improved traits (Tian et al., 2018).

Most current transformation efforts rely heavily on tissue culture for plant regeneration. Due to the difficulty inherent in tissue culture of wheat, future endeavors with transformation may benefit from in planta techniques. Researchers have attempted inoculation of stem tips with *Agrobacterium* carrying various genes with some success. *Agrobacterium*-mediated transformation can also be achieved with floral organs but suffers some when applied to immature embryos, due to browning

and death of the tissue. This response may be highly genotype dependent, though, and shows promise with cultivars such as Yumai66 and Lunxuan208. Progress toward mature embryo transformation with *Agrobacterium* also looks promising, as these tissues are always available and lack a time-sensitive nature like immature embryos. These advances, in addition to the discovery and use of additional strains of *Agrobacterium* for use with monocots, show great future promise for this technology; as does a lack of comparative gene silencing seen in *Agrobacterium*-mediated approaches (Li et al., 2012). Biolistic transformation, however, retains its ability to transform organelles such as mitochondria and chloroplasts where *Agrobacterium* is unable to achieve transformation (Jones, 2015).

Regardless of the transformation method used, other marker genes that pose fewer environmental concerns and may confer greater efficiencies are desirable. Some of the alternatives so far have been plant-derived, whereas others are considered improvements over current technology. This is true in the case of *pmi* versus *bar* as selectable marker genes. *Pmi* is regarded as safer for ecological systems in that it is a positive selection gene, rather than a negative one, and is therefore less prone to environmental escape. Regardless of what marker is used, strides have been made to separate the marker genes from genes for trait improvement so that marker genes can be more readily bred out (Li et al., 2012). Beyond marker genes there has been consideration of the constructs themselves. Promotor investigation for various genes of interest has allowed for tissue-specific expression and induction by various influences, which can save plant resources, over the purely constitutive promotors of the past (Jones, 2015).

A novel transformation technology is the use and insertion of minichromosomes. This technique has the potential advantage of being able to integrate many genes at once and to have faithful replication within an organism independent of host chromosomes. This would limit the issues of variable expression based on host chromosome integration position effects. Virtually any number of genes could be combined in this way and regulated as desired (Li et al., 2012).

Wheat must meet many new demands in the coming years beyond just the enormous increase in production predicted to meet consumer demands of 2050. Other demands for wheat improvement include increased tolerance to disease, pests, and environmental conditions such as unprecedented heat and drought. Great strides, including the sequencing of the wheat genome, have been made to better understand wheat genetics. Research in other areas is bound to come up with other novel genes useful for improvement. All of this opens the doors to possibilities in molecular breeding and biotechnology in not just wheat, but other crop and horticultural plants (Jones, 2015). R genes, those are resistance genes, such as the one cloned by Zhang et al. (2019), could prove extraordinarily useful

for disease resistance development – particularly when liberated from linkage drag experienced in hexaploid backgrounds (C. Zhang et al., 2019). This liberation could be achieved through introduction of the R genes via transformation.

Chapter 2: Tissue Culture Response of Common Wheat

Abstract

The tissue culture response was tested in six hexaploid wheat varieties in an eight-week long replicated experimental design. The wheat varieties tested included two spring genotypes (Fielder and UI Platinum), two facultative varieties (Florida 301 and Florida 303), and two winter types (Brundage and Florida 302). Testing for each genotype was repeated at least four times with 100 or more immature embryos per replicate. Callus induction was calculated at the proportion of embryogenic-callus-producing embryos to all embryos plated; regeneration was calculated as the proportion of the derived embryogenic calli that produced shoots 0.5 cm or longer among all calli obtained for the replicate; possible regeneration calculation included those calli that had dark green regions indicative of future shoot development given more time, in addition to the regenerated calli. Data analysis revealed that Brundage was the least regenerable genotype with a regeneration rate of 9.90% and Fielder was the most regenerable variety with a regeneration rate of 51.72%. The tested varieties were grouped into a highly regenerable group (including Fielder, Florida 303, and UI Platinum), and a less regenerable group (including Florida 301, Florida 302 and Brundage) by the Fisher's Least Significant Difference test conducted with R software version 3.6.1.

Introduction

Plant tissue culture can be an essential part of a plant improvement system or breeding program for staple food crops such as wheat and rice. Different plant species show genotype-dependent tissue culture responses. Testing must be done to validate an *in vitro* process for regenerating fertile plants (Alikina et al., 2016; Sears & Deckard, 1982). Tissue culture of wheat allows for embryo rescue from interspecies crosses, transgenic production, induced mutation breeding and genome-editing methods. Many explant types such as protoplasts, anthers, leaf segments, nodes, coleoptiles, shoot apical meristems, and immature inflorescence have been attempted with varying responses to culture. The most common explant tissue is the scutella of immature embryos, since this tissue is the most effective explant type for regeneration of whole plants (Khan et al., 2015; Kumar et al., 2017). A genotypes' ability to regenerate whole plants can be calculated based on a cultivar's ability to regenerate shoots and roots *in vitro* from excised scutella.

Researchers have attempted to find correlations between a wheat variety's agronomic traits and its tissue culture amenability and thereby circumvent the lengthy and laborious need to develop a specific tissue culture regime based on individual genotypes. This would be beneficial to many transformation laboratories. However, many of these efforts have been only marginally successful.

Nonetheless, weak correlations have been drawn between grain yield and callus formation, kernel number per spike to both regenerative calli and plant number per embryo. Of the traits explored, only productive tillering showed a direct positive effect on all analyzed tissue culture traits. These associations were drawn from experiments with 96 wheat cultivars (Dodig et al., 2008). Regarding spring wheat, relationships have been estimated between callus regeneration and kernel number per spikelet, effective tillering and longevity. These correlations were stronger with these traits and mature embryo culture versus their relationships to immature embryo culture (Li et al., 2012). In a study of 38 European winter wheats, regeneration conclusions were drawn based on relationships between genotype and callus induction period length. Conclusions indicated that a 3-week callus induction period versus 9-weeks was better, with statistically significant reductions in regenerated plants with the longer period (Varshney & Altpeter, 2002).

Spring and winter wheats differ in their sensitivity and need for cold-temperature periods at the seedling stage in order to achieve competency for seed-setting. Spring cultivars lack a need for an extended period of cold treatment, whereas winter wheat seedlings require four to eight weeks of cold treatment at an ideal temperature of 0°C to 7°C to be competent for reproductive growth. A lack of cold treatment prohibits winter wheats from forming spikes and therefore seeds. Facultative varieties have variable need for vernalization, requiring a fewer number of days at a cold temperature and/or vernalization requirements being met at slightly higher temperatures than obligate winter types. Regardless of differing sensitivities of the myriad winter and facultative wheat genotypes, a 50-day cold treatment is commonly accepted as sufficient (“Spring Wheat Growth Stage”).

The materials and methods below outline a similar two-step process to that of Alikina et al. (2016) – a four-week callus induction period in a dark, warm environment followed by a four-week treatment on regeneration media in a light chamber of the same temperature. More akin to the technique of Kumar et al. (2017), however, the regeneration media used in this study contained a mixture of synthetic auxin, cytokinin and an induction agent that has been shown to promote shoot growth, rather than use of a medium containing no plant growth regulators at all. The method presented here did not include a third, two-week, root-inducing treatment similar to that of Kumar et al., as calli producing sufficient shoots were deemed to be regenerated (Alikina et al., 2016; Kumar et al., 2017). Khan et al. (2015) notes maturity of the embryos, ranging in size from 0.8 to 1.5 mm, and auxin concentration of media are critical factors in achieving successful tissue culture of wheat scutella.

Materials and Methods

Plant Materials

Six hexaploid wheats were selected for testing of the tissue culture response of isolated immature embryos, their ability to produce embryogenic calli and viable shoots. The varieties included spring wheats Fielder and UI Platinum, winter types Brundage and Florida 302, and facultative varieties Florida 301 and Florida 303. Their pedigrees and GRIN reference numbers are contained in Table 2.1. Brundage and UI Platinum were included in this study because they are varieties released from Idaho in recent years. UI Platinum could provide a spring-type comparison to Fielder, and so the data may be used to inform future transformation experiments.

Another consideration of genotype selection was a desire to have a diversity of traits represented in the different wheats included in this study. Fielder (CItr 17268), another Idaho variety released in 1974, is a semi-dwarf, stiff-strawed variety of soft white spring wheat that has a medium maturity. It has been used as a model in past transformation experiments by other researchers (Li et al., 2012). UI Platinum (PI 672533) is a short, hard white spring wheat with good end-use quality and early maturity. The facultative wheats, Florida 301 and Florida 303 (CItr 17769, PI 601807) are soft red wheats. Florida 303 is awned and Florida 301 is awnletted. Winter wheat Florida 302 (PI 601163) is also a soft red but with the longer vernalization requirement and having a good grazing quality. Lastly, Brundage (PI 599193) was chosen due to its regional heritage and susceptibility to rust. It is awnletted with a waxy blue-green foliage. It is a soft white winter wheat with a short semi-dwarf habit and excellent straw strength.

Donor plants were grown in the greenhouse at University of Idaho, Moscow, Idaho, USA throughout the testing period. Varieties were sown on approximately seven to fourteen-day intervals to assure regular availability of plant material. Plant health was of high priority with watering every two to three days, weekly fertilization, and pest control as necessary. Six donor plants were grown in a 17x17 cm pot. Greenhouse temperatures ranged 22°C to 25°C during the day and between 15°C to 20°C at night. A 16-hour photoperiod was achieved with supplemental 400 W high-pressure sodium lights.

For winter and facultative varieties, seeds were rolled in damp germination paper and were kept in water in a dark 4°C refrigerator for up to two months. Germinated seeds that had greenish-yellow shoots protruding from the top were taken for transplanting into the greenhouse as donor material. This satisfied those genotypes' vernalization requirements so they could set seed. Spring varieties were sown directly into 17x17 cm pots in the greenhouse. Six seedlings were contained in one pot.

For this study secondary tillers of donor plants were left intact rather than stripped away as Sparks & Jones had done (Sparks & Jones, 2009). Priority was given, however, to the newest tillers that were healthy and of a normal morphology at the correct maturity. Plants were used for donor purposes within thirty days post-anthesis, and then discarded.

Media and Culture Conditions

Callus induction medium (CI) (1L): Basic media (Table 2.2) plus 49 µL copper sulfate (0.1M), and 400 µL 2,4-D (5 mg/mL). Embryos and resultant calli were kept at 23°C, in the dark, for a total of four weeks with one media refreshment midway.

Regeneration medium (RM-) (1L): Basic media plus 49 µL copper sulphate (0.1M), 40 µL 2,4-D (5 mg/mL), and 100 µL 6-benylaminopurine (1 mg/mL). Calli were kept in a growth chamber at 23°C with a 16-hour photoperiod for an additional four weeks with one media refreshment midway.

Immature Embryo Extraction

Spikes were harvested 14 to 16 days post-anthesis. If used after the day of harvest, they were kept in a dark, 4°C refrigerator for up to seven days. In preparation for embryo excision, immature seeds were removed from glume, lemma and palea. Approximately 20 mL worth of seeds were harvested in this way to assure ample material to provide enough immature embryos to fill two 15x100 mm petri dishes with spacing of ~1 cm between each embryo. Usually approximately 110 to 150 healthy embryos per petri dish were arranged. This spacing allowed enough space for callus development without loss of visual differentiation of the individuals (Figure 2.1). Prior to excision, seeds were surface sterilized with twice their volume of a 70% ethanol, 0.05% Tween20, and 18.2MΩ water solution for five minutes at 250 rpm at room temperature, then a 10% bleach, 0.05% Tween20, and 18.2MΩ water solution for an additional 20 minutes at 250 rpm at room temperature. After draining, sterilized seeds were rinsed via hand shaking with sterile deionized water at least three times. Developed calluses were transferred every two weeks to fresh media of the appropriate composition for experimental timings.

When excising immature embryos, one must remove the axil of the embryo to avoid germination of meristematic tissue prior to callus growth, which forms primarily from the scutella. Without complete axil removal, precocious shoots and roots develop from the region, which precludes callus development. Excised embryos are placed with the cut side in contact with the medium, so the scutella are exposed. If precocious germination did occur, the tissue was removed during the first or second subculture at the time of appearance, thereby leaving only undifferentiated cells. If removal of

this sprouted tissue failed to leave an adequate grouping of cells for continued callus growth, then the individual embryo was discarded.

Embryo excision was performed with the aid of a stereoscopic microscope. Excision and subsequent media changes were done in a laminar flow hood which was sterilized prior to procedures by using 100% ethanol spray and/or a 15-minute UV light exposure. All tools were autoclaved regularly and sterilized in a bead-sterilizer prior to every use.

At least four replicates of approximately 100 embryos for each genotype was completed. Four replicates were made in order to account for seasonal differences, variation in the suitability of plant material, and to lend statistical validity. According to a sample size estimation table, one can estimate a population of over 250,000 with 95% confidence and a 5% margin of error with a sample size of 384 (The Research Advisors).

Analysis

Embryos/calli were counted at excision and every subsequent medium change. Doing so allowed for calculation of callus induction. Callus induction was calculated as the proportion of successful calli among embryos excised and plated on the medium. The count of successful calli, for the purpose of callus induction calculation, was taken at the time of transfer to the second plate of CI. The final callus number was used to determine regeneration rates. The regeneration rate was calculated as the number of calli producing recognizable shoots and leaf tissue of at least 0.5 cm in length, divided by the total number of calli at the time of evaluation, then multiplied by one hundred. Calculation of the possible regeneration rate combined the total number of calli with shoots as described above and added those calli featuring dark green regions indicative of distinguishable plant growth. These were considered “possible” because given additional time on the regeneration medium they had a strong likelihood of producing plants (Figure 2.1).

Comparisons of callus induction across all varieties was made using Analysis of Variance (ANOVA). Analysis of Variance was also used to compare regeneration rates across all varieties. Because a difference was detected in the group means for regeneration rates, Fisher’s Least Significant Difference (LSD) testing was used to evaluate how groups varied from one another and to reveal the significance of those differences. Results were confirmed using Bonferroni testing. These tests were completed using R software version 3.6.1 (R Core Team (2019)).

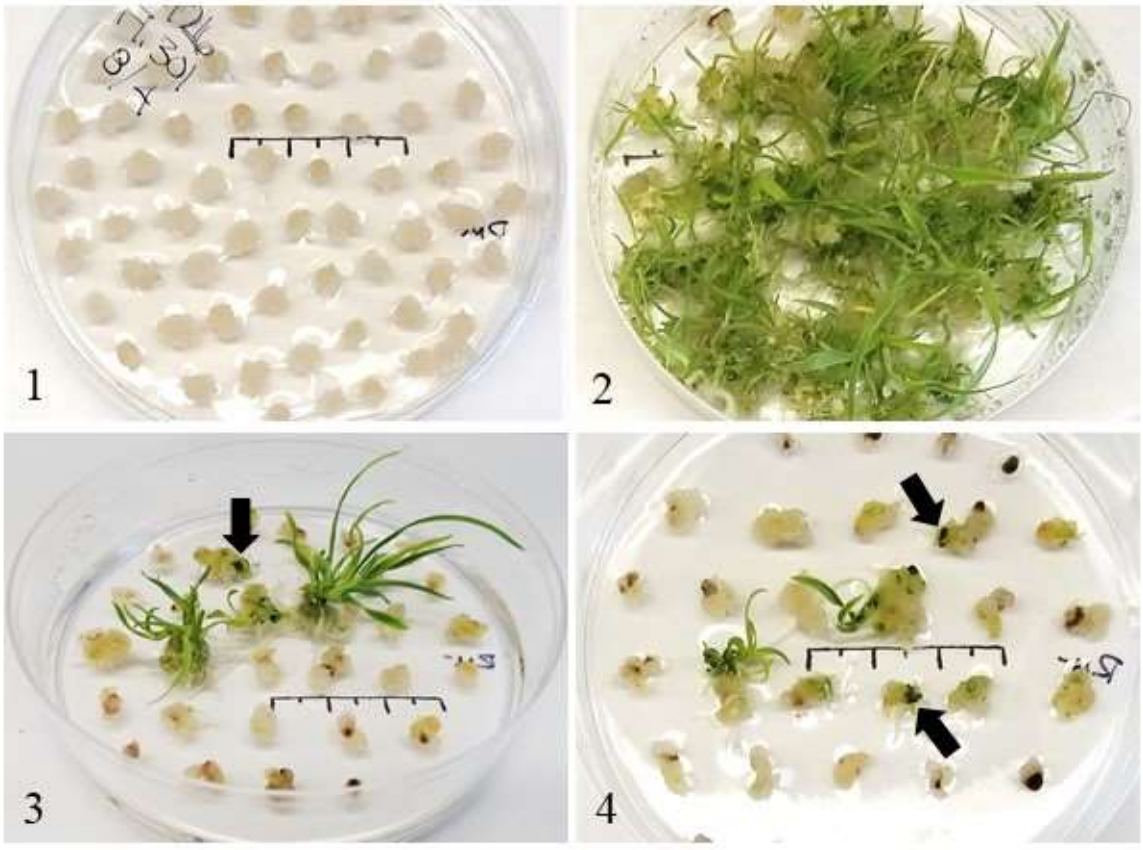


Figure 2.1 Tissue culture examples

The scale for the images is by the centimeter, with 0.5 cm demarcations. 1: This image typifies the spacing and size of healthy calli after two weeks of growth on CI media, indicating the original spacing of embryos as placed on to the medium immediately following excision. One can see that the calli appear to be mostly white and of a fairly uniform size. 2: A plate of calli of the Fielder variety at the time of evaluation featuring vigorous regeneration of many calli. Many calli produce a great number of shoots and leaves, which have a healthy appearance and green color. 3: Three calli that were counted as regenerated with varying degrees of vigor as one can see from the difference in the length and density of produced tissue, and one (indicated by black arrow) considered a possible regenerant. 4: Two counted regenerated calli are featured in this image and two counted as possible ones as indicated by black arrows. Calli that had some green coloration but that were lacking the indicative dark-green hue of shoot/leaf tissue, were not considered to have the possibility of future regeneration.

Results

A total of 33 experiments of the eight-week treatment were initiated, of those only 31 were carried to full completion. The failure of experiments 5 and 8 was due to a high percentage of precocious germination. Sprouting likely occurred due to the researcher's own excision technique and therein the failure to completely remove the embryos' axils during those replicates. The initiation of the replicates for each genotype were within a time range encompassing April of 2019 through September of 2019. The researcher attempted to space replicates so that a possible seasonal effect might be mitigated, however, embryo availability was limited by the maturity and health of available plant material. Figure 2.2 gives a graphical display of when replicates where initiated. Timing of replicates is further discussed in a later section.

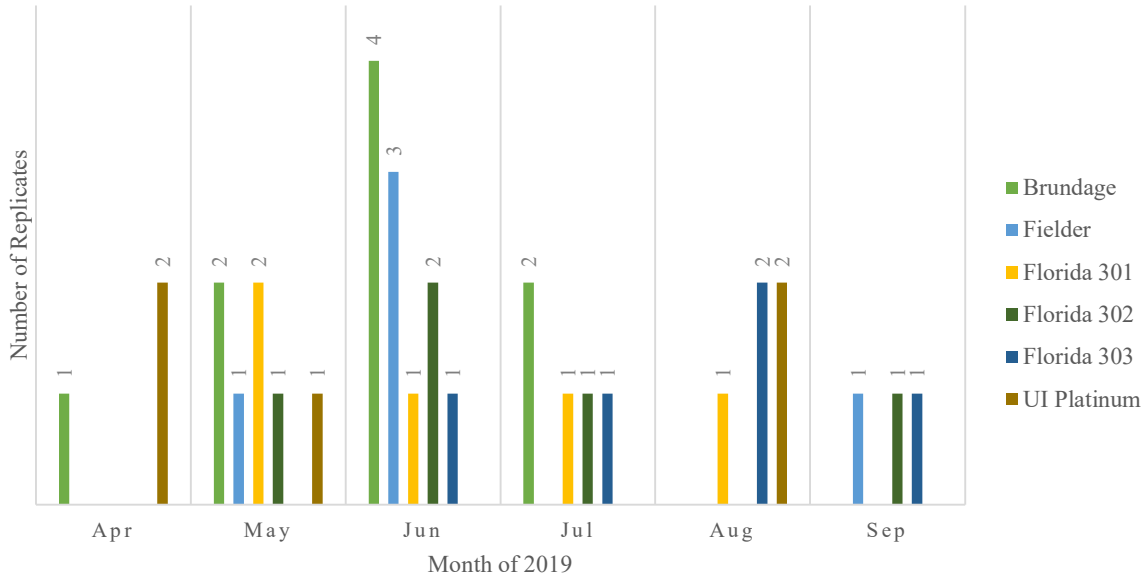


Figure 2.2 Tissue culture initiation

Depicted are the months of 2019 when replicates for tissue culture testing were initiated, and the number of replicates for each genotype within that month. The initiations were dependent on the health and maturity of available plant materials and were therefore not completely regular. Ideally, one replicate per genotype initiated per month would have been conducted so that seasonal effects on the embryo health, viability, and tissue culture response on any of the genotypes would have been mitigated by experimental design. Also, the inclusion of every month of a year for tissue culture testing would have been desirable for these same reasons.

The callus induction rate was calculated as discussed previously (Figures 2.3 and 2.4) A Studet's t-test was used to determine if rates of callus induction of any of the genotypes differed significantly from 100%. When a Student's t-Test was completed in R, the results indicated that the null hypothesis was rejected – that the true mean was not 100%. The calculated mean of callus induction for all genotypes was 94.03%. When Analysis of Variance (ANOVA) testing was used to determine if any of the genotype's group means varied significantly from one another the researcher failed to reject the null hypothesis and accepted that no group mean was significantly different from one another. The assumptions for ANOVA were assured to have been satisfactorily met. The results

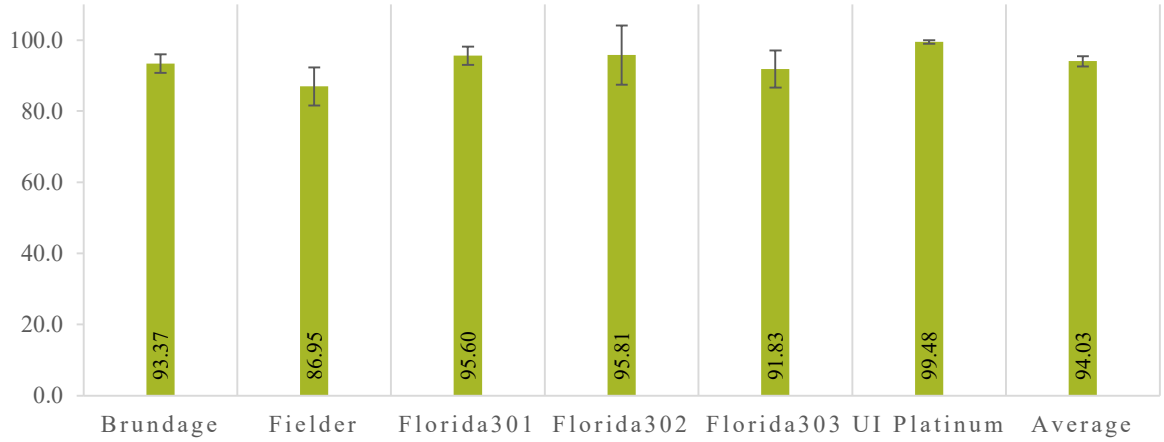


Figure 2.3 Average rate of callus induction by genotype

Graph represents the average for callus induction across all replicates, grouped by genotype, with bars indicating standard error of the mean. Callus induction was calculated based on the number of embryos of a genotype that were excised and plated compared to the number of embryogenic calli that formed as a result, as shown in another figure. (Figure 2.4).

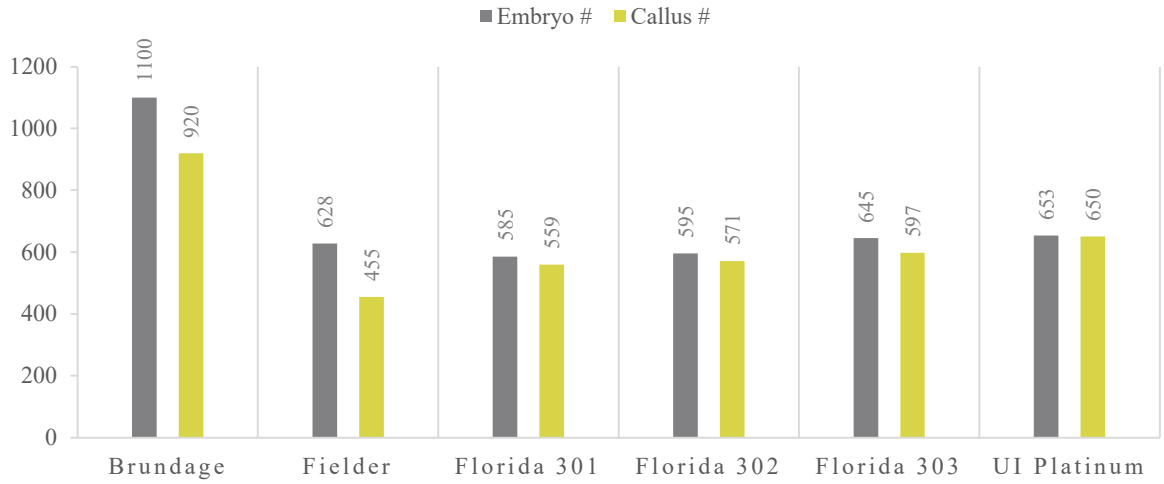


Figure 2.4 Sums of embryos excised and embryogenic calli formed per genotype

Graph shows the total number of embryos of each genotype that were plated and the resultant number of embryogenic calli from them. These figures were used to calculate the rate of callus induction for each genoytpe (Figure 2.3). With a total of nine replicates, Brundage had a greater number of embryos that were excised and resultant calli thereof. Other genotypes had four to five replicates of approximatly 100 embryos per replicate.

indicate that there was no difference between the spring, facultative and winter wheats tested in regard to callus induction rates, as there was no significant difference between any of the genotypes' means. Callus induction average rate was above 90% for all genotypes except Fielder, which was 86.95%. This could possibly be explained by contamination at the time of the first media transfer in some of the early experiments, as there was no adjustment made for these figures. Regardless, these means are all satisfactorily high in their indication of callus induction for the purpose of regeneration. UI Platinum had the highest rate of callus induction. A visualization for average rates of callus induction by genotype is in Figure 2.3, which was derived from embryo and callus counts as seen in Figure 2.4. These averages were taken from Table 2.4.

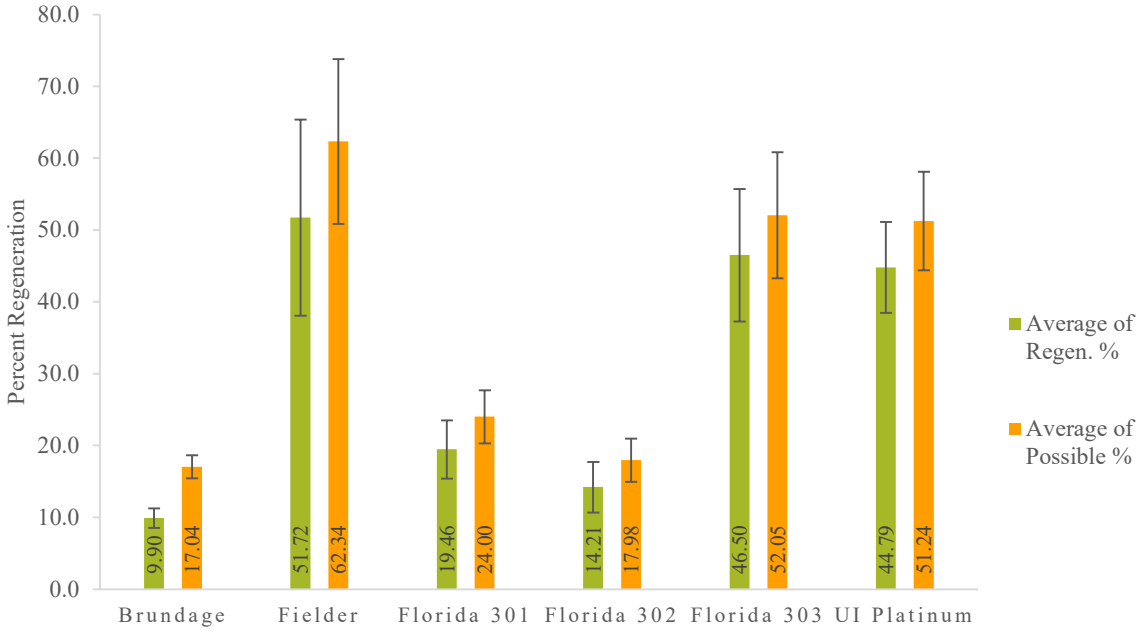


Figure 2.5 Regeneration rates by genotype

Overall regeneration and possible regeneration rates are averaged for each genotype. Regeneration rates are based on the number of embryogenic calli that produced shoots and/or leaf tissue of 0.5 cm or greater. Possible regeneration rates also included in this calculation calli that had dark green regions that were indicitive of production of leaf tissue or shoots if they were given more time on the regeneration medium (Figure 2.1). The error bars indicate standard error of the mean for each set of data.

Visualizations of the percent of actual regeneration rate and the calculations of possible regeneration rate are represented in Figure 2.5 and Figure 2.6, which provides a breakdown by replicate. Corresponding Table 2.5 is located at the end of the chapter. From this data one can see that Fielder, Florida 303 and UI Platinum appeared to be more regenerable than the other varieties. Statistical conclusions supported this with calculated regeneration means from R at 51.71% for Fielder, 46.50% for Florida 303, and 44.79% for UI Platinum. It appeared that Fielder and Florida 303 may have had some regeneration sensitivity based on seasonality due to earlier replicates having relatively higher

regeneration and possible regeneration rates, versus later replicates of the same genotype. Based on this data, Brundage appeared to be the least regenerable genotype.

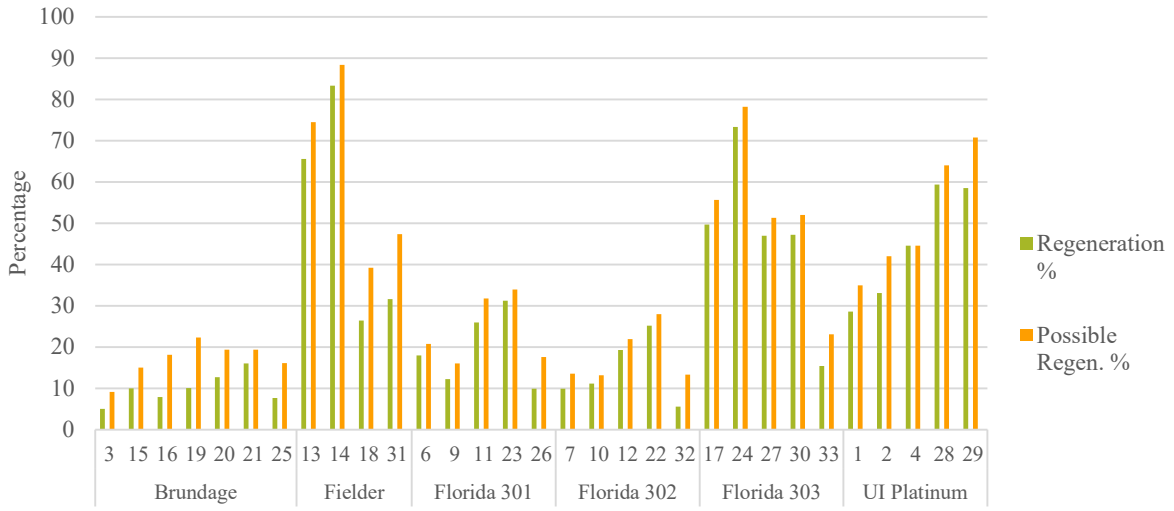


Figure 2.6 Regeneration rates by replicate

Figure is based on replicate identifier (number). Each green bar represents the regeneration rate of a particular replicate, and the orange bar is the possible regeneration rate. Fielder appears to be the most regenrable genotype, and Brundage the least regenerable genotype.

ANOVA analysis determined that there was a significant difference between genotype means with a very small p-value at an α of 0.05. All assumptions for ANOVA were verified to have been met. When Fisher's LSD test was used to compare regeneration percentages based on genotype, results were grouped into two categories: 'a' and 'b' (Figure 2.7). Group means were: 51.72% for Fielder, 46.50% for Florida 303, 44.80% for UI Platinum, 16.46% for Florida 301, 14.21% for Florida 302, and 9.90% for Brundage. This grouping indicated that, statistically, the rates of regeneration percentage did not differ between Fielder, Florida 303, and UI Platinum in group 'a'. Additionally, the rates did not differ significantly between Florida 301, Florida 302, and Brundage in group 'b'. However, there was a significant difference between the two groups, with the means being higher in group 'a' than group 'b'. These data indicated that regeneration was better for the genotypes in group 'a'. When exploring statistical conclusions for the possible regeneration rate by genotype, the group conclusions were the same.

These comparisons were validated with Bonferroni testing. The statistical significance was calculated with an α of 0.05. Any comparison with a p-value below 1.0 is significant. The Brundage-Fielder p-value was 0.0018. Florida 301-Brundage was 1.0, Florida 301-Fielder was 0.0463 (Indicating significance, but not to the level of the Brundage-Fielder comparison). Florida 302 compared to

Brundage and Florida 301 produced insignificant p-values of 1.0. Whereas Florida 302 compared to Fielder yielded a p-value of 0.0121. Florida 303 had significance values of 0.0038, 0.01118, and 0.0280 respectively when compared to Brundage, Florida 301 and Florida 302. Lastly, UI Platinum had significant p-values when compared to Brundage, Florida 301, and Florida 302, at 0.0064, 0.1723, and 0.0442, respectively. The closer the p-value to 1.0, the less significant the difference between genotypes was regarding regeneration rate.

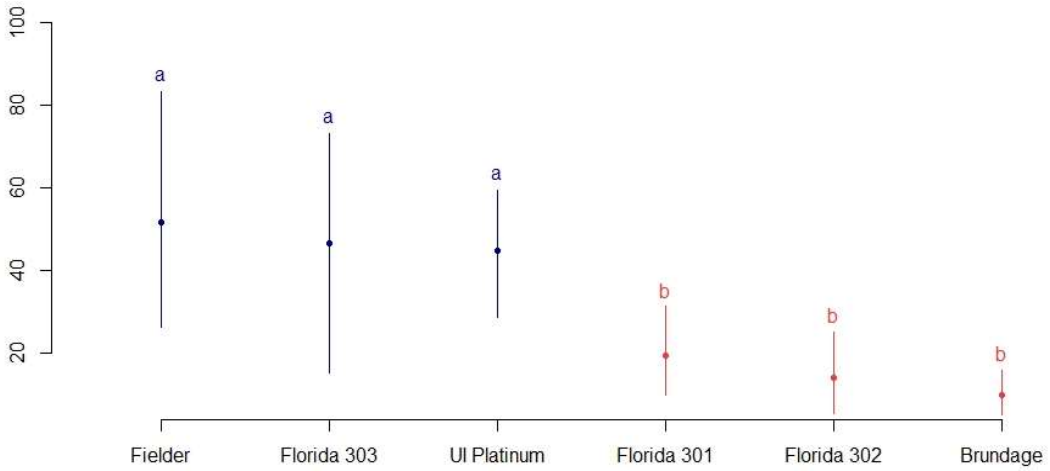


Figure 2.7 Comparison of regeneration rate by genotype based on Fisher's LSD test

This is the graphical output by R based on the Fisher's LSD test. One can see that the genotypes Fielder, Florida 303, and UI Platinum are grouped into the 'a' group, which has higher regeneration rate means than the 'b' group that includes genotypes Florida 301, Florida 302 and Brundage. The points in each line represent the mean for that genotype and the line itself represents the range of rates of regeneration for each.

Statistically, the most significant difference was between Brundage and Fielder regarding actual regeneration rate with a p-value of 0.0018 at an α of 0.05. These data were interpreted to signify that the spring wheats tested had a higher regeneration capacity than the winter wheats included, but that the facultative wheats' regeneration capacities were significantly different between them.

Discussion

The appropriate maturity of embryos for the best possible success rate of regeneration must be determined and, therefore, for transformation. A trained eye can determine when immature seeds are close to the optimal maturity for harvest. Prior to gaining this experience, trainees are advised to mark stems when plants are observed to be pollinating – displaying yellow anthers expelled from central spikelets, which is generally indicative of anthesis – and checking maturity of embryos by visual inspection beginning 11 days thereafter. Checking embryo maturity can be done by liberation of one of the central spikelets' immature seeds and using one's thumbnail to pop the embryo out. Ideal embryo size is 0.5 mm to 1.5 mm long.

Although statistically unnecessary, calli counts at every transfer provide an indication of levels of contamination. It is highly recommended to take notes regarding the rooting status of calli considered regenerated. These notes might have included length measurements of the roots produced and comments on appearance of the roots themselves.

Sears & Deckard (1982) weighed calli before and after a twelve-day growth period and at five different stages thereafter. A similar measure in this study could have quantified the growth rate of calli of different genotypes. Additionally, similar to the technique of those researchers and others, at least one replicate for each genotype could have been carried to full plant regeneration. Doing so would have served to assure that shoots considered regenerated in this study could indeed form whole, morphologically normal regenerants. Also a potential for future consideration are variations of 2,4-D levels in media for those genotypes that did were less responsive to regeneration efforts (Kumar et al., 2017; Sears & Deckard, 1982).

The initial goal of the research was to collect at least five replicates for each genotype, unfortunately, only four replicates of Fielder were completed due to a lack of availability of heathy Fielder donor material of the appropriate maturity within the time needed to complete experimentation. Due to an abundance of Brundage donor material in June, and the ease of completing tissue culture replications at the same time as excision for bombardment, many replicates were initiated in that month. In retrospect, a more even distribution of replicate initiation, such as one replicate for each genotype in each month, would have been desirable. Additionally, having replicates spread throughout an entire year would have also been desirable as this would have even more greatly mitigated any seasonal effects on the regeneration capability of calli formed, or on the health of donor embryos.

Based on the results of other researchers' comparisons of regeneration and many having noted that spring varieties perform better than winter ones, it is not surprising that Fielder and UI Platinum are in the top group for regeneration capabilities. Perhaps more interesting is the significant difference between the rates of regeneration for the facultative varieties Florida 301 and Florida 303. This disparity farther reinforces the conclusions of prior literature that regeneration is highly genotype dependent. Brundage, of the tested varieties, was conclusively the least regenerable. Although, given more time on regeneration media, Brundage may have performed better. This is indicated by the possible regeneration percent mean of 17.04%. This possible regeneration percent mean, however, is still lower than the actual regeneration rate of any other genotypes tested.

Table 2.1 Plant material information

Table includes the variety names, wheat type, pedigrees and GRIN Global Accession numbers of wheat materials tested in these experiments. Typically, winter type wheats require a vernalization treatment of 30 to 60 days in within a temperature range of 0°C to 5°C to be competent to set seed, whereas facultative types can suffice between 15 to 30 days at a range between 3° to 15°C (Braun & Sãulescu, 1998). Spring types do not require a vernalization treatment for flowering.

Variety	Type	Pedigree	Accession #
Brundage	Winter	Stephens/Geneva	PI 599193
Fielder	Spring	Yaktana 54A*4//Norin 10/Brevor/3/2*Yaqui 50/4/Norin 10/Brevor//Baart/Onas	CItr 17268
Florida 301	Facultative	Holley//Olesen/Purdue 64212A3-23	CItr 17769
Florida 302	Winter	Coker 65-20//P4946A4-18-2-10-1/Hadden/3/Vogel/5/Anderson//P4946A4-18-2-10-1/Hadden	PI 601163
Florida 303	Facultative	Coker 65-20/8/(Norin 33-3/6/(Fairfield/4/Fultz/Hungarian*2//PI94587 durum/3/Fultz/Hungarian, Pd39153A1-11-1-1)/5/(Trumbull*3//Hope/Hussar, Pd3932A7-3-1-2)/3/Newsar, PD4946A4-18-2-10-1)/7/Hadden/9/(Norin 10/Brevor/4/Anderson/3/(Coker 55-9, Chancellor*2//T. timopheevi/Steinweidel), Vogel 5)/8/(Norin 33-3/6/(Fairfield/4/Fultz/Hungarian*2//PI94587 durum/3/Fultz/Hungarian, Pd39153A1-11-1-1)/5/(Trumbull*3//Hope/Hussar, Pd3932A7-3-1-2)/3/Newsar, Pd4946A4-18-2-10-1)/7/Hadden/10/Coker 797	PI 601807
UI Platinum	Spring	Blanca Grande/Jerome	PI 672533

Table 2.2 Basic media composition

Basic media were used as the basis to create all the specific media types used in these experiments. The specific media types were created with the additions of other minor components at specific concentrations in various amounts. Added components depended on media type but included sucrose, $CuSO_4$, 2,4-D, glufosinate-ammonium, and 6-BA.

Component	Amount per liter
Murashige & Skoog salts*	4.3 g
Maltose monohydrate	40 g
Thiamine hydrochloride solution	10 mL
Asparagine monohydrate	0.15 g
18.2MΩ water	To 1 L

Adjust PH to 5.8 with potassium hydroxide (1M)

Phytagel is added to bottles prior to autoclaving at 122.8°C for 30 minutes. Media is allowed to cool to approximately 60°C before minor components are added, mixed, and poured into sterile 15x100 mm petri dishes.

*Catalog No. M524 (PhytoTechnology Laboratories, USA)

Table 2.3 Raw data for tissue culture tests

RT	Variety	Excised	Embryo	Callus	CallusInduction	Final	Evaluation	Shoot #	Regen.	#Potential	Total	Possible	Comments
1	UI Platinum	18-Apr	126	126	100.00	126	13-Jun	36	28.57	8	44	34.92	
KJC*	Brundage	18-Apr				161	15-Jun	27	16.77	12	39	24.22	Unbombarded Control
2	UI Platinum	23-Apr	115	112	97.39	112	18-Jun	37	33.04	10	47	41.96	
3	Brundage	11-May	113	113	100.00	99	7-Jul	5	5.05	4	9	9.09	
4	UI Platinum	16-May	128	128	100.00	110	11-Jul	49	44.55	0	49	44.55	
5	Fielder	16-May	102								0		Too many precocious sprouts
6	Florida 301	27-May	106	106	100.00	106	22-Jul	19	17.92	3	22	20.75	
7	Florida 302	31-May	117	114	97.44	111	26-Jul	11	9.91	4	15	13.51	
8	Brundage	31-May	113								0		Too many precocious sprouts
9	Florida 301	31-May	114	106	92.98	106	26-Jul	13	12.26	4	17	16.04	
10	Florida 302	4-Jun	100	99	99.00	99	3-Aug	11	11.11	2	13	13.13	
11	Florida 301	7-Jun	120	104	86.67	104	3-Aug	27	25.96	6	33	31.73	
12	Florida 302	7-Jun	121	116	95.87	114	3-Aug	22	19.30	3	25	21.93	
13	Fielder	11-Jun	126	96	76.19	90	6-Aug	59	65.56	8	67	74.44	
14	Fielder	13-Jun	151	120	79.47	120	8-Aug	100	83.33	6	106	88.33	
15	Brundage	13-Jun	147	120	81.63	120	8-Aug	12	10.00	6	18	15.00	
16	Brundage	15-Jun	147	128	87.07	127	11-Aug	10	7.87	13	23	18.11	
17	Florida 303	15-Jun	154	151	98.05	151	11-Aug	75	49.67	9	84	55.63	
18	Fielder	21-Jun	132	125	94.70	125	19-Aug	33	26.40	16	49	39.20	
19	Brundage	21-Jun	144	139	96.53	139	19-Aug	14	10.07	17	31	22.30	
20	Brundage	26-Jun	155	150	96.77	150	22-Aug	19	12.67	10	29	19.33	
21	Brundage	9-Jul	150	150	100.00	150	3-Sep	24	16.00	5	29	19.33	
22	Florida 302	17-Jul	144	144	100.00	143	10-Sep	36	25.17	4	40	27.97	
23	Florida 301	17-Jul	113	112	99.12	112	10-Sep	35	31.25	3	38	33.93	Small embryos
24	Florida 303	17-Jul	104	101	97.12	101	10-Sep	74	73.27	5	79	78.22	Small embryos
25	Brundage	30-Jul	131	120	91.60	118	24-Sep	9	7.63	10	19	16.10	
26	Florida 301	4-Aug	132	131	99.24	131	29-Sep	13	9.92	10	23	17.56	
27	Florida 303	9-Aug	153	143	93.46	115	4-Oct	54	46.96	5	59	51.30	
28	UI Platinum	10-Aug	152	152	100.00	150	4-Oct	89	59.33	7	96	64.00	
29	UI Platinum	13-Aug	132	132	100.00	130	8-Oct	76	58.46	16	92	70.77	
30	Florida 303	23-Aug	126	125	99.21	125	18-Oct	59	47.20	6	65	52.00	
31	Fielder	4-Sep	117	114	97.44	114	31-Oct	36	31.58	18	54	47.37	
32	Florida 302	4-Sep	113	98	86.73	90	31-Oct	5	5.56	7	12	13.33	Contamination ~ 7 embryos (adjusted callus count from 91)
33	Florida 303	4-Sep	108	77	71.30	65	31-Oct	10	15.38	5	15	23.08	Contamination ~ 12 embryos (adjusted callus count from 65)

How to read the table: RT = Regeneration Test, that is, replicate number (identifier). Excised = the date embryos were plated. Callus = number of induced embryogenic calli. CallusInuction = callus induction rate as calculated by dividing the number of produced embryogenic calli by the number of excised embryos and multiplying by 100. Final = final callus number. Shoot # = Calli with shoots/leaf tissue of 0.5 cm or greater. Regen. = regeneration rate as calculated by dividing the number of calli that produced roots by the final number of calli at the time of evaluation multiplied by 100. #Potential = Calli featuring dark green regions indicative of leaf or shoot tissue growth. Total = the number of calli producing shoots in addition to the number of calli deemed potential. Possible = possible rate of regeneration as calculated by the number of calli with shoots, plus the number with dark green regions, divided by final number of calli at that time, multiplied by 100.

Table 2.4 Average rate of callus induction

Total embryo number that was excised and plated by genotype, and the number of embryogenic calli that were formed thereof were used to calculate the average rate of callus induction. Also included is the standard error of the mean.

Variety	Embryo #	Callus #	Average %	Std. Error of Mean
Brundage	1100	920	93.37	2.63
Fielder	628	455	86.95	5.34
Florida 301	585	559	95.60	2.57
Florida 302	595	571	95.81	8.32
Florida 303	645	597	91.83	5.22
UI Platinum	653	650	99.48	0.52
Totals	**4206**	**3752**	**94.03**	**7.69**

Table 2.5 Regeneration by genotype and replicate

Rate of regeneration and possible regeneration of calli is shown below as determined by tissue growth 0.5 cm or greater, or the dark green color indicative of such tissue growth, compared to the final number of calli at evaluation. Data is divided by replicate identifier (number) and in average per genotype. Standard error of the mean is also included from the data.

Genotype/Replicate	Regeneration % (Std. Error of Mean)	Possible Regeneration % (Std. Error of Mean)
Brundage	**9.90** (1.36)	**17.04** (1.60)
3	5.05	9.09
15	10.00	15.00
16	7.87	18.11
19	10.07	22.30
20	12.67	19.33
21	16.00	19.33
25	7.63	16.10
Fielder	**51.72** (13.66)	**62.34** (11.48)
13	65.56	74.44
14	83.33	88.33
18	26.40	39.20
31	31.58	47.37
Florida 301	**19.46** (4.04)	**24.00** (3.70)
6	17.92	20.75
9	12.26	16.04
11	25.96	31.73
23	31.25	33.93
26	9.92	17.56
Florida 302	**14.21** (3.53)	**17.98** (3.00)
7	9.91	13.51
10	11.11	13.13
12	19.30	21.93
22	25.17	27.97
32	5.56	13.33
Florida 303	**46.50** (9.21)	**52.05** (8.77)
17	49.67	55.63
24	73.27	78.22
27	46.96	51.30
30	47.20	52.00
33	15.38	23.08
UI Platinum	**44.79** (6.32)	**51.24** (6.86)
1	28.57	34.92
2	33.04	41.96
4	44.55	44.55
28	59.33	64.00
29	58.46	70.77
Average	**29.06** (3.93)	**35.32** (3.97)

Chapter 3: Transformation of *Yr28* Gene in Wheat for Stripe Rust Resistance

Abstract

Five wheat genotypes were transformed with *Yr28*, a gene imparting stripe rust resistance from *Aegilops tauschii*, via particle bombardment of immature embryos. The primary genotype was Brundage, and others were tested to compare transformation efficiency including Fielder, Florida 301, Florida 302, and Florida 303. The fosmid, F2-1, carrying the gene of interest lacked a plant selection marker and therefore co-bombardment was done with the plasmid PC174 carrying the *bar* gene. Presence of the *bar* gene was assayed for by painting a portion of the lower leaves with herbicide with the active ingredient glufosinate-ammonium. PCR aplification confirmed the presence of *Yr28* in putative transgenic plants, and gene expression was confirmed with reverse transcription PCR. Of 63 putative transformants a total of 24 plants were confirmed to be transformed, 11 of which were of the Brundage genotype. The average transformation efficiency across all genotypes was 0.24%, with Fielder having the highest efficiency at 0.43% and Florida 303 the lowest at 0.13%. ANOVA analysis revealed that there was no significant difference in transformation efficiency between genotypes. Fisher's Least Significant Difference testing confirmed this. Ten of the 24 transgenic T_0 plants displayed stripe rust resistance. Plants progressed to the T_1 generation segregated for the transgene. Seven T_1 plants of the Brundage-derived #10 family displayed stripe rust resistance, and evidence of expression for *Yr28*.

Introduction

Wheat transformation can improve target varieties with agronomically viable traits in a shorter period of time than traditional breeding methods (Sparks & Jones, 2004; Sparks & Jones, 2009). Molecular testing can determine whether the gene of interest is present in the genome of a given transgenic line, regardless of whether that gene is phenotypically expressed. Many independently transformed lines must be produced in order to have plants with reasonable expression of the transgene, as locations and copy numbers of transgenes can lead to variable expression (Altpeter et al., 1996). The reasonable expression of a transgene must be phenotypically validated – in the case of this study demonstrating resistance to wheat stripe rust.

Stripe rust resistance is a prime candidate for this transformation, as it is a highly destructive disease, particularly in the Northwest United States (Chen et al., 2002; Murray et al., 2005). Stripe rust is a fungal disease of cereals. The pathogen that infects wheat is *Puccinia striiformis* f. sp. *tritici* (*Pst*) and is also known as yellow rust due to the color of the spores. The disease is typically more destructive

than the other rusts. The spores are generally dispersed by wind and can travel great distances, thereby infecting large areas of susceptible varieties. Infection requires high humidity for a period of time followed by disease development and eventually the pustules erupt through the leaf tissue. Although treatable with fungicides, these applications are expensive and labor-intensive and therefore it is best to reduce disease spread by planting resistant varieties (Murray et al., 2005).

Yr28 is a traditional R (resistance) gene that is derived from *Aegilops tauschii* and imparts all-stage resistance to stripe rust infection. It was previously mapped to the 4DS chromosome of *Ae. tauschii* subspecies *stangulata* but has since been located in accessions of the subspecies *tauschii* as well (Liu et al., 2013; Singh et al., 2000; C. Zhang et al., 2019). The gene was cloned by C. Zhang et al. and published in September 2019 (C. Zhang et al., 2019). The gene features multiple splicing patterns that occur partially due to a duplicated 3' untranslated region, which are believed to impart the gene's resistance characteristics. There are known homologues in Chinese Spring with 86% to 94% identity, and therefore similar homologues are likely in other wheat varieties, that lack the duplicated 3' untranslated region (C. Zhang et al., 2019). These homologues provide a potential opportunity for future gene editing with clustered regularly interspaced short palindromic repeats (CRISPR). Regardless, the cloning of Yr28 grants the opportunity to explore functionality and possible background repression of the gene in various wheat genotypic backgrounds through transformation.

Materials and Methods

Much of the protocol presented in this chapter is similar to that described by Sparks & Jones (2009). As with most modern particle bombardment experiments, the Bio-Rad PDS-1000/He device was used to accelerate microscopic metal particles coated in desirable DNA at target tissues where that DNA may be incorporated into the target's genome. There are some key differences in the following protocol to that of Sparks & Jones (2009). These differences include utilization of excised scutella directly without culturing them for one or more days prior, in order to form calli, pre-bombardment. Additionally, the media compositions used herein are slightly different, such as inclusion of osmotic pre- and post- bombardment treatments of the embryos. A larger volume and slightly higher concentration of DNA was used to coat gold particles, in addition to a larger volume of gold solution applied to carrier discs. The gene gun was set to a higher rupture disc pressure coupled with a longer flight distance. Lastly, selection for transformed cells was done at a different point in the regeneration process.

Plant Material

Five hexaploid wheat genotypes were utilized for the following transformation experiments and included Brundage, Fielder, Florida 301, Florida 302 and Florida 303 (Table 3.1). Brundage was the

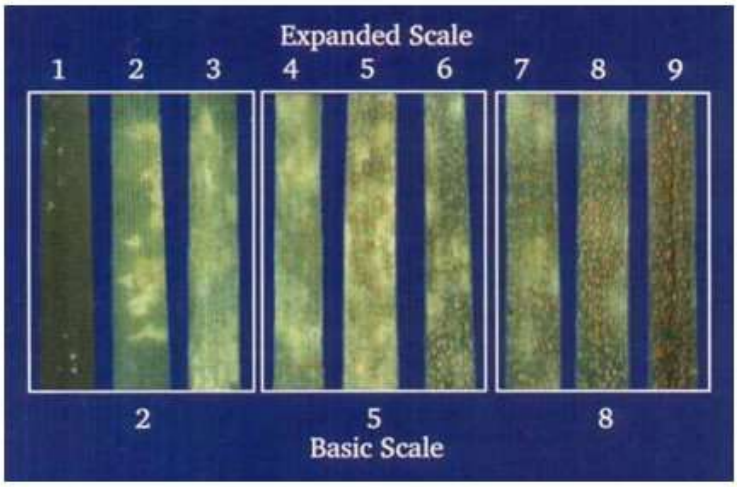
target genotype with the goal of imparting stripe rust resistance, because it is a regionally important variety with high susceptibility to the stripe rust disease. Donor plants for all varieties were grown in a controlled greenhouse environment in Moscow, Idaho, USA, throughout the experiments with a 16-hour photoperiod achieved between the hours of 7:00 a.m. and 9:00 p.m with supplemental lighting (400 W high-pressure sodium), a daytime temperature range of 22°C to 24°C, and a nighttime range of 15°C to 20°C. Seeds of winter and facultative types were rolled in damp germination paper and kept in water in a 4°C refrigerator until sprouted and ready to use (30 days or more to achieve competency for seed-setting). Donor plants were planted in the greenhouse every 10 to 14 days, with six plants to a 17x17 cm pot. Plants were kept in the best health possible with regular watering and pest control schedules. Spikes were collected 12 to 16 days post-anthesis. Spikes could be kept in water in a 4°C refrigerator up to seven days prior to threshing but were used as soon as possible upon removal from donor plants in order to maintain embryo health.

Prior to embryo excision, the immature seeds were threshed from the spikes by hand. They were then surface sterilized with a solution of 70% ethanol, 0.05% Tween 20, and deionized water for five minutes at 250 rpm at room temperature. After draining a solution of 10% bleach, 0.05% Tween 20 and deionized water was added and shaken at 250 rpm for 20 minutes at room temperature. The seeds were rinsed, with hand shaking, and drained three times with sterilized deionized water. For bombardment preparation, embryos were extracted to osmotic media (OS) (Table 3.4). In regeneration testing, conversely, embryos were extracted directly to callus induction medium (CI). Treatments on osmotic medium have been shown by other researchers to limit cell damage done by bombardment in the form of extrusion of the protoplasm, and therefore the treatments improve future regeneration (Altpeter et al., 1996; Vain et al., 1993). Embryos were allowed to “rest” on this OS medium for at least four hours prior to bombardment procedures. During this time the embryos were gathered into a 1.5 cm circle in the center of the plate, scutella-side up, as flush as possible with the medium surface, and with as little space as possible between individuals, but with little-to-no overlap. This optimized the scutella surface area for receiving the DNA-coated gold particles during bombardment. All media used a basic medium composition as the base (Table 3.3).

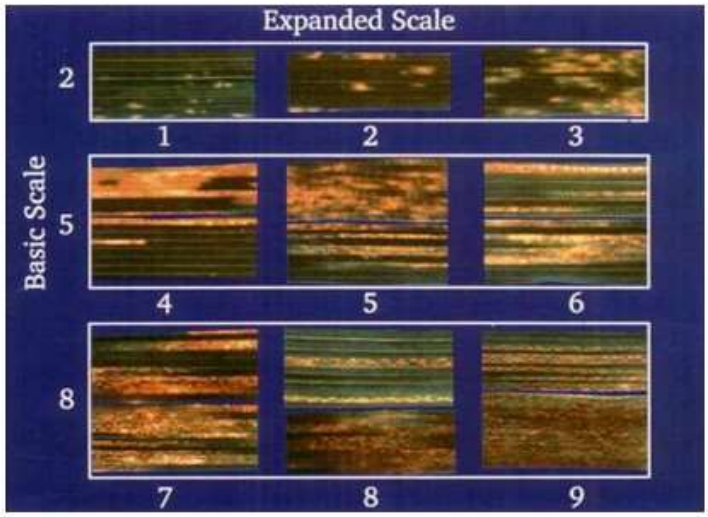
Disease Evaluation

All genotypes utilized for these experiments were tested at Washington State University’s Department of Plant Pathology by Dr. Meinan Wang for their susceptibility to four U.S. stripe rust races, listed later. Seeds were provided and planted on May 27th, 2019 in a rust-free environment. Plants in the one-leaf stage were inoculated on June 7th, 2019 and kept in a dark dew chamber at 10°C for 24 hours, then were transferred to a growth chamber with a 16-hour photoperiod and temperatures

ranging daily from 4°C to 22°C (Personal communication with Dr. Wang). Twenty days post-inoculation the inoculated plants were scored based on their susceptibility or resistance to races PSTv-4, Pstv-37, PSTv-39 and PSTv-41. The scoring scale (Table 3.5) utilized for this test, and all subsequent evaluations, utilize a range (Figure 3.1) presented in a USDA pamphlet published in February 1992 (Line, 1992). Per the test performed by Dr. Wang, all wild-type non-transformed genotypes scored an 8 for every race tested. This score corresponded with a susceptible rating.



Visual scale for seedling stripe rust infection type



Visual scale for adult plant stripe rust infection type

Figure 3.1 Visual scales for recording stripe rust infection types

As taken from Line, R. F. (1992). *Virulence, aggressiveness, evolution, and distribution of races of Puccinia striiformis (the cause of stripe rust of wheat) in North America, 1968-87*.
Top image depicts the scale and visualizations of the disease at its range of severity in wheat seedlings; the bottom image corresponds to the range as it appears in older/adult plants. A rating of 1 to 3 corresponds to a resistant rating, 4 and 5 indicates moderately resistant, 6 corresponds with moderate susceptibility, 7 and 8 indicate susceptibility, and a 9 is highly susceptible.

Plasmids

F2-1 and PC174 from the work of C. Zhang (C. Zhang et al., 2019) were provided to the researcher. Also used was PC1164, linearized by enzyme digestion, described below (Figure 3.2).

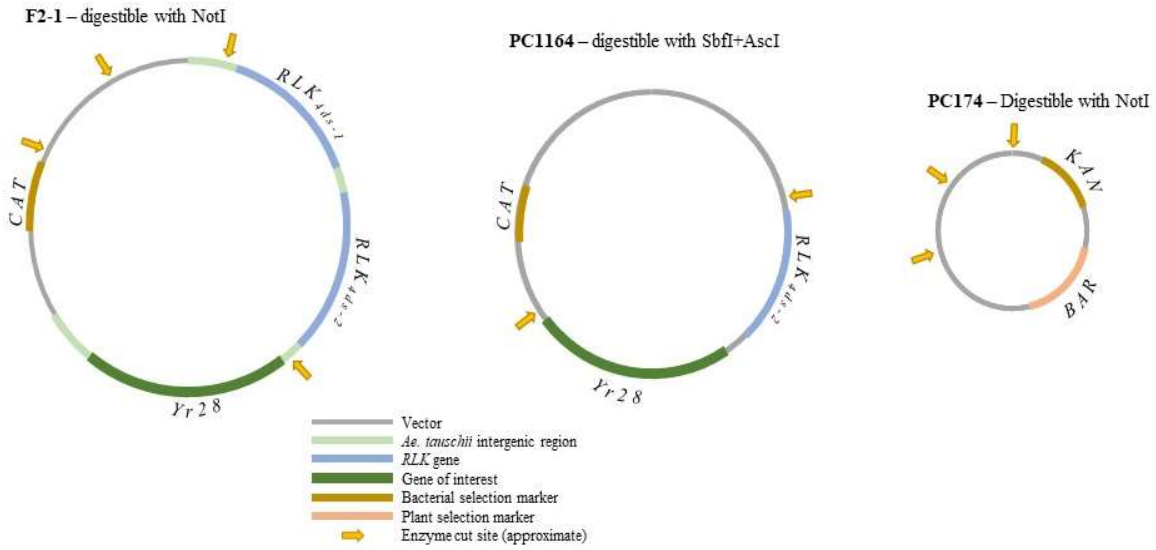


Figure 3.2 Plasmid diagrams indicating genes of interest and marker genes

F2-1 is approximately 47.7 kb total, digestible by NotI into segments of approximately 7.5 kb, 7.5 kb, 11 kb and 21.5 kb. PC1164 is digestible with a combination of SbfI and AscI into segments approximately 2.8 kb and 12 kb and contains two of three native genes from *Ae. tauschii* that are contained in F2-1. Neither of these constructs contains a plant selectable marker, only bacterial selectable markers. PC174 is approximately 10.2 kb digestible by NotI into segments 1.2 kb, 1.5 kb and 7.5 kb and contains only bacterial and plant selectable markers – it was co-bombarded with the other constructs in order to introduce the *bar* gene to transformed wheat. Diagrams are not to scale.

The fosmid F2-1 is nearly 47.7 kb and contains three native genes and intergenic regions from *Aegilops tauschii* with intact promotors and terminators, the third of which is *Yr28*. PC174 contains the *bar* gene under the CaMV *35S* promotor to confer resistance to glufosinate-ammonium. PC174 also features hygromycin resistance, but this was not tested in these experiments. F2-1's insert region is approximately 39.5 kb contained in backbone pCC1FOS (Epicentre Technologies Corp., Chicago, IL, USA) that contains the bacterial gene, *cat*, for chloramphenicol resistance. PC174 is approximately 10 kb with a bacterial resistance to kanamycin. For a few of the early experiments with Brundage, a plasmid containing $RLK_{4DS\text{-}2}$ and *Yr28* on pC414 backbone, corresponding to the second and third genes in the series described above, was linearized via enzyme digestion by SbfI and AscI (hereafter referred to as PC1164/EC). F2-1 and PC1164/EC were both co-bombarded with PC174 for wheat transformation.

DH5α *E. coli* (Zymo Research, USA) were transformed with each construct as follows: 50 µL bacterial suspension of competent cells (thawed over ice) was added to a 1.5 mL tube. Five µL of plasmid DNA was added to the competent cells and cooled on ice for 30 minutes. The tube was heat-

shocked by immersion into a 42°C water bath for 45 seconds and chilled for five more minutes. Then 450 µL LB broth (Table 3.2) was added and the tube was placed in a 37°C shaker for one hour at 200 rpm. In a clean bench, 100 µL of the inoculated competent cell culture was spread on an agar plate containing the bacterial selection antibiotic appropriate for the construct. This plate was incubated at 37°C for 12 to 15 hours. Pre-prepared were two 25 mL tubes with 3.5 mL LB, plus 3.5 µL selection agent in each tube – a colony was sampled with a sterile pipette tip from the culture plate and was then added to the LB mixture which was then cultured for an additional 14 to16 hours at 37°C at 250 rpm.

Then the Zymo Miniprep Kit was used to extract the plasmid DNA. Enzyme digestion and gel electrophoresis confirmed the plasmid identities (Figure 3.3). Once confirmed, a larger volume of LB was prepared at a ratio of 1 mL LB solution to 1 µL selection agent up to 250 mL. Then 50 µL of the bacterial storage solution was added to this volume and incubated at 37°C for 12 to 16 hours at 225 rpm. After this large-scale increase, the Zymo Maxiprep Kit (Zymo Research, Irvine, CA, USA) with minor adjustments was used to extract the plasmid DNA.

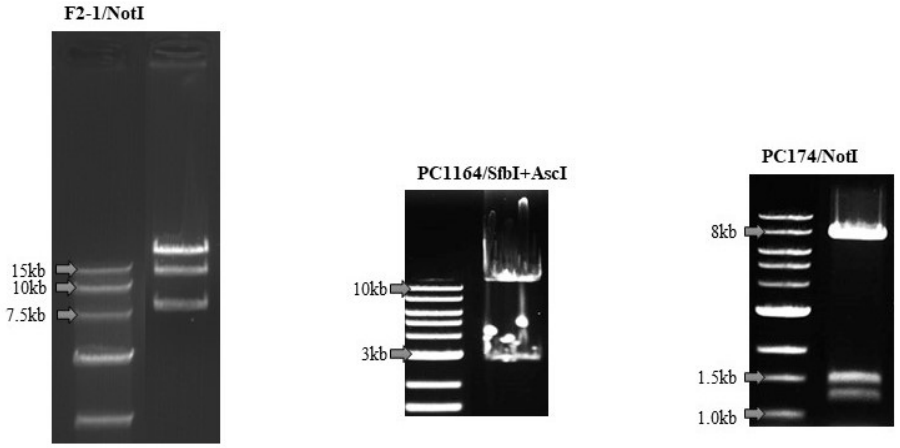


Figure 3.3 Plasmid verification via enzyme digestion

All samples were run on 1% agarose gels for 70 minutes at 110V. F2-1 fragments = 7.5 kb + 7.5 kb + 11 kb + 21.5 kb with a 15kb DNA ladder (Rovalab, Teltow, Germany). PC1164 = 2.8 kb + 12 kb and PC174 = 1.2 kb + 1.5 kb + 7.5 kb – both with a 1 kb DNA ladder (New England BioLabs, Ipswich, MA, USA)

The first large-scale increase of F2-1 was only moderately successful, with concentrations under 300 ng/µL. For transformation it is desirable to have concentrations of over 1,000 ng/µL. A SpeedVac Concentrator (ThermoFisher Scientific, Waltham, MA, USA) was used to increase the concentration to 1,336.1 ng/µL, but this reduced the volume and therefore more DNA increase was needed. In following increases, a Fosmid Autoinduction Solution (Epicentre Technologies Corp., Chicago, IL, USA) was added at a rate of 2 µL autoinduction solution to 1 mL LB to help boost the bacterial

output of the DNA, and various experiments were done to test the optimal time period for incubation. The most successful protocol involved a 5 mL volume of LB pre-cultured with 100 µL bacterial suspension, 10 µL autoinduction solution and 5 µL chloramphenicol (12.5 mg/L) at 37°C, 250 rpm, for 14 hours. That ~5 mL volume was added to a volume increase of 250 mL LB with 1000 µL autoinduction solution and 500 µL chloramphenicol for 8 more hours at 37°C at 250 rpm. This protocol resulted in concentrations around 2,000 ng/µL following use of the extraction kit.

DNA Precipitation on Gold Particles

Gold particles, 0.06-micron size, were prepared in individual aliquots ahead of time in a clean bench. They were prepared at 2 mg gold per 40 µL ddH_2O in individual 1.5 mL tubes. Tubes were kept on ice for five minutes then centrifuged at 12,000 rpm for five seconds at 4°C. The water supernatant was removed then 50 µL 100% ethanol was added, mixed thoroughly by pipetting and then alternately chilled and vortexed for 30 seconds. Tubes were then chilled for an additional one minute before centrifuging again for five seconds. Supernatant was removed and tubes were left to dry in a clean bench. After fully dried, aliquots were stored at -20°C until use.

In order to precipitate plasmid DNA onto the gold aliquots as prepared above, the aliquot was removed from cold storage, suspended with 200 µL ddH_2O and vortexed before chilling for five minutes. Then the water supernatant was discarded, and the plasmid DNA was added. Amounts for each plasmid of the co-precipitation varied based on concentration and molecular size of the DNA but was usually approximately 10 µL each. After mixing by pipette, 225 µL ddH_2O, 250 µL $CaCl_2$, and 50 µL spermidine were quickly added and mixed before alternately chilling and vortexing for 15 minutes. The mixture was centrifuged, and the supernatant discarded. The DNA/gold pellet was rinsed twice with ethanol, then resuspended in ethanol before the mixture was coated on the carrier discs. See Appendix A-1 for protocol specifics. Suspension must be chilled and is best used within two hours.

Bombardment

The PDS-1000/He system by Bio Rad (Bio-Rad Laboratories, Hercules, CA, USA) was used for all bombardment procedures. The settings were a 2.5 cm gap from rupture disc to carrier disc, a 5.5 cm target distance from the stopping screen to embryos and a 0.8 cm distance between the carrier disc and the stopping screen. A pressure of 1,100 psi to break the rupture disc was achieved by setting the regulator pressure to 1,300 psi. A 26” Hg (-8.6 bar) vacuum pressure was used inside the chamber for each bombardment.

All elements of the device had to be sterilized before each use. Sterilization was done with a liberal application of 100% ethanol spray inside and out of the machine and dip sterilization of all discs, screens and other removable parts in 100% ethanol. All sterilized elements were allowed to air-dry in the clean hood prior to assembly and use. Care was taken to maintain a sterile environment during assembly and bombardments. The embryo circles gathered on osmotic medium (OS) were centered inside the machine on the target level with lids removed. Bombardment itself was carried out per the manufacturer's directions.

Media Compositions and Cultures

After bombardment, embryos remained on OS at 23°C in the dark. The initial transfer to callus induction medium (CI) occurred 16h post-bombardment. Calli were transferred to callus induction media plus a selection agent (CI+) after two weeks on CI in order to limit cell growth of untransformed cells. Regeneration medium (RM) that was used for the four weeks thereafter, with one media refreshment between, also contained the same level of selection agent as CI+ media. CI and CI+ cultures were maintained at 23°C in the dark, whereas RM cultures were kept at 23°C with a 16-hour photoperiod, as were root development (RD) cultures in glass vessels. Table 3.4 contains the media compositions.

After sufficient development of shoots (generally 1 cm or greater), possible regenerants were transferred to RD, that also contained the herbicide selection agent. The amount of time in RD varied from two weeks to one month to allow for adequate root growth, which was generally up to 3 cm or greater. When shoots had failed to form in four weeks on the second RM, a third media refreshment was performed and maintained up to an additional four weeks, to account for later development of shoots.

Transfer to Soil

Putatively transformed seedlings that survived selection in the RM and RD stages were transferred to potting mix after sufficient root development (as described above). In order to allow the acclimation and development of tender plant tissues such as a cuticle, a humid environment for the seedlings was achieved by covering seedling pots with a plastic film tent for approximately five days. After film removal, seedlings were carefully watered by hand so as not to bury basal tissue until such time as the established seedlings could withstand regular watering and were large enough to allow for leaf painting with herbicide, then vernalization and/or stripe rust inoculation was performed.

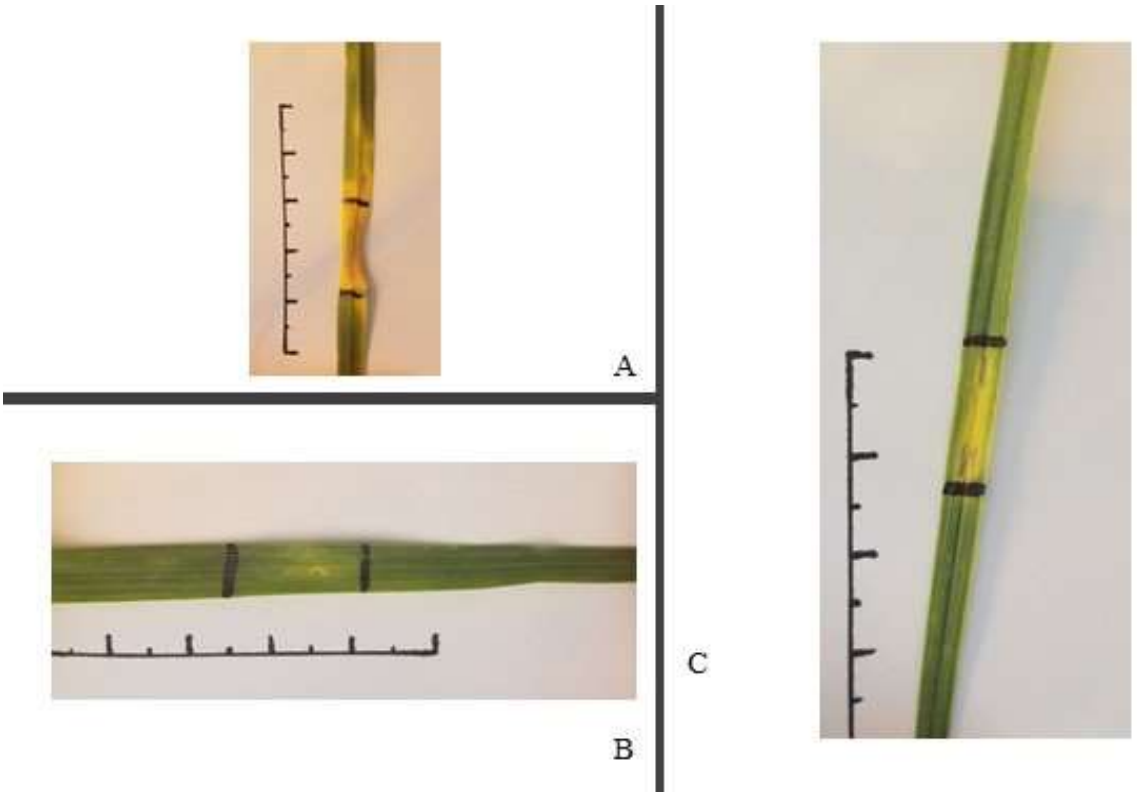


Figure 3.4 Tissue responses to leaf painting examples

In order to assay for the presence of the *bar* gene in putative transgenic seedlings, a solution of glufosinate-ammonium (0.12 g/L) was painted on the front and back of an area about 1.5 cm to 2 cm square of one of the lower leaves of every tiller. Four to five days thereafter cell death would occur on plants that lacked the *bar* gene expression, and plants that had an active *bar* gene would have tissue that remained healthy. Images feature leaves of different genotypes. A: Definitive susceptible reaction characterized by cell mortality indicating *bar* gene is either not present or not active. B: Tolerant reaction indicative of the expression of the *bar* gene. C: Early indication of susceptibility to the herbicide.

Leaf Painting with Herbicide

Presence of the *bar* gene was tested for by painting a portion of the lower leaves of seedlings at the 3-leaf stage with a glufosinate-ammonium and water solution (0.12 g/L). Plants were tested at the three-leaf stage: any tillers that had at least three leaves and were large enough to test were included. An area of approximately 1 to 2 cm square was demarcated with a permanent marker on a secondary leaf and the herbicide solution was applied with a swab within this area to both front and back of leaves. Five days post-application, tolerance was determined by healthy, normal-looking, undamaged tissue, whereas susceptibility was determined by marked tissue discoloration, wilting, and necrosis. Plants displaying tolerance were deemed to possess the *bar* gene which confers herbicide tolerance by coding for phosphinothricin acetyltransferase which inactivates the primary toxin of the herbicide (D'halluin et al., 1995).

Vernalization

Winter and facultative cultivars were given a vernalization treatment after the status of the *bar* gene was determined and prior to inoculation. The vernalization treatment involved storage of the plants in a chamber with an 8-hour photoperiod and a constant temperature of 3°C. All applicable plants were given a treatment of at least 30 days, winter varieties were given 60 days or greater as time allowed. This treatment is required for these varieties to have a full life cycle and set seed. The exception to this order was the Brundage #10 T_1 family and plants #37 through #40 that were vernalized after inoculation due to time constraints and the desire to have phenotype validation data for those plants as soon as possible.

DNA and RNA Extraction

DNA extraction was done with one of three methods: SDS, CTAB or MagBead (Pacific Biosciences, Menlo Park, CA, USA) extraction with the KingFisher device (ThermoFisher Scientific, Waltham, MA, USA). Separate tissue samples were collected, and RNA extraction was done with the TRIzol (ThermoFisher Scientific, Waltham, MA, USA) method. The RNA then underwent DNaseI treatment prior to cDNA synthesis to form a more stable double-stranded product. The protocols utilized are presented as A-3 through A-6 in Appendix A. RNA extraction through cDNA synthesis are all listed as a single protocol.

PCR Testing

Polymerase chain reaction and agarose gel electrophoresis were used to detect the presence of *Yr28* in putative transgenic plants. The forward primer for DNA confirmation was located within the gene's promotor region and had a 5' to 3' sequence of TGTGTCATGTTTGGTCGATAGG. The reverse primer was within the first exon of the gene, with a 5' to 3'sequence of TCCTCCCTTGTAGCTTCACG. The amplified fragment should have been 727 bp if the gene was present. After DNA extraction, PCR was carried out with the components as stipulated in Appendix A-7, and the conditions below.

Components included an enzyme-DNA buffer, ultrapure water, forward and reverse primers, Taq DNA polymerase, dNTP's, and template DNA. The reaction set-up for DNA PCR was 40 cycles of 94°C denaturation for 30 seconds followed by 59°C annealing temperature for 30 seconds and extension at 72°C for 45 seconds and followed by a hold temperature of 15°C indefinitely until samples were retrieved.

Samples were separated on 1% agarose gels at 120 V to 170 V for 20 to 35 minutes depending on gel size and space between well-rows. All PCR testing contained a positive control of PI 511383 DNA,

which natively contains the *Yr28* gene, a negative control of the wild-type non-transgenic plants' genotype, as well as a negative control of water.

cDNA was produced from plant total RNA using reverse transcriptase (M-MLV RT) as described in Appendix A-6. RNA testing involved two consecutive PCRs; products were separated by agarose gel electrophoresis. Prior to first round PCR, the plants' cDNA was diluted 2x, then that dilution was used as the template DNA for the reaction. The PCR components for the PCR reactions included 13.5 µL ultrapure water, 4 µL 5X Taq reaction buffer, 1 µL template DNA, 0.3 µL Taq DNA polymerase (house-made), 0.4 µL dNTP's, and 0.4 µL forward and reverse primers (Appendix A-8). The first cycle used a primer that was within in the gene's fourth exon with a 5' to 3' sequence of TAGTTCAAGCGTGAGCAAACC. The reverse primer was within the seventh exon with a 5' to 3' sequence of CCATGTTTCTTCACCAGCTG. This should result in a 3,933 bp fragment if the gene were expressed. *Actin* was used as the control to verify the reactions functioned properly. The primers for that gene had the 5' to 3' sequences of TATGCCAGCGGTCGAACAAC and GGAACAGCACCTCAGGGCAC and should have resulted in a 438 bp fragment.

The second round of PCR utilized a forward primer with the 5' to 3' sequence of CTGTAGTTGAACTCGAATTGGG and the reverse primer 5' to 3' sequence of ATGGCTGATGCTTTTCCCCG. The forward primer fell in the fifth exon and the reverse in the sixth. This should have resulted in a 679 bp fragment if *Yr28* were expressed in the plants tested.

Reaction one set-up was 35 cycles of 94°C denaturation for 30 seconds followed by 58°C annealing temperature for 30 seconds and extension at 72°C for 150 seconds and followed by a hold temperature of 15°C indefinitely until samples were retrieved.

Reaction two set-up was 35 cycles of 94°C denaturation for 30 seconds followed by 60°C annealing temperature for 30 seconds and extension at 72°C for one minute and followed by a hold temperature of 15°C indefinitely until samples were retrieved. Resulting PCR products were separated on 1% agarose gels for 30 minutes at 170 V.

Inoculation

Inoculation of transgenic plants and their untransformed wild-type counterparts as controls was done with field-collected spores as a ratio of 1:20 with talcum powder. Total volume varied per number of plants to be inoculated but ranged from 10 mL to over 20 mL. Plant leaves were misted with water to create a simulation of dew, then the powder mix was applied with a small hand aerosol duster. The plants were then enclosed in a large tub connected to a humidifier. The humidifier was set to maintain a humidity level of 100% with misting at a high level, this meant that mist was introduced constantly

into the dark tub, simulating a dew chamber. This was carried out in a chamber set to a constant 10°C. This setup was maintained for approximately 36 hours, after which the plants were taken out of the tub and let to progress in the chamber with a regular 12-hour light cycle.

In initial inoculation procedures for plants #2 though #16, before and after the 36-hour 10°C treatment, plants were kept at a diurnal range of 5°C to 20°C with a 12-hour photoperiod. Experiments thereafter had to be conducted differently due to a failure of that chamber during which it got up to 50°C and did not cool. All inoculations thereafter had no temperature differential and were therefore maintained at 10°C with a 12-hour photoperiod for the duration of disease development until shortly after phenotypic evaluation. When moving plants from the chambers to the greenhouse, plants were acclimatized after evaluation if the environments were significantly different between the chamber and greenhouse. Immediately after moving plants into the greenhouse, fungicide was applied to allow plants to complete their life-cycle disease-free.

The exact race composition of inoculum for plants #2 though #21 is unknown, as testing was not done. However, based on race surveys done in the region by the Washington State University ARS Extension (Pullman, WA, USA) the top races were likely composed primarily of PSTv-37, PSTv-52, PSTv-47, and PSTv-322 (Chen, 2017). These spores were hand collected from Dr. Daolin Fu's plots at Parker Farm (Moscow, ID, USA) in June of 2017. Inoculations of plants #22 and beyond was done with spores that were hand collected from WSU stripe rust test plots (Pullman, Washington, USA) on July 3rd, 2019 by the researcher. Testing of races from that field indicated predominant races again included PSTv-37 and PSTv-47 (courtesy of Dr. Meinan Wang – WSU). Collected spores were desiccated in a drying vessel at 4°C for approximately five days, then stored in -80°C until use.

Phenotype Scoring

The scales in Figure 3.1 and Table 3.4 were used to score plants approximately 21 days after inoculation. Photographs of leaf samples were taken as a verification of score, but plants were scored based on the evaluation of the whole plant. If plants did not have enough leaves to warrant removal of one or two for photographs, but the plants could be scored, no photographs were taken.

Senescence and Harvest

After phenotypic evaluation, plants were moved back into the greenhouse and treated with fungicide as needed to eliminate stripe rust and other fungal pathogens such as powdery mildew or leaf rust, which were sometimes present as well. Plants were kept healthy with regular watering, fertilization and pest control as needed until natural senescence. Once plants were fully desiccated, spikes were individually harvested by hand and recorded. All seeds were hand threshed to reduce the loss of seeds

due to breakage that occurs with machine threshing, and to eliminate the possibility of environmental escape of genetically modified material. Any remaining plant material and associated soil was autoclaved at 121.8°C for at least 30 minutes prior to disposal.

Appendix A-2 diagrams a Workflow Chart that gives a visual layout of the basic steps of the transformation process as described in this work. There was some minor variation on the timings listed in the chart due to inherent differences in plants development and other factors.

Data Analysis

Comparisons of mean transformation efficiency across all varieties was made using Analysis of Variance (ANOVA). Fisher's Least Significant Difference (LSD) test was used to evaluate how groups varied from one another and to determine the significance of those differences, if any. Results were confirmed using Bonferroni testing. These tests were completed using R software version 3.6.1 (R Core Team, 2019).

Results

A total of 33 independent experiments with 142 bombarded plates of embryos on OS media were carried to completion. These experiments equated to more than 11,000 embryos bombarded with DNA, of which over 2,900 were with PC1164/EC+PC174 and the remainder with F2-1+PC174 (Table 3.6). In terms of the number of embryos bombarded, Brundage far exceeded the other genotypes, with nearly seven times more than the next highest variety. Brundage was the genotype of choice with the goal of introducing stripe rust resistance. The other genotypes largely served to provide transformational data comparisons, as they are currently agronomically unimportant varieties. All genotypes included could also provide data for possible future transformation experiments.

Sixty-three total T_0 plants were produced and tested from these experiments, but only 24 of them were positive for *Yr28* through PCR assay of the DNA. Although the experimental efficiency overall seems to be 0.56% when all plants produced are counted, the true overall transformation efficiency is only 0.21%, which is very similar to that achieved by Vasil et al. (1992) of 0.20%. The calculation adjustment from 0.56% to 0.21% accounts for those plants that were assured, through PCR assay, to be transgenic. Presence of the *bar* gene was tested *in vivo* for these experiments via leaf painting rather than through PCR investigation. Comparison of assays for the presence of the *bar* gene to the PCR results for *Yr28* showed that there were 27 plants tolerant to herbicide, and 24 plants that tested positive for *Yr28* in PCR investigation. Some of these were not the same plants, however. Tables 3.6, 3.7, and 3.8 provide information about transformation, resulting transformed lines and results of assays for the *bar* gene. The results suggest that the two co-bombarded constructs did not co-integrate

in the genome in every transformation event, although further testing would be needed to confirm this conclusion. Table 3.9 indicates there were 63 plants, up to #45 produced, but any plants beyond #40A, B, and C could not be adequately phenotype or RNA tested due to time constraints.

DNA was extracted from every plant produced and tested with the primers described previously. These primers targeted within the promotor region of *Yr28* and the gene's first exon to create a band that was equal to 727 base pairs, if the gene were present. This PCR confirmation (Figure 3.5) features the 24 lanes showing an insertion of *Yr28* for those plants. Other bands that are easily distinguishable are those of PI 511383, which acted as the positive control. Note that #31B and #32 were retested in the second to last gel (orange arrows), and that for #32 only two of the four tested tillers possessed the gene of interest.

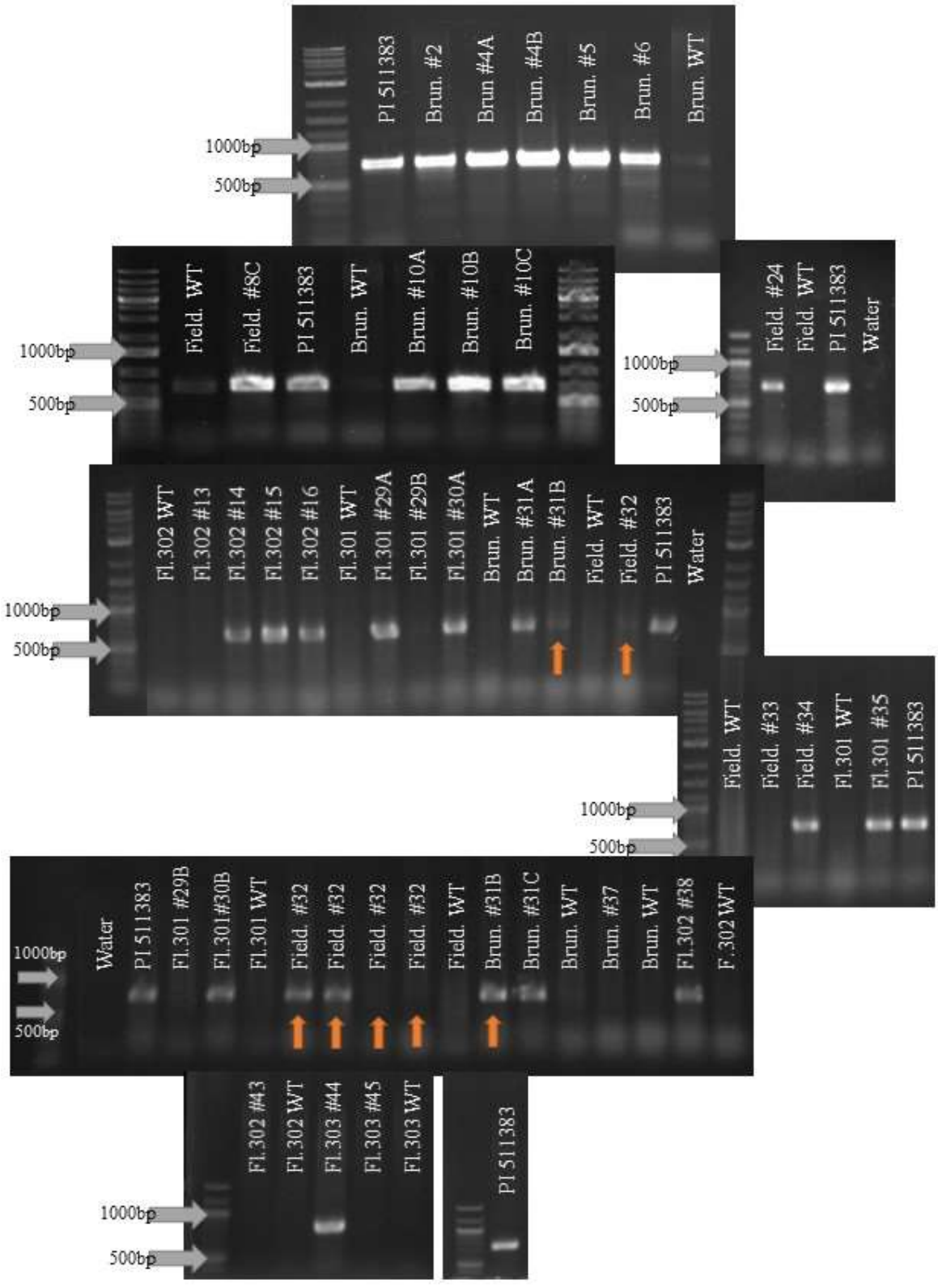
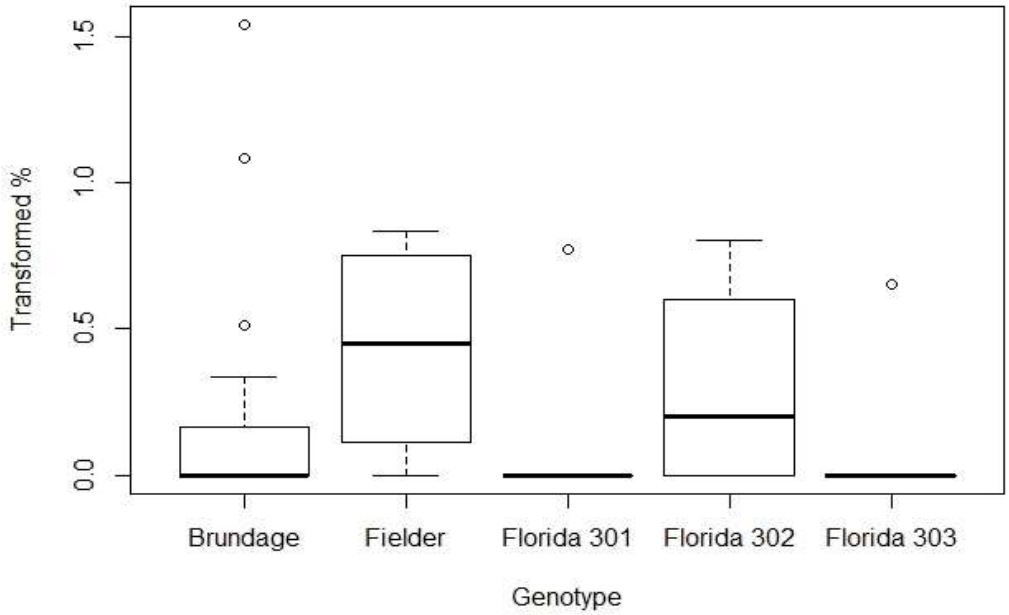


Figure 3.5 PCR results of T_0 plants #2 through #45 positive for Yr28 in PCR investigation
The selection of plant DNA tested with primers that resulted in a 727 bp fragment from *Yr28* is shown. Orange arrows (top) indicate plants where the likelihood was that only one tiller of more than one initially sampled for DNA extraction was positive for the gene, and retesting (arrows below) concluded this was true.

When statistical analysis was done in R regarding transformation with *Yr28* DNA, it was determined by a Student's t-test that the mean transformation rate was not 0% but was instead calculated to be 0.23%. Calculated mean transformation rates from R were 0.43% for Fielder, 0.30% for Florida 302, 0.23% for Brundage, 0.17% for Florida 301, and 0.13% for Florida 303. A boxplot of the transformation rates for each genotype show that most replicates for Brundage, Florida 301, and Florida 303 had a transformation rate of 0% (Figure 3.6, A). The mean transformation rate of replicates of Fielder and Florida 302 was above 0% but less than 0.5%. ANOVA analysis determined there was no significant difference between the transformation rates of the genotypes. The assumptions for ANOVA were verified to satisfaction with a mean of residuals approximating 0, homogeneity of variance, and relative normality of residuals (these can be visualized with graphs produced by the software (not shown)). Additionally, Fisher's LSD test was performed. One can see in a plot of transformation rate ranges (Figure 3.6, B) that all genotypes are within a single group, and therefore there was no significant difference between them.

A:



B:

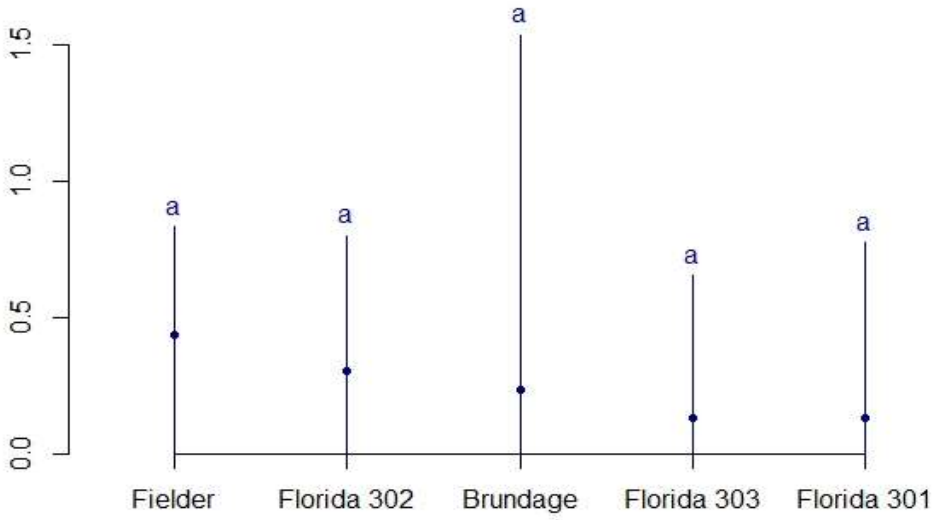


Figure 3.6 R plots of transformation rate data

Transformation rates are derived from the number of transgenic plants produced in an experiment compared to the number of embryogenic calli. A: Boxplot of transformation rate data. For Brundage, Florida 301, and Florida 303 the heavy line indicates that the median of the data is at 0%. Medians for Fielder and Florida 302 are higher, but still below 0.5%. Dots indicate outlier data points, of which there were three experiments for Brundage that surpassed 0.5%, and one each for Florida 301 and Florida 303 which represent the only experiments for those particular genotypes that yielded plants. B: LSD plot of transformation rate ranges, below, indicated all genotypes were within a single group and therefore had no significant difference. Lines represent the range of transformation rates for each genotype, whereas the dots within the line are the calculated mean of the data.

cDNA expression testing was done on plants affirmed to have *Yr28* with DNA PCR. In Figure 3.7 one can see bands of the appropriate length in the second lane of the gels for T_0 plants #2, #4A and #4B, #5, #6 (Image A), #10A, #10B, and #10C (Image B), #24 (Image C), #31A (Image F) #31B, #31C (Image D), #32 (Image E, sample taken from tiller two of the four DNA lanes shown in Figure

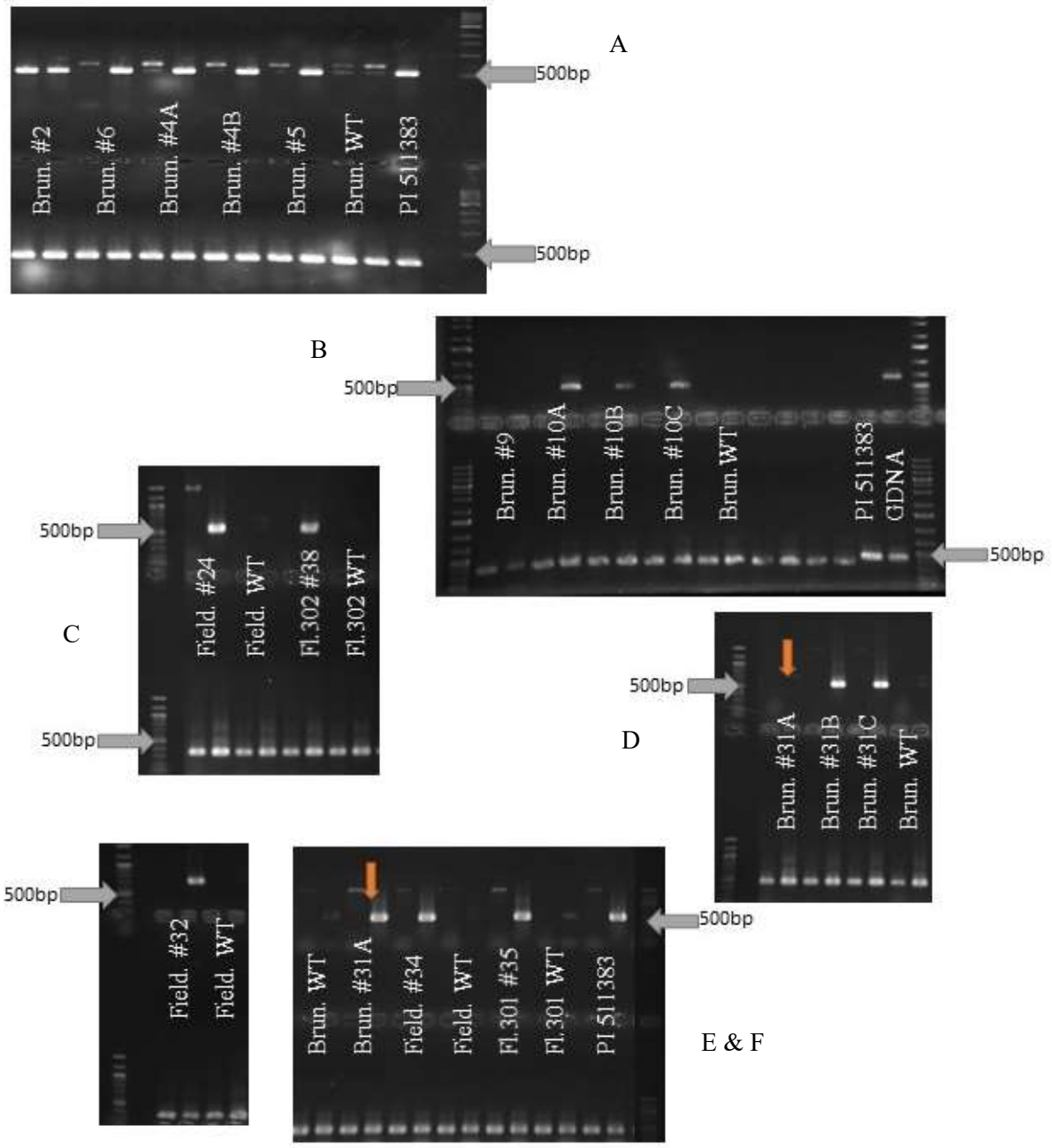


Figure 3.7: cDNA PCR assay for expression of *Yr28* in T_0 transformed plants

Each plant's results occupy two lanes due to the two-cycle PCR testing. The initial amplification is not generally visible (one can see the first round product for plants #24 in image C and #35 in image F for example), but the presence of a band for *Actin* at 438 bp confirmed that the amplification was sucessful for all samples. The orange arrow in image D indicates where the initial testing for plant #31A was not sucessful, but a fresh tissue sample was taken, and more care was taken during extraction and thereby yielded results featured in image F.

3.5 prior), #34, #35 (Image F), and #38 (Image C). The lower row features the results for *Actin*. For the control on the #10 gel (Image B), PI 511383 gDNA was used rather than cDNA, and therefore the band was larger, as it contained intron sequences as well as exon sequences. cDNA expression could not be tested for plants #41 through #45 in time for publication.

Despite the number of plants produced, there were only 10 plants were resistant to stripe rust. Unfortunately, not all plants were reliably inoculated with the disease due to reasons explored in later discussion. Regardless, plants that were given phenotypic scores indicative of resistance were believed to be reliable. This conclusion was drawn due to the inclusion, and subsequent comparison, of control plants of each tested genotype in addition to whole-plant evaluation. Some notable results will be shown in figures to follow. Of the ten T_0 plants that showed an increased resistance to the disease, three were possible clones of #10 and three were possible clones of #31, all of the Brundage background. Two of those ten plants were of the Fielder background (#24 and #34) and the last two were from Florida 301 (#36) and Florida 302 (#38) respectively.

For comparison purposes the phenotypic responses of T_0 plants #2 through #6 are included. Although these plants were positive for the DNA and cDNA PCR assays for *Yr28*, they showed no increased resistance to the disease (Figure 3.8). The difference between leaf samples was unremarkable, and likewise differences in the whole plant comparisons were undetectable. Plants #2, #4A, #4B, #5 and #6 scored as 8, 7, 8, 7, and 8 on the USDA scale, respectively. The wild-type (WT) Brundage controls scored as 8. When later plants were inoculated and evaluated, however, there was a detectable difference in phenotypic responses. Three plants: #10A, #10B and #10C showed a hypersensitive phenotypic disease response after inoculation, which is indicative of resistance. This inoculation occurred on May 6th, 2019. Hypersensitive response is demonstrated in Figure 3.9. These plants were reinoculated (June 14th, 2019) fourteen days after the initial evaluation. This assured that leaves in the adult plant stage would be reliably exposed to the disease. At evaluation thereafter plants scored 4, 6, and 5 on the USDA scale (#10C did not have enough leaves to warrant the removal of one for a photograph but was scored nonetheless) versus 8 for the WT control (Figure 3.10).

Figure 3.8 Leaf samples from Brundage-derived T_0 plants #2 through #6

Photos taken of leaves from the plants listed above are indicative of whole plant disease response approximately twenty-one days post-inoculation. There was no remarkable disease resistant phenotype shown amongst these plants.

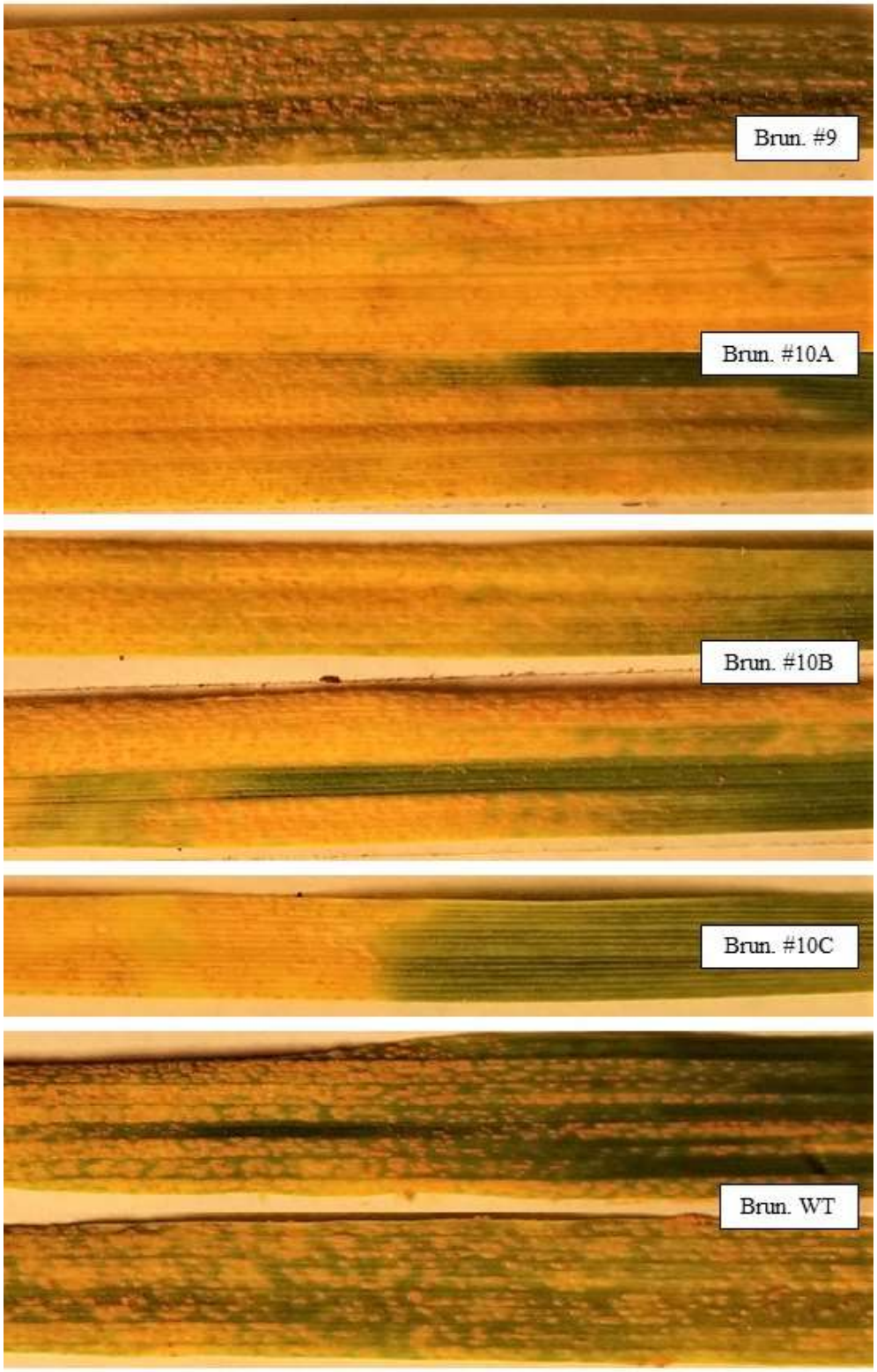


Figure 3.9 Leaf samples from Brundage-derived T_0 plants #9 and #10

Plants #10A through #10C show an early hypersensitive response when evaluated approximately twenty-three days post-inoculation, which was exemplified by the chlorosis present in the samples.

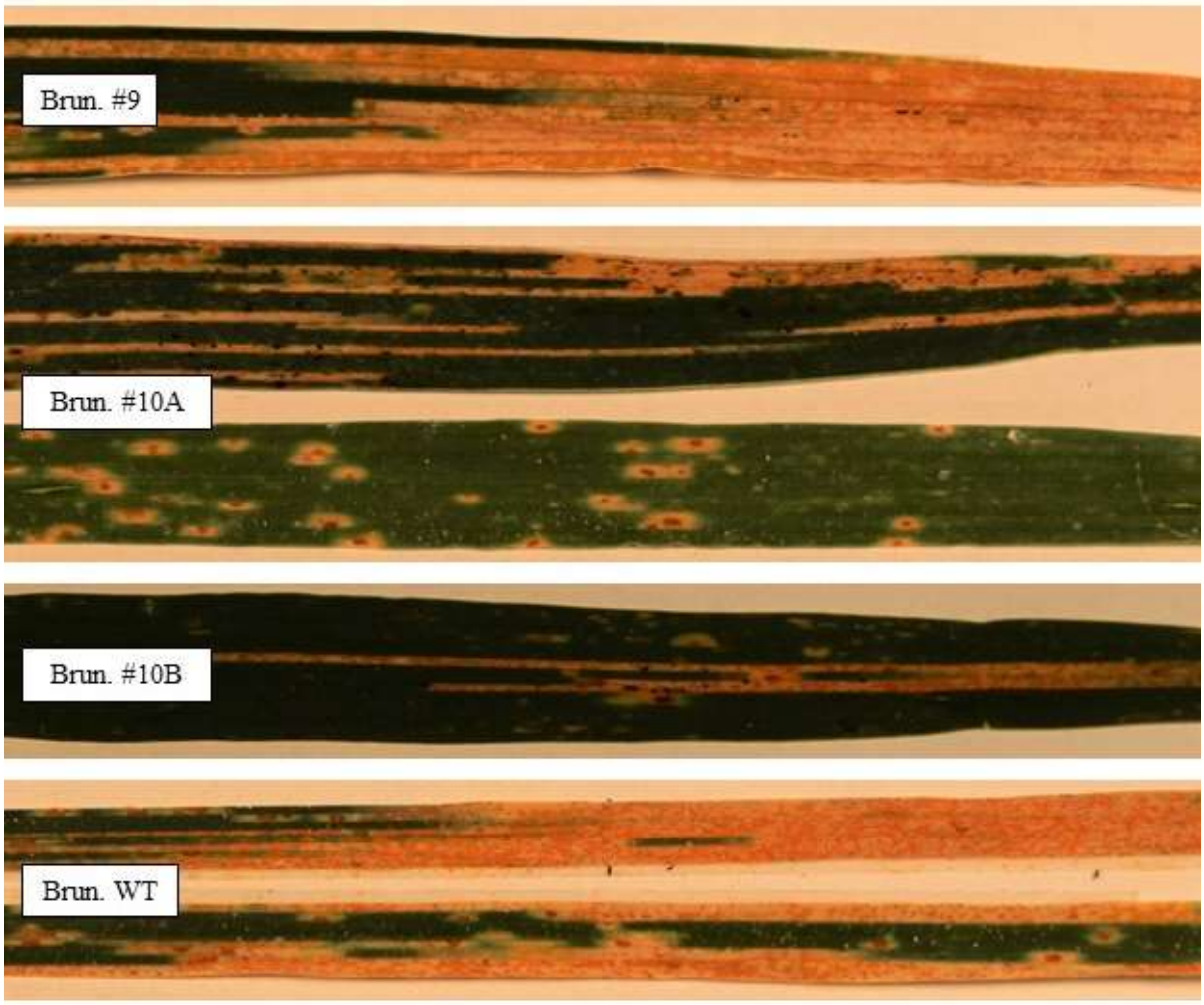


Figure 3.10 Leaves from Brundage-derived T_0 plants #9 and #10, after second inoculation

After a second inoculation and following the twenty-one-day period, the resistant response is indicated by smaller regions occupied by stripe rust pustules and necrosis, or by a lack of stripe rust spores (as was the case for the lower #10A leaf sample, where only leaf rust spores were present) when compared to the control plant or the untransformed plant #9.

Another example of disease resistance was later present in plant #24 which was produced from the Fielder genotype. It was a result of the transformation procedure KJ18, which produced 8 putative plants but only one that was transformed. In many cases, plants resulting from a single procedure had similar maturities and were therefore included in the same round of inoculation. Having many plants to test at once allowed for reliable comparison of the different plants' phenotypic responses to the disease. In Figure 3.11 the plants underwent inoculation on the same date. A lack of pustules and sporulation of stripe rust was evident on plant #24 at the time of evaluation. Plants in the replicate were scored as 7 or 8, with the exception of plant #24, which scored 2.

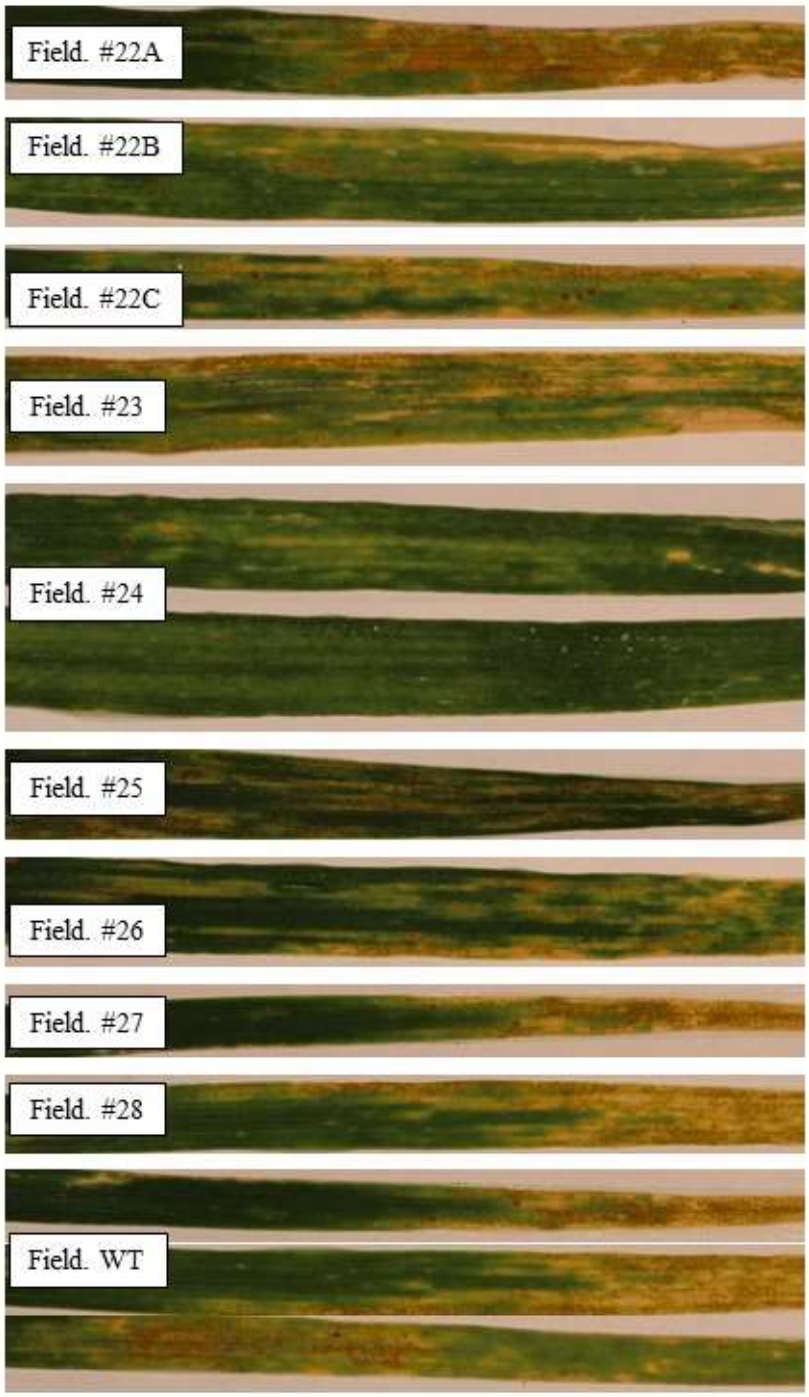


Figure 3.11 Leaves from Fielder-derived T_0 plants #22 through #28

Leaves from plant #24 have no stripe rust sporulation (there is slight sporulation present from leaf rust) and have very mild chlorosis, versus prolific sporulation and chlorosis in other samples.

Plants #31A, #31B, #31C, #34, #35 and #38 also featured distinguishable phenotypically resistant responses. Plants #31A-C, of the Brundage background, all scored at 3. Plants #34 and #35, of the Fielder and Florida 301 backgrounds, scored 3 and 4 respectively, and plant #38, of the Florida 302 background, was scored 6. Although more difficult to immediately distinguish, the pustules and sporulation on plant #38 were more infrequent and the fungal colonies less dense than that of the control plants which scored 7. As a rating of 6, this classifies plant #38 as moderately susceptible, rather than the control plants that were definitively susceptible at ratings of 7 and 8.

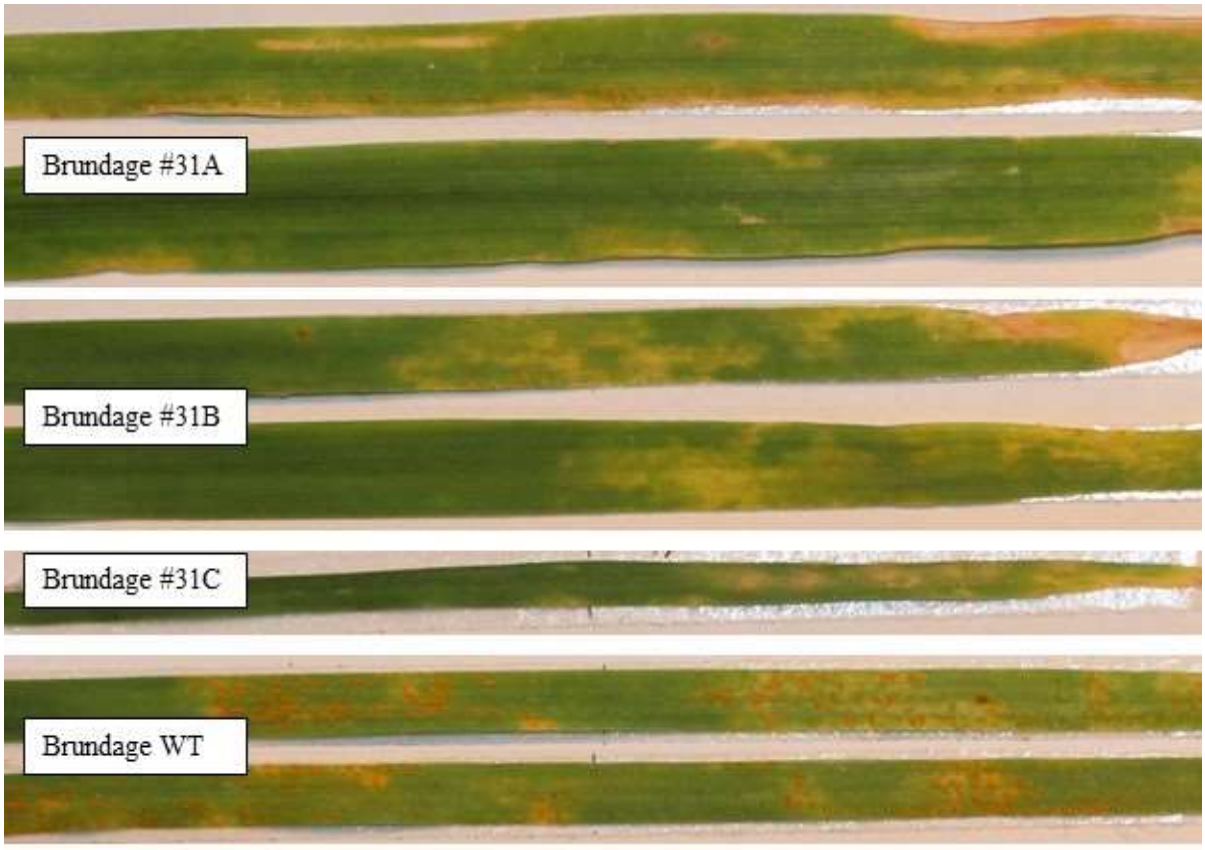


Figure 3.12 Leaves from plants Brundage-derived T_0 plants #31A, B, and C

Leaf samples of transformed Brundage-derived plants displayed reduced stripe rust infection, distiguished by less sporulation when compared with control (WT) leaf examples below. The pattern of chlorosis seen on the transformed leaf samples was indicative of a hypersensative phenotypic response to the disease.

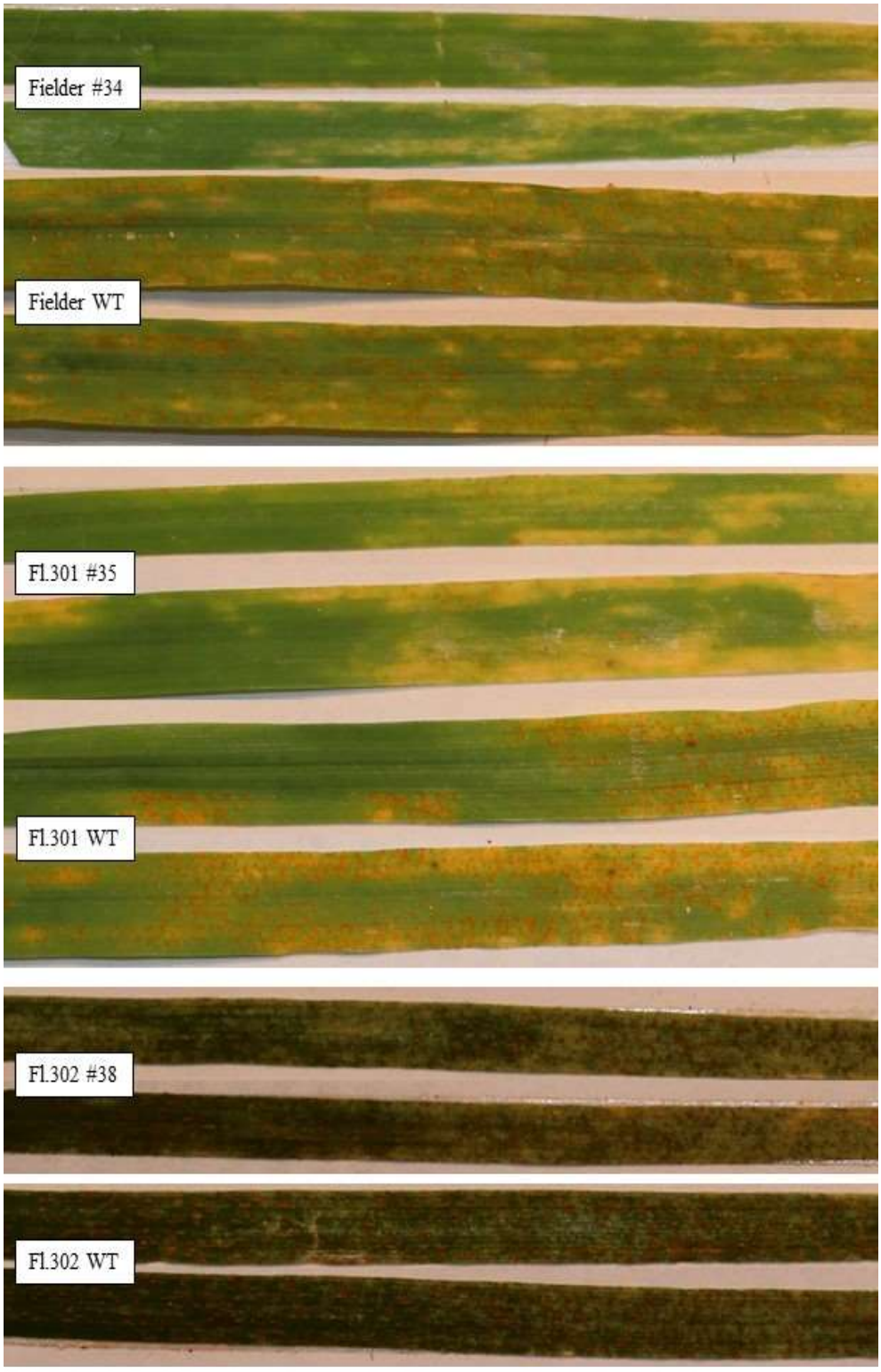


Figure 3.13 Leaves from T_0 plants #34, #35 and #38, and controls

One can see distict differences between plants #34 and #35 and their untransformed WT counterparts. Though less visually obvious from the photographs, #38 displayed a reduction in spoulation based on colony size and frequency. Plant #38 was scored a 6, which classifies it as moderatly susceptable, versus the control plants that were scored at 8, classifying them as susceptable.

Some plants were carried into the T_1 generation for additional testing. Seeds from Brundage-derived plants #2 through #6, and Fielder-derived plants #7 and #8 were collected, recorded, and all planted for the T_1 generation. Each seed was counted as an individual and numbered based on which spike from the parent plant it came from. For example, plant #8C had four spikes that produced seeds so the numbering system would record the third seed from the second spike as 8C-S2-3. This T_1 generation from plants #2 through #6 died because of a chamber failure wherein the chamber got up to 50°C and did not cool for over 12 hours just after inoculation. Fortunately, tissue was collected prior to plant death. Therefore, phenotype data was not available for those plants. Although the T_1 generation from Fielder-derived plants #7 and #8 were inoculated together and did not die, the inoculation was not satisfactory and reliable phenotype data was not obtained for those plants. Despite these drawbacks, genotypic investigation was conducted.

Although the T_1 generation of Brundage from plants #2 through #6 died, thereby excluding the possibility for evaluation of phenotypic responses to the disease, tissue was gathered prior to their death. This allowed for testing of DNA and expression for *Yr28*. Figure 3.14 demonstrates that there were individuals that carried the gene of interest. There were 20 productive spikes from the T_0 plants and testing concluded that 11 were positive for *Yr28*. Although this is not a Mendelian 3:1 ratio, the

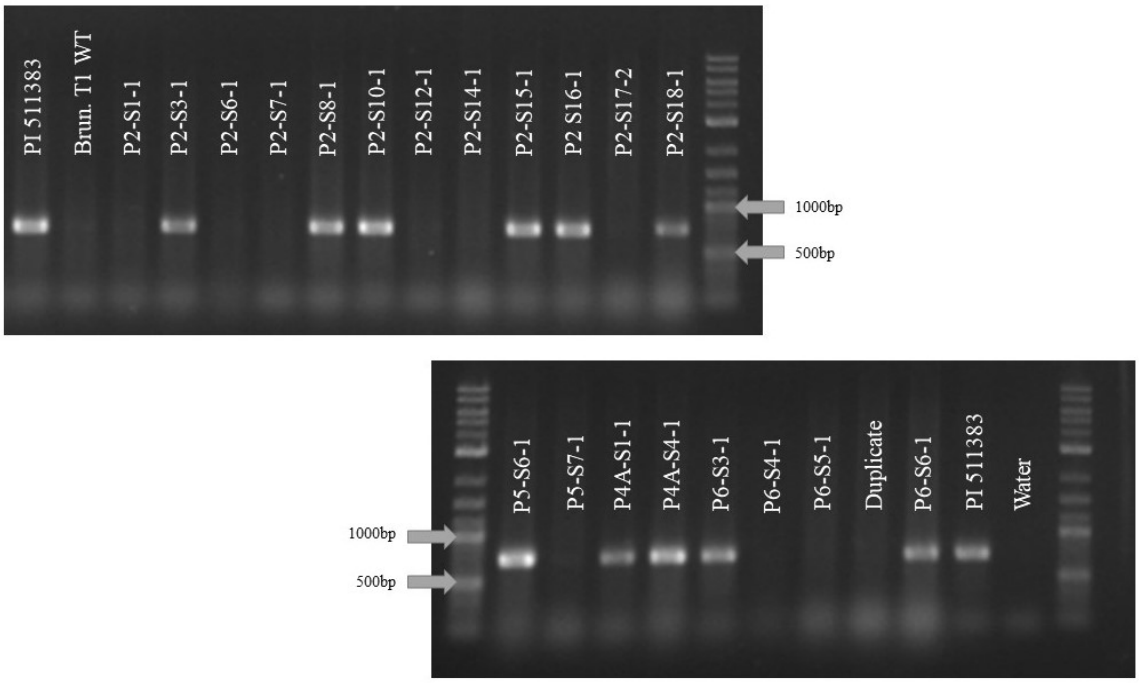


Figure 3.14 DNA results from Brundage #2-6 T_1 generation

One individual plant produced from each productive spike of T_0 Brundage-derived plants #2, #4A, #4B, #5, and #6 was tested with a PCR assay for *Yr28* DNA

tested individuals did not encompass the entire population, and there was a possibility that some of the T_0 plants had untransformed spikes.

Individuals were selected at random from spikes that tested positive for *Yr28* DNA in order to extract RNA. Approximately half the total number of individuals of positive spikes were included in this testing. For example, Brundage-derived T_0 #2 plant's spike 8 produced 20 successful T_1 progeny, and therefore RNA was extracted from nine of them. In the case of T_0 plant #6's spike 1, it produced three successful T_1 progeny and two of their RNA was extracted, converted to cDNA via reverse transcriptase and then tested.

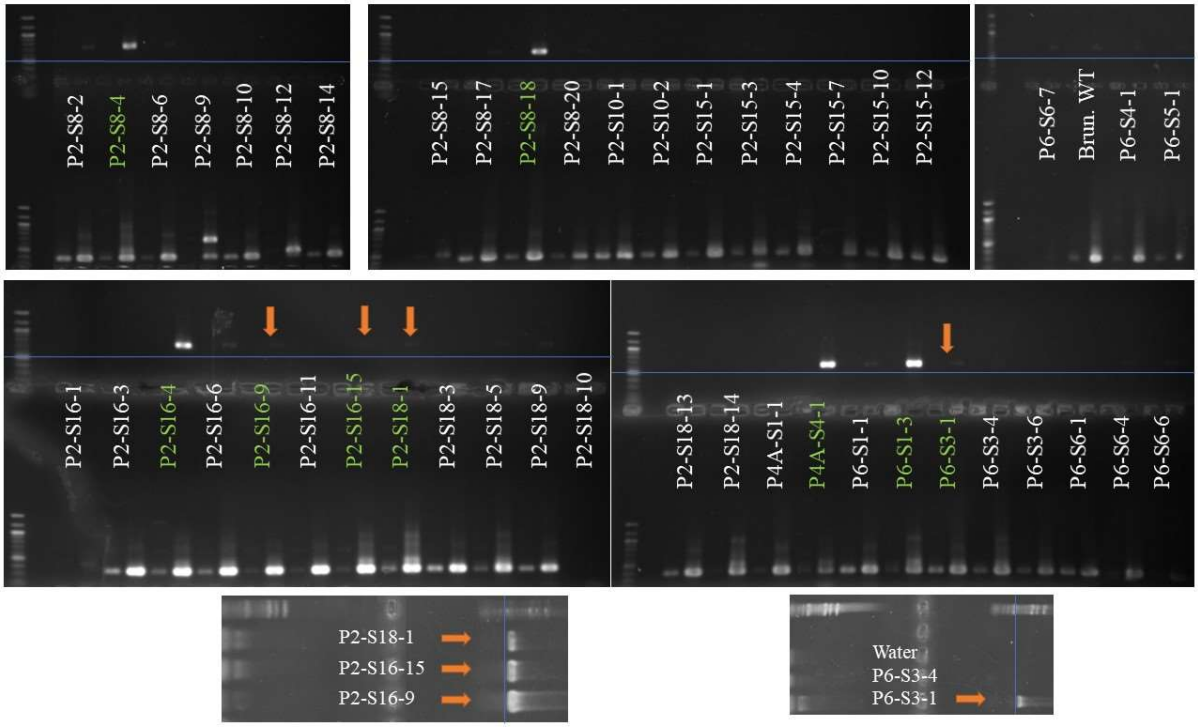


Figure 3.15 cDNA results from Brundage-derived #2 through #6 T_1 generation

Plants with detectable cDNA are highlighted in green. Blue lines indicate 500 bp marker of the ladders. Orange arrows indicate individuals that were suspected to be positive due to shadows in the original gels, were therefore subsequently retested, and produced positive results.

Results from cDNA testing of Brundage-derived #2 though #6 T_1 generation is indicative of segregation. This segregation fails to meet a 3:1 ratio, however, but this may be due to a number of factors including incomplete transformation, gene silencing, and the lack of data for all T_1 individuals of a given T_0 spike. The results of other T_0 plants in this study indicates that a plant may have the DNA for the gene of interest, but the cDNA was absent – this is indicative of silencing and will be discussed later. Some T_0 spikes failed to produce progeny that had detectable cDNA from *Yr28*, such as P2-S3, P2-S10, P2-S15, P4A-S1, P5-S6, and P6-S6.

For plant #2's spike 8, two of the nine tested individuals had detectable cDNA. Plant #2 spike 16 had three of the seven tested plants with detectable cDNA. Some of these plants' RNA was retested via a DNase I retreatment and reverse transcription and then PCR for a second time based on the suspicion that initial RNA samples had low concentrations due to incomplete recovery following RNA extraction. Orange arrows in Figure 3.15 indicate those individuals that resulted in detectable cDNA following retesting – other individuals, such as P2-S16-6 and P2-S18-9, were also retested but without positive results. Resulting ratios were 1:2, as positive for cDNA detection to lacking cDNA detection for *Yr28*, or below, for most tested T_1 plants – with the exception of 4A-4, because there was only one T_1 progeny plant from that spike (Figure 3.16).

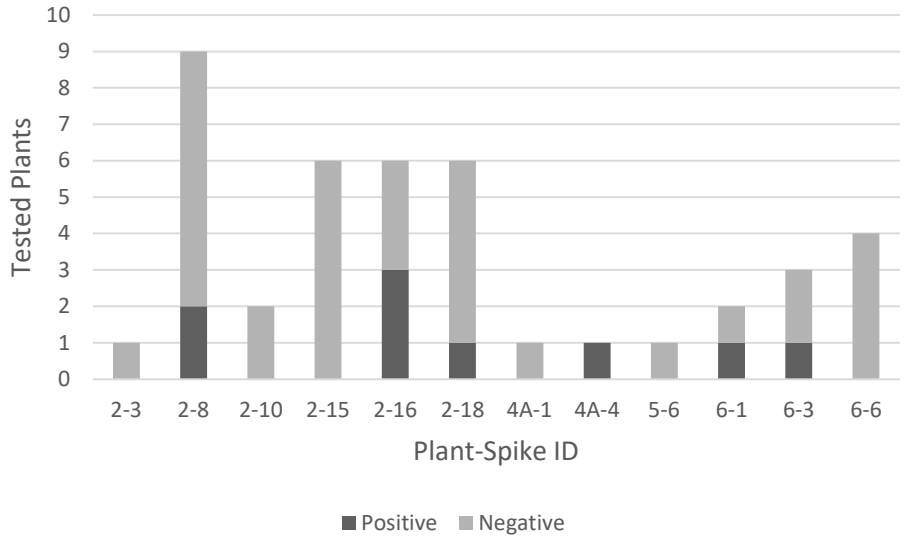


Figure 3.16 Visualization of cDNA detection ratios for Brundage-derived #2-#6 T_1 generation

Visual ratios represent only the T_1 progeny plants that were tested, rather than the whole population. Ratios deviate from 3:1, for individuals positive for the *Yr28* cDNA to those lacking the expression, in all cases. The best ratio was 1:0 in the case of 4A-4.

The DNA results of Fielder T_0 Plants were such that only 8C was positive for *Yr28*. Nevertheless, each spike produced from plants #7A, C, D, and #8A, B, and C, was tested for assurance that all other plant lacked the desired DNA (results not shown). PCR investigation confirmed that only 8C-S3 was positive for *Yr28*. Despite this indication, every individual from all four spikes of #8C were tested. Those results definitively demonstrated that only individuals from 8C-S3 had the gene of interest, and of those 9 individuals, only 3 contained the gene: namely 8C-S3-5, 8C-S3-8, and 8C-S3-9 (Figure 3.18). As with the Brundage T_1 population discussed previously, it may have been that some spikes resulted from untransformed cells. Likely, shoots for T_0 plant #8C arose from independents cells: T_1 seeds from the transformed cell segregated for *Yr28*, but others from untransformed cells were all negative for *Yr28*.

The Fielder-derived T_0 #8C plant showed no expression of the *Yr28* gene, and therefore expression in the T_1 progeny was not tested.

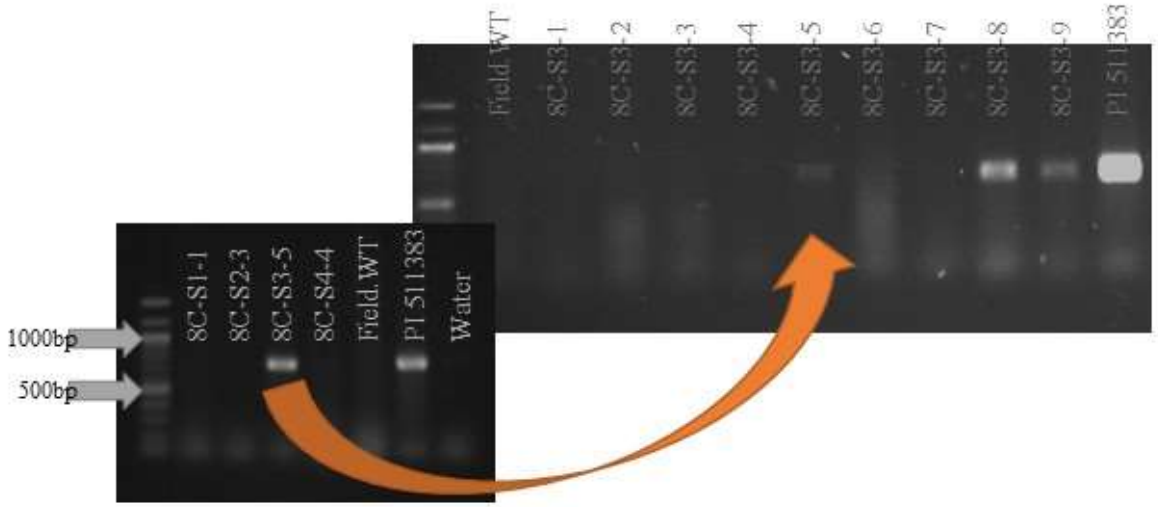


Figure 3.17 DNA results from Fielder-derived T_1 generation progeny from #8C

The image on the left features the PCR product from just one T_1 individual from each #8C T_0 spike. The orange arrow relates the relationship of the PCR product from the same T_1 individual (i.e. 8C-S3-5). Of nine T_1 plants from T_0 spike 3, only three had *Yr28*. Alhtough this indicated segregation of the transgene, it failed to fulfill a typical 3:1 ratio of a single Medelian gene.

Other T_1 plants that underwent testing ware from Brundage-derived T_0 plants #10A, #10B and #10C and were a result of germination of half of the seeds from each spike of those plants. The other half of the seeds were not germinated and tested due to time constraint and remained in storage for later evaluation. This was done to protect against an unforeseen failure killing all resultant individuals, as occurred with a previous T_1 population.

The DNA investigation into the Brundage-derived #10 T_1 plants yielded evidence of segregation for the transgene. These segregation ratios were not the expected 3:1, however. The ratios varied and plants from 10A-S1's segregation ratio was 4:3 as having the gene to devoid of it, 10A-S2's was 8:3 (which is approaching a 3:1 Mendelian segregation), 10A-S3's was 4:3, 10A-S4's was 2:1 (also close to expected), 10B-S1's was 1:1, 10B-S2's was 2:3, and 10C-S1's was 2:9. The deviation from the expected ratio may have to do with a lack of data for all individuals in the T_1 generation, because T_1 seeds are limited and only half of them were tested.

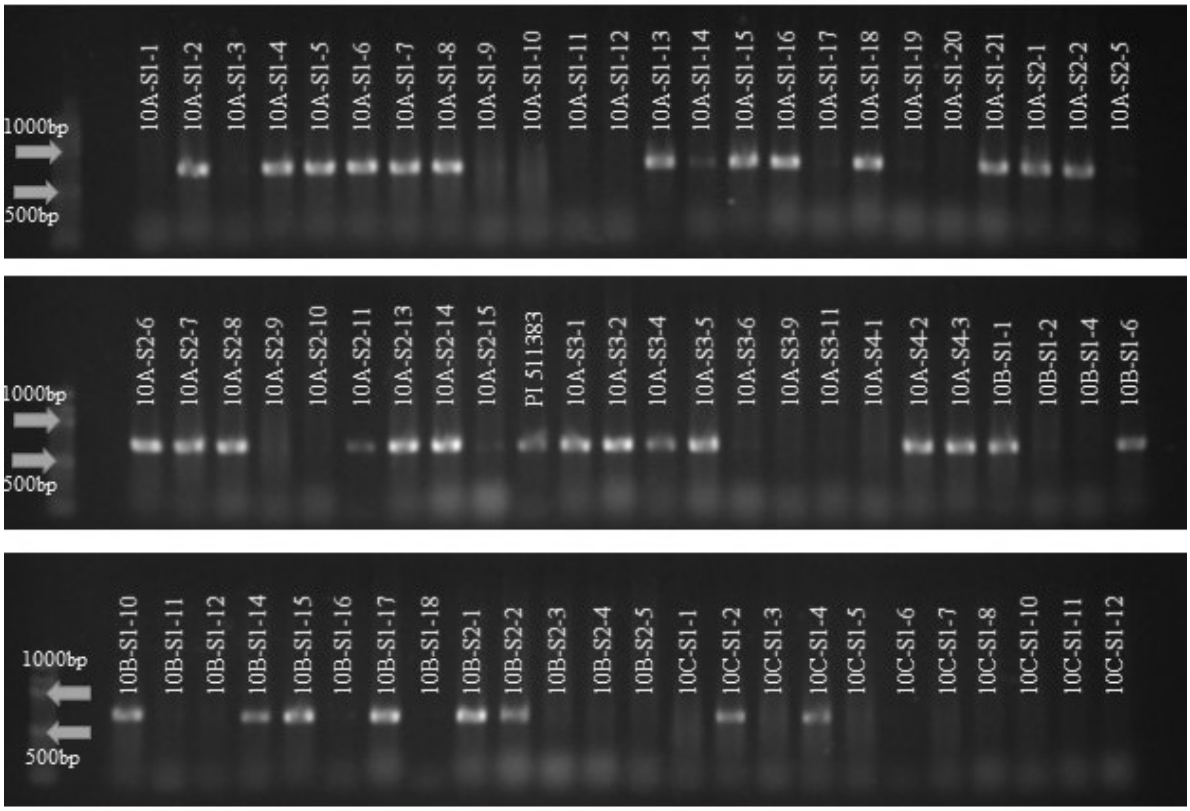


Figure 3.18 PCR results for *Yr28* DNA in Brundage-derived #10 T_1 plants

PCR results from every individual plant of the Brundage #10 T_1 population that was sucessfully started. Of the 71 individials, 37 had detectable DNA for *Yr28*. This represents only half the population from Brundage-derived plants #10A through C. The other half was stored and reserved for later testing, so that an unforseen circumstances could not destroy the entirety of the population. Segregation ratios are therefore incomplete, but near the expected 3:1, as containing the gene of interest to lacking it, for the progeny of some tillers.

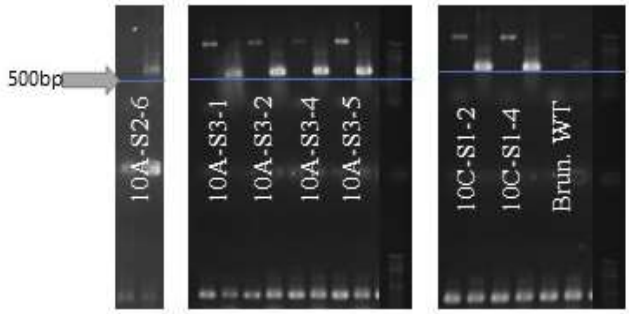


Figure 3.19 PCR results of *Yr28* cDNA in Brundage-derived #10 T_1 plants

All T_1 plants from #10 T_0 plants were tested. The selection of T_1 plants that clearly had cDNA for *Yr28* are shown, indicative of expression of the gene. The blue line indicated the 500bp hatch mark from the 100bp ladder (New England BioLabs, Ipswich, MA, USA). *Actin* products (438 bp), in the bottom row, served as the control and indicated sucessful amplification under the PCR conditions used.

All the T_1 progeny of #10 were tested for their tolerance to herbicide by leaf-painting, as had been done for the T_0 parents. The individuals that were tolerant of the herbicide did not perfectly correspond to those that were assayed as having *Yr28* DNA. This suggests that, at least in some cases, the constructs used in bombardment may have integrated at different genomic loci and therefore segregated separately.

Every individual of the Brundage-derived #10 T_1 population that assayed as having DNA for the gene of interest was tested for expression. RNA was extracted and converted to cDNA as previously described, then two-cycle PCR testing was done (Appendix A-8). Of the 37 plants that tested positive for *Yr28* DNA, only seven of them assayed as having expression of that gene (Figure 3.19). Of these seven, however, they all showed a stripe rust resistant phenotype. They were all rated between 2 and 3 putting them all in the resistant category. The WT controls were rated 7 and 8 (Figure 3.20).

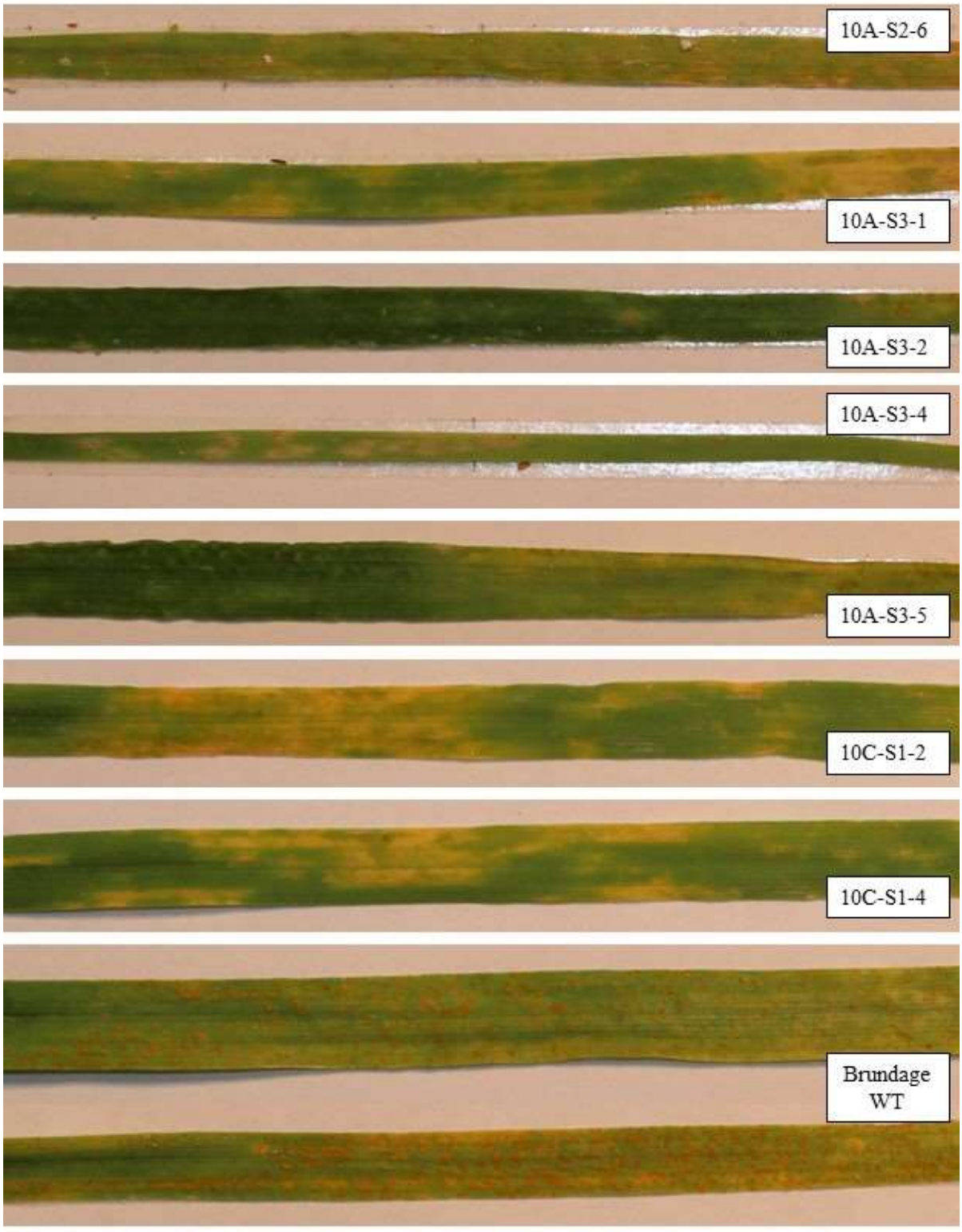


Figure 3.20 Leaf samples from #10 T_1 plants that tested positive for *Yr28* cDNA

All transformed T_1 plants in the image were rated 3, 3, 3, 2, 3, 3, and 2 with respect to the order in which they are shown top to bottom. These ratings correspond to solidly resistant phenotypes in terms of stripe rust infection. Plants 10C-S1-2 and 10C-S1-4 were noted as having hypersensative responses, much as the #10 individuals in the T_0 generation from which they descended, displayed. The WT control plants were rated 7 and 8 respectively.

Discussion

In the exploration of cultivars to include in this study, beyond Brundage, the researcher relied on varieties known to be successful in historical transformation experiments. These genotypes allowed for comparison of transformation efficiency to Brundage, for which there is a lack of published data regarding transformation success. In searching the USDA's repository for winter wheat varieties, namely Florida, mentioned in reviewed papers, the researcher discovered three options: 301, 302 and 303, which were all included in the testing outlined above. For transformation, the variety UI Platinum, which was tested in tissue culture, was excluded because of an innate resistance to stripe rust, *Puccinia striiformis* f. sp. *tritici* (*Pst*), which would make phenotypic disease response validation difficult. Additionally, this genotype did not undergo susceptibility testing at Washington State University to give a baseline susceptibility score. Future transformation experiments with this variety can be informed by the tissue culture data produced in the present study. Another genotype considered for testing but excluded was the variety Jagger. This winter wheat, which has been used in other researchers' transformation experiments prior, was not included due to its own innate resistance to stripe rust, along with difficulty by this researcher in germinating the seeds provided by the USDA.

Unlike tissue culture experimentation, the rate of callus induction was not calculated during the transformation experiments. The first count of individual calli was not taken until immediately following transfer to CI+ medium. This meant that embryos that did not form successful calli were disregarded. Data on this aspect of regeneration of bombarded tissue may have been an interesting measure to contrast with data gained from tissue culture experimentation.

The overall transformation efficiency achieved in this experimentation was lower than expected. The results were similar to those achieved in 1992 when particle bombardment of wheat was first reported (Vasil et al., 1992). This research did, however, include recalcitrant genotypes including Brundage, in addition to the reportedly highly transformable spring genotype Fielder. Despite an overall rate of transformation of 0.23% for Brundage, the researcher was able to introgress a robust R gene with resistance to the damaging disease of stripe rust. These lines have the potential to be useful for breeding possibilities in the future, and also demonstrate that introgression of *Yr28* in this background does not suffer from genetic repression of the gene's stripe rust resistance characteristics.

There is a possibility of clonal material in the explanation of plants produced via these experiments. When bombarded calli formed shoots, sometimes there was more than one shoot produced per callus. If this were the case the researcher would carefully separate shoots with enough root to do so – if there were not enough root to divide the shoots they were left together and treated as a single plant. The plants resulting from these divisions were labelled as clones: such as, A, B, C, and so on of the

original plant #. For simplicity, and because confirmation of clonal relationships was not done via sequencing or other testing methods, statistical conclusions were drawn as if those possible clones were individual plants. If one were to consider the clonal relationships, only 18 possible new individual genotypes could have been produced - that is assuming that shoots from one callus resulted from a single transformed cell, which is how the individuals would share identical genotypes.

Inoculation was a difficult aspect of testing to make consistent. Although the same procedure was carried out by the researcher for every inoculation, the results varied greatly. It is not clear why this might have been. Although the viability of the spores was considered, even with relatively fresh spores sometimes the inoculation failed to result in consistent infection. In later rounds of testing, plants that did not exhibit consistent infection symptoms were inoculated a second time, so long as the plants were not too large to fit into the tubs used to create the dew-chamber environment. In this way plant disease-response phenotypes could be more accurately analyzed.

According to the statistical analysis done in R, none of the genotype's rate of transformation is significantly different. Although the rates of transformation vary some between the genotypes, all are below 0.5%. Perhaps with a more stringent significance level, the ANOVA and LSD test results may have detected a statistical difference between the genotypes. However, Fielder was the most transformable variety with an average rate of 0.43%, accounting for four plants produced from 1,009 embryogenic calli. If calculated based on the number of transgenic plants produced from the total number of embryogenic calli for each genotype, rather than averaging the rates of transformation across all experiments, the figure for Fielder-derived transgenics becomes 0.40%, and makes Florida 301 the most transformable variety with a rate of 0.43% accounting for four plants produced from 933 calli. Regardless of how the rate was calculated, the transformation efficiencies of all genotypes remained below 0.5%.

When it came to the number of escapes, all varieties suffered from some level of plants that were untransformed having survived and been promoted through the selection process. Brundage had a total of 22 plants that were carried through to leaf-painting and genotypic validation, but only 11 of those were positive for *Yr28*. This equates to a ratio of 2:1. Other ratios were 9:2 for Fielder, 7:4 for both Florida 301 and 302, and 9:1 for Florida 303. From tissue culture testing, Florida 303 ranked as the second most regenerable genotype, but this data indicates that regeneration capability does not always equate to transformability.

There were many plants that assayed as having DNA for the gene of interest, but that failed to demonstrate expression of the gene via cDNA detection. In the T_0 plants there were seven plants

where the cDNA assay failed to return detection. Similarly, of those plants that were/could be tested for their resistance phenotype to stripe rust, none of them showed any increased resistance to the disease. These results are indicative of gene silencing, or a lack of produced gene product (mRNA), that can occur due to positional effects in the genome, amongst others. There were five of the early T_0 plants, #2 through #6 as well as plant #32 for a total of six, that showed the presence of RNA product for the gene (via cDNA assay) but did not demonstrate any increase in disease resistance when compared to untransformed control wild-type plants. The primers for the cDNA assay were within middle exons of the gene sequence, but this placement would not account for all the possible splicing patterns for *Yr28*, which have been hypothesized as the drive for how the gene functions to impart resistance. Therefore, it would be of value to do additional investigation into the transcript variants produced by plants that demonstrated expression of the gene. It would be interesting to compare the transcript variants that may or may not be present in those plants that demonstrated phenotypic resistance, versus those that did not. Of the T_0 plants that had expression of the gene in the form of detectable cDNA, 10 of the 16 showed increased phenotypic disease resistance.

The future of genetic modification (GM) in wheat is uncertain. There are, however, other genetically modified crops that have gained some public acceptance, such as soybean and corn. With the looming demands of growing population and climate change, there is a possibility that genetic modification of other crops, such as wheat, may gain traction with the public. If that were to be the case, regulation still demands that the GM products be vector-sequence and marker-gene free. Data in this study suggests that co-integration of co-bombarded constructs is not always the case, which would provide the possibility of relatively simple removal of superfluous marker genes and associated vector sequence through backcrossing to the parent variety. There is also the future possibility of editing introduced DNA via CRISPR such that only the desired genes remain. These are desirable approaches for a top-quality variety such as Brundage. Brundage was largely replaced in the Pacific Northwest grower's market by other wheat varieties in the last five years. The choice of other varieties was not due to a lack of quality of the Brundage wheat, but rather that other varieties had greater stripe rust resistance.

Table 3.1 Genotype information

Table includes the variety names, wheat type, pedigrees and GRIN Global Accession numbers for every genotype tested in these experiments. Typically, Winter type wheats require a vernalization treatment of 30 to 60 days in length within a temperature range of 0° to 5°C to be competent to set seed, whereas Facultative types can suffice with a temperature range between 3° to 15°C for between 15 to 30 days (Braun & Sãulescu, 1998). Spring types do not require a vernalization treatment for flowering.

Variety	Type	Pedigree	Accession #
Brundage	Winter	Stephens/Geneva	PI 599193
Fielder	Spring	Yaktana 54A*4//Norin 10/Brevor/3/2*Yaqui 50/4/Norin 10/Brevor//Baart/Onas	CItr 17268
Florida 301	Facultative	Holley//Olesen/Purdue 64212A3-23	CItr 17769
Florida 302	Winter	Coker 65-20//P4946A4-18-2-10-1/Hadden/3/Vogel/5/Anderson//P4946A4-18-2-10-1/Hadden	PI 601163
Florida 303	Facultative	Coker 65-20/8/(Norin 33-3/6/(Fairfield/4/Fultz/Hungarian*2//PI94587 durum/3/Fultz/Hungarian, Pd39153A1-11-1-1)/5/(Trumbull*3//Hope/Hussar, Pd3932A7-3-1-2)/3/Newsar, PD4946A4-18-2-10-1)/7/Hadden/9/(Norin 10/Brevor/4/Anderson/3/(Coker 55-9, Chancellor*2//T. timopheevi/Steinweidel), Vogel 5)/8/(Norin 33-3/6/(Fairfield/4/Fultz/Hungarian*2//PI94587 durum/3/Fultz/Hungarian, Pd39153A1-11-1-1)/5/(Trumbull*3//Hope/Hussar, Pd3932A7-3-1-2)/3/Newsar, Pd4946A4-18-2-10-1)/7/Hadden/10/Coker 797	PI 601807

Table 3.2 LB medium composition

LB medium was used for the growth of bacteria transformed with the experimental plasmids so that DNA could be procured for bombardment experiments. After autoclaving and cooling, the medium would be inoculated with the transformed bacteria, then cultured at 37°C in a shaker at 200 rpm for 12 to 15 hours while bacteria would multiply. Another process would lyse the bacteria and purify the plasmids carrying the DNA for future transformation.

Components	Amount per liter
Tryptone	10g
Yeast extract	5g
Sodium chloride	10g
Agar powder	15g

Measure powders and add MΩ water to volume, mix well.
Autoclave at 121.8°C for at least 30 minutes then let cool.

Table 3.3 Basic medium composition

Basic medium was used as the basis to create all the specific media types used in these experiments. The specific media types were created with the additions of other minor components at specific concentrations in various amounts. Additives depended on media type but included sucrose, $CuSO_4$, 2,4-D, glufosinate-ammonium, and 6-BA.

Component	Amount per liter
Murashige & Skoog salts*	4.3g
Maltose monohydrate	40g
Thiamine hydrochloride solution	10mL
Asparagine monohydrate	0.15g
18.2MΩ water	To volume
Adjust PH to 5.81-5.85 with potassium hydroxide (1M)	
Phytagel is added to bottles prior to autoclaving at 122.8°C for 30 minutes. Media is allowed to cool to approximately 60°C before minor components are added, mixed, and poured into sterile 15x100mm petri dishes.	

*Catalog No. M524 (PhytoTechnology Laboratories,Overland Park, KS, USA)

Table 3.4 Medium compositions

Table lists the minor components that were added to the basic medium to create each type of specific medium for the various stages of transformed cell development.

Media type	Shorthand	Basis	Supplements	Amount per liter
Callus Induction	CI	Basic Media 2.5 g Phytagel	$CuSO_4$ (0.1M) 2,4-D (5mg/mL)	49 µL 400 µL
Callus Induction with Selection	CI+	Basic Media 2.5 g Phytagel	$CuSO_4$ 2,4-D Bialaphos (5mg/mL)	49 µL 400 µL 600 µL
Regeneration	RM-	Basic Media 2.5 g Phytagel	2,4-D $CuSO_4$ 6-benylaminopurine (1mg/mL)	40 µL 49 µL 100 µL
Regeneration with Selection	RM	Basic Media 2.5 g Phytagel	2,4-D $CuSO_4$ 6-benylaminopurine Bialaphos	40 µL 49 µL 100 µL 600 µL
Root Development	RD	½ Basic Media 3.5 g Phytagel	Bialaphos	600 µL
Osmotic	OS	Basic Media 3.5 g Phytagel	sucrose 2,4-D	171.18 g 400 µL

PH of all media were adjusted to 5.81-5.85 with potassium hydroxide (1 M) prior to autoclaving. Components were added after media was autoclaved at 122.8°C for 30 minutes and allowed to cool to ~60°C. Osmotic medium was poured into sterile 15x60 mm petri dishes, root development medium was poured into 6 oz tissue culture jars which were autoclaved with their plastic lids at the time of the media, and all others were poured into sterile 15x100 mm petri dishes. Bialaphos is a product of Gold Biotechnology, St. Louis, MO, USA. It provides the glufosinate-ammonium component of herbicide. Phytagel is a product of Sigma-Aldrich, St. Louis, MO, USA.

Table 3.5 Scale for recording stripe rust infection types

Numerical scale, the description, and corresponding category used for rating the severity of stripe rust infection in plants tested during these experiments.

Infection types	Signs and symptoms for infection types*	Category rating
0	No visible signs or symptoms	Highly Resistant
1	Necrotic and/or chlorotic flecks; no sporulation	Resistant
2	Necrotic and/or chlorotic blotches or stripes; no sporulation	Resistant
3	Necrotic and/or chlorotic blotches or stripes; trace sporulation	Resistant
4	Necrotic and/or chlorotic blotches or stripes; light sporulation	Moderately Resistant
5	Necrotic and/or chlorotic blotches or stripes; intermediate sporulation	Moderately Resistant
6	Necrotic and/or chlorotic blotches or stripes; moderate sporulation	Moderately Susceptible
7	Necrotic and/or chlorotic blotches or stripes; abundant sporulation	Susceptible
8	Chlorosis behind sporulating area; abundant sporulation	Susceptible
9	No necrosis or chlorosis; abundant sporulation	Highly Susceptible

*Blotches occur on seedlings and stripes occur on plants in later stages of growth.

Adapted from: Line, R. F. (1992):
Virulence, aggressiveness, evolution, and distribution of races of Puccinia striiformis (the cause of stripe rust of wheat) in North America, 1968-87

Table 3.6 Procedure breakdown by wheat genotype and plasmid used for bombardment

Table outlines the number of procedures done for each genotype based on the plasmid used for bombardment (if not specifically noted, the experimental construct was F2-1; Brundage was the only genotype to be bombarded with the enzyme digested plasmid PC1164), the number of embryogenic calli that resulted from this number of procedures and how many plates of embryos were bombarded per genotype.

Genotype	Total procedures	Total calli	Total plates bombarded
Brundage	**15**	**7,171**	**84**
1164/EC	3	2,910	33
F2-1	12	4,261	51
Fielder	**5**	**1,009**	**15**
Florida 301	**6**	**933**	**17**
Florida 302	**4**	**1,220**	**14**
Florida 303	**5**	**952**	**12**
Grand Total	**35**	**11,285**	**142**

Table 3.7 Plant transformation data by each procedure

Record of the number of plants produced and the number of transgenic plants that resulted from each transformation experiment, the experimental DNA that was used for bombardment, and the number of embryogenic calli that resulted, which in turn allowed for the calculation of the rates of plant production and transgenic plant production.

PDS #	Genotype	Vector	Callus #	Plants Produced	Transgenic Plants	Produced %	Transformed %
25	Brundage	F2-1	1,050			0.00	0.00
26	Brundage	F2-1	390	2	2	0.51	0.51
35	Brundage	1164/EC	1,140			0.00	0.00
45	Brundage	1164/EC	870			0.00	0.00
46	Brundage	1164/EC	900	3	3	0.33	0.33
KJ1	Fielder	F2-1	120	7	1	5.83	0.83
KJ3	Brundage	F2-1	195	4	3	2.05	1.54
KJ4	Florida 301	F2-1	90			0.00	0.00
KJ5	Florida 303	F2-1	270	1	0	0.37	0.00
KJ6	Florida 302	F2-1	270			0.00	0.00
KJ7	Brundage	F2-1	80			0.00	0.00
KJ8	Fielder	F2-1	150			0.00	0.00
KJ9	Florida 301	F2-1	129			0.00	0.00
KJ10	Florida 303	F2-1	195			0.00	0.00
KJ11	Brundage	F2-1	218	1	0	0.46	0.00
KJ13	Brundage	F2-1	390			0.00	0.00
KJ14	Florida 302	F2-1	375	4	3	1.07	0.80
KJ15	Brundage	F2-1	354	3	0	0.85	0.00
KJ16	Florida 301	F2-1	135	2	0	1.48	0.00
KJ16	Florida 303	F2-1	131	2	0	1.53	0.00
KJ17	Brundage	F2-1	370			0.00	0.00
KJ18	Fielder	F2-1	441	8	1	1.81	0.23
KJ19	Brundage	F2-1	378			0.00	0.00
KJ20	Florida 301	F2-1	388	5	3	1.29	0.77
KJ21	Florida 302	F2-1	328	1	0	0.30	0.00
KJ22	Fielder	F2-1	298	3	2	1.01	0.67
KJ23	Brundage	F2-1	277	3	2	1.08	0.72
KJ24	Brundage	F2-1	319	5	0	0.31	0.00
KJ25	Florida 302	F2-1	247	2	1	0.40	0.00
KJ26	Brundage	F2-1	240			0.00	0.00
KJ27	Florida 301	F2-1	121			0.00	0.00
KJ28	Florida 303	F2-1	203	4	0	1.97	0.00
KJ29	Florida 301	F2-1	70			0.00	0.00
KJ29	Florida 303	F2-1	153	2	1	1.31	0.65
	Totals/Average		**11,285**	**63**	**24**	**0.76**	**0.24**

How to read table: PDS # = procedure number (identifier). Vector = the experimental construct used for bombardment, which was co-bombarded with PC174 for additional introduction of the plant selectable marker: the *bar* gene. Callus # = the number of embryogenic calli that formed from the bombarded embryos in each experiment. Plants Produced = the number of putative transgenic plants that resulted. Plants Transformed = the number of plants, of the putative ones, that were confirmed to be transgenic. Produced % = the calculation of the rate of plants produced from each procedure based on the number of calli. Transformed % = the calculation of the rate of production of plants that were confirmed to be transformed based on the number of calli that were produced in that procedure.

Table 3.8 Individual plant data

Detail of T_0 plant data by individual identifier. See 'How to read table' information below.

PDS #	Plant ID	Genotype	Herb.	PCR	Disease Rating
26	2	Brundage	Yes	+	8
46	5	Brundage	No	+	7
26	6	Brundage	Yes	+	8
46	4A	Brundage	No	+	7
46	4B	Brundage	No	+	8
KJ1	7A	Fielder	Yes	-	N/A
KJ1	7C	Fielder	Yes	-	N/A
KJ1	7D	Fielder	Yes	-	N/A
KJ1	8A	Fielder	No	-	N/A
KJ1	8B	Fielder	No	-	N/A
KJ1	8C	Fielder	No	+	N/A
KJ3	9	Brundage	No	-	8
KJ3	10A	Brundage	Yes	+	4
KJ3	10B	Brundage	Yes	+	6
KJ3	10C	Brundage	Yes	+	5
KJ5	11	Fl.303	No	-	7
KJ11	12	Brundage	No	-	N/A
KJ14	13	Fl.302	No	-	N/A
KJ14	14	Fl.302	Yes	+	N/A
KJ14	15	Fl.302	Yes	+	N/A
KJ14	16	Fl.302	Yes	+	N/A
KJ15	17	Brundage	No	-	8
KJ15	18A	Brundage	No	-	8
KJ15	18B	Brundage	No	-	8
KJ16	19	Fl.303	No	-	8
KJ16	20A	Fl.301	No	-	8
KJ16	20B	Fl.301	No	-	8
KJ16	21	Fl.303	No	-	8
KJ18	22A	Fielder	No	-	8
KJ18	22B	Fielder	No	-	7
KJ18	22C	Fielder	No	-	7
KJ18	23	Fielder	No	-	8
KJ18	24	Fielder	No	+	2
KJ18	25	Fielder	No	-	8
KJ18	26	Fielder	No	-	7
KJ18	27	Fielder	No	-	8
KJ18	28	Fielder	No	-	8
KJ20	29A	Fl.301	Yes	+	8
KJ20	29B	Fl.301	No	-	8
KJ20	30A	Fl.301	Yes	+	8
KJ20	30B	Fl.301	Yes	+	9
KJ23	31A	Brundage	Yes	+	3
KJ23	31B	Brundage	Yes	+	3

PDS #	Plant ID	Genotype	Herb.	PCR	Disease Rating
KJ23	31C	Brundage	Yes	+	3
KJ22	32	Fielder	Yes	+	8
KJ22	33	Fielder	No	-	7
KJ22	34	Fielder	Yes	+	3
KJ20	35	Fl.301	No	+	4
KJ21	36	Fl.302	No	-	8
KJ24	37	Brundage	Yes	-	8
KJ25	38	Fl.302	No	+	6
KJ28	39	Fl.303	Yes	-	Early
KJ28	40A	Fl.303	Yes	-	Early
KJ28	40B	Fl.303	Yes	-	Early
KJ28	40C	Fl.303	No	-	Early
KJ23	41	Brundage	No	-	N/A
KJ24	42A	Brundage	Yes	-	N/A
KJ24	42B	Brundage	Yes	-	N/A
KJ24	42C	Brundage	Yes	-	N/A
KJ24	42D	Brundage	Yes	-	N/A
KJ25	43	Fl.302	No	-	N/A
KJ29	44	Fl.303	No	+	N/A
KJ29	45	Fl.303	No	-	N/A

How to read table: PDS # = procedure number (identifier). Genotype = plant's base variety for genetic makeup. Plant ID = seedling identifier (number) assigned when plants were moved into potting soil in the greenhouse. Herb. = indicates plant's tolerance of herbicide – 'yes' indicates no/minimal cell death from the assay for the *bar* gene via leaf-painting with a glufosinate-ammonium solution. PCR = indicates the results of the assay of the DNA's status for *Yr28* – '+' means the gene was detected. Disease rating = the numerical value of disease severity based on whole-plant evaluation approximately 21 days after inoculation with the stripe rust pathogen – 'N/A' signifies a rating could not be given due to circumstances at the time, and 'Early' means that although infection was present on the plants at the time of publication, that it was too early (prior to 21 days post-inoculation) to give a well-informed rating.

Table 3.9 Transformation procedure data

Record keeping method for data, including dates, organized by procedure identifier. See 'How to read table' information below.

PDS #	Genotype	DNA	Plates Bomb	Date Bombarded	Calli	Rooting Medium	Rtg. Late	Soil	Soil Later	Seedling ID	Plant Total	Herbicide Testing	Herb Ltr.
25	Brundage	F2-1	12	4/11/2018	1050	6/23/2018							
26	Brundage	F2-1	6	4/13/2018	390	6/23/2018	7/25/2018	7/17/2018	8/30/2018	2,6	2	8/6/2018	
35	Brundage	1164/EC	10	4/28/2018	1140								
45	Brundage	1164/EC	11	5/18/2018	870	7/26/2018							
46	Brundage	1164/EC	12	5/22/2018	900	7/26/2018		8/8/2018		4a,b;5	3	8/24/2018	
KJ1	Fielder	F2-1	3	10/5/2018	120	12/11/2018		12/26/2018		7a,b,c,d,e; 8a,b,c	7	1/11/2019	
KJ2	Fielder	F2-1	3	10/24/2018	0								
KJ3	Brundage	F2-1	3	11/26/2018	195	2/1/2019		2/15/2019	3/2/2019	10a,b,c; 9	4	3/2/2019	3/13/2019
KJ4	Florida 301	F2-1	3	11/29/2018	90								
KJ5	Florida 303	F2-1	3	12/13/2018	270	2/22/2019		3/15/2019		11	1	3/30/2019	
KJ6	Florida 302	F2-1	3	12/18/2018	270								
KJ7	Brundage	F2-1	3	12/22/2018	80								
KJ8	Fielder	F2-1	3	12/27/2018	150								
KJ9	Florida 301	F2-1	2	¼/2019	129								
KJ10	Florida 303	F2-1	3	1/7/2019	195								
KJ11	Brundage	F2-1	3	1/29/2019	218	4/21/2019		5/5/2019		12	1	6/3/2019	
KJ12	Brundage*	F2-1	2	2/14/2019	0								
KJ13	Brundage	F2-1	6	3/5/2019	390								
KJ14	Florida 302	F2-1	5	3/15/2019	375	5/14/2019	5/26/2019	5/30/2019	6/10/2019	13,14,15,16	4	6/14/2019	6/22/2019
KJ15	Brundage	F2-1	3	4/4/2019	354	5/31/2019	6/19/2019	6/14/2019		17;18a,b	3	6/30/2019	
KJ16	Florida 301	F2-1	2	4/10/2019	135	6/19/2019		7/3/2019		20a,b	2	7/18/2019	
KJ16	Florida 303	F2-1	1	4/10/2019	131	6/7/2019	6/19/2019	6/22/2019	7/3/2019	19;21	2	7/6/2019	7/28/2019

PDS #	Genotype	DNA	Plates Bomb	Date Bombarded	Calli	Rooting Medium	Rtg. Late	Soil	Soil Later	Seedling ID	Plant Total	Herbicide Testing	Herb Ltr.
KJC*	Brundage	N/A	3	4/18/2019	161					0.1677			
KJ17	Brundage	F2-1	3	4/23/2019	370								
KJ18	Fielder	F2-1	3	5/1/2019	441	6/28/2019		7/15/2019		22a,b,c;23;24;25;26;27;28	8	7/28/2019	8/3/2019
KJ19	Brundage	F2-1	3	5/3/2019	378	9/1/2019							
KJ20	Florida 301	F2-1	5	5/27/2019	388	7/18/2019	9/1/2019	7/28/2019	9/26/2019	29a,b;30a,b;35	5	8/8/2019	10/3/2019
KJ21	Florida 302	F2-1	3	6/4/2019	328	9/1/2019		9/26/2019		36	1		10/3/2019
KJ22	Fielder	F2-1	3	6/11/2019	298	8/17/2019	9/1/2019	9/3/2019	9/26/2019	32;33;34	3	9/10/2019	10/3/2019
KJ23	Brundage	F2-1	3	6/15/2019	277	8/14/2019	9/10/2019	9/3/2019	10/29/2019	31a,b,c;41	4	9/10/2019	
KJ24	Brundage	F2-1	3	7/9/2019	319	9/23/2019	10/11/2019	10/10/2019	10/29/2019	37;42a,b,c,d	5	10/19/2019	
KJ25	Florida 302	F2-1	3	7/17/2019	247	9/23/2019		10/10/2019		38;43	2	10/19/2019	
KJ26	Brundage	F2-1	3	7/30/2019	240								
KJ27	Florida 301	F2-1	4	8/4/2019	121								
KJ28	Florida 303	F2-1	3	8/9/2019	203	10/11/2019		10/19/2019		39;40abc	4	10/29/2019	
KJ29	Florida 301	F2-1	1	8/23/2019	70								
KJ29	Florida 303	F2-1	2	8/23/2019	153	10/17/2019		10/29/2019		44;45	2	11/12/2019	

How to read table: PDS # = procedure number (identifier). DNA = the plasmid used for the experiment. Plates Bomb = the number of plates of embryos (~50 to 100 embryos per plate depending on embryo size) that were bombarded in the procedure. Date Bombarded = the date that bombardment occurred – transfer to CI medium occurred approximately 16 hours post-bombardment, which was typically the next morning. Calli = the number of embryogenic calli that resulted from the bombarded embryos – count was taken at the time of transfer to CI+ medium. Rooting Medium = the date when shoots, if there were any, were transferred to RD medium. Rtg. Later = if calli were given more time on RM, then formed shoots later this was the date those later shoots were transferred to RD medium. Soil = when shoots from RD had ~3 cm of root tissue they were planted in the greenhouse and tented for ~five days with plastic film for acclimation and cuticle development. Seedling ID = the identifier that successful seedlings through the process this far were assigned. Plant Total = the number of seedlings that survived up to the point of herbicide testing and inoculation. Herbicide Testing = when the leaf painting assay for the *bar* gene was done with a glufosinate-ammonium solution – results were generally distinguishable within five days. Herb Ltr. = testing date for seedlings of the same procedure that developed later with the leaf-painting assay

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Appendix A

Appendix A-1: Gold/DNA-Coating Protocol

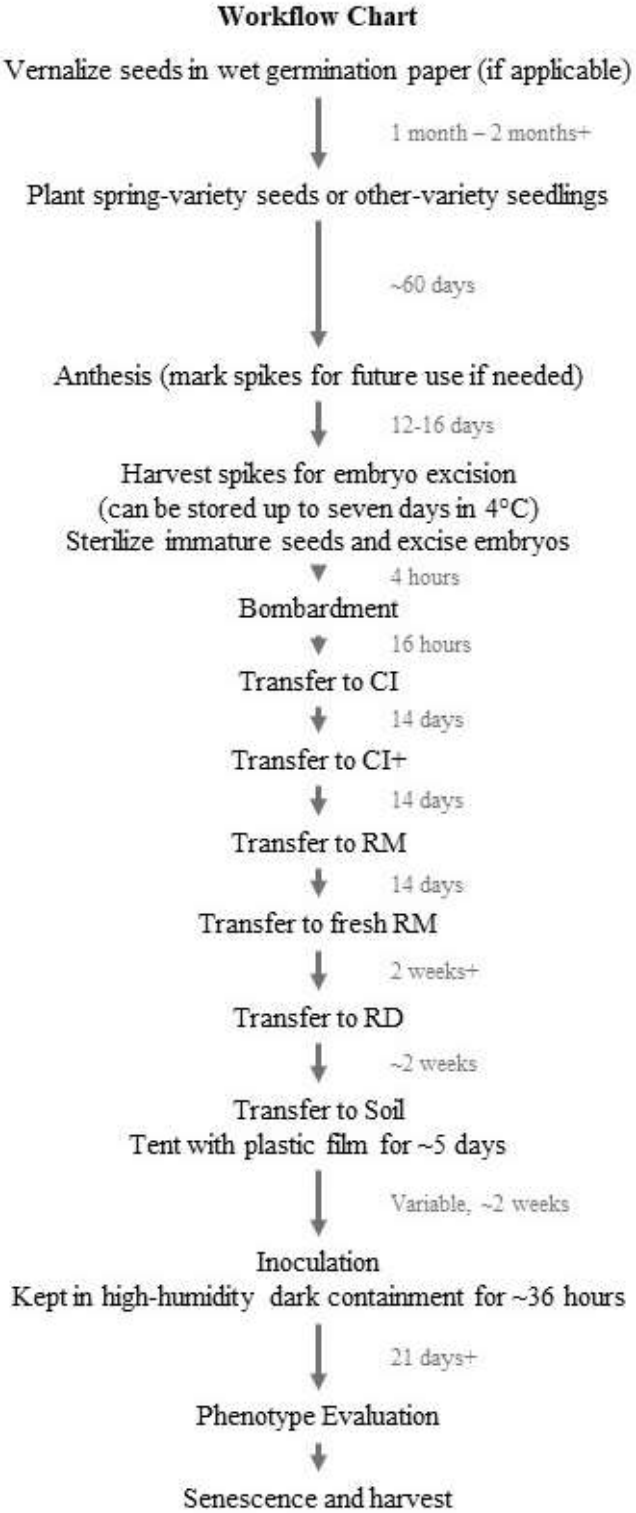
The following steps were completed on ice for DNA-coating:

1. Suspend the gold aliquot with 200 µL ddH_2O – vortex to mix
2. Chill 5 minutes
3. Centrifuge at 12,000 rpm at 4°C for 5 seconds
4. Remove and discard supernatant
5. Add plasmid DNA* – 20 µL total
6. Add 225 µL ddH_2O and mix by pipetting
7. Quickly add 250 µL $CaCl_2$ (2.5 M)
8. Quickly add 50 µL (1 M) spermidine and mix
9. Vortex and chill alternatingly for 15 minutes – chill an additional 5 minutes
10. Centrifuge for 10 seconds
11. Remove and discard supernatant
12. Add 50 µL 100% ethanol – mix well by hand then vortex
13. Chill for 1 minute
14. Centrifuge for 5 seconds
15. Remove and discard supernatant
16. Add 45 µL 100% ethanol and mix well by hand
17. Vortex for 30 seconds just prior to spreading on carrier disc
18. Spread 13 µL on each of three carrier discs, attempting to make gold evenly distributed

Use suspension within two hours. Keep chilled prior to use.

*Plasmid DNA concentrations and molecular sizes were used to calculate how much of each were needed with co-bombardment. An approximate molar ratio of 1.5:1 was used for plasmid-of-interest to marker plasmid.

Appendix A-2: Workflow Chart Diagram



Appendix A-3: SDS DNA Extraction Method

1. Collect leaf tissue in 2 mL tubes. Freeze in liquid nitrogen and grind tissue to a fine powder
2. Add 800 µL DNA extraction buffer (See note for composition**) and mix well by vortex
3. Add 5µL RNAseA (10mg/mL) and incubate in a 37°C bath for 30 minutes
4. Add 800µL phenol:cholorform:isoamyl alcohol (25:24:1) and shake well
5. Centrifuge at 12,000 rpm for 10 minutes
6. Add 650 µL chloroform and mix gently
7. Transfer the upper phase, clear and aqueous, to a 1.5 mL fresh tube
8. Add 45 µL sodium acetate (3M, pH 4.8) and 450 µL isopropanol – mix gently to precipitate DNA
9. Rest in -20°C for 30 minutes
10. Centrifuge at 12,000 rpm for 10 minutes
11. Pour off supernatant
12. Resuspend pellet in 600 µL 70% ethanol then centrifuge again for 10 minutes
13. Pour off the supernatant, use a pipette to remove all traces, then dry for ~30 minutes in a clean hood
14. Resuspend pellet in 100 µL of nuclease-free water – allow DNA to dissolve for 12-24 hours at 4°C then store at -20°C

    **Note: 1 L extraction buffer is composed of 33 mL 30% sarkosyl & 1% N-lauroyl sarcosine, 12.1 g tris base, 5.8 g sodium chloride, 3.2 g disodium ethylenediaminetetraacetate dihydrate in 18.2MΩ water with the final pH adjusted to 8.5 with hydrogen chloride (1 M)

Appendix A-4: MagBead DNA Extraction Method

1. Collect ~200 mg of leaf tissue in a 2 mL tube with 3 metal bb's (4.5 mm, zinc-plated steel, available from sporting goods stores)
2. Add 800 µL CTAB buffer*
3. Place tubes in the Tissue Lyser machine (Qiagen, Germantown, MD, USA) set at a frequency of 30/sec for 13 minutes – repeat this step as needed to pulverize tissue as completely as possible
4. Incubate in a 65°C bath for 30 minutes – shake tubes every 10 minutes
5. Centrifuge at 12,000 rpm for 10 minutes
6. Transfer ~400 µL of the supernatant (clear-green and aqueous) to a deep-well plate
7. Add 400µL 10% polyethylene glycol and MagBead solution (created as 40:1 PEG to MagBead suspension) to each well (MagBead solution: Zymo Research, Irvine, CA, USA)
8. Put 200 µL ultra-pure deionized water in to each well of a shallow-well elution plate
9. Put 800 µL 80% ethanol into each well of a deep-well wash plate
10. Load the DNA extraction program for the KingFisher Flex (ThermoFisher Scientific, Waltham, MA, USA)
11. Machine prompts you to load each plate: DNA sample (with PEG & beads), wash, elution and tip covers
12. Let program run, then seal elution plate with foil and store at -20°C

*CTAB buffer is composed to 30 mL with 3 mL tris-hydrochloride (1 M, pH 6), 9 mL sodium chloride (5 M), 3.75 mL hexadecyltrimethylammonium bromide (16% w/v), 6 mL ethylenediaminetetraacetic acid (EDTA) (250 mM, pH 7) and 0.231 g dithiothreitol (DTT) in 18.2MΩ water

Appendix A-5: CTAB DNA Extraction Method

1. Collect leaf tissue in 2 mL tubes with 2 metal bb's (4.5 mm, zinc-plated steel, available from sporting goods stores)
2. Add 600 µL CTAB buffer (*see composition prior) per sample
3. Use the Tissue Lyser at a setting of 30/sec for 1 minute – repeat as needed to pulverize tissue
4. Place tubes in a 65°C bath for 30 minutes, shaking every 10 minutes
5. Centrifuge short at 12,000 rpm, then add 450 µL 24:1 chloroform to isoamyl alcohol
6. Shake vigorously then let rest at room temperature for 5 minutes
7. Centrifuge at 12,000 rpm for 10 minutes
8. Remove ~300 µL supernatant to a fresh 1.5 mL tube (only remove clear, aqueous top layer)
9. Add 30 µL sodium acetate (3 M) and 300 µL isopropyl alcohol – shake tubes vigorously
10. Place in -20°C for at least 1.5 hours
11. Centrifuge at 12,000 rpm for 10 minutes
12. Pour off supernatant
13. Add 500 µL 70% ethanol and resuspend pellet
14. Centrifuge at 12,000 rpm for 10 minutes
15. Pour off supernatant
16. Repeat steps 13 through 15 to wash pellet again
17. Let pellet dry in clean hood for ~30 minutes.
18. Resuspend pellet with 100 to 200 µL of nuclease-free water and let DNA dissolve 12 to 24 hours in 4°C, then store at -20°C

Appendix A-6: TRIzol RNA Extraction, DNAseI Treatment, and cDNA Synthesis

1. Freeze and grind tissue in 2 mL tubes
2. Add chilled TRIzol reagent (usually 1000 µL – recommended 1 mL per 50 to 100 mg plant tissue) to sample over ice (ThermoFisher Scientific, Waltham, MA, USA)
3. Chill on ice for 15 minutes
4. Add 200 µL chloroform and shake vigorously for ~15 seconds – let rest at room temperature for 15 minutes
5. Centrifuge at 10,000 rpm at 4°C for 15 minutes
6. Remove clear, aqueous upper phase to a new DEPC-treated 1.5 mL tube
7. Add 500 µL isopropanol (per 1 mL TRIzol earlier) and mix gently
8. Let rest in -20°C for 30 minutes
9. Centrifuge at 10,000 rpm at 4°C for 15 minutes
10. Pour off supernatant – centrifuge short at 10,000 rpm, then use a pipette to remove the remainder of the supernatant
11. Wash pellet with 1,000 µL (per 1 mL TRIzol) cold 75% ethanol (made with DEPC-treated water) and vortex
12. Centrifuge 4 to 5 minutes
13. Repeat steps 10 through 12 for a second wash
14. Dry pellet in a clean hood for ~5 minutes
15. Dissolve pellet in 50 µL DEPC-treated water
16. Incubate in oven at 65°C for 10 minutes, then transfer immediately to ice for 5 minutes
17. Test concentration and proceed to DNAseI treatment (ThermoFisher Scientific, Waltham, MA, USA) or store samples at -80°C
18. In a PCR tube mix 1 µL RNA sample (or 2 µL if RNA concentration is less than 500 ng/µL), 1 µL DNAseI buffer, 1 µL DNAseI enzyme (40 U/µL) and 9 to 10 µL DEPC-treated water
19. Let rest at room temperature for 15 minutes
20. Add 1 µL ethylenediaminetetraacetic acid (EDTA) (50mM)
21. Incubate in 65°C oven for 10 minutes
22. Add 1 µL oligo-DT's (145.5 mM) and 1 µL dNTP's (10 mM)
23. Incubate an additional 5 minutes at 65°C
24. Spin down briefly

25. Add 5x first-strand buffer, 2 µL dithiothreitol (DTT) (100 mM), and 1 µL RNAse out inhibitor (40 U/µL) (buffer and inhibitor by ThermoFisher Scientific, Waltham, MA, USA) – mix by pipette then spin down
26. Let rest at room temperature for 2 minutes
27. Add 0.6 µL M-MLV RT (200 U/µL) (ThermoFisher Scientific, Waltham, MA, USA) and mix by pipette
28. Use a PCR machine to incubate sample at 37°C for 50 minutes, then inactivate the reaction with 70°C for 15 minutes
29. Can be stored directly at -20°C, a volume should be diluted to 10% cDNA for future use with PCR testing.

Appendix A-7: DNA PCR Components

Volume to 20 µL

Nuclease-free water: 13.2 or 11.2µL (based on amount of DNA template)

6x Taq buffer with loading dye: 6 µL

Taq polymerase 1:8: 0.5 µL

dNTP's (10 mM): 0.3 µL

Forward primer (1 µM): 0.5 µL

Reverse primer (1 µM): 0.5 µL

Template DNA: 1 or 3 µL (depending on quality and concentration

Appendix A-8: cDNA PCR Components

Volume to 20 µL

Nuclease-free water: 13.5

5x Taq buffer: 4 µL

Taq polymerase 1:8: 0.3 µL

dNTP's: 0.4 µL

Forward primer (1 µM): 0.4 µL

Reverse primer (1 µM): 0.4 µL

Template DNA: 1 µL

The product from the first-round reaction was diluted 500x then used as the template DNA in the second set-up which otherwise had the same components as the first.