GENETIC ANALYSIS AND FUNCTIONAL GENOMIC TOOL DEVELOPMENT TO CHARACTERIZE RESISTANCE GENE CANDIDATES IN WHEAT

(Triticum aestivum L.)

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation/thesis of HARVINDER SINGH BENNYPAUL find it satisfactory and recommend that it be accepted.

Chair
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GENETIC ANALYSIS AND FUNCTIONAL GENOMIC TOOL DEVELOPMENT TO
CHARACTERIZE RESISTANCE GENE CANDIDATES IN WHEAT

*(Triticum aestivum* L.)*

Abstract

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Wheat (*Triticum aestivum* L.) is the most widely cultivated food crop in the world. Biotic factors such as insects and pathogens cause a significant loss in wheat yield worldwide. Deployment of resistance genes is usually the most effective, economical and environmentally sound method of managing pest-related losses. Linked molecular markers can help in accurate and targeted deployment of resistance genes.

Majority of the cloned plant resistance genes belong to the NBS-LRR class of resistance genes. Analyses of NB/LRR-containing sequences, PCR amplified from various crops, have suggested that only a small fraction of the *R*-gene-like genomic sequences may be functional, and this fraction is expected to be even smaller in crop plants with larger genomes. Therefore, resistance gene-like sequences amplified from the expressed fraction of the genomes may be better markers if not the resistance genes itself. In a previous study, 184 *R*-gene candidates (RGCs) were amplified from the expressed portion of the wheat genome of which 121 physically mapped to 18 regions containing 82 wheat resistance genes. With the overall goal to
characterize the RGCs, the main objectives of this study were to test linkage of RGCs to known resistance genes and to develop reverse genetic tools for transient and stable RNAi to facilitate characterization of the RGCs.

Linkage relationship of 14 phenotypically characterized resistance genes was investigated with 47 RGCs mapping in their reported chromosomes/arms/regions. Chromosomal location of nine resistance genes, $H13$, $H22$, $H24$, $H25$, $Lr9$, $Lr16$, $Yr10$, $Pm3a$ and $Pm20$, was determined relative to RGCs. Seven of the nine genes were physically mapped. In total, 26 RGCs were found linked ($\leq 30$ cM) with nine resistance genes. Nine RGCs showed close linkage ($\leq 10$ cM) with seven resistance genes of which $BF482366$ is 3.8cM from $H24$, $BE492937$ and $BF474204$ are 4.7cM and 5.6cM from $Yr10$, respectively.

Virus-induced gene silencing (VIGS), using barley stripe mosaic virus based vector, was optimized for transient silencing of genes in important wheat tissues including leaf, root and developing seed. Monitored using green fluorescent protein (GFP), spread of VIGS vector was observed in inoculated leaf tissue, phloem and root cortex at 10 and 17 days post-inoculation, but was absent in apical meristems and reproductive tissues. Compared to control plants, an antisense construct of the wheat coronatine insensitive1 ($TaCOI1$) gene reduced $TaCOI1$ transcripts by 60% in roots and 65% in foliage of cultivar Scarlet. The seed-specific granule bound starch synthase ($GBSS$) gene was successfully silenced with antisense and hairpin constructs which resulted in up to 81% reduction in amylose content. Antisense and hairpin constructs produced similar levels of gene silencing except in one case where hairpin construct showed a significantly higher level of silencing. Monitored by typical symptoms of $PDS$ gene silencing, 8 to 11% of the progeny of the inoculated plants showed transmission of gene
silencing. In the next generation, however, 53 to 61% of the selfed progeny, of the plants that showed silencing in previous generation, showed silencing.

Stable RNAi approach involving Agrobacterium-mediated transformation with hpRNA expressing construct was optimized in wheat. A clear and repeatable procedure was standardized whereby, starting with immature embryos, transformants can be produced at a frequency of up to 5.5% in eight to ten weeks. In one experiment, the optimized procedure produced 22 transgenic plants from 400 immature embryos. The Gateway® cloning technology based RNAi vector pHELLSGATE 8 was investigated for its use in high throughput construction of hpRNA constructs and for gene silencing. Nine sequences, ranging in length from 225 – 633bp, were successfully cloned in sense and antisense orientation to make hpRNA constructs. Irrespective of the length of the sequence, 50 – 67% of the constructs had sequences cloned in proper orientation. Plants transformed with pHELLSGATE 8-based RNAi construct showed 80 – 100% reduction in transcript level of targeted wheat expressed sequence tag BE405778.
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DISSERTATION OUTLINE

First chapter describes objectives and hypotheses building for the study based on the available literature. The second chapter provides detailed methods that are also briefly described in the manuscripts included in this dissertation. Third chapter is written in the format of “The Plant Journal” and it describes virus-induced gene silencing for functional analysis of genes expressing in various wheat tissues. The fourth chapter describes mapping of resistance genes relative to expressed resistance gene candidates (RGCs). The fifth chapter explains RNAi gene silencing in hexaploid wheat by Agrobacterium-mediated transformation.
Dedication

This dissertation/thesis is dedicated to my parents – S. Ajaib Singh and Mrs. Mohinder Kaur, my wife Harwinder and daughters Jasmine and Simar
CHAPTER ONE

INTRODUCTION

Bread or common wheat (*Triticum aestivum* L.) is one of the most important cereal crops in the world and provides about 23% of the dietary energy supply (http://faostat.fao.org/). It is the staple food for about 35% of the world’s population and provides more calories and protein in the world’s diet than any other crop (http://faostat.fao.org/). Since 1960s, wheat consumption in the developing countries has risen at an average rate of 5% per year, mainly because of rising population and changes in dietary patterns due to increasing wealth. By 2020, the demand for wheat is expected to be 40% greater than its current level of 630 million tons per year (Cassman 1999; Fufa et al. 2005). To meet this demand, global wheat production must increase by at least 2% per year. Increase in productivity, from continuously shrinking arable land base, could be accomplished by increasing yield and/or by protecting the 25% of the crop that is lost each year to biotic (pests) and abiotic (drought, heat, cold and salinity) stress factors. Genetic resistance to pests is usually the most effective, economical, and environmentally sound method of disease/pest management.

1.1.0 Wheat genome

1.1.1 Organization

Bread wheat is an allohexaploid (*2n = 6x = 42, AABBDD*) that evolved from a hybridization event of a tetraploid species (*Triticum turgidum* L.; *2n = 4x = 28, AABB*) with a diploid species *Aegilops tauschii* Coss. var. *strangulata* (Eig) Tzvelev (syn. *T. tauschii, A. squarrosa* (*2n = 2x = 14, DD*) (Dvorak et al. 1993; Kihara 1944; McFadden and Sears 1946;
Sarkar and Stebbin 1956). *T. turgidum* originated from a hybridization event between *Triticum urartu* Tumanian ex Gandilyan (2n=14, AA) and *Aegilops speltoides* Tausch (2n=2x=14, SS»BB) or its closely related species about 500,000 years ago (Huang et al. 2002). Wheat belongs to the tribe Triticeae in the grass family Poaceae that also contains major crops like rice (*Oryza sativa* L.), maize (*Zea mays* L.), barley (*Hordeum vulgare* L.) and oat (*Avena sativa* L.). Among the major crops of the grass family, hexaploid wheat has the largest genome that is about 35 times larger than that of rice and six times the size of maize (Arumuganathan and Earle 1991). Bread wheat, being hexaploid, has genome size about three times that of the diploid cultivated barley.

### 1.1.2 Gene distribution

Despite such a large size only 1 - 5% of the wheat genome appears to contain genes (Sidhu and Gill 2004). Physical mapping of gene markers on an array of chromosome deletion lines has revealed that most wheat genes are present in clusters that occur more frequently in the distal parts of the chromosomes (Gill et al. 1996b; Gill et al. 1996a; Sandhu and Gill 2002a). More than 85% of the wheat genes are present in 48 gene rich regions (GRRs) that cover less than 10% of the genome (Erayman et al. 2004). These GRRs are interspersed with gene-poor regions predominantly consisting of retrotransposon-like repetitive DNA sequences and pseudogenes (Barakat et al. 1997; Feuillet and Keller 1999; Sandhu and Gill 2002a; SanMiguel et al. 1996).

### 1.1.3 Recombination
Comparisons of genetic distances among C-bands have shown that distribution of recombination is uneven along the wheat chromosomes (Curtis and Lukaszewski 1991; Dvorak et al. 1984). Comparisons of wheat physical and genetic linkage maps conclusively showed uneven distribution of both genes and recombination, and established a general correlation between the two (Faris et al. 2000; Gill et al. 1993; Gill et al. 1996b; Gill et al. 1996a; Kota et al. 1993; Sandhu et al. 2001; Weng et al. 2000). According to Erayman et al. (2004), recombination in wheat mainly occurs in the GRRs and the rate can vary as much as 140-fold among various GRRs. In some cases, regions around eukaryotic centromeres and telomeres have been reported to suppress recombination rates (Puechberty et al. 1999; TAGI 2000; Tanksley et al. 1992). More than 30% of the wheat genes are in recombination-poor regions and thus are inaccessible to map-based cloning (Erayman et al. 2004).

1.2.0 Mapping in wheat

Physical and genetic mapping are the two main strategies that have been used to map protein, DNA and morphological markers on wheat chromosomes. Physical maps show the specific physical locations of its genes and/or markers on each chromosome, whereas, genetic linkage maps show relative position of markers along a chromosome based on frequencies of recombination among markers.

1.2.1 Physical mapping

In wheat, physical maps are constructed using aneuploid stocks. Due to polyploid buffering, bread wheat can tolerate deficiencies of whole chromosomes, chromosome arms, or sub-arm regions, allowing the development of a vast array of cytogenetic stocks (Endo 1988;
Endo and Gill 1996; Sears 1954; Sears and Sears 1978). The three main types of aneuploid stocks that are invaluable for physical mapping include nullisomic-tetrasomic (NT), ditelosomic (DT), and single-break deletion lines in a common background of a spring wheat cultivar, ‘Chinese spring’ (CS). The NT lines have a pair of chromosomes missing the deficiency of which is compensated for by a double dose of either of its two homoeologous chromosomes. Ditelosomics lines are deficient in a pair of chromosome arms. A total of 436 chromosome deletion lines for all of the 21 chromosomes are available in wheat (Endo, 1996). These aneuploid stocks have been extensively used for inter- and intra-chromosomal mapping to develop cytogenetic physical maps of the 21 wheat chromosomes (Gill et al. 1993; Gill et al. 1996b; Gill et al. 1996a; Kota et al. 1993; Mickelson-Young et al. 1995; Weng et al. 2000; Werner et al. 1992). The same approach was recently used to physically map about 9,400 wheat ESTs (Conley et al. 2004; Hossain et al. 2004; Linkiewicz et al. 2004; Miftahudin et al. 2004; Munkvold et al. 2004; Peng et al. 2004; Randhawa et al. 2004) (http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi). In another study, Erayman et al. (2004) physically mapped 3025 loci including 252 phenotypically characterized genes and 17 quantitative trait loci (QTLs) relative to 334 deletion breakpoints. Dilbirligi et al. (2004) mapped 121 expressed resistance gene candidates (RGCs), sequences similar to cloned resistance genes and amplified from expressed portion of the wheat genome, to 26 chromosomal regions encompassing about 16% of the wheat genome. In another study 137 resistance gene like sequences, kinase analogs and NBS-LRR resistance gene analogs, amplified from genomic DNA were physically and genetically mapped on 20 wheat chromosomes (Maleki et al. 2003).

1.2.2 Genetic mapping
The construction of genetic linkage maps is important for the efficient exploration of genetic potential of any organism. Genetic linkage maps of many plant species have been constructed and utilized for gene tagging and genome organization and evolutionary studies.

Genetic linkage mapping in wheat can be complicated because of its polyploidy nature. Another complicating factor is the low levels of polymorphism that is probably due to wheat’s recent origin from a few hybridization events (Kihara 1944; Sears 1954, 1966). Genetic linkage maps of bread wheat have been developed using ‘restriction fragment length polymorphisms’ (RFLPs) (Anderson et al. 1992; Chao et al. 1989b; Devos et al. 1992; Gale et al. 1995; Gill et al. 1996b; Gill et al. 1996a; Liu and Tsunewaki 1991; Mingeot and Jacquemin 1999; Nelson et al. 1995b; Nelson et al. 1995c; Nelson et al. 1995a; Van Deynze et al. 1995), simple sequence repeat (SSR) (Roder et al. 1998; Somers et al. 2004), amplified fragment length polymorphisms’ (AFLPs) and ‘randomly amplified DNAs’ (RAPDs) (Chalmers et al. 2001; Semagn et al. 2006). Over 137 genetic maps of various homoeologous groups are available in wheat (http://wheat.pw.usda.gov/GG2/index.shtml). The genetic map based on a recombinant inbred lines (RILs) population from a cross between synthetic hexaploid wheat W-7984 and bread wheat cultivar Opata 85 and coordinated by the International Triticeae Mapping Initiative (ITMI), has the most number of markers with over 1000 RFLP (Marino et al. 1996; Nelson et al. 1995b; Nelson et al. 1995c; Nelson et al. 1995a; Van Deynze et al. 1995) and 1150 SSR (Roder, 1998; Roder et al. unpublished results) loci.

1.3.0 Molecular markers
DNA marker technology developments in the last two decades have provided a large number of environmentally insensitive genetic markers (Gupta et al. 1999; Peleman and van der Voort 2003). Three broad categories of DNA markers are available that include; (i) hybridization-based (RFLPs); (ii) polymerase chain reaction (PCR) based including RAPDs, AFLPs, SSRs or microsatellites, sequence-tagged sites (STS) and sequence characterized amplified regions (SCARs); and (iii) hybridization and/or PCR-based markers like single nucleotide polymorphisms (SNPs).

The RFLPs were among the first markers used for large scale gene mapping and tagging (Autrique et al. 1995; Borner et al. 1997; Botstein et al. 1980; Hartl et al. 1995; Hartl et al. 1993; Ma et al. 1993; Ma et al. 1994; Nelson et al. 1997; Paull et al. 1995; Williams et al. 1994). More than 14,000 RFLP probes have been developed for wheat (Conley et al. 2004; Hossain et al. 2004; Linkiewicz et al. 2004; Miftahudin et al. 2004; Munkvold et al. 2004; Peng et al. 2004; Randhawa et al. 2004). Detailed RFLP linkage maps have been published for all 21 wheat chromosomes (Chao et al. 1989a; Marino et al. 1996; Nelson et al. 1995b; Nelson et al. 1995c; Nelson et al. 1995a; Van Deyne et al. 1995; Xie et al. 1993). Time-consuming and laborious process for RFLP analysis, low levels of polymorphisms in wheat (Bryan et al. 1999), and the use of radioactive isotopes, reduced the applicability of these markers in genetic mapping.

In wheat, about 200 RAPD markers have been used in studies dealing with genetic diversity (Chague et al. 1999; Joshi and Nguyen 1993; Vierling and Nguyen 1992) and gene mapping (Demeke et al. 1996; Dubcovsky et al. 1998; Dweikat et al. 1997; Penner et al. 1995). The RAPD markers fell out of use due to their inability to distinguish homozygous and heterozygous individuals, utility across cultivars, and reproducibility issues. Use of AFLPs in mapping studies has also been restricted because of the same reasons although these markers are
especially useful for targeted high density mapping of a region of interest (Ma and Lapitan 1998). The AFLPs have been successfully used to map *Lr46* and *Yr29* (William et al. 2003), *Yr31* (Singh et al. 2003), *Pm4b* (Hartl et al. 1998), *Pm22* (Singrun et al. 2003), and *Pm24* (Huang and Roder 2003).

With an objective to develop user-friendly reliable makers linked to a gene of interest, it is a common practice to convert RFLPs, RAPDs or AFLPs into STS (Sequence Tagged Sites) or SCAR (Sequence Characterized Amplified Region) markers. These type of markers have been developed for wheat resistance genes *Lr1* (Feuillet et al. 1995), *Lr9*, *Lr10*, *Lr24* (Schachermayr et al. 1997; Schachermayr et al. 1995; Schachermayr et al. 1994), *Lr19* (Cherukuri et al. 2003; Prins et al. 2001), *Lr24* (Dedryver et al. 1996), *Lr28* (Naik et al. 1998), *Lr35* (Seyfarth et al. 1999); *Pm2* (Mohler and Jahoor 1996), *Pm4a* (Ma et al. 2004) *Pm13* (Cenci et al. 1999), *Pm21* (Liu et al. 1999); *Sr2* (Johnston et al. 1998), *Sr24*, *Sr26* (Mago et al. 2005); *Yr17* (Robert et al. 1999), *YrMoro* (Smith et al. 2000b); *H6* (Dweikat et al. 2002), *H9* (Zhu et al. 2005); and *Cre1* (Williams et al. 1996).

Among PCR based markers, SSRs have emerged as markers of choice owing to their highly polymorphic nature, abundance, codominant inheritance and reproducibility (Prasad et al. 2000; Roder et al. 1995). According to Röder et al. (1995), SSRs (GA)*n*/(GT)*n*, (AC)*n* and (AG)*n* are found approximately every 270, 292 and 212 kb of the wheat DNA, respectively. In bread wheat, > 2100 SSRs have been genetically mapped, of which about 1100 have been physically mapped (Roder et al. 1998; Somers et al. 2004; Song et al. 2005; Sourdille et al. 2004; Varshney et al. 2005).

A number of important resistance genes of wheat have been tagged with SSR markers. These include resistance genes for leaf rust, *LrTr* (Aghaee-Sarbarzeh et al. 2001), *LrTt1*
(Leonova et al. 2004), $Lr16$ (McCartney et al. 2005), $Lr39$ (Raupp et al. 2001); powdery mildew, $Pm3$, $Pm5e$ (Huang et al. 2004; Huang and Roder 2003), $Pm16$ (Chen et al. 2005); stripe rust, $Yr5$ (Sun et al. 2002), $Yr10vav$ (Bariana et al. 2002), $Yrns-B1$ (Borner et al. 2002); septoria tritici blotch, $Stb1$, $Stb2$, $Stb3$, $Stb4$ (Adhikari et al. 2004a, 2004b, 2004c); Hessian fly, $H9$, $H10$, $H11$, $H13$ (Liu et al. 2005a; Liu et al. 2005b; Liu et al. 2005c); Russian wheat aphid, $Dn1$, $Dn2$, $Dn4$, $Dn5$, $Dn6$, $Dn8$, $DnX$ (Liu et al. 2002b; Liu et al. 2000); and cereal cyst nematode $Cre3$ (Jahier et al. 2001), $Cre5$ (Martin et al. 2004).

1.4.0 Plant resistance genes

So far more than 46 resistance genes ($R$ genes) conferring resistance to various insect pests, nematodes, bacteria, fungi and viruses have been cloned from different plant species (Dilbirligi 2003). Most of these genes follow the gene-for-gene model of host-parasite interaction and seem to encode components of signal transduction pathway of resistance to plant pathogens (Michelmore and Meyers 1998). According to the gene-for-gene model (Flor 1971), a specific host $R$ gene interacts with a specific avirulence ($Avr$) gene in the pathogen to provide resistance. A lack of interaction results in susceptibility. In the simplest model for the gene-for-gene resistances, the $R$ gene protein interacts with the pathogen $Avr$ protein, either directly or via another protein (Staskawicz et al. 1995). Recognition of specific gene product initiates a signal transduction cascade of host genes and resistance is often manifested in the form of hypersensitive response (Hammond-Kosack and Jones 1997; Staskawicz et al. 1995). Hypersensitive response is locally induced programmed cell death leading to inhibition of pathogen growth at the site of infection.
Among the cloned resistance genes, \textit{HM1} from maize, \textit{Mlo} from barley and \textit{RPW8} from Arabidopsis, do not follow the gene-for-gene model of interaction. The \textit{HM1} gene encodes a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent HC-toxin reductase, which detoxifies the toxin made by race 1 of \textit{Cochliobolus carbonum} (Johal and Briggs 1992). Barley powdery mildew resistance gene, \textit{Mlo}, encodes an integral membrane protein that presumably inhibits programmed cell death. In the event of penetration attempt by the pathogen, the recessive allele \textit{mlo} results in spontaneous cell death thus provides a broad-spectrum resistance against most of the \textit{Blumeria graminis} f.sp. \textit{hordei} races (Buschges et al. 1997; Shirasu and Schulze-Lefert 2000). Similarly, the Arabidopsis \textit{RPW8} gene confers a broad-spectrum resistance against pathogenic races of powdery mildew (Xiao et al. 2001).

A majority of the resistance genes characterized to date show a high level of sequence similarity and contain one or more of the four major protein domains, namely nucleotide binding site (NB), receptor-like transmembrane kinase (RLK), cytoplasmic protein kinase (PK) and leucine rich repeat (LRR). These domains are also found in other receptors and signal transduction proteins (Staskawicz et al. 2001). Most of the cloned \textit{R} genes can be grouped into four distinct classes based on their domain(s). The most abundant with 31 of the 46 \textit{R} genes is the group containing NB and LRR domains (NB/LRR). This class of resistance genes can be further divided into two subclasses based on a conserved NH\textsubscript{2}-terminus domain. One class has a coiled-coil domain (CC) at the NH\textsubscript{2}-terminus (\textit{Bs2, Dm3, Gpa2/Rx1, I2, Mi, Mla, Pip, Pi-ta, Prf, Rp1, RPM1, RPP8/HRT, RPP13, RPS2, RPS5, Rx2, Sw-5, Xa1}) and the other has a TIR (Toll/interleukin receptor) domain (\textit{L, M, N, P, RPP1, RPP5, RPS4}) (Sun et al. 2001). The TIR domain shares homology to the cytoplasmic domain of the Toll protein of \textit{Drosophila} and its mammalian homolog, interleukin-1 receptor (IL-1R). The CC domain, which is sometimes
formed of leucine zipper (LZ), is more common in the NBS-LRR proteins of cereals. The TIR domain has not yet been identified in the NB/LRR containing R genes of monocots.

DNA sequence analysis revealed 166 putative NB-LRR genes in *Arabidopsis thaliana* and about 600 in rice (*Oryza sativa*) (Goff et al. 2002; Richly et al. 2002; TAGI 2000). The LRRs are found in many plant and animal proteins and are usually involved in protein-protein interactions (Kobe and Deisenhofer 1994). However, the combination of NBS-LRR domains has only been shown to function in disease resistance pathways (Meyers et al. 1999). Second class of R genes encodes transmembrane proteins containing extracellular LRRs (Dixon et al. 1998). Some of the important members of this class are Cf-2, Cf-4, Cf-5 and Cf-9 that provide race-specific resistance in tomato against *Cladosporium fulvum*. Third class includes genes like *Xa21* of rice that contains LRR, PK and RLK domains and encodes a transmembrane protein joined with an extracellular LRR receptor and a cytoplasmic serine-threonine kinase (Song et al. 1995). Fourth class of R genes contains only PK but lacks both LRR and NB domains. Included in this class is the *Pto* gene of tomato, which is a cytoplasmic serine-threonine kinase conferring resistance to a bacterial pathogen *Pseudomonas syringae pv. tomato/avrPto* (Martín et al. 1993). *Pto* was the first gene to be cloned that follows the gene-for-gene model of interaction. Kinases modulate phosphorylation of the serine-threonine residues in order to control the protein activity (Loh and Martin 1995).

Several other R genes have been cloned and sequenced that do not fall in any of the above-mentioned classification e.g. *HM1* (Johal and Briggs 1992), *Hs1prot* (Cai et al. 1997), *Rpw8* (Xiao et al. 2001), *Ve* (Kawchuk et al. 2001), and *Rpg1* (Brueggeman et al. 2002). The tomato verticillium wilt-resistance gene (*Ve*) seems to encode a new group of R genes with cell surface glycoprotein, signals for receptor-mediated endocytosis, and LZ or PEST sequences
[PEST, region rich in proline (P), glutamate (E), serine (S), and threonine (T)] (Kawchuk et al. 2001). Barley stem rust resistance gene *Rpg1*, which encodes a receptor kinase-like protein with two tandem protein kinase domains, is a novel plant disease resistance gene (Brueggeman et al. 2002). Another recently cloned barley gene, *Rpg5*, consists of a unique structure encoding three typical plant disease resistance protein domains: nucleotide-binding site, leucine-rich repeat, and serine threonine protein kinase (Brueggeman et al. 2008).

1.4.1 Motifs/domains of R genes and their functions

Major domains present in the plant *R* genes contain one or more motifs with highly conserved amino acid sequences. The LRR domain contains 9 to 41 imperfect repeats, each about 25 amino acids long with a consensus amino acid sequence of XX(L)X(L)XXXX (Cooley et al. 2000). The PK domain of both *Pto* and *Xa21* contains up to 25 amino acids long motifs, where the first three (DFG) and the last two (PE) residues are highly conserved. An internal threonine (T) and a serine (S) residue are also conserved and are essential for autophosphorylation (Liu et al. 2002a). The NB domain of NB-LRR class of *R*-genes is also present in several other kinases such as ATP/GTP binding proteins. This domain contains three motifs: a kinase-1a (p-loop), kinase-2, and a putative kinase-3a (Tameling et al. 2002; Traut 1994). These motifs in *R*-genes have a consensus sequence of GXXGXGK(T/S)T, LXXXDDVW and GXXXXTXR for p-loop, kinase-2, and putative kinase-3a, respectively. The P-loop motif of *R* genes, which plays a role in phosphate binding, is remarkably different from that present in other NB-encoding proteins (Hammond-Kosack and Jones 1997; Meyers et al. 1999). The GLPL, RNBS-D and MHD are other motifs present in the NB domain of NB-LRR-type *R* genes (Meyers et al. 1999). The sequences interspersing these motifs and domains can be
very different even among homologs of an $R$ gene (Michelmore and Meyers 1998; Pan et al. 2000).

The LRR domain contains multiple repeat (about 10 to 40) motifs that are about 24 amino acids in length with leucines or other hydrophobic amino acids, proline and asparagines, present at regular intervals (reviewed in Jones and Jones, 1997). A high degree of sequence variation has been observed in the LRR encoding region (Ellis et al. 2000). This variation probably occurs due to point mutations, recombination, deletions and duplication events. Nucleotide changes in resistance gene sequences can either be synonymous (Ks) that do not change the amino acid sequence; or can be non-synonymous (Ka) that result in amino acid sequence changes. An alignment of 26 LRR units of the L6 rust resistance protein depicts that the sequence variation is more in the XXLXLXX (L=leucine or other aliphatic amino acid, X=any amino acid) motif (18.7%) as compared to the regions interspersing the LRR domain (6.6%). Furthermore, it has been shown that the synonymous changes are more for the non-LRR-encoding regions compared to the LRR-encoding regions, especially the XXLXLXX motif where non-synonymous changes are more (Ellis et al. 2000). Increased selection for amino acid variation at this site supports the assumption that this motif is actively involved in binding different pathogen-derived ligands and hence is involved in the control of resistance gene specificity (Ellis et al. 2000).

It has been proposed that the ligand produced directly or indirectly by the $Avr$ gene may be recognized by the extracellular receptors such as LRR domains of $R$ genes and such recognitions can trigger a signal transduction cascade leading to disease resistance (Dixon et al. 1996; Staskawicz et al. 1995). The $L2$ and $L10$ alleles of flax rust resistance gene $L$ that mainly differ in the amino acid sequence of the LRR regions, recognize different $Avr$ effectors. The
observation that replacing the LRR of \textit{L10} with that of \textit{L2} changes its specificity for effector binding (Ellis et al. 1999), again supports role of the LRR as receptor of effectors. Variation in number of direct repeats of LRR units in different alleles at the \textit{Rpp5} locus of Arabidopsis (Parker et al. 1997), \textit{Cf-2} and \textit{Cf-5} loci of tomato (Dixon et al. 1998), \textit{L} and \textit{M} genes of flax (Anderson et al. 1997), and \textit{Rp1} locus of maize has been reported. Recombination events in the \textit{Rp1} complex have been associated with the generation of novel race specificities against the \textit{Puccinia sp.} (Richter et al. 1995).

1.5.0 Resistance genes of wheat

Biotic and abiotic stress accounts for nearly 25% of the yield losses and approximately half of the losses are due to the biotic factors such as insects, diseases, viruses and nematodes (http://pseru.ars.usda.gov). About 285 genes conferring resistance to various pests have been identified in wheat or its wild relatives (McIntosh et al. 2000). Of the 285 wheat resistance genes; 156 have been mapped to chromosome arms, 69 to chromosomes and genomic location of 64 genes is not yet known (McIntosh et al. 2000).

Monosomic analysis has been extensively used to map genes on to wheat chromosomes and arms. Monosomic plants are 2n – 1 missing one of the 42 chromosomes. A complete set of monosomics for all wheat chromosomes is available in cultivar CS (Sears 1954) and in few other cultivars (Morris et al. 1972). To map a dominant resistance gene ‘A’ by monosomic analysis for example, a monosomic series in a cultivar that lacks the gene ‘A’ is used as a female parent (as the transmission of n – 1 gametes is about 75% through the egg cell and only about 4% through the pollen). Crosses with all the non-critical monosomics (‘A’ gene carrying chromosome not in monosomic condition) will show normal F$_2$ phenotypic segregation ratio of
3 (resistant): 1 (susceptible). However, a critical cross (involving monosome which carries gene ‘A’) will show a deviation from that ratio and a majority of the plants will be resistant. Monosomic analysis is a reliable approach for mapping genes as long as the identity of monosome is confirmed by cytology. Monosomic shift, where monosomy shifts to another chromosome, can happen due to imperfect transmission of certain chromosomes (Person 1956). For example, a plant monosomic for chromosome 1A can give rise to progeny disomic for 1A but monosomic for 1B. If undetected, monosomic shift can result in mapping of a gene to a wrong chromosome.

Now the most common method of localizing a gene is by mapping it relative to molecular marker(s) with known map location. Of the 156 wheat resistance genes originally mapped on chromosome arms using monosomic analysis, 120 have now been localized relative to molecular markers. Genomic location of 22 resistance genes was revised when mapped relative to molecular markers (McIntosh et al. 2007). For example, Hessian fly (Mayetiola destructor Say) resistance genes H9, H10 and H11, which were assigned to chromosome 5A by monosomic analysis (Carlson et al. 1978; Stebbins et al. 1982), have now been reassigned to chromosome 1AS by mapping relative to microsatellite markers (Liu et al. 2005b). Using a similar approach, another Hessian fly resistance gene H13 that was originally mapped to chromosome 6DL by monosomic and telocentric analysis (Gill et al. 1987), has been re-assigned to the short arm of chromosome 6D (Liu et al. 2005b).

Although more accurate than conventional cytological mapping, marker-based mapping can also assign a gene to a wrong genomic location as the mapping location of a gene is as correct as the location of the reference marker loci. Russian wheat aphid resistance gene Dn2 have been mapped to 7DL (Miller et al. 2001) and 7DS (Liu et al. 2000) based on the same SSR
marker *Xgwm111*. According to Miller et al. (2001) the exact position of *Xgwm111* on the map is ambiguous, which is similar to its status on the Roder et al.'s (1998) map.

Precise physical location of a marker is very important for its use in gene mapping and cloning. Resistance gene-like sequences (Resistance Gene Analogs amplified from genomic DNA and Resistance Gene Candidates amplified from cDNA) have been shown to cluster around the known resistance genes in a number of crops including soybean (Kanazin et al. 1996; Yu et al. 1996), maize (Xiao et al. 2007), Barley (Madsen et al. 2003) and wheat (Dilbirligi et al. 2004; Maleki et al. 2003). Resistance gene-like sequences can be good markers for resistance genes because of sequence similarity and proximity to known resistance genes.

1.6.0 **Resistance gene-like sequences**

Using degenerate primers designed from the conserved regions of the cloned *R* genes, polymerase chain reaction (PCR) based approaches have been used to clone genomic copies of resistance gene like sequences from rice (Mago et al. 1999), wheat (Spielmeyer et al. 1998), potato (Leister et al. 1996), soybean (Kanazin et al. 1996; Yu et al. 1996), barley (Collins et al. 2001b), lettuce (Shen et al. 1998), maize (Collins et al. 1998), common bean (Rivkin et al. 1999), sunflower (Gentzbittel et al. 1998), pepper (Pflieger et al. 1999), and Arabidopsis (Aarts et al. 1998; Speulman et al. 1998).

Resistance gene analogs (RGAs) have been shown to be linked to many disease resistance genes (Collins et al. 2001a; Collins et al. 1998; Leister et al. 1998). Barley RGAs on Chromosomes 2H and 6H showed close linkage with quantitatively inherited powdery mildew and leaf rust resistance genes, and on 3H and 7H with seedling resistance to powdery mildew (Madsen et al. 2003). In wheat, RGAs linked to stripe rust resistance genes *Yr5* and *Yr9*, have
been identified (Shi et al. 2001; Yan et al. 2003). In Arabidopsis, RGAs showed genetic linkage to 21 known disease resistance loci (Speulman et al. 1998). Similarly, many soybean genomic RGAs amplified with primers based on NBS region of N and RPS2, mapped in the vicinity of known genes for resistance to potyviruses (Rsv1 and Rpv), phytophthora root rot (Rps1, Rps2, and Rps3), and powdery mildew (rmd) (Yu et al. 1996). So far, none of the RGAs has been shown to be an R gene by functional complementation of a susceptible genotype. It could be because of the reason that majority of RGAs have been amplified from genomic DNA and RGAs from pseudo - R genes may outnumber that from functional R genes. Most of the cloned R genes have been shown to be single or few copies that will be out-competed by multi-copy non-functional RGAs during random cloning and sequencing of the amplified genomic fragments (Dilbirligi et al. 2004).

Analysis of NB-LRR containing genomic sequences from various plant species suggested that only a small fraction of the R genes may be functional (Chin et al. 2001; Shen et al. 2002; Sun et al. 2001). Transcripts for only nine of the 173 RGAs isolated from six major crops are represented by the corresponding ESTs (Dilbirligi and Gill, 2003). Over one million wheat ESTs are present in various databases (http://www.nebi.nlm.nih.gov/) but none represents any of the cloned wheat resistance genes including powdery mildew resistance gene Pm3b, leaf rust resistance genes Lr10 and Lr1. Similarly, no EST is available for the stem rust resistance gene RPG1 of barley. Proportion of the expressed R genes is expected to be even smaller in wheat because of the larger genome size. Therefore, the gene cloning approaches that specifically target the expressed part of the genome are expected to be more effective.

Dilbirligi et al. (2003) used cDNA, from leaves of resistant wheat varieties inoculated with various pathogens, as template to amplify R gene candidates (RGCs) by using degenerate
primers for the conserved p-loop (GVGKTT), hydrophobic domain (GLPLAL) and poly-T primers with two selective bases (T primers). The amplified fragments were size separated on 5% denaturing polyacrylamide-urea gels. Thus, unlike other similar studies, fragments differing even by a few base pairs were differentiated thus were individually cloned and sequenced. Blast search of 220 unique sequences in the NCBI database led to the identification of 184 RGCs. These included 87 NB-LRR type, 16 receptor-like kinases, and 13 Pto-like kinases. The remaining were seven HMI and two Hs1pro homologs, 17 pathogenecity-related, and 42 unique NB/kinases. From 220 sequences, 121 RGCs were physically mapped using 339 wheat deletion lines (Dilbirligi et. al., 2003). These RGCs mapped in 26 chromosomal regions, of which 18 contained 92 of the 98 morphologically characterized wheat R genes. In the present study, 92 of the 121 RGCs mapping in six chromosomal regions were chosen for linkage analysis with 22 resistance genes mapping on the corresponding chromosomes.

1.7.0 Wheat transformation

Two different approaches to transfer foreign DNA into wheat cells have been used: (i) direct gene transfer method by particle bombardment (Vasil et al. 1992); and (ii) an agrobacteium-mediated gene transfer (Cheng et al. 1997). Particle bombardment (biolistic transformation) is currently the most widely used technique for direct gene transfer including RNAi-triggering DNA sequences (Loukoianov et al. 2005; Travella et al. 2006; Yan et al. 2004). As compared to Agrobacterium-mediated transformation, the main advantages of particle bombardment are higher transformation efficiency and the absence of biological incompatibilities that are encountered when using biological vectors such as Agrobacterium tumefaciens.
The main disadvantage of the particle bombardment method is that it often results in multiple and complex insertion loci that often cause silencing of the target gene (Dai et al. 2001; Hammond 1999). In maize, more than 90% of the Agrobacterium-mediated transgenic events produced less than three copies per event compared to more than three copies that were produced by particle bombardment with some having as many as 100 copies (Shou et al. 2004). Insertion site mutations, deletion and re-arrangements of plant genomic DNA and insertion of contaminating bacterial DNA are common disadvantages of both methods but the extent and severity of these events is relatively less in Agrobacterium-mediated system (Arencibia et al. 1998). A transformation method that results in minimal disturbance to plant genome is very important for RNAi studies so that the loss of function phenotypes can be attributed to the targeted genes.

Agrobacterium-mediated transformation has been successfully used to transfer genes or RNAi-inducing DNA sequences in *Arabidopsis thaliana*, *Coffea arabica*, and *Oryza sativa* (Chuang and Meyerowitz 2000; Miki et al. 2005; Ogita et al. 2004; Stoutjesdijk et al. 2002; Wesley et al. 2001). Agrobacterium–mediated transformation of wheat, with transformation efficiency of 0.9% (Cheng et al. 1997) and 1-2% (Peters et al. 1999), has been repeated (Khanna and Daggard 2003; Wu et al. 2003) but has not been adapted for RNAi probably due to a lower transformation efficiency and lack of repeatability of the published procedures (Wu et al. 2003). This is partly due to lack of detailed, clearly-written, publicly-available wheat transformation protocols. One of the objectives of the present study was to optimize Agrobacterium-mediated wheat transformation and develop a detailed easy-to-follow protocol for transformation and RNAi.
1.8.0 Gene cloning strategies for wheat

Understanding molecular basis of trait development is important for genetic improvement of crop plants. The foremost step towards understanding molecular basis of a trait is the cloning of the genes responsible for the trait. Cloning is also important to understand functional regulation of the gene and its interaction with other components of the pathway. In addition, gene cloning is useful for finding gene-derived markers for marker-assisted breeding.

In wheat, a larger genome size (16,000 Mbp), hexaploid nature and the presence of a large amount of repetitive DNA make gene cloning a monumental task. Map-based cloning of wheat genes Lr1 (Cloutier et al. 2007), Lr10 (Feuillet et al. 2003), Lr21 (Huang and Roder 2003), Pm3b (Yahiaoui et al. 2004), Cre3 (Lagudah et al. 1997), VRNI (Yan et al. 2003) and VRN2 (Yan et al. 2004) have proven to be time consuming and laborious. More than 30% of the wheat genes are in recombination-poor regions and thus are inaccessible to map-based cloning (Erayman et al. 2004; Qi et al. 2004). Furthermore, in addition to the non-transcribing retroelement type repeated DNA, most wheat genes have orthologs and in some cases paralogs. Non-functional or diverged-function gene copies may further complicate gene cloning with aforesaid strategies.

Reverse genetics, an approach to go from sequence to function, is particularly appealing for plants like wheat. In wheat and barley however, it has only been used to confirm identity of the cloned genes (Brueggeman et al. 2008; Lacomme et al. 2003; Scofield et al. 2005). In addition to being a confirmation tool, reverse genetics has an enormous potential in aiding gene discovery in crop plants. Wheat expressed sequence tags (over a million) and gene sequences from closely related species are important resources that can be utilized for cloning wheat genes using reverse genetic approaches. Even though transient RNAi has been shown to work in wheat
leaves through VIGS (Scofield et al. 2005) but its utility in other important tissues has not been shown. Similarly, stable RNAi have been shown by transforming wheat with biolistic transformation (Travella et al. 2006) but RNAi through Agrobacterium-mediated transformation using a high throughput silencing vector still needs to be optimized.

1.9.0 RNA interference (RNAi)

1.9.1 Process of RNAi

In the last decade, RNAi has emerged as a tool of choice for reverse genetic studies. It is variously termed as; quelling in fungi, posttranscriptional gene silencing (PTGS) and RNA interference in plants and animals. Discovered by Fire et al. (1998) in Caenorhabidits elegans, RNAi is a manifestation of a broad class of RNA silencing phenomena that involves homology-dependent degradation of mRNA in the cytoplasm (Hamilton and Baulcombe 1999). A double-stranded RNA (dsRNA) with sequence homology to the target gene, acts as a trigger (Elbashir et al. 2001a; Elbashir et al. 2001b; Fire 1999; Hammond et al. 2001; Sharp 2001). This kind of RNA-silencing is a highly conserved mechanism found in almost all eukaryotes and is believed to serve as a defense mechanism against exogenous (e.g., viruses) and endogenous (e.g., mobile genetic elements) genetic parasites (Carthew 2001; Cogoni and Macino 2000; Elbashir et al. 2001a; Matzke et al. 2001; Plasterk and Ketting 2000; Sharp 2001).

In general terms, RNAi machinery uses the sequence information in the dsRNA to generate a protein-RNA complex that destroys the homologous mRNA. According to the available details of this phenomenon dsRNA is cleaved into small interfering (21 to 23 nucleotides) RNAs (siRNAs) by an RNase III like enzyme, known as Dicer (Bernstein et al. 2001; Ketting et al. 2001; Knight and Bass 2001; Tabara et al. 2002). Biochemical evidence
indicates that these siRNAs are incorporated into a nuclease containing multisubunit protein complex, the RNAi-induced silencing complex (RISC). The incorporated siRNAs then direct RISC to the target complimentary mRNA (McManus and Sharp 2002). RNAi is highly specific as mismatches of $>1$-$2$ bp within the $21$- to $23$-nt siRNA are known to effectively disrupt degradation of the target mRNA (Elbashir et al. 2001a; Elbashir et al. 2001b). Single nucleotide polymorphisms can be used to exclusively silence a sequence as was shown for the silencing of a mutant Machado-joseph disease/spinocerebellar ataxia type 3 allele while the expression of the wild type allele remained unaffected (Yokoyama and Miller 2003).

1.9.2 Types of RNAi

RNAi in plants can either be transient or stable/heritable. Stable RNAi is achieved by integration of the dsRNA expressing construct into a genome by transformation of plants with RNAi construct via either Agrobacterium or particle bombardment. This type of RNAi is heritable and often stable over generations. For transient RNAi, construct expressing dsRNA is not integrated into the genome and thus this type of silencing is non-heritable. Virus-induced gene silencing (VIGS) is an example of transient RNAi. Large-scale functional genomics first needs transient RNAi to quickly shortlist the candidates followed by stable RNAi to study the genes in more detail.

1.9.2.1 Transient RNAi

For transient gene silencing in plants, RNAi-inducing sequence can be introduced into cells by particle bombardment, agro-infiltration or virus infection. Delivery systems for transient RNAi are fast and simple as compared to inefficient and cumbersome process of genetic
transformation that is needed for stable RNAi. Using particle bombardment as a delivery system for RNAi plasmids, Schweizer et al. (2000) showed that transient expression of double-stranded RNA interferes with gene function at the single cell level in wheat, barley and maize. Infiltration of Agrobacterium, carrying RNAi-plasmid, has been shown to cause silencing of the target green fluorescent protein (GFP) gene in *Nicotiana benthamiana* (Voinnet and Baulcombe 1997; Voinnet et al. 1998).

Transient RNAi can be accomplished using virus induced gene silencing (VIGS) by expressing RNAi-inducing sequence as a part of the viral genome. VIGS was first demonstrated against invading viral genomes where host tissue infected with a recombinant viral vector, carrying target gene sequence, showed degradation of the endogenous gene (Vance and Vaucheret 2001). A VIGS system was first applied in plants to silence the phytoene desaturase (*PDS*) gene in *Nicotiana benthamiana* (Kumagai et al. 1995). The *PDS* gene is essential for carotenoid biosynthesis, and its suppression in barley (*Hordeum vulgare*) results in photobleaching of chlorophyll (white streaks) in the affected tissue (Holzberg et al. 2002). The VIGS system of (Kumagai et al. 1995) used an infectious strain of tobacco mosaic virus (TMV) carrying an antisense *PDS* sequence. Since then, several modified viral genomes have been used as VIGS vectors for targeting genes in leaf tissue of barley (Holzberg et al. 2002; Lacomme et al. 2003; Brueggeman et al. 2008) and wheat (Scofield et al. 2005), flowers and leaves of peas (*Pisum sativum*) (Constantin et al. 2004), fruit of tomato (*Lycopersicon esculentum*) (Fu et al. 2005), and roots and meristems of tomato, tobacco and *Arabidopsis thaliana* (Valentine et al. 2004).

Barley stripe mosaic virus (BSMV) and Brome mosaic virus (BMV) vectors have been developed for gene silencing in monocot species, including barley, wheat, maize (*Zea mays*) and
rice (*Oryza sativa*) (Ding, 2006; (Ding et al. 2006; Holzberg et al. 2002; Lacomme et al. 2003; Scofield et al. 2005). BSMV is a single-stranded RNA virus consisting of a tripartite genome (α, β and γ) (Petty et al. 1989). Target sequence in antisense or hairpin construct is inserted in the γb open reading frame of the γ RNA (cDNA) downstream of an introduced translation stop codon. BSMV-based VIGS has been successfully used to study the function of several genes involved in disease resistance pathways in barley and wheat leaves; *Rar1, Sgt1*, and *Hsp90* (Holzberg et al. 2002; Lacomme et al. 2003; Scofield et al. 2005). Application of this exciting technology remains to be demonstrated for important tissues such as roots and meristems of monocots, and for developing seeds of both monocots and dicots.

1.9.2.2 Stable RNAi

Stable RNAi can be achieved by transforming an organism with gene construct encoding hairpin RNA (hpRNA). In wheat, stable silencing of *Phytoene destaurase* (Travella et al. 2006), *VRN2* and *VRN3* (Yan et al. 2006; Yan et al. 2004) have been achieved by particle bombardment-based transformation. In another study, Agrobacterium-mediated transformation of hairpin expressing construct resulted in 85% and 90% knockdown in *phytoene desaturase* (*PDS*) gene in rice and Arabidopsis, respectively (Wesley et al. 2001). Stable RNAi is a reliable and reproducible tool for gene silencing (Regina et al. 2006; Smith et al. 2000a; Travella et al. 2006), however, this approach generally is not efficient for large-scale functional analysis in crop plants such as wheat due to lack of efficient and reproducible transformation methods.
1.10 Objectives

The main goal of this study was to characterize previously cloned RGCs (Dilbirligi, 2003). Thus, the objectives of this study were to: 1) physically and genetically map known resistance genes relative to RGCs, and 2) optimize reverse genetic tools for transient and stable RNAi to facilitate characterization of the RGCs.
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CHAPTER TWO

MATERIALS AND METHODS

2.1 DNA gel blot analysis

Plant genomic DNA extraction and gel blot analysis was performed following the procedure described by Gill et al. (1993). Leaf tissue was ground in liquid nitrogen and incubated in DNA extraction buffer (0.1 M Tris pH 7.0, 0.7 M NaCl, 10 mM EDTA pH 8.0, 1% Hexadecyltrimethylammonium Bromide, 0.14 M 2-Mercaptoethanol) at 60°C for 3 hours. The slurry was extracted with equal volume of chloroform-octanol (24:1 v/v). The DNA was precipitated from the supernatant by adding two-third volume of isopropanol. Precipitated DNA was washed first with 76% ethanol + 0.2 M sodium acetate for 30 minutes and then with 76% ethanol + 10 mM ammonium acetate. The DNA was air dried and then dissolved in TE containing RNase (2 µg/ml). The DNA was extracted with phenol: chloroform (1:1) and recovered as precipitates by adding 0.3 M sodium acetate (pH 5.2) + equal volume of isopropanol. The DNA pellet was washed with 70% ethanol for 30 minutes, air dried, and dissolved in TE.

For each sample, 5 µg of genomic DNA was quantified using DyNA Quant200 fluorometer (Hoefer) and digested overnight with 20 units of restriction enzymes in the presence of spermidine (3 mM) and Bovine Serum Albumin (0.1 mg/ml). The DNA was digested at the temperature recommended by the restriction enzyme manufacturer. Digested DNA was size separated by electrophoresis on a 36-lane 0.8% agarose gel in 1X TBE (Tris-borate-EDTA) buffer at a constant voltage of 35V for 18 hours.
‘Southern’ blotting onto nylon membrane (Micron Separations Inc. MA), DNA immobilization, and hybridization were performed following manufacturer’s recommendations. Gels were stained with ethidium bromide and treated with 0.25N HCl for 12 minutes with gentle shaking. After washing with distilled water, gels were treated with denaturing solution (0.4N NaOH, 0.6M NaCl) for 30 minutes followed by neutralizing solution (0.5M Tris, 1.5M NaCl, pH 7.5) for 30 minutes. The DNA was transferred to nylon membrane by capillary method for 16 hours in 20X SSC (0.3 M sodium citrate, 3 M sodium chloride). After the transfer, the membranes were soaked in 5x SSPE (3 M sodium chloride, 0.2 M sodium phosphate monobasic, 0.02 M EDTA) at 65°C for 5 minutes, air dried, and baked at 80°C for 1 hour. The membranes were exposed to 20,000 µJoules of UV energy in the UV stratalinker (Spectrolinker, Fisher) in order to crosslink DNA to the membranes. Prehybridization was performed in 35 x 150 mm glass bottles containing 50 ml of prehybridization solution (6X SSPE, 5% Denhardt’s solution, 2.5% SDS, 0.5% Salmon sperm DNA), incubated at 65°C for 5 - 18 hours in a hybridization rotisserie (Hybaid). For hybridization, about 30 ng of probe DNA was labeled with 30 µCi of ³²P-dCTP following the random primer labelling technique (Feinberg and Vogelstein 1983). Unincorporated nucleotides were removed using Sephadex G-50 spin column method. Hybridizations were performed in 10 ml of solution containing 5% dextran sulfate, 6x SSPE, 5% Denhardt’s solution, 2.5% SDS, 0.5% Salmon sperm DNA. The blots were washed at 65°C in 2X SSPE, 0.5% SDS for 30 minutes. After rinsing with 2X SSC solution the blots were wrapped in plastic and exposed to X-ray film with two intensifying screens at -80°C for three to seven days depending upon the radioactive signal.

2.2 Making VIGS constructs using oligonucleotides
Complementary oligonucleotides for the target sequence, with \textit{Pac1} and \textit{Not1} sites at the 5’ and 3’ ends respectively, were synthesized by Operon Biotechnologies, Huntsville, Alabama, US. Commercially synthesized oligonucleotides were dissolved in 0.1X TE at a concentration of 10 µg/µl. Complementary oligonucleotides were mixed (10 µg each), heated at 65°C for 20 minutes and incubated at room temperature (RT) for 1 hour. The annealed products were cleaved using 20 units each of \textit{Pac1} and \textit{Not1} at 37°C overnight. Restriction cleavage products were purified by phenol: chloroform (1:1) extraction and then precipitated by adding 2 volumes of ethanol and 1/10th volume of 3 M sodium acetate (pH 5.2). The precipitates were washed in 70% ethanol, air dried, and dissolved in 0.1X TE. The cleaved products were mixed in equal amounts (50 -100 ng) with \textit{Pac1} - \textit{Not1} restricted \textit{pγ}.bPDS4 plasmid and were ligated following pGEM\textsuperscript{®}-T Easy Vector System protocol (Promega Corp. USA).

2.3 \textit{Making viral RNAs and inoculation}

Infectious RNAs were prepared by transcribing the three linearized plasmids (two common plasmids \textit{pα}, \textit{pβΔβ}a and a target specific BSMV\textit{γ}-based plasmid) using the mMessage mMachine T7 \textit{in vitro} transcription kit (Ambion, Austin, TX, USA) according to the manufacturer’s recommendations.

Plasmids \textit{pα}, \textit{pβΔβ}a and the target specific \textit{pγ} were linearized with restriction enzymes \textit{MluI}, \textit{SpeI} and \textit{SwaI}, respectively. Linearized plasmid DNA was treated with proteinase K (100-200 µg/ml) and 0.5% SDS for 30 minutes at 50°C, followed by standard phenol/chloroform extraction and ethanol precipitation. The plasmid DNA was resuspended in 0.1X TE at 125 ng/µL concentration. For RNA transcription from each linearized plasmid, a 2.5 µL reaction was set using 80 ng DNA, 0.25 µL of the enzyme mix, 0.25 µL of 10X buffer and 1.25 µL of 2X
NTP/CAP (ribonucleotides). The reactions were incubated at 37°C for 60 minutes. Equal volumes of transcribed RNAs from all three plasmids (2.5 µL each) were combined with 45 µL FES (0.1 M glycine pH 8.9, 0.06 M K$_2$HPO$_4$, 1% sodium pyro-phosphate, 1% Celite, 1% bentonite) and was used for plant inoculations.

2.4 **Total RNA extraction**

Total RNA was isolated using TRIzol® reagent according to the manufacturer’s recommendations (Invitrogen Corp., Carlsbad, CA, USA). All non-disposable laboratory ware were rinsed with 0.01% v/v diethyl pyrocarbonate and autoclaved for 45 minutes prior to use. Plant tissue (0.5 to 1 g fresh weight) was pulverized to a fine powder in liquid nitrogen using a mortar and pestle and transferred to chilled ‘Oak Ridge’ tubes containing 8 ml of TRIzol® reagent. Tubes were inverted 30 – 40 times for mixing the contents and then incubated at room temperature (RT) for 5 – 60 minutes. Samples were centrifuged at 12,000 g at 4°C for 10 minutes in a Beckman JA-17 fixed-angle rotor. Supernatant was transferred to clean Oak Ridge tubes and mixed with 2 ml of chloroform. Samples were vortexed for 30 seconds and incubated at RT for 2 - 3 minutes. After incubation, the samples were centrifuged at 10,000 g at 4°C for 15 minutes in Beckman JA-17 fixed-angle rotor. The aqueous phase was transferred to clean Oak Ridge tubes and mixed with 2 ml each of Isopropanol and citrate NaCl (0.8 M Sodium citrate, 1.2 M Sodium Chloride). The resulting solution was mixed by inverting the tubes several times and then incubated at RT for 10 minutes. Total RNA was pelleted by centrifugation at 10,000 g at 4°C for 15 minutes in the previously mentioned rotor. The supernatant was gently discarded and the RNA pellet was washed with 80% chilled ethanol. After centrifugation at 7,500 rpm @ 4°C for 10 minutes, the supernatant was discarded and the RNA pellet was resuspended in
DEPC-treated MilliQ water. RNA concentration was estimated by diluting 5 µl of RNA with 495 µl of water and absorbance at 260 nm was measured using a Bio-Rad SmartSpec 3000 (Bio-Rad Laboratories, Hercules, CA, USA).

2.5 First strand cDNA synthesis

Approximately 5 µg of RNA was combined with 2.5 mM oligo dT_{20} (Invitrogen) in a total volume of 11 µl of DEPC-treated nanopure water. The mixture was heated at 65°C for 5 minutes and then chilled on ice. The RNA mixture was supplemented with 8 µl containing 1X First Strand buffer (Invitrogen), 10 mM DTT, 500 mM of each dNTP, and 40 units of RNaseOUT (Invitrogen) in a final volume of 25 µl, and was heated to 55°C in a thermocycler. After 1 minute, cDNA synthesis was initiated by adding 200 units of SuperScript™ III reverse transcriptase (Invitrogen) and carried out for an additional 2 hours at 55°C. Newly synthesized cDNA was precipitated by adding 2.5 volumes of ethanol along with 0.3 M sodium acetate, pH 5.2, and the pallet was resuspended in 20 µl of nanopure water. Approximately 7.5 to 8 µg of cDNA was obtained from each synthesis.

2.6 Amylose content estimation

Upon maturity, seeds were harvested from the spikes and were ground using a pestle and mortar. Amylose content was measured in 20 mg of flour using the Amylose/Amylopectin assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland). Estimation for each sample was replicated three times.

Briefly, 20 mg of flour sample was added to 1 mL of dimethyl sulfoxide (DMSO) and mixed gently using vortex mixer. For complete dispersion, the samples were heated in a boiling
water bath for about 1 minute. Once gelatinous lumps of starch were completely dissolved, samples were vigorously mixed and were then incubated in a boiling water bath for 15 minutes. During incubation samples were intermittently mixed by vigorous vortexing. After incubation samples were stored at RT for about 5 minutes. To each sample, 6 mL of 95 % (v/v) ethanol was added with continuous stirring on a vortex mixer. Sample tubes were mixed by inverting and then allowed to stand for 15 minutes. After centrifugation at 2,000 g for 5 minutes, the supernatant was discarded and the tubes were drained on tissue paper for 10 minutes. To the recovered starch pellet 2 mL of DMSO was added with gentle vortex mixing. Each tube was placed in a boiling water bath for 15 minutes with occasional mixing. On removing the tubes from the boiling water bath, 4 mL of Concanavalin (Con) A solvent (anhydrous sodium acetate (0.18 M), sodium chloride (0.9 M), calcium chloride (0.68 mM), magnesium chloride (1.03 mM) and manganese chloride (1.06 mM), pH 6.4) was immediately added and the samples were mixed thoroughly. The samples were transferred to 25 mL volumetric flasks by repeated washing with the same volume (for each sample) of Con A solvent each time. This solution was referred to as “Solution A”. One mL of “Solution A” was transferred to 2.0 mL Eppendorf® microfuge tube and 0.5 mL of Con A solution (Concanavalin A - Sigma Type V, dissolved in diluted 10 ml Con A solvent) was added to each tube. The tube contents were mixed gently by repeated inversion and allowed to stand for 1 hour at RT. After centrifugation at 14,000 g for 10 minutes at RT, 1 mL of the supernatant was transferred to a 15 mL centrifuge tube containing 3 mL of 100 mM sodium acetate buffer (pH 4.5). After mixing the contents, tubes were lightly stoppered (with a marble) and heated in a boiling water bath for 5 minutes to denature Con A. Thereafter the tubes were placed in a water bath at 40°C and allowed to equilibrate for 5 minutes. 100 µL of amyloglucosidase/ α-amylase enzyme mixture (amyloglucosidase (200 units
on p-nitrophenyl β-maltoside) + fungal α-amylase (500 units in 2 ml solution)) was added to each sample and the tubes were again incubated at 40°C for 30 minutes. The samples were centrifuged at 2,000 g for 5 minutes. From each sample, 1 mL aliquots of the supernatant were mixed with 4 mL of GOPOD reagent (glucose oxidase (> 12000 units) + peroxidase (> 650 units) + 4-aminoantipyrine (80mg) powder mixed with GOPOD reagent buffer made of potassium phosphate buffer [(1 M, pH 7.4), p-hydroxybenzoic acid (0.22 M) and sodium azide (0.22 % w/w)] and incubated at 40°C for 20 minutes. Reagent Blank and the D-Glucose controls (supplied with the kit) were also incubated. Absorbance of each sample and the D-glucose controls was measured at 510 nm against the reagent blank.

For determining total starch, 0.5 mL of “Solution A” was mixed with 4 mL of 100 mM sodium acetate buffer, pH 4.5. 100 µL of amyloglucosidase/α-amylase enzyme mixture was added to each sample and the mixture was incubated at 40°C for 10 minutes One mL aliquots (in duplicate) were transferred to glass test tubes containing 4 mL of GOPOD reagent. After thorough mixing, the samples were incubated at 40°C for 20 minutes. Absorbance of each sample and the D-glucose controls was measured at 510 nm against the reagent blank. Amylose content was calculated using the formula:

\[
\text{Amylose content} = \frac{(\text{Absorbance (Con A Supernatant)})}{(\text{Absorbance (Total Starch Aliquot)})} \times 66.8
\]

2.7 Making RNAi constructs using Gateway® Recombination Technology

RNAi constructs through Gateway® technology (http://www.invitrogen.com) were made in two steps; cloning attB-flanked PCR product into entry vector (pDONR™201) by BP reaction, and shuttling cloned fragment from entry vector to destination vector (pHELLSGATE 8) by LR reaction.
Sequences to be cloned were amplified with specific primers having attB sites 5’ ends (forward primer with attB1 site, GGG GAC AAG TTT GTA CAA AAA AGC AGG CT and reverse primer with attB2 site, GGG GAC CAC TTT GTA CAA GAA AGC TGG GT. For BP reaction, about 150 ng attB-PCR product, 150 ng of pDONR™ 201 vector and 2 µl of 5X BP Clonase™ reaction buffer were added to a 1.5 ml centrifuge tube. The volume was adjusted to 8 µl with TE Buffer, pH 8.0. BP Clonase™ enzyme mix was thawed on ice for about 2 minutes and then mixed briefly twice (2 seconds each time). Two µl of BP Clonase™ enzyme mix was added to the tube (containing PCR product, pDONR™ 201 vector and 5X BP Clonase™ reaction buffer) and the contents were mixed well by briefly vortexing twice. After a quick spin in microcentrifuge, the tubes were incubated at 25°C for 1 hour. The reaction was terminated by adding 1 µl of Proteinase K (2 µg/µl) solution followed by incubation at 37°C for 10 minutes.

For transformation, 2 µl of each BP reaction and 50 µl of DH5α competent cells were mixed and incubated on ice for 30 minutes. Cells were heat-shocked by incubating at 42°C for 30 seconds. About 250 µl of S.O.C. medium (20 g bacto-tryptone, 5g bacto yeast extract, 2 ml of 5 M NaCl, 2.5 ml of 1 M KCl, 10 ml of 1 M MgCl2, 10 ml of 1 M MgSO4, 20 ml of 1 M glucose and water to volume of 1 litre, autoclaved for 15 minutes at 121°C) was immediately added to the heat-shocked cells and the mixture was incubated at 37°C for 1 hour with gentle shaking. Two volumes, 20 µl and 100 µl, of each transformation reaction was plated onto separate LB plates (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar and volume to 1 litre with water, pH adjusted to 7.5 with NaOH and autoclaved at 121°C for 15 minutes) amended with 100 µg/ml Kanamycin. Single bacterial colonies were selected and grown by shaking overnight in 2 ml LB media amended with 100 µg/ml Kanamycin. Plasmid DNA was isolated using alkaline lysis method (Sambrook et al. 1989) and confirmed by restriction
digestion. Clones giving expected restriction pattern served as entry clones for the next cloning step.

The LR reaction between an entry clone and the destination vector pHELLSGATE 8 was performed in a similar fashion as BP reaction except LR Clonase™ was used instead of BP Clonase™. RNAi clones, resulting from LR reaction, were checked with restriction mapping with the appropriate restriction enzymes.

2.8 Transformation of Agrobacterium tumefaciens with RNAi constructs

Frozen electro-competent cells of Agrobacterium strain C58C1 were thawed on ice. About 20 µl competent cells were transferred to a 0.5 ml tube containing 10 – 30 ng (in 1 µl) of plasmid DNA kept on ice. After gentle mixing, the mixture was dispensed into a chilled sterile electroporator cuvette. The cuvette was tapped gently to drop mixture to the bottom of the cuvette and then was placed between the electrodes of the electroporator (MicroPulser®, Biorad). Electroporation program “AGR” was selected, each sample was pulsed once and 200 µl LB or S.O.C medium was added immediately into the pulsed cuvette. Mixture was transferred from the cuvette to 10 ml snap-cap tube and shaken at 28°C at 200 rpm for 1 to 1.5 hours. About 100 µl of the culture was spread on LB media amended with 100 µg/ml spectinomycin. The plates were incubated at 28°C. Transformed bacterial colonies appeared after 2-3 days.

2.9 Agrobacterium-mediated wheat transformation
Six seeds of Bobwhite (line 31208) were sown in a six-inch pot containing sunshine #1 potting mix (SunGro Horticulture, Bellevue, WA). After germination only 4 plants per pot were kept. After the plants reached 2-leaf stage, 20-20-20 Peter’s fertilizer (J.R. Peters, Inc., Allentown, PA, USA) was applied twice a week at a concentration of 100 ppm. Plants were grown in a Conviron growth chamber equipped with high-intensity discharge lamps, with light intensity ranging from 500 – 700 µmol m$^{-2}$ s$^{-1}$, programmed for 16 hours with 20$^\circ$C during the day and 13$^\circ$C at night. Young plants were watered every other day and the older plants (>1 month old) every day. Particular care was taken to avoid exposure of plants to stresses such as diseases, insects and drought. Heads were tagged at the first sign of anthesis (anthers showing from the middle of the ear) with different colored tags for each separate date. After 12th day of tagging, few seeds from each tagged-head were chosen to see if embryos reached at the right stage (not too starchy but just beginning to get starchy).

For embryo dissection, immature seeds were disinfected by immersing in 70% ethanol for one minute and then in 10% bleach for 15 minutes. After three washings with sterile water, embryos were dissected out using sterile scalpels under aseptic conditions in a laminar flow hood.

Dissected embryos were cultured on “Callus Initiation Media for Embryos” (see sections 2.10 for all wheat transformation media recipes), scutellum side up (flat side up), in the dark at 24$^\circ$C for four days. On the same day, Agrobacterium culture (containing RNAi plasmid) was streaked on the LB plates (amended with antibiotics Rifampicin, Gentamycin @ 50 mg/L and Spectinomycin, Streptomycin Sulfate @ 100 mg/L) and was grown at 28$^\circ$C. After four days of culturing, embryos showing formation of embryogenic tissue were selected using a
stereomicroscope. Selected embryos were inoculated with Agrobacterium culture as described below.

A day before inoculation day (at about 4:30 pm), 2 ml culture of Agrobacterium (containing RNAi plasmid) was started in YEP medium (amended with antibiotics Rifampicin, Gentamycin @ 50 mg/L and Spectinomycin, Streptomycin Sulfate @ 100 mg/L) at 28°C with shaking at 200 rpm. On the inoculation day (around 8:00 am), 2 ml culture (overnight grown) was used to inoculate 50 ml YEP medium amended with antibiotics for Agrobacterium strain and RNAi plasmid). When the culture reached an OD reading of 0.6 – 0.8 (about 1:00 - 2:00 pm), a bacterial pelleted was obtained by centrifuging at 3500 rpm for 10 minutes. The pellet was resuspended in 25 – 30 ml of “Inoculation Medium” at an OD of 0.6 – 0.8. Embryos were inoculated by immersing into 3 ml of Agrobacterium containing inoculation medium. After 30 minutes, embryos were removed from inoculation medium and placed on “Co-cultivation Medium” (scutellum side up) for 2 days at 24°C in the dark. After 2 days embryos were moved to “Callus Initiation Medium for Agrobacterium”. Inoculated embryos were kept in the dark for 2 weeks at 24°C.

After 2 weeks, callus tissue was broken into small pieces (about 2 mm in size) under dissecting microscope. While breaking calli, special care was taken to avoid breaking newly developing shoots. Calli pieces were moved to “Regeneration medium 1” in a growth chamber set at 16 hours light (Gro-Lux bulbs) with 8 hours of dark at 24°C. After 14 days, calli were moved to “Regeneration Medium 2”. Regenerated plants showing nice root system extending into the medium were moved to “Regeneration Medium 2” in the Phytatrays®. Plants were moved to soil after the development of nice root and shoot system.
## 2.10 Media and solution recipes for wheat transformation

<table>
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<tr>
<th>MS Major Salts (10X)*</th>
<th>Component</th>
<th>To make 1 liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>19 gm</td>
<td></td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>16.5 gm</td>
<td></td>
</tr>
<tr>
<td>MgSO₄ . 7H₂O</td>
<td>3.7 gm</td>
<td></td>
</tr>
<tr>
<td>CaCl₂ . 2H₂O</td>
<td>4.4 gm</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.7 gm</td>
<td></td>
</tr>
</tbody>
</table>

*Use NanoPure water to make this solution and store at 4°C.

<table>
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<th>Modified MS Vitamins (100X)*</th>
<th>Component</th>
<th>Amount/500 ml</th>
<th>Units</th>
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</thead>
<tbody>
<tr>
<td>Glycine</td>
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<td></td>
</tr>
<tr>
<td>Myo-inositol</td>
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<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>20 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyridoxine HCL</td>
<td>20 gm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiamine HCL</td>
<td>40 mg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Make this solution in nano-pure water and store at 4°C.

<table>
<thead>
<tr>
<th>MS Minor Salts (100X)*</th>
<th>Component</th>
<th>To make 1 liter</th>
<th>Units</th>
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</thead>
<tbody>
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<td>MnSO₄ . H₂O</td>
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</tr>
<tr>
<td>KI</td>
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</tr>
<tr>
<td>H₃BO₃</td>
<td>620 mg</td>
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<td></td>
</tr>
<tr>
<td>ZnSO₄ . 7H₂O</td>
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</tr>
<tr>
<td>CuSO₄ . 5H₂O</td>
<td>2.5 mg</td>
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</tr>
<tr>
<td>Na₂MoO₄ . 2H₂O</td>
<td>25 mg</td>
<td></td>
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</tr>
<tr>
<td>CoCl₂ . 6H₂O</td>
<td>2.5 mg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Make this solution in nano-pure water and store at 4°C.
**MS Vitamins (100X)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/500 ml</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>100</td>
<td>mg</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>5</td>
<td>gm</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>25</td>
<td>mg</td>
</tr>
<tr>
<td>Pyridoxine HCL</td>
<td>25</td>
<td>mg</td>
</tr>
<tr>
<td>Thiamine HCL</td>
<td>5</td>
<td>mg</td>
</tr>
</tbody>
</table>

**YEP Media\(^{a}\)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10 gm</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>5 gm</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 gm</td>
</tr>
<tr>
<td>Type A Agar</td>
<td>15 gm</td>
</tr>
<tr>
<td>(^{a})Rifampicin 50 mg/L</td>
<td>1 ml</td>
</tr>
<tr>
<td>(^{a})Streptomycin sulfate 100 mg/L</td>
<td>1 ml</td>
</tr>
<tr>
<td>(^{a})Gentamycin 50 mg/L</td>
<td>1 ml</td>
</tr>
<tr>
<td>(^{b})Spectinomycin 100 mg/L</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

\(^{a}\) Before adding Agar, pH was adjusted to 7.0. Media was auto-claved at 121°C for 15 minutes and filter sterilized antibiotics were added after media cooled down to 60°C. \(^{a}\) = antibiotics for Agrobacterium, \(^{b}\) = antibiotics for RNAi plasmid.

**Iron Stocks (200X)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/250 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(_2)EDTA(^{a}) 0.5 M, pH 8.0</td>
<td>10.2 ml</td>
</tr>
<tr>
<td>FeSO(<em>4)(</em>{7})H(_2)O(^{b})</td>
<td>1.45 gm</td>
</tr>
</tbody>
</table>

\(^{a}\) = store at 4°C, \(^{b}\) = store at 4°C wrapped in foil.
## Callus Initiation Medium for Agrobacterium

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/liter</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS Major (10X)</td>
<td>100</td>
<td>ml</td>
</tr>
<tr>
<td>MS minor (100X)</td>
<td>10</td>
<td>ml</td>
</tr>
<tr>
<td>2,4-D (1 mg/ml)</td>
<td>0.5</td>
<td>ml</td>
</tr>
<tr>
<td>FeSO₄ (200X)*</td>
<td>5</td>
<td>ml</td>
</tr>
<tr>
<td>NaEDTA (200X)*</td>
<td>5</td>
<td>ml</td>
</tr>
<tr>
<td>Maltose</td>
<td>40</td>
<td>gm</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.75</td>
<td>gm</td>
</tr>
<tr>
<td>MES</td>
<td>1.95</td>
<td>gm</td>
</tr>
<tr>
<td>pH 5.8 (with conc. KOH)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytagel</td>
<td>2</td>
<td>gm</td>
</tr>
<tr>
<td>Final volume</td>
<td>975</td>
<td>ml</td>
</tr>
</tbody>
</table>

Mix the following components, filter sterilize in hood, and add 25 mls / litre to the autoclaved mixture of above ingredients

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/liter</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS vitamins (100X)</td>
<td>10</td>
<td>ml</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.5</td>
<td>gm</td>
</tr>
<tr>
<td>Casein hydrolystate</td>
<td>0.1</td>
<td>gm</td>
</tr>
<tr>
<td>Ascorbic acid (50 mg/ml)</td>
<td>2</td>
<td>ml</td>
</tr>
<tr>
<td>Picloram (1 mg/ml)</td>
<td>2.2</td>
<td>ml</td>
</tr>
<tr>
<td>Geneticin (25 mg/ml)</td>
<td>0.4</td>
<td>ml</td>
</tr>
<tr>
<td>Final volume with ddH₂O</td>
<td>25</td>
<td>ml</td>
</tr>
<tr>
<td>Cefotaxime (100 mg/ml)</td>
<td>0.5</td>
<td>ml</td>
</tr>
<tr>
<td>Vancomycin (100 mg/ml)</td>
<td>0.5</td>
<td>ml</td>
</tr>
<tr>
<td>Ticarcillin (250 mg/ml)</td>
<td>0.2</td>
<td>ml</td>
</tr>
</tbody>
</table>

*mix and incubate these ingredients at 60°C till color turn yellow, then add to the mixture of other ingredients before autoclaving.
### Callus Initiation Medium for Embryos

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/liter</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS Major (10X)</td>
<td>100</td>
<td>ml</td>
</tr>
<tr>
<td>MS minor (100X)</td>
<td>10</td>
<td>ml</td>
</tr>
<tr>
<td>2,4-D (1 mg/ml)</td>
<td>0.5</td>
<td>ml</td>
</tr>
<tr>
<td>FeSO4 (200X)*</td>
<td>5</td>
<td>ml</td>
</tr>
<tr>
<td>NaEDTA (200X)*</td>
<td>5</td>
<td>ml</td>
</tr>
<tr>
<td>Maltose</td>
<td>40</td>
<td>gm</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.75</td>
<td>gm</td>
</tr>
<tr>
<td>MES</td>
<td>1.95</td>
<td>gm</td>
</tr>
</tbody>
</table>

**pH 5.8 (with conc. KOH)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/liter</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytagel</td>
<td>2</td>
<td>gm</td>
</tr>
<tr>
<td>Final volume</td>
<td>975</td>
<td>ml</td>
</tr>
</tbody>
</table>

Mix the following components, filter sterilize in hood, and add 25 mls / litre to the autoclaved mixture of above ingredients.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/liter</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS vitamins (100X)</td>
<td>10</td>
<td>ml</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.5</td>
<td>gm</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>0.1</td>
<td>gm</td>
</tr>
<tr>
<td>Ascorbic acid (50 mg/ml)</td>
<td>2</td>
<td>ml</td>
</tr>
<tr>
<td>Picloram (1 mg/ml)</td>
<td>2.2</td>
<td>ml</td>
</tr>
</tbody>
</table>

**Final volume with ddH₂O**

25 ml

*mix and incubate these ingredients at 60°C till color turn yellow, then add to the mixture of other ingredients before autoclaving.
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Component*</th>
<th>Amount/500 ml</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MS major (10X)</td>
<td>5 ml</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>MS minor (100X)</td>
<td>0.5 ml</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2,4-D (1g/ml)</td>
<td>0.25 ml</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>FeSO₄ (200X)</td>
<td>0.25 ml</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>NaEDTA*(200X)</td>
<td>0.25 ml</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Maltose</td>
<td>20 gm</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>MgCl₂</td>
<td>0.375 gm</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>MES</td>
<td>1.95 gm</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Gelrite</td>
<td>1 gm</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>MS vitamins (100X)</td>
<td>6 ml</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Glutamine</td>
<td>0.3 gm</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Casein hydrolysate</td>
<td>0.06 gm</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Glucose</td>
<td>6 gm</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Picloram (98%) (1mg/ml)</td>
<td>1.32 ml</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Ascorbic acid (50 mg/ml)</td>
<td>1.2 ml</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Acetosyringone (200 µm)</td>
<td>23.4 mg</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Final volume with ddH₂O</td>
<td>30 ml</td>
<td></td>
</tr>
</tbody>
</table>

* mix components 1-8 (3 and 4 are mixed together and heated at 60°C for 10 minutes before mixing with rest of the components). After adjusting pH, volume is made to 475mls. The solution is divided to 380 ml (part A) and 95 ml (part B). One gm of Gelrite is added to part A and part A and B are autoclaved at 121°C for 15 minutes. Components 10 -17 are mixed and filter sterilized and then added, 5 ml to part B and 25 ml to part A. Part A portion is co-cultivation media and part B is inoculation media.
2.11 REFERENCES


CHAPTER THREE

Virus-induced Gene Silencing for Functional Analysis of Genes Expressing in Various Wheat Tissues

(Under Review The Plant Journal)

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\textsuperscript{2}USDA ARS, Root Disease and Biological Control Research Unit, Pullman, WA, 99164, USA

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Email: ksgill@wsu.edu

Phone: 509-335-4666
3.1 ABSTRACT

Barley stripe mosaic virus (BSMV)-based virus induced gene silencing (VIGS) has been shown to be an effective strategy for rapid functional analysis of genes in leaf tissue of wheat and barley. To expand the potential of VIGS in wheat, we investigated gene silencing in roots, meristems and reproductive tissues, compared silencing in nine cultivars, and characterized the transmission of silencing in two selfed generations of inoculated plants. Optimal conditions for gene silencing included a growth regimen of 22°C, 16 hr day under 500 to 700 µmol m$^{-2}$s$^{-1}$ supplemental lighting and 18°C, 8 hr night. Of nine cultivars, Zak was the most responsive to silencing of the phytoene desaturase ($PDS$) gene. Incidence of $PDS$ silencing ranged from 8 to 11% in progeny of the inoculated generation and from 53 to 61% in the first selfed generation. Spread of the VIGS vector, monitored using green fluorescent protein (GFP), was observed in inoculated leaf tissue, phloem and root cortex at 10 and 17 days post-inoculation, but was absent in apical meristems and reproductive tissues. An antisense construct of the wheat coronatine insensitive1 ($TaCOI1$) gene reduced $TaCOI1$ transcripts by 60% in roots and 65% in foliage of cultivar Scarlet, compared to control plants. The seed-specific granule bound starch synthase ($GBSS$) gene was successfully silenced with antisense and hairpin constructs which resulted in up to 81% reduction in amylose content. Antisense and hairpin constructs produced similar levels of gene silencing except one case where hairpin construct showed a significantly higher level of silencing.

Keywords: VIGS, Wheat, Functional Genomics, Roots, Seed, BSMV
3.2 INTRODUCTION

Most of the wheat (*T. aestivum* L.) grown in the world is allohexaploid (2n=6X=42), having three closely related genomes designated A, B and D. The polyploid inheritance, large genome size (16,000 Mb) (Arumuganathan and Earle, 1991) and large amounts of repetitive DNA render wheat recalcitrant to reverse genetics and genomic approaches compared to other plant species. More than 855,000 wheat expressed sequence tags (ESTs) corresponding to 38,566 unigenes are available in public databases (http://www.ncbi.nih.gov) but their functions are largely unconfirmed due to a lack of efficient reverse genetics tools. Posttranscriptional gene silencing (PTGS) has emerged as a promising tool to target specific sequences in wheat. In addition, PTGS has the potential to simultaneously suppress homoeologous genes in polyploid species like wheat (Travella *et al.*, 2006). Stable RNA interference (RNAi), a form of PTGS, is a reliable and reproducible tool for gene silencing (Regina *et al.*, 2006; Smith *et al.*, 2000; Travella *et al.*, 2006), however, this approach generally is not efficient for large-scale functional analysis in crop plants such as wheat because of a lower genetic transformation frequency.

Virus-induced gene silencing (VIGS), which avoids the inefficient and cumbersome process of genetic transformation, is ideal for a rapid and high-throughput functional analysis of genes (Burch-Smith *et al.*, 2004). VIGS utilizes a PTGS-based plant defense mechanism first demonstrated against invading viral genomes (Kumagai *et al.*, 1995; Vance and Vaucheret, 2001), in which host tissue infected with a recombinant viral vector carrying target gene sequences results in degradation of the endogenous gene (Cai *et al.*, 2006). A VIGS system was first applied in plants (Kumagai *et al.*, 1995) to silence the phytoene desaturase (*PDS*) gene in *Nicotiana benthamiana*. *PDS* is essential for carotenoid biosynthesis, and its suppression in barley (*Hordeum vulgare*) results in photo-bleaching of chlorophyll (white streaks) in the
affected tissue (Holzberg et al., 2002). The VIGS system of Kumagai et al. (1995) used an infectious strain of tobacco mosaic virus (TMV) carrying an antisense *PDS* sequence. Since then, several modified viral genomes have been used as VIGS vectors for targeting genes in leaf tissue of barley (Holzberg et al., 2002; Lacomme et al., 2003) and wheat (Scofield et al., 2005), flowers and leaves of peas (*Pisum sativum*) (Constantin et al., 2004), fruit of tomato (*Lycopersicon esculentum*) (Fu et al., 2005), and roots and meristems of tomato, tobacco and *Arabidopsis thaliana* (Valentine et al., 2004).

Barley stripe mosaic virus (BSMV) and Brome mosaic virus (BMV) vectors have been developed for gene silencing in monocot species, including barley, wheat, maize (*Zea mays*) and rice (*Oryza sativa*) (Holzberg et al., 2002; Lacomme et al., 2003; Scofield et al., 2005; Ding et al., 2006). BSMV is a single-stranded RNA virus consisting of a tripartite genome (α, β and γ) (Petty et al., 1989). Antisense or hairpin sequences, from the plant gene to be targeted for silencing, are inserted in the γb open reading frame of the γ RNA (cDNA) downstream of an introduced stop codon; the stop codon prevents translation of the insert. BSMV-based VIGS has been successfully used to study the function of several genes involved in disease resistance pathways in barley and wheat leaves (Holzberg et al., 2002; Lacomme et al., 2003; Scofield et al., 2005). Application of this exciting technology remains to be demonstrated for important tissues such as roots and meristems of monocots, and for developing seeds of both monocots and dicots. As BSMV is seed transmitted in wheat and barley, it will interesting to see if gene-silencing BSMV vector will be able to transmit gene silencing to next generations. This will provide an option to target genes involved in processes, like seed germination, which can not be targeted in the same generation as these processes are over by the time plant reaches the stage suitable for inoculation with VIGS vector.
The objectives of this study were; 1) to investigate optimum conditions for BSMV-based VIGS in adapted wheat cultivars, 2) to examine VIGS potential in roots, meristems and reproductive tissues and 3) to investigate if VIGS is inherited from one generation to next. Photo-bleaching, resulting from silencing of $PDS$ was used as a visual marker for optimizing VIGS and green fluorescent protein (GFP) was used to monitor the spread of GFP-encoding infectious RNAs to various tissues of wheat. In addition, we targeted the silencing of three specific genes, $coronatine$-$insensitive1$ ($TaCOI1$) in roots and leaves, $granule$-$bound$ $starch$ $synthase$ ($GBSS$ or $waxy$) in developing seeds, and meristem-expressed $vernalization2$ ($VRN2$), a repressor of flowering that normally is down-regulated by cold treatment (Yan et al., 2004). In Arabidopsis, $COI1$ is required for cellular responses to the phytohormone jasmonic acid, which regulates defense against insects and pathogens, wound healing, and pollen fertility (Xie et al., 1998). Wheat roots and leaves express two distinct $COI$ homologues, $TaCOI1$ and $TaCOI2$ (our unpublished data). An antisense DNA construct of $TaCOI1$ was designed using a full-length mRNA (cDNA). $TaCOI1$ mRNAs shares 80% nucleotide sequence identity with $TaCOI2$ mRNA, and 76% nucleotide sequence identity with rice $COI1$ (GenBank AY168645) (our unpublished results). Starch, a main component of wheat grain, is a polymer composed of amylose and amylopectin. $GBSS$, also known as the ‘waxy’ protein (Sivak, 1995), catalyzes the synthesis of amylose. Wheat harbors three homoeologous $waxy$ genes, $Wx-A1$, $Wx-D1$ and $Wx-B1$, present on chromosomes 7AS, 7DS and 4AL, respectively (Murai et al., 1999). Here, we report that $PDS4$-based photo-bleaching and GFP-BSMV translocation correlate to silencing of $TaCOI1$ in wheat leaves and roots and silencing of $waxy$ in the endosperm.

3.3 RESULTS
3.3.1 VIGS optimization and varietal differences

We used photo-bleaching caused by silencing of the *PDS* gene as a visual marker to optimize BSMV-mediated VIGS in various wheat tissues, and to compare VIGS in nine cultivars of hexaploid wheat. A temperature regimen of 22ºC day/18ºC night was found to be optimal for VIGS in all tissues of wheat. At day temperatures of 24ºC and above, viral symptoms in the form of mottling/mosaic and narrowing of leaves obscured the photo-bleaching phenotype.

Seven of the nine cultivars, Zak, Scarlet, Bobwhite, Thatcher, Moro, Eltan and Jagger, showed loss of chlorophyll pigmentation or streaks of white (photo-bleaching) on the third true leaf at 7 to 9 days post inoculation (dpi). The extent and intensity of photo-bleaching varied among the cultivars, but peaked in the fifth and sixth leaves at 21 to 22 dpi in all seven, and declined gradually in the later stages of plant growth. Among the tested cultivars, Zak showed the maximum degree of photo-bleaching (Fig.1). In comparison, photo-bleaching in Bobwhite, Scarlet, Thatcher, Moro, Eltan and Jagger covered less leaf area and was less intense (data not shown).

Chancellor and Kavkaz did not show photo-bleaching. Instead, both cultivars showed severe symptoms of BSMV infection in the form of dwarfing, rosette formation and excessive tillering. Leaves had a burnt or necrotic appearance and plants eventually died.

Cultivar Zak was found to be the best cultivar for optimizing VIGS, thus was used for the rest of the experiments, except for the *TaCOI* and *VRN2* gene silencing studies. Scarlet was used for *TaCOI* silencing experiments as damage to Zak foliage was too severe to obtain high-quality RNA. Zak has spring growth habit thus was not used to study vernalization gene *VRN2*. Instead, winter wheat varieties Eltan and Jagger were used for *VRN2* silencing experiments.
3.3.2 Comparison of inoculation methods for gene silencing at the boot stage

For optimizing an inoculation method for silencing genes expressing in reproductive tissues, we monitored PDS silencing in spikes of cultivar Zak after inoculation of flag leaves with PDS-silencing infectious RNAs. The hand rubbing method of inoculation, which worked well to inoculate 8-10 day old plants, did not produce any infection on leaves or spikes when flag leaves of five plants were inoculated at boot stage (data not shown). Therefore, we infiltrated PDS-silencing infectious RNAs around the midrib of the flag leaf using a needle-less syringe. The syringe method resulted in significant photo-bleaching of the spike at 12 to 13 dpi (Fig. 2) in all five inoculated plants. Spikes of the inoculated tiller showed the highest intensity of bleaching, whereas in emerging side tillers photo-bleaching generally was less severe and was more variable (data not shown).

3.3.3 VIGS in root and reproductive tissues

The utility of BSMV-mediated VIGS for wheat will depend upon the ability of the virus to spread to various tissues. To examine this, we monitored movement of pγ.GFP in root and crown tissue using confocal microscopy. GFP expression was detected in root tissue, particularly in the vascular bundles of roots at 10 dpi (Fig.3b, 4b). At 17 dpi, GFP expression was observed in root cortical cells surrounding the vascular bundles (Fig.4c). No GFP was detected in the root tip or shoot apices at either 10 or 17 dpi (Fig. 4c, 5a and 5b respectively).

We monitored the translocation of pγ.GFP to reproductive tissues of the spike, including the ovary, stigma, anthers, pollens and developing seeds, at 15 and 23 dpi following syringe inoculation of tiller flag leaves. In spikes that exhibited photo-bleaching (Fig. 2), no GFP
expression was found in the awn, lemma, palea, stigma, anther and pollen, even after 23 dpi (Fig. 5).

3.3.4 Silencing TaCOII in root and shoot tissue

To determine whether movement of infectious RNA particles to roots was accompanied by changes in gene expression, we targeted the coronatine-insensitive1 (TaCOII) gene homologue in roots and leaves of cultivar Scarlet. Infection of leaves with pγTaCOIIas resulted in a 50 to 70% decrease in TaCOII transcript level in roots and a 63 to 68% decrease in leaves (Table 1).

In wheat, TaCOI gene family consists of two members TaCOII and TaCOI2 which show a sequence similarity of 74.2%. The sequence we used for the VIGS construct showed 76.2% similarity between the two sequences. We also quantified the expression of TaCOI2 in TaCOII-silenced plants to examine the effect of TaCOII-targeted VIGS on gene family members. We observed a 31 to 58% suppression of TaCOI2 transcripts in roots and 29 to 54% suppression in the foliage (Table 1). Similar trends in TaCOII silencing were observed in Bobwhite and Zak (data not shown), although viral symptoms sustained by these cultivars were more severe than for Scarlet, and the leaf tissue generally was not suitable for RNA extraction.

3.3.5 Silencing granule-bound starch synthase (GBSS) genes in grains

The GBSS (waxy) genes were targeted using both antisense (pγ.wWxas) and hairpin (pγ.wWxhp) constructs. As a measure of waxy gene silencing, amylose levels in the seeds from the infected tiller (i.e. main tiller infected via inoculating flag leaf) and uninfected or pγ.GFP-infected control tiller were compared. The level of amylose was significantly reduced in the
seeds from spikes inoculated with \textit{waxy} constructs compared to those of the non-inoculated or \textit{py}.GFP-inoculated plants (Table 2). Reduction in amylose content ranged from 30.5\% to 39.1\% among the five plants that were inoculated with \textit{py}.wWxas. The corresponding hairpin construct (\textit{py}.wWxhp) caused a 34.2\% to 37.6\% reduction in amylose content among four of the inoculated plants. The fifth plant showed 81.3\% reduction in amylose content.

3.3.6 \textit{Silencing VRN2 in shoot meristems}

The \textit{vernalization2} (\textit{VRN2}) gene was targeted for silencing to determine if BSMV-based VIGS is operational in wheat shoot meristems. Double inoculations (first inoculation when plant is 7-9 days old and then second inoculation after 15 days of first inoculation) were done to insure that \textit{VRN2} was suppressed for at least six weeks, the interval needed for initiation of flowering. All the inoculated plants showed typical mosaic symptoms, indicating adequate BSMV infection (data not shown). Both controls (vernalized single and double inoculated) flowered at the expected time, indicating BSMV infection dose not interfere with flowering (Table 3). Successful silencing of the \textit{VRN2} gene was expected to cause early flowering (Yan \textit{et al}., 2004). However, none of the plants flowered within the experimental time frame when inoculated either once or twice with \textit{py}.wVrnas (antisense construct) or \textit{py}.wVrnhp (hairpin construct).

3.3.7 \textit{Transmission of PDS silencing through seed}

To study the percentage of seeds that transmitted infectious BSMV RNA to the next generation and to determine whether such RNA can exert a silencing phenotype, we examined two generations of plants derived from tillers of inoculated plants. In the first generation
following inoculations, 8 to 11.1% of the plants showed photo bleaching typical of \textit{PDS} gene silencing. In the second generation, however, \textit{PDS} silencing persisted in 52.63\% to 61\% of the plants that showed silencing in the first generation.

### 3.4 DISCUSSION

Assigning function to the large number of putative genes from EST and genome sequencing projects is perhaps the single most important challenge in post-genomics era of biology. Gene knockouts, functional complementation and other approaches have been used to assign function, but these involve the generation and screening of a large number of transgenic lines, which technically is demanding and inefficient in many plants, including wheat. The frequency of \textit{Agrobacterium}-mediated stable transformation in wheat ranges from 0.9 to 2\% (Cheng \textit{et al.}, 1997). Although the particle bombardment method is more efficient, it often results in complex insertion events that may cause unwanted gene silencing and may complicate functional analysis (Dai \textit{et al.}, 2001; Hammond \textit{et al.}, 1999). VIGS is a transient knock-out system that has been optimized for genes expressed in leaf tissue of wheat and barley (Holzberg \textit{et al.}, 2002; Scofield \textit{et al.}, 2005). Here, we report the development of VIGS for wheat roots and seeds, two of the most important tissues for targeting agronomic traits.

In our system, photo-bleaching first appeared on the third true leaf 7 to 9 days after inoculation of the second true leaf of 8- to 10-day-old plants. The intensity and extent of photo-bleaching peaked in the fifth and sixth leaf at 21 – 22 dpi, gradually declined onwards, and was never observed on the spikes inoculated at the two-leaf stage. These results differed from those of Scofield \textit{et al.} (2005) who rarely observed photo-bleaching on the fifth leaf of either wheat or barley. However, our findings were similar to that of Holzberg \textit{et. al.} (2002) who observed
maximum intensity and spread of photo-bleaching on the fifth leaf of barley after 17 to 19 dpi. The use of different cultivars or growing conditions might account for the variation in PDS silencing.

We found that photo-bleaching and viral symptoms are cultivar- and temperature-dependent. As reported by Atabekov (1989) for a barley-BSMV interaction, we observed a range of symptom intensities, from mild photo-bleaching to necrosis and stunting. In contrast to the temperature regimens of 20 to 25 °C and 26.5 to 29 °C suggested by Scoefield et al. (2005) and Holzberg et al. (2002), respectively, we found that 18 to 22 °C to be the optimal growing temperature for VIGS in wheat. Our results agree with those of Fu et al. (2005) for tomato, which showed enhancement of silencing at lower temperature and humidity. In our studies, day temperatures of 24 °C and above decreased photo-bleaching and increased viral symptoms. As our optimization was based on visual viral symptoms, its relationship with actual suppression of the target gene was not tested thus is not clear. Although no supporting data is presented, in our experience plants showing mild viral symptoms usually show the highest level of target gene suppression (data not shown).

Wild-type seed borne BSMV strains, including ND18, are known to spread to male and female reproductive tissues in barley, such as the megaspore, pollen mother cells, egg and pollen (Carroll, 1976a, 1976b). BSMV has been observed in the pollen and seed of the majority of gramineous host plants (Bennett, 1969) and can survive up to 20 years in wheat seed (McNeal, 1976). In experiments aimed at investigating the ability of recombinant GFP-BSMV (derived from strain ND18) to spread in different wheat tissues, GFP expression was only found in inoculated leaf tissue, phloem and root cortex. The absence of GFP in reproductive tissue from spikes that displayed conspicuous viral symptoms could be due to instability of GFP in these
tissues of wheat plants. Lawrence and Jackson (2001) rarely observed GFP at 7-9 dpi in the leaves of inoculated barley plants, even though those leaves clearly exhibited chlorotic symptoms. In tobacco, GFP is relatively more stable and can be viewed in uninoculated leaves distal to the site of inoculation of inoculated plant. Our waxy gene experiment showed clearly that VIGS does work in developing seed suggesting problem with GFP expression in reproductive tissue rather than lack of silencing.

Until now, BSMV-based VIGS has not been demonstrated in roots and seeds of any plant. At least 10 days were required before GFP was detected in root vascular tissue, and an additional 7 days for movement into cells surrounding the vascular tissue. This time frame delineates some constraints when targeting silencing to root tissues; seedlings must be old enough for inoculation (two-leaf stage), and functional analysis must be carried out after infectious RNAs have reached the root cortex and have been expressed. As VIGS is transient, a double-inoculations scheme is required to silence genes in roots for periods longer than 17 days. Despite these constraints, BSMV-based VIGS was successful for TaCOI1 silencing in seedling roots.

Our results with TaCOI1 show that VIGS can be effectively used to silence gene families. The antisense TaCOI1 construct also resulted in silencing of TaCOI2 expression. Of the 398 nucleotides used to target TaCOI1, 303 nucleotides were identical with TaCOI2, although longest stretch of perfect homology was only 13 bp. Considering that 21 bp of perfect homology is reported to be the minimum required for efficient RNAi (Elbashir et al., 2002), we were surprised to observe up to 58% suppression of TaCOI2 in roots and 54% in leaves. A study by Jackson et al. (2003) reported that a 11-bp match was sufficient for off-target effects. For an
allopolyploid such as wheat, a low threshold for off-target effects might be useful for targeting homoeologous copies.

Both antisense and hairpin constructs successfully silenced *waxy* gene expression and caused as much as 81.33% reduction in amylose content in a plant inoculated with the hairpin construct. A higher level of gene silencing by hairpin constructs compared to antisense, has also been observed in other systems as well (Smith *et al.*, 2000; Wesley *et al.*, 2001; Lacomme *et al.*, 2003). However, the extent of gene silencing in the remaining (four) plants inoculated with hairpin-encoding constructs was similar to antisense-encoding constructs. This could be due to conversion of hairpins to antisense or sense RNA via recombination, a phenomenon commonly observed in recombinant viruses (Christophe Lacomme, personal communication). Recombinant viruses are known to excise foreign inserts during systemic spread due to selection imposed during replication, cell-to-cell movement through plasmodesmata, and entry/exit into the phloem translocation stream (Etessami *et al.*, 1989; Hayes *et al.*, 1989; Elmer and Rogers, 1990; Gilbertson *et al.*, 2003). Lacomme *et al.* (2003) compared the abilities of sense, antisense and hairpin constructs to silence *PDS* in barley leaf tissue, and obtained the highest level of silencing with hairpin constructs. In contrast, our study targeted seed tissue which was relatively distal from the inoculation point. We hypothesize that the farther the recombinant virus has to travel to the target tissue, the greater are the chances that it will recombine (due to recombination imposed by the translocation) and overtake the original construct.

BSMV strain ND18, on which the BSMV-VIGS vector is based, is known to be seed-transmitted in barley (Carroll, 1972). Up to 11% of the first-generation progeny of plants inoculated with pγ.bPDS4 showed the *PDS*-silenced (photo-bleaching) phenotype, and substantially more (52 to 61%) second-generation progeny (from the first generation progeny
showing photo-bleaching) also displayed photo-bleaching. The higher rate of transmission to the second generation may be explained by the findings of (Eslick, 1955) that rate of seed transmission is influenced by host age at the time of inoculation. In barley, seed transmission is a cultivar-specific phenomenon and transmission rate is variable for different cultivars; however, this needs to be confirmed for wheat.

We did not observe any GFP in meristems of roots, stems. The absence of GFP in root and shoot meristems of plants displaying conspicuous viral symptoms could be due to instability of GFP in these tissues of wheat plants. In barley, GFP was rarely observed at 7-9 dpi in the leaves of inoculated plants, even though those leaves clearly exhibited chlorotic symptoms. On the other hand, GFP is relatively more stable in tobacco and can be viewed in uninoculated leaves distal to the site of inoculation of inoculated plant (Lawrence and Jackson, 2001). It is also possible that due to inefficient infection of meristems, GFP expression is so low that it is not visually detectable. Lin (1984) has shown that distribution of BSMV is highly uneven in barley shoot meristems and viral titer is significantly lower compared to other tissues.

Targeting of VRN2, a gene expressed in the shoot meristem, did not produce expected silencing phenotype in winter wheat cultivars Jagger and Eltan. The result was not the expected silencing phenotype reported in a previous VRN2 study (Yan et al., 2004), in which about 50% of stably-silenced Jagger displayed earlier flowering time. Lin (1984) has shown that BSMV is allowed into meristems but the distribution of the virus is highly uneven and its titer is significantly lower compared to other tissues. Exclusion of viruses from plant meristems also is a well-known phenomenon, e.g. Tobacco mosaic virus and Potato virus X are excluded from the growing points or vegetative meristems (Ratcliff et al., 2001; Hull, 2002). Absence of the expected phenotype for VRN2 silencing in meristems in the current study could result from
inefficient meristems infection by the BSMV-based VIGS vector or by physical or biochemical alterations, either direct or indirect, in tissues of this highly developmentally-regulated organ.

In conclusion, we have shown that BSMV-based VIGS constructs are translocated to roots and specific reproductive tissues, and can effectively silence genes in roots and wheat grains. Our findings provide a new tool to study the functions of genes expressed in these important tissues. We have extended previous reports on VIGS in monocots that address gene silencing after the two-leaf stage to inoculations at the boot stage and to transmission of VIGS to second-generation progeny of inoculated plants. The genetic/epigenetic basis for transmission of the _PDS_ silencing has yet to be elucidated. Our findings indicate that BSMV-mediated VIGS potentially can be used for targeting genes that are expressed as early as germination. We will continue to refine the targeting of root- and seed-expressed genes for studies on grain quality and pathogen resistance.

### 3.5 EXPERIMENTAL PROCEDURES

#### 3.5.1 BSMV-based VIGS constructs

In every gene silencing experiment in this study, infectious RNAs derived from three BSMV-based plasmids were used. Two plasmids, pα and pβΔβa, were common in all the experiments and the third plasmid (pγ based) varied according to the gene targeted. Plasmids pγ.bPDS4, pγ.wWxas, pγ.wWxhp, pγ.wVrnhp and pγ.wVrnas were used for targeting _PDS, GBSS_ and _VRN2_ genes respectively. Plasmids pα, pβΔβa and pγ.bPDS4 are described earlier by Holzberg et al. (2002). The pγ based constructs used for silencing _GBSS_ and _VRN2_ were constructed as follows: Antisense DNA segments containing 5’ _Pac1_ and 3’ _Not1_ restriction sites were generated using synthetic oligonucleotides, and cloned into pγ.bPDS4 (from which
PDS fragment was removed by Pac1-Not1 digestion). Antisense constructs of seed-expressed granule bound starch synthase (waxy) genes from wheat, designated pγ.wWxas, consisted of 91-bp amplicons from a conserved region of all three waxy homoeologues, Wx-7A (GenBank accession number AB019622, 1345-1437bp), Wx-4A (GenBank accession number AB019623, 1351-1443bp) and Wx-7D (GenBank accession number AB019624, 1428-1520bp). Waxy hairpin constructs (pγ.wWxhp) consisted of conserved direct and inverted repeats (45 bp each) separated by 9 bp loops from Wx-7A (1393-1437bp), Wx-4A (1399-1443bp) and Wx-7D (nt 1476-1520bp); all hairpin sequences were internal to the sequence used for the antisense constructs. Complementary oligonucleotides for a target sequence were dissolved in 0.1X TE at a concentration of 10 µg/µl. Oligonucleotide mixtures (10 µg each) were heated at 65°C for 20 minutes, and incubated at room temperature for 1 hr. The annealed products were cleaved using 20 units each of Pac1 and Not1 at 37°C overnight. Restriction cleavage products were precipitated using 3M sodium acetate (pH 5.2) prior to cloning into Pac1-Not1 restricted pγ.bPDS4.

For targeting the VRN2 gene in shoot meristems, a hairpin construct (pγ.wVrnhp) and an antisense construct (pγ.wVrnmas) were constructed as described for waxy using a 45-bp sequence (corresponding to 147-191bp) and a 97-bp sequence (91-189bp) of VRN2 (GenBank accession number AY485969), respectively.

A 398-bp TaCOI1 antisense segment was generated from a full-length cDNA clone using PCR and primers TaCOI1F 5’-ATG CGG CCG CCT GCT CAG GGG CTG CAC CAA G- CTTC-3’ and TaCOI1R 5’-AGT TAA TTA AAA CGC AGG GCT CCC CAT CTT CCA TC-3’. Pac1 and Not1 terminal sites were introduced using the primers for ligation to Pac1-Not1 restricted pγ.bPDS4. The resulting construct was named pγTaCOI1as.
Orientation and sequences of all cloned inserts were confirmed using ABI Big Dye chemistry and ABI 310 or ABI 377 sequencers (Applied Biosystems, Foster City, CA, USA) at the Laboratory for Biotechnology and Bioanalysis, Washington State University, Pullman, WA.

3.5.2 Growth conditions and cultivars used for VIGS

For VIGS optimization, plants of cultivar Zak were grown in 4- or 6-inch pots of Sunshine #1 potting mix (SunGro Horticulture, Bellevue, WA, USA) supplemented with 14 g Nutricote 14-14-14 fertilizer (Plantco Inc., Brampton, Ontario, Canada) per liter of soil. Plants were grown in a Conviron PGR15 growth chamber equipped with high-intensity discharge lamps under 16 hours light at 500-700 µmol m\(^{-2}\) s\(^{-1}\). Three different day temperatures, 22°C, 24°C and 26°C, and a constant night temperature of 18°C were used to test suitability for VIGS of each of nine wheat cultivars, Zak, Bobwhite, Moro, Scarlet, Eltan, Jagger, Thatcher, Chancellor and Kavkaz. Plants were fertilized with 100 ppm of 20-20-20 Peter’s fertilizer (J.R. Peters, Inc., Allentown, PA, USA) twice a week throughout the experiments.

3.5.3 Transcription and inoculation of viral RNAs

Infectious RNAs were transcribed from three linearized plasmids (two common plasmids pα, pβΔβa and a target specific BSMV\(\gamma\)-based plasmid) containing the recombinant tripartite BSMV genome using the mMmessage mMmachine T7 in vitro transcription kit (Ambion, Austin, TX, USA) according to the manufacturer’s recommendations. Equal volumes of transcribed RNAs (2.5 µL each) were combined with 45 µL FES as described by Pogue et al. (1998).

For monitoring the spread of the GFP VIGS reporter construct in leaves, roots and meristems, 8- to 10-day-old wheat plants were inoculated at the two-leaf stage. Inoculations
were done by rubbing the infectious RNA mixture along the second leaf using two gentle strokes. For targeting genes in the developing seeds and observing spread GFP of VIGS reporter construct in the reproductive tissues, the flag leaf of the main tiller was inoculated when the spike was at the boot stage. Inoculations were performed either by hand rubbing or by pressure infiltration using a 1-cc needle-less syringe. Inoculated plants were lightly misted with water and covered with plastic bags for 16 to 18 hours.

Two inoculation schemes were carried out for the VRN2 silencing constructs. Single inoculations were performed at the two-leaf stage, and double inoculations at both the two-leaf stage and 15 days after the first inoculation. VRN2 experiments consisted of culivars Jagger and Eltan each inoculated with either pγ.wVrnas, or pγ.wVrnhp along with an uninoculated control. Two plants of each cultivar were inoculated per treatment, and treatments were replicated twice.

3.5.4 GFP confocal microscopy

GFPc3 activity encoded by pγ.GFP was monitored in inoculated wheat tissues using a Bio-Rad MRC 1024 laser scanning confocal microscope and 25-mW krypton/argon laser (Bio-Rad, Thornwood, NY, USA) at 488 nm excitation and 510 nm emission. Leaf and root tissue were examined at 10 days post-inoculation (dpi) and wheat spikes at 15 to 17 dpi. Tissues, specifically leaf, root, ovary, stigma, anther, pollen, lemma, palea, awns and developing seed, were mounted in water under cover slip. Images were acquired using the LaserSharp2000 software (Bio-Rad, Thornwood, NY, USA) and reconstructed using Photoshop software (Adobe, Mountain View, CA, USA).

3.5.5 RNA isolation, cDNA synthesis and TaCOI1 real-time PCR assays
Wheat seedlings were grown in sand for 8 to 10 days, inoculated with pγTaCOI1as or pγ.GFP (infection control), and maintained at 22°C day and 18°C night with supplemental lighting for up to 21 dpi. Non-inoculated plants served as negative controls. Plants were watered with 0.94 g/L autoclaved Miracle-Gro solution (Scotts, Port Washington, NY, USA) as needed, usually 3 to 4 times per week. Plants were gently removed from sand, and the roots were rinsed in autoclaved nanopure water. Root and foliar tissues of each plant were separated using a sharp razor blade, harvested in liquid nitrogen, and stored at -80°C.

For RNA preparations, tissue (about 0.5 to 1 g fresh weight) was pulverized to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was isolated using 8 mL of TRIZOL reagent (Invitrogen Corp., Carlsbad, CA, USA) per sample according to the manufacturer’s recommendations. All non-disposable laboratory ware were rinsed with 0.01% v/v diethyl pyrocarbonate and autoclaved for 45 min prior to use. RNA was quantified by absorbance at 260 nm using a Bio-Rad SmartSpec 3000 (Bio-Rad Laboratories, Hercules, CA, USA).

RNA (5 μg) was combined with 2.5 μM oligo dT20 (Invitrogen) in a total volume of 11 μL of DEPC-treated nanopure water, heated at 65°C for 5 minutes, and chilled on ice. The RNA mixture was supplemented with 8 μL containing 1x First Strand buffer (Invitrogen), 10 mM DTT, 500 μM of each dNTP, and 40 Units of RNaseOUT (Invitrogen) in a final volume of 25 μL that was heated to 55°C in a thermocycler. After 1 minute, cDNA synthesis was initiated by the addition of 200 units of SuperScript™ III reverse transcriptase (Invitrogen) and carried out for an additional 2 hours at 55°C. cDNA was precipitated in 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate, pH 5.2, and resuspended in 20 μL of nanopure water. Approximately 7.5 to 8 μg of cDNA was obtained from each synthesis.
Real-time PCR analysis of TaCOI1 and TaCOI2 transcripts was performed using the DNA Master SYBR Green 1 chemistry and the LightCycler real-time PCR thermocycler (Roche Diagnostics, Indianapolis, IN, USA). PCR primers for TaCOI1 cDNA were: COI73-F2 5’ CCGAGGTTGCTCTATAC 3’ and JA1-R1 5’ CCGACGTTACCAGAAG 3’. TaCOI2 primers were: TaCOI2-F5 5’ CTTTGCGATAGGATGCCCA and TaCOI2-R5 5’ CTCCTTCAAGCGTACAG 3’. Internal (constitutively-expressed) controls for root and leaf samples were the wheat homologue of the maize glyceraldehyde 3-phosphate dehydrogenase gene (GPDH43; GenBank no. Q43247) and the wheat homologue of barley glyceraldehyde 3-phosphate dehydrogenase gene (GPDH26; GenBank no. P26517), respectively. Primers for GPDH43 transcripts were: GPDH43-F1 5’ CTCCAACGCTAGTTGC 3’ and 5’ GPDH43-R1 5’ AACTTTCCGGTAAAGCTCTG 3’ and for GPDH26 were: GPDH26-F1 5’ GAATCAACGGTTGAAG 3’ and GPDH26-R1 5’ CCACAACGTAAATCGGC 3’. Each PCR reaction consisted of about 200 ng of cDNA, 5 pmol each of forward and reverse primer, 3 mM of supplemental MgCl2, and SYBR Green I reagents (Roche) in a total volume of 10 µL. The amplification profile for all three cDNAs was 95°C for 10 minutes followed by 35 cycles of 95°C 10 seconds, annealing for 5 seconds, and 72°C for 10 seconds. Annealing temperatures for TaCOI1, GPDH43, and GPDH26 were 57°C, 53°C and 53°C, respectively. Amplicon melting profiles were generated over a range of 65 to 98°C, with a temperature change of 0.1°C sec⁻¹ and fluorescence monitoring every 0.3°C. TaCOI1 mRNA levels were normalized to GPDH43 (for roots) and GPDH26 (for shoots) using the ΔΔCₜ method (Livak and Schmittgen). VIGS was expressed as a ratio of TaCOI1 mRNA (normalized to G3PDH mRNA) in pγTaCOI1as-inoculated plants to that in pγ.GFP (infection control) plants.
3.5.6 Amylose content estimation

Each experiment consisted of three treatments, the waxy antisense construct pγ.wWxas, the waxy hairpin construct pγ.wWxhp, and the GFP negative control, pγ.GFP. On maturity, seeds were harvested from the spike of the inoculated tiller. Seeds were ground using a pestle and mortar, and 20 mg of flour from each plant (replicate) was used for amylose content determination. Amylose content was measured using the Amylose/Amylopectin assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland). Five replications of each treatment were measured.

3.5.7 VIGS transmission through seed

The PDS silencing phenotype was used as an indicator of VIGS transmission through seeds in the wheat cultivar Zak. Flag leaves were inoculated with the PDS silencing construct pγ.bPDS4 at boot stage and mature grains were harvested from the spike of inoculated tillers. 72 seeds (24 from each of three main tiller heads) were sown and the foliage of the first-generation progeny was scored for chlorophyll bleaching (white streaks) at the two-leaf stage. First generation plants showing the PDS silencing phenotype were allowed to mature and produce seed. 128 seeds from three mature heads (from main tiller of each of three plants) were further sowed and plants scored for photo-bleaching at two leaf stage.

Acknowledgments

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supported by Washington Wheat Commission, The Vogel Endowment funds, and USDA ARS Project Number 5248-22000-012-00D (P.O.).
3.6 FIGURES AND TABLES

Table 3.1 Silencing of TaCOI1 and TaCOI2 expression in root and leaf tissue of pyTaCOI1as–inoculated Scarlet, evaluated using real-time PCR.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Root expression(^\dagger)</th>
<th>Leaf expression(^\dagger)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TaCOI1</td>
<td>TaCOI2</td>
</tr>
<tr>
<td>Control</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>pyTaCOI1as</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant 1</td>
<td>0.30</td>
<td>0.42</td>
</tr>
<tr>
<td>Plant 2</td>
<td>0.50</td>
<td>0.69</td>
</tr>
</tbody>
</table>

\(^\dagger\) TaCOI transcript abundance, normalized to G3PDH, in pyTaCOI1as-inoculated plants expressed as a fraction of TaCOI transcript abundance in control (uninoculated) plants. Two of the least infected plants, based on viral symptoms, were selected for real-time PCR assays.

Table 3.2 Analysis of amylose content/waxy expression suppression by BSMV-VIGS in the seeds of wheat variety Zak

<table>
<thead>
<tr>
<th>% Reduction in amylose content(^a)</th>
<th>Number of plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Waxy-Antisense infected</td>
</tr>
<tr>
<td>30 - 32</td>
<td>2</td>
</tr>
<tr>
<td>34 - 36</td>
<td>1</td>
</tr>
<tr>
<td>36 - 38</td>
<td>0</td>
</tr>
<tr>
<td>38 - 40</td>
<td>2</td>
</tr>
<tr>
<td>80 – 82</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) Reduction in amylose content was expressed as percentage of VIGS-GFP control. 20 mg flour from spike of inoculated tiller of each plant was used for amylose content measurement.
Table 3.3 BSMV-based VIGS (virus-induced gene silencing) of *VRN2* gene failed to induce flowering in winter wheat varieties Jagger and Eltan

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1st Inoculation</th>
<th>2nd Inoculation</th>
<th>Flowering</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vernalized control †</td>
<td>Yes</td>
<td>No</td>
<td>At normal time ‡</td>
</tr>
<tr>
<td>Vernalized control †</td>
<td>Yes</td>
<td>Yes</td>
<td>At normal time ‡</td>
</tr>
<tr>
<td>pγ.wVrnhp</td>
<td>Yes</td>
<td>No</td>
<td>No flowering</td>
</tr>
<tr>
<td>pγ.wVrnhp</td>
<td>Yes</td>
<td>Yes</td>
<td>No flowering</td>
</tr>
<tr>
<td>pγ.wVrnas</td>
<td>Yes</td>
<td>No</td>
<td>No flowering</td>
</tr>
<tr>
<td>pγ.wVrnas</td>
<td>Yes</td>
<td>Yes</td>
<td>No flowering</td>
</tr>
</tbody>
</table>

*a* = at 2 leaf stage, *b* = 15 days after 1st inoculation, † = inoculated with GFP-expressing VIGS vector, ‡ = 90 – 100 days after germination. Each treatment consisted of 3 plants except vernalized controls that had one plant each.
Figure 3.1 *PDS* silencing in the fifth leaf of Zak at 17 days post inoculation.

Figure 3.2 *PDS* silencing in wheat spikes: viral symptoms on control inoculated with GFP expressing vector (a), (b) photo-bleaching on treatment inoculated with *PDS*-knockdown vector.
Figure 3.3 BSMV-based VIGS vector expressing GFP in vascular tissue of wheat roots. (a) Un-inoculated control, (b) inoculated with GFP expressing VIGS vector. Arrows in figure b indicate GFP expression.

Figure 3.4 BSMV-based VIGS vector expressing GFP in root tissue of wheat. (a) Uninoculated control, (b) inoculated with GFP expressing VIGS vector. Confocal merged (transmitted + GFP fluorescence) images taken at 10 days post inoculation (DPI) (a) & (b) and 17 DPI (c).
3.7 REFERENCES


Mapping Various Resistance Genes of Wheat Relative to Expressed Resistance Gene Candidates (RGCs)

Harvinder Singh Bennypaul and Kulvinder S. Gill
4.1 ABSTRACT

We localised nine important resistance genes of wheat relative to 47 expressed resistance gene candidates (RGCs) present in six wheat gene-rich regions. Out of the nine mapped genes; H13, H22, H24 and H25 are Hessian fly (M. destructor Say) resistance genes, Lr9 and L16 are leaf rust (Puccinia triticina Eriks) resistance genes, Pm3a and Pm20 are powdery mildew (Blumeria graminis (DC.) E.O. Speer f. sp. tritici) resistance genes and Yr10 is stripe rust (Puccinia striiformis f. sp. tritici Westend) resistance gene.

Six of these genes mapped within 10 cM and three within 20 cM of RGC loci. H13 showed linkage to 4 RGC loci with the closest proximal and distal loci at 7.9 cM and 15.1 cM respectively. Similarly, H22 showed linkage to 4 RGC loci with the closest proximal and distal loci at 13.1 cM and 10.1 cM respectively. Five RGC loci were found linked to H24 with the closest proximal and distal loci at 14.4 cM and 3.8 cM respectively. H25 showed linkage to two RGC loci, both located distally at 6.7 cM and 21.1 cM. Lr9 shared linkage group with seven RGC loci with the closest flanking loci at 21.6 cM and 12.4 cM respectively. Five RGC loci were found linked to Lr16 with the closest proximal and distal loci at 8.6 cM and 24.8 cM respectively. Yr10 and Pm3a showed linkage to five and eleven RGC loci respectively. Yr10 is flanked by proximal and distal loci at 5.6 cM and 4.7 cM respectively, whereas Pm3a is flanked by proximal and distal loci at 6.0 cM and 10.6 cM respectively. Seven RGC loci showed linkage to Pm20 with the closest proximal and distal loci at 12.4 cM and 10.5 cM respectively.

Except one, physical location of all genes was revealed by physically mapping the flanking markers. H22, Yr10 and Pm3a mapped in the gene rich region (GRR) 1S0.8; H22 on chromosome 1DS, Yr10 on 1BS and Pm3a on 1AS. Pm20 and Lr9 mapped in the GRR 6L0.9 on chromosome 6BL. Lr16 mapped in the GRR 2S0.9 on short arm of chromosome 2B. H13, H24
and 

$H25$ mapped in the distal 21%, 19% and 15% of chromosome 6DS, 3DL and 4AL respectively. Four genes ($H22$, $Yr10$, $Pm3a$, $H13$) mapped in gene-rich regions with higher recombination rates (<165 Kb/cM) and two ($Pm20$, $Lr9$) in a region with moderate recombination rate (825 Kb/cM). Leaf rust resistance gene $Lr16$ mapped in a region with a very low recombination rate (1300 Kb/cM) thus is not a good target for map based cloning.
4.2 INTRODUCTION

Bread or common wheat (*T. aestivum* L.) is the most widely cultivated food crop in the world, with a total production of over 600 million tons annually (http://faostat.fao.org/). Biotic and abiotic stress accounts for nearly 25% of the yield losses and approximately half of the losses are due to biotic factors. Genetic resistance to pests (diseases, insects, viruses) is usually the most effective, economical and environmentally sound method of control.

About 285 genes conferring resistance to various pests have been identified in wheat or in its wild relatives (McIntosh et al. 2000). Of these, 156 have been mapped to chromosome arms, 69 to chromosomes and genomic location of 64 genes is yet to be determined (McIntosh et al. 2000). Of the 156 resistance genes mapped on chromosome arms 120 have been localized relative to molecular markers. Mistakes in gene mapping are frequent as 22 wheat resistance genes have been reassigned to new chromosomal locations (McIntosh, 2007). Although a majority of the mistakes were made by monosomic analysis, mistakes were also made by mapping relative to molecular markers. For example, Russian wheat aphid resistance gene *Dn2* was mapped to the long arm of chromosome 7D by Miller (2001) but to the short arm by Liu (2000).

Use of molecular markers in marker-assisted selection (MAS) can assist conventional plant breeding to speed up cultivar development. In a MAS-based breeding strategy, markers are used as chromosome landmarks to facilitate selection of chromosomal segments carrying useful agronomic traits. MAS-based screening is particularly useful for hard to score traits and for accumulation of multiple resistance genes into a popular cultivar.
Recombination is highly uneven on the wheat and other eukaryotic chromosomes (Sidhu and Gill 2004). Detailed analysis of wheat homeologous group 1 chromosomes revealed that recombination occurs mainly in gene-rich regions encompassing ~13% of the genome (Sandhu and Gill 2002b). Distribution of recombination is very similar in other wheat chromosomes (Erayman et al. 2004). Most of the recombination occurs in the distal 50% of the chromosomes. There may be as much as a 20-fold difference in the rate of recombination even among various gene-rich regions (Sandhu and Gill 2002a). In many plants, R genes have been usually shown to cluster on the highly recombinogenic subtelomeric ends of chromosomes (Akhunov et al. 2003; Botella et al. 1997; Brueggeman et al. 2002; Ernst et al. 2002; Goff et al. 2002; Halterman et al. 2001; Meyers et al. 1999; Richly et al. 2002; Sakamoto et al. 1999). Recombination in regions immediately around the R genes, however, is usually suppressed (Chin et al. 2001; Noel et al. 1999; Shen et al. 2002; Sun et al. 2001). Because of the massive differences in recombination rate particularly around the R genes, the linkage-based analysis alone may be misleading. Therefore, physical mapping of the R genes is also important for gene mapping and cloning especially in wheat where distribution of genes and recombination is highly uneven along the chromosomes.

Resistance gene like sequences (Resistance Gene Analogs and Resistance Gene Candidates) have been shown to cluster around known resistance genes in a number of crops including soybean (Kanazin et al. 1996; Yu et al. 1996), maize (Xiao et al. 2007), barley (Madsen et al. 2003) and wheat (Dilbirligi and Gill 2003; Maleki et al. 2003). As compared to Resistance Gene Analogs (RGAs) that are amplified from genomic DNA, Resistance Gene Candidates (RGCs) cloned from the expressed portion of the genome may be better markers and candidates for resistance genes. Dilbirligi and Gill (2003) amplified 184 RGCs from the
expressed portion of the wheat genome by using degenerate primers for the conserved p-loop (GVGKTT) and the hydrophobic domain (GLPLAL) of the cloned resistance genes. In addition to these two very important motifs, the RGCs shared two other characteristics common to the cloned R genes: (1) significant sequence similarity to NBS-LRR (Nucleotide Binding Site – Leucine Rich Repeat) and PK (protein kinase) domains and (2) conserved number of nucleotides between domains (Dilbirligi and Gill 2003). These RGCs physically mapped to 26 chromosomal regions, which included 20 regions where known resistance genes were mapped by comparative mapping. Linkage relationship of the RGCs with the known resistance genes is however not known. In the present study, 22 resistance genes (H13, H22, H24, H25, Pm1, Pm2, Pm3a, Pm6, Pm8, Pm9, Pm13, Pm20, Lr2a, Lr2c, Lr9, Lr11, Lr14a, Lr16, Lr17, Lr18, Lr26, and Yr10) were selected for linkage analysis with 92 RGCs.

Hessian fly (M. destructor Say) is one of the most destructive pests of wheat worldwide with annual losses reaching $100 million in the US alone (Ratcliffe and Hatchett 1997). The resistance genes H13, H22, H24 and H25 provide high and stable level of antibiosis against a wide range of Hessian fly biotypes and geographic populations (Bouhssini et al. 1999; Gill and Raupp 1987; Hatchett and Gill 1981; Ratcliffe and Hatchett 1997). The H13 gene was mapped to chromosome 6DL with telocentric analysis (Gill and Raupp 1987) but to 6DS when mapped relative to SSR markers (Liu et al. 2005a). The H22 gene was mapped to 1D by monosomic analysis (Raupp et al. 1993) and to the distal 30% of 1DL when mapped relative to SSR markers (Raupp et al. 1993; Zhao et al. 2006). Gene H24 has been mapped to chromosome 3D by monosomic analysis (Wilson et al. 1989) and to 3DL by linkage analysis using RFLP markers (Ma et al. 1993). Gene H25 is an interstitial translocation from Secale cereale to chromosome 4AL of bread wheat (Friebe et al. 1991).
Leaf rust (\textit{P. triticina} Eriks.) and stripe rust (\textit{P. striiformis} Westend f. sp. \textit{tritici} Eriks.) are among the most important diseases of wheat (\textit{T. aestivum} L.) and other small grains around the world. Severe leaf rust infection can reduce yield by as much as 40\% (Knott 1989). Stripe rust can cause 100\% yield loss if infection occurs early and if the disease continues to develop during the growing season (Chen et al. 2002). More than 50 leaf rust resistance genes (McIntosh et al. 2007) and 70 stripe rust resistance genes (Chen 2005) have been reported in wheat.

The \textit{Lr2} gene was mapped to short arm of chromosome 2D by monosomic analysis (Soliman et al. 1964) but was later changed to chromosome 1B (McIntosh and Baker 1968). The \textit{Lr16} has been mapped to chromosome 4B by Dyck and Kerber (1971) but was later reassigned to the short arm of chromosome 2B by McCartney (2005). The \textit{Lr17} (Dyck and Samborski 1968) has been mapped to the short arm of chromosome 2A. The \textit{Lr26} (Friebe et al. 1993) and the \textit{Yr10} (Metzger and Silbaugh 1970) genes have been mapped to the long and the short arms of chromosome 1B, respectively. The \textit{Lr9} has been mapped to the long arm of chromosome 6B (Friebe et al. 1996). The linkage block containing the \textit{Lr14a}, the stem rust resistance gene \textit{Sr17} and the powdery mildew resistance genes \textit{Pm5} have been assigned to chromosome 7BL by cytogenetic analysis (Law and Johnson 1967). \textit{Triticum timopheevii} derived \textit{Lr18} was mapped to T5BS.5BL-5G#1 translocation on chromosome 5BL (Friebe et al. 1996).

(Robert et al. 1999; Seah et al. 2001), Yr26 (Venter et al. 2001), YrH52 (Peng et al. 2000) and Yrns-B1 (Borner et al. 2002) have been identified. In all of the aforementioned studies, the markers have been reported to be closely linked to the respective gene. Applicability of these markers in practical breeding scenarios differs widely as not all of these markers can detect the particular gene in diverse genetic backgrounds. For example, the STS marker for Lr9 worked well in the Australian breeding material (Schachermayr et al. 1997) but did not work well in the Indian germplasm as it amplified non-specific fragments (Gupta et al. 2005). Similarly, marker linked to Lr1 at <1 cM (Feuillet et al. 1995) has been reported to be effective only in about 50% of the breeding lines as the other 50% lines carry the Lr1 specific allele but do not show polymorphism for the marker. Therefore, it is better to have additional markers even for the well-marked genes.

Powdery mildew, caused by Blumeria graminis (DC.) E.O. Speer f. sp. tritici (Bgt.), is an important foliar disease of wheat (T. aestivum L.) in cool areas of the world (Bennett 1984). Race-specific resistance usually controlled by qualitatively inherited genes has been extensively used in breeding programs around the world. Mapped to 35 loci (Pm1-Pm35) on various wheat chromosomes, 51 powdery mildew resistance genes/alleles have been identified (Hsam et al. 2003; Liu et al. 2002; Miranda et al. 2007; Miranda et al. 2006; Xie et al. 2003; Zeller et al. 2002; Zhu et al. 2005). Hsam et al. (1998) mapped four alleles (Pm1a – Pm1d) to Pm1 locus in the distal part of chromosome 7DL. The Pm9 has also been mapped to the distal part on chromosome 7DL (Schneider et al. 1991). The Pm2 gene has been mapped to chromosome 5D using monosomic analysis (McIntosh and Baker 1970) and to chromosome 5DS by linkage analysis with Xcfd81-5D, an RFLP marker located 2 cM from the gene (Qiu et al. 2006). Ten alleles (Pm3a-Pm3j) have been identified at the Pm3 locus and have been mapped to the short
arm of chromosome 1A relative to morphological, biochemical and molecular markers (Briggle and Sears 1966; Hart et al. 1993; Huang et al. 2004; Ma et al. 1994; McIntosh and Baker 1968; Sourdille et al. 1999; Yahiaoui et al. 2004). The Pm3b allele encodes a coiled coil-nucleotide binding site-leucine rich repeat (CC-NBS-LRR) protein and is a member of a large family of resistance gene-like (RGL) sequences spread over approximately 1Mb (Yahiaoui et al. 2004). The T. timopheevii derived Pm6 was mapped at a distance of $1.5 \pm 1.4$ cM from an RFLP marker Xbcd135-2B on chromosome 2B (Liu et al. 1998). The Pm8 gene was transferred from rye to wheat and was mapped to T1BL.1RS wheat-rye translocation (Mohle et al. 2001). Ceoloni et al. (1992) used monosomic and C-banding analysis to map Pm13, derived from the short arm of chromosome 3S1 of Aegilops longissima, to introgressed chromosome segments on short arm of either 3B or 3D. Pm20, derived from the long arm of chromosome 6R of Secale cereale, has been physically mapped by C-banding analysis to the distal third of the recombined translocation chromosome T6BS 6RL rec (Heun and Friebe 1989). A precise genetic map location of the Pm20 is not known.

This study was undertaken with the objectives to physically and genetically map 22 resistance genes relative to 92 RGCs.

### 4.3 MATERIALS AND METHODS

#### 4.3.1 Plant material

A soft spring wheat cultivar Zak was used as a common susceptible parent for all the crosses with lines carrying various resistance genes. The resistance gene donor lines were TA4115 (Lr2a), TA4117 (Lr2c), TA3983 (Lr9), TA4123 (Lr11), TA3950 (Lr16), TA3854 (Lr17), TA4125 (Lr14a), TA5562 (Lr18), TA3987 (Lr26), TA5011 (H13), TA5005 (H22),
TA5016 (H24), TA5033 (H25), TA3896 (Pm1, Pm2, Pm9), TA3893 (Pm2, Pm6), TA3887 (Pm3a), TA2910 (Pm8), TA5561 (Pm13), TA5041 (Pm20) and Moro (Yr10). Nullisomic-tetrasomic (NT) lines (Sears 1954) were used for chromosomal location of DNA fragment bands. The NT lines lack a pair of homologous chromosomes, the deficiency of which is compensated for by a double dose of either of the two homoeologous chromosomes. For example, in nulli1B/tetra1A (N1B/T1A), the chromosome 1B pair is missing but chromosome 1A is in tetra dose. A DNA fragment band missing in N1B/T1A but present in N1D/T1B or N1A/T1D, was localized to chromosome 1B. The aneuploid stocks and the resistant parent lines for leaf rust, powdery mildew and Hessian fly mapping populations were kindly provided by the Wheat Genomic Resources Center, Kansas State University, Manhattan, Kansas, USA. Resistant parent line for stripe rust mapping population was kindly provided by Dr. Xianming Chen, USDA-ARS and WSU, Pullman, Washington, USA.

4.3.2 Phenotypic screening

4.3.2.1 Hessian fly resistance genes

Screening for the Hessian fly resistance genes was performed by Dr. Ming-Shun Chen (USDA-ARS, GMPRC, 20 Waters Hall, KSU, Manhattan, KS, USA). Hessian fly biotype L culture was supplied by S. E. Cambron, USDA-ARS, West Lafayette, IN, USA. The insects were maintained on seedlings of cultivars Ike (H3), Magnum (H5), Caldwell (H6) and Seneca (H7/H8) sequentially. The Hessian fly pupae along with infested wheat plants were stored at 4°C until needed.

The Hessian fly screening was performed following previously described method (Hatchett and Gill 1981; Maas et al. 1987) with modifications. For non-segregating material (parents, F1s), five plants were screened for each line whereas for segregating plants (F2,3...
families), 12 - 15 plants were screened. Briefly, seeds were sown in uniformly spaced rows (12 rows or 24 half-rows per flat) in flats (52 x 36 x 10 cm) containing a mixture of soil and vermiculite in a 1:1 ratio. The plants were grown in growth chambers at 18°C with 14 hours light. Each flat of 8 - 9 days old seedlings was infested by confining ≈ 200 newly mated females under a cheesecloth tent. The seedlings were scored for the Hessian fly reaction after 14 days of infestation. The resistant plants grew normally with light green leaves and dead larvae between leaf sheaths. The susceptible plants were stunted with dark green leaves and harbored live larvae or pre-pupae. Cultivars susceptible to biotype L, Karl 92 and Ike, were used as positive control whereas resistant cultivars Molly, carrying H13, and Hamlet, carrying H21, were used as negative controls of infestation. Based on reaction of F3 families, F2 plants were classified as homozygous or heterozygous for the resistance gene in question.

4.3.2.2 Leaf rust resistance genes

For non-segregating material (parents, F1s), five plants were screened for each line where as for segregating plants (F2:3 families), 10 - 12 plants were screened. Plants were grown in 24-cell trays filled with Sunshine # 1 potting mix (SunGro Horticulture, Bellevue, WA, USA). After emergence, all seedlings were given 100 ppm of 20-20-20 Peter’s fertilizer (J.R. Peters, Inc., Allentown, PA, USA) and watered as required. Seedlings, grown in a greenhouse operating at 22°C 16 hour light and 18°C 8 hour dark cycle, were inoculated at full emergence of primary leaves, approximately 8 – 9 days after germination. Mostly freshly multiplied inoculum was used in screening, however, if frozen (at -20°C), inoculum was given a heat shock treatment for 5 minutes at 40°C before inoculating plants. For inoculation, urediniospores mixed with 10-fold quantity of talcum powder, were uniformly dusted over the plants. The plants were lightly
misted with water, covered with plastic box to maintain a higher level of relative humidity (RH), and were incubated overnight in a growth chamber set at 16°C without lights and at 100% RH. The plants were then transferred to a greenhouse operating at 22°C 16 hours light and 18°C 8 hours dark cycle.

Ten to twelve days after inoculations, the infection types (ITs) of plants were classified according to a 0 - 4 scale of Long and Kolmer (1989); 0 = immune (no hypersensitive flecks or uredinia), 0; = faint hypersensitive flecks, ; = distinct hypersensitive flecks, 1= small uredinia surrounded by distinct necrosis, 2 = small uredinia surrounded by distinct chlorosis, 3 = intermediate-size uredinia lacking chlorosis, and 4 = large uredinia lacking chlorosis. Plants showing ITs of 0 to 2 were classified as resistant and of 3 to 4 as susceptible. The *P. triticina* races were kindly provided by Cereal Disease Laboratory, St. Paul, Minnesota, USA.

4.3.2.3. *Stripe rust resistance genes*

Screening for *P. striiformis* f. sp. *tritici* was performed using race PST-78. The number of plants used and the plant growing conditions were similar to that used for the leaf rust scoring. Seedlings at two-leaf stage (10 - 11 days old) were inoculated by uniformly dusting with 1:10 mixture of urediniospores and talcum powder followed by placing the plants in a dew chamber at 10°C for 16 - 18 hours. The plants were then transferred to a growth chamber operating at 16 hours light and 8 hours dark with temperatures gradually changing from 8°C to 16°C in 6 hours, staying at 16°C for 6 hours, ramping down to 8°C in 6 hours, staying at 8°C and again repeating the cycle. Infection type (IT) data were recorded 18 - 21 days after inoculation based on a 0 - 9 scale of McNeal et al. (1971). Infection types 0 - 4 were considered as resistance reaction and 5 - 9 as susceptible reaction; 0 = no visible symptom, 1 = necrotic
and/or chlorotic flecks, 2 = necrotic and/or chlorotic blotches or stripes without sporulation, 3 and 4 = necrotic and/or chlorotic blotches or stripes with trace sporulation, 5 - 9 = moderate to abundant sporulation with or without chlorosis or necrosis. The *P. striiformis* f. sp. *tritici* race was provided by Dr. Xianming Chen, WSU, Pullman, Washington, USA.

4.3.2.4 **Powdery mildew resistance genes**

Plant screening for powdery mildew resistance was performed by Dr. S. L. K. Hsam (Technische Universitat Munchen, Institut fur Pflanzenbau und Pflanzenzuchtung, Freising-Weihenstephan, Germany) following the protocol of (Lutz et al. 1992). On an average, five plants for the non-segregating material and 12 – 17 for the segregating material were screened. The *Bgt* isolates used in this study were single-spore progenies of various collections made from different parts of Europe (Zeller et al. 2002).

Powdery mildew resistance tests were carried out on segments of primary leaves of 10-day old plants grown in phytotron set at 20°C and continuous light of 200 μE/m²s. A three cm leaf segment per plant was cut into 7 - 9 pieces and cultured in a Petri-dish on 6 g/l agar with 35 mg/l benzimidazole. The inoculum was produced on wheat cv. ‘Kanzler’ and was spread in a settling tower onto the leaf segments at densities of 400 – 500 spores/cm². Petri-dishes carrying inoculated segments were incubated at 17°C under continuous lighting at 10 μE/m²s. Disease reaction was assessed 10 days post inoculation and three main classes of host reaction were distinguished: r = resistant (immune response), i = intermediate (up to 50% infection), s = susceptible (>50% infection). Intermediate and susceptible were combined into the susceptible class.
4.3.3 DNA extraction, Southern blotting and marker analysis

Plant genomic DNA extraction and gel blot analysis was performed following the procedure described by Gill et al. (1993). Leaf tissue was ground in liquid nitrogen and incubated in DNA extraction buffer (0.1 M Tris pH 7.0, 0.7 M NaCl, 10 mM EDTA pH 8.0, 1% Hexadecyltrimethylammonium Bromide, 0.14 M 2-Mercaptoethanol) at 60°C for 3 hours. The slurry was extracted with equal volume of chloroform-octanol (24:1 v/v). The DNA was precipitated from the supernatant by adding two-third volume of isopropanol. The DNA pellet was washed twice, once with 76% ethanol + 0.2 M sodium acetate for 30 minutes and then with 76% ethanol + 10 mM ammonium acetate. Air dried pellet was dissolved in Tris EDTA buffer (TE) containing RNase (2 μg/ml). The DNA was extracted with phenol: chloroform (1:1) and recovered by adding 0.3 M sodium acetate (pH 5.2) and an equal volume of isopropanol. Finally, the DNA pellet was washed with 70% ethanol for 30 minutes, air dried and dissolved in TE.

For gel blot DNA analysis, 5μg of genomic DNA was digested with 4 units of restriction enzymes per μg of DNA. The digested DNA was size separated by electrophoresis on 0.8% agarose gels. ‘Southern’ blotting onto nylon membrane (Micron Separations Inc. MA), DNA immobilization, and hybridization were performed following manufacturer’s recommendations. Up to 12 enzymes were used to detect chromosome specific polymorphism for the critical chromosomes. In case where none of the enzymes gave chromosome-specific polymorphism, restriction enzyme giving maximum polymorphism was used for making blots.

4.3.4 Data Analysis
Chi-square ($\chi^2$) tests were conducted to determine the goodness-of-fit of plant segregation ratios to theoretical Mendelian segregation ratios. Linkage analysis was performed using the mapping software Map Manager QTXb20 (Manly et al. 2001). Linkage was determined with the criteria of LOD $\geq 3.0$ ($P = 0.05$) and a maximum recombination fraction of 0.40. Marker orders were estimated using the Kosambi mapping function (Kosambi 1944). Physical location of the genes was determined by the physical location of the bracketing RGCs. Physical location of RGCs was previously reported by Dilbirligi et al. (2004). Direction of centromere was inferred by comparison of the maps with the existing physical and genetic maps. Two bands (one from each parent) were considered non-allelic if both bands are missing in any of the progeny.

4.4 RESULTS AND DISCUSSION

The 22 wheat lines carrying various resistance genes and cultivar Zak were screened with various races of *P. triticina*, *P. striiformis* f. sp. *tritici*, *B. graminis* f. sp. *tritici* and biotype L of *M. destructor* for absence or presence of resistance genes. Of these, 14 comparisons showed contrasting phenotype with Zak showing susceptible reaction (Table 4.2, 4.3, 4.11). For the remaining eight comparisons Zak did show the susceptible reaction but the resistant lines didn’t show the expected resistant reaction thus were not studied further. The 47 RGCs (Table 4.1, 4.4) physically mapping in the chromosomes/arms/regions known to carry the 14 resistance genes were used for the mapping.

4.4.1 Powdery Mildew

4.4.1.1 Screening for powdery mildew resistance genes
Zak and powdery mildew resistance gene carrying lines TA3896 (Pm1, Pm2, Pm9), TA3887 (Pm3a), TA3893 (Pm6), TA2910 (Pm8), TA5561 (Pm13) and TA5041 (Pm20) were screened with various B. graminis f. sp. tritici isolates (Table 4.3). Zak was susceptible to all the isolates suggesting it dose not have Pm1, Pm2, Pm3a, Pm6, Pm8, Pm9, Pm13 and Pm20. TA3896, TA2910 and TA5561 gave susceptible reaction indicating absence of powdery mildew resistance genes in question. TA3887, TA3893 and TA5041 showed resistant reaction indicating presence of Pm3a, Pm6 and Pm20 respectively. Resistance gene carrying lines showing expected resistance reaction were crossed with susceptible Zak for developing F2 mapping populations.

All powdery mildew resistance gene F2 populations, except Pm6, showed 1: 2: 1 single gene segregation (Table 4.10) which is consistent with previous reports for Pm3a (Johnson 1979) and Pm20 (Friebe et al. 1994) based resistance. The χ² (df = 2, P = 0.05) value for Pm3a and Pm20 was 4.19 and 3.00 respectively. Pm6 F2 populations did not show single gene segregation for resistance to powdery mildew. In earlier report Pm6 has been shown to follow 1: 2: 1 segregation ratio (Jorgensen and Jensen 1973). In our study Pm6 F2 populations segregated 10:32:31 (homozygous resistant: heterozygous resistant: homozygous susceptible). Number of homozygous susceptible plants was more than expected and number of homozygous resistance plants was less than expected. This could be due to presence of suppressors of Pm6 expression in Zak. Suppressor genes inhibiting the expression of Pm8 in cv. Disponent and of Pm17 in line Helami-105 have been reported by Zeller et. al (1996).

4.4.1.2 Linkage analysis of IS0.8 GRR-specific RGCs with Pm3a
Powdery mildew resistance gene *Pm3a* has been mapped to wheat chromosomes 1AS (McIntosh and Bennett 1978). Eight RGCs, physically mapping in the GRR 1S0.8 (Dilbirligi et al. 2004) on short arm of group 1 chromosomes (Table 4.7), were used for linkage analysis with *Pm3a*. The RGCs were *BE492937, BE405778, BF474204, BF475048, BF483881, BG606866, BE518223 and BM135313*. These eight RGCs detected 56 bands in both Chinese spring (CS) and Zak, and 53 in *Pm3a* donor TA3887. The NT analysis with CS lines localized 38 of 56 bands; 22 to chromosome 1A, 12 to chromosome 1B and four to chromosome 1D (Table 4.7). All the RGCs except *BF483881* detected bands on more than one homoeologue.

Restriction enzymes *BglII, DraI, EcoRI, EcoRV, HindIII* and *ScaI* were used for polymorphism survey between the two parents (Table 4.7). All RGCs detected a total of 32 bands between resistant and susceptible parent with *EcoRI, HindIII* and *ScaI*. Six of eight RGCs detected chromosome 1A-specific polymorphism with *HindIII*, whereas chromosome-1A specific polymorphism for RGCs *BF474204* and *BF483881* was only detected by *EcoRI* and *ScaI* respectively. As *Pm3a* has been mapped on chromosome 1AS (McIntosh and Bennett 1978), enzymes giving 1A specific polymorphism were used in gel blot analysis.

A genetic map showing linkage between *Pm3a* and the 1S0.8-specific RGCs is given in figure 4.1. RGCs *BE135313, BE518223, BG606866, BG606866, BF475048 and BF474204* were found linked to *Pm3a* gene in a 68.9 cM region. *BM135313, BE405778* and *BE518223* detected four, three and two linked loci respectively. *BG606866, BF475048* and *BF474204* detected one linked locus each. All the fragment bands linked to *Pm3a* mapped on chromosome 1A by NT analysis as these bands were missing in N1AT1B but present in N1DT1B and N1BT1A (data not shown). Direction of centromere was deduced using mapping information of *BF475048* in relation to RFLP marker *Xbcd1434* from genetic map of *Dn7* in rye chromosome.
arm 1RS (Lapitan et al. 2007). *Xbcd1434* is perfectly linked to *Pm3a* (Sandhu et al. 2001) and this marker is proximal to *BF475048* in genetic map of *Dn7*. Assuming a conserved order between wheat and rye for markers around *Pm3a*, *BF475048* is distal to *Pm3a* as it mapped distal to *BM135313d*. The marker *BF474204* is therefore proximal to *Pm3a*. The *Pm3a* gene is flanked by *BF474204* (6.0 cM) on the proximal side and *BM135313d* (10.6 cM) on the distal side.

RGC *BE492937* and *BF483881* did not map in the linkage group of *Pm3a*. Both the RGCs were physically mapped in the same chromosomal region as *Pm3a* by Dilbirligi et al. (2004). This could be due to high rate of recombination in 1S0.8 GRR, where *Pm3a* and the linked RGCs are mapped in this study. This GRR is a recombination hot spot accounting for 86% of arms recombination (Sandhu and Gill 2002a). The GRR 1S0.8 is only ∼7 Mb in size but spans about 45 – 50 cM genetic distance in wheat, barley, rye and oat (Sandhu and Gill 2002b).

### 4.4.1.3 Linkage analysis of RGCs mapping in the distal region of 6BL with *Pm20*

Powdery mildew resistance gene *Pm20* has been mapped to wheat chromosomes 6BL (Heun and Friebe 1989). Nine RGCs (Table 4.7) mapping in distal region of 6BL (Dilbirligi et al. 2004) were used for linkage analysis with *Pm20*. The RGCs were *Unl154*, *Unl194*, *BE403251*, *BE405507*, *BE426789*, *BE426790*, *BE500158*, *BF199778* and *BM137173*.

The nine RGCs detected 41, 42 and 43 bands in CS, Zak and *Pm20* donor TA5041 respectively. The NT analysis with CS lines localized 25 of 41 bands; seven to chromosome 6A, 16 to chromosome 6B and two to chromosome 6D (Table 4.7). *BE405507*, *BE500158*, *BM137173* and *Unl154* detected bands on more than one homoeologue.
Restriction enzymes *BamHI, DraI, EcoRI, EcoRV, HindIII* and *XbaI* were used for polymorphism survey between the parents (Table 4.7). None of the enzymes detected any polymorphism for RGCs *BF199778, BE426789, Unl194* and chromosome 6B-specific polymorphism for *BM137173*. *EcoRV* detected chromosome-6B-specific polymorphisms for RGCs *BE403251, BE426790, BE500158* and *BamHI, DraI* detected 6-B specific polymorphism for *BE405507* and *Unl154* respectively. As *Pm20* has been mapped to chromosome 6BL (Heun and Friebe 1989), restriction enzymes giving 6B specific polymorphism were used for gel blot analysis.

A genetic map showing linkage between *Pm20* and the RGCs is given in figure 4.2. RGCs *BE403251, BE405507, BE426790* and *BE500158* were found linked to *Pm20* gene in a 70.2 cM region in the GRR 6L0.9. *BE403251, BE405507* and *BE426790* detected 2 linked loci each. All the fragment bands linked to *Pm20* mapped on chromosome 6B by NT analysis as these bands were missing in N6BT6A but present in N6AT6B and N6DT6B (data not shown). The *Pm20* gene is flanked by *BE426790b* at 10.5 cM on one side and *BE403251b* at 12.4 cM on the other side. All the markers genetically mapped in this study have been previously physically mapped in the same region of wheat genome using deletion lines (Dilbirligi et al. 2004). *BF199778, BE426789, BM137173, Unl154* and *Unl194*, which were mapped in the same chromosomal region by Dilbirligi et al. (2004), could not be confirmed to map in the same region due to absence of chromosome 6B-specific polymorphism with all the restriction enzymes tried. According to best of our knowledge this is the first genetic map of powdery mildew gene *Pm20*. This GRR is a ≈ 45 Mb in size and is present on long arm of homeologous group 6.
4.4.2 Hessian fly

4.4.2.1 Screening for Hessian fly resistance genes

The Hessian fly resistance gene parents TA5011 ($H13$), TA5005 ($H22$), TA5016 ($H24$), and TA5033 ($H25$) showed resistance reaction to the L biotype (Table 4.11). All five plants of the susceptible parent Zak showed susceptible reaction. All five Hessian fly resistance genes showed dominance inheritance as all five $F_1$ plants of the crosses ZakXTA5011, ZakXTA5005, ZakXTA5013, ZakXTA5016 and ZakXTA5033 exhibited complete resistance.

Among the 79 $F_{2:3}$ families for $H13$, 12 were homozygous resistant, 19 susceptible, and 48 were heterozygous. Similarly 73 $F_{2:3}$ families for $H22$ segregated into 21 homozygous resistant, 17 susceptible and 35 were heterozygous. The $H24$ segregatd for three resistant, 18 susceptible and 40 heterozygous progeny. The 67 $F_{2:3}$ families for the $H25$ population segregated into 16 homozygous resistant, 12 susceptible and 39 heterozygous families.

Hessian fly resistance genes $H13$, $H22$ and $H25$ showed inheritance of a single dominant gene as the Chi square values (at $P = 0.05$) to test segregation ratio against 1: 2: 1 were 4.90, 0.56, 2.28 for $H13$, $H22$ and $H25$, respectively (Table 4.11). These results are consistent with the previous reports for these genes (Friebe et al. 1991; Gill and Raupp 1987; Raupp et al. 1993). In contrast to a previous report (Ma et al. 1993), gene $H24$ did not show a 1: 2: 1 segregation as the Chi square value at $P = 0.05$ was 13.29. The main reason for this distortion was a higher than expected number of heterozygotes and a lower number of homozygous resistant plants. On an average 12 plants per $F_{2:3}$ were screened and some of the heterozygous resistant families showed only 1 or 2 susceptible plants. This could have happened due to contamination of avirulent Hessian fly biotype L with other biotype that is virulent for $H24$ (Dr. Ming-shun Chen, KSU, personal communication). This segregation distortion seems to be
caused by misscoring of the heterozygotes as the Chi square for a test against 3:1 ratio (by combining homozygous resistant and heterozygous resistant) was only 0.66. Therefore, H24 phenotypic data was scored both as 1: 2: 1 as well as 3:1 for map construction.

4.4.2.2 Linkage analysis of 1S0.8 GRR-specific RGCs with H22

The resistance gene H22 was transferred to common wheat from the wheat D genome donor *Aegilops tauschii* Coss., by direct crossing (Gill and Raupp 1987; Ratcliffe and Hatchett 1997). Since H22 was previously localized to the distal 30% of the short arm of chromosome 1D by mapping relative to SSR markers (Raupp et al. 1993), nine RGCs mapping in the GRR 1S0.8 were used for the linkage analysis. The RGCs were *BE492937, BE405778, BF474204, BE499561, BE498831, BF475048, BF483881, BG606866* and *BE518223*. These nine RGCs detected 58 fragment bands in CS and 55 each in Zak and H22 donor line TA5005 (Table 4.8). The NT analysis localized 45 of the 58 fragment bands; 17 to chromosome 1A, 21 to chromosome 1B and seven to chromosome 1D. Except *BE499561*, all RGCs detected more than one band on at least one of the homoeologue.

Restriction enzymes *BamHI, BglII, DraI, EcoRI, EcoRV, HindIII, KpnI, NcoI, PstI, SacI, Scal and XbaI* were used for polymorphism survey between Zak and TA5005 (H22). With enzymes *BglIII, DraI, XbaI* and *PstI* eight of the nine RGCs detected 19 polymorphic bands between the two parents (Table 4.8). RGC *BE498831* did not detect any polymorphism between the two parents even with 12 restriction enzymes. The RGCs *BE405778, BF474204, BF475048, BF483881* and *BG606866* did detect polymorphism between the parents but none of the fragment bands was chromosome 1D-specific. RGCs *BE492937, BF483881, BG606866,*
BE518223 and BE499561 detected 1D-specific polymorphism, first three with enzyme BglII and the last two with XbaI.

A 1DS map around the H22 is given in figure 4.3. All RGCs linked to the H22 gene detected one locus each on 1D except for BE499561 that detected two loci. All the fragment bands linked to H22 mapped on 1D by nulli-tetra analysis as these bands were missing in N1DT1B but present in NIAT1B and N1BT1A (data not shown). Direction of centromere was found by comparing the marker order of H22 map with Yr10 map (Figure 4.9). Before comparing H22 and Yr10 maps, location of RGC BE518223 (which is present H22 map but absent in Yr10 map) on Yr10 map was decided by comparison with Pm3a map (Figure 4.1). RGC BE518223 was found to be distal to BE492937 on Yr10 map. Comparison of H22 and Yr10 maps revealed that BE492937 is proximal to BE518223. RGC BE518223 is located 10 cM distal to H22, whereas, BE492937, BE499561a and BE499561b are proximal to the gene at 13.1 cM, 25.4 cM and 28.5 cM, respectively. Zhao et al. (2006) mapped H22 to distal 30% region on short arm of chromosome 1D. Based on somatic metaphase chromosome size distal 30% of 1DS corresponds to about 70 Mb (Gill et al. 1991) - a relatively large region. However, our results indicate that H22 is located within the GRR 1S0.8 as all the markers, both proximal and distal, found linked to H22 in this study have been previously mapped to this GRR (Dilbirligi and Gill 2003). The 1S0.8 region, flanked by deletions 1BS-19 and 1BS-4, is about 7 Mb in size and is a recombination hot-spot with 86% of arm’s recombination (Erayman et al. 2004). 1S0.8 is one of the largest gene-rich regions of wheat which contains agronomically important genes, including 12 genes conferring resistance to diseases like leaf rust (Lr10, Lr21, Lr26), stem rust (Sr31, Sr33), powdery mildew (Pm3, Pm8) and stripe rust (Yr9, Yr10, Yr15) (Sandhu et al. 2002b).
Another cluster of Hessian fly resistance genes containing H9, H10, H11 and Hdic was also mapped to the distal region on chromosome 1AS (Liu et al. 2005b).

4.4.2.3 Linkage analysis of chromosome 6D-specific RGCs with H13

H13 was transferred to common wheat from Aegilops tauschii Coss., an important source for resistance (Gill and Raupp 1987; Ratcliffe and Hatchett 1997). H13 is a dominant resistance gene with a very high and stable level of antibiosis against wide range of Hessian fly biotypes and geographic populations (Bouhssini et al. 1999; Gill and Raupp 1987; Hatchett and Gill 1981; Ratcliffe and Hatchett 1997).

H13 was previously mapped to chromosome 6D using monosomic analysis and later to 6DL (35.0 ± 8.0 recombination units from the centromere) by telocentric analysis (Gill and Raupp 1987). Recently, this gene has been mapped to distal cluster on short arm of chromosome 6D based on linkage analysis of simple sequence repeat (SSR) markers (Liu et al. 2005a). As there are two mapping locations reported for H13 on chromosome 6D, 14 RGCs, mapping in two distal clusters, one on long arm and the other on short arm of homoeologous group 6, were chosen for linkage analysis with the gene in question. Of the 14 candidates Unl190, BF20008, BF478914 and BG607637 were mapped in a cluster distal to deletion breakpoint 6BS-8 on short arm and Unl154, Unl194, BE403251, BE405507, BE426789, BE426790, BE500158, BE638124, BF199778 and BM137173 were mapped to another cluster distal to deletion breakpoint 6AL-8 (Dilbirligi and Gill 2003).

The 14 RGCs detected 86 bands in CS, 85 bands in the susceptible parent Zak and 89 bands in H13 donor TA5011 (Table 4.8). 52 of 86 bands in CS were mapped with the help of NT analysis; 13 on chromosome 6A, 31 on chromosome 6B and eight on chromosome 6D.
Seven of 14 RGCs detected bands on chromosome 6D where \( H13 \) was mapped in earlier studies. RGCs \( BE405507, BE426789, BE638124 \) and \( Unl194 \) detected bands on single homoeologue whereas \( Unl190, BF20008, BF478914, BG607637, Unl154, BE403251, BE426790, BE500158, BE638124, BF199778 \) and \( BM137173 \) detected bands on multiple homoeologues. Number of bands per homologue varied from zero to six.

Restriction enzymes \( BamHI, BglII, DraI, EcoRI, EcoRV, HindIII \) and \( XbaI \) were used for polymorphism survey between the two parents (Table 4.8). Of 14 RGCs 11 detected polymorphism between the parents with \( BamHI, DraI, EcoRI, EcoRV \) and \( HindIII \), however, chromosome 6D specific polymorphism was found only in case of \( BE403251, BF199778, BF478914, BF200008 \) and \( Unl154 \). \( EcoRV, HindIII \) and \( DraI \) detected chromosome 6D specific polymorphism for three, one and one RGCs respectively. As \( H13 \) has been previously mapped to chromosome 6DL (Gill and Raupp 1987) and 6DS (Liu et al. 2005a) restriction enzymes giving 6D specific polymorphism was used for gel blot analysis.

A map of \( H13 \) and the linked RGCs is given in figure 4.4. None of the 10 RGCs mapping in the distal cluster on long arm of chromosome 6D showed linkage to \( H13 \). Two, \( BE200008 \) and \( BF478914 \), of the four RGCs in the distal cluster on short arm of chromosome 6 were found linked to \( H13 \). Each of these RGCs detected two loci that are linked to \( H13 \). All the fragment bands linked to \( H13 \) mapped on chromosome 6D by nulli-tetra analysis as these bands were missing in N6DT6B but present in N6AT6B and N6BT6A (data not shown). Direction of centromere is inferred from the fact that \( H13 \) is recently mapped between deletion breakpoints 6DS-4-FL0.79 and 6DS-6-FL0.99 (Liu et al. 2005a). As RGC \( BF478914 \) has been previously mapped distal to 6DS-6-FL0.99 (Dilbirligi and Gill 2003) that means \( H13 \) is proximal to this candidate. \( BE200008a \) is closest and proximal to the gene at 7.9 cM. Both loci detected by
BF478914 are distal to the gene, BF478914a at 15.1 cM and BF478914b at 21.8 cM. RGCs BG607637 and Unl190, eventhough mapped in same region in an earlier study, could not be mapped due lack of chromosome 6D-specific polymorphism between mapping population parents. Mapping location of H13 found in this study agrees with the previous monosomic mapping result that this gene is located on chromosome 6D, but disagree with the ditelosomic arm mapping results that mapped H13 on 6DL (Gill and Raupp 1987). Our results, however, agree with Liu et al. (2006) who also mapped this gene on 6DS in wheat genome.

According to Raupp et al. (1993), H23 and H13 are linked at a distance of 25 ± 5.0 map units. Ma et al. (1993) used RFLP markers to map H23 15.6 cM distal to XksuG48a. A study by Pestsova et al. (2000) placed XksuG48a between the markers Xgdm132 and Xgdm141 and the latter two markers are mapped distal to H13 on chromosome 6DS (Liu et al. 2005a). In the same region a defense response gene Ppo (polyphenol oxidase), with close linkage and proximal to XksuG48a, has been mapped by Li et al. (1999). Malik et al. (2003) mapped the wheat curl mite resistance gene Cmc4 between XksuG48a and Xgdm141. All these observations show that a cluster of insect-pest resistance genes is present in the distal region of 6DS.

Plant resistance to Hessian fly is controlled by the mechanism of antibiosis (Painter 1951) in which larvae die after feeding on plants. Antibiosis is also a common mechanism of resistance in majority of plant-parasitic mites (Archer et al. 1997). Polyphenol oxidases play a defensive in plant – pest interactions. It is interesting to note that resistance genes with a common mode of action are clustered together in this distal region. According to Richter and Ronald (2000), race-specific disease resistance genes in plants are commonly clustered in linked array, and most likely evolved from the progenitor resistance gene through duplication and
diversification. This gene rich cluster prove the observation made by Gill et al. (1996) that genes are present in gene rich and gene poor regions in wheat genome.

The RGCs found linked to H13, BF200008 and BF478914, have NB/LRR type structure and show 40-60% similarity to cloned NB/LRR type resistance genes. These RGCs can be useful for the cloning of H13 and other genes present in this distal cluster or for finding closely linked markers for marker-assisted breeding.

4.4.2.4 Linkage analysis of 3DL-specific RGCs with H24

H24 was transferred to common wheat from Aegilops tauschii Coss. (Gill and Raupp 1987; Ratcliffe and Hatchett 1997). This gene has been mapped to chromosome 3D by monosomic analysis (Wilson et al. 1989) and to long arm of chromosome 3D by linkage analysis with RFLP markers (Ma et al. 1993). As H24 is reported to be mapped to chromosome 3D or 3DL, seven RGCs mapping on long arm of chromosome 3D were chosen for linkage analysis with H24. These RGCs were Unl132, Unl150, BE482366, BE607045, BE499910, BE405711 and BE591154. Unl150 and BE607045 have been physically mapped in the region flanked by breakpoints 3DL-1 (FL value = 0.23) and 3DL-2 (FL value = 0.27), whereas Unl130, Unl132, BE482366, BE499910, BE405711 and BE591154 have been mapped distal to deletion breakpoint 3AL-5 (FL value = 0.78) (Dilbirligi, 2004).

The seven RGCs detected 39, 38 and 37 bands in Zak, CS and TA5016 (H24 donor parent) respectively (Table 4.8). NT analysis with CS lines mapped 28 of 38 bands; five to chromosome 3A, 13 to chromosome 3B and ten to chromosome 3D. All RGCs detected bands on at least two homoeologues.
Restriction enzymes *BamHI, Dral, EcoRI, EcoRV, HindIII, NcoI, Scal* and *XbaI* were used for polymorphism survey between the parents (Table 4.8). Six RGCs detected 13 polymorphic bands between susceptible and resistant parent with *Dral, EcoRI, EcoRV, HindIII, NcoI and Scal*. All RGCs, except *Unl150*, detected chromosome 3D specific polymorphism between two parents. *Unl150* did not detect any polymorphism between the two parents with the eight enzymes used in this study. As *H24* has been mapped on chromosome 3D (Wilson et al. 1989) and 3DL (Ma et al. 1993) restriction enzyme giving 3D specific polymorphism were used for gel blot analysis.

Linkage analysis found *H24* linked to the RGCs *BE607045, BE499910, Unl132*, and *BF482366* mapping in the distal cluster on long arm of chromosome 3D (Figure 4.5). All RGCs linked to *H24* detected one locus on 3D except for *BF482366* that detected two loci. All the fragment bands linked to *H24* mapped on chromosome 3D by nulli-tetra analysis as these bands were missing in N3DT3B but present in N3AT3B and N3BT3D (data not shown). Direction of centromere was decided based on the physical mapping information of *BE607045* relative to other RGCs. Dilbirligi et al. (2004) mapped *BE607045* proximal to *Unl132, BE482366* and *BE499910*. *Unl132, BE482366, BE499910* and *BE607045* showed linkage with *H24*, whereas, *Unl150, BE405711* and *BE591154* did not show any linkage with gene in question. All linked RGCs detected single locus in the region except *BE482366* which detected 2 loci. Both loci, *BE482366a* and *BE482366b*, are distal to the gene at 3.8 cM and 14.7 cM respectively. *Unl132, BE499910* and *BE607045* are all proximal to the gene at 14.4 cM, 33.3 cM and 40.7 cM respectively.

*H24* flanking markers, *Unl132* and *BE482366a*, are located distal to the breakpoint of CS deletion 3DL-3-FL0.81 (Dilbirligi and Gill 2003). This means *H24* is located in the distal
19% of long arm of chromosome 3D. Ma et al. (1993) mapped \( H24 \) 5.9 cM proximal to markers \( XcnlBCD451 \) and \( XcnlCDO482 \) which are also located distal to breakpoint of CS deletion 3DL-3-FL0.81 on long arm of chromosome 3D (Dilbriligi et al. 2006). Due to lack of proximal linked marker, these authors were unable to locate \( H24 \) precisely.

Like \( H13 \) and \( H22 \), \( H24 \) is also present in a distal cluster along with other Hessian fly resistance genes. \( H26 \) (Wang et al. 2006) and \( H32 \) (Sardesai et al. 2005) have also been mapped to distal 19% of long arm of chromosome 3D. Leaf rust resistance gene \( Lr24 \) and stem rust resistance gene \( Sr24 \) are also reported to be present in the distal region of long arm of chromosome 3D (Boyko et al. 1999).

RGCs found linked to \( H24 \) (\( BE607045 \), \( Unl132 \), \( BE482366 \), and \( BE499910 \)) show >40% sequence similarity to \( Hm1 \) or NB/LRR type resistance genes (Table 4.8). RGCs found linked with \( H24 \) can be useful for cloning of the gene or finding other closely linked markers for marker-assisted breeding. More importantly, unlike previous studies (Ma et al. 1993) which only found distal markers, we were able to find markers proximal and distal to \( H24 \), a prerequisite for isolation of genes by positional cloning.

4.4.2.5 Linkage analysis of RGCs mapping on long arm of chromosome 4AL with \( H25 \)

\( H25 \) conditions resistance to biotypes Great Plains `GP’ and biotypes A through L of the Hessian fly (Friebe et al. 1991; Friebe et al. 1990; Mukai et al. 1993; Ratcliffe and Hatchett 1997). This gene was derived from the rye (\( Secale cereale \)) cultivar Balbo and was transferred to wheat by radiation treatment. In the germplasm TA5033, \( H25 \) is present on an interstitial rye segment in the 4AL arm of the wheat-rye translocation chromosome Ti4AS·4AL-6R#1L-4AL (Friebe et al. 1991). Currently there are no published reports about linked molecular markers for
this gene. Markers linked to \textit{H25} will be useful in genetic mapping, MAS breeding and ultimately cloning of the gene.

RGCs \textit{Unl124}, \textit{Unl129}, \textit{Unl163}, \textit{Unl164}, \textit{Unl181}, \textit{BE201403}, \textit{BE444404} and \textit{BE497251} mapping on long arm of homoeologous group 4 (Dilbirli and Gill 2003) were chosen for linkage analysis with \textit{H25}. These 8 RGCs detected 50 bands each in CS, Zak and TA5033 (\textit{H25}). Using nullisomic-tetrasomic CS lines for homoeologous group 4, 25 of 50 bands were mapped to homoeologous chromosomes of group 4; 17 bands on chromosome 4A, three on chromosome 4B and five on chromosome 4D (Table 4.8). \textit{BE201403}, \textit{BE444404} and \textit{Unl181} detected bands on one homoeologue, whereas \textit{Unl124}, \textit{Unl129}, \textit{Unl163}, \textit{Unl164} and \textit{BE497251} detected bands on multiple homoeologues.

Restriction enzymes \textit{DraI}, \textit{EcoRI}, \textit{EcoRV}, \textit{HindIII} and \textit{ScaI} were used for polymorphism survey between the two parents (Table 4.8). All RGCs except \textit{Unl181} detected a total of 13 polymorphic bands between the parents and number of polymorphic bands per RGCs ranged from one to three. \textit{DraI} detected chromosome 4A-specific polymorphism in 6 of 8 RGCs. As \textit{H25} is reported to be present on chromosome 4A, restriction enzymes detecting chromosome 4A-specific polymorphism were preferred for gel blot analysis.

A linkage map of \textit{H25} and linked RGCs is given in figure 4.6. Among eight RGCs, only two, \textit{Unl163} and \textit{BE497251}, were found linked to \textit{H25}. Each of these linked RGCs detected a single locus on chromosome 4A. All the fragment bands linked to \textit{H25} mapped on chromosome 4A by nulli-tetra analysis as these bands were missing in N4AT4D but present in N4BT4A and N4DT4B (data not shown). Both of these linked RGCs are distal to \textit{H25} as they are located in the distal 15\% of chromosome 4AL, whereas \textit{H25} is located on interstitial rye segment which is approximately located in the middle of the arm (Friebe et al. 1991). \textit{Unl163} is located between
deletion breakpoints 4DL - 12 (FL = 0.71) - 4DL-14 (FL = 0.86) and BE497251 is located distal to breakpoint 4AL-1 (FL = 0.85) (Dilbirligi, 2004). Unl163 is located at 6.7 cM from H25, whereas BE497251 is located at 21.1 cM from the gene (Figure 4.6). RGCs BE201403, BE44404, Unl124 and Unl129 did not show any linkage with H25 even though they have been previously physically mapped to distal 15% of group 4 (Dilbirligi et al. 2004) and detected 4A specific polymorphism between resistant and susceptible parent in this study. The GRR 4L0.9 encompass distal 14% of group 4 and accounts for 68% of long arm’s recombination (Erayman et al. 2004). Our results suggest that linked marker BE497251 is physically closer to H25 and a recombination hot spot is present between this RGC and other unlinked RGCs physically mapped in this region.

In conclusion we have identified markers linked to H13, H22, H24 and H25 genes which can be useful in cloning of linked genes or finding other closely linked markers for marker-assisted selection. Our study confirmed that; H13 is located in a distal cluster on 6DS not 6DL, H25 is located on long arm of 4A. More importantly we have localized H22 to the GRR 1S0.8 (7Mb region) and H24 to distal 19% of long arm of chromosome 3D.

4.4.3 Leaf rust and stripe rust

4.4.3.1 Screening for leaf rust and stripe rust resistance genes

Common susceptible parent Zak is known to have moderate adult-plant resistance to leaf rust (Kidwell et al. 2002). However, there is no information available on the leaf rust resistance genes present in this cultivar. Zak and resistant lines TA4115 (Lr2a), TA4117 (Lr2c), TA3983 (Lr9), TA4123 (Lr11), TA3950 (Lr16), TA3854 (Lr17), TA4125 (Lr14a), TA5562 (Lr18) and TA3987 (Lr26) were checked with P. triticina races MBB, TLGF, TNRJ and TNTL to confirm
presence of resistance genes in resistant line (Table 4.2). Avirulence / virulence formulae of leaf and stripe rust races are given in table 4.5.

Zak was found to be susceptible to all the leaf rust races used in this study indicating this cultivar dose not have Lr2a, Lr2c, Lr9, Lr10, Lr11, Lr14a, Lr16, Lr17, Lr18, Lr24, Lr26 and Lr30. Resistant lines TA4123 (Lr11), TA4125 (Lr14a) and TA5562 (Lr18) showed susceptible reaction suggesting absence of Lr gene in question. Resistant lines carrying Lr2a, Lr2c, Lr9, Lr17 showed resistant reaction consistent with reported by Long and Kolmer (1989) (Figures 4.10, 4.11 and Table 4.2). Resistant lines carrying Lr16 and Lr26 did express resistant reaction to leaf rust races but the infection type was higher (relatively less resistant) than reported (Figures 4.11, 4.12 and 4.13). This could be due to a different genetic background of resistant lines (Lr16 is in cultivar Exchange background and Lr26 is in Kavkaz) in our study as compared to Long and Kolmer (1989) where these genes were in cv. Thatcher background. Infection type, for an Lr gene and rust race combination, can vary depending on the genetic background of the cultivar. The Lr2 alleles and Lr3 gene express most resistance in the Thatcher background and least resistance in Red Bobs (Dyck and Samborski 1974). Pretorius et al. (1990) reported similar effects on the expression of Lr22a. Six resistant lines showing expected resistant reaction were used for developing mapping populations (Table 4.9).

Race MBB, avirulent to Lr2a, Lr2c, Lr9, Lr16, Lr17, was used for screening the populations of crosses ZakXTA4115 (Lr2a), ZakXTA4117 (Lr2c), ZakXTA3983 (Lr9), ZakXTA3950 (Lr16) and ZakXTA3854 (Lr17). Race TNRJ, avirulent to Lr26, was used for screening population from cross ZakX3987 (Lr26). All leaf rust resistance gene F2 populations, except Lr26, showed 1: 2: 1 single gene segregation (Table 4.9) which is consistent with the previous observations (Long and Kolmer 1989). The $\chi^2$ ($df = 2, P = 0.05$) value for Lr2a, Lr2c,
Lr9, Lr16 and Lr17 was 0.33, 0.48, 0.63, 2.13, 0.25 respectively. Lr26 F2 populations did not show single gene segregation for resistance to leaf rust. Number of homozygous susceptible plants was more than expected and number of homozygous resistance plants was less than expected. In earlier reports Lr26 based resistance has been shown to be controlled by single gene in both the cases (McIntosh et al. 1983). One of the reasons for this observation could be the presence of resistance suppressors in susceptible parent Zak’s background. In a previous study, Bai and Knott (1992) reported D-genome based resistance suppressors for 10 leaf rust resistance genes. McIntosh and Dyck (1972) found that Thatcher has a gene that inhibited the expression of Lr23 when tested with P. triticina isolates from Canada and partially inhibited the resistance when tested with isolates from Australia.

Zak is reported to be susceptible to stripe rust race PST-78 indicating that it dose not have resistance gene Yr10 (Chen XM personal communication). Our screening results agreed with this observation. Zak displayed susceptible phenotype (infection type >5) while Moro and F1 plants displayed resistance phenotype (infection type 1) to stripe rust race PST-78. Our infection type data for Yr10 agrees with that of Macer (1975). Yr10 F2 population phenotype ratio showed single gene segregation (Table 4.9). The $\chi^2$ ($df = 2, P = 0.05$) value was 0.08. This is consistent with reports of Chen (2005).

4.4.3.2 Linkage analysis of RGCs mapping in the distal region of long arm of chromosome 6B with Lr9

The Aegilops umbellulata derived Lr9 gene, located on chromosome 6BL, has been reported to be an effective gene in wheat breeding (McIntosh 1995; Murphy et al. 2004; Schachermayr et al. 1994). RGCs BE403251, BE405507, BE426789, BE426790, BE500158,
BE638124, BF199778, BM137173, Unl154 and Unl194 mapping in the distal region of chromosome 6BL (Dilbirligi et al. 2004) were selected for linkage analysis with \textit{Lr9}.

Nine of ten RGCs detected 57, 59 and 55 bands in CS, susceptible parent Zak and resistant parent TA3983 respectively (Table 4.6). \textit{BM137173} could not be mapped due to high background. Using nullisomic-tetrasomic CS lines for homoeologous group 6, 43 of 57 bands were mapped to homoeologous chromosomes of group 6; nine bands on chromosome 6A, 21 on chromosome 6B and 13 on chromosome 6D (Table 4.6).

Restriction enzymes \textit{BamHI, DraI, EcoRI, EcoRV, HindIII, SacI} and \textit{XbaI} were used for polymorphism survey between the two parents (Table 4.6). All RGCs except \textit{Unl194} detected 19 polymorphic bands between susceptible and resistant parent with \textit{DraI, EcoRV and HindIII}. \textit{DraI} detected polymorphism for four RGCs, whereas \textit{EcoRV} and \textit{HindIII} detected polymorphism in three and one RGCs respectively. As \textit{Lr9} gene has been mapped on chromosome 6BL previously (Murphy et al. 2004), enzymes giving 6B specific polymorphism were used for gel blot analysis.

A 6BL map around the \textit{Lr9} is given in figure 4.7. Five RGCs, \textit{Unl154, BE403251, BE426789, BE426790} and \textit{BE500158} were found linked to \textit{Lr9}. All the RGCs linked to \textit{Lr9} detected single locus except for \textit{Unl154} and \textit{BE426790} that detected two loci each. All the fragment bands linked to \textit{Lr9} mapped on chromosome 6B by nulli-tetra analysis as these bands were missing in N6BT6A but present in N6AT6D and N6DT6B (data not shown). \textit{Lr9} is flanked by marker loci \textit{Unl154a} and \textit{BE426789} at 12.4 cM and 21.6 cM respectively. Both of these RGCs are located in the GRR 6L0.9 (Dilbirligi et al. 2004) meaning \textit{Lr9} is located in this GRR. The mapping location is consistent with earlier reports (McIntosh 1995; Murphy et al. 2004; Schachermayr et al. 1994). RAPD markers \textit{Xpsr546} and \textit{Xcmwg684}, located in the GRR.
6L0.9 (Erayman et al. 2004), have been reported to be linked to Lr9 at 8 ± 2.4 cM and 0 cM respectively (Schachermayr et al. 1994). This GRR is about 45Mb in size and with 72% of long arm’s recombination it is the 3rd highest in recombination after GRRs 1S0.8 and 6S1.0 (Erayman et al. 2004). Dilbirligi et al. (2004) mapped Lr3, Lr38, Sr11, Sr13, Sr26 and Sr29 in the GRR 6L0.9 via comparative mapping. All of the RGCs mapping in this region show 40 – 60% similarity to NBS-LRR class of resistance genes and thus can be good candidates or markers for the gene in the region.

4.4.3.3 Linkage analysis of RGCs mapping in the distal region of short arm of group 2 chromosomes with Lr2, Lr16 and Lr17

Leaf rust resistance genes Lr2, Lr16 and Lr17 provide race-specific resistance to P. triticina at seedling stage. These genes, along with others, have been extensively used in breeding programs around the world (Kolmer 1996). The Lr2 locus in wheat has three alleles, Lr2a, Lr2b and Lr2c. Lr2 was first mapped to chromosome 1B using monosomic analysis (Soliman et al. 1964) and later re-assigned to short arm of chromosome 2D (Luig and McIntosh 1968; McIntosh and Baker 1968). Lr17 locus has two alleles, Lr17a and Lr17b, and is located on short arm of chromosome 2A (Dyck and Kerber 1977; Singh et al. 2001). Lr16 was originally assigned to chromosome 4B using the Rescue monosomic series (Dyck and Kerber 1971) and was later re-assigned to chromosome 2BS with CS (McIntosh et al. 1998). Rescue and CS differ by a 2B–4B translocation (McIntosh, personal communication). Dilbirligi et al. (2004) localized Lr2, Lr16 and Lr17 to the distal region on short arm of group 2 chromosomes with comparative mapping. Eight RGCs (Table 4.6), mapping in the distal region of short arm of...
group 2 chromosomes (Dilbirligi et al. 2004), were selected for linkage analysis with \textit{Lr2a}, \textit{Lr2c}, \textit{Lr16} and \textit{Lr17}.

RGCs \textit{Unl145}, \textit{Unl216}, \textit{BE442854}, \textit{BE442802}, \textit{BE497251}, \textit{BE499523}, \textit{BF200166} and \textit{BF484266} detected 40, 39 and 40 bands in CS, susceptible parent Zak and resistant parent TA4115 (\textit{Lr2a}) respectively. The NT analysis with CS lines localized 22 of 40 bands; four to chromosome 2A, 14 to chromosome 2B and four to chromosome 2D (Table 4.6). Except \textit{BE638124} all RGCs detected bands on more than one homoeologue.

Restriction enzymes \textit{BamHI}, \textit{DraI}, \textit{EcoRI}, \textit{EcoRV}, \textit{HindIII}, \textit{ScaI} and \textit{XbaI} were used for polymorphism survey between susceptible parent Zak and resistant line TA4115 (\textit{Lr2a}). All RGCs except \textit{BF200166} and \textit{BF484266} detected 13 polymorphic bands between susceptible and resistant parent with \textit{DraI}, \textit{EcoRI}, \textit{EcoRV} and \textit{XbaI} (Table 4.6), however, chromosome 2D specific polymorphism was found only in case of \textit{Unl154}, \textit{Unl216} and \textit{BE497251}. As \textit{Lr2a} was mapped to chromosome 2D previously (Luig and McIntosh 1968; McIntosh and Baker 1968), enzymes giving chromosome 2D specific polymorphism were used for gel blot analysis.

In \textit{Lr2c} mapping study, RGCs \textit{Unl145}, \textit{Unl216}, \textit{BE442854}, \textit{BE442802}, \textit{BE497251}, \textit{BE499523}, \textit{BF200166} and \textit{BF484266} detected 53, 54 and 52 bands in CS, susceptible parent Zak and resistant parent TA4117 (\textit{Lr2c}) respectively (Table 4.6). The NT analysis with CS lines localized 27 of 53 bands; three to chromosome 2A, 18 to chromosome 2B and six to chromosome 2D (Table 4.6). Except \textit{BE499523} and \textit{BF200166}, all RGCs detected bands on more than one homoeologue.

Restriction enzymes \textit{BamHI}, \textit{DraI}, \textit{EcoRI}, \textit{EcoRV}, \textit{HindIII}, \textit{ScaI} and \textit{XbaI} were used for polymorphism survey between susceptible parent Zak and resistant line TA4117 (\textit{Lr2c}). Among eight RGCs used in this study, \textit{BE442854}, \textit{BE497251}, \textit{Unl145} and \textit{Unl216} detected
polymorphism between susceptible and resistant parent with \textit{DraI}, \textit{EcoRV} and \textit{HindIII} (Table 4.6). As gene \textit{Lr2c} has been mapped to chromosome 2D (Luig and McIntosh 1968; McIntosh and Baker 1968) restriction enzymes giving chromosome 2D specific polymorphism were used for gel blot analysis.

In \textit{Lr16} mapping study, \textit{Unl216}, \textit{BE442854}, \textit{BE442802}, \textit{BE497251}, \textit{BE499523}, \textit{BF200166} and \textit{BF484266} detected 52, 53 and 52 bands in CS, susceptible parent Zak and resistant parent TA3950 (\textit{Lr16}) respectively (Table 4.6). \textit{Unl154} could not be mapped due high background. The NT analysis with CS lines localized 26 of 52 bands; five to chromosome 2A, 16 to chromosome 2B and five to chromosome 2D (Table 4.6). Except \textit{BE499523} all RGCs detected bands on more than one homoeologues.

Restriction enzymes \textit{BamHI}, \textit{DraI}, \textit{EcoRI}, \textit{EcoRV}, \textit{HindIII}, \textit{SacI} and \textit{XbaI} were used for polymorphism survey between susceptible parent Zak and resistant line TA3950 (\textit{Lr16}). All RGCs detected a total of 16 polymorphic bands between susceptible and resistant parent with \textit{DraI} and \textit{HindIII} (Table 4.6). \textit{DraI} detected 2B-specific polymorphism for \textit{Unl216}, \textit{BE442802}, \textit{BE497251}, \textit{BF200166}, whereas \textit{HindIII} detected 2B-specific polymorphism for \textit{BE442854}, \textit{BE499523} and \textit{BF484266}. As \textit{Lr16} has been mapped to chromosome 2BS (McIntosh et al. 1998) restriction enzymes giving chromosome 2B specific polymorphism were used for gel blot analysis.

In \textit{Lr17} mapping study, \textit{Unl145}, \textit{Unl216}, \textit{BE442854}, \textit{BE442802}, \textit{BE497251}, \textit{BF200166} and \textit{BF484266} detected 32, 35 and 36 bands in CS, Zak and resistant parent TA3854 (\textit{Lr17}) respectively (Table 4.6). The NT analysis with CS lines localized 18 of 32 bands; four to chromosome 2A, nine to chromosome 2B and five to chromosome 2D (Table 4.6). \textit{BE499523}
could not be mapped due high background. Except BE442854 all RGCs detected bands on more than one homoeologues.

Restriction enzymes BamHI, BglII, DraI, EcoRI, EcoRV, HindIII, NcoI, SacI, Scal and XbaI were used for polymorphism survey between susceptible parent Zak and resistant line TA3854 (Lr17). All RGCs except BE442802 and BE499523 detected a total of 11 polymorphic bands between susceptible and resistant parent, however, only Unl145 and Unl216 detected 2A specific polymorphism (Table 4.6). As Lr17 is reported to map on chromosome 2A (Dyck and Samborski 1968), restriction enzyme giving 2A specific polymorphism were used for gel blot analysis.

None of the RGCs were found linked to Lr2a or Lr2c. This could be due to low level of chromosome 2D specific polymorphism between resistant and susceptible parents for RGCs. Only one RGC in case of Lr2a and three RGCs in case of Lr2c, detected polymorphism specific to chromosome 2D where both alleles of Lr2 locus are mapped (Table 4.6). It is also possible that this Lr2 locus is not present on chromosome 2D. Originally this locus was mapped to 1B (Soliman et al. 1964) and later re-assigned to 2D (Luig and McIntosh 1968; McIntosh and Baker 1968). There is a need to confirm the mapping location of Lr2 locus by using more marker from chromosome 2D and also from 1B.

Similarly none of the RGCs detected linkage with Lr17. This was expected observation as Lr17 is mapped to chromosome 2A (Dyck and Samborski 1968) and we could not find chromosome 2A specific polymorphism between susceptible and resistant parent despite using up to 12 restriction enzymes. It is a known fact that hexaploid bread wheat shows relatively low levels of polymorphism for RFLP loci, most likely as a result of its narrow genetic base (Chao et al. 1989). Usually <10% of all RFLP loci are polymorphic in an intraspecific context (Roder et
Conversion of RGCs to other marker types, with more intraspecific polymorphism, may be required to truly understand their linkage relationship with resistance genes, especially *Lr2* and *Lr17*.

A 2BS map around *Lr16* is given in figure 4.8. Of seven RGCs, *Unl216*, *BE499523*, *BE442854* and *BE497251* were found linked to *Lr16* within 59.6 cM on short arm of chromosome 2B. All RGCs linked to the *Lr16* gene detected a single locus on 2B except for *BE442854* that detected two loci. All the fragment bands linked to *Lr16* mapped on chromosome 2B by nulli-tetra analysis as these bands were missing in N2BT2D but present in N2AT2B and N2DT2A (data not shown). Direction of centromere is inferred form the fact that *Unl145* is physically mapped proximal to *Unl216* (Dilbirligi et al. 2004) and these markers are tightly linked at 1.5 cM (from *Lr2a* population linkage analysis in this study). *Unl216*, *BE499523*, *BE442854a* and *BE442854b* are proximal to the gene while *BE497251* is located distally. *BE442854* and *BE497251* flank *Lr16* at 8.6 cM and 24.8 cM respectively. As all the linked RGCs have been previously physically mapped in the GRR 2S0.9 (Dilbirligi et al. 2004), this suggests that *Lr16* is located within this distal GRR on 2BS. This map location is consistent with other reports based on linkage with other genes on 2BS (McIntosh et al. 1998).

McCartney et al. (2005) showed tight linkage of microsatellite marker loci *Xwmc764* to *Lr16* in the distal region of 2BS, where, *Xwmc764* was mapped at 1 cM, 3 cM and 9 cM from *Lr9* in three different populations. These authors were unable to find a distal linked marker for *Lr16*. A distal marker will be useful for marker-assisted selection of *Lr16* in crosses where the recombination rate between *Lr16* and *Xwmc764* is relatively high. Distal marker *BE497251* found in our study may not be too useful as it located at 24.8 cM distal from gene but it can be useful in finding other closely linked markers that are distal to the gene.
4.4.3.4 Linkage analysis of 1S0.8-specific RGCs with Yr10

Stripe rust resistance gene *Yr10* was originally found in Turkish wheat line PI 178383. *Yr10* has been mapped to short arm of chromosome 1B, 2.0 ± 0.3 cM from *Rg1* controlling brown glume colour (Metzger and Silbaugh, 1970), and 5.0 ± 2.2 cM from *Gli-1B* (Payne et al. 1986) which codes for gliadin proteins. Since *Yr10* was mapped on short arm of chromosome 1B, ten RGCs (Table 4.6) mapping in the GRR 1S0.8 (Dilbirligi et al. 2004) on short arm of group one chromosomes were used for linkage analysis with *Yr10*. The RGCs were *BE492937*, *BE405778*, *BF474204*, *BE499561*, *BE498831*, *BF475048*, *BF483881*, *BG606866*, *BE518223* and *BM135313*. These ten RGCs detected 71, 69 and 70 bands in CS, susceptible parent Zak and resistant parent Moro respectively (Table 4.6). The NT analysis with CS lines localized 44 of 71 bands; 18 to chromosome 1A, 17 to chromosome 1B and nine to chromosome 1D (Table 4.6). All the RGCs except *BE499561* detected bands on more than one homoeologue.

Restriction enzymes *BglII, DraI, EcoRI, EcoRV, HindIII* and *XbaI* were used for polymorphism survey between the two parents (Table 4.6). All RGCs except *BE499561* detected a total of 23 polymorphic bands between susceptible and resistant parent. Restriction enzymes *HindIII, EcoRI* and *DraI* detected chromosome 1B specific polymorphism for two, three and three RGCs respectively. *XbaI* detected polymorphism in only one case. As *Yr10* has been mapped on chromosome 1BS (Metzger and Silbaugh, 1970) enzymes giving 1BS specific polymorphism were used in gel blot analysis.

A 1BS map around *Yr10* is given in Figure 4.9. Of ten, five RGCs clustered around *Yr10* in 25.1cM on short arm of chromosome 1B. All RGCs linked to *Yr10* detected one locus on chromosome 1BS. All the fragment bands linked to *Yr10* mapped on chromosome 1B by nulli-
tetra analysis as these bands were missing in N1BT1A but present in N1AT1B and N1DT1B (data not shown). Direction of centromere was decided by comparing markers order with \textit{Pm3a} map (Figure 4.1). RGCs \textit{BE405778}, \textit{BF475048} and \textit{BF474204} were common between the genetic maps of \textit{Yr10} and \textit{Pm3a}. By comparison of these two maps, \textit{BE492937}, \textit{BE498831}, \textit{BF475048} and \textit{BE405778} mapped distally to \textit{Yr10}, whereas \textit{BE474204} mapped proximal to the gene. \textit{BE492937}, \textit{BE474204}, \textit{BE498831}, \textit{BF475048} and \textit{BE405778} map 4.7 cM, 5.6 cM, 9.5 cM, 11.1 cM and 19.5 cM respectively from \textit{Yr10}. These five RGCs physically map between deletion breakpoints 1BS-4/1BL-19 (FL value = 0.54) and 1BS-18 (FL value = 0.50) in the GRR 1S0.8 on short arm of chromosome 1B (Dilbirligi et al. 2004). Metzger and Silbaugh (1970) mapped \textit{Yr10} 2.0 ± 0.3 cM from \textit{Rg1} locus controlling brown glume color. Payne et al. (1986) mapped \textit{Yr10} at a distance of 5.0 ± 2.2 cM from \textit{Gli-1B}, a locus that codes for gliadin proteins. Both \textit{Rg1} and \textit{Gli-1B} map in the GRR 1S0.8 on short arm of chromosome 1B (Erayman et al. 2004). Wang et al. (2002) reported microsatellite marker \textit{Xpsp3000}, located on the end of chromosome 1BS, as linked to \textit{Yr10} at a distance 1.2 cM. Mapping order of RGCs in \textit{Yr10} map is consistent with the map of powdery mildew gene \textit{Pm3a} that also map in the GRR 1S0.8 (Bennypaul and Gill, this study).

\textit{Yr10} and linked RGCs are located in a ≈ 7Mb size GRR (1S0.8) which also contains agronomically important genes, including 12 genes conferring resistance to leaf rust (\textit{Lr10}, \textit{Lr21}, \textit{Lr26}), stem rust (\textit{Sr31}, \textit{Sr33}), powdery mildew (\textit{Pm3}, \textit{Pm8}) and stripe rust (\textit{Yr9}, \textit{Yr10vav}, \textit{Yr15}) (Sandhu et al. 2001). A cluster of Hessian fly resistance genes containing \textit{H9}, \textit{H10}, \textit{H11} and \textit{Hdic} was also mapped to the distal region on chromosome 1AS (Liu et al. 2005b). RGCs found linked to \textit{Yr10} will be useful in marker-assisted breeding and cloning of the \textit{Yr10} and other genes present in this distal cluster on short arm of chromosome 1B.
4.5 Resistance Gene-Like Sequences: Gene candidates or?

Many genes including \( R \) genes have been cloned by targeting gene candidates in plants e.g. \( bmr \) ((Bout and Vermerris 2003; Feuillet et al. 1997; Li et al. 2006; Sawers et al. 2006) and \( Rp1-D \) (Collins et al. 2001). Resistance gene-like sequences (RGLs), showing sequence similarity with motifs/domains of cloned resistance genes, represent gene candidates for resistance genes. An initial step towards the isolation of plant disease resistance genes is the linkage analysis for finding the candidates that cosegregate with known resistance loci.

Madsen et al (2003) mapped 30 RGAs belonging to 10 families in barley. Of 30, two RGAs mapped in close proximity to resistance genes \( rpg4, \, RPG1, \, Rh2, \, mlt \) and one RGA was found tightly linked to \( Ha2 \) gene at 2.7 cM. Yao et al. (2007) did not find tight linkage between RGs \( BE405003 \) (9.3 cM), \( BE405531 \) (5.5 cM), \( BQ171700 \) (23.9 cM) and \( BE405507 \) (no linkage) and two powdery mildew resistance genes, \( Mlm80 \) and \( Mlm2033 \), mapping in the same wheat GRR (7L1.0). In a recent study, McFadden et al. (2006) mapped 49 NBS-LRR RGA-ESTs on 21 wheat chromosomes and none of the RGA-ESTs showed co-segregation with resistance gene (\( Yr7/Sr9, \, Sr30 \)) segregating in this population.

In the current study, 47 RGCs were analysed for linkage with phenotypically characterized wheat resistance genes. 26 RGCs were found linked to nine resistance genes and the closest RGC mapped 3.8 cM from the resistance gene. Of 21 RGCs that did not show linkage to genes studied, 10 did not detect loci on the chromosome (where gene under study is mapped) or polymorphism between the resistant and susceptible parent. Of 26 RGCs showing linkage with resistance genes, 19 shows 40 – 60% sequence similarity with motifs/domains of cloned resistance genes and five show more than 60% sequence similarity with motifs/domains of cloned resistance genes. In addition to sharing sequence similarity, these RGCs have also
been mapped physically (Dilbirligi et al. 2004) and genetically (this study) in the vicinity of known resistance genes.

Despite sequence and mapping location similarity, none of the tested RGCs showed perfect linkage to the genes in this study. This could be partly due to the fact that not all the bands detected by a candidate could be scored due to absence of relevant polymorphism (chromosome specific polymorphism where gene in question is mapped). It is a known fact that hexaploid bread wheat shows relatively low levels of polymorphism for RFLP loci, most likely as a result of its narrow genetic base (Chao et al. 1989). Usually <10% of all RFLP loci are polymorphic in an intraspecific context (Roder et al. 1998).

It is also possible that these RGCs are not the candidates for any of the nine genes studied. This could be true considering that many cloned resistance genes are expressed at very low level (Hulbert SH 2001) and thus may be out-competed during amplification of RGCs. Like many other cloned resistance gene, ESTs of powdery mildew gene Pm3b from wheat and stem rust resistance gene RPG1 from barley are not present in EST databases (Dr. Beat Keller and R. Brueggeman, personal communication). We also checked sequence of RGCs against cloned resistance genes Lr21, Pm3b and Lr10 from 1S0.8 GRR of wheat genome. None of RGCs, except BF474204, showed any sequence similarity to these resistance genes. BF474204 shares 78% (e value = 5e-78) sequence similarity with Lr21.

In a number of species, resistance genes have been shown to cluster in a multiallelic structure or as genetically separable loci. Examples of such complex resistance loci are found in flax (Ellis et al. 1995), lettuce (Witsenboer et al. 1995), barley (Jorgensen 1992), and common bean (Geffroy et al. 1999). In wheat, resistance gene clusters have also been reported on various chromosomes, for example, a cluster of 14 resistance genes exist in the GRR 1S0.8 on short arm
of group one. This cluster includes resistance genes giving resistance to biotypes/races of Hessian fly, leaf rust, stripe rust and powdery mildew (Liu et al. 2005b; Sandhu et al. 2001). Majority of RGCs analysed in this study map in the regions where clusters of resistance genes have been reported (Dilbirligi et al. 2004), for example, 42 resistance genes map in 6 regions in the proximity of analysed RGCs. Linkage relationship of only nine of 42 genes have been investigated in this study.

These RGCs have the potential to be the candidate genes for other genes mapping in the region. \textit{Rps1} locus in soybean is known to have five functional resistance genes, \textit{Rps1-a}, -\textit{b}, -\textit{c}, -\textit{d}, and -\textit{k}, for resistance against various biotypes of \textit{Phytophthora sojae} (Schmitthenner 1989). This locus has recently been reported to have 38 copies of CC-NBS-LRR type expressed resistance gene-like sequences which are presumed to play role in the evolution of new resistance gene specificities (Bhattacharya et al. 2005). A similar role for RGCs of wheat could also be possible.
Figure 4.1 Genetic linkage map of GER 1S9 8-specific resistance gene candidates and powdery mildew resistance gene Pm3a of wheat. The deletion line breakpoints and FL (fraction length) of the retained arm is marked by an arrow.
Figure 4.2 Genetic linkage map GRR 6L.0.9-specific resistance gene candidates and powdery mildew resistance gene 
Pm20 of wheat. GRR 6L.0.9 is located in the distal region of long arm of chromosome 6R.
Figure 4.3 Genomic linkage map of GRR 180.3-specific resistance gene candidates and Hessian fly resistance gene H22 of wheat. The deletion line breakpoints and FL (fraction length) of the retained arm is marked by arrows.
Figure 4.4 Genetic linkage map of 6DS-specific resistance gene candidates and Hessian fly resistance gene H13 of wheat. The deletion line breakpoints and FL (fraction length) of the retained arm is marked by arrows.
Figure 4.5 Genetic linkage map of 3DL-specific resistance gene candidates and Hessian fly resistance gene H24 of wheat. The deletion line breakpoints and FL (fraction length) of the retained arm is marked by an arrow.
Figure 4.6 Genetic linkage map of 4AL-specific resistance gene candidates and Hessian fly resistance gene H23 of wheat. The deletion line breakpoints and FL (fraction length) of the retained arm is marked by an arrow.
Figure 4.7 Genomic linkage map of 6BL-specific resistance gene candidates and leaf rust resistance gene \( Lr9 \) of wheat.
Figure 4.8 Genetic linkage map of GRR 280.9-specific resistance gene candidates and leaf rust resistance gene \textit{Lr16} of wheat. The deletion line breakpoints and FL (fraction length) of the retained arm is marked by arrows.
Figure 4.9 Genetic linkage map of GRR 1S0.8-specific resistance gene candidates and stripe rust resistance gene Yr10 of wheat. The deletion line breakpoints and FL (fraction length) of the retained arm is marked by arrows.
Figure 4.10 Leaf rust reaction of Zak (1, 4), TA4115 (3), TA4115 (2), $F_1$ – Zak × TA4115 (5) and $F_1$ – Zak × TA4117 (6)
to MB2 race of *Puccinia tritici*. Zak is the susceptible parent and TA4115 and TA4117 carry genes $Lr2a$ and
$Lr2c$ respectively.
Figure 4.11 Leaf rust reaction of Zak (1), TA3854 (2), TA3950 (3) and TA3983 (4) to MBB race of *Fusarium triticum*. Zak is the susceptible parent and TA3854, TA3950 and TA3983 carry *Lr17*, *Lr16* and *Lr9* genes respectively.
Figure 4.12 Leaf rust reaction of Zak (1), TA3987 (2), F₁ – ZakX3987 (3) to TNRJ race of *Puccinia triticina*. Zak is the susceptible parent and TA3987 carry *Lr26* resistance gene.
Figure 4.13 Disease reaction of Zak (1), TA3987 (2), TA4123 (3), TA5562 (4) to TNBJ race of leaf rust
(*Puccinia triticina*). TA3987, TA4123 and TA5562 are reported to carry *Lr26*, *Lr11* and *Lr18* respectively.
Table 4.1 Resistance gene candidates and Hessian fly resistance genes mapping in or near gene-rich regions of wheat

<table>
<thead>
<tr>
<th>Gene-rich region&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Chromosome group</th>
<th>Hessain fly resistance gene(s) in or around the GRR&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Resistance gene candidates in the Gene-rich region&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1S0.8</td>
<td>1</td>
<td>$H3^<em>, H5, H6^</em>, H9^{<em>'}, H10^{</em>'}, H11^{†}$, $H15^*, H22^{†'}$ and $Hdic^{†'}$</td>
<td>$BE492937, BE405778, BF474204$ $BE499561, BE498831, BF475048$ $BF483881, BG606866, BM135313$ $BE518223, Unl143$</td>
</tr>
<tr>
<td>3L0.9</td>
<td>3</td>
<td>$H24, H26^*$ and $H32$</td>
<td>$BE405711, BE499910, BF482366$ $BE591154, Unl132, Unl130$</td>
</tr>
<tr>
<td>4L0.9</td>
<td>4</td>
<td>$H25$ and $H26^*$</td>
<td>$BF201403, BE444404, BE497251$ $Unl124, Unl163, Unl129$</td>
</tr>
<tr>
<td>6S1.0</td>
<td>6</td>
<td>$H13^{<em>'}$ and $H23^</em>$</td>
<td>$BF478914, BG607637, BF200008$ $Unl190$</td>
</tr>
<tr>
<td>6L0.9</td>
<td>6</td>
<td>$H13^<em>, H23^</em> and H24$</td>
<td>$BE500158, BF199778, BE403251$ $BE426789, BE426790, BM137173$ $BE638124, BE405507, Unl154$ $Unl194$</td>
</tr>
</tbody>
</table>

* = multiple genomic locations reported
† = mapping in the gene-rich region
<sup>a</sup> = According to Erayman et al. 2004
<sup>b</sup> = McIntosh et al. 2007
<sup>c</sup> = according to Dilbirligi et al. 2003
Table 4.2 Infection types produced by various cultivars or accession used for development of mapping populations of leaf rust and stripe rust resistance genes

<table>
<thead>
<tr>
<th>Cultivar/Accession&lt;sup&gt;a&lt;/sup&gt; (Lr/Yr gene)</th>
<th>Leaf or stripe rust race (s)</th>
<th>Infection type observed</th>
<th>Infection type Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zak (-)</td>
<td>MBB, TNRJ, TLGF, TNTL</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>TA4115 (Lr2a)</td>
<td>MBB</td>
<td>;</td>
<td>;</td>
</tr>
<tr>
<td>TA4117 (Lr2c)</td>
<td>MBB</td>
<td>;1</td>
<td>;1</td>
</tr>
<tr>
<td>TA3983 (Lr9)</td>
<td>MBB</td>
<td>0;</td>
<td>0;</td>
</tr>
<tr>
<td>TA4123 (Lr11)</td>
<td>TNRJ</td>
<td>4</td>
<td>2;</td>
</tr>
<tr>
<td>TA4125 (Lr14a)</td>
<td>TNTL</td>
<td>4</td>
<td>;</td>
</tr>
<tr>
<td>TA3950 (Lr16)</td>
<td>MBB</td>
<td>2</td>
<td>;1N</td>
</tr>
<tr>
<td>TA3854 (Lr17)</td>
<td>MBB, TLGF</td>
<td>;12</td>
<td>;12</td>
</tr>
<tr>
<td>TA5562 (Lr18)</td>
<td>TNRJ</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>TA3987 (Lr26)</td>
<td>TNRJ, MBB</td>
<td>;1</td>
<td>;</td>
</tr>
<tr>
<td>Moro (Yr10)</td>
<td>PST-78</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Leaf rust infection types according to Long and Kolmer (1989): 0 = no uredinia or other sign of infection, ; = no uredinia but hypersensitive necrotic flecks present, 1 = small uredinia often surrounded by necrosis, 2 = small to medium uredinia often surrounded by necrosis, N = more necrosis than usual for infection type, 4 = large uredinia without chlorosis or necrosis. Stripe rust infection types according to McNeal and Konzak (1971): 0 = immune, 1 = necrotic flecks, 2 = necrotic flecks without sporulation, 3-4 = necrotic and chlorotic areas with restricted sporulation, 5-6 = moderate sporulation with necrosis and chlorosis, 7-8 = sporulation with chlorosis, 9 = abundant sporulation without chlorosis. <sup>a</sup> = Leaf rust/stripe rust gene present in line in parenthesis, - = not known.
Table 4.3 Reaction of Zak and resistant parents to *B. graminis* f. sp. *tritici* (Bgt) isolates

<table>
<thead>
<tr>
<th>Triticum accession (TA) / Cultivar</th>
<th>Powdery mildew resistance gene</th>
<th>Bgt isolate(s)</th>
<th>Disease reaction observed</th>
<th>Disease reaction expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zak</td>
<td>?</td>
<td>2, 5, 6, 9, 10, 13, 14, 15, 17, 94, 121, J12</td>
<td>Susceptible</td>
<td>Susceptible</td>
</tr>
<tr>
<td>TA3896</td>
<td><em>Pm1</em></td>
<td>2, 10,</td>
<td>Susceptible</td>
<td>Resistant</td>
</tr>
<tr>
<td>TA3896</td>
<td><em>Pm2</em></td>
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</tr>
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<td>Resistant</td>
</tr>
<tr>
<td>TA3893</td>
<td><em>Pm6</em></td>
<td>J12, 14</td>
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<td>Resistant</td>
</tr>
<tr>
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<td><em>Pm8</em></td>
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<td>TA5561</td>
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<td>Resistant</td>
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<tr>
<td>TA5041</td>
<td><em>Pm20</em></td>
<td>10, 13</td>
<td>Resistant</td>
<td>Resistant</td>
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Table 4.4 Resistance gene candidates and leaf rust, stripe rust and powdery mildew resistance genes in or near three gene rich regions of wheat

<table>
<thead>
<tr>
<th>GRR</th>
<th>Chromosome group</th>
<th>Leaf or stripe rust resistance gene in or around the GRR</th>
<th>Resistance gene candidates in the Gene-rich region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1S0.8</td>
<td>1</td>
<td>Lr10‡, Lr21†, Lr26†, Yr9‡, Yr10‡, Yr15‡, Pm3a, Pm8</td>
<td>BE492937, BE405778, BF474204 BE499561, BE498831, BF475048 BF483881, BG606866, BM135313, BE518223, Unl143</td>
</tr>
<tr>
<td>2S0.9</td>
<td>2</td>
<td>Lr2*, Lr15*, Lr16†, Lr17†, Lr22†, Lr23*, Lr28, Lr38†, Yr17†</td>
<td>BE442854, BE442802, BE499523 BE497251, BF200166, BF484266 Unl145, Unl216</td>
</tr>
<tr>
<td>6L0.9</td>
<td>6</td>
<td>Lr3, Lr9, Lr38, Pm20</td>
<td>BE500158, BF199778, BE403251 BE405507, BE426789, BE426790, BE638124, BM137173 Unl154, Unl194</td>
</tr>
</tbody>
</table>

a = Gene rich region  
* = multiple genomic locations reported  
† = mapping in the gene-rich region  
a = According to Erayman et al. 2004  
b = McIntosh et al. 2007  
c = according to Dilbirligi et al. 2003
Table 4.5 Avirulence / virulence formulae of leaf rust (*P. triticina*) and stripe rust (*P. striiformis f. sp. tritici*) races

<table>
<thead>
<tr>
<th>Rust Race</th>
<th>Avirulence⁵</th>
<th>Virulence⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBB</td>
<td><em>Lr2a, Lr2c, Lr9, Lr16, Lr24, Lr26, Lr3ka, Lr11, Lr17, Lr30</em></td>
<td><em>Lr1, Lr3a</em></td>
</tr>
<tr>
<td>TLGF</td>
<td><em>Lr10, Lr16, Lr24, Lr26, Lr3ka, Lr17, Lr30, LrB</em></td>
<td><em>Lr1, Lr2a, Lr2c, Lr3a, Lr9, Lr11, Lr14a, Lr18</em></td>
</tr>
<tr>
<td>TNRJ</td>
<td><em>Lr16, Lr26, Lr17, Lr18, LrB</em></td>
<td><em>Lr1, Lr2a, Lr2c, Lr3a, Lr9, Lr3ka, Lr24, Lr30, Lr10, Lr14a, Lr11</em></td>
</tr>
<tr>
<td>TNTL</td>
<td><em>Lr16, Lr26, Lr10, Lr14a, Lr18</em></td>
<td><em>Lr1, Lr2a, Lr2, Lr3a, Lr11, Lr3ka, LrB</em></td>
</tr>
<tr>
<td>PST-78</td>
<td>Chinese 166, Moro, Hyak, Stephens, Druchamp, Paha, Produra, Yamhill, Tyee, Tres</td>
<td>Fielder, Lee, Compair, Clement, Heines VII</td>
</tr>
</tbody>
</table>

All the races except PST-78 are leaf rust races. PST-78 is stripe rust race. *Lr* = leaf rust resistance gene, *Yr* = stripe rust resistance gene. ⁵ = Avirulence/virulence phenotype for 1) leaf rust races based on Long and Kolmer (1989) and 2) for stripe rust race based on Line and Qayoum (1991) and Chen et al. (2002).
Table 4.6 Number of loci, chromosomal location and polymorphism of resistance gene candidates mapping in the region of wheat genome containing leaf rust (*Lr2a, Lr2c, Lr9, Lr16, and Lr17*) and stripe rust (*Yr10*) resistance genes.

<table>
<thead>
<tr>
<th>Resistance gene</th>
<th>Resistance gene candidate</th>
<th>Enzyme</th>
<th># of loci</th>
<th>Chromosomal location (NT analysis)</th>
<th># of polymorphic bands between Zak&lt;sup&gt;c&lt;/sup&gt; &amp; R gene donor&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
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<td></td>
<td>CS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Zak&lt;sup&gt;c&lt;/sup&gt;</td>
<td>R gene donor&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<td>BE518223*</td>
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<td>Unl216*</td>
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Table 4.6 (Contd.)

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<th>Resistance gene</th>
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<th># of loci</th>
<th>Chromosomal location (NT analysis)</th>
<th># of polymorphic bands between Zak &amp; R gene donor&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
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<tr>
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<td>Zak&lt;sup&gt;c&lt;/sup&gt;</td>
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Lr17

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<th>Zak&lt;sup&gt;c&lt;/sup&gt;</th>
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<th>B</th>
<th>D</th>
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<td>Unl216*</td>
<td>EcoRV</td>
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<td>1</td>
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<td>1</td>
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<sup>a</sup> = Chinese spring, <sup>c</sup> = Susceptible parent, <sup>b</sup> = Moro (Yr10), TA4115 (Lr2a), TA4117 (Lr2c), TA3983 (Lr9), TA3950 (Lr16), TA3854 (Lr17), - = not mappable due to high background.

* = NBS/LRR (Nucleotide Binding Site/Leucine Rich Repeat) type sequence structure, ‡ = PK (Protein Kinase) like sequence structure, † = PR (Pathogenesis-related protein) like sequence structure as described by Dilbirligi et al. (2003).
Table 4.7 Number of loci, chromosomal location and polymorphism of resistance gene candidates mapping in the region of wheat genome containing powdery mildew resistance genes *Pm3a* and *Pm20*.

<table>
<thead>
<tr>
<th>Resistance gene</th>
<th>Resistance gene candidate</th>
<th>Enzyme</th>
<th># of loci</th>
<th>Chromosomal location (NT analysis)</th>
<th># of polymorphic bands between Zak(^c) &amp; R gene donor(^b)</th>
</tr>
</thead>
<tbody>
<tr>
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\(^*\) = sequence has NBS/LRR (Nucleotide Binding Site/Leucine Rich Repeat) type structure, \(^\dagger\) = sequence has PK (Protein Kinase) like structure, \(^\ddagger\) = sequence has PR (Pathogenesis-related protein) like structure, \(^a\) = Chinese spring, \(^b\) = TA3887 (*Pm3a*), TA5041 (*Pm20*), \(^c\) = Susceptible parent.
Table 4.8 Number of loci, chromosomal location and polymorphism of resistance gene candidates mapping in the region of wheat genome containing Hessian fly resistance genes (H13, H22, H24, and H25).

<table>
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<th>Resistance gene</th>
<th>Resistance gene candidate</th>
<th>Enzyme</th>
<th># of loci</th>
<th>Chromosomal location (NT analysis)</th>
<th># of polymorphic bands between Zak&lt;sup&gt;c&lt;/sup&gt; &amp; R gene donor&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td>CS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Zak&lt;sup&gt;c&lt;/sup&gt;</td>
<td>R gene donor&lt;sup&gt;b&lt;/sup&gt;</td>
<td>A</td>
</tr>
<tr>
<td>H22</td>
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<td>0 1 2</td>
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<td>BF474204*</td>
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<tr>
<td></td>
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<tr>
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<td>BE607045&lt;sup&gt;i&lt;/sup&gt;</td>
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<tr>
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<tr>
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<td>2 1 0</td>
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<td>Unl164&lt;sup&gt;j&lt;/sup&gt;</td>
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<td>2</td>
</tr>
<tr>
<td></td>
<td>Unl181&lt;sup&gt;j&lt;/sup&gt;</td>
<td>DraI</td>
<td>2 2 2</td>
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</tbody>
</table>

<sup>a</sup>= NBS/LRR (Nucleotide Binding Site/Leucine Rich Repeat) type sequence structure, <sup>i</sup>= PK (Protein Kinase) like sequence structure, <sup>f</sup>= PR (Pathogenesis-related protein) like sequence structure, <sup>§</sup>= NB/Kinase type sequence structure, <sup>¥</sup>= Hm1 like sequence structure as described by Dilbirli et al. (2003). <sup>‡</sup>= Chinese spring, <sup>c</sup>= Susceptible parent, <sup>b</sup>= TA5005 (H22), TA5011 (H13), TA5016 (H24) and TA5033 (H25).
Table 4.9 Disease response patterns of wheat mapping populations to leaf rust (*P. triticina*) and stripe rust (*P. striiformis* Westend).

<table>
<thead>
<tr>
<th>Cross</th>
<th>Resistance gene</th>
<th># of F$_{2:3}$ families evaluated</th>
<th>Response</th>
<th>$\chi^2$ (1:2:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zak X TA4115</td>
<td><em>Lr2a</em></td>
<td>64</td>
<td>14</td>
<td>25</td>
</tr>
<tr>
<td>Zak X TA4117</td>
<td><em>Lr2c</em></td>
<td>70</td>
<td>17</td>
<td>33</td>
</tr>
<tr>
<td>Zak X TA3983</td>
<td><em>Lr9</em></td>
<td>60</td>
<td>17</td>
<td>27</td>
</tr>
<tr>
<td>Zak X TA3950</td>
<td><em>Lr16</em></td>
<td>64</td>
<td>15</td>
<td>28</td>
</tr>
<tr>
<td>Zak X TA3854</td>
<td><em>Lr17</em></td>
<td>67</td>
<td>15</td>
<td>35</td>
</tr>
<tr>
<td>Zak X TA3987</td>
<td><em>Lr26</em></td>
<td>70</td>
<td>13</td>
<td>25</td>
</tr>
<tr>
<td>Zak X Moro</td>
<td><em>Yr10</em></td>
<td>68</td>
<td>17</td>
<td>33</td>
</tr>
</tbody>
</table>

*a* = Crosses were made between cultivar Zak and *Lr/Yr* gene carrying Triticum accessions (TA) from WGRC, KSU, Manhattan, Kansas.  
*b* = Race MBB used for screening leaf rust populations and race PST78 used for screening *Yr10* population. $\chi^2$ ($df = 2, P = 0.05$) = 5.99, $\chi^2$ ($df = 1, P = 0.05$) = 3.84.
Table 4.10 Disease response patterns of wheat *Pm3a* and *Pm20* mapping populations to *B. graminis* f. sp. *tritici* isolates

<table>
<thead>
<tr>
<th>Cross</th>
<th>Resistance gene</th>
<th># of F&lt;sub&gt;2:3&lt;/sub&gt; families evaluated</th>
<th>Response</th>
<th>( \chi^2 ) (1:2:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zak X TA3887</td>
<td><em>Pm3a</em></td>
<td>67</td>
<td></td>
<td>4.19</td>
</tr>
<tr>
<td>Zak X TA5041</td>
<td><em>Pm20</em></td>
<td>67</td>
<td></td>
<td>3.00</td>
</tr>
<tr>
<td>Zak X TA3893</td>
<td><em>Pm6</em></td>
<td>73</td>
<td></td>
<td>13.19</td>
</tr>
</tbody>
</table>

\( a \) = Crosses were made between cultivar Zak and Pm gene carrying Triticum accessions (TA) from WGRC, KSU, Manhattan, Kansas. 
\( b \) = Isolates used for screening: *Pm3a* = Bgt 2, Bgt 6; *Pm20* = Bgt 13, Bgt 10. \( \chi^2 (df = 2, P = 0.05) = 5.99 \).
Table 4.11 Reaction of Zak and resistant parents to Hessian fly (*M. destructor* Say) biotype L

<table>
<thead>
<tr>
<th>Triticum accession (TA) / Cultivar</th>
<th>Hessian fly resistance gene</th>
<th>Observed disease reaction</th>
<th>Expected disease reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zak</td>
<td>?</td>
<td>Susceptible</td>
<td>Susceptible</td>
</tr>
<tr>
<td>TA5011</td>
<td><em>H13</em></td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td>TA5005</td>
<td><em>H22</em></td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td>TA5016</td>
<td><em>H24</em></td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td>TA5033</td>
<td><em>H25</em></td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
</tbody>
</table>
Table 4.12 Disease response patterns of wheat mapping populations to Hessian fly (*M. destructor* Say) biotype L

<table>
<thead>
<tr>
<th>Cross</th>
<th>Resistance gene</th>
<th># of F$_{2:3}$ families evaluated</th>
<th>Response</th>
<th>$\chi^2$</th>
<th>(1:2:1)</th>
<th>(3:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zak X TA5011</td>
<td><em>H13</em></td>
<td>79</td>
<td>12 48 19</td>
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<td>-</td>
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<tr>
<td>Zak X TA5005</td>
<td><em>H22</em></td>
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<td>21 35 17</td>
<td>0.5616</td>
<td>-</td>
<td></td>
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<tr>
<td>Zak X TA5016</td>
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<td>61</td>
<td>03 40 18</td>
<td>13.29</td>
<td>0.662</td>
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<tr>
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<td>67</td>
<td>16 39 12</td>
<td>2.28</td>
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<td></td>
</tr>
</tbody>
</table>

$^{a}$ Crosses were made between cultivar Zak and Hessian fly resistance gene carrying Triticum accessions (TA) from WGRC, KSU, Manhattan, Kansas. $^{b}$ Biotype L was used for screening all the populations, $\chi^2 (df = 2, P = 0.05) = 5.99, \chi^2 (df = 1, P = 0.05) = 3.8$. 

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CHAPTER FIVE

RNAi Gene Silencing in Hexaploid Wheat by Agrobacterium-mediated Transformation

Harvinder S. Bennypaul and Kulvinder S. Gill
5.1 ABSTRACT

Agrobacterium-mediated genetic transformation has been shown to be an effective strategy for direct gene transfer and functional genomics in a number of crop plants. Being relatively less disturbing to the genome, this method of transformation is more suitable for gene silencing studies as compared to the biolistic transformation method which in itself is known to cause gene silencing. Despite the advantages, the Agrobacterium-mediated transformation method has not been widely adapted in wheat mainly due to the lack of repeatability of the reported procedures. In this study a clear and repeatable procedure was optimized whereby, starting with immature embryos, transformants can be produced at an efficiency of up to 5.5% in eight to ten weeks. The optimized procedure produced 22 transgenic plants from 400 immature embryos. The Gateway® cloning technology based RNAi vector pHELLSGATE 8 was investigated for its use in high throughput construction of hpRNA constructs and gene silencing. Nine sequences, ranging in length from 225 – 633bp, were successfully cloned in the sense and the antisense orientation to make hpRNA constructs. Irrespective of the length of the cloned sequence, 50 – 67% of the constructs had sequences cloned in the right orientation, whereas 33 – 50% constructs showed intron reversal. pHELLSGATE 8-based construct pHGBE405778 was used for RNAi of resistance gene candidate BE405778. Twenty two putative transformants were obtained from 100 embryos. Of 22, two plants were found to be positive with Southern analysis. Two plants, one from T1 progeny of each transformant, were tested for expression of BE405778 with Real-Time RT-PCR. Both transformants showed 80 – 100% reduction in transcript level of the target gene.
5.2 INTRODUCTION

Understanding molecular basis of biological processes is poised to bring future improvements in agronomic performance of crop plants. Gene cloning is important for that and for developing ultimate markers for marker-assisted selection (MAS). In wheat, large genome size (16,000 Mbp), hexaploid nature and presence of a large amount of repetitive DNA makes gene cloning a monumental task. Map-based cloning of wheat genes \( Lr1 \) (Cloutier et al. 2007), \( Lr10 \) (Feuillet et al. 2003), \( Lr21 \) (Huang et al. 2003a), \( Q \) (Simons et al. 2006), \( VRN1 \) (Yan et al. 2003), \( VRN2 \) (Yan et al. 2004), \( VRN3 \) (Yan et al. 2006) and \( Pm3b \) (Yahiaoui et al. 2004) has proven to be time consuming and laborious. Wheat genes located in recombination-poor regions are very difficult if not impossible to clone using map based or positional cloning approaches (Erayman et al. 2004; Qi et al. 2004). Furthermore, presence of non-transcribing retroelement type repeated DNA, orthologs, paralogs and non-functional or diverged-function gene copies may further complicate map-based gene cloning.

A promising alternative to map-based gene cloning is the candidate gene approach, where cloned genes from other species are used to clone their homologues with similar function, from the target species. \textit{Arabidopsis thaliana} meristems identity gene \( APETALA1 \), which initiates the transition from vegetative to the reproductive apex, is similar in sequence and function to wheat \( VRN1 \) gene (Yan et al. 2003). Similarly \textit{Arabidopsis thaliana} \( APETALA2 \) (\( AP2 \)) gene, which is involved in the establishment of floral meristems identity (Bowman et al. 1993; Irish and Sussex 1990), floral organ identity (Komaki et al. 1988; Bowman et al. 1989; Kunst et al. 1989; Jofuku et al. 1994), and temporal and spatial regulation of floral homeotic gene expression (Drews et al. 1991), shows sequence similarity to the \( Q \) gene that is responsible
for the free-threshing character of wheat (Simons et al. 2006). In order for the candidate gene approach to be successful, a highly efficient plant transformation method is needed both for functional complementation as well as for reverse genetic approaches like RNA interference (RNAi).

Biolistic transformation (particle bombardment) is currently the most widely used technique both for direct gene transfer and for RNAi (Loukoianov et al. 2005). In wheat, silencing of Phytoene desaturase (Travella et al. 2006), VRN2 (Yan et al. 2004) and VRN3 (Yan et al. 2006) have been achieved by biolistic transformation of wheat plants with constructs expressing dsRNA. Biolistic transformation, however, often results in complex insertion loci (3-100 copies) that can cause silencing of the target gene (Dai et al. 2001; Hammond 1999). For RNAi however, a transformation method, which results in minimal disturbance to the plant genome is very important so that loss of function phenotypes can be attributed to the targeted gene. Agrobacterium-based transformation has been reported to produce simple insertion loci (<3 copies) in a majority (>90%) of the transgenic events (Shou et al. 2004). Insertion site mutations, deletion and re-arrangements of plant genomic DNA and insertion of contaminating bacterial DNA are common with both transformation methods but the extent and severity of these events is relatively less in Agrobacterium-based system (Arencibia et al. 1998).

Agrobacterium-mediated transformation has been successfully used to transfer RNAi-inducing DNA sequences in Arabidopsis thaliana, Coffea arabica and Oryza sativa (Chuang and Meyerowitz 2000; Miki et al. 2005; Ogita et al. 2004; Stoutjesdijk et al. 2002; Wesley et al. 2001). In wheat, Agrobacterium–mediated tranformation with efficiencies of 0.9% (Cheng et al. 1997) and 1-2% (Peters et al. 1999a) have been reported (Cheng et al. 1997; Khanna and Daggard 2003; Peters et al. 1999b; Wu et al. 2003). In wheat, Agrobacterium-mediated
transformation has not been widely used for RNAi and gene transfer mainly due to a lower transformation efficiency and unreproducibility of the published transformation procedures (Wu et al. 2003).

In order to efficiently utilize candidate gene approach and to benefit from wheat expressed sequence tags (about 1,250,927), an efficient vector system to produce RNAi constructs in a high throughput manner, and a robust, less disturbing (clean) and reliable transformation method are needed. This study was undertaken with the objectives to: 1) standardize a simple, easy to follow and repeatable protocol for Agrobacterium-mediated transformation of wheat; 2) investigate potential of the pHELLSGATE 8 as a high throughput vector for making RNAi constructs; and 3) optimize an RNAi-based gene silencing approach in hexaploid wheat.

5.3 MATERIALS AND METHODS

5.3.1 Vector construction

The RNAi constructs were made using the pHELLSGATE 8 (pHG8) vector (Wesley et al. 2001) that is based on the Gateway Recombination technology (http://www.invitrogen.com/content.cfm) and is ideal for cloning polymerase chain reaction (PCR) amplified sequences. With this technology, a PCR fragment (bordered with recognition sites attB1 and attB2) is recombined \textit{in vitro} into a plasmid (pDONR201) containing attP1 and attP2 sites. The PCR fragment, now bordered with recognition sites attL1 and attL2 (recombination between attB and attP sites give rise to attL site) in pDONR201, is directionally recombined \textit{in vitro} into pHG8 containing attR1 and attR2 sites (Fig. 5.2). The BP (between sites attB and attP) and the LR (between sites attL and attR) recombination reactions are done
using a commercially available BP clonase and LR clonase enzymes (http://www.invitrogen.com). Final construct (Fig. 5.1) has the PCR fragment cloned in sense and antisense directions separated by PDK (pyruvate orthophosphate dikinase) gene intron and this arrangement, under the control of 35S promoter, leads to transcription of a hairpin molecule.

Sequences for cloning were amplified from 50 – 100 ng DNA of the corresponding clones (plasmids with cloned EST sequences) using primers containing attB1 and attB2 sites (Table1). The PCR was performed at 94°C for 2 minutes; followed by 35 cycles of 94°C 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds. Final extension was performed at 72°C for 10 minutes. The PCR products were gel-purified using the Q-Biogene GENECLEAN® kit. Entry clones were made by cloning PCR product into pDONR201 vector using the Gateway® BP Clonase Enzyme. The entry clones were further recombined with pHG8 to make RNAi constructs using the Gateway® LR Clonase Enzyme. The BP and the LR recombination reactions were carried out following the manufacturer’s recommendations.

Cloning efficiency of the BP and the LR recombination was checked by transformation of the entry clones or the RNAi constructs into the DH5α strain of *E. coli*. For transformations, 2 µl of the BP or the LR reaction was mixed with 50 µl of quickly thawed frozen competent cells. The mixture was given a heat shock at 42°C for 30 seconds. Heat shocked cells were immediately mixed with 250 µl of S.O.C. medium (at room temperature) and were grown in a shaker at 37°C for 1 – 1.5 hours. About 100 µl of the bacterial culture was spread on LB plates (supplemented with 50 mg L⁻¹ kanamycin in case of entry clone or 100 mg L⁻¹ spectinomycin in case of RNAi construct) and was allowed to grow overnight at 37°C. Cultures from single colonies were grown overnight at 37°C in 2 ml LB media supplemented with the appropriate
antibiotics. The plasmid DNA was isolated by the alkaline lysis method (Maniatis et al. 1982). Accuracy of each entry clone was checked by restriction analysis of 4 – 6 clones with appropriate restriction enzymes. Accuracy of each RNAi construct was first checked by restriction analysis of 5 - 8 clones with enzymes XhoI and XbaI followed by sequencing analysis of a representative clone by automated dye-terminator sequencing using ABI 310 or 377 sequencers at the WSU sequencing facility.

5.3.2 Transformation of Agrobacterium tumefaciens

Originally tri-parental mating (Fig. 5.9) was used to transfer pPTN290 binary plasmid to C58C1 strain of Agrobacterium tumefaciens (Ditta et al. 1980). Tri-parental mating is a form of bacterial conjugation where a conjugative plasmid (coding for genes required for conjugation and DNA transfer) present in one of the bacterial strains (helper strain) assists the transfer of a mobilizable plasmid present in a second bacterial strain (donor strain) into a third bacterial strain (recipient strain). Cultures of Agrobacterium strain C58C1, E. coli helper strain pRK2013 and E. coli donor strain (containing plasmid pPTN290) were started on LB agar plates containing appropriate antibiotics; 50 mg/ml rifampicin and gentamycin for Agrobacterium, 50 mg/ml kanamycin for the helper strain and 50 mg/ml spectinomycin for the donor strain. Plates were incubated at 28°C and 37°C in case of E. coli and Agrobacterium, respectively. After 3 – 4 days, a single colony was selected from each plate and 5 ml LB culture was started using an appropriate temperature and antibiotic. Next day, 100 µl each of C58C1, pRK2013 and the donor strain were mixed and incubated at room temperature for 30 seconds. Mixture was centrifuged briefly to pellet the cells that were then resuspended in 100µl of fresh LB. The resulting bacterial culture was spotted on the LB agar plate and incubated at 28°C. The
following day, a loop of culture was taken from the plate and mixed with 1 ml LB. About 100µl of this culture was spread on LB plate containing 50 mg/ml each of rifampicin, gentamycin and spectinomycin. Plates were incubated at 28°C until individual colonies can be seen.

We optimized an electroporation-based method to directly transfer plasmids to *A. tumefaciens*. Briefly, electrocompetent cells (prepared using the standard methods) of the strain C58C1 were transformed with the pPTN290 by electroporation. About 20 µl of quickly thawed frozen competent cells were mixed with 10 - 30 ng of the vector DNA in a pre-chilled electroporation cuvette. Electroporations were carried out at 1.25kV/cm, 200 Ohm using a Bio-Rad MicroPulser®. Electroporated cells were immediately mixed with 1ml of LB medium (at room temperature) and were grown in a shaker at 28°C for 1 - 2 hours. About 100 µl of the bacterial culture was spread on LB plates (supplemented with 50 mg L⁻¹ rifampicin, 50 mg L⁻¹ gentamycin and 50 mg L⁻¹ spectinomycin) and was allowed to grow for 3 - 4 days at 28°C. Cultures from single colonies were grown overnight at 28°C in LB media supplemented with previously mentioned antibiotics.

Transformation of *A. tumefaciens* cells was confirmed by first extracting the vector DNA by the alkaline lysis method (Maniatis et al. 1982) from a 5ml overnight grown culture. Extracted vector DNA was then transfected into competent cells of *E.coli* strain DH5α cells. Transfection into *E.coli* was needed as it is difficult to recover enough DNA from a 5ml *A. tumefaciens* culture. A 2 ml culture of the transfected DH5α cells was grown overnight and the vector DNA was extracted. Identity of the extracted RNAi vector was confirmed by restriction digestion using appropriate enzymes.

### 5.3.3 Plant material and growing conditions
Spring wheat cv. BobWhite sister line 31208 (from CIMMYT) was used for the transformation and RNAi experiments. Plants (4 per pot) were grown in 6 inch pots containing sunshine # 1 potting mix (SunGro Horticulture, Bellevue, WA) in a Conviron growth chamber equipped with high-intensity discharge lamps, with light intensity ranging from 500 – 700 µmol m\(^{-2}\) s\(^{-1}\), programmed for 16 hours at 20ºC during the day and 13ºC at night. The 20-20-20 Peter’s fertilizer (J.R. Peters, Inc., Allentown, PA, USA) was applied twice a week at a concentration of 100 ppm. Plants were watered every other day for the first month and then every day after that. Particular care was taken to avoid exposure of the plants to chemicals or to various stresses. Spikes were harvested when embryos were slightly starchy (12 - 16 days post anthesis). The immature seeds were disinfected by immersing in 70% ethanol for one minute and then in 10% bleach for 15 minutes. After three washings with sterile water, embryos were dissected out using sterile scalpels under aseptic conditions in a laminar flow hood.

5.3.4 Embryo culture

The media used for callus initiation, inoculation, co-cultivation and regeneration were based on Clemente and Mitra (2004). The immature embryos were cultured, with scutellum side up, for 4 days at 24 ± 1ºC in the dark on callus initiation medium (CIM) containing Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog 1962) supplemented with 0.5 mg L\(^{-1}\) 2,4-dichlorophenoxyacetic acid (2,4-D); 2.2 mg L\(^{-1}\) picloram; 40 gm L\(^{-1}\) maltose; 0.75 gm L\(^{-1}\) magnesium chloride; 1.95 gm L\(^{-1}\) MES; 2 gm L\(^{-1}\) phytagel; 0.5 gm L\(^{-1}\) glutamine; 0.1 gm L\(^{-1}\) casein hydrolysate; and 100 mg L\(^{-1}\) ascorbic acid. Media pH was adjusted to 5.8 with conc. KOH.
5.3.5 Inoculation and co-cultivation

For making the inoculation media, a 2 ml A. tumefaciens culture containing appropriate construct, was grown for 16 hours at 28°C with shaking (150 rpm) in Yeast Extract Peptone (YEP) media (10 gm L\(^{-1}\) yeast extract, 5 gm L\(^{-1}\) peptone, 5 gm L\(^{-1}\) NaCl, pH 7.0) containing 50 mg L\(^{-1}\) rifampicin, 50 mg L\(^{-1}\) gentamycin and 100 mg L\(^{-1}\) spectinomycin. The 2 ml culture was used to inoculate 50 ml of YEP media containing the three antibiotics. After growing to an OD\(_{260}\) of 0.6 – 0.8, the A. tumefaciens cells were pelleted by centrifugation at 3500 rpm for 10 minutes and then resuspended in the inoculation medium (1/10X MS salts, 1X MS vitamins, 0.5 mg L\(^{-1}\) 2,4-D, 2.2 mg L\(^{-1}\) picloram, 1% glucose, 200 µm acetosyringone, pH 5.4) at an OD\(_{260}\) of 0.6 – 0.8.

The immature embryos grown on the callus initiation media (CIM) for four days were immersed in the above-mentioned inoculation media for 30 minutes. Thereafter, the embryos were placed, scutellum side up, on the co-cultivation media at 24°C in dark. Co-cultivation media is the same as the inoculation media except it also contains phytogel or gelrite at the rate of 2 gm L\(^{-1}\). After two days, the embryos were transferred to CIM supplemented with 10 mg L\(^{-1}\) Geneticin®, 50 mg L\(^{-1}\) ticarcillin, 50 mg L\(^{-1}\) cifotaxime and 50 mg L\(^{-1}\) vancomycin, and were incubated at 24°C in dark for 2 weeks.

5.3.6 Regeneration and selection of putative transgenic plants

After 2 weeks, each callus was divided into several small pieces (about 2 mm each) and moved to the regeneration medium 1 (MS medium, 0.2 mg L\(^{-1}\) 2-4,D, 4% maltose, 25 mg L\(^{-1}\) Geneticin® and 50 mg L\(^{-1}\) each of ticarcillin, cifotaxime and vancomycin) under 16 hour light (Gro-Lux Bulbs) and 8 hours dark at 24°C. After 14 days the calli were moved to the
regeneration medium 2 (same as regeneration medium 1 but without 2,4-D) under the same light and temperature conditions. After 2 weeks, regenerating plants showing a nice shoot (>3cm long) and root systems (extending into the medium) were transferred to Phytatrays (Sigma) containing the regeneration medium 2. Plants with a normal looking leaf and root systems were transferred from Phytatrays (Sigma) to soil and where then grown at 22°C days (16 hour light period) and 18°C nights (8 hours dark period) in a green house.

5.3.7 Selecting transgenic plants

Three methods used to identify transgenic plants are described below.

5.3.7.1 NPTII ELISA

The T-DNA region of the pHG8 vector based RNAi constructs contains the NPTII gene as a selectable marker. The NPTII ELISA assay is an effective colorimetric assay to screen for transgenic plants (Nagel et al. 1992). The PathoScreen NPTII ELISA assay kit (Agdia, Elkhart, IN) was used to detect the NPTII protein in leaf sample extracts of the putative transgenics. Leaves were macerated in freshly prepared PEB1 extraction buffer (in a volume 10 times the weight of a leaf sample) and the test for each sample was performed in triplicate as per manufacturer's recommendations. Visual quantification of the color intensity was done by diluting the samples with PEB1 extraction buffer till the color intensity matched with that from the untransformed control plants. Relative color intensity was calculated by the number of dilutions it took to bring color intensity similar to that of the control.

5.3.7.2 PCR analysis
The PCR analysis was performed to amplify a 620bp sequence of the \textit{NPTII} gene (539-1159 bp) using 50 – 60 ng of genomic DNA of each of the putative transgenic plant along with a known transgenic plants as a positive control (transformed with pPTN290 plasmid having \textit{NPTII} gene, kindly provided by Dr. Tom Clemente, UNL, Nebraska) and BobWhite sister line 31208 as a negative control. The PCR screen was first optimized on the controls before using it to screen the putative transgenic plants. Primers for this analysis were \textit{NPTIIF} – ATG ACT GGG CAC AAC AGA CA and \textit{NPTIIR} – AAT ATC ACG GGT AGC CAA CG. The reactions were performed at 94°C for 2 minutes; followed by 35 cycles of 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds. Final extension was performed at 72°C for 10 minutes.

5.3.7.3 DNA Gel-blot analysis

Genomic DNA for the gel-blot analysis was isolated following the SDS-based method (Anderson \textit{et al.} 1992). For each sample, 5 µg of genomic DNA was digested with \textit{Not}1 restriction enzyme and was electrophoretically separated on a 0.8% agarose gel, as previously described (Gill \textit{et al.} 1993). ‘Southern’ blotting onto nylon membrane (Micron Separations Inc. MA), DNA immobilization, and hybridization were performed following the membrane manufacturer’s recommendations and as described earlier (Gill \textit{et al.} 1993). The membranes were then hybridized with a $^{32}$P-labeled \textit{BamH1-XbaI} fragment from the plasmid (Fig. 5.1). Number of integrated T-DNA copies was inferred from the number of bands. From the \textit{Not}1 digested genomic DNA, the \textit{BamH1-XbaI} probe is expected to detect two bands from each of the T-DNA insertion site (Fig. 5.1). The \textit{Not}1 – \textit{Not}1 band, from the T-DNA, will be common to all copies whereas the other chimeric band, including part of T-DNA and genomic DNA from
integration site, will be different for each integration event. Number of the bands (not considering common band) will correspond with number of T-DNA copies integrated.

5.3.8 RNA procedures and the real-time assay

All non-disposable laboratory ware were soaked in 0.01% v/v diethyl pyrocarbonate (DEPC) overnight followed by autoclaving for 45 minutes. For RNA isolation, 0.5 to 1 g fresh leaf tissue was pulverized to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was isolated using 8 mL of the TRIzol® reagent (Invitrogen Corp., Carlsbad, CA, USA) per sample according to the manufacturer’s recommendations. RNA quality was checked by formaldehyde denaturing gel electrophoresis (Sambrook et al. 1989) and quantity by absorbance at 260 nm using a Jasco V530 spectrophotometer (JASCO Inc., Easton, MD, USA).

About 2 µg of RNA was combined with 1 µg oligo dT₁₅ (Promega) and the volume was increased to 15 µL using DEPC-treated nanopure water. The mixture was heated to 70°C for 5 minutes and then chilled on ice. After a quick spin, the RNA mixture was supplemented with 5 µL 5x M-MLV RT buffer (Promega), 1.25 µL of 10 µM dNTP (Promega), 200 units of M-MLV RT (Promega) and 30 Units of RNaseOUT (Invitrogen) in a final volume of 25 µL that was incubated at 42°C for 1 hour in a thermocycler. Reaction was terminated by heating it to 70°C for 15 minutes. The newly synthesized cDNA was precipitated by adding 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate, pH 5.2, and was resuspended in 20 µL of nanopure water after centrifugation.

Real-time RT-PCR analysis was performed on a Rotor-Gene 2000 thermocycler (Corbett Research, Mortlake, NSW, Australia) using the Qiagen QuantiTect SYBR Green Master Mix (Valencia, CA, USA). PCR primers for the analysis were: BE405778-F (5’-CCA GTA AGG
TGG TCC AAC TCC T-3’) and BE405778-R (5’-TGT AGA GAC CGC GAA GGG A C-3’). Constitutively-expressed wheat Actin gene (NCBI, NID = AY423548) was used as an internal control for normalizing the gene expression. A 173bp sequence of the internal control was amplified using primers Act-F (5’ -TGT GCT TGA TTC TGG TGA TGG TGT G-3’) and Act-R (5’-CGA TTT CCC GCT CAG CAG TTG T-3’).

For each sample, transcript level was quantified in 210 ng and 350 ng cDNA. In addition to the cDNA, each reaction consisted of 5 pmol each of the two primers, 10 µL of 2X QuantiTect SYBR Green PCR Master Mix and nuclease-free water in a total volume of 20 µL. PCR amplification was performed at 50°C for 2 minutes; hot start at 94°C for 10 minutes followed by 35 cycles of 94°C 40 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 45 seconds. Amplicon melting profiles were generated over a range of 72°C to 99°C with 0.5°C increments, holding for 5 seconds at each increment to determine the melting temperature of the specific product.

Absolute quantification of mRNA was performed using external cDNA standards. The mRNA concentrations in the samples were determined from the respective standard curves. The mRNA level in the control and the treatment was normalized with respect to the Actin mRNA. Gene silencing was expressed as percent reduction in the transcript of the target gene in the transgenic plants compared to that in the untransformed plants.

5.4 RESULTS AND DISCUSSION

5.4.1 Transformation of Agrobacterium tumefaciens

Tri-parental mating has been commonly used for the tranformation of the Agrobacterium strain C58C1 with a binary plasmid or RNAi constructs (Dickman et al. 2001; Sullivan and
Quesenberry 2006). This process takes 10 – 11 days for the completion and involves a number of steps for culturing helper and donor bacterial strains. In contrast, electroporation does not require a helper or a donor strain and the transfection of the plasmid can be verified in the recipient strain in two days. However, transformation efficiency of C58C1 with electroporation was not known at the time of this study. To compare tri-parental mating and electroporation for transformation efficiency, Agrobacterium strain C58C1 was transformed with the vector pPTN290.

Both methods resulted in a large number of normal looking colonies on LB (supplemented with spectinomycin 50 mg/ml) agar plates. Restriction digestion with HindIII and NotI of two random clones from each method showed the expected restriction pattern (Fig. 5.10). These data suggest that electroporation is equally efficient for transformation of C58C1 strain but it is much faster and easier than the tri-parental mating method. As compared to 10 – 11 days required for tri-parental mating, electroporation can produce transformed colonies of C58C1 in two days. Therefore, electroporation was used for the rest of the experiments.

5.4.2 The pHG8 as a high-throughput RNAi vector for wheat

Large scale RNAi experiments can only become feasible if the RNAi constructs can be readily assembled. Traditional cloning technology that relies on restriction, digestion and ligation is often laborious and time consuming. The pHG8 vector is a high throughput, hpRNA encoding, binary vector based on Gateway® recombination system for quick and efficient cloning of PCR products (Wesley et al. 2001). We investigated the ease and efficiency of making RNAi constructs using the pHG8 vector.
Nine EST sequences (Table 1) of lengths varying from 225 – 633bp were used for testing the ease and efficiency of making hpRNA constructs using the Gateway® Recombination Technology (Invitrogen Corporation, Carlsbad, California, USA) (Fig. 5.2). The vector pHG8 was used as a parent vector to make constructs pHGBE492937, pHGBE405778, pHGBF474204, pHGBF475048, pHGBF483881, pHGBG606866, pHGBE518223, pHGBE591154 and pHGBE498831 using sequences from the corresponding ESTs (Table 1).

To check efficiency of BP recombination reaction, 44 entry clones corresponding to nine ESTs were restricted with enzymes specific to each cloned sequence. All the clones gave expected restriction pattern confirming that this cloning step worked to 100% efficiency (Table 2). Entry clones were used for LR recombination reaction with pHG8 vector to make RNAi constructs. Efficiency and accuracy of this cloning step was checked by restriction digestion of 5 - 8 clones of each construct with XhoI and XbaI. From each cloning experiment, two to three clones showed a wrong restriction pattern and ≥ 3 clones showed the expected pattern (Table 3). Number of clones giving correct restriction pattern ranged from 50 – 67%, irrespective of the EST sequence and length of sequence used. Surprisingly, in all the cases of aberrant restriction pattern, fragment size was about 800bp more than expected size (Fig. 5.3).

Sequencing of the aberrant clones showed the PDK intron to be present in a reverse orientation. Clones showing intron reversal ranged from 33 – 50% and this phenomenon was found to be independent of the sequence or the sequence length (Table 2). Contrary to our results, Wesley et al. (2001) recovered correct clones in 100% of the cases while cloning 200nt or 400nt sequences. Orientation of the PDK intron, with respect to CaMV S35 promoter, is important for the proper functioning of the pHG8 – based constructs (Helliwell et al. 2003).
During transcription the \textit{PDK} intron splices out leaving few basepairs in the middle which form loop in the hpRNA.

Despite the problem of intron reversal $\geq 50\%$ right constructs were obtained in 100\% of the cloning experiments. As a single right clone is sufficient for each experiment, pHG8-based method of making hpRNA constructs is very effective and efficient.

\subsection*{5.4.3 Agrobacterium-mediated wheat transformation}

The pHG8-based vector pHGBE474204, carrying 400 bps of EST BE474204, was used for the optimization of Agrobacterium-mediated transformation.

\subsubsection*{5.4.3.1 Plant regeneration}

Plant regeneration through tissue culture is a prerequisite for tissue culture based transformation technologies like Agrobacterium-mediated and biolistic transformation. One of the most transformation-responsive material reported so far is spring wheat line 'BobWhite' and its sister lines (Bliffeld \textit{et al.} 1999). A number of Spring wheat cv. BobWhite sister lines (from CIMMYT) have been reported to be good for tissue culture (Pellegrineschi \textit{et al.} 2001). We used one of those sister lines, 31208, for our transformation and RNAi studies.

No systematic data was collected but it was observed that embryos harvested after 12 -16 days of anthesis ranged in size from 0.8 – 2.5 mm and the ones in the size range 0.8 – 1.5 mm were found to respond better with regards to scutellum embryogenesis. Embryos $>2$ mm in size performed poorly and rarely showed scutellum embryogenesis.

During root and shoot generation three types of plants were observed; plants with bushy and bright green foliage having multiple roots extending into the media (Figure 5.6B, plant 1), plants with green foliage with 1 or 2 roots extending into the media (Figure 5.6B, plant 2), and
plants with dull green foliage with none or few shallow roots (Figure 5.6B, plant 3). The dull green plants with shallow root system eventually died. Nice green shoot system with multiple roots extending into the media is generally a sign of transformed plant (personal communication, Dr. Tom Clemente, Plant Transformation Core Research Facility, University of Nebraska).

We recovered 78 putative transformants out of the 400 embryos that were initially used. Of 78 putative transformants, 23 produced bushy bright green foliage with nice root system (Figure 5.6B, plant 1), whereas rest of the putative transformants produced one or two shoots (Figure 5.6B, plant 2) with one or two roots extending into the media. Putative transformants showed variation in various characters like abnormal leaf shapes, bushy stunted growth, early flowering and heading, empty florets and fewer tillers These types of abnormalities are commonly observed in transformation techniques involving tissue culture (Singh et al. 1998). These variations, also called as somaclonal variations, can be manifested as either somatically or meiotically stable events. Meiotically stable events are transmitted from generation to generation, whereas, somatically stable events are not seed transmissible. Both types of events can interfere with phenotypic assessment of primary transformants (T₀). Interference from somatically stable events can be avoided by studying T₁ or T₂ progeny as these type of variations are usually not seed transmissible. Thus, fertility of transgenic plants is very important for their use in basic research or as genetic material in breeding programs.

Particle bombardment was reported to produce <35% fertile primary rice transformants (Dai et al. 2001). In another study dealing with RNAi-based silencing of phytene desaturase (PDS) gene in wheat, none of the T₀ plants (which were produced by particle bombardment and showed strong PDS knock-down albino phenotype) were able to set seed (Travella et al. 2006).
In our study all 78 putative transformants (100%) produced seed but all the plants were partially fertile as not all of the florets set seed. All but two plants produced > 5 seeds per plant. One of those two plants produced four seeds and the other one just two seeds. Our results agree with Dai et al. (2001) who reported that transgenic plants produced via Agrobacterium-based transformation show better fertility than transgenic plants produced by particle bombardment.

5.4.3.2 Screening for the transgenic plants

The NPTII gene ELISA assay has been extensively used as a selectable marker to screen for transgenic plants during transformation experiments (Nagel et al. 1992). Xing-Guo et al. (2005) have shown NPTII ELISA (using PathoScreen kit) as an effective assay for selecting transformants in wheat. This assay is routinely used for selecting transformants in a number of plant transformation facilities (personal communication, Dr. Ravi Chibbar, University of Saskatchewan; Dr. Tom Clemente, Plant Transformation Core Research Facility, University of Nebraska)

All 78 putative T₀ transformants, produced via Agrobacterium-based transformation of pHGBE474204 construct, were evaluated by the NPTII ELISA assay using PathoScreen kit (Agdia, Elkhart, IN). Of these, 23 plants showed 3 - 5 times color development as compared to the control plants. These are the same 23 plants that showed nice shoot and root system development during regeneration. The rest of the plants did not show any visually detectable difference in color development as compared to the control. These plants either have the NPTII gene altered or are false positives which survived the selection of antibiotic Geneticin® (at 50 mg L⁻¹) during transformation process. Geneticin® has been reported to be better selection agent than kanamycin and paromomycin for stopping growth of nontransformed wheat plants at
concentrations of 10 to 50 mg L\(^{-1}\) (Clemente and Mitra 2004). However, relatively older immature embryos and germinated somatic embryos are very tolerant to this antibiotic (Witrzens \textit{et al.} 1998). As florets in single wheat head mature at slightly different rates, embryos harvested from one head are mixture of young and old. We believe, in this study, the majority of the false positives have regenerated from older immature embryos in this study.

The \textit{NPTII} PCR assay to confirm transformants was first optimized using a known transformant (transformed with \textit{NPTII} carrying binary vector pPTN290) as a positive control, along with the BobWhite line. The PCR reaction amplified a 620 bp band out of the known transformant and the band was missing in BobWhite sisterline 31208 (Fig. 5.4). Except one (lane #18), all 23 NPTII ELISA positive plants showed the diagnostic 620 bp band (Fig. 5.5) and the amplification was missing in the negative control BobWhite 31208 (lane 34 and 35). Wells 28 – 33 were left blank to avoid carryover of PCR products to negative control wells.

The method optimized in this study produced transformants at a transformation efficiency of 5.5% in 8 – 10 weeks starting from immature embryos. Our method represents an improvement over other published Agrobacterium–mediated transformation procedures which reported production of transformants in 9 – 12 weeks at a transformation efficiency of 0.9 - 2% (Cheng \textit{et al.} 1997; Khanna and Daggard 2003; Peters \textit{et al.} 1999b; Wu \textit{et al.} 2003).

\textit{5.4.4 RNAi using pHG8-based vector}

The pHG8 is a high throughput and high efficiency RNAi vector which has been reported to give 66 -100% silenced plants in tobacco, Arabidopsis, cotton and rice (Wesley \textit{et al.} 2001). This vector has not yet been used in wheat. We tried RNAi of RGC BE405778 using pHG8-based vector pHGBE405778. 100 embryos were used for transformation wheat with
pHGBE405778. Out of 100 embryos 22 putative transgenic plants grew on the selection media. T₀ plants and number of integrated T-DNA copies were confirmed with DNA gel blot analysis using NotI as restriction enzyme. After blotting, membrane was probed with ³²P-labeled BamHI – XbaI fragment from the T-DNA of RNAi construct (Fig. 5.1). NotI restriction releases 5808 bp band in pHG8 (Fig. 5.1). NotI restriction of pHGBE405778 RNAi constructs will release a smaller band of 4404 bp as two stretches 1179 bp and 1169 bp, between attR₁ and attR₂ sites, of pHG8 were replaced by 467 bp sense and antisense sequence during construction of pHGBE405778.

Two plants (BE405778-1 in lane 12 and BE405778-2 in lane 24) were found to be transformed with the T-DNA of RNAi construct pHGBE405778 (Fig. 5.7). As expected these plants shared a common 4404bp band, as expected in case of T-DNA of RNAi construct pHGBE405778. In addition to the common band, two bands were detected in BE405778-1 and three bands were detected in BE405778-2 indicating integration of two and three T-DNA copies in former and latter case respectively. This is also corroborated by higher intensity of common band in BE405778-2 as this band is a mixture of three bands rather than two bands in BE405778-1. Both of these transgenic plants were allowed to set seed. Two T₁ plants, one from the progeny of each transformant, were used for checking expression of targeted RGC BE405778 using Real-Time RT-PCR. Untransformed plant of BobWhite sisterline 31208 was used as a positive control for the expression of BE405778.

Expression of BE405778 was compared in transgenic plants and untransformed control. For each plant, BE405778 mRNA level was quantified in 210 ng and 350 ng cDNA. Two concentration of cDNA were used to increase the chance of detecting even low level of expression. BE405778 mRNA level in control and treatment was normalized with respect to
wheat Actin gene mRNA. Absolute quantification of the EST BE405778 and reference gene Actin mRNAs was performed using external cDNA standards. Concentrations of the EST BE405778 and reference gene mRNAs in samples were determined from the respective standard curves. Silencing of BE405778 was expressed as % reduction in transcript of BE405778 in transgenic plants to that in untransformed plant.

In 210 ng cDNA, no BE405778 transcripts were detected in both transgenic plants whereas untransformed control did show presence of transcripts (Fig. 5.8 and Table 1.1). In 310 ng cDNA, BE405778-1-1 and BE405778-1-2 showed >80% and 100% reduction in transcript level as compared to untransformed control (Fig. 5.8 and Table 1.1). This is in agreement with other RNAi reports that Agrobacterium-based transformation produce higher percentage of plants showing knockdown as compared to particle bombardment. In Arabidopsis thaliana, 87-100% plants showed knockdown phenotype in Agrobacterium-mediated transformation of double-stranded RNA-expressing constructs corresponding to four genes, AGAMOUS, CLAVATA3, APETALA1, and PERIANTHIA (Wambutt et al. 2000). Yan et al. (2004) and Loukoianov et al. (2005) used particle bombardment to transform wheat with RNAi constructs targeting VRN2 and VRN1 respectively. In both the cases only one out of three transgenic plants showed knockdown phenotype. Similarly, Travella et al. (2006) reported only about 40% and 33% of wheat transgenic plants showing knockdown phenotype of Phytoene deaustarase (PDS) and Ethylene Insensitive 2 (EIN2) genes respectively.

In RNAi studies, level of transcript knockdown is important especially for those cases where phenotypes are less sensitive to the level of gene expression and a reduced expression is still sufficient to confer proper phenotype. One such case was reported by Travella et al. (2006) where RNAi, via particle bombardment, gave 30 – 50% knockdown of EIN2 without any effect.
on phenotype. In gene-silencing studies, level of transcript knockdown has been shown to depend on expressed conformation of targeting sequence (antisense, sense, hairpin or intron-spliced hairpin) and various other factors including transformation methodology (Wesley et al. 2001). pHG8, a hairpin RNA expressing vector, has been reported to give robust silencing of PDS in tobacco, Arabidopsis and rice (Wesley et al. 2001). Goyer et al. (2004) reported 83 – 92% knockdown of Sarcosine Oxidase (SOX) gene transcript using pHG8 in Arabidopsis thaliana. We have achieved 80 -100% knockdown of wheat EST BE405778 with pHG8 RNAi via Agrobacterium-mediated transformation of wheat. This level of silencing should be able to produce knockout phenotype in majority of genes.

In conclusion we have optimized the methodology of Agrobacterium-mediated wheat transformation. Using this system, stable transgenic plants with one or few copies of T-DNA can be produced in as little as 8 – 10 weeks. Agrobacterium-mediated wheat transformation with pHG8-based RNAi construct gives consistent and effective silencing of wheat genes. Based on our data, we also believe that other variations of pHELLSGATE like pStartgate (with Ubiquitin promoter), pOpOff (dexamethasone-inducible RNAi) will be useful in functional genomics in wheat.
5.5 REFERENCES


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Figure 5.1 T-DNA region of RNAi vector pHGB405778. RB, right border; 1, 35S promoter; 2 & 8, attR site; 4 & 6, attR2 site; 5, PDK (pyruvate orthophosphate dikinase) intron; 9, OCS (octopine synthase terminator); 10, NOS (nopaline synthase) promoter; NPT II (neomycin phosphotransferase II); 11, NOS terminator. LB, left border; 3 & 7, EST sequence in sense and antisense orientations. Probe 1 (generated by XbaI & BamHI restriction) used for screening of transformants by Southern analysis.
Figure 5.2 A schematic diagram of DNA cloning using Gateway® Recombination technology

(Figure used with permission from CSIRO, Australia)
Figure 5.3  *PEK* (pyruvate orthophosphate dikinase) intron reversal in pHELLSGATE 8 during making of RNAi construct. 517bp PCR product amplified from wheat EST EE405778 was cloned in the vector using Gateway® technology (Invitrogen, Carlsbad, CA). 6 colonies were checked and each of them was restricted with *XhoI* and *XbaI* (separately) to make sure the presence of cloned sequence in sense and antisense orientations respectively. Colony 1 and 4 show intron reversal, colony 6 shows construct with proper orientation of intron, colonies 2, 3, 4 and 5 shows mixture of constructs with proper or reversed intron orientation. M = 1kb extension ladder (Invitrogen)
Figure 5.4  PCR analyses for selecting NPTII gene primers for screening transformants. Progenies of transgenic plants (transformed with plasmid pPIN230 with NPTII gene as selectable marker) were screened with primers NPTIIF (ATGACTGGGCACAACAGACA) and NPTIIR (AATATCAGG GTAGCACAACG). These primers amplify 620 bp sequence (539 - 1159 bp) of NPTII gene. Lanes 1 = 1Kb extension ladder marker, 2 -16 = progeny of transformants, 17&18 = BobWhite 31208 (negative control used for transformation). An unexpected 1000 bp band was amplified from the genome of cultivar used for transformation.
Figure 5.5 PCR analyses of NPTII-ELISA positive putative transformants. RNAi vector, pHELLSGATE 8's selectable marker NPTII gene's 620 bp sequence (539 – 1159 bp) was amplified using primers NPTII (ATG-ACTGGCCACAAACAGACA) and NPTII (AATATCACGGGTAGCCAACG). Lanes 1&21 = 1Kb extension ladder marker, 2 - 18&22 - 27 = putative transformants, 19&20 = positive control, 28 - 33 = empty wells, 34&35 = BobWhite 31208 (negative control used for transformation)
Figure 5.6 Agrobacterium-mediated transformation of wheat. A. Embryogenic calli showing shoot regeneration on regeneration medium 1. B. Root regeneration on regeneration medium 2. B. Plant 1 shows bushy, bright green foliage with multiple roots extending into the media, plant 2 shows single bright green shoot with one root extending into the media and plant 3 shows yellow shoot with single shallow root.
Figure 5.7 Southern analysis of putative transgenics. DNA from 22 putative transgenics (lanes 03 - 24) was restricted with XbaI and the resulting fragments were resolved by electrophoresis and transferred to membrane. The membrane was probed with α-32P-labelled DNA probe corresponding to BamHI - XbaI portion of T-DNA (Fig. 1). Lanes 12 and 24 shows transformed plants and Lane 1 and 2 are negative and positive controls respectively. Negative control consisted of untransformed plant and positive control (0.4 µg DNA from untransformed plant + 50 µg of RNAi plasmid pHELLSGATE 8). 5818 bp is expected band after XbaI digestion of pHELLSGATE 8 and 4404 bp is expected common bands between all transgenic plants containing T-DNA of RNAi construct pHGB205778. Asterisks show location of bands other than the common band.
Figure 5.8  Real-Time RT-PCR analysis of mRNA levels of EST BE405778 in transgenic plants transformed with RNAi construct expressing hairpin RNA for targeting BE405778. Transgenics BE405778-1-1 and BE405778-1-2 are T1 progeny of independent primary transformants. Control is untransformed plant of the Bob White sister lines used for transformation. mRNA level of BE405778 is normalized with reference to Actin gene.
Figure 5.9 Restriction map of plasmid pPTN290.
Figure 5.10 Comparison of electroporation and tri-parental mating for transformation of *Agrobacterium tumefaciens* strain C58C1. Plasmid DNA was extracted from C58C1 cells transformed with pPTN290 via tri-parental mating or electroporation. Plasmid DNA was restricted with *Hind*III and *Nco*I for confirming plasmid identity. 1 = 1Kb ladder, 2 & 3 = *Hind*III digested DNA from electroporated cells, 4 & 5 = *Hind*III digested DNA from tri-parental mated cells, 6 & 7 = *Nco*I digested DNA from electroporated cells, 8 & 9 = *Nco*I digested DNA from tri-parental mated cells.
Table 5.1 Primers used for amplification of EST sequences for making RNAi constructs.

| Primer Name   | Sequence (5’ – 3’)
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BE492937_F</td>
<td>C CAG GCC GAA GTG GAG ATA ATT</td>
</tr>
<tr>
<td>BE492937_R</td>
<td>G AGG ATT TGA TGT CTC GGT GGA</td>
</tr>
<tr>
<td>BE405778_F</td>
<td>C CAG TAA GGT GGT TCC AAC TCC T</td>
</tr>
<tr>
<td>BE405778_R</td>
<td>T GTA GAG ACC GCG AAG GGA C</td>
</tr>
<tr>
<td>BF474204_F</td>
<td>C GGG TAC TCG CTG TTG CTT T</td>
</tr>
<tr>
<td>BF474204_R</td>
<td>C AAG GCC ATT GAT TAC CAG ATT G</td>
</tr>
<tr>
<td>BE498831_F</td>
<td>A TGC TCA TTT CTT CAG CAA GGA GGA G</td>
</tr>
<tr>
<td>BE498831_R</td>
<td>C CAA TCC AAG GAT C TT TTC AGT AAT C</td>
</tr>
<tr>
<td>BF475048_F</td>
<td>G TAC AAT CAT CTC CGG AGC GAA CTA T</td>
</tr>
<tr>
<td>BF475048_R</td>
<td>C TAA CCC TGA AGA GCT CAT CCC TCT C</td>
</tr>
<tr>
<td>BF483881_F</td>
<td>A CAC ACT CTC ATA ATA TAT GTT GTT GAA AG</td>
</tr>
<tr>
<td>BF483881_R</td>
<td>T TTG GAG TGA GAT CAG CCT GC</td>
</tr>
<tr>
<td>BG606866_F</td>
<td>T CAC GTC AGT GAA GGA AGT GC</td>
</tr>
<tr>
<td>BG606866_R</td>
<td>C CAC CAC AAT ACA TAT AAA CCC AGA</td>
</tr>
<tr>
<td>BE518223_F</td>
<td>G CTC TTG CTC CAG TGA GGC</td>
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<td>G CTC AGA TTA AGT ATA GCC TGG ACA</td>
</tr>
<tr>
<td>BE591154_F</td>
<td>C GAA CAA TGA TCA TGT CAA GGC AGT</td>
</tr>
<tr>
<td>BE591154_R</td>
<td>T CCG TGC AGT ATT CTC TTT CCA TCC</td>
</tr>
</tbody>
</table>

\(^a\) = For cloning the amplified product into the pDONR201 vector, all forward primers have attB1 and all reverse primers have attB2 sequence included at their 5’ end. attB1 sequence = GGG GAC AAG TTT GTA CAA AAA AGC AGG CT, attB2 sequence = GGG GAC CAC TTT GTA CAA GAA AGC TGG GT, \(^b\) = product size (bp)
Table 5.2 Efficiency of BP recombination reaction for cloning PCR products into pDONR201.

<table>
<thead>
<tr>
<th>Entry clone</th>
<th>Restriction enzyme(s) used</th>
<th>Number of clones tested</th>
<th>Number of clones with right restriction pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDBE405778</td>
<td>DpnI, AvaI</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>pDBE474204</td>
<td>DpnI, SacI, AvaI</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>pDBF483881</td>
<td>DpnI, AvaI, HindIII</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>pDBE591154</td>
<td>DpnI, AvaI, ClaI</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>pDBG606866</td>
<td>DpnI, AvaI</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>pDBE498831</td>
<td>DpnI</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>pDBE492937</td>
<td>Sau3A</td>
<td>6</td>
<td>6</td>
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<td>pDBF475048</td>
<td>Sau3A</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>pDBE581223</td>
<td>Sau3A</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 5.3 *PDK* intron reversal in LR recombination reaction during making of pHELLSGATE 8 based RNAi constructs.

<table>
<thead>
<tr>
<th>RNAi construct</th>
<th>Length of sequence cloned (bp)</th>
<th>Number of clones examined</th>
<th>Number of clones showing correct <em>PDK</em> orientation</th>
<th>% Clones showing <em>PDK</em> intron reversal$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHGBE498831</td>
<td>225</td>
<td>6</td>
<td>4</td>
<td>33.0</td>
</tr>
<tr>
<td>pHGBE492937</td>
<td>261</td>
<td>5</td>
<td>3</td>
<td>40.0</td>
</tr>
<tr>
<td>pHGBF475048</td>
<td>291</td>
<td>5</td>
<td>3</td>
<td>40.0</td>
</tr>
<tr>
<td>pHGBE518223</td>
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<td>6</td>
<td>4</td>
<td>33.0</td>
</tr>
<tr>
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<td>6</td>
<td>4</td>
<td>33.0</td>
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<tr>
<td>pHGBF483881</td>
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<td>6</td>
<td>3</td>
<td>50.0</td>
</tr>
<tr>
<td>pHGBF474204</td>
<td>400</td>
<td>8</td>
<td>5</td>
<td>37.5</td>
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<tr>
<td>pHGBE405778</td>
<td>467</td>
<td>7</td>
<td>4</td>
<td>42.8</td>
</tr>
<tr>
<td>pHGBE591154</td>
<td>633</td>
<td>6</td>
<td>4</td>
<td>33.0</td>
</tr>
</tbody>
</table>
Table 5.4 Transcript level of EST BE405778 in transgenic plants transformed with RNAi construct expressing hpRNA targeting BE405778.

<table>
<thead>
<tr>
<th>Plant(^{a})</th>
<th>Transcript level in 210 ng cDNA</th>
<th>Transcript level in 350 ng cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.000007766</td>
<td>0.000017143</td>
</tr>
<tr>
<td>BE405778-1-1</td>
<td>0.0</td>
<td>0.000003296</td>
</tr>
<tr>
<td>BE405778-1-2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

\(^{a}\) = BE405778-1-1 and BE405778-1-2 are T\(_{1}\) progeny of independent primary transformants. Control is untransformed plant of the BobWhite sisterline used for transformation. mRNA level of BE405778 is normalized with reference to wheat Actin gene.