Investigation of *Un8*-Mediated Barley Loose Smut Resistance

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By

Wen Zang

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ABSTRACT

Barley loose smut, caused by the basidiomycete pathogen *Ustilago nuda* (Jens.) Rostr. (*U. nuda*), is a common fungal disease throughout Canadian barley growing regions and can be effectively controlled by the *Un8* resistance gene. The first study (Chapter 3) was designed to isolate the *Un8* gene by map-based cloning. The *Un8* gene was delimited to a 0.108 cM interval on chromosome arm 1HL and a minimal tiling path consisting of two overlapping bacterial artificial chromosomes was identified. Sequence analysis identified a *Un8* candidate gene predicted to be a putative protein kinase with two kinase domains. Twenty-six cultivated and eight wild barley accessions with diverse genetic backgrounds were collected for the second study (Chapter 4) and sequence alignment revealed that all resistant accessions from Canada shared the same amino acid sequence with the landrace accession, CN91953, which was reported as the donor of *Un8* to North American barley breeding programs.

The remaining three studies focused on elucidating the mechanisms underlying *Un8*-mediated loose smut resistance. In Chapter 5, a simple and reliable diagnostic method was developed to examine the infection processes of *U. nuda* within barley seeds and it was found that the early seedling stage might be the most important time point for *Un8*-conditioned loose smut resistance. Seedling mortality was also observed in resistant and susceptible lines which led to questions as to whether this was a function of the high inoculum concentration used to evaluate disease response. To further investigate this resistance, the commonly used inoculation method was improved by reducing the inoculum concentration (Chapter 6). During this study a large fitness cost (i.e. seedling mortality) previously observed was associated only with resistant lines carrying the *Un8* gene. In the final study (Chapter 7), expression analysis was undertaken to better understand *Un8*-mediated resistance and seedling mortality observed. Two barley genes involved in cytokinin regulation, *CKX1* and *CKX2.1*, which encode cytokinin oxidase/dehydrogenase (CKX) enzymes to irreversibly degrade cytokinins, were significantly up-regulated at time points that coincided with early seed germination. This indicated that the cytokinin pathway may be involved in the loose smut resistance conditioned by the *Un8* gene.

Taken together, this study has provided deeper insight into the long-lived *Un8* loose smut resistance gene, including a possible role for cytokinins in barley loose smut resistance.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
AFB	Auxin signaling F-box
AOS	Allene oxide synthase
ATG	Autophagy
BAC	Bacterial artificial chromosome
BAK1	Brassinosteroid insensitive 1-associated receptor-like kinase 1
BIN	Brassinosteroid-insensitive
bp	Base pairs
BR	Brassinosteroid
BZR	Brassinazole resistant
CAPS	Cleaved amplified polymorphic sequence
Cas9	CRISPR-associated protein 9
CC	Coiled coil
CDC	Crop Development Centre
CDD	Conserved domain database
CK	Cytokinin
CKX	Cytokinin oxidase/dehydrogenase
cM	CentiMorgan
CRISPR	Clustered regularly interspaced short palindromic repeats
CTAB	Cetyltrimethylammonium bromide
cv.	Cultivar
DH	Doubled-haploid
Dpi	Days post-inoculation
Dps	Days post-sowing
EFR	EF-Tu receptor
EF-Tu	Elongation factor Tu
ET	Ethylene
ETI	Effector triggered immunity
FLS2	FLAGELLIN SENSING 2
FPC	Fingerprinted contigs
GA	Gibberellin
Gb	Gigabases
GBS	Genotyping-by-sequencing
GH3.2	Gretchen Hagen 3.2
Hb	*Hordeum bulbosum* L.
Hs	*Hordeum vulgare* ssp. *spontaneum* C. Koch.
Hv	*Hordeum vulgare ssp. vulgare* L.
IAA	Indole-3-acetic acid
Indel	Insertion/deletion
ITS	Internal transcribed spacer

JA	Jasmonate acid
JAK	Janus kinase
JAZ	Jasmonate ZIM-domain
Kb	Kilobase
LOX	Lipoxygenase isozyme
LRR	Leucine-rich repeat
MKK3	Mitogen-activated protein kinase kinase 3
MMAS	Molecular marker-assisted selection
MTP	Minimum tiling path
Mya	Million years ago
NaOH	Sodium hydroxide
NBS	Nucleotide binding site domain
NGS	Next-generation sequencing
NLS	Nuclear localization signal
NPR1	Non-expressor of pathogenesis-related genes 1
OPR	Oxophytodienoate reductase
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PR	Pathogenesis-related
PRRs	Pattern recognition receptors
Pst	*Pseudomonas syringae* pathovar (pv.) *tomato*
PTI	PAMP triggered immunity
qRT-PCR	Quantitative real-time PCR
QTL	Quantitative trait loci
R	Resistance gene
R	Resistance protein
RAD-seq	Restriction-site associated DNA sequencing
RFLP	Restriction fragment length polymorphism
RIL	Recombinant inbred line
SA	Salicylic acid
SLN1	Slender 1
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeat
TILLING	Targeting induced local lesions in genomes
TIR	Toll-interleukin-1-receptor
TOR	Target of rapamycin
TrD	Transmembrane domain
TuBA	Alpha tubulin
UTR	Untranslated region
WRKY	W=Tryptophan, R=Arginine, K=Lysine, Y=Tyrosine
β-glucan	Beta-glucan

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CHAPTER 1

General Introduction

1.1 Background

Cultivated barley (*Hordeum vulgare* ssp. *vulgare* L.) (2n = 2x = 14) arose from its wild progenitor (*Hordeum vulgare* ssp. *spontaneum* C. Koch.) approximately 10,000 years ago (Badr et al. 2000) and is used mainly for animal feeding and malting. It is one of the founder crops of Old World agriculture for Neolithic food production with excellent adaptation to different agro-ecological zones and is the fourth most important cereal crop after maize, rice, and wheat (Badr et al. 2000; Mayer et al. 2012). Due to its diploid nature and suite of genomic tools, including a draft genome sequence (Mayer et al. 2012; Mascher et al. 2017), cultivated barley is considered a good model for genomic studies of other Triticeae crops, including polyploid wheat and rye (Schulte et al. 2009).

In Canada, barley is the second most important cereal crop after wheat in terms of production, consumption, and exports. Alberta and Saskatchewan are the two main production areas and account for 51% and 34%, respectively, of total barley production in Canada (Statistics Canada, 2006 - 2015 average). Barley can be classified into two- and six-rowed barley according to the arrangement of fertile spikelets within the head, and into food, feed, and malting classes based on end use. In Saskatchewan, since 1980, 56 barley cultivars have been released by the Crop Development Centre (CDC) within the Department of Plant Sciences at the University of Saskatchewan.

Within Canada, barley is infected by a number of important diseases, such as net blotch (*Pyrenophora teres* Drechs.), spot blotch (*Cochliobolus sativus* (S. Ito & Kurib.) Drechsler ex Dastur), Fusarium head blight (*Fusarium graminearum* Schwabe), powdery mildew (*Blumeria graminis* (DC) Speer f. sp. *hordei* EM. Marchal), stem rust (*Puccinia graminis* Pers.:Pers. f. sp. *tritici* Eriks. & E. Henn. and *Puccinia graminis* Pers.:Pers. f. sp. *secalis* Eriks. & E. Henn.), scald (*Rhynchosporium commune* (Oudem.) J. J. Davis) and several smuts. Barley smuts are easily recognized, destructive seed-borne diseases (Thomas

1997; Menzies et al. 2014) that are classified into three distinct diseases, covered smut, false loose smut, and loose smut, caused by *Ustilago hordei* (Pers.) Lagerh (*U. hordei*), *Ustilago nigra* Tapke (*U. nigra*), and *Ustilago nuda* (Jens.) Rostr (*U. nuda*), respectively. Yield losses caused by smut pathogens are directly proportional to the percentage of infected heads in which seeds will be completely replaced by black teliospores during flower development (Thomas 1997). It is assumed that smut diseases were introduced to Canada on seed imported by European settlers and initially caused great losses in barley production before the development of effective controls, such as fungicide-based seed treatments and smut resistance breeding (Menzies et al. 2014).

Loose smut of barley is a common seed-borne disease in the prairie provinces of Canada that can be found in about 50% of barley fields (Menzies et al. 2014) and is the only smut disease which infects the developing barley kernel. Loose smut can be effectively controlled by growing cultivars carrying loose smut resistance (*Un* genes) and also by the use of systemic seed treatment fungicides. Yield losses in barley attributable to loose smut are generally less than 1% (Thomas 1997; Menzies et al. 2014). As a result, there have been only a few studies of loose smut over the past few decades. However, there is currently renewed interest in research of seed-borne diseases because of the development of fungicide resistance and the increased prevalence of organic farming, where the utilization of fungicides to treat seed is not allowed. For these reasons growing resistant cultivars is preferred.

Plant hormones, also known as phytohormones, are small organic molecules that regulate almost every aspect of plant growth, development, reproduction, and immune responses at low concentrations (Bari and Jones 2009; Pieterse et al. 2009). The roles of jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) in plant immunity have been well established. JA and ET are primarily involved in defense against necrotrophic pathogens, whereas SA plays a critical role in defense against biotrophic pathogens, and JA/ET and SA signaling pathways are generally antagonistic to each other (Pieterse et al. 2009). In addition, other phytohormones such as auxins, brassinosteroids, cytokinins, and gibberellins have also been found to regulate plant defense (Bari and Jones 2009; Naseem et al. 2014). Interestingly, plant pathogens have the ability to synthesize compounds that are similar to plant hormones

or have the ability to manipulate phytohormone pathways to facilitate their infection (Kazan and Lyons 2014; Chanclud and Morel 2016). For example, the corn smut pathogen *Ustilago maydis* is able to produce CKs which act as virulence factors (Morrison et al. 2017).

With the advent of genomics, many disease resistance (*R*) genes have been isolated, providing better insight into their function and evolution. In barley, the first disease resistance gene isolated by positional cloning was the *Mlo* powdery mildew resistance locus (Büschges et al. 1997). Other resistance genes, such as the stem rust-resistance gene *Rpg1* (Brueggeman et al. 2002) and the leaf stripe resistance gene *Rdg2a* (Bulgarelli et al. 2010), have also been isolated by the same approach. The barley loose smut resistance gene *Un8* has been used in barley breeding in Canada for over 60 years and genetic mapping of the *Un8* locus was initiated in early 1990s. In the current work, attempts were made to isolate the *Un8* gene to better understand this durable resistance and investigate mechanisms underlying the *Un8*-mediated resistance.

1.2 Research hypotheses

i. There is a high degree of synteny among barley, rice and Brachypodium, in the *Un8* region which can be exploited to isolate the *Un8* gene;

ii. *U. nuda* infection is completely inhibited in resistant lines at the early seedling stage;

iii. Resistance to *U. nuda* is mediated via the cytokinin pathway.

1.3 Objectives

i. To isolate a *Un8* candidate gene using an F4 recombinant inbred line (RIL) population derived from the cross TR09398 (Resistant) × TR07728 (Susceptible);

ii. To characterize alleles of the *Un8* candidate gene from cultivated and wild barley;

iii. To study the infection processes to understand the distribution of *U. nuda* mycelia present in mature embryos obtained from resistant and susceptible lines after inoculation;

iv. To identify the growing stages and plant tissues associated with *Un8* resistance to *U. nuda*;

v. To understand the defense pathway associated with *Un8* resistance.

CHAPTER 2

Literature Review

2.1 Barley

The genus *Hordeum* is mainly characterized by the presence of a three-flowered spikelet at each rachis joint of the inflorescence. *Hordeum* species belong to one of the most economically important plant groups on Earth, the tribe Triticeae in the grass family Poaceae (von Bothmer and Komatsuda 2011) along with wheat (*Triticum*, several species), rye (*Secale cereale* L.), and triticale (*Triticosecale* Wittm.). Several valuable perennial forage grasses, such as crested wheatgrass (*Agropyron cristatum* (L.) Gaertn.), intermediate wheatgrass (*Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey), and Russian wildrye (*Psathyrostachys juncea* (Fisch.) Nevski), are also placed in this tribe (Asay 1992; von Bothmer and Komatsuda 2011). All species have large genomes with a basic chromosome number x = 7, for example barley (2n = 2x = 14, 5.1 gigabases (Gb)), rye (2n = 2x = 14, 8.1 Gb), and bread wheat (*Triticum aestivum* L.) (2n = 6x = 42, 16 Gb) (von Bothmer and Komatsuda 2011; Mayer et al. 2012; Martis et al. 2013; Chapman et al. 2015). Given the self-compatible diploid nature and diversified genetic stocks, barley has been proposed as an ideal model in genomic studies of other Triticeae crops (Schulte et al. 2009). Divergence of the Avenae tribe (oat) and Triticeae (barley and wheat) is estimated to have occurred approximately 25 million years ago (Mya), with genera *Hordeum* and *Triticum* diverging around 13 Mya (Gaut 2002).

Within the genus *Hordeum*, cultivated barley (*Hordeum vulgare ssp. vulgare* L.; subsequently *Hv*) is the most economically important species (Baik and Ullrich 2008; von Bothmer and Komatsuda 2011). Two- and six-rowed barley are the two basic types that are differentiated based on spikelet morphological differences in which the two lateral spikelets are fertile in six-rowed barley and sterile in two-row barley. Hulless (or naked) barley differs from hulled (or covered) barley by the easily separable lemma and palea upon threshing. Differences in other characters, such as growing season and starch composition, are also used

to distinguish cultivars giving rise to spring or winter barley and waxy or normal barley (Pourkheirandish and Komatsuda 2007; Baik and Ullrich 2008).

2.1.1 Barley and its related species

The genus *Hordeum* is widely distributed over a large geographic area throughout the world and some species, such as *H. jubatum* L., *H. marinum* Huds., and *H. murinum* L., are common weeds (von Bothmer and Komatsuda 2011). The genus *Hordeum* comprises 31 species, including diploid (2n = 2x = 14), tetraploid (2n = 4x = 28), and hexaploid (2n = 6x = 42) forms (von Bothmer and Komatsuda 2011), and all species can be classified into four different haplomes based on meiotic chromosome behavior in interspecific hybrids, with the **H** haplome in *Hv*, *H. vulgare* ssp. *spontaneum* C. Koch. (subsequently *Hs*) and *H. bulbosum* L. (subsequently *Hb*), $\mathbf{X_u}$ haplome in *H. murinum* L., $\mathbf{X_a}$ haplome in *H. marinum* Huds., and **I** haplome in all other *Hordeum* species (Blattner 2009).

Transferring desirable agronomic characteristics from other *Hordeum* species to cultivated barley is an important aspect of barley breeding. *Hordeum* genetic resources are classified into three different genetic pools based on their relation to cultivated barley and feasibility of gene transfer (von Bothmer and Komatsuda 2011). The primary gene pool of barley consists of closely related species including cultivars, landraces, breeding lines, and genetic stocks, together with the ancestral form of domesticated barley *Hs*. Hybridization of *Hv* with other members of the primary gene pool show almost no sterility barriers for gene transfer (von Bothmer and Komatsuda 2011). Identification and incorporation of desirable new alleles from *Hs* into *Hv* has often been used by breeders. Accessions of *Hs* have been utilized to increase *Hv* performance, especially with respect to disease resistance (Dreiseitl and Bockelman 2003; Roy et al. 2010; Friedt et al. 2011; Dreiseitl 2014).

Barley's secondary gene pool contains only *Hb* which occurs as both diploid and tetraploid forms (von Bothmer and Komatsuda 2011). Gene transfer from *Hb* to *Hv* is possible, with some difficulty because *Hb* shows partial incompatibility with *Hv*, but this can be overcome by modifying environmental conditions and using specific genotypes (Pickering 1984). *Hb* is well-established for producing doubled haploid plants based on the selective

elimination of the *Hb* chromosomes after crossing with *Hv* (Kasha and Kao 1970) and is of particular interest as a source of various disease resistances (Scholz et al. 2009; Hickey et al. 2011; Johnston et al. 2013; Wendler et al. 2015).

All other wild species constitute the tertiary gene pool of barley. The potential of the tertiary gene pool for barley improvement, unfortunately, is very limited because of the strong reproductive barriers present between this gene pool and *Hv* (von Bothmer and Komatsuda 2011). However, some exceptions exist, such as *H. lechleri* which has very good compatibility with *Hv* (von Bothmer et al. 1983). Successful applications have been achieved in wheat breeding programs using the diploid wild barley *H. chilense*, which has good compatibility with both durum and bread wheat and has resulted in the creation of the synthetic cereal species, *Tritordeum*, created by crossing *H. chilense* with tetraploid durum wheat (Martín et al. 1999). *H. marinum* also has attracted attention as a potential gene donor for wheat breeding due to its excellent stress tolerance and compatibility with wheat (Munns et al. 2011).

Table 2.1 Taxa of the genus *Hordeum* (Blattner 2009; von Bothmer and Komatsuda 2011).

Taxon	Subspecies	Ploidy[a]	Life Form[b]	Haploid Genome
Section *Vulgare*				
H. vulgare L.	*vulgare*	2x	a	H
	spontaneum	2x	a	H
H. bulbosum L.		2x, 4x	p	H, HH
H. murinum L.	*glaucum*	2x	a	Xu
	murinum	4x	a	XuXu
	leporinum	4x, 6x	a	XuXu, XuXuXu
Section *Anisolepis*				
H. chilense Roemer & Schultes		2x	p	I
H. cordobense Bothmer *et al.*		2x	p	I
H. euclaston Steudel		2x	a	I
H. flexuosum Steudel		2x	a/p	I
H. intercedens Nevski		2x	a	I
H. muticum Presl		2x	p	I
H. pusillum Nuttal		2x	a	I
H. stenostachys Godron		2x	p	I
Section *Critesion*				
H. comosum Presl		2x	p	I
H. pubiflorum Hooker f.	*pubiflorum*	2x	p	I
	breviaristatum	2x	p	I
H. jubatum L.		4x	p	II

Table 2.1. (continued).

Taxon	Subspecies	Ploidy[a]	Life Form[b]	Haploid Genome
Section *Critesion*				
H. arizonicum Covas		6x	a/p	III
H. lechleri (Steudel) Schenck		6x	p	III
H. procerum Nevski		6x	p	III
Section *Stenostachys*				
H. marinum Hudson	*marinum*	2x	a	Xa
	gussoneanum	2x, 4x	a	Xa, XaXa
H. bogdanii Wilensky		2x	p	I
H. roshevitzii Bowden		2x	p	I
H. erectifolium Bothmer *et al.*		2x	p	I
H. patagonicum (Haumann) Covas	*magellanicum*	2x	p	I
	mustersii	2x	p	I
	patogonicum	2x	p	I
	setifolium	2x	p	I
	santacrucense	2x	p	I
H. capense Thunberg		4x	p	IXa
H. depressum (Scribn. & Sm.) Rydb.		4x	a	II
H. fuegianum Bothmer *et al.*		4x	p	II
H. guatemalense Bothmer *et al.*		4x	p	II
H. secalinum Schreber		4x	p	IXa
H. tetraploidum Covas		4x	p	II
H. brachyantherum Nevski	*californicum*	2x	p	I
	brachyantherum	4x, 6x	p	II, IIXa
H. brevisubulatum (Trinius) Link	*brevisubulatum*	2x, 4x	p	I, II
	nevskianum	2x, 4x	p	I, II
	violaceum	2x, 4x	p	I, II
	iranicum	4x, 6x	p	II, III
	turkestanicum	4x, 6x	p	II, III
H. parodii Covas		6x	p	III

[a]x = 7;

[b]a = annual; p = perennial.

2.1.2 Origin and adaptation of cultivated barley

Barley was among the first domesticated cereal grains, along with einkorn and emmer wheat, to be used as human food in the Fertile Crescent area of the Near East (Badr et al. 2000). From archaeological evidence, the earliest remains of barley grains date back as far as 10,000 years ago (Badr et al. 2000). In recent years, studies have pointed out that an area approximately 1,500 to 3,000 km further east of the Fertile Crescent (Morrell and Clegg 2007) and the Tibet Plateau (Dai et al. 2012) have also contributed to barley domestication.

The intermediate ancestor of *Hv*, *H. vulgare* ssp. *spontaneum* (C. Koch) Thell., was discovered by the German botanist Carl Koch in Turkey who regarded it as a separate

species, however it is now considered to be a subspecies (Komatsuda 2014). Populations of *Hs* are still widely distributed in the Near East Fertile Crescent area, one of the domestication centres for *Hv* (Badr et al. 2000). However, the true progenitor of *Hv* is still debated and *Hs* might be derived from its ancestors, perhaps *Hb* or *H. murinum* with developed lateral florets (Zhou 2010). *Hs* is an annual species with the same diploid genome as *Hv* and displays a large amount of genetic diversity. Moreover, it is easy to transfer desirable genes to *Hv* from *Hs* because they are fully interfertile (Nevo et al. 1979; Jakob et al. 2014). The main feature differentiating *Hs* from *Hv* is the brittle rachis of *Hs* which allows mature spikelets to easily shatter during maturation (Pourkheirandish et al. 2015).

Barley is a short-season and early-maturing crop known as 'the last crop before the desert' (Russell et al. 2016). It is adapted to a wide range of environments and cultivated in regions with higher latitudes and altitudes, and farther into deserts than other cereal crops (Ullrich 2011). For example, barley is well adapted to the harsh weather conditions in the Mediterranean area where low rainfall and cold winter temperatures are common (van Oosterom and Acevedo 1992). Barley can thrive further south toward the Sahara where it is more competitive than drought-tolerant durum wheat (Ullrich 2011). In the highlands of the Tibetan Plateau, hulless barley has been the staple food for millennia (d'Alpoim Guedes et al. 2015). A number of genes associated with barley's excellent ability to tolerate diverse environmental stresses have been identified, such as *CBF* genes for cold and frost tolerance, *Dhn* genes for drought tolerance, *HSP17.8* for heat stress tolerance, and *HvCEN* for flowering responses (Dawson et al. 2015; Russell et al. 2016).

2.1.3 Genetic basis for barley domestication

Prior to the advent of human settlements, foraging for wild cereals, as well as seeds and nuts, was a common activity. During the shift to cultivation from foraging, only a few plant species were chosen as food crops and the resulting human intervention introduced a number of changes as part of the plant domestication process (Purugganan and Fuller 2009). Barley domestication involved the selection for three key traits, non-brittle rachis, six-rowed head, and naked caryopsis. Seed dormancy was also reduced during this process (Salamini et al. 2002). Modifications to vernalization requirement and photoperiod response by mutations

and recombination accelerated the spread of barley to different geographic areas far from its original place of domestication (Pourkheirandish and Komatsuda 2007).

2.1.3.1 Non-brittle rachis

A very critical step in the domestication process is the modification of the seed dispersal system. In *Hs*, interruption of specific abscission zones at each rachis node greatly contributes to seed dispersal during maturation, however this makes grain harvesting difficult (Haberer and Mayer 2015). Histological analysis of the rachis at anthesis shows an expansion of five to six cell layers with dramatically reduced thickness of primary and secondary cell walls in *Hs* which facilitates the formation of brittle rachis (Pourkheirandish et al. 2015). The non-brittle rachis is achieved by a recessive mutation in one of two genes, *Btr1* and *Btr2* on chromosome 3HS, preventing rachis nodes from forming such expanded cells and thereby retaining grain on the head after maturity (Pourkheirandish et al. 2015).

2.1.3.2 Six-rowed head

The barley head is composed of three single-flowered spikelets (one central and two lateral), named triplets, attached alternately at each rachis node. The identity of either the two-rowed type or the six-rowed type is determined according to fertility differences of the lateral spikelets (Komatsuda et al. 2007). In six-rowed barley, all three spikelets are fully fertile and are able to develop into grains, while both lateral spikelets are sterile in two-rowed barley (Komatsuda et al. 2007; Koppolu et al. 2013). *Hs* and early *Hv* were two-rowed types and the arrow-shaped spikelet facilitated burial in the soil (Pourkheirandish and Komatsuda 2007), while six-rowed barley did not come into cultivation until 8,800-8,000 years ago (Komatsuda et al. 2007). Fertility of the two lateral spikelets in the six-rowed head is determined by at least five independent recessive alleles, the naturally occurring *six-rowed spike1* (*vrs1*) and *intermedium-c* (*int-c* or *vrs5*) and artificially induced mutants *vrs2*, *vrs3*, and *vrs4*, located on chromosomes 2HL, 4HS, 5HL, 1HL, and 3HS, respectively (Lundqvist et al. 1997; Koppolu et al. 2013; Muñoz-Amatriaín et al. 2014).

2.1.3.3 Naked caryopsis

Wild and most cultivated barleys have a hulled caryopsis and barley cultivars with weaker attachment of the hull (lemma and palea) to the caryopsis are known as naked or hulless barley. Hulless barley first occurred around 8,000 years ago at Ali Kosh in Iran (Newman and Newman 2006; Pourkheirandish and Komatsuda 2007; Taketa et al. 2008). The covered/naked trait is under the control of the *Nud* locus on chromosome 7HL located in a ~17 Kb (kilobase) region which is completely deleted in naked barley (Taketa et al. 2008). However, in collections from Tibet, Yu et al. (2016) found a novel *Nud* allele (*nud1.g*) in three naked barleys. The main difference between *Nud* and *nud1.g* is a non-synonymous SNP in *nud1.g* presumably resulting in the structural change of Nud, thereby converting the caryopsis type. The *Nud* gene encodes a transcription factor belonging to the ethylene response factor family which regulates a lipid biosynthesis pathway and is expressed predominantly in the testa of the ventral side of the caryopsis, thus affecting hull adhesion (Taketa et al. 2008).

2.1.3.4 Reduced dormancy

Seed dormancy has generally been reduced by selection for weak dormancy which has led to uniform emergence, but is also associated with pre-harvest sprouting (Derera 1989; Nakamura et al. 2016). Uniform germination is essential during the malting process where dormancy is not desirable. However, stringent selection against seed dormancy can produce barley cultivars susceptible to the preharvest sprouting resulting in lower grain quality (Prada et al. 2004). Barley seed dormancy is a quantitative trait and many quantitative trait loci (QTL) have been identified. Among them, two common QTL, *SD1* and *SD2* both located on chromosome 5H, are believed to play important roles in determining seed dormancy. *SD1* is the major QTL locus which explains much of the phenotypic variance observed for seed dormancy (Sato et al. 2016a). The recently isolated gene designated as *Qsd2-AK* for the *SD2* locus is a *Mitogen-activated protein kinase kinase 3* (*MKK3*) (Nakamura et al. 2016).

2.1.3.5 Vernalization requirement and photoperiod insensitivity

Hs is generally regarded as having a winter growth habit requiring vernalization and long-day conditions to induce reproductive growth (von Bothmer and Komatsuda 2011; Comadran et

al. 2012). Time of flowering is the most critical factor for reproductive success during barley's spread to different geographic areas and is mainly associated with the interaction between vernalization (prolonged exposure to low temperatures) and photoperiod (day length) (Pourkheirandish and Komatsuda 2007; Nitcher et al. 2013). Many genes are involved in flowering regulation, with *VRN-H1*, *VRN-H2*, and *VRN-H3* affecting vernalization and *PPD-H1* and *PPD-H2* responding to photoperiod (Dawson et al. 2015). Allelic variation of *HvCEN* is also associated with differences in flowering time (Comadran et al. 2012).

2.2 Barley end-uses and production in Canada

2.2.1 Barley end-uses

Barley is a multi-purpose crop grown primarily as a feed-grain for animals and grain for malting. Approximately two-thirds of production in Canada is used for animal feed and one-third for malting, with a very small amount used for human food (Baik and Ullrich 2008). As a versatile and high-quality feed crop, barley grain is commonly utilized in the diet of animals, such as cattle, poultry, and swine (Blake et al. 2011). Barley cultivars suitable for fish feed have also been developed (Bregitzer et al. 2007). In North America, most of the barley fed to cattle is malting barley that fails to produce high quality malt (Blake et al. 2011). Barley grain typically contains a large amount of soluble fiber called beta-glucan (β-glucan) that can bind water in the intestine and increase digesta viscosity, thereby affecting the absorption of nutrients. β-glucan can be hydrolyzed by two cellulases: 1,3-1,4-β-glucanase and 1,4-β-glucanase (Fernandes et al. 2016). Studies have shown that addition of enzymes, such as 1,3-1,4-β-glucanase, to cleave the mixed linked β-glucan has positive outcomes on a barley-based diet by effectively reducing the degree of polymerization (Ribeiro et al. 2012). Fernandes et al. (2016) suggested that 1,3-1,4-β-glucanase improves the nutritive value of barley-based diets than 1,4-β-glucanase.

Green barley biomass is also harvested as forage for feeding purpose with preference for smooth-awned cultivars (Park et al. 2008). It has also been suggested that two-row barley is more suitable than six-row types for forage production in Western Canada (Gill et al. 2013). Hooded barley, which is characterized by the development of a modified awn on the

central floret bearing a rudimentary inverted floret (caused by a 305 base pairs (bp) duplication within an intron sequence of *Knox3* gene) (Müller et al. 1995; Osnato et al. 2010) has also found a niche use as forage.

The production of malt, predominantly for brewing beer (but also used in the production of whiskey), brings the largest value-added use for barley. The brewing industry needs high quality grain to produce malt which typically is composed of 60-65% starch and 10.5-13.5% protein (Schwarz and Li 2011). In North America the cv. 'Harrington', released by the CDC at the University of Saskatchewan in 1981 was the malt industry standard for two-rowed malting barley for 20 years (Bregitzer et al. 2007), but has since been replaced by cultivars such as 'AC Metcalfe' and 'CDC Copeland'. Among the many important parameters affecting malting quality are plumpness, grain protein content, malt extract percentage, ratio of wort soluble protein to total malt protein, diastatic power, α-amylase activity, wort β-glucan content, and dormancy (Zale et al 2000; Muñoz-Amatriaín et al. 2010; Mohammadi et al. 2015). Over 280 distinct QTL, located across all seven barley chromosomes, have been associated with malt quality (Wei et al. 2009). Recently, new loci for malt quality were detected by genome-wide association studies (Matthies et al. 2014; Mohammadi et al. 2015). Schmidt et al. (2016) suggested that genomic selection could be a valuable approach for malting barley breeding to increase selection efficiency.

Barley was historically used for human nutrition, but human consumption has decreased dramatically throughout the world with the increased exploitation of other crops (wheat and rice) in human diets. However, in some parts of the world, for example the highlands of Tibet, Ethiopia, and Andean countries, barley is still a major food source (Arendt and Zannini 2013). Even though barley is not a preferred food, it has a number of potential health benefits, including lowering coronary heart disease risk by decreasing cholesterol level, regulating blood glucose and insulin levels, and colon health (Wood 2007; Baik and Ullrich 2008; Baik et al. 2011). The popularity of barley grain and its components in various foods, such as flours for bread making, noodles, pasta, and soups, is growing in developed countries (Jilal et al. 2008; Baik et al. 2011).

2.2.2 Canadian barley production

Canada is currently the fourth largest barley producer after the Russian Federation, Germany, and France according to FAOSTAT (http://faostat3.fao.org/browse/Q/QC/E) (Fig. 2.1). In Canada, barley production ranks third among cereals after wheat and maize (Statistics Canada, CANSIM table 001-0010). During the last 10 years, the seeded area and production of barley have decreased in Canada due to competition from other crops, such as wheat, canola, corn, and pulses, but average yields have increased (Statistics Canada, CANSIM Table 001-0017) (Fig. 2.2). The major growing areas in Canada are the Prairie provinces, mainly Alberta and Saskatchewan, and to a lesser extent Manitoba. In recent years, these three provinces accounted for 51%, 34%, and 8%, respectively (Fig. 2.3) (Statistics Canada, CANSIM Table 001-0017).

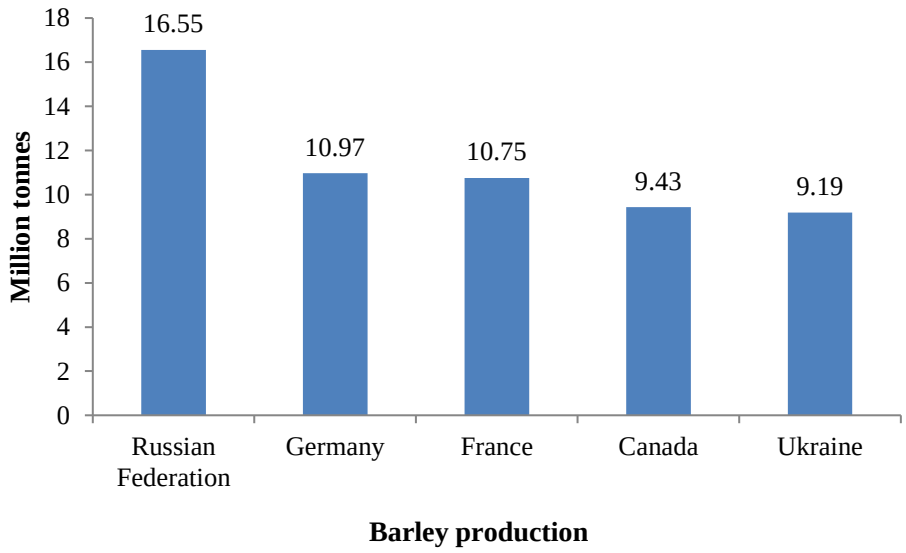

Fig. 2.1 Top five barley producers worldwide (ten year average from 2005-2014, FAOSTAT).

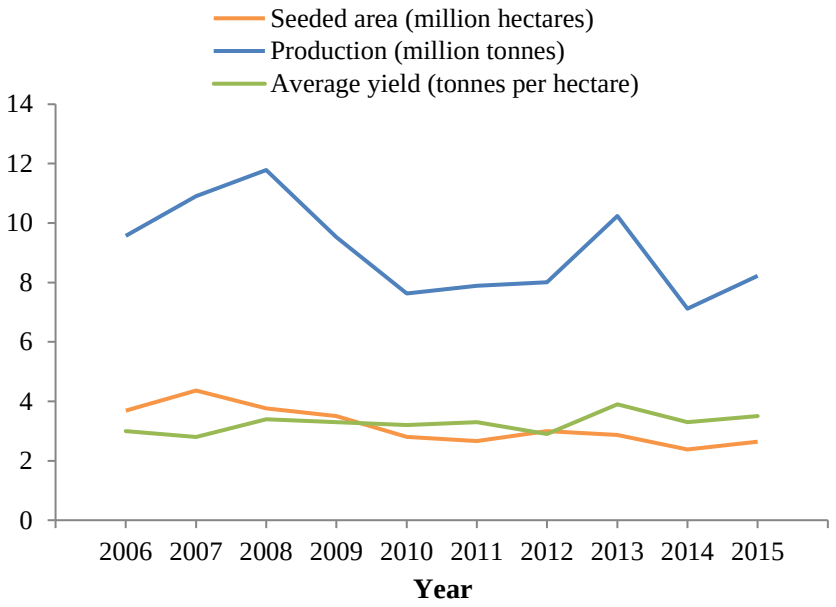

Fig. 2.2 Canadian barley seeded acres, production and yield by year (2006-2015, Statistics Canada).

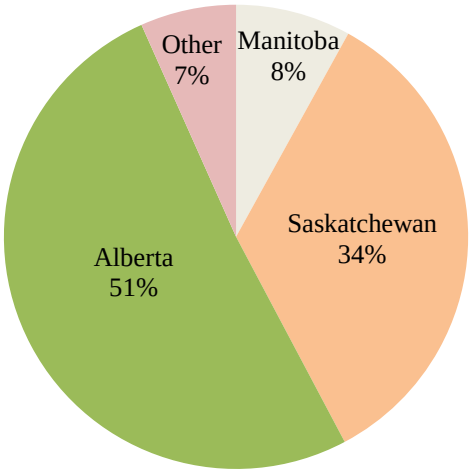

Fig. 2.3 Barley production within Canada (ten year average from 2006-2015, Statistics Canada).

2.3 Loose smut of barley

2.3.1 The life cycle of barley loose smut

The life-cycle of loose smut has been described in detail previously (Malik and Batts 1960a; Thomas 1997). Upon germination, the mycelium is present within the embryo and passes to

the crown node near the soil surface, penetrates the node and then enters the growing point of the tillers. During the elongation of successive internodes, the process of infection, beginning from the lowest node, keeps pace with the growing point. Normally, the fungus can invade all young tissues within the inflorescence (head), except the rachis, and may even sporulate on the flag leaves (Malik and Batts 1960b). As the head develops, hyphae differentiate and transform into masses of teliospores. The growing spore masses come together and when the head emerges the smutted florets can be distinguished. Between heading and maturity, the symptoms of loose smut are clearly observable because the diseased heads are initially dark brown and clearly visible among the green heads of unaffected plants. By maturation, the heads are entirely transformed into a dry, olive brown spore mass except for a delicate pericarp membrane which encloses teliospore-bearing sori and ruptures shortly after the heads emerge. Teliospores are subsequently dispersed and blown into nearby open florets by wind. As a result, within a few days, only the uninfected rachis remains (although deformed awns may remain on some heads). Lastly, teliospores from infected heads germinate after landing in open florets and the dikaryotic infecting hyphae penetrate through the ovary wall progressing into the developing embryo to establish in the scutellum and embryo.

2.3.2 Management of loose smut of barley

2.3.2.1 Chemical control and fungicide-tolerant isolates of loose smut

Loose smut can be controlled effectively by fungicidal seed treatments with systemic fungicides, such as carboxin (5, 6-dihydro-2-methyl-1, 4-oxathiin-3-carboxanilide) (von Schmeling and Kulka 1966), or through a foliar application of a systemic fungicide, such as triadimefon (Jones 1997). To avoid the risk of loose smut developing tolerance or resistance to carboxin, a fungicide rotation scheme has been suggested consisting of carboxin seed treatments alternated with ergosterol demethylation inihibitors (Menzies et al. 2005).

Carboxin, which can stop the mycelium growing within the infected embryo and was developed in the 1960s, has been the most popular systemic fungicide worldwide for controlling loose smut and reduces this disease to low levels (von Schmeling and Kulka 1966; Menzies 2008). However, the long-term use of a fungicide increases the incidence of

fungicide-tolerant or resistant isolates of loose smut. Leroux (1986) suggested that the induction of carboxin-resistant isolates by the use of carboxin as a seed treatment to control loose smut would occur rapidly. On winter barley, Leroux and Berthier (1988) identified carboxin- and fenfuram-resistant isolates of loose smut in France. To identify fungicide resistant isolates, plants of the susceptible cv. 'Regal' were inoculated with 20 different loose smut isolates from Europe and Canada and the harvested seeds treated with carboxin to identify the carboxin-resistant isolates. A second study was conducted using the same 20 isolates to determine if carboxin-resistant isolates could be identified by the use of an *in vitro* test alone and it was found that the carboxin-resistant and susceptible isolates could effectively be distinguished at a fungicide concentration of 0.1 µg/ml (Newcombe and Thomas 1991). Menzies et al. (2005) reported that an isolate of loose smut (97-255) collected from central Italy showed more carboxin-resistance than a wild type isolate (72-66) collected from Canada. Using a teliospore germination assay on carboxin-amended media, isolate 99-204 from Manitoba and 99-32B from Saskatchewan showed resistance to carboxin (Menzies 2008). Subsequently, an *in planta* assay was conducted using the susceptible cv. 'Regal' to quantify fungicide resistance in these two isolates. After being inoculated with loose smut isolates 99-204 and 99-32B from Saskatchewan, the seeds were then treated with carboxin at two concentrations using Vitavax Single Solution. One was the recommended concentration at 240 ml/100 kg seed (55 g active ingredient carboxin/100 kg seed, equivalent to 2.3×10^5 µg/ml) and the other was twice the recommended concentration at 110 g active ingredient carboxin/100 kg seed. There was no significant difference between the carboxin-treated and untreated plants under the recommended rate in the percentage of smutted heads produced from treated seeds using either isolates. However, significantly fewer smutted heads were produce with isolate 99-204 compared to 99-32B after being treated with the higher concentration of carboxin, suggesting that 99-32B was much more resistant to carboxin than 99-204 (Menzies 2008).

2.3.2.2 Control through genetic disease resistance

The most economical and preferred way to control loose smut and avoid fungicide resistance is to grow cultivars with genetic resistance. Prior to 1949, in North America the dominant

cultivar used as a source of genetic resistance in barley breeding programs was cv. 'Trebi' (C.I. 936) (Tapke 1955). Resistance in cv. 'Trebi' was governed by a single dominant gene *Un* (Livingston 1942; Robertson et al. 1947). In cv. 'Missouri Early Beardless', Livingston (1942) identified a weak gene for resistance and subsequently designated it as *Un2*. Two-independent dominant genes, *Un3* (Schaller 1949) and *Un6* (Skoropad and Johnson 1952), were found in cv. 'Jet' (C.I. 967) which provided resistance to isolates avirulent on cv. 'Trebi' (Tr) and virulent on cv. 'Trebi' (Ts). In cv. 'Dorsett' (C.I. 4821) and a hybrid (X 173-10-5-6-1), Schaller (1949) identified two dominant genes, *Un4* and *Un5*, respectively. The resistance of cv. 'Anoidium' (C.I. 7269) to a smut isolate was governed by a single recessive gene (Andrews 1956) and was assigned the symbol *Un7*. Metcalfe and Johnston (1963) identified a new single dominant gene governing resistance to loose smut in accession PR28, derived from the winter barley line C.I. 4966. Metcalfe (1966) assigned the symbol *Un8* to this gene. *Un8* gene was mapped to barley chromosome 1H and has been the most effective and long-lived resistance against most known isolates of loose smut in Western Canada (Eckstein et al. 1993; Eckstein et al. 2002).

Other loose smut resistance genes have been found, such as *Un11*, *Un12*, *Un13*, and *Un15* (Mueller 2006). Recently, a loose smut resistance gene in the cv. 'Morex' was mapped to chromosome 3H using the mapping population 'Steptoe' × 'Morex' (Menzies et al. 2010), but it was uncertain whether this gene was identical with *Un6* which was derived from cv. 'Jet' and previously mapped on chromosome 3HL (Pomortsev et al. 2000). Thomas and Metcalfe (1984) identified two loose smut resistant lines (C.I. 9973 and C.I. 14099) from Ethiopia, in which the gene(s) responsible for resistance differed from *Un8*. After artificially inoculating 23 spring barley accessions with eleven populations of loose smut from Europe, Mueller (2006) found that these loose smut populations were virulent on all accessions except 'Jet' which contained the *Un3* and *Un6* genes, 'CDC Freedom' with the *Un8* gene, CIho9973 with quantitative resistance, and 'Lino' and 'GangTuoQuingKeHao1' with undefined resistance genes. Compared with other susceptible accessions, K-19907 with the *Un13* gene and 'Roland' with the *Un15* gene showed a lower percentage of infected plants (Mueller 2006). Both dry conditions and higher temperatures were thought to enhance the resistance of the *Un12* gene (Mueller 2006).

2.3.3 Other loose smut species

False loose smut of barley caused by *U. nigra* closely resembles loose smut. However, false loose smut is a surface borne smut as teliospores are borne on the seed surface. It infects the barley plant by penetrating the seedling after germination. According to Chełkowski et al. (2003), only one gene (*Ung*) is known to confer resistance to false loose smut. By the time heads of infected plants emerge, the smutted heads have been replaced by a mass of dark-brown to olive-black spores which are eventually dispersed by the wind (Chełkowski et al. 2003). *Ustilago avenae* (Pers.) Rostr. is a pathogen that causes loose smut of oat, and like false loose smut, is surface borne (Menzies et al. 2003). While *Ustilago tritici* (Pers.) infects wheat causing similar disease symptoms to loose smut of barley and is also systemic, there are some differences between them. For example, the penetrating hyphae of loose smut of barley are much finer and can frequently be seen in endosperm, while the hyphae of wheat loose smut rarely penetrate this tissue (Malik and Batts 1960c). Another difference is that barley loose smut can enter the embryo from the ventral side of the grain while wheat loose smut is only found to enter the embryo from dorsal side of the grain (Batts 1955; Malik and Batts 1960c). As with barley loose smut, resistance to wheat loose smut is under monogenic control. Several resistance genes have been identified and localized in hexaploid wheat (Procunier et al. 1997). Three markers (one sequence characterized amplified regions (SCAR) marker and two restriction fragment length polymorphism (RFLP) markers) were developed and linked to the loose smut resistance gene *Ut-X* (Procunier et al. 1997) on chromosome 2BL. In durum wheat the *Utd1* resistance gene was located to chromosome 5BS and several markers were developed in two durum wheat populations, DT662 × D93213 and 'Sceptre' × P9162-BJ08*B (Randhawa et al. 2009).

2.4 Barley genomics

Barley has a haploid genome size of 5.1 Gb with seven distinct chromosome pairs denoted 1H-7H (Linde-Laursen 1996). Sequencing the whole genome of this economically important crop is highly challenging. To assemble whole-genome shotgun sequence from reads (obtained by next-generation sequencing, NGS) into an ordered, overlapping and contiguous sequence, whole-genome framework tools such as genetic and physical maps will greatly

facilitate the process since a large proportion of repetitive DNA exists within the genome (~84%) and the short length of NGS reads introduce complexity into barley genome assembly (Mayer et al. 2012; Zhou et al. 2016).

2.4.1 Genetic maps for sequence assembly

High-density genetic maps have provided an important foundation for QTL mapping, marker-assisted selection, and physical map construction in barley. During the past few decades, a number of technologies have been applied to accelerate the development of genetic maps. Southern hybridization-based RFLP markers were firstly employed to construct first generation genetic maps for barley (Graner et al. 1991; Kleinhofs et al. 1993; Sherman et al. 1995). DNA markers, such as sequence-tagged site, SSR (simple sequence repeat), and Diversity Arrays Technology, based on the polymerase chain reaction (PCR) emerged soon afterwards to avoid the laborious procedures associated with RFLP markers (Stein 2014).

Illumina GoldenGate Single Nucleotide Polymorphism (SNP) assays, a high-throughput genotyping platform, were developed for barley and initially contained 2,943 SNPs located to 975 unique positions within the genome, which provided an opportunity to produce high density consensus maps covering larger proportions of the barley genome and assess genetic variation within germplasm pools (Close et al. 2009; Szücs et al. 2009; Muñoz-Amatriaín et al. 2011).

With the incorporation of Next Generation Sequencing technologies, the barley Infinium iSelect 9K chip was produced which consisted of 7,864 SNPs and has been widely used in barley genotyping, consensus map construction, and association studies (Comadran et al. 2012; Alqudah et al. 2014; Muñoz-Amatriaín et al. 2014; Maurer et al. 2015; Silvar et al. 2015). Based on NGS technology, two other methods of *de novo* SNP detection, restriction-site associated DNA sequencing (RAD-seq) and genotyping-by-sequencing (GBS), were developed to reduce genome complexity and significantly improve the efficiency of SNP identification (Chutimanitsakun et al. 2011; Poland et al. 2012; Honsdorf et al. 2014; Zhou et al. 2015).

2.4.2 Physical maps for sequence assembly

Although a number of software packages can be used for sequence assembly of NGS reads, it is still difficult to establish the correct linear order of contigs along individual chromosomes, especially for species with large and complex genomes without a framework of genome wide physical maps (Schulte et al. 2011; Mascher et al. 2013). The North American six-rowed spring malting cultivar 'Morex' was the first cultivar used to construct a bacterial artificial chromosome (BAC) library with 6.3× haploid genome coverage (Yu et al. 2000). An additional five Morex BAC libraries were generated, either by restriction enzyme digestion or random shearing, to provide sufficient coverage for whole genome physical map construction (Schulte et al. 2011).

571,000 BAC clones derived from the above six BAC libraries representing ~14-fold haploid genome coverage were used to construct the barley physical map by high-information-content fingerprinting and contig assembly (Mayer et al. 2012). This barley physical map comprised 9,265 BAC contigs with a cumulative length of 4.98 Gb (more than 95% of the barley genome) and could be represented by a minimum tiling path (MTP) of 67,000 BAC clones. Subsequently, by a newly developed population sequencing method (Mascher et al. 2013) for genetic anchoring of physical maps, a genome-wide physical map of the barley genome was constructed which contained more than half a million BAC clones and provided a framework ready for clone-by-clone sequencing of the barley genome (Ariyadasa et al. 2014).

2.4.3 Barley genome sequencing

The International Barley Sequencing Consortium initiated a project in 2006 aimed at developing a high quality reference sequence using NGS technologies (Schulte et al. 2009). On November 29, 2012, the International Barley Sequencing Consortium reached a milestone with the release of a sequence-enriched physical and genetic framework for cv. 'Morex', revealing that ~84% of the barley genome is comprised of repetitive DNA (Mayer et al. 2012). However, this initial barley genome assembly for cv. 'Morex' was highly fragmented with only 6,278 BACs sequenced within the physical map. Further efforts were made to

improve the reference sequence making the entire genome sequence accessible. Currently, a high-quality reference genome sequence of barley cv. 'Morex', including sequences in the pericentromeric region, has recently been released (Mascher et al. 2017).

In addition to the whole genome assemblies for 'Morex', 'Barke', and 'Bowman' (Mayer et al. 2012), two whole genome sequences for 'Haruna Nijo' (Sato et al. 2016b) and a Tibetan hulless barley (Zeng et al. 2015) have been released. Currently, a number of barley genomic resources can be accessed online, such as HarvEST (Close et al. 2004), IPK Blast Server (Mayer et al. 2012), and the Barley Draft Genome Explorer BARLEX (Colmsee et al. 2015).

2.5 Molecular marker-assisted selection (MMAS)

Molecular marker-assisted selection (MMAS) is genotype-based selection used to accelerate cultivar development in a breeding program. This method allows selection for traits with high heritability in early generations and pyramiding multiple desirable genes together into a single genotype at reduced cost (Collard and Mackill 2008; Miedaner and Korzun 2012). With MMAS, selecting desired lines is independent of large scale phenotypic tests once molecular markers are established for traits of interest (Collard and Mackill 2008). Xu and Crouch (2008) highlighted that MMAS might also be successfully employed in several breeding areas including traits that are difficult to evaluate and for backcrossing programs.

2.5.1 Molecular marker-assisted selection for barley disease resistance

Many studies have attempted to develop molecular markers for barley disease resistance genes such as the barley yellow mosaic virus complex (Tyrka et al. 2008; Sedlacek et al. 2010), leaf rust (Sedlacek and Stemberkova 2010), net blotch (Keiper et al. 2008), powdery mildew (Repkova et al. 2009; Sedlacek and Stemberkova 2010), and scald (Dizkirici et al. 2008). During fine mapping and gene cloning efforts some very tightly linked or intragenic markers for traits have been developed which have significantly increased reliability of MMAS, for example scald resistance genes *Rrs1* (Hofmann et al. 2013) and *Rrs2* (Hanemann et al. 2009), leaf stripe resistance gene *Rdg2a* (Bulgarelli et al. 2004; Bulgarelli et al. 2010),

leaf rust resistance gene *Rph20* (Hickey et al. 2011), and yellow mosaic virus disease resistance gene *rym11* (Lüpken et al. 2013; Yang et al. 2014).

Even though there are many publications on molecular markers, additional efforts are needed to increase the use of markers in breeding programs (Xu and Crouch 2008). In barley, about 50 genes can be routinely used for MMAS with the most successful applications being *Rpg1* resistance to stem rust (Steffenson and Smith 2006), *mlo* resistance to powdery mildew (Miedaner and Korzun 2012), *rym4*/*rym5* resistance to barley yellow mosaic virus complex (Miedaner and Korzun 2012), and *Un8* resistance to loose smut (Eckstein, personal communication). Other successful examples involve the resistance to cereal cyst nematode (Barr et al. 2000), stripe rust (Toojinda et al. 1998; Castro et al. 2003; Hayes et al. 2003), and spot form of net blotch (Eglinton et al. 2006).

2.5.2 Next-generation sequencing (NGS) for MMAS

Next-generation sequencing (NGS) technology is a very rapid strategy for genome-wide molecular marker discovery (Chutimanitsakun et al. 2011; Poland et al. 2012; Zhou et al. 2015). NGS can rapidly identify large numbers of SNPs and, moreover, can discover many markers in one sequencing run reducing the work of marker discovery (Yang et al. 2012a). Several NGS-based methodologies, such as reduced representation libraries, RAD-seq, and GBS have been established to reduce costs by sequencing a small part of the genome (Davey et al. 2011; Sonah et al. 2013).

Several recently published examples are selected here to emphasize the feasibility of NGS on MMAS. Within the grain legume crop *Lupinus angustifolius* L, Yang et al. (2012a) generated close to 40 molecular markers linked to the target gene and two flanking markers spanning the target gene. Yang et al. (2016) used NGS for the selection of flower sex, powdery mildew resistance and acylated anthocyanins in grape breeding. In rice, Zheng et al (2016) mapped the rice blast resistance gene *Pi65(t)* within a narrow genetic region and used linked markers to develop a new rice cultivar with both blast resistance and high yield. In barley, Liu et al. (2014) mapped genes associated with plant height and Honsdorf et al. (2014) greatly improved the genetic resolution of a number of QTL responsible for drought stress

tolerance. NGS has also been utilized to identify QTL for the rate of water uptake into barley grain (Cu et al. 2016).

2.5.3 Marker development for barley loose smut resistance

When evaluating loose smut resistance, florets must be individually hand-inoculated at early anthesis. This is very labor-intensive and time-consuming. The long life cycle of the fungus renders development of loose smut resistant cultivars difficult. Plants produced by the inoculated seeds cannot be assessed for the disease reaction until heading one generation later (Eckstein et al. 2002). In addition, false negatives (escapes) which result from the failure of artificial inoculation are not uncommon.

Disease escape is one of the most critical considerations with loose smut screening (Thomas and Metcalfe 1984). Unlike other barley smut diseases, *U. nuda* infects barley mainly at the flowering stage and the most accurate method to evaluate barley loose smut resistance is by direct inoculation of the floret (Menzies et al. 2009), even though it is tedious and time-consuming compared with seedling inoculation (Jones and Dhitaphychit 1991). For floret inoculation, timing is extremely important and the inoculation must be completed within few days during early anthesis to achieve a high infection rate (Oort 1939; Menzies et al. 2009). However, the infection rate by this method is still not highly efficient, is genotype dependent (Wunderle et al. 2012), and requires a high level of technical skill. Thus, further screening of symptomless lines may be required. Molecular markers linked to loose smut resistance genes allow the selection of breeding material based on genotype rather than phenotype. For these reasons, the development of molecular markers for the screening of loose smut resistance can accelerate the resistance breeding program significantly (Eckstein et al. 2002).

A restriction fragment length polymorphism (RFLP), based on the cDNA clone ABC261, was identified by Eckstein et al. (1993) in the DH mapping population ‘Harrington’ × TR306 as linked to the *Un8* loose smut resistant gene on the long arm of chromosome 5 (1HL). By sequencing the clone ABC261, Eckstein et al. (2002) were able to develop primers for a SCAR marker (Un8-700R) linked to *Un8*. The genetic distances between Un8-700R and

Un8 varied from 0 to 7.1 cM in five crosses. Since then a number of closer markers (Un8SNP1, Un8SNP4, and Un8SNP6) for *Un8* have been developed for MMAS (Eckstein, personal communication). Li et al. (2001) used a population of doubled-haploid (DH) lines from 'Harrington' × TR306 and bulked-segregant analysis to develop an SSR linked to the *Un8* loose smut resistance gene without the need of a large-insert genomic library. The distance between the SSR marker and *Un8* in populations of 'Harrington' × TR306, TR306 × Kao 22-3 and 'AC Oxbow' × 'Manley' range from 8.6 to 10.3 cM.

The Crop Molecular Genetics Laboratory at the University of Saskatchewan currently screens about 25,000 two-row barley breeding lines per year using real-time PCR with TaqMan assays for various resistance genes and quality traits in barley and oat, of which about 8,000 lines are screened for the presence of *Un8*. In addition to the much greater number of lines which can be evaluated using MMAS, the current cost of screening a line for *Un8* using MMAS is considerably less than using artificial field/greenhouse inoculations (Eckstein, personal communication).

2.6 Mechanisms of plant disease resistance

Plant disease is one of the main constraints affecting global food security. Changes in the genetic structure of the pathogen and introduction of diseases from other parts of world may cause significant yield losses in crops. For example, in 1998 in Uganda, a new devastating *Puccinia graminis* f. sp. *tritici* race (Ug99) virulent to the widely utilized wheat stem rust resistance gene *Sr31* was detected and spread into East Africa and Asia (Pretorius et al. 2000; Singh et al. 2011). This same race is virulent on the widely used *Rpg1* resistance gene and is thus also a risk to barley production since many cultivars contain *Rpg1* to achieve barley stem rust resistance (Kleinhofs et al. 2009; Steffenson et al. 2016). Similarly, canola club root, caused by the soil-borne pathogen *Plasmodiophora brassicae* Woronin, was likely initially introduced by early European settlers into Canada with fodder turnips, but has only posed a serious threat to Prairie canola production in Canada since 2003 (Howard et al. 2010).

Generally, plant disease resistance can be categorized into qualitative resistance and quantitative resistance. Compared to qualitative resistance, which usually results in complete

resistance, quantitative resistance is conferred by multiple genes or several loci instead of a single resistance (*R*) gene and usually gives more durable disease resistance (Kou and Wang 2010).

2.6.1 Innate immunity in plants

A plant's innate immunity system is rapidly activated after the detection of an attempted pathogen attack. Two types of innate immunity are deployed by plants: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector triggered immunity (ETI) (Jones and Dangl 2006). PTI is the first line of defense which recognizes and responds to pathogen/microbe-associated molecular patterns, which are conserved molecules found in many microbes. The recognition of PAMPs by pattern recognition receptors (PRRs) is responsible for initiating a series of defense signals (Jones and Dangl 2006; Newman et al. 2013). Several classical examples of the interaction between PAMPs and PRRs include the recognition of flagellin fragment flg22 by *Arabidopsis* FLAGELLIN SENSING 2 (FLS2), bacterial Elongation factor Tu (EF-Tu; elf18/26) by EF-Tu receptor (EFR) in *Arabidopsis* and other Brassicaceae, bacterial sulfated protein Ax21 by the Xa21 receptor in rice, and fungal chitin by chitin oligosaccharide elicitor-binding proteins together with chitin elicitor receptor kinase 1 in rice and *Arabidopsis* (Newman et al. 2013).

Pathogens can suppress PTI pathways by delivering effector proteins into host cells. However, the effectors can be recognized by resistance (R) proteins, which triggers ETI and usually results in hypersensitive cell death (Jones and Dangl 2006). Some of the best examples of this phenomenon come from bacterial pathogens, such as *Pseudomonas syringae* and *Xanthomonas campestris*, from which the virulence effectors are injected into host cells via the type III protein secretion system and compromise host plant defense (He et al. 2004). The first cloned plant disease resistance gene following the gene-for-gene interaction was *Pto* which encodes an intracellular serine/threonine protein kinase and confers resistance to *Pseudomonas syringae* pathovar (pv.) *tomato* (*Pst*) (Martin et al. 1993). Through direct recognition of effectors AvrPto or AvrPtoB by Pto, ETI can be effectively induced with the association of hypersensitive cell death in a *Prf*-dependent manner with more than 20 genes involved in the *Pto*-mediated resistance pathway (Oh and Martin 2011).

2.6.2 Main classes of plant disease resistance genes

A large number of plant disease resistance (*R*) genes have been isolated and most can be categorized into a few main classes based on the conserved structural motifs in their protein products (Gururani et al. 2012). The major group of R proteins contains both a nucleotide binding site domain and a leucine-rich repeat domain (NBS-LRR), which can be further divided into CC-NBS-LRR and TIR-NBS-LRR according to differences in the N-terminus. The CC-NBS-LRR subclass contain a coiled coil domain (CC), whereas the TIR-NBS-LRR subclass has a domain with homology to the mammalian toll-interleukin-1-receptor (TIR) (McHale et al. 2006). The *Pfr* gene required for *Pto* resistance and *R* genes located in the barley *Mla* locus are among the best characterized examples of the CC-NBS-LRR subclass (Pedley and Martin 2003; McHale et al. 2006). The tobacco *N* gene, flax *L6* gene, and *Arabidopsis RPS4* and *RPP5* genes are examples of the TIR-NBS-LRR subclass (McHale et al. 2006).

The second resistance gene class is the LRR-TrD which consists of extracellular leucine rich repeats (LRR) attached to a transmembrane domain (TrD) (Gururani et al. 2012). Examples within this group include the tomato genes, *Cf-2*, *Cf-4*, and *Cf-9*, recognizing *Cladosporium fulvum* (*C. fulvum*) race-specific effectors Avr2, Avr4, and Avr9, respectively (Wulff et al. 2009). The third *R* gene class encodes an additional intracellular kinase domain (TRR-TrD-Kinase). The rice *Xa21* which is effective against *Xanthomonas oryzae* pv. *oryzae*, *Arabidopsis* flagellin-induced complex of flagellin sensitive 2 (*FLS2*) and BRI1-associated receptor kinase 1 (*BAK1*) are all included in the LRR-TrD-Kinase class (Song et al. 1995; Chinchilla et al. 2007). *Pto* and *Rpg1* represent another class of *R* genes. *Pto*, the first isolated plant disease resistance gene, encodes a cytoplasmic serine/threonine protein kinase and *Rpg1* produces a protein with two tandem protein kinase domains (Martin et al. 1993; Brueggeman et al. 2002; Gururani et al. 2012). Besides the domain architectures listed above, examples of plant disease resistance proteins with unique structures have been identified. For example, the *Arabidopsis RPW8* gene belongs to the CC-TrD class (Xiao et al. 2001), *Arabidopsis RRS1-R* encodes a TIR-NBS-LRR protein with an additional NLS (Nuclear localization signal) and

WRKY (W=Tryptophan, R=Arginine, K=Lysine, Y=Tyrosine) domains (TIR-NBS-LRR-NLS-WRKY) (Deslandes et al. 2002).

2.6.3 Models for perception of plant pathogen effectors

Several different models have been postulated to explain the R-protein mediated effector perception mechanisms in plants. The receptor-ligand model, also known as the gene-for-gene model proposed by Flor in the 1940s, was the first hypothesis to explain the host-pathogen interaction (Flor 1971). Under this model, host defense responses can be triggered by direct interaction between the host resistance protein and the corresponding pathogen effector, such as AvrPita-Pi-ta in rice (Jia et al. 2000) and Avr567-L in flax (Dodds et al. 2006).

As there are limited examples for the receptor-ligand model, a second hypothesis called the guard model was formulated, initially to explain the *Pst* resistance in tomato (van der Hoorn and Kamoun 2008). Under this model, R proteins are proposed to guard the state of guardees, which are the molecules targeted and modified by pathogen effectors to circumvent disease resistance (Dangl and Jones 2001). Two R proteins under this classification are *Arabidopsis* proteins RIN4 (Jones and Dangl 2006; Spoel and Dong 2012) and PBS1 (Shao et al. 2003).

Reconciling evolutionary limitations imposed by the guard model promoted the formulation of the decoy model. In the decoy model, some proteins act as decoys to mimic pathogen virulence targets are acquired by hosts during evolution and are solely involved in effector perception (van der Hoorn and Kamoun 2008). The decoy model is consistent with most aspects of *Pto*-mediated resistance. *Pto*-encoded protein kinase, which is closely related to the kinase domains of FLS2 and chitin elicitor receptor kinase 1, can function as the target of AvrPto and AvrPtoB (Xiang et al. 2008; Dodds and Rathjen 2010).

However, the requirement of Pto kinase activity cannot be explained by the decoy model (Dodds and Rathjen 2010). As such, a modified decoy model named the bait and switch model was proposed which encompassed independence of modification of the host

target and comprised a two-step recognition event. The interaction between an effector and the accessory bait protein associated with the R protein facilitates the recognition of the effector resulting in switching of the R protein from an 'OFF' state to an 'ON' state and induction of a defense response (Collier and Moffett 2009).

2.6.4 Plant hormones and the plant defense response

Plant hormones, also known as phytohormones, such as abscisic acid (ABA), auxins, brassinosteroids (BRs), cytokinins (CKs), ethylene (ET), gibberellins (GAs), jasmonate acid (JA), and salicylic acid (SA), are small organic molecules that have been found to be essential in almost every aspect of plant growth, development, and stress response (Bari and Jones 2009; Pieterse et al. 2009). During plant response to biotic stresses, JA, ET, and SA are recognized as the three most important phytohormones. JA and ET are mainly associated with resistance toward necrotrophic pathogens, whereas SA plays a predominant role in the defense against biotrophic and hemi-biotrophic pathogens. Other phytohormones have also been identified as crucial in the response to biotic stresses, for example ABA, auxins, BRs, and CKs (Bari and Jones 2009; Naseem et al. 2014).

JA-related signaling cascades are controlled by Jasmonate ZIM-domain (JAZ) repressor proteins which are the targets of SCF^{COI1} (Pauwels and Goossens 2011). In the presence of the active hormone derivative jasmonate-isoleucine (JA)-Ile conjugate, JAZ proteins can be degraded through the interaction with SCF^{COI1} to activate the JA-responsive genes, such as *VSP2* and *PDF1.2* (Pieterse et al. 2009; Pauwels and Goossens 2011). ET is generally believed to work in plant defense response in concert with JA. Synergistic interactions between JA and ET to activate defense signaling have been found in a number of cases and *ERF1* and *ORA59* both regulating *PDF1.2* expression function as the important nodes connecting JA and ET pathways (Pré et al. 2008; Pieterse et al. 2009). In most cases, JA and SA pathways antagonistically regulate plant disease resistance responses. Non-expressor of pathogenesis-related genes 1 (*NPR1*) is one of the key components of the SA pathway and plays an important role in the suppression of JA induction (Pieterse et al. 2009; Bari and Jones 2009; Verma et al. 2016). Overexpression of some components downstream of

NPR1 can upregulate the expression of *PR1*, the SA-responsive marker gene, and suppress the JA-responsive marker gene *PDF1.2* (Bari and Jones 2009).

Phytohormones are essential integrators balancing plant development and defense responses and several review articles summarize the growth-defense tradeoffs during plant disease resistance (Huot et al. 2014; Lozano-Durán and Zipfel 2015; Naseem et al. 2015). Briefly, due to resource restrictions, plant pathogen attack can induce the energetically costly defense responses resulting in down-regulating expression of growth-related genes, thus compromising plant growth. For example, *FLS2*-mediated *Pst* resistance can suppress auxin signaling partially through microRNA miR393 (Huot et al. 2014). Yang et al. (2012b) demonstrated that restricted plant growth by JA defense signaling could be achieved through the interaction with the GA signaling cascade in rice. BR is an important phytohormone in promoting plant growth and Fan et al. (2014) found that *Arabidopsis* bHLH Transcription Factor HBI1 is a crucial node connecting growth and immunity through the BR signaling pathway. Deng et al. (2016) indicated that the BR-involved tradeoffs might be mediated through the inhibition of ROS (reactive oxygen species) production by BES1/BZR1 in *Nicotiana benthamiana*.

CHAPTER 3

Fine Mapping and Identification of a Candidate Gene for the Barley *Un8* Loose Smut Resistance Gene

Chapter 3 was prepared based on the publication:

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Abstract

In North America, durable resistance against all known isolates of barley loose smut, caused by the basidiomycete pathogen *Ustilago nuda* (Jens.) Rostr. (*U. nuda*), is under the control of the *Un8* resistance gene. Previous genetic studies mapped *Un8* to the long arm of chromosome 5 (1HL). Here, a population of 4,625 lines segregating for *Un8* was used to delimit the *Un8* gene to a 0.108 cM interval on chromosome arm 1HL, and assign it to fingerprinted contig 546 of the barley physical map. The minimal tiling path was identified for the *Un8* locus using two flanking markers and consisted of two overlapping bacterial artificial chromosomes. One gene located close to a marker co-segregating with *Un8* showed high sequence identity to a disease resistance gene containing two kinase domains.

3.1 Introduction

Loose smut of barley (*Hordeum vulgare* L.) is caused by the basidiomycete pathogen *Ustilago nuda* (Jens.) Rostr. (*U. nuda*). This seed-borne disease has been reported in about 50% of fields in the prairie provinces of Western Canada (Menzies et al. 2014) and is also common in the United States (Menzies et al. 2010). Yield reduction due to *U. nuda* infection is commonly less than 1%, however over 10% yield loss has been reported (Thomas 1997; Orr et al. 1998). After colonization of the florets, *U. nuda* can overwinter in the embryo of mature seeds as dormant mycelium. Upon seed germination, the pathogen will colonize tissue behind the growing point of the barley host and eventually infect the inflorescence where the

florets are replaced with the distinctive black teliospore masses which serve as the next source of inoculum (Thomas 1997).

Among the common disease control strategies, plant resistance is the most economical and effective strategy. The first resistance gene, *Un*, was reported in the cv. 'Trebi' in the 1940s (Livingston 1942; Robertson et al. 1947). Since then, a total of 15 resistance genes (Livingston 1942; Robertson et al. 1947; Schaller 1949; Skoropad and Johnson 1952; Andrews 1956; Metcalfe and Johnston 1963; Metcalfe 1966) associated with loose smut resistance have since been identified, with *Un11*, *Un12*, *Un13*, and *Un15* the most recent additions (Mueller 2006). Among these resistance genes, *Un8*, which was found in the PR28 derived from the winter barley line C.I. 4966 (Metcalfe and Johnston 1963; Metcalfe 1966), is the most effective and long-lived resistance, effective against all known loose smut isolates in Western Canada (Thomas and Menzies 1997).

Breeding for resistance to loose smut involves individual hand inoculation of florets at early anthesis and evaluating the phenotype at heading in the following growing season. This process is both labor-intensive and time-consuming and moreover, the occurrence of false negatives (escapes) resulting from the failure of artificial inoculation, necessitates several rounds of screening to ensure the presence of resistance. Molecular marker-assisted selection (MMAS) for loose smut resistance is one of the best examples of how markers can improve selection since significant increases in efficiency and accuracy are achievable. *Un8* was initially mapped onto the long arm of barley chromosome 5 (1HL) in linkage with the ABC 261 RFLP marker (Eckstein et al. 1993). Subsequently, microsatellite (Li et al. 2001) and sequence characterized amplified region (SCAR) (Eckstein et al. 2002) markers were developed for *Un8*.

When dense unigene-based single nucleotide polymorphism (SNP) maps became available for barley (Close et al. 2009) and the accompanying information deposited into databases (e.g. HarvEST:Barley), it was possible to develop additional markers for *Un8*. Barley unigenes 4245, 16527, and 14722 (HarvEST:Barley v. 1.83, assembly 35) were used to create three TaqMan® assays which defined a region of approximately 6.2 cM around *Un8* (Eckstein, personal communication). After several years of MMAS in the Crop Development

Centre barley breeding program in which over 20,000 lines were evaluated, it became apparent that despite the close linkage of the *Un8* markers initially identified, there was a higher recombination frequency in this region of the barley genome than indicated by the smaller populations used originally to define the *Un8* region (Eckstein et al. 2002). As a result, the usefulness of the markers was reduced.

Recent advances towards understanding the barley genome have provided a number of avenues to identify molecular markers in tighter linkage to the *Un8* gene. The existence and defining of micro-colinearity between barley and other model species, such as rice (*Oryza sativa* L.) and *Brachypodium distachyon* L. Beauv. (Brachypodium) (Mayer et al. 2011), permit the use of genomic sequences available in the syntenic regions for additional molecular marker development. This strategy has been exploited in barley to fine map the *sdw3* semi-dwarfing gene (Vu et al.2010), *dsp* spike density gene (Shahinnia et al. 2012), two novel QTL (Silvar et al. 2012) and *Ror1* (Acevedo-Garcia et al. 2013) conferring powdery mildew resistance, the BaMMV/BaYMV resistance gene *rym11* (Lüpken et al. 2013), *HvNax3* (Shavrukov et al. 2013) and *HvNax4* (Rivandi et al. 2011) which limit Na^+ accumulation, and *Ryd3* controlling tolerance to barley yellow dwarf virus (Lüpken et al. 2014). With respect to the barley *Un8* region, the syntenic regions in Brachypodium and rice are chromosomes 2 and 5, respectively (Mayer et al. 2011). Assembly of the 5.1 Gb barley genome which integrates physical and genetic information together with gene expression and bacterial artificial chromosome (BAC) clones (Mayer et al. 2012) provides a valuable tool for not only marker development, but also for the positional cloning of the *Un8* gene.

The objective of this study was to enrich the 6.2 cM interval harbouring the *Un8* loose smut resistance gene using a variety of strategies (EST data, SNP genotyping arrays, synteny, and BAC and whole-genome sequence data) which take advantage of the array of genomic tools available in barley, and to identify the candidate gene(s) for *Un8*. This would allow us to develop perfect markers diagnostic for the presence of the *Un8* gene which would assist our MMAS program.

3.2 Materials and methods

3.2.1 Plant materials and mapping populations

An F_4 recombinant inbred line (RIL) population (4,625 lines) derived from the cross TR09398 × TR07728 was used for genetic mapping. After the initial cross the population was advanced from the F_1 to F_4 generations using the bulk breeding method. The F_4 RILs used in this study were randomly selected individual seeds from the larger F_4 bulk seed sample (comprised of ~150,000 seeds). The F_4 population was screened with two co-dominant flanking TaqMan® markers, Un8SNP1 and Un8SNP6, using the Applied Biosystems® StepOnePlus™ Real-Time PCR System. Lines were selected if they showed recombination between Un8SNP1 and Un8SNP6 and were homozygous for both markers. Genomic DNA from 122 such lines was isolated from leaves using the modified cetyltrimethylammonium bromide (CTAB) method (Procunier et al. 1991). As well, seeds from each line were harvested separately to evaluate their reaction to loose smut. The phenotypic and genotypic information of the 'Harrington' (susceptible) × TR306 (resistant) doubled-haploid (DH) population (149 lines; Eckstein et al. 2002) was also used in this study to help position newly developed markers.

3.2.2 Evaluation of loose smut reaction

Reaction to loose smut was tested at flowering, as previously described by Eckstein et al. (2002), in the greenhouse and field by artificial inoculation using a mixture of loose smut pathotypes. All lines were evaluated at the North Seed Farm disease nursery (Saskatoon, SK, Canada) where lines were planted as hill plots (15 seeds/hill) (with susceptible checks throughout the nursery). In the greenhouse, three seeds of each line were sown in a pot. The cultivar 'CDC Austenson' was used as a susceptible control in the greenhouse experiments. For both field and greenhouse inoculations, 6-8 heads were inoculated at anthesis using a 3-ml syringe and at least 15 inoculated seeds were tested for disease reaction in the following generation. If a line showed susceptibility to the disease (i.e. smutted heads were observed) no further testing was done. If a line showed resistance (i.e. no smutted heads were

observed), two additional inoculations were conducted to confirm the resistance. A goodness of fit to a 1:1 ratio (resistant to susceptible) was tested using the Chi-squared test (χ^2).

3.2.3 Marker development and genotyping

Because *Un8* was previously assigned to chromosome arm 1HL between markers Un8SNP1 and Un8SNP6 (Eckstein, personal communication) which were designed based on EST unigene sequences 4245 and 14722, respectively, (HarvEST:Barley version 1.83, assembly 35), other unigenes located on the barley integrated map (HarvEST:Barley) within the interval flanked by these two markers were explored for marker development. EST unigene sequences were extracted from HarvEST:Barley, formatted as FASTA files and used to query the barley cv. 'Morex' whole genome assembly using the BLASTN basic search program within the ViroBLAST interface tool (http://www.webblast.ipk-gatersleben.de/barley/viroblast.php). Genomic DNA contig sequences identified through these queries were used to assist marker development.

The 9K Barley iSELECT Infinium SNP Assay was also used to develop markers in the *Un8* target region. Three loose smut resistance resources ('CDC Meredith', TR306, and TR09398) and four susceptible sources (TR07728, TR09397, 'Harrington', and 'CDC Kindersley') were genotyped with the 9K assay. Available information for sequences surrounding the SNPs which differentiated resistant from susceptible lines was used to identify additional sequence information contained in Morex BACs for marker development.

The syntenic relationship of barley with model species was exploited to identify markers in the *Un8* interval. Based on information within HarvEST:Barley, the putative orthologous genes to the genes from which Un8SNP1 and Un8SNP6 were developed were identified in rice (Os05g48422 and Os05g49030, respectively) and Brachypodium (Bradi2g16930 and Bradi2g16430, respectively). Once this region was defined in both reference genomes, all gene sequences located in the syntenic region were extracted from rice (http://www.ricemap.org/) and Brachypodium (http://www.brachypodium.org/g-mod/genomic/contigs). The gene sequences were queried against the barley EST database in HarvEST:Barley using the BLASTN function (*E* value $\leq e^{-10}$ and identity ≥ 80 %) to find the

putative orthologous unigenes in barley. Barley unigenes identified in this manner were also queried against the barley cv. 'Morex' whole genome assembly using the ViroBLAST interface tool (as mentioned above) to identify the genomic DNA contig sequence for further marker development (and to confirm the 1HL chromosome arm location).

After initial analysis of the phenotypic and genotypic data generated from the TR09398 × TR07728 population, it was determined that *Un8* was close to the Un8SNP4 marker. Based on that information the HarvEST:Barley database was queried to identify BAC clones spanning the Un8SNP4 marker. BAC clones HVVMRXALLhA0751D06 and HVVMRXALLhA0772N02 were found to co-locate with Un8SNP4, while BAC HVVMRXALLhA0498L15 was located only 0.7 cM away from Un8SNP4 on the barley integrated map (HarvEST:Barley). BAC clone sequences were downloaded from the HarvEST:Web (http://www.harvest-web.org/hweb/pickassy.wc) for marker development.

Once the genomic DNA sequence was obtained using the strategies above, PCR primers were designed using PrimerPremier 5.0 (PREMIER Biosoft International, Paulo Alto, CA, USA) to amplify a fragment of the genomic DNA to identify polymorphisms between TR09398 and TR07728. Standard PCR amplifications were performed in a 25 µl volume containing 1× Lucigen® PCR buffer, dNTPs (100 µM each), primers (0.2 µM each), 50 ng genomic DNA, and 1 U Taq DNA polymerase. PCR conditions were: 5 min at 94 °C for initial denaturation, followed by 35 cycles of 94°C for 45 s, 55-65°C for 45 s, 72°C for 1 min, and a final extension step at 72°C for 5 min. The amplification products were separated on 1% agarose gels and 5-6 clones of each amplicon were cloned into the TOPO® TA® Cloning Vector, Sanger Sequenced at the National Research Council (Saskatoon, SK, Canada) and aligned using DNAMAN v. 7 (Lynnon Biosoft, San Ramon, CA, USA) to ensure the consistency of the sequence data. All allele-specific, amplicon size shift and cleaved amplified polymorphic sequence (CAPS) markers developed were run under the standard PCR conditions listed above. Amplicon size shift markers resulted from the fortuitous design of the original PCR primers. Allele-specific markers were created by designing new PCR primers targeted against SNP sites identified in the originally sequenced PCR products. The allele-specific primers were designed with an additional mismatch nucleotide introduced into

the third nucleotide position from the SNP site at the 3′-end of the primer, according to the method described by Liu et al. (2012), to increase the SNP detection efficiency. For CAPS markers, PCR products were subsequently digested with 2 U of restriction endonuclease (NEB) corresponding to the SNP site identified in the originally sequenced PCR product. PCR products for all markers were separated on 1.5% agarose gels.

TaqMan® assays were developed to target SNP sites identified in the originally sequenced PCR products. TaqMan® SNP genotyping was performed with the ABI StepOnePlus™ Real-Time PCR System in a 10 µl volume which included 1× ABI TaqMan® GTXpress™ MasterMix, 0.36× ABI TaqMan® SNP Genotyping assay and 25 ng genomic DNA. PCR conditions were: 30 s at 60°C for pre-PCR read and 10 min at 94°C for hot-start activation, followed by 40 cycles at 94°C for 15 s, 60°C for 30 s and 60°C for 30 s for post-PCR read.

3.2.4 Linkage analysis

Linkage analysis was carried out by screening all the newly developed markers on the 122 F_4 lines derived from the TR09398 × TR07728 population which had been preselected for recombination between Un8SNP1 and Un8SNP6. Genetic distance was estimated according to the Kosambi mapping function (Kosambi 1944) based on a population size of 4,625 lines. This was the number of lines remaining after removing 211 lines which displayed a recombination between Un8SNP1 and Un8SNP6 in a heterozygous state (i.e. only one homologous chromosome was recombinant), from the original population of 4,836 lines evaluated for recombination between the two markers. The 211 lines were used for a second calculation of total genetic distance between Un8SNP1 and Un8SNP6, but since these lines were discarded after the initial screening with Un8SNP1 and Un8SNP6, they could not be evaluated with the subsequent markers developed and thus they did not contribute to the linkage map created. The linkage map was constructed with JoinMap 4.0 (Kyazma B.V., Wageningen, The Netherlands).

3.2.5 Physical map construction, BAC sequencing and assembly

Two markers, Un8SNP4 and 0498L15 F3/R3, were utilized to identify the fingerprinted contigs (FPC) in the physical map of barley (Mayer et al. 2012; Ariyadasa et al. 2014) which encompassed the *Un8* gene. The minimal tiling path (MTP) of the targeted FPC was then identified.

Shotgun sequencing of DNA from Morex BACs HVVMRXALLmA0180J17 and HVVMRXALLeA0154F16, which composed the MTP of the targeted FPC, was performed using the Illumina HiSeq 2000 (2 × 100 cycles) device essentially as described (Meyer and Kircher 2010). Individual assemblies for the targeted BACs were produced with clc Assembly Cell version 4.0.6beta.

Nextera mate pair sequencing libraries with insert sizes ranging between 3 and 10 kb were prepared following the instructions of the manufacturer (Illumina) and sequenced using the Illumina MiSeq (2 × 250 cycles) and HiSeq2000 (2 × 100 cycles) devices. Shotgun assemblies were scaffolded with mate pairs using SSPACE PREMIUM version2.3.

3.2.6 Gene prediction and annotation and protein domain annotation

Identification of all putative gene sequences within Morex BAC clones comprising the MTP was accomplished using several methods. The possible genes were located with the online-based tools GeneMark (http://www.opal.biology.gatech.edu/GeneMark/eukhmm.cgi) and GENSCAN (http://www.genes.mit.edu/GENSCAN.html). In addition, predicted genes from the HarvEST:Barley (v. 1.98, assembly 37) and recently released barley genome assembly (http://www.barleyflc.dna.affrc.go.jp/hvdb/index.html) were identified and compared with predictions from GeneMark and GENSCAN. Predicted genes were annotated using the BLASTP tool to query the NCBI and iTAK (plant transcription factor and protein kinase identifier and classifier) databases. Domain annotation of the deduced protein sequence of the *Un8* candidate gene was carried out by SMART (http://www.smart.embl-heidelberg.de/), PROSITE (http://www.prosite.expasy.org/), and the conserved domain database (CDD) (http://www.ncbi.nlm.nih.gov/cdd).

3.3 Results

3.3.1 Evaluation of loose smut reactions

After development of an F_4 population derived from the cross TR09398 (resistant, carries *Un8*) × TR07728 (susceptible, lacks *Un8*), a total of 4,836 lines from this population were screened with the Un8SNP1 and Un8SNP6 TaqMan markers with 122 recombinant lines identified. After inoculation with the mixture of loose smut pathotypes, 57 lines showed resistance and 65 lines showed susceptibility which was consistent with a single gene mode of resistance ($\chi^2 = 0.525$, $P = 0.4689$).

3.3.2 Marker enrichment and fine genetic mapping of the *Un8* interval

Un8 was initially confined to a 6.2 cM region on chromosome arm 1HL by the flanking markers Un8SNP1 (unigene 4245) and Un8SNP6 (unigene 14722) (Eckstein, personal communication). To enrich the *Un8* region, four different methods were used. First, sequences from 12 barley unigenes located between unigenes 4245 and 14722 were identified and used for marker development. After these unigene sequences were queried against the barley cv. 'Morex' whole genome assembly using the ViroBLAST interface tool to obtain larger genomic DNA sequence reads, polymorphisms between the mapping population parents, TR09398 and TR07728, were identified in five of these unigenes which became the basis of markers 8487, 1406, 0498L15 F3/R3, 3602 and 13742 (Appendix A).

Second, genotyping data obtained from three loose smut resistance sources ('CDC Meredith', TR306, and TR09398) and four susceptible sources (TR07728, TR09397, 'Harrington', and 'CDC Kindersley') using the 9K Barley iSELECT Infinium SNP Assay identified 21 SNP markers in the *Un8* interval. This resulted in the placement of two additional markers (48060 and 10924) in the *Un8* region (Appendix A). Third, colinearity between barley chromosome arm 1HL (location of *Un8*) and the syntenic regions on the long arm of rice chromosome 5 and Brachypodium chromosome 2 were exploited to find additional markers. Barley unigenes 4245 (Un8SNP1) and 14722 (Un8SNP6), which bracket the *Un8* gene, were used to define the orthologous regions in rice and Brachypodium. All rice

and Brachypodium genes contained in the orthologous interval were identified and queried against the HarvEST:Barley (assembly 35) database and the barley cv. 'Morex' whole genome assembly (using the ViroBLAST interface tool) to identify previously unidentified or unmapped barley unigenes in the *Un8* region. This produced two additional markers (17452 and 21217) in the *Un8* region (Appendix A). Finally, based on available BAC sequences in the *Un8* region, four markers were developed, 0751D06 F6/R6 from BAC HVVMRXALLhA0751D06, 0498L15 F8/R8 from BAC HVVMRXALLhA0498L15, and Un8SNP7 and HI1406 from BAC HVVMRXALLhA0772N02 (Appendix A).

Thirteen new markers were developed for the *Un8* interval using the 4,625 RILs derived from TR09398 × TR07728 which, along with the Un8SNP1, Un8SNP4, and Un8SNP6 markers, defined a 2.853-cM region (Fig. 3.1). Among the 122 lines used to create the linkage map spanning the *Un8* locus, a total of 132 recombinations were observed within the Un8SNP1 to Un8SNP6 interval because several of the lines contained multiple recombinations. When the additional 211 lines which contained a single recombinant chromosome in the *Un8* region were included in the calculation of genetic distance between Un8SNP1 and Un8SNP6, a value of 4.911 cM was obtained. After including the loose smut reaction data, two markers, 0751D06 F6/R6 and Un8SNP4, showed complete linkage with *Un8* in the TR09398 × TR07728 population (Fig. 3.1).

It was previously known that one line from the DH population derived from the cross 'Harrington' × TR306 (Eckstein et al. 2002) showed a recombination between the *Un8* gene and the Un8SNP4 marker. To determine if the 0751D06 F6/R6 marker co-segregating with the *Un8* gene in the TR09398 × TR07728 population was closer to *Un8* than the Un8SNP4 marker, this marker was screened on the 'Harrington' × TR306 population. The 0751D06 F6/R6 marker showed no recombination with *Un8*, indicating that it was the most closely linked marker to the *Un8* gene (Fig. 3.2a). Ultimately, the *Un8* gene was determined to be within a genetic interval flanked by markers Un8SNP4 and 0498L15 F8/R8 (Fig. 3.2a).

3.3.3 Synteny between barley, rice and Brachypodium

The syntenic regions between barley chromosome arm 1HL, rice chromosome 5 and Brachypodium chromosome 2 around the *Un8* gene were delimited by markers Un8SNP1 and Un8SNP6 and very few rearrangements of marker order were observed (Fig. 3.1). No orthologous sequences for the most closely linked distal marker, 0498L15 F8/R8, could be identified in either rice or Brachypodium (Figs. 3.1, 3.2a). Therefore, we used the 0498L15 F3/R3 and Un8SNP4 markers, which were 0.346 cM apart (Fig. 3.1), to calculate the physical distance of the syntenic regions in rice and Brachypodium. In rice, the orthologous region spanned approximately 24,000 bp and contained three genes, while the same region was less than 3,000 bp in Brachypodium and no genes were present (Fig. 3.1).

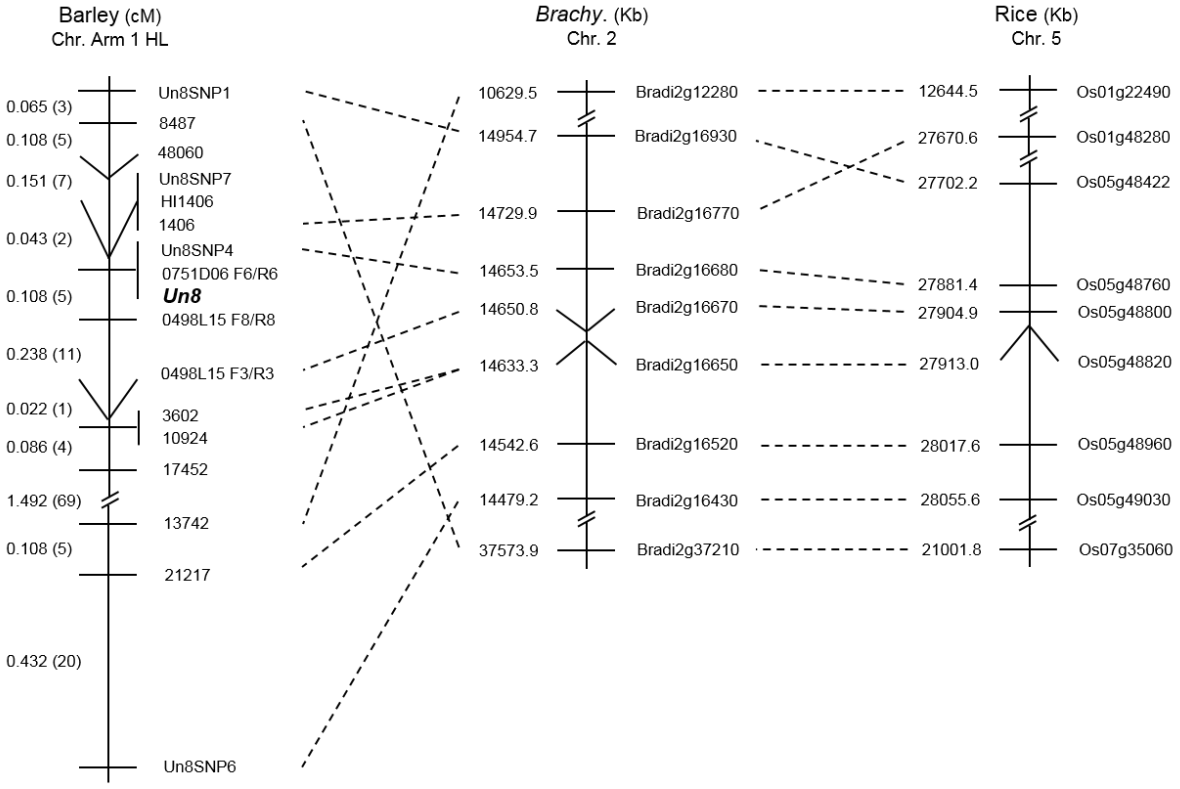


Fig. 3.1 Genetic map of the *Un8* region on barley chromosome arm 1HL created using 4,625 recombinant inbred lines derived from TR09398 (R) × TR07728 (S), and comparison of this interval with the physical maps of *Brachypodium distachyon* chromosome 2 and rice chromosome 5. Dashed lines connect putative orthologous genes. Marker names (barley) and gene names (*Brachypodium* and rice) are indicated to the right of each map while distance (cM and recombination events (in brackets) in barley, kb from the top of chromosome 2 in *Brachypodium* and the top of chromosome 5 in rice) are denoted to the left of each linkage group. The *Un8* gene is denoted in bold text. Double slashes within each linkage group represent a large interval, or alternate chromosome in the case of rice, not in scale with the remainder of the linkage group. Detailed information on these markers is presented in Appendix A.

3.3.4 Physical mapping and candidate gene prediction for *Un8*

Fingerprinted contig 546, part of the genome-wide physical map of barley (Mayer et al. 2012; Ariyadasa et al. 2014), was anchored to the *Un8*-targeted genetic map generated in this study by markers Un8SNP4 and 0498L15 F3/R3 and the MTP for FPC 546 was defined. The MTP was composed of two overlapping BACs, HVVMRXALLmA0180J17 (~160 kb) which contained marker Un8SNP4 and HVVMRXALLeA0154F16 (~150 kb) which contained 0498L15 F3/R3 (Fig. 3.2b). Subsequently, the 0498L15 F8/R8 marker was identified within BAC HVVMRXALLeA0154F16 and the 0751D06 F6/R6 marker was located within both BAC clones (Fig. 3.2b). Complete sequence for each BAC was contained in multiple scaffolds and contigs of varying size (Fig. 3.2c). BAC HVVMRXALLmA0180J17 was composed of one very large scaffold (J17_sc1; ~151 kb) and three small contigs (Fig. 3.2c), while BAC HVVMRXALLeA0154F16 was covered by two large scaffolds (F16_sc1 and F16_sc2; ~52 and ~45 kb) and seven smaller scaffolds and contigs (Fig. 3.2c).

DNA sequences of both BACs were analyzed for putative genes and a total of 17 were identified (Fig. 3.2c; Table 3.1). Only scaffolds and contigs containing putative genes are shown in Fig. 3.2c. Among this group, only two resistance associated genes were identified using GeneMark and GENSCAN. One was a cell wall invertase (β-fructofuranosidase, Fig. 3.2c; Table 3.1), but a CAPS marker designed for this gene identified one recombination between it and *Un8* within the 'Harrington' × TR306 population. The second predicted resistance-associated gene was a protein kinase containing two tandem kinase catalytic domains. It was co-located within the same two BAC clone scaffolds as the *Un8* co-segregating marker 0751D06 F6/R6 at a distance of ~3,000 bp (Fig. 3.2c; Table 3.1). The gene was also identified in HarvEST:Barley (v.1.98, assembly 37), denoted as MLOC_38442 (Mayer et al. 2012), and in the barley whole genome assembly as a RNA-Seq gene (denoted XLOC_040148; Mayer et al. 2012). The predicted function of this gene and its identification from multiple sources make it a good candidate for the *Un8* gene. According to the iTAK (plant transcription factor and protein kinase identifier and classifier) database, the best BLASTP hit for the *Un8* candidate gene was to a wall-associated protein kinase in *Oryza sativa* (BLASTE-score 2e-112, 41% identity (275/672) at the amino acid level with 55 gaps).

There were two additional predicted proteins present within the same BAC scaffold as the *Un8* co-segregating marker 0751D06 F6/R6 (Fig. 3.2c; Table 3.1) which cannot be fully disregarded as possible candidate genes. However, the lack of an annotated function for both and the absence of a corresponding RNA sequence (Mayer et al. 2012) for one of the predicted genes make them weaker candidates. No putative orthologous barley genes corresponding to the three rice genes which were located within the syntenic region identified by the Un8SNP1 and Un8SNP6 markers were present within the two BACs spanning the *Un8* locus.

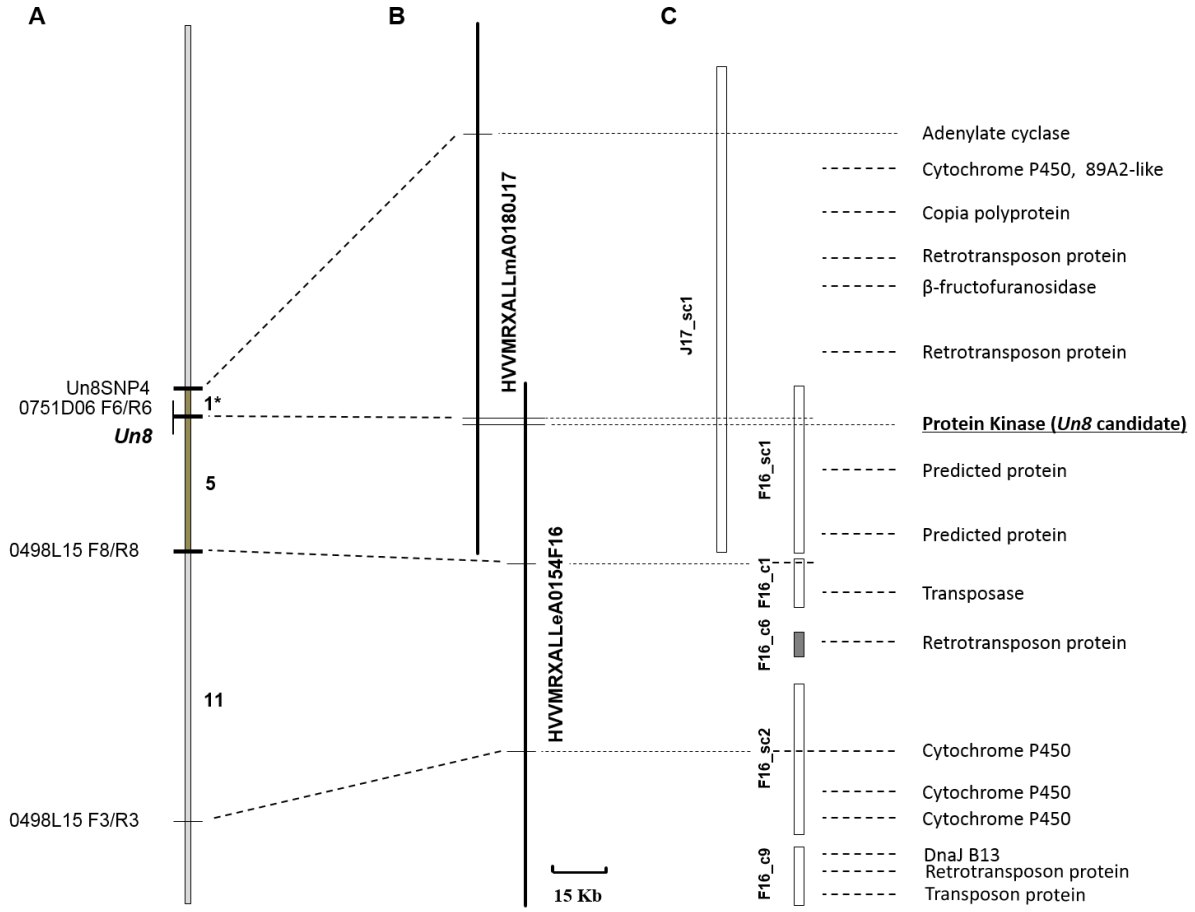


Fig. 3.2 Fine-scale orientation of the *Un8* region on barley chromosome arm 1HL indicating flanking markers, BAC clones spanning the *Un8* locus and location of all predicted genes within the BAC clones. (**a**) Genetic map displaying flanking markers which encompass the *Un8* locus (shaded region) with marker names to the left and recombination events observed in both the TR09398 (R) × TR07728 (S) and 'Harrington' × TR306 mapping populations indicated to the right. The asterisked number indicates a recombination between *Un8* and the Un8SNP4 marker observed in the 'Harrington' × TR306 population (Eckstein et al. 2002). Marker 0751D06 F6/R6 co-segregated with the *Un8* gene in both mapping populations. (**b**) Physical map of the *Un8* region. The *Un8* locus is spanned by two BACs (HVVMRXALLmA0180J17 and HVVMRXALLeA0154F16) with an overlapping region of ~50 kb in which the *Un8* locus resides. A scale bar for the physical map is provided at the bottom. (**c**) Predicted genes in the *Un8* region. Gene annotations are presented on the right and BAC scaffolds

('sc') and contigs ('c') are indicated to the left. J17 and F16 denote the corresponding BAC clone with which the scaffold or contig is associated. Only BAC scaffolds and contigs containing predicted genes are shown. The exact location of the F16_c6 contig (denoted with a shaded box) relative to F16_sc2 and F16_c1 has not been determined. The correct orientation of F16_c1, F16_c6, F16_sc2 and has not been resolved.

Table 3.1 Annotations, BLASTP ID and E-scores, gene ID, BAC ID and Morex contig associated with all predicted genes identified in BACs HVVMRXALLmA0180J17 and HVVMRXALLeA0154F16 which comprise the minimum tiling path spanning the *Un8* region.

Predicted Gene Annotation[a]	**BLASTP ID**[b]	**E-score**[b]	**Barley Gene ID**[c]	**BAC ID**[d]	**Morex Contig**
Adenylate cyclase	XP_006655586	0	MLOC_25774	HVVMRXALLmA0180J17_sc1	contig_171284
Cytochrome P450, 89A2-like	XP_005335357	$7e^{-111}$	-	HVVMRXALLmA0180J17_sc1	contig_58606
Copia polyprotein	AF466199	0	-	HVVMRXALLmA0180J17_sc1	-
Retrotransposon protein	ABB46931	0	-	HVVMRXALLmA0180J17_sc1	contig_84931
Beta-fructofuranosidase	XP_003575078	0	MLOC_5612	HVVMRXALLmA0180J17_sc1	contig_136454
Retrotransposon protein	BAA22288	0	-	HVVMRXALLmA0180J17_sc1	-
Protein kinase (*Un8* candidate)	EEE50557 (AAL25177)	$3e^{-179}$ ($2e^{-112}$)	MLOC_38442	HVVMRXALLmA0180J17_sc1 HVVMRXALLeA0154F16_sc1	contig_2550456
Predicted protein	AF427791	0	-	HVVMRXALLmA0180J17_sc1 HVVMRXALLeA0154F16_sc1	contig_1590184, contig_1973319, contig_243921
Predicted protein	BAK06775	0	MLOC_65367	HVVMRXALLmA0180J17_sc1 HVVMRXALLeA0154F16_sc1	contig_50087
Transposase	EMT30676	0	-	HVVMRXALLeA0154F16_c1	-
Tetrotransposon protein	BAA22288	0	-	HVVMRXALLeA0154F16_c6	-
Cytochrome P450	BAJ96841	0	MLOC_17557	HVVMRXALLeA0154F16_sc2	contig_1577063
Cytochrome P450	BAJ96841	0	MLOC_17557	HVVMRXALLeA0154F16_sc2	contig_1577063
Cytochrome P450	BAJ96841	0	MLOC_17557	HVVMRXALLeA0154F16_sc2	contig_1577063
DnaJ homolog subfamily B member 13	EMT30186	$5e^{-130}$	MLOC_23473	HVVMRXALLeA0154F16_c9	contig_140197, contig_162350
Retrotransposon protein	BAH79979	0	-	HVVMRXALLeA0154F16_c9	contig_223045
Transposon protein	AAP53844	$4e^{-74}$	-	HVVMRXALLeA0154F16_c9	-

[a]Predicted genes are presented in the same order as in Fig. 3.2;
[b]BLASTP ID and E-scores were determined using the NCBI database for all predicted genes. The BLASTP ID and E-score indicated in brackets for the *Un8* candidate gene were determined with the iTAK database;
[c]MLOC gene identifiers obtained from the IPK Barley Blast Server (http://webblast.ipk-gatersleben.de/barley/viroblast.php);
[d]The 'sc' or 'c' suffix indicates the scaffold or contig, respectively, on which the predicted gene resides within the associated BAC.

3.4 Discussion

Over the past 70 years, a minimum of 15 resistance loci conferring loose smut resistance were reported (Mueller 2006) and *Un8*, which was identified more than half of a century ago, is still the most effective (Metcalfe and Johnston 1963; Metcalfe 1966; Thomas and Menzies 1997). Barley lines harbouring *Un8* are resistant to all known loose smut isolates in Western Canada, making it the most valuable resource for loose smut resistance breeding. However,

the search for new sources of resistance continues, for example the identification of resistance on chromosome 3H (Menzies et al. 2010), which could provide alternative resistance should *Un8* resistance become ineffective with the evolution of new virulent pathotypes.

Fine mapping the barley *Un8* locus

Developing markers to *Un8* resistance has been instrumental to allow for MMAS of *Un8*-based resistance, but it also provided the initial tools towards map-based cloning of the underlying gene. Building on the 20 years of effort to genetically map the *Un8* loose smut resistance gene (Eckstein et al. 1993, 2002; Li et al. 2001; Eckstein, personal communication), this study has created a high resolution map of the *Un8* region consisting of sixteen markers and spanning a distance of 2.853 cM near the distal end of chromosome arm 1HL.

Positional cloning of genes in barley is hampered by the large genome (5.1 Gb) and high percentage of repetitive sequences (84%) (Mayer et al. 2012), however, the *Un8* gene is located near the distal end of chromosome arm 1HL where three recombination 'hot spots' were identified, with an estimated physical/genetic ratio of 0.6 Mb/cM (Künzel et al. 2000) which assisted fine mapping of the *Un8* gene in this study. The location of the *Un8* gene supports prior observations that many barley resistance genes are found distally in regions of high recombination (Mayer et al. 2012). In the present study, the interval spanning markers Un8SNP4 and 0498L15 F3/R3 is less than 265 kb and 16 recombinants were identified from the 4,625 F_4 lines screened (or 0.346 cM). This produced a physical to genetic distance ratio of 0.76 Mb/cM, similar to the estimate of Künzel et al. (2000).

Comparative studies in the *Un8* region with rice and Brachypodium

Rice diverged from barley approximately 50 million years ago (Dubcovsky et al. 2001; Paterson et al. 2004) which predated the divergence of barley and Brachypodium (Bossolini et al. 2007; International Brachypodium Initiative 2010). As such, Brachypodium shows a closer relationship with Triticeae than rice or other species like sorghum (International Brachypodium Initiative 2010) and may be a better model for comparative study (Huo et al.

2008; Mayer et al. 2011). For example, the analogue of the barley *Rpg1* stem rust-resistance gene can be found within the syntenic region in Brachypodium, but not in rice (Brueggeman et al. 2002; Drader and Kleinhofs 2010). Similarly, resistance gene analogues to the *Yr26* wheat stripe rust-resistance gene were located by syntenic mapping in Brachypodium, but no such genes were identified in the syntenic region of rice (Zhang et al. 2013). The syntenic relationship was conserved slightly better in Brachypodium than in rice for the *Un8* region. Only barley markers 13742 and 8487 localized to different locations on Brachypodium chromosome 2. These same two markers also localized to alternate chromosomes in rice, as did barley marker 1406 (Fig. 3.1). However, no resistance genes (or analogues) were identified in the *Un8* syntenic region of either Brachypodium or rice.

Un8 candidate gene

Only two of the 17 genes predicted to exist within the BAC clones spanning the *Un8* locus appeared to play a role related to disease resistance and both were located within the interval delimited by Un8SNP4 and 0498L15 F8/R8. One of these two genes was predicted to be a cell wall invertase (β-fructofuranosidase). These genes are up-regulated in response to pathogen infection and, via the import of hexose sugars to the site of infection, help increase plant metabolism to mount an effective defence (Proels and Hückelhoven 2014). However, a recombination event was identified in the 'Harrington' × TR306 mapping population between *Un8* and the predicted cell wall invertase gene located in BACHVVMRXALLmA0180J17. The second disease-related gene was a predicted protein kinase resistance gene analogue that was located close to the 0751D06 F6/R6 marker cosegregating with *Un8*. Protein kinases, such as receptor-like protein kinases and mitogen-activated protein kinases, are representatives of one of the main protein classes associated with plant disease resistance. In barley, most of the kinase containing resistance genes located on both arms of chromosome 1H tended to cluster distally (Mayer et al. 2012).

Domain annotation of the deduced protein sequence of the *Un8* candidate gene showed that it contained two tandem protein kinase domains. Both of the catalytic domains were classified into the tyrosine kinase subfamily using the SMART database. However, the CDD database placed the first catalytic domain into the tyrosine-specific kinase subfamily

(smart00219) while the second domain contained an apfam00069 protein kinase domain similar to that found in *Rpg1* (Brueggeman et al. 2002). If the *Un8* candidate gene is ultimately proven to be the *Un8* resistance gene, then it and *Rpg1* would be the only barley resistance proteins reported to contain two protein kinase domains, although they do not share a high degree of similarity with only 26% (186 of 703 amino acid residues) overall identity at the protein level (26% within the protein kinase I domain and 30% within the protein kinase II domain). Moreover, *Rpg1* is classified as a receptor-like protein kinase and the *Un8* candidate gene as a wall-associated protein kinase. However, because both the genes mediate durable resistance to barley biotrophic pathogens (lasting over 50 years in both cases) it would be interesting to determine if the longevity of their resistances is based on a similar mechanism.

The predicted wall-associated protein kinase gene identified in this study is a strong candidate to be the *Un8* gene due to its prediction from multiple sources, the presence of a corresponding RNA sequence aligned with the candidate gene position within the cv. 'Morex' whole genome assembly. However, because the BAC clones used in this study are derived from 'Morex', a susceptible variety, it is also possible that 'Morex' does not contain a *Un8* allele and the gene thus would not be present within the clones. Additionally, there were two predicted genes within the interval delimited by Un8SNP4 and 0498L15 F8/R8 which could also be the *Un8* gene. However, these two genes were considered weak candidates since no annotated function was associated with either and there was no RNA sequence identified for one of them.

Concluding remarks

In this study, a physical map surrounding the *Un8* loose smut resistance gene was constructed and one putative disease resistance gene analogue sequence was found in the region which was considered a strong candidate for the *Un8* gene. Until such time as a perfect marker is created for *Un8*, the 0751D06 F6/R6 marker, which not only cosegregates perfectly with *Un8* in the TR09398 × TR07728 mapping population, but also in a broader spectrum of barley populations (Eckstein, personal communication), will be very useful for MMAS efforts as it will alleviate some of the prior issues related to recombination between *Un8* and previous

markers which caused incorrect phenotypic predictions in barley breeding lines at the CDC. Next steps will focus on allele characterization of the *Un8* candidate, expression analysis of the candidate gene, and transformation of the candidate gene into a susceptible barley line to definitively prove that it is the *Un8* gene.

CHAPTER 4

Allele Characterization of the *Un8* Candidate Gene

Abstract

The barley *Un8* gene that confers resistance to loose smut caused by *Ustilago nuda* (Jens.) Rostr. (*U. nuda*) has protected Canadian barley production for over 60 years. Map-based cloning has defined the *Un8* resistance locus to a small region of high recombination (0.76 Mb/cM). A *Un8* candidate gene was identified within this region and was predicted to encode a protein kinase containing two tandem kinase domains. In this study sequence analysis was carried out to characterize the coding and promoter region of the *Un8* candidate gene within a diverse collection of 26 cultivated (*Hordeum vulgare* ssp. *vulgare* L.) and eight wild (*Hordeum vulgare* ssp. *spontaneum* C. Koch.) barley accessions. The six resistant accessions (all *H. vulgare*) shared the same DNA sequence, while eight different alleles were identified among the 28 susceptible accessions, including four which were unique to the wild accessions. Thirteen amino acid variations in the coding region were detected between resistant and susceptible accessions. Among these, four amino acids were predicted to be associated with changes in protein function. Sequence variation in the promoter region and coding sequence indicated that differences in functionality of the *Un8* candidate gene in resistant or susceptible barley lines might result from either transcriptional regulation or gain/loss of protein function.

4.1 Introduction

Ustilago nuda (Jens.) Rostr. (*U. nuda*) is a basidiomycete fungus which is the causal agent of barley loose smut. This common fungal pathogen in the prairie provinces of Western Canada can produce significant yield losses in the absence of effective management. *U. nuda* infects barley during flowering and becomes dormant within the mature embryo of infected seeds. Most tillers of plants from infected seeds do not produce seed, thus yield losses caused by loose smut are directly related with the percentage of infected plants (Thomas 1997).

At least 15 resistance genes against loose smut have been identified in barley germplasm (Mueller 2006). The *Un8* loose smut resistance gene (Metcalfe 1966) was

identified in the winter barley accession CN91953 collected from Azerbaijan (Metcalfe and Johnston 1963). It has been used to protect Canadian barley cultivars such as 'AC Oxbow', 'AC Metcalfe' and 'CDC Freedom' from yield losses for more than 60 years and remains very effective in both Canada and Europe (Metcalfe and Johnston 1963; Mueller et al. 2006; Menzies et al. 2014). However, very few studies have been carried out to investigate the *Un8*-mediated resistance mechanisms (Gabor and Thomas 1987). *Un8* was initially mapped onto the long arm of barley chromosome 5 (1HL) in linkage with the ABC 261 RFLP marker (Eckstein et al. 1993) and subsequently delimited to a 6.2 cM region as defined by barley unigenes 4245 and 14722 (HarvEST:Barley v. 1.83, assembly 35) (Eckstein, personal communication). As presented in Chapter 3, the *Un8* resistance locus was further defined to a 0.108 cM interval and eventually a candidate gene was identified which was predicted to encode a protein kinase containing two tandem kinase domains.

Additional evidence that the candidate gene identified in Chapter 3 is *Un8* can be obtained by sequencing alleles of this gene from an array of resistant and susceptible germplasm, including wild accessions, to determine if the resistant allele is conserved and if there are amino acid residues specific to the resistant allele which differentiates it from the susceptible allele(s). This was the case with another durable barley resistance gene, *Rpg1*, which has protected North American barley against stem rust, caused by *Puccinia graminis* f. sp. *tritici* and *Puccinia graminis* f. sp. *secalis*, for over 70 years. It was determined that all resistant cultivars shared the same amino acid sequence for Rpg1 and almost all alleles from susceptible lines contained a serine to arginine conversion at position 320 and phenylalanine insertion at position 321 (Brueggeman et al. 2002; Mirlohi et al. 2008).

Around 10,000 years ago, cultivated barley (*Hordeum vulgare* ssp. *vulgare* L., subsequently *Hv*) (2n = 2x = 14) was domesticated from its wild progenitor (*Hordeum vulgare* ssp. *spontaneum* C. Koch., subsequently *Hs*) (2n = 2x = 14) in the Near East Fertile Crescent (Badr et al. 2000). *Hs* has been a valuable source for improving cultivated barley in breeding programs, especially as a source of resistance to various diseases (Fetch et al. 2003), such as leaf rust (*Puccinia hordei* Otth.) (Ivandic et al. 1998; Fetch et al. 2003; Steffenson et al. 2007), net blotch (*Pyrenophora teres* f.sp. *teres*) (Fetch et al. 2003), powdery mildew

(*Blumeria graminis* f.sp. *hordei*) (Fetch et al. 2003; von Bothmer and Komatsuda 2011), scald (*Rhynchosporium secalis*) (von Bothmer and Komatsuda 2011), and spot blotch (*Cochliobolus sativus*) (Roy et al. 2010). It is unclear if the *Un8* resistance allele was present with *Hs* or if it arose through mutations after domestication. A preliminary attempt to answer this question was made by sequencing the *Un8* candidate gene and evaluating for reaction to loose smut in a limited set of *Hs* accessions.

The objective of this study was to sequence the *Un8* candidate gene, including the promoter region, in barley accessions (both *Hv* and *Hs*) derived from different regions of the world to: a) uncover additional alleles of the *Un8* candidate gene from *Hv* and *Hs*; b) identify polymorphisms within the *Un8* candidate gene that differentiate resistant from susceptible alleles to determine regions within the gene critical for governing loose smut resistance; c) identify polymorphisms within the promoter region of the *Un8* candidate gene to identify sequences that might result in differential expression; d) sequence the *Un8* candidate gene in the barley accession CN91953 to confirm that it is the original source of *Un8* used in Canada for loose smut resistance breeding.

4.2 Materials and methods

4.2.1 Plant materials and disease phenotyping

Accessions of *Hv* and *H*s were obtained from the Crop Development Centre (University of Saskatchewan, Saskatoon, SK, Canada) and from Plant Gene Resources of Canada (Saskatoon, SK, Canada). Reaction to loose smut was evaluated as described in Chapter 3. For accessions that required vernalization, seeds were germinated on damp cotton balls and seedlings at the one to two leaf stage were kept at 4°C in the dark for seven weeks before transferring to soil.

4.2.2 Allele sequencing

Genomic DNA was isolated from young leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Procunier et al. 1991) and primer pairs were designed by Primer Premier 5.0 software (PREMIER Biosoft International, USA) to produce overlapping

amplicons that encompassed the full genomic sequence of the *Un8* candidate gene including the ~2,100 bp promoter sequence. The sequence was obtained from the cv. 'Morex' available from BARLEX (http://barlex.barleysequence.org). Primer sequences are provided in Table 4.1 and their relative positions upstream and within the *Un8* candidate gene coding region are shown in Fig. 4.1.

Standard PCR amplifications were performed in a 25 µl volume containing 1 × Lucigen® PCR buffer, dNTPs (100 µM each), primers (0.2 µM each), 50 ng genomic DNA, and 1 U Taq DNA polymerase. PCR conditions were: 5 min at 94°C for initial denaturation, followed by 35 cycles of 94°C for 45 s, 54-60°C for 45 s, 72°C for 1 min, and a final extension step at 72°C for 5 min. The amplification products were separated on 1% agarose gels and the desired DNA fragments were purified from gels using the QIAquick Gel Extraction Kit (Qiagen). The purified fragments were cloned into the TOPO® TA® cloning vector (Invitrogen) and 5-6 clones of each amplicon were Sanger Sequenced by Eurofins Genomics (Louisville, KY, USA) to ensure the consistency of the sequence data. Sequence alignment was conducted by DNAMAN v.7 software (Lynnon Biosoft) and the upstream promoter region was analyzed using the Web-based tool PlantPAN 2.0 (http://plantpan2.itps.ncku.edu.tw/) (Chow et al. 2015) for the presence of *cis*-acting regulatory regions.

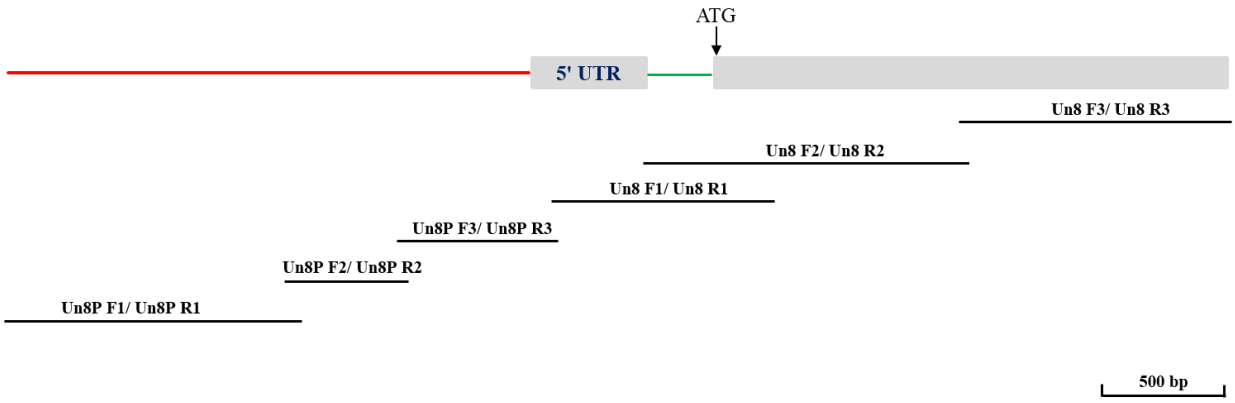


Fig. 4.1 Relative positions of PCR amplicons (indicated by black lines) used to sequence the upstream promoter and coding regions of the *Un8* candidate gene. Sequence was obtained from cv. 'Morex'. PCR primer names are indicated above each PCR amplicon. Red line: promoter sequence immediately upstream of the 5' untranslated region (UTR); Green line in the gene: intron; Grey boxes: exons of the *Un8* candidate gene. The translation start site is located in the second exon. The full length of the two exons and intron in 'Morex' is 2,763 bp.

Table 4.1 Information for PCR primers used to sequence the *Un8* candidate gene.

Primer Name	Sequence (5' - 3')	Position[a] (bp)	Annealing Temperature (°C)	Amplicon Size[a] (bp)
Un8P F1	AGGGTAAGGTTGCCCGTAAT	-934	60	1200
Un8P R1	GGCGAAGTGGAAGCAGAA	-2105		
Un8P F2	AGCCTTCTCAACTTAGCCTGGT	-504	54	500
Un8P R2	CTTTACACTTGGGGCACTACACTA	-987		
Un8P F3	TGCTAGTGTGGCTCACTCGTAC	128	57	650
Un8P R3	AACCAGGCTAAGTTGAGAAGGC	-526		
Un8 F1	GTGCTCAAGTGGCAATCCT	70	56	900
Un8 R1	ACCCATTGGTCGTCGTCA	941		
Un8 F2	GATCCTCGTGTACTCAGGTCTC	441	57	1300
Un8 R2	AAATGCTACTCCAGGCTACG	1768		
Un8 F3	ACAAACAGGTTGGCGATTC	1689	58	1100
Un8 R3	TGACATCTTTGTGGCATTACC	2829		

[a]Primer pair position and amplicon size were based on sequence obtained from cv. 'Morex'.

4.3 Results

4.3.1 Loose smut reaction among the cultivated and wild barley accessions

Among the 26 *Hv* accessions which were evaluated for reaction to loose smut only six showed a resistant reaction. Five of these accessions were lines derived from breeding programs located in Western Canada, while the sixth accession was the landrace CN91953 confirming it is likely the source of *Un8* used in North American breeding programs (Table 4.2). All eight wild *Hs* accessions displayed susceptibility to the *U. nuda* pathogen (Table 4.2).

4.3.2 Alignment of the deduced amino acid sequence for *Un8* among cultivated and wild barley accessions

The *Un8* candidate gene from 26 cultivated and eight wild barley (*Hs*) accessions representing diverse geographic regions was sequenced and the deduced amino acid sequences aligned. The accessions were classified into nine different groups based on a variety of amino acid substitutions and indels, as well as a drastically truncated sequence due to the presence of a premature stop codon. All of the resistant accessions, including the landrace CN91953, shared an identical amino acid sequence (Group I) which indicates a single origin for the *Un8* gene within breeding programs (Table 4.2, Fig. 4.2). Among the

resistant accessions, TR12135 carries two alleles, one matching the resistant accessions while the other matched the Group III susceptibility allele. This is probably because TR12135 is heterogeneous at the *Un8* locus, having been derived at the F4 stage (Beattie, personal communication). Group II and Group III alleles were identified from *Hv* accessions and represented the dominant haplotypes associated with susceptibility to loose smut, while Group VI alleles were found in a smaller percentage of *Hv* accessions. Four alleles (Group IV, V, VII, and VIII) were found only in wild barley accessions, Group VIII alleles containing a premature stop codon. Only a portion of the *Un8* candidate in the first protein kinase domain could be isolated from the Group IX accessions (data not shown), indicating that a more significant deletion had occurred.

Thirteen amino acid variations were identified which differentiated resistant from susceptible accessions with most of the amino acid differences located in the kinase II domain (Fig. 4.2). Among these differences, serine (S) residues at positions 190 and 532 and glutamine (Q) residues at positions 513 and 530 in the resistant (R) protein, all of which are associated with predicted functional residues, were deleted or converted into other amino acids which may result in a loss of function (Fig. 4.2).

Table 4.2 Origin, loose smut reaction and *Un8* candidate gene allele carried by the F4 recombinant inbred line mapping population parents (TR09398 and TR07728) and 32 cultivated and wild barley accessions of diverse origin.

Line	Species	Pedigree	Origin	Loose Smut Reaction	*Un8* Candidate Gene Allele Group
'AC Metcalfe'	*H. v.*	'AC Oxbow'/'Manley'	Canada	R	Group I
CN91953[a, b]		N/A	Azerbaijan	R	
HB11316		'CDC Rattan'/SH041242	Canada	R	
TR09398		TR238/Wpg8412-9-2-1//'Baronesse'/TR336	Canada	R	
TR11698		'Ponoka'/H93102002	Canada	R	
TR12135		TR253/BM9216-4//SM04261	Canada	R	
TR07728	*H. v.*	'Salute'/'Xena'	USA	S	Group II
'Bowman'		'Klages'//'Fergus'/'Nordic'/3/ND1156/4/'Hector'	USA	S	
'OAC 21'[a]		Selection from manchurian introduction	Canada	S	
'Morex'[a]		'Cree'/'Bonanza'	USA	S	
TR12737		'Xena'/'Sebastian'	USA	S	
'Calcule'		97-7207-484/'Zenobia'	Germany	S	
'Streif'		'Pasadena'/'Aspen'	Germany	S	

Table 4.2. (continued).

Line	Species	Pedigree	Origin	Loose Smut Reaction	*Un8* Candidate Gene Allele Group
'Barke'		'Libelle'/'Alexis'	Germany	S	
'Baudin'		'Stirling'/'Franklin'	Australia	S	
'Carisima'		'Femina'/O6306//L5184/'Prisma'	South America	S	
'Champlain'[a]	*H. v.*	'Moore'/'Montcalm'	Canada	S	Group III
CN5658[b]		N/A	Iran	S	
CN62649[a]		'Keowee'/'Volbar'	USA	S	
'Jet'		N/A	Ethiopia	S	
'Montcalm'[a]		Michigan 31604/Common 6-Rowed 4307 MC// Mandscheuri 1807 MC	Canada	S	
CN72631	*H. s.*	N/A	Ethiopia	S	Group IV
CN48980	*H. s.*	N/A	Turkey	S	Group V
'Ayelen'		G6066/'Quilmes Alfa'	South America	S	
'CDC Austenson'	*H. v.*	TR128//TR236/WM862-6/3/94Ab12271	Canada	S	Group VI
'Optic'		'Corniche'/'Force'//'Chad'	UK	S	
CN49142	*H. s.*	N/A	Turkey	S	Group VII
CN48518		N/A	Jordan	S	
CN49323	*H. s.*	N/A	Greece	S	Group VIII[c]
CN50037		N/A	Greece	S	
'CDC Copeland'	*H. v.*	WM861-5/TR118	Canada	S	
CN5708[a, b]	*H. v.*	N/A	Iran	S	Group IX[d]
CN46365	*H. s.*	N/A	Syria	S	
CN49887	*H. s.*	N/A	Israel	S	

[a]Six-row barley;
[b]CN5658, CN5708, and CN91953 are landrace accessions;
[c]Alleles contains a premature stop codon;
[d]Allele is partially deleted.

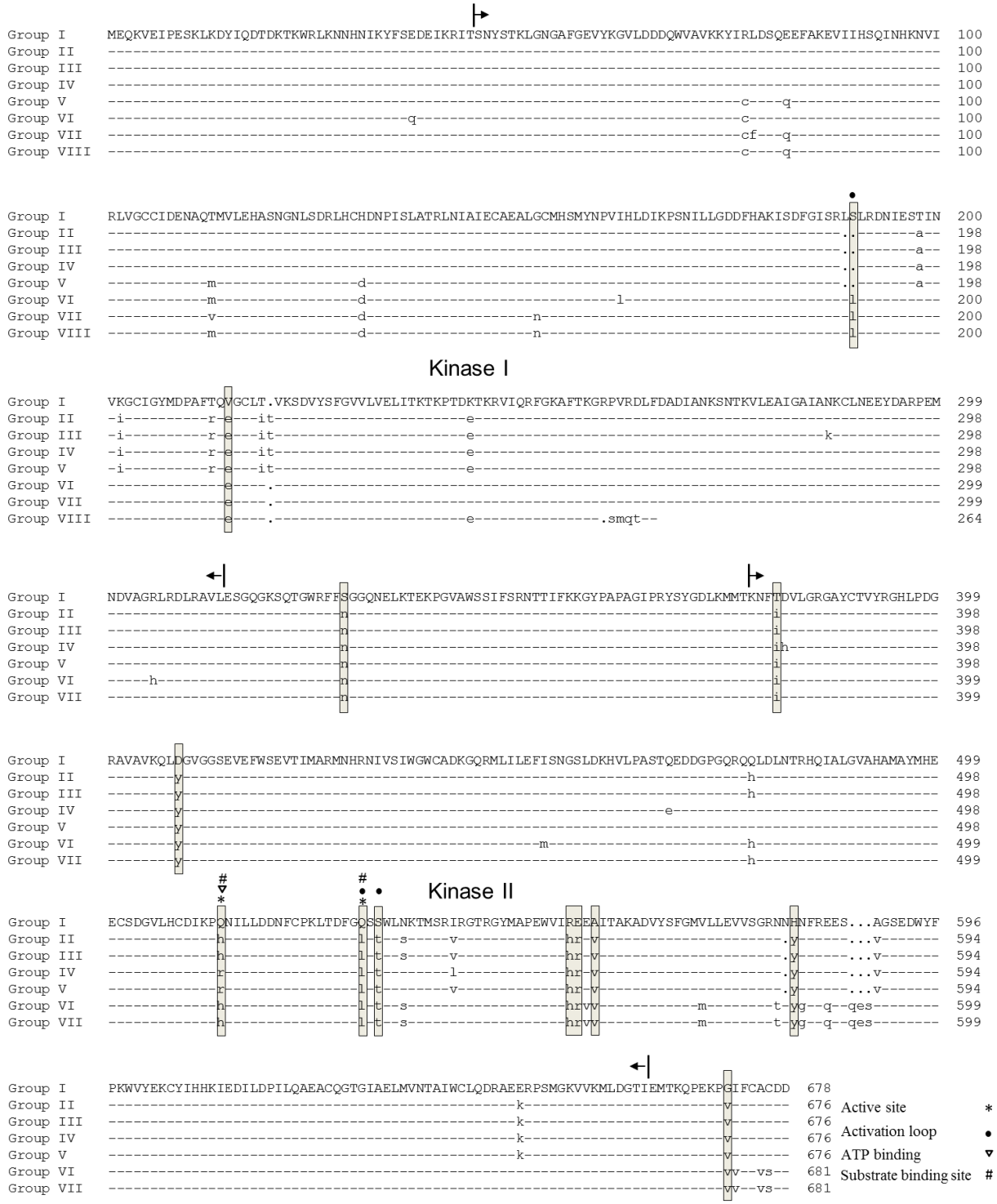
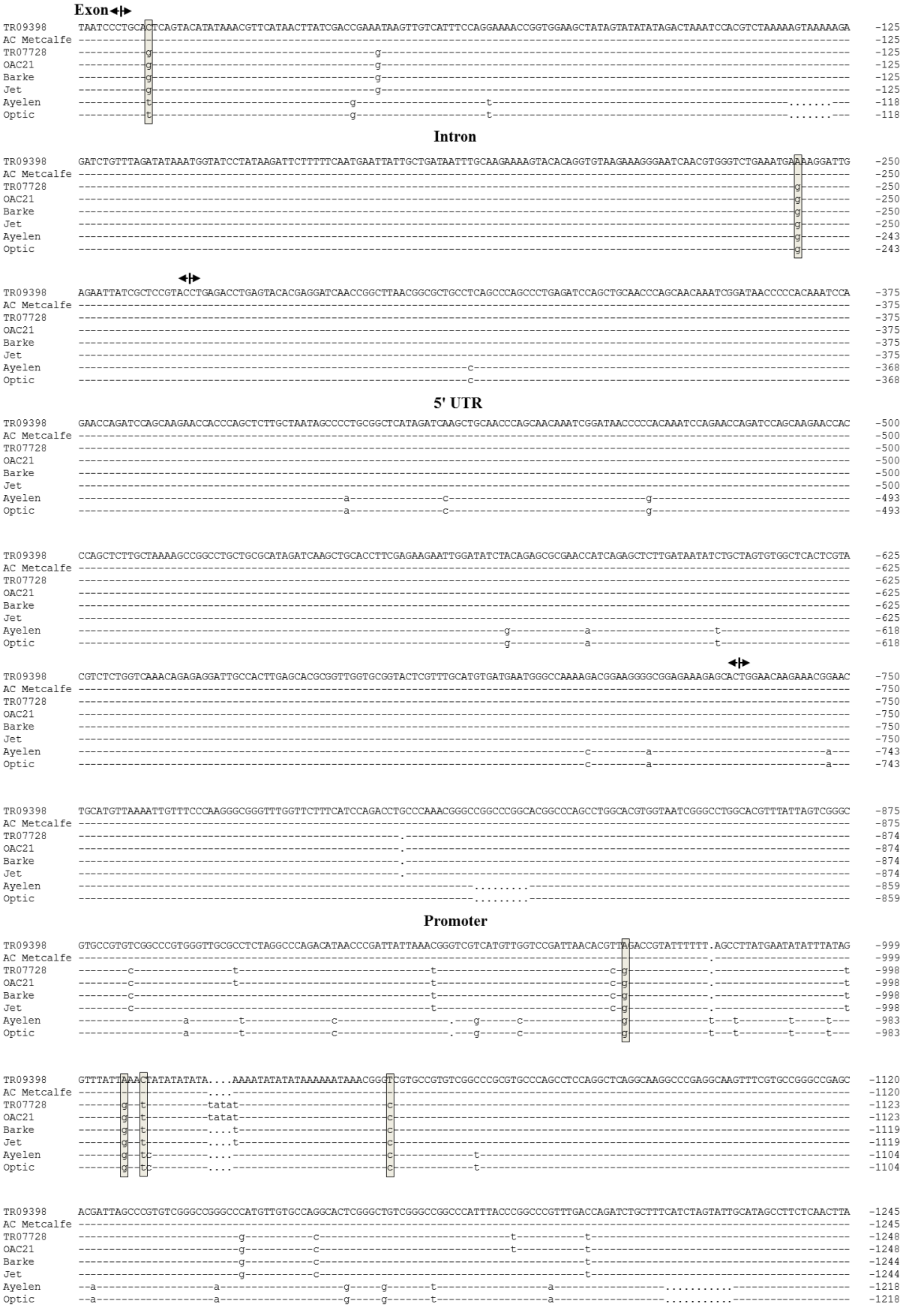


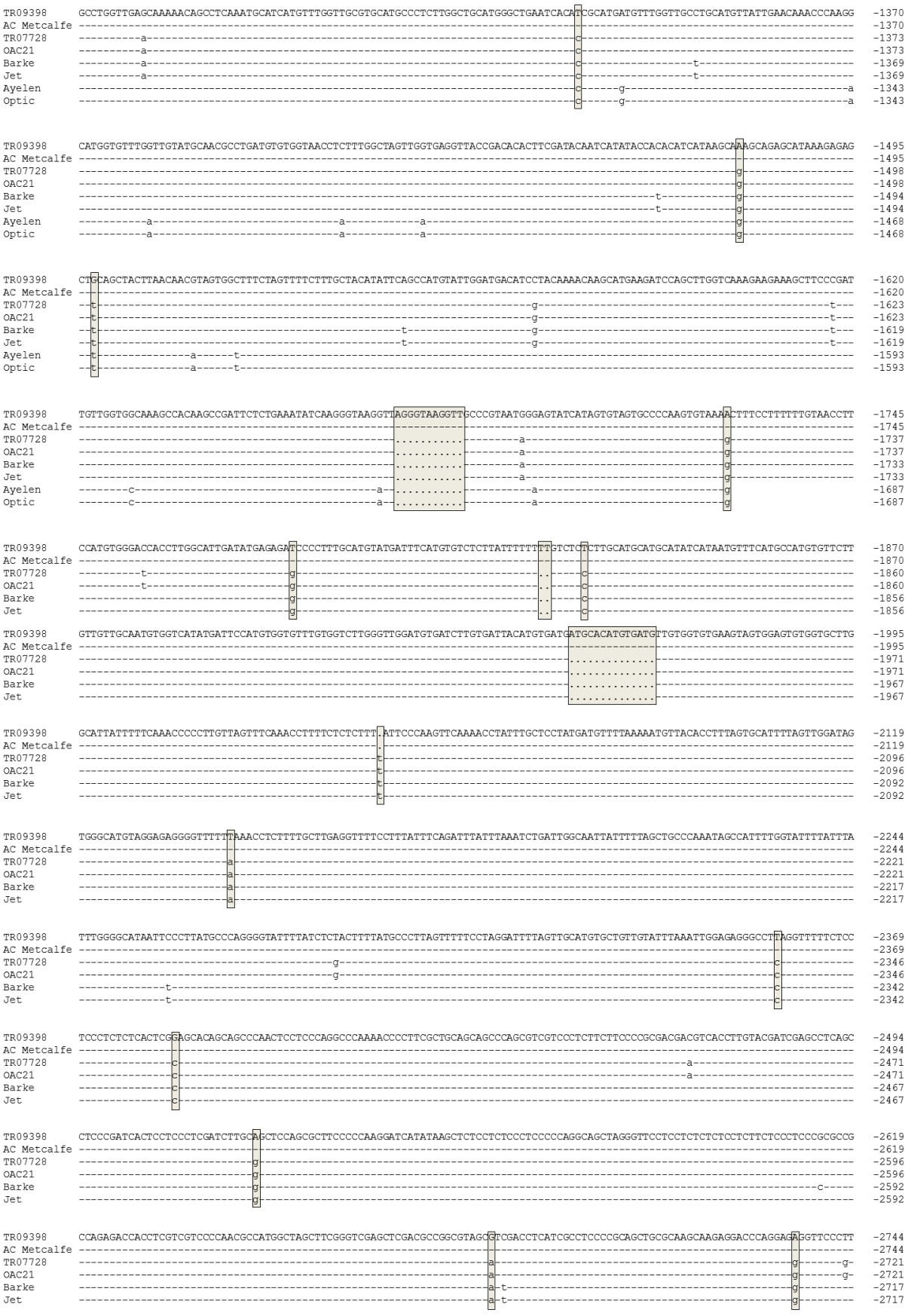
Fig. 4.2 Amino acid sequence alignment of the *Un8* candidate gene alleles derived from thirty-four cultivated and wild barley accessions. The Group I allele is associated with resistance while Groups II-VIII are found in susceptible accessions. The dominant features of the deduced protein are the two tandem kinase domains (each of which is delimited by forward and reverse-pointing arrows). Thirteen amino acid variations between resistant and susceptible accessions (indicated by boxes) were identified and seven amino acid differences were found only in the wild barley accessions. Most of the sequence differences (9/13) were found in the second protein kinase domain.

4.3.3 Sequence alignment upstream of the *Un8* coding sequence among cultivated barley lines

Based on the deduced amino acid sequence alignment for the *Un8* candidate gene, eight cultivated barley lines (two lines/Group) (resistant: TR09398 and 'AC Metcalfe'; susceptible: TR07728, 'OAC21'; 'Barke', 'Jet'; 'Ayelen', and 'Optic') were chosen for sequencing of the region upstream of the translation start site. Four primer pairs (Un8P F1/Un8P R1, Un8P F2/ Un8P R2, Un8P F3/ Un8P R3, Un8 F1/ Un8 R1) were designed for this purpose (Fig. 4.1). For 'Ayelen' and 'Optic' (Group VI), the amplicons yielded by Un8P F1/ Un8P R1 were not of the expected size and DNA sequence alignment indicated the amplicons were not derived from the correct location (1HL) but from chromosome 2H (http://webblast.ipk-gatersleben.de/barley/viroblast.php). These findings could indicate that this fragment might have been translocated in both Ayelen and Optic.

DNA sequence alignment showed that only two polymorphic sites distinguished resistant from susceptible lines within the intron. None were found within the first exon (5' UTR) (Fig. 4.3). In the promoter region, a total of 22 polymorphisms, including 19 SNPs and three insertion/deletion (indels), were identified between resistant and susceptible accessions, with the most common SNP being an A/G transition (Fig. 4.3).


Exon
Intron
5' UTR
Promoter
TR09398
AC Metcalfe
TR07728
OAC21
Barke
Jet
Ayelen
Optic


TR09398 GCCTGGTTGAGCAAAAACAGCCTCAAATGCATCATGTTTGGTTGCGTGCATGCCCTCTTGGCTGCATGGGCTGAATCACATCGCATGATGTTTGGTTGCCTGCATGTTATTGAACAAACCCAAGG -1370
AC Metcalfe ---------- -1370
TR07728 ----------a----------c---------- -1373
OAC21 ----------a----------c---------- -1373
Barke ----------a----------c----------t---------- -1369
Jet ----------a----------c----------t---------- -1369
Ayelen ----------c-----g----------a -1343
Optic ----------c-----g----------a -1343
TR09398 CATGGTGTTTGGTTGTATGCAACGCCTGATGTGTGGTAACCTCTTTGGCTAGTTGGTGAGGTTACCGACACACTTCGATACAATCATATACCACACATCATAAGCAAAGCAGAGCATAAAGAGAG -1495
AC Metcalfe ---------- -1495
TR07728 ----------g---------- -1498
OAC21 ----------g---------- -1498
Barke ----------t----------g---------- -1494
Jet ----------t----------g---------- -1494
Ayelen ----------a----------a----------a----------g---------- -1468
Optic ----------a----------a----------a----------g---------- -1468
TR09398 CTGCAGCTACTTAACAACGTAGTGGCTTTCTAGTTTTCTTTGCTACATATTCAGCCATGTATTGGATGACATCCTACAAAACAAGCATGAAGATCCAGCTTGGTCAAAGAAGAAAGCTTCCCGAT -1620
AC Metcalfe ---------- -1620
TR07728 ----------g----------t--- -1623
OAC21 ----------g----------t--- -1623
Barke ----------t----------g----------t--- -1619
Jet ----------t----------g----------t--- -1619
Ayelen ----------a-----t---------- -1593
Optic ----------a-----t---------- -1593
TR09398 TGTTGGTGGCAAAGCCACAAGCCGATTCTCTGAAATATCAAGGGTAAGGTTAGGGTAAGGTTGCCCGTAATGGGAGTATCATAGTGTAGTGCCCCAAGTGTAAAACTTTCCTTTTTTGTAACCTT -1745
AC Metcalfe ---------- -1745
TR07728 ----------..........----------a----------g---------- -1737
OAC21 ----------..........----------a----------g---------- -1737
Barke ----------..........----------a----------g---------- -1733
Jet ----------..........----------a----------g---------- -1733
Ayelen --------c----------a-----..........----------a----------g---------- -1687
Optic --------c----------a-----..........----------a----------g---------- -1687
TR09398 CCATGTGGGACCACCTTGGCATTGATATGAGAGATCCCCTTTGCATGTATGATTTCATGTGTCTCTTATTTTTTTTGTCTCTCTTGCATGCATGCATATCATAATGTTTCATGCCATGTGTTCTT -1870
AC Metcalfe ---------- -1870
TR07728 ----------t----------g----------..----------c---------- -1860
OAC21 ----------t----------g----------..----------c---------- -1860
Barke ----------g----------..----------c---------- -1856
Jet ----------g----------..----------c---------- -1856
TR09398 GTTGTTGCAATGTGGTCATATGATTCCATGTGGTGTTTGTGGTCTTGGGTTGGATGTGATCTTGTGATTACATGTGATGATGCACATGTGATGTTGTGGTGTGAAGTAGTGGAGTGTGGTGCTTG -1995
AC Metcalfe ---------- -1995
TR07728 ----------.......... -1971
OAC21 ----------.......... -1971
Barke ----------.......... -1967
Jet ----------.......... -1967
TR09398 GCATTATTTTTCAAACCCCCTTGTTAGTTTCAAACCTTTTCTCTCTTTCATTCCCAAGTTCAAAACCTATTTGCTCCTATGATGTTTTAAAAATGTTACACCTTTAGTGCATTTTAGTTGGATAG -2119
AC Metcalfe ----------. -2119
TR07728 ----------t---------- -2096
OAC21 ----------t---------- -2096
Barke ----------t---------- -2092
Jet ----------t---------- -2092
TR09398 TGGGCATGTAGGAGAGGGGTTTTTTAAACCTCTTTTGCTTGAGGTTTTCCTTTATTTCAGATTTATTTAAATCTGATTGGCAATTATTTTTAGCTGCCCAAATAGCCATTTTGGTATTTTATTTA -2244
AC Metcalfe ---------- -2244
TR07728 ----------a---------- -2221
OAC21 ----------a---------- -2221
Barke ----------a---------- -2217
Jet ----------a---------- -2217
TR09398 TTTGGGGCATAATTCCCTTATGCCCAGGGGTATTTTATCTCTACTTTTATGCCCTTAGTTTTTCCTAGGATTTTAGTTGCATGTGCTGTTGTATTTAAATTGGAGAGGGCCTTAGGTTTTTCTCC -2369
AC Metcalfe ---------- -2369
TR07728 ----------g----------c---------- -2346
OAC21 ----------g----------c---------- -2346
Barke ----------t----------c---------- -2342
Jet ----------t----------c---------- -2342
TR09398 TCCCTCTCTCACTCGGAGCACAGCAGCCCAACTCCTCCCAGGCCCAAAACCCCTTCGCTGCAGCAGCCCAGCGTCGTCCCTCTTCTTCCCCGCGACGACGTCACCTTGTACGATCGAGCCTCAGC -2494
AC Metcalfe ---------- -2494
TR07728 ----------c----------a---------- -2471
OAC21 ----------c----------a---------- -2471
Barke ----------c---------- -2467
Jet ----------c---------- -2467
TR09398 CTCCCGATCACTCCTCCCTCGATCTTGCAGCTCCAGCGCTTCCCCCAAGGATCATATAAGCTCTCCTCTCCCTCCCCCAGGCAGCTAGGGTTCCTCCTCTCTCTCCTCTTCTCCCTCCCGCGCCG -2619
AC Metcalfe ---------- -2619
TR07728 ----------g---------- -2596
OAC21 ----------g---------- -2596
Barke ----------g----------c----- -2592
Jet ----------g---------- -2592
TR09398 CCAGAGACCACCTCGTCGTCCCCAACGCCATGGCTAGCTTCGGGTCGAGCTCGACGCCGGCGTAGCGTCGACCTCATCGCCTCCCCGCAGCTGCGCAAGCAAGAGGACCCAGGACAGGTTCCCTT -2744
AC Metcalfe ---------- -2744
TR07728 ----------a----------g-------g- -2721
OAC21 ----------a----------g-------g- -2721
Barke ----------a-t----------g---------- -2717
Jet ----------a-t----------g---------- -2717

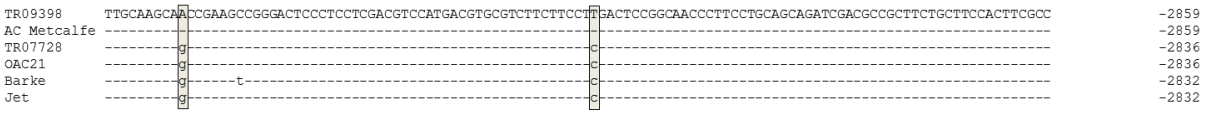


Fig. 4.3 DNA sequence variations identified upstream of the translation start site among eight cultivated barley lines representing four different *Un8* candidate gene alleles. TR09398 and 'AC Metcalfe' are resistant accessions (Group I) while TR07728 and 'OAC21' (Group II), 'Barke' and 'Jet' (Group III), and 'Ayelen' and 'Optic' (Group VI) are susceptible accessions. DNA sequence variations between resistant and susceptible accessions are indicated by boxes.

4.3.4 *Un8* candidate gene promoter sequence variation

Approximately 2,000 bp of the upstream promoter region were analyzed for the presence of *cis*-acting regulatory regions in which sequence variation was present that differentiated resistant from susceptible alleles. A total of 15 such *cis*-regulatory elements were identified which contained SNPs or indels which have the potential for altered transcriptional regulation of the *Un8* candidate gene (Table 4.3). Among them, five *cis*-regulatory elements (AP2, bHLH, bZIP, Homeodomain (TALE), and WRKY) are related with biotic stress (Table 4.3).

Table 4.3 *Cis*-elements identified in the ~2,000 bp region upstream of the *Un8* candidate gene from a set of eight resistant and susceptible cultivated barley accessions.

***Cis*-element Name**	**Description**		**Position**[a]	**Sequence of TF Binding Site**[b]	**Similarity Score**
AP2	Ethylene-responsive transcription factor. Probably acts as a transcriptional activator. Binds to the GCC-box pathogenesis-related promoter element and also involved in carotenoid biosynthesis regulation.	TF_motif_seq_0254	963-	TAGAC	0.8
			1008+	AACTA	0.8
			1776-	GAGAT	0.8
		TFmatrixID_0623	1663-	ggTAAGGtta	0.9
B3	Iron-deficiency response.	TF_motif_seq_0256	1949+	GATGC	0.8
bZIP	Plays a role in plant immunity, abiotic stress responses, and seed dormancy control.	TFmatrixID_0193	1039-	aaaCGGGT	0.75
		TF_motif_seq_0271	960-	CGTTA	0.8
			1948+	TGATG	0.8
			2685-	CGTCG	0.8
			2804+	TGACT	0.8
bHLH	Common transcription factor of light, abscisic acid (ABA), and jasmonic acid (JA) signaling pathways. In cooperation with MYB2 is involved in the regulation of ABA-inducible genes under drought stress conditions.	TF_motif_seq_0298	1953+	CACATg	1
		TF_motif_seq_0300	1953+	CACATg	0.8
		TF_motif_seq_0301	1953+	CACATg	1

Table 4.3. (continued).

Cis-element Name	Description		Position[a]	Sequence of TF Binding Site[b]	Similarity Score
Dehydrin	Low temperature responsive element.	TF_motif_seq_0258	1045-	GTCGT	0.8
			2381-	CTCGG	0.8
			2686-	GTCGA	0.8
Dof	Acts as a negative regulator in phytochrome-mediated light responses.	TFmatrixID_0236	959-	aCGTTAga	1
		TFmatrixID_0472	1476+	AAAGCa	1
		TFmatrixID_0638	2036-	ctcTCTTT(T)at	0.99
		TF_motif_seq_0239	1725-	ACTTT	1
			2353-	GCCTT	1
			2800-	TCCTT	1
GATA; tify	No function indicated.	TF_motif_seq_0237	1324-	CATCG	1
			1778-	GATCC	1
			1948+	TGATG	1
Homeodomain; TALE	Core of the TGAC-containing W-box. Parsley WRKY proteins bind specifically to TGAC-containing W box elements within the Pathogenesis-related Class10 (PR-10) genes. Required for shoot apical meristem (SAM) formation during embryogenesis.	TF_motif_seq_0246	2804+	TGACT	1
Homeodomain; HB-PHD	Recognizes a DNA fragment of the light-induced cab-E.	TFmatrixID_0285	2143+	tTAAACctct	1
Myb	Binds preferentially double-stranded telomeric repeats, but can bind to the single G-rich telomeric strand.	TFmatrixID_0363	1670+	tTAGGGta	0.98
		TFmatrixID_0365	1668+	ggtTAGGGta	1
		TFmatrixID_0551	2752-	CAACCgaa	0.95
NAC; NAM	Involved in anther development and response to desiccation.	TFmatrixID_0382	2801-	ccTTGACtc	1
Storekeeper	Unknown function.	TFmatrixID_0417	1042-	cgGGTCGtg	0.99
TCR; CPP	Plays a role in development of both male and female reproductive tissues.	TFmatrixID_0224	2141+	tTTTAAac	0.99
		TF_motif_seq_0251	1042-	CGGGT	1
		TF_motif_seq_0266	964+	AGACC	0.75
			1043+	GGGTC	0.75
			1044+	GGTCG	0.75
			1778+	GATCC	0.75
			2384-	GGAGC	0.75
		TF_motif_seq_0431	961-	gttAGACC	0.75
Trihelix	Myb/SANT-like DNA-binding domain.	TF_motif_seq_0267	961+	GTTAG	0.75
			1669+	GTTAG	0.75
			2751-	GCAAC	0.75

Table 4.3. (continued).

Cis-element Name	Description		Position[a]	Sequence of TF Binding Site[b]	Similarity Score
WRKY	WRKY DNA-binding domain; involved in the control of processes related to senescence and pathogen defense.	TF_motif_seq_0270	2804+	TGACT	1
		TF_motif_seq_0339	2803+	TTGACt	1
		TFmatrixID_0445	2802-	cTTGACtc	1
ZF-HD	Regulates floral architecture and leaf development. Regulates the abscisic acid (ABA) signal pathway that confers sensitivity to ABA in an ARF2-dependent manner.	TF_motif_seq_0241	1004+	ATTAA	1

[a]Position denoted based on the sequence from resistant Group I allele. The start of analyzed promoter sequence was designated as -733;
[b]Red text indicates sequence variation specific to the resistant Group I allele; bracketed base(s) are not present in the resistant Group I allele.

4.4 Discussion

Work carried out in Chapter 3 identified two overlapping bacterial artificial chromosome (BAC) clones spanning the *Un8* locus and a *Un8* candidate gene was found by positional cloning and sequencing of the two BACs. In the current study, *Hv* accessions from diverse geographic regions and *Hs* accessions mainly from the Near East Fertile Crescent (recognized as the barley domestication centre) were sequenced and analyzed to characterize *Un8* candidate gene alleles, identify important amino acid residues associated with gene function and confirm the original accession from which the *Un8* candidate gene was originally identified.

A significant number of polymorphisms in the *Un8* candidate gene were detected among the selected accessions within both the open reading frame and upstream promoter region. Nine different alleles were identified based on deduced amino acid sequence variation with all resistant accessions harbouring the same Group I allele. It is not surprising that a high level of gene conservation was found in resistant resources given that the *Un8* resistance used in all Canadian breeding programs is believed to have been derived from the same landrace, CN91953 (Fig. 4.2, Table 4.2). CN91953 was collected in 1927 from Azerbaijan (http://pgrc3.agr.ca/cgi-bin/npgs/html/acchtml.pl?107547), a region close to barley's domestication centre. This region has previously been found to be associated with mutations relevant to the domestication of barley. For example, the ancestral allele of *SD2*, in which a

single mutation in the *Mitogen-activated protein kinase kinase 3* (*MKK3*) gene decreases MKK3 kinase activity and is thought to be responsible for the lack of seed dormancy in non-dormant cultivars, was identified in Azerbaijan (Nakamura et al. 2016). In the limited sample size used for this study, none of the *Hs* and landrace accessions carried a Group I allele associated with resistance. Thus, a larger-scale effort to screen germplasm (both *Hs* and landraces) derived from or around the Azerbaijan area is necessary to identify the ancestral allele of the *Un8* candidate gene and to determine if this region is indeed the origin of the *Un8* candidate gene.

Based on the deduced amino acid sequence alignment for the *Un8* candidate gene, most of the *Hs* accessions tested were classified into distinct allele groups from *Hv* accessions. The exceptions were *Hs* accessions CN46365 and CN49887 which were grouped with 'CDC Copeland' and *Hv* accession CN5708 as it appeared that the same portion of the *Un8* candidate in these accessions was deleted. In addition, some *Hs* accessions contained rare alleles which were not found in *Hv*, for example the premature stop codon in the Group VIII allele of CN49323 and CN50037 collected from Greece. These observations together indicate that most of the *Hs* alleles have likely been lost prior to barley domestication. The fact that different alleles for the *Un8* candidate are present in *Hs* indicates this gene did exist in the wild barley genepool and that the origin of the *Un8* candidate gene (originally identified in CN91953) possibly arose after barley domestication by the accumulation of mutations. Similarly, it is interesting to note that an intact and functional *Rpg1* gene has yet to be found in any *Hs* accession and may also have developed after domestication (Mirlohi et al. 2008). However, as only eight *Hs* accessions were tested in this study, additional *Hs* accessions are needed to support this hypothesis.

Amino acid sequence alignment showed that most of the variations (9/13) which differentiated the resistant Group I allele from susceptible alleles were present in the second kinase domain, indicating that the second kinase domain is probably more important in explaining resistance/susceptibility, possibly via differences in total kinase activity. In barley, *Rpg1* is the only other known resistance gene which has the same general structure as the *Un8* candidate gene. Interestingly, both are durable resistance genes that contain two tandem

protein kinase domains (Brueggeman et al. 2002). Even though both kinase domains in *Rpg1* were deemed essential for stem rust resistance, only the second was catalytically active (Nirmala et al. 2006). In mammals, the Janus Kinases, which are important for cytokine signalling networks, carry two kinase domains with the first kinase domain catalytically inactive (Yamaoka et al. 2004).

A total of 13 amino acid variations in the *Un8* candidate gene differed between resistant and susceptible alleles. Several studies (Bryan et al. 2000; Nakamura et al. 2016; Sato et al. 2016a; Yu et al. 2016) have suggested that even a few changes in amino acid sequence could significantly affect protein function. For example, variation in barley seed dormancy is a consequence of a single mutation in *Qsd1* and *Qsd2* (Sato et al. 2016a; Nakamura et al. 2016). The change from covered to naked caryopsis is achieved by a single mutation in the dominant allele of the *Nud* gene, which is involved in the lipid biosynthesis pathway (Yu et al. 2016). In rice, the loss of blast disease resistance was reported to be the result of an amino acid change in the Pi-ta resistance protein (Bryan et al. 2000). Therefore it is possible that any of the variations identified in the *Un8* candidate gene could play a role in the loss of resistance. Four residues (S190 in Kinase I; Q513, Q530, and S532 in Kinase II) in particular were the most promising candidates responsible for loss of resistance as they were located within predicted functional sites. Further investigation revealed that the three sites (S190 in Kinase I, Q530 and S532 in Kinase II) within the activation loops may be most relevant as this loop is essential for the autophosphorylation of protein kinase (Nolen et al. 2004).

Alignment of the intron and 5' UTR across the cultivated accessions showed a significant amount of sequence conservation among groups. This high level of conservation might imply an important function operating at the post-transcriptional level for the *Un8* candidate gene, such as for mRNA stability and translation efficiency (Wilkie et al. 2003; Zou et al. 2003; Kim et al. 2014). After analyzing the upstream promoter region, 15 *cis*-regulatory motifs were identified which contained variation between resistant and susceptible alleles that might result in altered gene expression. The AP2, bHLH, bZIP, Homeodomain (TALE), and WRKY *cis*-regulatory motifs were of particular interest because of their roles in plant disease

resistance response. The bZIP (Wei et al. 2012a) and WRKY (Wei et al. 2012b) families are especially relevant as they have been shown to be involved in the maize-*Ustilago maydis* interaction.

Taken together, the data presented in this study suggests that the *Un8* candidate gene present in loose smut resistant Canadian breeding programs is likely derived from a single landrace source (CN91953). Although preliminary, it appears that the mutation(s) in the *Un8* candidate gene giving rise to resistance may have arisen after domestication. Based on sequence variation within and upstream of the candidate gene, two possible mechanisms may be responsible for the *Un8* candidate gene-mediated resistance: one is related to gene regulation as variation in several *cis*-regulatory motifs was identified; the other is associated with protein function as variation in several amino acid residues associated with predicted functional domains were identified. Additional work will be needed to study expression of the *Un8* candidate gene in resistant and susceptible lines to further explore the possibility of altered expression as the basis of the resistance mechanism.

CHAPTER 5

Assessment of *Ustilago nuda* Infection of Barley by Histological and PCR Analysis

Abstract

Barley loose smut, caused by *Ustilago nuda* (Jens.) Rostr. (*U. nuda*), is a widespread disease in Canadian barley production areas. Susceptible barley plants infected by *U. nuda* display no obvious symptoms until the heading stage when most floral parts and seeds are replaced by the teliospores of *U. nuda*. To reduce the incidence of loose smut, development of cultivars with genetic resistance using such long-lived and effective genes as *Un8* is highly desirable. Therefore, the availability of simple and reliable diagnostic methods to detect the presence of *U. nuda* within barley seeds is important for loose smut resistance breeding. In this study, a modified diagnostic method was used to observe the development of *U. nuda* mycelium within infected barley seeds obtained from both resistant and susceptible lines with the goal of identifying diagnostic differences between resistant and susceptible reactions. After staining with trypan blue, the mycelium of *U. nuda* could be clearly identified in the scutellum of embryos and almost every part of the embryo in hand-cut sections with no significant visual differences between resistant and susceptible lines. In contrast to previous reports (Gabor and Thomas 1987), no obvious tissue necrosis within the embryo was associated with the presence of *Un8*. Inoculated seeds of both resistant and susceptible lines frequently produced stunted seedlings that failed to grow, in addition to normal seedlings, which complicated the ability to differentiate the resistance reaction from possible tissue damage caused by excessive inoculum. Thus there are no histological or visual observations that differentiate the *U. nuda*-barley interaction in resistant or susceptible lines during the first six days post-germination. As expected, given the systemic nature of loose smut, when tested with PCR primers specific to the *U. nuda* pathogen all tissues of six day-old seedlings of the inoculated susceptible line were positive for *U. nuda* DNA. However, normal seedlings from inoculated seeds of the resistant line were negative for *U. nuda* DNA. While *U. nuda*-free seedlings from inoculated seeds of the resistant line may represent the normal phenotype of a resistant response, it is more likely that they are “escapes” from unsuccessful inoculation. Poorly germinated seeds and stunted seedlings from inoculated seeds, while possibly the result of a heavy

dose of inoculum as occurred in some seedlings of the inoculated susceptible seeds, could possibly be a result of the *Un8* resistance mechanism.

5.1 Introduction

The biotrophic fungus *Ustilago nuda* (Jens.) Rostr. (*U. nuda*), which is the cause of barley loose smut, attacks florets at the flowing stage and colonizes the plant without showing any obvious symptoms until the flowering period in the next growing season at which point most heads are replaced by a mass of teliospores (Thomas 1997). Thus, often no seeds are produced and considerable losses in yield result. The infection process of *U. nuda* in barley has been analyzed and described previously in susceptible barley lines and indicated that the pathogen was present in most parts of the embryo, including the scutellum, leaf primordia, and coleoptile, but less frequently in the radicle (Malik and Batts 1960a; Malik and Batts 1960c; Wunderle et al. 2012).

Embryo examination is the most common method to evaluate infection of barley by *U. nuda* (Morton 1961; Wunderle et al. 2012). According to Mobasser et al. (2012), after treatment with sodium hydroxide (NaOH) the separated embryos can be cleaned by lactic acid solution and the mycelium within the embryo will become visible with the use of a binocular microscope. Eibel et al. (2005) attempted to develop an enzyme-linked immunosorbent assay to detect *U. nuda* in the seedling stage, but it was determined to be unsuitable because of the poor correlation between the enzyme-linked immunosorbent assay results and microscopic examination of embryos, and the labor- and time-intensive nature of the assay. To study the infection process of *U. nuda* within the plant, fluorochrome Blankophor® was recently used to clearly show the presence of *U. nuda* within barley tissues after seed germination and a PCR-based method was developed and applied at the early growing stage to distinguish between healthy and infected plants from susceptible lines (Wunderle et al. 2012).

To date at least 15 loose smut resistance genes have been identified in barley (Mueller 2006). Among these, the *Un8* gene is effective against all known isolates of loose smut and

has been used in Canadian breeding programs for over half a century. However, very few reports are available to describe the mechanism of *Un8*-mediated loose smut resistance. One such study carried out by Gabor and Thomas (1987) attempted to elucidate when and in which tissues resistance conditioned by several loose smut resistance genes, *Un*, *Un3*, *Un6*, and *Un8*, was expressed. Based on histological observations, Gabor and Thomas (1987) found that in some embryos cell necrosis in the growing point, leaf primordium, and scutellum, and cell wall reinforcement within the embryo resulted from *Un8*-conditioned resistance. However, more than half of inoculated seeds harbouring the *Un8* gene still contained mycelia in the embryo which suggested that other growing stages/tissues could also be involved in *Un8*-mediated resistance (Gabor and Thomas 1987).

Wheat loose smut, caused by *Ustilago tritici* (Pers.) Rostr. (*U. tritici*), has a disease cycle similar to *U. nuda* (Wunderle et al. 2012). In contrast to the *Un8*-mediated barley loose smut resistance, no single resistance gene appears to be effective against all isolates of *U. tritici* (Kassa et al. 2014; Kassa et al. 2015). In contrast to observations made by Gabor and Thomas (1987), Popp (1959) pointed out that in highly resistant wheat lines, *U. tritici* was present only in the scutellum of the embryo. This was in agreement with the observations of Batts and Jeater (1958) that *U. tritici* was unable to proceed from the scutellum to the growing point in resistant lines. Additionally, in lines classified as immune, the mycelium of *U. tritici* could not even be found in the embryo (Popp 1959).

The main objectives of this work were to: 1) study the infection processes of *U. nuda* in both resistant and susceptible lines to clearly describe the distribution and relative quantity of mycelia in mature embryos; and 2) identify characteristic features of *Un8*-mediated resistance that restrict growth of *U. nuda* to certain tissues or growth stages which can be used as an early diagnostic tool to identify resistant barley lines.

5.2 Materials and methods

5.2.1 Plant materials and inoculation method

Five loose smut resistant lines ('AC Metcalfe', HB11316, TR09398, TR11698, and TR12135) and two susceptible lines (TR07728 and 'CDC Copeland') were grown in a growth chamber in the University of Saskatchewan phytotron under a 16 h light (22°C, 285 µmol photons m^{-2} s^{-1})/8 h dark (18°C) cycle at 50% relative humidity. Lines were inoculated at early anthesis as described in Chapter 3 and the inoculated lines were grown to maturity and seed harvested from the inoculated florets.

5.2.2 Microscopic observation of *U. nuda* within barley embryos

Ustilago nuda-infected barley embryos were extracted from mature seeds following the methods described by Mobasser et al. (2012) with several minor modifications. Briefly, seeds were placed in freshly prepared 5% (m/v) NaOH aqueous solution, with 0.01% (w/v) trypan blue, and kept at room temperature (~22°C) for 20 h. Seeds were then transferred into a container and washed in warm water to separate the embryo from the rest of the seed. Separated embryos were collected using a sieve with 1 mm^2 mesh and transferred to a lactic acid solution (lactic acid: glycerol: water; 1:1:1 ratio) maintained at boiling for 6 min to clear the stain from the embryos. The cleared embryos were kept in a 1:1 glycerol to water solution prior to observation with a dissecting microscope.

As the extracted embryos frequently become very fragile after boiling, making it impossible to study the infection processes, a modified preparation method was developed. Seeds were initially soaked in distilled water for 2-3 h, at which point longitudinal hand-cut sections were prepared with razor blades. Sections were treated with the 5% NaOH solution with 0.01% (w/v) trypan blue for 3 h and then transferred into the boiling lactic acid solution for 1 min to clear the embryo for observation.

5.2.3 Tissue sampling and DNA extraction for the detection of *U. nuda*

Inoculated seeds from resistant and susceptible lines (~36 seeds for each) were sown in pots (12 seeds/pot) at a depth of 3 cm (to encourage growth of larger sub-crown internodes) and grown in growth chambers (phytotron, University of Saskatchewan) under the conditions described above. Once plants had reached the one leaf stage (~6 days after sowing), each seedling was carefully removed from the soil and washed to remove soil from the roots. It was observed that seedlings tended to have three distinct growth morphologies, regardless of whether they were from resistant or susceptible lines, and were subsequently grouped according to their appearance. Seedlings could be grouped as: normal (Group I), seedlings that produced a coleoptile which emerged from the soil but no further development occurred and eventually the seedling died (Group II), and seedlings that produced a severely stunted coleoptile that did not emerge from the soil (Group III). After removal of the coleoptile, seedlings were dissected into the scutellum tissue, the lower region of sub-crown internode above where the scutellum attaches, and the ~1 cm upper region of the sub-crown internode (containing the crown node). DNA was extracted from tissue arising specific to these three regions using the modified cetyltrimethylammonium bromide (CTAB) method (Procunier et al. 1991).

The polymerase chain reaction (PCR) was used to detect the presence of *U. nuda* within the dissected tissues using a modified primer pair, (ITSUnF/ITSUnR: 5'-TGTGGCTCGCACCTGTCCAACTAA-3'/5'-TTCTCCTTGCGTCGCGCTGTTTGA-3'), which specifically amplify the *U. nuda* internal transcribed spacer (ITS) region. These primers were developed based on sequence data from Wunderle et al. (2012). PCR conditions were: 5 min at 94°C for the initial denaturation, followed by 35 cycles of 94°C for 45 s, 62°C for 45 s, 72°C for 1 min, and a final extension step at 72°C for 5 min.

Ustilago nuda DNA was also extracted from pure culture to provide a positive control sample for PCR. Teliospores were grown on potato dextrose agar for 7 d to produce mycelia which was dried, harvested by vacuum filtration and ground in liquid nitrogen for DNA extraction with the DNeasy Plant Mini Kit (Qiagen).

5.3 Results

5.3.1 Microscopic examination of *U. nuda* infection within whole embryos

Embryos were extracted from 20 randomly selected inoculated seeds of each line. After microscopic observation, the infection rate (i.e. the presence of *U. nuda* mycelium) was found to vary from 50% ('AC Metcalfe') to 90% (HB11316) (Table 5.1). There did not appear to be any difference between resistant and susceptible lines with respect to infection rate. The absence of mycelium in some seeds confirms the presence of escapes using this inoculation technique and the need to test multiple seeds of a given line before a determination of resistance or susceptibility can be made (Fig. 5.1A).

Microscopic examination of trypan blue-stained embryos revealed that in both susceptible and resistant lines mycelium could be observed in the scutellum (Fig. 5.1B). Additionally, different amounts of mycelium were observed in embryos from both resistant and susceptible lines (data not shown), likely a result of the inherent variability of the inoculation technique.

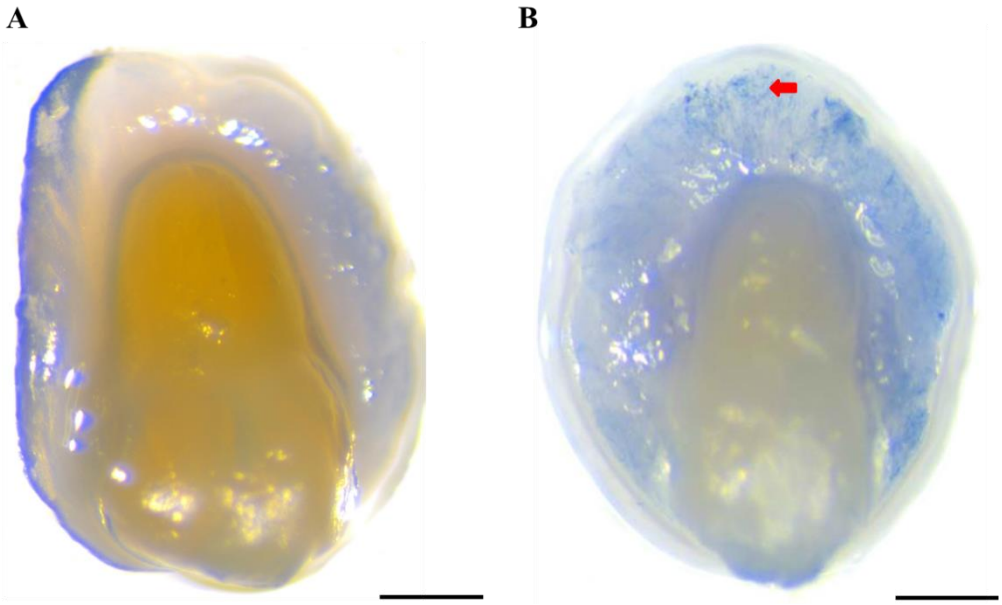


Fig. 5.1 Representative images of barley embryos infected by *U. nuda*. Embryos from susceptible and resistant lines had the same appearance. **(A)** inoculated embryo with no *U. nuda* mycelia present (i.e. an escape); **(B)** inoculated embryo with *U. nuda* mycelia present. Red arrow indicates the presence of *U. nuda* mycelium in the scutellum (stained dark blue). Bar = 0.5 mm.

Table 5.1 Proportion of embryos in which *U. nuda* mycelia were observed using a set of resistant and susceptible barley lines.

	Resistant Lines					Susceptible Lines	
	'AC Metcalfe'	**HB11316**	**TR09398**	**TR11698**	**TR12135**	**TR07728**	**'CDC Copeland'**
Embryos extracted	14	19	19	18	17	20	16
Embryos infected	7	17	15	11	10	16	11

5.3.2 Microscopic examination of *U. nuda* infection within cross-sectioned embryos

Free-hand sections of embryos derived from inoculated florets revealed that in both resistant and susceptible lines mycelia could be found throughout the embryo, including the scutellum, leaf primordia, scutellar node region, coleoptile, and coleorhiza (Fig. 5.2). In terms of mycelial development, the mycelia in most of the examined embryos had already progressed beyond the scutellar node into the area immediately behind the shoot growing point (Fig. 5.2C). In addition, no obvious difference was detected between resistant and susceptible lines in terms of the amount or distribution of *U. nuda* mycelia throughout the embryos.

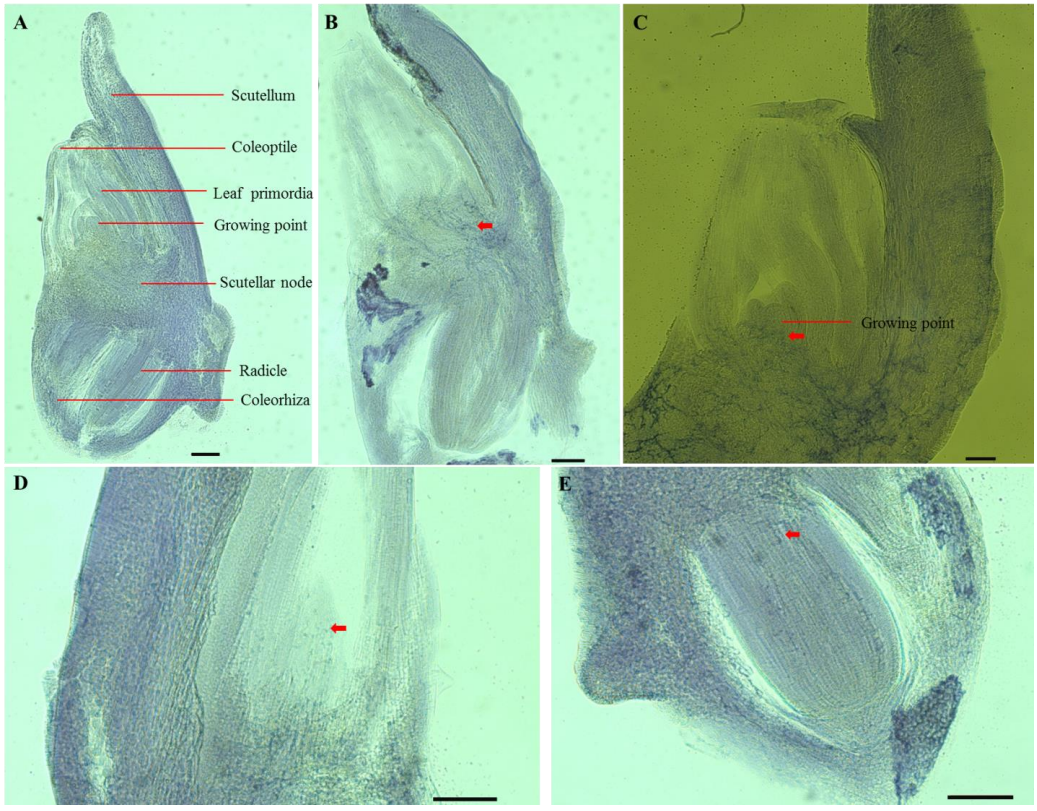


Fig. 5.2 Representative images of *U. nuda* infection within barley embryos. Embryos from resistant and susceptible lines had the same appearance. **(A)** un-infected embryo with tissues structure labelled; **(B)** infected

embryo; **(C)** mycelia present below the growing point; (**D**) mycelia present in the leaf primordia; **(E)** mycelia present in the radicle. Red arrows indicate *U. nuda* mycelia (stained dark blue). Bar = 0.2 mm.

5.3.3 PCR Detection of *U. nuda* in barley tissue

After five to six days, the inoculated seeds derived from both resistant and susceptible lines had several distinct germination outcomes: a) normal, healthy growing seedlings (Fig. 5.3A Group I); b) seedlings that produce a coleoptile which emerged from the soil, but developed no further and eventually died (Fig. 5.3A Group II); c) seeds that germinated, but produced a severely stunted coleoptile that did not emerge from the soil (Fig. 5.3A Group III). In addition, some seeds failed to germinate (data not shown).

Using the *U. nuda*-specific PCR primers, *U. nuda* could be detected in the three dissected tissues (scutellum, the lower region of sub-crown internode above where the scutellum attaches, and the ~1 cm upper region of the sub-crown internode) (Fig. 5.3B) in abnormally developed seedlings (Groups II and III) from inoculated resistant (Fig. 5.3C) and susceptible lines (Fig. 5.3D). However, *U. nuda* could not be detected in any of the three dissected tissue types from the resistant line which produced normal seedlings (Group I; Fig. 5.3C), but could be detected in all three tissue types from the susceptible line which produced normal seedlings (Group I; Fig. 5.3D). It also appeared that within the resistant line there was less *U. nuda* biomass in the upper region of the sub-crown internode than in the other two tissues dissected (Fig. 5.3C, lane 3 compared to lanes 1 and 2 in Groups I and II). This was not observed in the susceptible line (Fig. 5.3D, compare lane 3 to lanes 1 and 2 in Groups I and II).

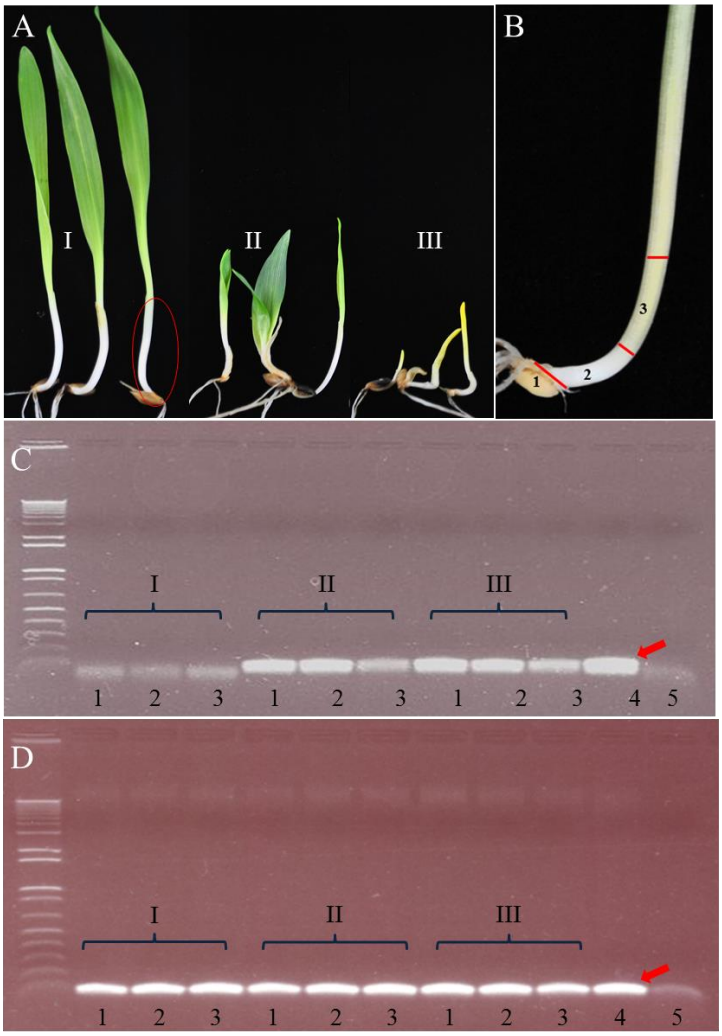


Fig. 5.3 Sampling and PCR detection of *U. nuda* from three different seedling tissues six days after sowing. (**A**) Three distinct germination outcomes after sowing of inoculated seeds from both resistant and susceptible lines: Group I, normal seedlings; Group II, seedlings that produce a coleoptile which emerges from the soil, but develops no further and eventually dies; Group III, seedlings that produce a stunted coleoptile that does not emerge from the soil and eventually dies. (**B**) The three different seedling tissues dissected and used for PCR detection of the pathogen (shown from a Group I seedling): 1, scutellum; 2, the lower region of sub-crown internode above where the scutellum attaches; 3, the ~1 cm upper region of the sub-crown internode (containing the crown node if possible). These three tissues could also be obtained from Group II and III seedlings. (**C** and **D**) PCR detection of *U. nuda* DNA in the three seedling tissues from resistant (**C**) and susceptible (**D**) lines. The faint lower PCR bands in **C** and **D** are non-specific PCR products which can also be seen in the mock inoculated plants (negative control; lane 5). The fainter PCR band in lane 3 of Group II and III resistant seedlings (**C**) indicates less *U. nuda* biomass in comparison to Group II and III susceptible seedlings (**D**). Lane 4, *U. nuda* DNA extracted from pure culture (positive control).

5.4 Discussion

The *Un8* loose smut resistance gene has protected barley production in Canada for over 50 years and yet only one publication (Gabor and Thomas 1987) has investigated the resistant mechanism(s) related to this durable resistance gene. In this study, histological observations

of loose smut infection and PCR detection of *U. nuda* in both resistant and susceptible lines were used to better clarify the growth stage and tissue associated with the expression of *Un8*-conditioned loose smut resistance.

According to the results of this study, expression of the *Un8*-mediated loose smut resistance occurs mainly at the young seedling stage. Three hypotheses of when resistance is expressed following infection of *U. nuda* were presented by Gabor and Thomas (1987): 1) at the initial point of penetration into the embryo, 2) within the embryo following mycelial penetration beyond the scutellum in the form of cell necrosis (hypersensitive response) which prevented mycelial growth beyond the scutellar node, and 3) during the seedling stage (Gabor and Thomas 1987). Gabor and Thomas (1987) suggested that *Un8*-conferred resistance was associated with embryo necrosis (hypothesis 2), however they also identified mycelia beyond the scutellar node and were uncertain if the growth of mycelia eventually stopped. Unfortunately, they did not observe the reaction to *U. nuda* in a susceptible line to use as a basis of comparison. According to the histological results in this study, no significant difference between resistant and susceptible lines in terms of the distribution and amount of mycelia within embryos could be observed and also no obvious necrosis could be detected within the embryos derived from *Un8*-carrier lines. These visual observations were confirmed by PCR detection of *U. nuda* in scutellum tissue, the lower region of the sub-crown internode above where the scutellum attaches, and the ~1 cm upper region of the sub-crown internode (containing the crown node). This indicated that the critical time point for *Un8*-mediated resistance may not be during seed maturation, since one would not see the presence of mycelia in these tissues within resistant lines if the resistance mechanism was expressed at an earlier stage.

The PCR assays conducted to detect *U. nuda* in the three tissues dissected from both resistant and susceptible lines within the three groups of seeds that were classified based on germination phenotype provided insight into timing of expression of the resistance reaction. According to the PCR results (Fig. 5.3), all tissues, except from normal seedlings of inoculated seeds of the resistant lines, of six day-old seedlings from inoculated lines were positive for *U. nuda* DNA. Also, it was observed that the PCR amplicon was weaker in the

upper region of the sub-crown internode of resistant lines compared to susceptible lines (Fig. 5.3D lane 3 in Groups II and III), indicating less *U. nuda* biomass. This may have been due to reduced entry of *U. nuda* into these tissues by the *Un8*-mediated resistance. This observation is similar to that of the recently isolated *ZmWAK* maize smut resistance gene which significantly lowers the incidence of maize head smut caused by the endophytic pathogen *Sporisorium reiliana* (*S. reiliana*) (Zuo et al. 2015). Quantification of the *S. reiliana* biomass within different tissues through quantitative PCR revealed that *S. reiliana* was arrested in the mesocotyl which made it difficult for the pathogen to reach the shoot meristem (Zuo et al. 2015).

Ustilago nuda-free seedlings produced from inoculated seeds of resistant lines may be the result of the *Un8*-mediated resistant response, or they may represent "escapes" from unsuccessful inoculation because infection by floret inoculation is not highly efficient, is genotype dependent (Wunderle et al. 2012), and requires a high level of technical skill. Considering that the reduced *U. nuda* biomass quantified in the upper region of the sub-crown internode in the resistant line is similar to that observed during the expression of maize smut resistance (Zuo et al. 2015), it is reasonable that Groups II and III seedlings are the result of true *Un8* resistance and Groups I seedlings are "escapes".

Although it is likely that a high inoculum concentration could help prevent disease escapes when testing, it seemed that the commonly used inoculum concentration of 1 g spores/1 liter distilled water is excessive. It affected the viability of both resistant and susceptible seedlings from inoculated seed due to tissue damage within the embryo and thus confounds what appears to be the mechanism by which the *Un8* gene prevents transmission of loose smut.

In conclusion, this study found that there were no anatomical or visual observations that distinguished the *U. nuda*-barley interaction in resistant or susceptible lines during the first six days post-germination. The currently recommended inoculum concentration reduces the viability of seedlings from inoculated seed of both resistant and susceptible lines. As all normal seedlings from inoculated seeds of the resistant line were free of mycelium it is possible that they could arise from failure of the inoculation technique. If so, then some of the

poorly germinating/stunted seedlings that carried mycelium at the six-day stage, may exhibit those symptoms, not because of excess inoculum, but rather as a result of the resistance mechanism of the *Un8* gene. Additional studies are necessary to optimize the inoculation technique to produce fewer poorly germinating/stunted seedlings to improve disease screening, but also to help understand the *Un8* mediated disease response under the assumption that Group I seedlings represent the normal phenotype of a resistant response.

CHAPTER 6

Influence of Inoculum Concentration when Screening for Barley Loose Smut Resistance by Artificial Inoculation

Abstract

In Canada, barley loose smut caused by *Ustilago nuda* (Jens.) Rostr. (*U. nuda*) can be effectively controlled through genetic resistance by the *Un8* gene. It was observed during previous experiments (Chapter 5) that seed of both resistant and susceptible lines which had been artificially inoculated produced many seedlings with stunted coleoptile growth and eventually died. It was speculated that this was due to excessively high inoculum concentrations of *U. nuda*, which caused damage to the embryos/seedlings in both resistant and susceptible lines. In this study the inoculum concentration used most commonly to evaluate barley loose smut resistance (1 g teliospores/1 L distilled water) was compared with two lower inoculum concentrations (0.1 g/L and 0.01 g/L) with respect to disease prevalence and occurrence of damaged seedlings. A clear phenotypic distinction was found between resistant and susceptible lines at the one-leaf stage with reduced inoculum concentrations, in which inoculated seeds of resistant lines exhibited reduced germination and emergence, whereas seeds from susceptible lines germinated normally. The improved germination did not compromise the infection rate in the susceptible lines. This method of differentiating resistant and susceptible lines at a much earlier stage will improve the efficiency and reliability of identifying resistant lines in barley breeding programs and also provides an insight into the mechanism of action of *Un8*.

6.1 Introduction

Barley loose smut caused by *Ustilago nuda* (Jens.) Rostr. (*U. nuda*), together with covered smut caused by *U. hordei* (Pers.) Lagerh and false loose smut caused by *U. nigra* Tapke, are three smut diseases of barley (Thomas 1997). Historically, before resistance breeding, smut diseases could result in 10-25% yield loss and up to 75% of the plants could be infected in some fields (Menzies et al. 2014). Unlike the two surface-borne smuts, covered smut and false loose smut, *U. nuda* infects developing seeds during flowering and survives in the mature seeds as dormant mycelium (Thomas 1997). In most cases, the mycelium can

penetrate to just below the apical meristem in mature seeds (Chapter 5). Upon seed germination, the mycelium breaks dormancy and infects the growing point during the early seedling stage (Wunderle et al. 2012).

In Western Canada, loose smut was identified in 90% of the barley fields surveyed in 1985 and, from 1972 to 2009, the mean annual percentage of fields with plants infected by loose smut was close to 50% (Menzies et al. 2014). However, with effective management including the application of systemic seed treatment fungicides and use of loose smut resistant cultivars, yield loss is usually less than 1% (Menzies et al. 2014). When comparing these two control methods, systemic fungicides have the potential to increase the incidence of fungicide-tolerant strains of *U. nuda* (Menzies 2008), increase the cost of barley production, and cannot be used for organic production, so the development of loose smut resistant cultivars is considered a more economical and environmentally-friendly option.

The inoculation method is the most important step when screening barley germplasm for loose smut resistance (Menzies et al. 2009). Two inoculation methods, floret and seedling, have been developed to evaluate barley resistance (Jones and Dhitaphichit 1991). Although seedling inoculation in which the teliospore suspension is introduced into decapitated coleoptiles by vacuum-assisted inoculation (Kavanagh 1964; Jones and Dhitaphichit 1991) is more efficient because only one generation is needed to obtain the phenotypic data, floret inoculation is more effective at achieving high incidence of infection (Jones and Dhitaphichit 1991).

When conducting floret inoculation, the age of the floret is among the most critical factors for successful establishment of disease (Jones and Dhitaphichit 1991; Menzies et al. 2009). Inoculation should be carried out just prior to anthesis and florets individually hand-injected with the teliospore suspension. Once inoculation is complete, seeds must be allowed to mature and the phenotype cannot be evaluated until the next flowering stage. Despite direct infection of spores into the florets, disease escapes occur, thus a second screening of putative resistant lines is needed which is very time-consuming and labor-intensive (Eckstein et al. 2002; Menzies et al. 2009).

A second critical factor that must be considered for successful disease establishment is the teliospore concentration used. It was observed that high inoculum concentrations, which help prevent disease escape, were often associated with decreased plant survival (Oort 1939; Gabor and Thomas 1987; Jones and Dhitaphichit 1991; Chapter 5). After evaluating various concentrations, Jones and Dhitaphichit (1991) found 1 g teliospores/1 L distilled water was the most appropriate inoculum concentration. However, based on the results in Chapter 5, floret inoculation using this concentration is associated with low seedling survival rates. In this study, seedling survival and infection rate were evaluated at three different teliospore concentrations in an attempt to optimize barley loose smut resistance screening.

6.2 Materials and Methods

6.2.1 Plant materials and optimization of inoculum

To understand the effect of inoculum concentration on seedling mortality and symptom development when screening for barley loose smut resistance, three concentrations of inoculum were used to infect the susceptible lines 'CDC Austenson', TR12137, and 'Barke' and resistant line TR11698.

Spore concentrations of 1 g teliospores/L distilled water (1×10^7 spores/ml), the commonly used concentration (Mueller 2006), 0.1 g/L (1×10^6 spores/ml), and 0.01 g/L (1×10^5 spores/ml), were used. Each floret was injected with approximately 15 µl of teliospore suspension, which would mean that approximately 150,000, 15,000, 1,500 spores were injected into each floret at the 1.0 g/L, 0.1 g/L, 0.01 g/L concentrations, respectively. A mixture of local *U. nuda* isolates collected from the field (as described in Chapter 3) was used for inoculations. Plants were grown in the growth chamber in the University of Saskatchewan phytotron under a 16 h light (22°C, 285 µmol photons m^{-2} s^{-1})/8 h dark (18°C) cycle at 50% relative humidity.

At early anthesis, barley heads were artificially inoculated using a 3-ml syringe and heads inoculated with the three concentrations of spores were harvested separately. To determine the degree of infection, three replications (each consisting of 15 seeds per pot) of

each inoculum concentration were assessed for each of the three susceptible lines to observe seedling development and loose smut symptoms on mature plants (mature plants were only assessed for 'CDC Austenson'). Plants were grown in growth chambers under the growth conditions described above. Another sixteen inoculated seeds from 'CDC Austenson' and TR11698 were germinated at room temperature on cotton balls moistened with tap water and grown for 6 days to show the phenotypic difference between susceptible and resistant lines at a spore concentration of 0.01 g/L.

Another experiment was performed in which 15 seeds of 'CDC Austenson' and TR11698 inoculated with a 0.01 g/L were placed on moistened filter paper within petri plates at room temperature and germination observed over the course of 6 days. Seedlings were then transferred into soil and grown in a growth chamber under the conditions above to evaluate seedling mortality.

6.3 Results

6.3.1 Seed size variation after inoculation

It has previously been observed with both resistant and susceptible lines that inoculation of most florets at very high inoculum concentration (2 g/L) resulted in almost no seed formation (data not shown). After inoculation with various concentrations, there were no differences in seed appearance at the three inoculum concentrations in either resistant or susceptible lines (Fig. 6.1). Seeds derived from florets inoculated at the 0.01 g/L concentration were similar in size to seeds derived from mock inoculated florets (Fig. 6.1). By contrast, seeds formed from florets inoculated at the normal 1 g/L concentration appeared smaller with greater amounts of dark tissue (Fig. 6.1).

Fig. 6.1 Representative seeds produced from florets inoculated with three different teliospore concentrations along with a mock inoculated seed sample for comparison. The normally used concentration is 1 g/L (1×) with the other two concentrations being 0.1 g/L (0.1×) and 0.01 g/L (0.01×).

6.3.2 Effects of inoculum concentration on screening for loose smut disease resistance

Variation in seed germination rate and adult plant infection rate were observed for three susceptible lines with the three inoculum concentrations used (Table 6.1). In contrast to 1 g/L, the germination outcomes using 0.1 g/L and 0.01 g/L were significantly improved, with most of the inoculated seeds producing normal seedlings (i.e. Group I in Fig. 5.3A) (Table 6.1). Even though seed germination rates were similar between 0.1 g/L and 0.01 g/L, a higher infection rate was observed at 0.01 g/L when grow to maturity (Table 6.1).

Table 6.1 Impact of *U. nuda* spore concentration on seedling performance and disease development in three susceptible lines (Values represent the mean ± standard deviation obtained from three replications, each replication consisted of 15 seeds).

Line	Spore Concentration (g/L)[a]	Normal Seedlings	Infected Plants[b]
'CDC Austenson'	1.0	5.5 ± 2.1	2.7 ± 0.6
	0.1	14.0 ± 0.0	7.7 ± 1.5
	0.01	14.0 ± 0.0	10.0 ± 1.0
TR12737	1.0	4.3 ± 1.2	N/A
	0.1	9.0 ± 2.0	
	0.01	10.7 ± 2.1	
'Barke'	1.0	4.7 ± 1.2	N/A
	0.1	8.7 ± 1.5	
	0.01	11.0 ± 1.7	

[a]1.0 g/L: 1×10^7 spores/ml; 0.1 g/L: 1×10^6 spores/ml; 0.01 g/L: 1×10^5 spores/ml;
[b]Only 'CDC Austenson' was grown to maturity to determine the number of infected plants.

6.3.3 Seedling reaction to *U. nuda* infection in resistant and susceptible lines

Seeds from susceptible ('CDC Austenson') and resistant (TR11698) lines inoculated with 0.01 g spore/L were used to investigate the seedling reaction to *U. nuda*. Six days after sowing, a clear difference between the susceptible and resistant lines was observed, with 14/16 inoculated seeds from the susceptible line germinating and producing normal seedlings (Group I in Fig. 5.3A) (Fig. 6.2), while 15/16 seedlings from the resistant line failed to germinate normally (Group II and III in Fig. 5.3A) and eventually died (Fig. 6.2). Only one seed of TR11698 produced a normal seedling.

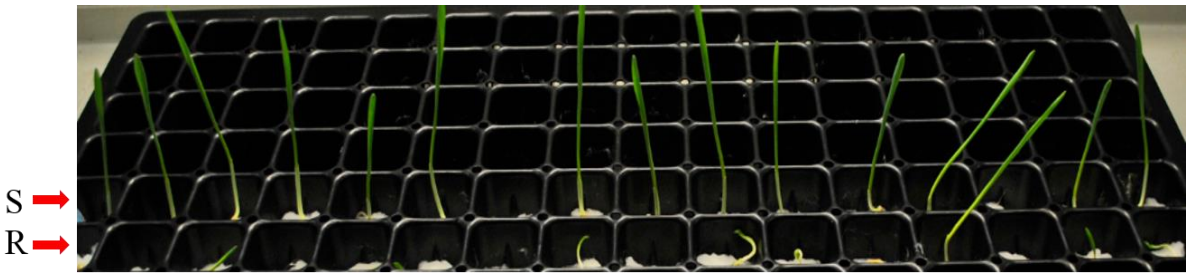


Fig. 6.2 Phenotypic differences observed between 'CDC Austenson' (susceptible, S) and TR11698 (resistant, R) at six days post sowing. After inoculation with 0.01 g spore/L, seedling vigour in the resistant accession was significantly reduced. Each accession contained 16 seeds.

Further evidence of the difference in germination and seedling growth from inoculated seeds of susceptible and resistant lines is shown in Fig. 6.3. After inoculation with 0.01 g spore/L, almost every seed of the susceptible cultivar 'CDC Austenson' produced a normal seedling, while most of seeds of the resistant line TR11698 either failed to germinate or did not germinate normally, failing to grow vertically and many exhibiting early leaf expansion. The difference between 'CDC Austenson' and TR11698 could be observed as early as 2 dps (day post sowing of inoculated seeds). In 'CDC Austenson', unlike TR11698, about 50% of the seeds had started to germinate with a clearly visible coleoptile and roots. At 4 and 6 dps nearly all seedlings of 'CDC Austenson' exhibited normal growth while in TR11698 those few seedlings that had germinated exhibited distorted growth. After being transferred into soil, all abnormal seedlings from TR11698 failed to develop further and eventually died at the seedling stage (data not shown).

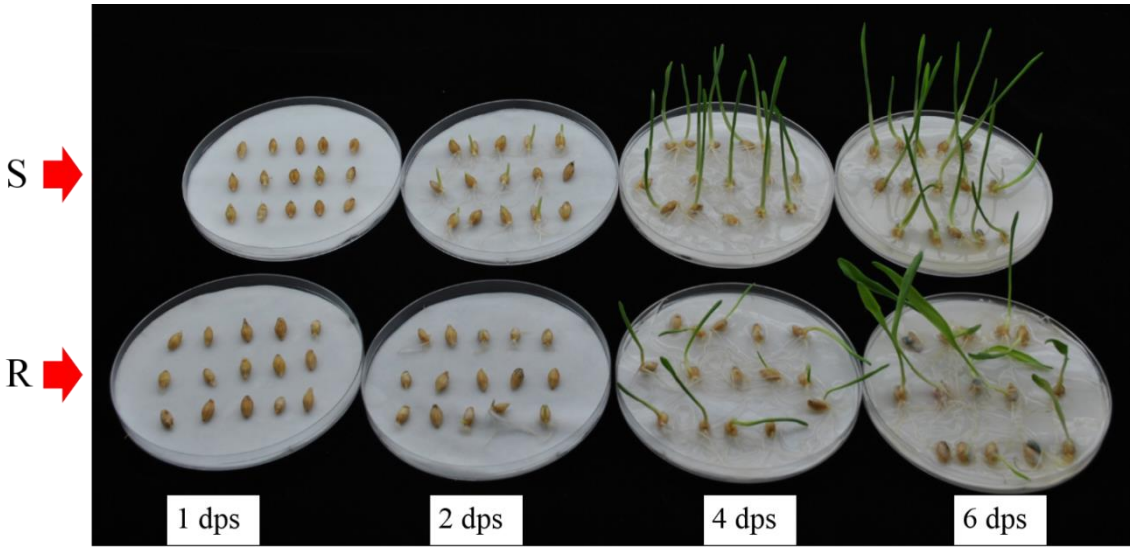


Fig. 6.3 Germination of inoculated seeds of 'CDC Austenson' (susceptible, S) and TR11698 (resistant, R) at six days after seed sowing. Seeds (15) in each petri dish were randomly selected. dps, day post sowing of inoculated seeds.

6.4 Discussion

Approximately 50 years have passed since the assignment of gene symbol *Un8* to this long-lived loose smut resistant gene originally identified from a landrace (Metcalfe 1966). However, very few investigations have been carried out to understand the resistant mechanisms conferred by *Un8* (Gabor and Thomas 1987). One observation that has been repeatedly noted is that after inoculation numerous plants, whether they carried the *Un8* gene or not, would fail to germinate or display seedling mortality (Oort 1939; Gabor and Thomas 1987; Jones and Dhitaphichit 1991). This observation with high spore concentrations suggested a significant amount of damage may be imposed by the growing mycelium which develops from the large number of teliospores.

In an effort to balance improved germination and seedling survival without compromising the infection rate, various concentrations of inoculum were evaluated. It was consistently observed that smaller seeds were associated with higher inoculum concentration (especially using 1 g/L) between resistant and susceptible lines. Although no statistical analysis was done to compare seed weight, it appears that seed formation is greatly affected by inoculum concentration. Moreover, a distinct improvement in germination and seedling survival was achieved in susceptible plants by lowering the inoculum concentration,

especially for cv. ‘CDC Austenson’, without compromising infection rate. These observations indicate that 0.01 g/L is the better concentration to use for evaluation of barley loose smut disease resistance. Interestingly, this lower concentration did not improve germination and seedling survival in resistant plants. This may provide an insight as to how *Un8*-mediated resistance is expressed.

In Chapter 5, although no *U. nuda* DNA could be detected in normal seedlings that developed from resistant lines at the 1 g/L, it was uncertain if this was due to *Un8* resistance or disease escape. Here, the absence of a high mortality in susceptible lines when inoculum concentration was low, but not in resistant lines, indicated that mortality under these conditions was likely a result of the host resistance response mediated by *Un8*. As such, normal seedlings from resistant lines in Figs. 5.3, 6.2, and 6.3 are postulated to be the result of disease escape rather than the expression of *Un8* resistance.

If the poor germination of the inoculated resistant lines was truly a fitness penalty arising from *Un8* resistance in response to *U. nuda*, it is possible the fitness cost is expressed through a change in regulation of genes involved with barley seedling growth. Such phenomena have been documented in other species. For example, restricted growth of rice plants by JA defense signaling, which is important for plant defense against necrotrophic pathogens and insects, is achieved through interaction with the gibberellin signaling cascade which is involved in growth promotion (Yang et al. 2012b). During *FLS2*-mediated *Pseudomonas syringae* pathovar *tomato* resistance, the signaling pathway of auxin can be suppressed by microRNA miR393 (Huot et al. 2014).

This study indicated that loose smut resistance may be evaluated in the seedling stage after inoculation based on poor germination and seedling mortality rated on a small sample of individual seeds. If the use of markers for *Un8* was not possible, this would save time and resources by not having to grow inoculated seeds to maturity to observe loose smut symptoms within the barley inflorescence. While the symptoms observed in resistant plants when exposed to 0.01 g spore/L may be an artifact of inoculum concentration that was still too high, it is more likely the true expression of *Un8*-mediated resistance (i.e. seedling mortality) caused by plant hormone crosstalk.

CHAPTER 7

Towards the Molecular Basis for the Mode of Action of *Un8*-Mediated Barley Loose Smut Resistance

Abstract

In Canada, barley loose smut caused by *Ustilago nuda* (Jens.) Rostr. (*U. nuda*) can be effectively controlled through genetic resistance from the *Un8* gene. Previously, a *Un8* candidate gene with two protein kinase domains was isolated by map-based cloning (Chapter 3) with only one allele associated with resistance and several susceptible alleles identified from a set of cultivated and wild barley accessions (Chapter 4). Furthermore, the most critical growth stage associated with *Un8*-mediated resistance was determined to be during the early seedling stage (Chapters 5 and 6). It was observed that *Un8* resistance is associated with a fitness cost which is expressed in the form of 'low seedling survival' of infected seeds (Chapter 6). In this study, gene expression studies were conducted at several post-infection growth stages to determine if this would reveal the mode of action of *Un8*. Expression analysis for genes involved in various pathways was carried out on young seedlings from inoculated resistant and susceptible genotypes. Ultimately two barley genes, *CKX1* and *CKX2.1*, which encode cytokinin oxidase/dehydrogenase (CKX) enzymes that are responsible for degradation of endogenous barley cytokinins, were identified as possible regulators of *Un8*-mediated loose smut resistance. Degradation of endogenous cytokinins, which play a central role in growth and development of plants, could impact seedling development through cell cycle regulation (Werner et al. 2001). Regulation of resistance through CKX and the associated disruption of barley cytokinin levels may provide a clue to the molecular basis for the poor germination of seedlings carrying *Un8* when infected by *U. nuda*.

7.1 Introduction

Plants are constantly subjected to biotic stresses over their life cycle. Unlike vertebrates, plants cannot mount a defense response through adaptive immunity, but instead depend on innate immunity to detect and prevent damage from various herbivorous insects and plant pathogens, such as bacteria, fungi, and viruses (Jones and Dangl 2006). Two types of innate immunity are deployed by plants: pathogen-associated molecular pattern (PAMP)-triggered

immunity (PTI) and effector triggered immunity (ETI). PTI is the first line of plant innate immunity and is activated via pattern-recognition receptors (PRRs) upon detection of conserved PAMPs. However, PTI can be suppressed by pathogens through the delivery of effectors into host cells. Effectors can in turn be recognized by plant resistance (R) proteins which elicit ETI, the second layer of plant innate immunity, which is usually associated with hypersensitive cell death (Jones and Dangl 2006). During plant innate immune responses, plant hormones (phytohormones), such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), are believed to play a central role in the plant immune response (Pieterse et al. 2009).

The barley *Un8* resistance gene is effective against loose smut disease caused by *Ustilago nuda* (Jens.) Rostr. (*U. nuda*). However, the underlying gene and related molecular mechanisms responsible for the durable *Un8* resistance are poorly documented. To address these deficiencies, a *Un8* candidate gene containing two tandem protein kinase domains was identified through map-based cloning (Chapter 3) and evidence to validate this candidate gene was provided through allele sequencing (Chapter 4). Previous studies indicated that the most critical growth stage associated with loose smut resistance conferred by *Un8* was during seed maturation (Gabor and Thomas 1987), however, in Chapter 5 and Chapter 6 evidence was provided that the seedling stage may be more important.

Resistance and susceptibility alleles in plants are commonly found to coexist in nature over long periods of time despite disease resistance seeming to be an obvious advantage which would lead to selection and the loss of susceptibility alleles from the population (Tian et al. 2003). To understand this phenomenon, Tian et al. (2003) hypothesized that the cost of resistance might explain their co-existence. The concept of a fitness cost associated with disease resistance can be described as the activated defense response having a corresponding negative impact on plant growth. It is hypothesized that allocating more energy and resources into plant disease resistance pathways would decrease their availability for other activities (Brown and Rant 2013; Huot et al. 2014). Brown and Rant (2013) suggested that fitness costs could be classified as those associated with expressing a defence reaction, the simple presence of a resistance gene, and disease escape (which is less common).

The negative effects on plant fitness due to the presence of *R* genes have been documented primarily in the model plant *Arabidopsis* and are often associated with smaller plants or yield penalties (Vogel et al. 2002; Tian et al. 2003; Heidel et al. 2004; Orgil et al. 2007; Todesco et al. 2010; Kato et al. 2011; Karasov et al. 2014). Other examples of negative side effects have been observed in flax (Howles et al. 2005) and rice (Shimono et al. 2007; Tang et al. 2011; Takatsuji 2014). In barley, the extensively utilized *mlo* gene for resistance to powdery mildew in Europe also resulted in significant yield losses (Brown 2002; Brown and Rant 2013). Another example in barley demonstrated that transgenic lines containing the wheat leaf rust resistance gene *Lr34* produced negative effects on growth (Chauhan et al. 2015).

In recent years, the role of phytohormones as communication molecules between different biochemical pathways has emerged as an important research area to help elucidate the molecular mechanisms underlying the negative effects of *R* genes on plant fitness. Apart from the three classic defense phytohormones, SA, JA, and ET, other phytohormones, such as auxins, brassinosteroids (BRs), cytokinins (CKs), and gibberellins (GAs), which were originally described for their roles in regulating plant growth and development, have also been shown to exert direct and/or indirect effects on plant-microbe interactions. Auxin is an important plant growth-promoting hormone and evidence suggests that its signaling pathway can be suppressed by a plant's innate immunity through microRNA miR393 (Robert-Seilaniantz et al. 2011; Huot et al. 2014). Yang et al. (2012b) observed in *Arabidopsis* and rice that resources were re-allocated from growth to JA-mediated defense through interference of the GA pathway. Other interactions among different phytohormones have been described in several review papers (Santner and Estelle 2009; Denancé et al. 2013; Lyons et al. 2013; Huot et al. 2014).

Additional work to elucidate whether differences in functionality of the *Un8* candidate gene in resistant or susceptible barley lines might result from transcriptional regulation through gene expression studies was provided by this study. Barley lines containing the *Un8* gene had low seedling survival after inoculation (Chapter 6), which may have been an extreme example of an *R* gene-associated fitness cost. To examine this, expression analyses

of genes involved in a number of phytohormone biosynthesis/signaling and metabolic pathways was conducted to provide initial clues into the mechanisms behind loose smut resistance and its associated fitness penalty.

7.2 Materials and Methods

7.2.1 Plant materials

Florets from resistant (TR11698) and susceptible ('CDC Austenson') lines were inoculated at early anthesis with a mixture of teliospores collected from local *U. nuda* field isolates (as described in Chapter 3) using a 3-ml syringe at a concentration of 0.01 g/L (1×10^5 spores/ml) in distilled water. Mock inoculated florets of each line were injected with distilled water to act as a control. Plants were grown in a growth chamber in the University of Saskatchewan phytotron under a 16 h light (22°C, 285 μmol photons m^{-2} s^{-1})/8 h dark (18°C) cycle at 50% relative humidity.

7.2.2 RNA preparation and cDNA synthesis

Immature seeds from teliospore and mock (control) injected florets were collected from both lines at 20, 25, and 30 days post-inoculation (dpi) (during seed maturation) and the embryos were removed for RNA expression analysis. In addition, mature seeds from teliospore and mock (control) injected florets were germinated from both lines on sterile cotton balls moistened with distilled water and incubated at room temperature (22°C) for 1, 2, 4, and 6 days post-sowing of inoculated seeds (dps) (during seedling development). At 1 and 2 dps the scutellum and young shoots were bulked together for RNA expression analysis, while at 4 and 6 dps the lower 1 cm of the coleoptile (containing the growing point) and the scutellum were harvested together for RNA expression analysis.

In germinated inoculated seeds of the resistant line it was difficult to distinguish at 1 and 2 dps seeds which would produce seedlings with normal growth morphology (Group I) versus those that would eventually die (Groups II and II) (Fig. 5.3A). As such, the expression analysis of the resistant line at these stages may represent expression occurring within both escapes and infected seedlings. At later time points (4 and 6 dps) when it was clear that

seedlings were either Group I or Groups II and III, the Group I seedlings from inoculated resistant seeds were excluded for gene expression analysis under the assumption that these seedlings represented escapes. Three biological replicates for each time point were prepared with each biological replicate consisting of bulked seeds (at least 12) collected from different heads.

All plant tissues were immediately frozen in liquid nitrogen and stored at -70°C. Samples were then ground in liquid nitrogen with a pre-chilled mortar and pestle prior to RNA extraction using the RNeasy Plant Mini Kit (Qiagen). RNA integrity was checked by agarose gel electrophoresis and purity evaluated by the A260/280 ratio using a NanoDrop-8000 spectrophotometer (Thermo Fisher Scientific Inc). The QuantiTect Reverse Transcription Kit (Qiagen) was used to convert 0.5 micrograms of total RNA into first strand cDNA according to the manufacturer's protocol and contaminating genomic DNA was removed. The cDNA samples obtained were diluted 5 times for expression analysis.

7.2.3 Quantitative real-time PCR (qRT-PCR)

The qRT-PCR was carried out with an Applied Biosystems (ABI) 7900 HT Fast Real-Time PCR System in 96-well optical reaction plates. Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2×) was used for the qRT-PCR reaction which was carried out in a 10 µl volume containing 1 µl of cDNA, 5 µl Master Mix, and 0.6 µl of a 5 µM stock of each primer. The qRT-PCR conditions were: 2 min at 50°C for pre-PCR read and 10 min at 95°C for hot-start activation, followed by 40 cycles at 95°C for 15 s, 60-62°C for 1 min.

The relative quantification of target and reference genes was performed in separate reactions and the results were based on three technical replicates for each biological sample. The threshold cycle (Ct) values generated from the SDS RQ Manager software (ABI) were used to calculate the expression levels of target genes in inoculated samples relative to the control using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) allowing for comparison between samples. Briefly, the Ct values of the target genes were normalized to the Ct value of the reference gene (*TUBA*), thus $\Delta\Delta Ct = \Delta Ct(\text{inoculated sample}) - \Delta Ct(\text{control})$ where $\Delta Ct = Ct(\text{target}) - Ct(\textit{TUBA})$. Twenty-five barley genes representing different phytohormone

biosynthesis/signaling and metabolic pathways were initially investigated (Appendix B). Because of the large number, expression analysis was carried out using only one biological replicate per time point to obtain preliminary results. Based on this information, a subset of genes representing the SA, JA, and CK pathways was selected for further examination through analysis of two additional biological replications.

7.3 Results

7.3.1 *Un8* candidate gene expression in response to *U. nuda* infection

It was found that the most striking difference between resistant and susceptible lines occurred at 30 dpi. At that time point, 2.2-fold higher expression of the *Un8* candidate allele was observed in the resistant line as compared with the mock-inoculated line (Fig. 7.1). No such up-regulation of the *Un8* candidate gene at this time point was observed in the susceptible line. However, a significant difference in expression between resistant (1.2×) and susceptible (1.8×) lines was found at 6 dps.

7.3.2 Phytohormone biosynthesis/signaling and metabolic pathway gene expression in response to *U. nuda* infection

Gene expression of critical regulators in phytohormone biosynthesis/signaling and metabolic pathways, including IAA, GA, BR, CK, TOR (target of rapamycin), and autophagy, were investigated across all time points. The genes which showed the greatest differential expression level in response to *U. nuda* infection between the resistant and susceptible lines were those from the cytokinin pathway (Appendix C, Fig. 7.1).

Five cytokinin oxidase/dehydrogenase genes (*CKXs*), which are responsible for the irreversible degradation of CK were investigated to study the role of the cytokinin pathway in *Un8*-mediated resistance. All five genes showed differential expression between the resistant and susceptible lines at at least one time point and at levels greater than all other genes tested (Appendix C, Fig. 7.1). However, *CKX1* and *CKX2.1* were selected for further investigation since the timing of their expression was correlated with the abnormal seedling growth observed in resistant lines in Chapter 5 (Group II and III seedlings shown in Fig. 5.3).

Expression of *CKX1* reached the highest level at 2 dps in the resistant line, which was 12.5-fold stronger than transcript abundance in mock-inoculated samples (Fig. 7.1). Following up-regulation of *CKX1* at 2 dps, transcript levels of *CKX2.1* were elevated at 4 dps and 6 dps, which were 64.9- and 27.3-fold higher than mock-inoculated resistant samples, respectively (Fig. 7.1).

7.3.3 Expression of barley defense-related genes in response to *U. nuda* infection

The roles of defense-related genes in the *U. nuda*-barley interaction were investigated by assaying transcript abundance of *NPR1*, *PR1b*, *PR2*, *PR5*, *AOS*, *LOX2a*, *LOX2b*, and *OPR*. After analyzing the preliminary results (Appendix C), *PR1b*, *PR2*, *PR5*, *LOX2a*, *LOX2b*, and *OPR* genes appeared to be the most likely candidates to be involved in the *U. nuda*-barley interaction. These six genes were investigated further and the results of their time-course expression are provided (Fig. 7.1).

All six genes (*LOX2a*, *LOX2b*, *OPR*, *PR1b*, *PR2*, and *PR5*) were expressed at significantly higher levels in the resistant line at 25 dpi. A significantly stronger transcriptional activation of all three genes of the JA pathway (*LOX2a*, *LOX2b*, and *OPR*) was also observed in the resistant line at 4 dps and 6 dps. A general trend among the JA-related genes appeared to be an up-regulation in expression within the resistant line, the one exception was increased expression of *LOX2a* at 1 dps in the susceptible line. The pattern of expression of the three pathogenesis-related genes (*PR1b*, *PR2*, and *PR5*) was less clear with higher expression levels noted in both resistant and susceptible lines at various time points. The one consistent observation was the elevated expression of all three genes in the susceptible line at 30 dpi.

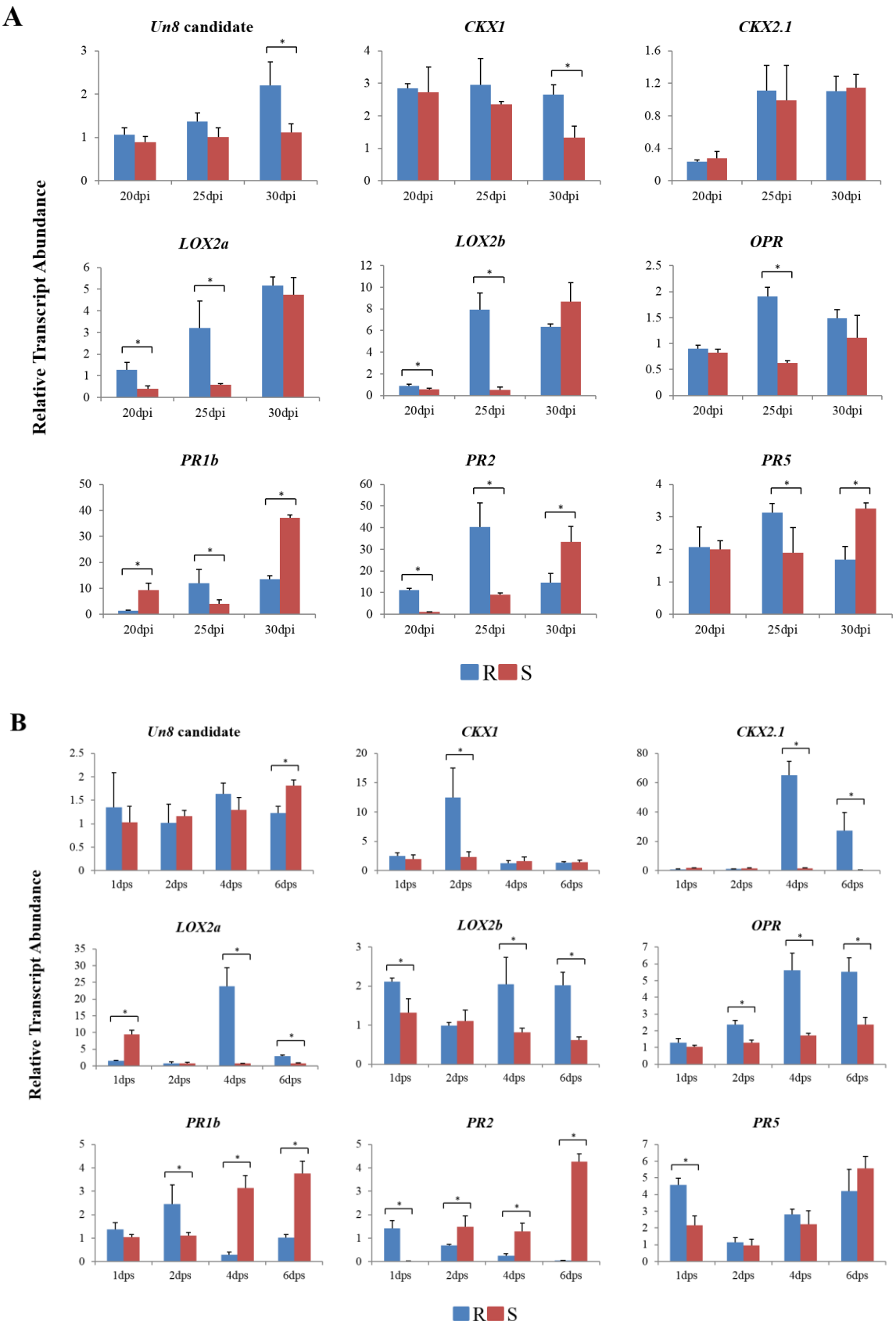


Fig. 7.1 Summary of gene expression analysis.

(A) Relative transcript abundance of selected barley genes in response to *U. nuda* infection during seed maturation.

(B) Relative transcript abundance of selected barley genes in response to *U. nuda* infection at the seedling stage.

LOX2a, *LOX2b*, and *OPR* are markers for the jasmonic acid (JA) defense pathway. *PR1b*, *PR2*, and *PR5* are defense-related genes. The barley *TuBA* gene was used as the internal control and expression values were normalized to the mock-inoculated samples in which expression was set to 1. Expression values for each gene are presented as the mean of three biological replicates with three technical replicates for each biological replicate. Error bars indicate the standard deviation and asterisks above the bars indicate a significant difference between resistant and susceptible lines at $P < 0.05$ (Student's *t* test). *CKX*, cytokinin oxidase/dehydrogenase; *LOX*, lipoxygenase isozyme; *OPR*, oxophytodienoate reductase; *PR*, pathogenesis-related. Dpi, day post inoculation. Dps, day post sowing of inoculated seeds.

7.4 Discussion

Approximately 50 years have passed since the identification of *Un8* (Metcalfe 1966). However, very few investigations have been carried out to understand the resistant mechanism conferred by the *Un8* gene (Gabor and Thomas 1987). The first goal of this study was to provide additional evidence, via gene expression analysis, that the *Un8* candidate gene is responsible for loose smut resistance expressed at the seedling stage (as observed in Chapters 5 and 6). Secondly, expression analysis for genes involved in various signaling pathways was carried out to help elucidate the molecular mechanisms underlying the fitness cost, as observed by 'low seedling survival' of infected seeds (Chapter 6), associated with *Un8*-mediated resistance.

Regulation of gene expression is not the basis of the *Un8* candidate gene mediated resistance

Identification of sequence variation in the promoter and coding regions between the resistant allele of the *Un8* candidate gene and various susceptible alleles led to the postulation that gene expression or gain/loss of protein function were both possible explanations for differences in *Un8* candidate gene mediated resistance between resistant and susceptible lines (Chapter 4). A strong case for gene expression as the basis of *Un8*-mediated resistance is difficult to make based on the results in this study. A significant up-regulation of *Un8* candidate gene in the resistant line was only observed at 30 dpi and only up to a 2-fold level. Additionally, the gene was observed to be upregulated in the susceptible line at 6 dps. It is possible that a 2-fold difference in expression is sufficient to differentiate a resistant from

susceptible reaction if the transcript is stable (and thus likely to be translated more), but one might expect the elevated transcript levels to exist beyond a single time point in the analysis. Similarly, no differences in expression of the *Rpg1* stem rust resistance gene, the only other example of a barley resistance gene containing two protein kinase domains (Brueggeman et al. 2002) which is also involved in durable resistance to a biotrophic pathogen (*Puccinia graminis* f. sp. *tritici*), were observed with incompatible interactions between host and pathogen (Rostoks et al. 2004). It was subsequently found that *Rpg1*-mediated resistance is regulated at the protein level instead of at gene expression (Nirmala et al. 2007).

The cytokinin pathway may be responsible for the negative impacts of *Un8*-mediated resistance on growth

The fitness costs to a plant when mounting a resistant response to pathogens are often assumed to be correlated with the diversion of energy from growth and development towards defense signaling pathways (Brown and Rant 2013; Huot et al. 2014). In the present study, a large fitness cost associated with *Un8*-mediated loose smut resistance was observed after seed germination in the form of low seedling survival. Previous studies have identified several phytohormones, such as auxins, BRs, and GAs, which may mediate disease resistance-related fitness costs (Yang et al. 2012b; De Bruyne et al. 2014; Huot et al. 2014). However, no significant differential expression of key regulators for these phytohormones, such as *BIN2* and *BZR1* for BR, *AFBs* for auxin and *SLN1* for GA, could be detected.

Cytokinins are a group of phytohormones that have been implicated in resistance-related fitness costs (Giron et al. 2013; Albrecht and Argueso 2017). Cytokinins play critical roles in the regulation of gap transitions during cell cycle progression, and controlling plant meristem activity, morphogenesis, and yield (Werner et al. 2001; Ashikari et al. 2005; Murray et al. 2012; Zhang et al. 2016; Albrecht and Argueso 2017). Therefore, the role of CKs was evaluated by investigating the expression of two *CKX* genes which are responsible for the irreversible degradation of CKs (Werner et al. 2001; Ashikari et al. 2005; Mrízová et al. 2013). Expression of *CKX1* at 2 dps and *CKX2.1* at 4 dps and 6dps were significantly up-regulated in the resistant line, which would conceivably reduce endogenous CK levels and thus might repress shoot meristem growth of barley seedlings at these very early growth

stages. This assumption is supported by Mrízová et al. (2013) who observed poor shoot generative ability from barley calli that overexpressed *ZmCKX1* and *HvCKX9*. The timing of *CKX1* and *CKX2.1* up-regulation is interesting given that the critical time point at which *U. nuda* mycelia grow into the shoot apex is 7-10 days after sowing (Wunderle et al. 2012). If CKXs are involved in *Un8*-mediated resistance, their inhibition of plant growth would represent an extreme response to pathogen infection.

More than one defense pathway may be involved in the *U. nuda*-barley interaction

Salicylic acid- and JA/ET-regulated signaling pathways are known to be involved in a range of resistant reactions, with SA and JA/ET contributing to defense responses against biotrophic and necrotrophic pathogens in *Arabidopsis*, respectively (Pieterse et al. 2009). Unlike *Arabidopsis*, gene expression analysis revealed JA was the main defense pathway in the resistance to barley covered smut, caused by *U. hordei*, which is a biotrophic pathogen (Gaudet et al. 2010). The role of the JA pathway in the barley-loose smut interaction was also investigated by analyzing the expressions of *LOX2* and *OPR* genes which are required for the biosynthesis of JA (Turner et al. 2002; Kouzai et al. 2016). According to the expression analysis there appeared to be a general trend of up-regulation among the JA-related genes within the resistant line across most time points, with a consistent up-regulation seen for all three JA-related genes at 25 dpi, 4 dps, and 6 dps. Besides the JA pathway, additional gene expression studies were undertaken to monitor the transcript levels of three *PR* genes (*PR1* (unknown secreted protein), *PR2* (β-1,3-glucanase), and *PR5* (thaumatin-like protein)), which encode small secreted antimicrobial proteins and are marker genes for the SA pathway in *Arabidopsis* (Pieterse et al. 2009; Rivas-San Vicente and Plasencia 2011; Spoel and Dong 2012). However, the roles of these three *PR* genes in the barley SA pathway are not as clear as in *Arabidopsis* as the expression of barley *PR1b* can be activated by the application of SA, JA, and ET (Gaudet et al. 2010). According to this study, a pattern of both up and down regulation of the *PR* genes was noted within the resistant line at various time points that did not indicate a clear association with resistance. Despite the importance of the JA pathway and PR proteins in modulating inducible defenses in other pathosystems, the results from this expression study suggest less obvious roles for JA and PR proteins, in comparison to CK, for

mediating barley loose smut resistance conditioned by *Un8*. Additional efforts are needed to characterize the role of SA in the *U. nuda*-barley interaction by measuring the concentration of SA in resistant and susceptible lines.

The target of rapamycin and autophagy pathway may be not involved in *Un8*-mediated resistance

Alternative mechanisms of loose smut resistance were investigated, including target of rapamycin and autophagy. The target of rapamycin (TOR) pathway was investigated because one of the members within the signaling network, an S6 protein kinase, also contains two protein kinase domains and this signaling network may regulate growth-defense trade-offs along with autophagy. The TOR protein kinase positively regulates S6 protein kinase activity which promotes plant growth and is also a negative regulator of autophagy, which is believed to contribute to plant defense by playing a 'pro-survival' or 'pro-death' role in programmed cell death (Menand et al. 2002; Deprost et al. 2007; Bassham 2009; Lenz et al. 2011; Minina et al. 2014). To investigate the involvement of the TOR and autophagy pathways in *Un8*-mediated resistance, the expression of two TOR-related genes and three autophagy (ATG) genes were monitored. However, the lack of altered mRNA indicated that neither may be relevant to *Un8* resistance.

Possible mechanism for *Un8*-mediated loose smut resistance

The significant up-regulation of *CKX1* and *CKX2.1* observed in *Un8* resistant plants provided an interesting clue into smut resistance mediated by this gene. It has been reported that CKs are not only crucial to the plant, but are also important for plant pathogens as they have the ability to alter the source-sink relationship in favour of the pathogen and act directly as an effector to inhibit plant defenses (Chanclud et al. 2016). Several plant pathogens, such as *Ustilago maydis*, *Claviceps purpurea*, and *Magnaporthe oryzae*, are able to produce CKs (Bruce et al. 2011; Hinsch et al. 2015; Chanclud et al. 2016), which in some cases (e.g. *Rhodococcus fascians*, *Ustilago maydis*, and *Magnaporthe oryzae*) are required for pathogen virulence (Pertry et al. 2009; Chanclud et al. 2016; Morrison et al. 2017). Interestingly, it has

been suggested that disease resistance could be achieved by engineering plants to overexpress *CKX* genes (Siemens et al. 2006), the very phenomenon observed in this study.

Given that *U. nuda* and *U. maydis* cause smut disease in barley and maize, respectively, and *U. maydis* is known to synthesize CKs, which are important for its virulence (Bruce et al. 2011; Morrison et al. 2017), it is reasonable to postulate that CKs might also be synthesized by *U. nuda* and play an important role in facilitating infection of the barley host. Thus, it is logical that barley has evolved a defence strategy to *U. nuda* that might recognize *U. nuda*-produced CKs resulting in the over-expression of the host CK pathway, including *CKX1* and *CKX2.1*, which will degrade CKs from both host and pathogen. While this would effectively halt pathogen growth and achieve resistance, the expression of *CKX1* and *CKX2.1* at the early growing stage may also disrupt the homeostasis of host CK resulting in a significant fitness cost in the form of hindered plant growth (thus the observation of Group II and III seedlings) (Fig. 7.2).

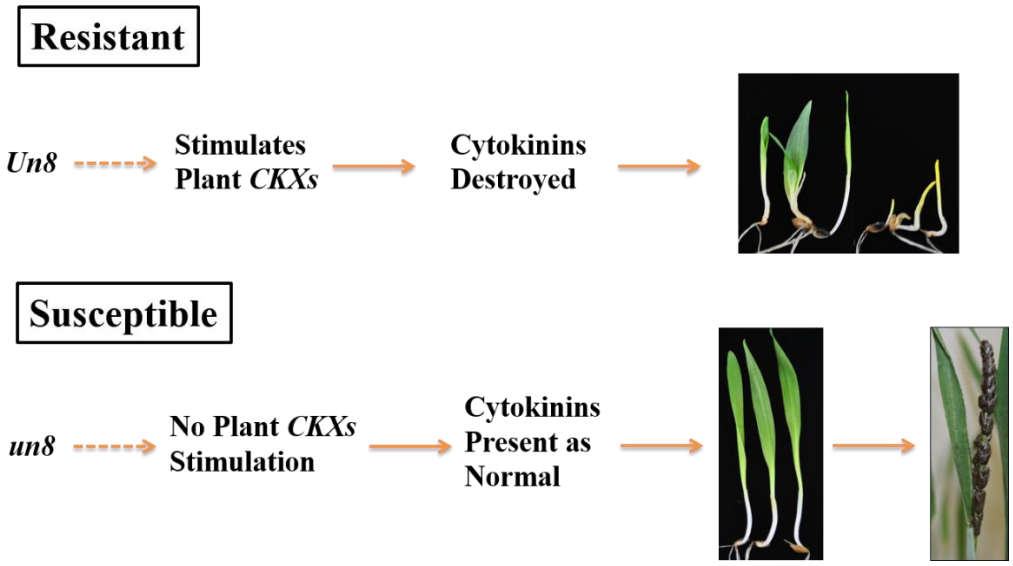


Fig. 7.2 Simple model of *Un8*-mediated loose smut resistance. During entry of *U. nuda* into the barley growing point at the seedling stage, *U. nuda*-derived cytokinins are detected by the *Un8* gene, which triggers the over-expression of the host CK pathway, including *CKX1* and *CKX2.1*, which degrades cytokinins from both host and pathogen resulting in a significant plant fitness cost in the form of hindered plant growth to effectively halt pathogen growth. In the absence of *Un8*, *U. nuda* can successfully penetrate the growing point without activating the over-expression of plant *CKX* genes and disturbing the homeostasis of host CKs, thus keeping pace with the growing point. Solid and dash lines indicate known and unknown connections between two parts, respectively. *CKX*, cytokinin dehydrogenase/oxidase.

Concluding remarks

Evidence for an atypical resistance mechanism associated with the durable *Un8* loose smut resistance gene was identified. Cytokinins might play important roles in regulating plant growth and appear to mediate *Un8* loose smut resistance. Elevated expression levels of two CK pathway genes were noted in the seedling stage, again supporting the observation that this growth stage may be critical for resistance (as concluded in Chapter 5). This work links the CK pathway (specifically *CKX1* and *CKX2.1*, which oxidize CKs) to barley loose smut resistance to provide insight into this host-pathogen interaction. Moreover, a working hypothesis is presented that uses the CK signaling pathway to explain the link between *Un8*-mediated disease resistance and plant fitness.

CHAPTER 8

General Discussion

Barley loose smut caused by *Ustilago nuda* (Jens.) Rostr. (*U. nuda*) was historically a serious disease problem; however, it can be controlled with fungicide-based seed treatments and smut resistant cultivars. In North American breeding programs, the barley *Un8* resistance gene has been deployed for over 50 years against loose smut. The current study aimed to isolate the *Un8* gene by map-based cloning to provide a deeper understanding of *Un8*-mediated loose smut resistance.

8.1 Conclusions

8.1.1 The *Un8* candidate gene encodes a putative protein kinase with two tandem kinase domains

Using two segregating populations with informative recombinants near the *Un8* locus, a *Un8* candidate gene was identified that encodes a putative protein with two tandem kinase domains (Chapter 3). Map-based cloning efforts delineated the *Un8* locus between two overlapping bacterial artificial chromosomes (BACs) using two flanking markers (Chapter 3). This was accomplished without the need of traditional chromosome walking, because of: i) high resolution achieved by using a large mapping population containing close to 5,000 lines; ii) a high degree of synteny among barley, Brachypodium, and rice at the *Un8* locus; iii) informative recombination events within the two mapping populations; and iv) the *Un8* gene is located near the distal end of chromosome 1H in which there is a high recombination frequency. The relationship between gene density and recombination frequency has been clarified in barley with the identification of some regions with relatively high gene density embedded within areas with suppressed recombination (Muñoz-Amatriaín et al. 2015). These low-recombination regions of barley, such as the pericentromeric regions, have hindered isolation of genes such as *rym11* (Lüpken et al. 2013), *Ryd3* (Lüpken et al. 2014), and *Spt1* (Richards et al. 2016).

Many plant disease resistance (*R*) genes have been characterized and most encode nucleotide-binding site-leucine-rich repeat (NBS-LRR) proteins. The other main classes of *R* genes include protein kinases and proteins containing a transmembrane domain and extracellular leucine rich repeats (Gururani et al. 2012). The first plant *R* gene isolated was *Pto*, a kinase conferring resistance to *Pseudomonas syringae* pv. *tomato* (Martin et al. 1993). Since then many *R* genes encoding protein kinases have been identified including the recently isolated northern corn leaf blight resistance gene *Htn1* (Hurni et al. 2015) and the maize head smut resistance gene *ZmWAK* (Zuo et al. 2015). However, it is uncommon for R proteins to have two protein kinase domains. Currently, the only known examples are the *Rpg1* stem rust resistance gene which was also identified from barley (Brueggeman et al. 2002). Thus, the newly identified *Un8* candidate gene from this study may expand our knowledge of protein kinases in disease resistance.

8.1.2 Allele sequencing supports the *Un8* candidate gene as the true loose smut resistance gene

Sequencing the *Un8* candidate allele in a collection of *Hordeum* germplasm (Chapter 4) revealed sequence conservation among resistant accessions such that only one allele associated with resistance was identified. The *Un8* candidate gene sequence from the winter barley accession CN91953 was identical to the other resistance alleles sequenced from modern cultivars and elite breeding lines, confirming that this landrace collected from Azerbaijan is the source of *Un8* resistance (Metcalfe and Johnston 1963).

Eight alleles were identified among the 28 susceptible accessions sequenced, including four unique to the wild accessions surveyed in the study and one common to both wild and cultivated accessions. Thirteen amino acid variations in the coding region were detected between resistant and susceptible accessions, with four amino acids predicted to be associated with changes in protein function. Within the 2,000 bp upstream of the 5' UTR, a total of 22 polymorphisms, including 19 SNPs and three insertion/deletion (indels), were identified between resistant and susceptible accessions. Seventeen of these polymorphisms were present within the *cis*-regulatory elements identified.

Based on sequence variation observed both within and upstream of the candidate gene, it was possible that the *Un8* candidate gene-mediated resistance resulted from the altered regulation of expression or altered protein function. However, given that up-regulation of the *Un8* gene in a resistant line was limited to one time point (among seven time points sampled from late seed maturation to early seedling development) and was only 2-fold greater than expression observed in a susceptible line (Chapter 7), it would seem that altered gene expression was an unlikely explanation.

8.1.3 The seedling stage may be critical for *Un8*-mediated loose smut resistance

Based on observations made in Chapters 5, 6, and 7, it appears that *Un8* resistance was mainly expressed during the seedling stage. This was in contrast to previous reports that *Un8*-mediated resistance was expressed within the embryo based on the observation of tissue necrosis (Gabor and Thomas 1987). No such tissue necrosis was observed in this study and no differences in the location and quantity of mycelium were observed between resistant and susceptible barley lines. However, the possibility that *Un8*-conditioned loose smut resistance may also be expressed during seed maturation, as indicated by Gabor and Thomas (1987), cannot be completely excluded without closer observations during this time.

The current observations were consistent with previous reports that other loose smut resistance genes, such as *Un3* and *Un6*, govern resistance at the seedling stage (Gabor and Thomas 1987). Similarly, seedling resistance was also noted in wheat lines resistant to loose smut caused by *U. tritici* (Batts and Jeater 1958). According to Ton et al. (2009), three phases of plant defense may occur against plant pathogens: Phase I occurring as a pre-invasive defense barrier; Phase II is expressed as an early post-invasive defense after successful penetration; while Phase III is a late post-penetration defense barrier. Different defense mechanisms are associated with each phase. For example, during Phase III further invasion by the pathogen is halted by a complex network of defense signaling cascades which can include salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) dependent defense pathways (Ton et al. 2009). Commonly, JA and ET are primarily involved in defense against necrotrophic pathogens, whereas the SA signaling pathway is often required for resistance against biotrophic pathogens (Pieterse et al. 2009). However, gene expression analysis from

this study indicated the CK pathway might be responsible for barley loose smut resistance conditioned by *Un8*.

8.1.4 *Un8*-mediated barley loose smut resistance is associated with an extreme fitness cost upon infection

The observation of abnormally growing seedlings arising from inoculated resistant and susceptible plants (Chapter 5) complicated the ability to differentiate the resistant reaction from possible tissue damage caused by excessive inoculum. As a result, in Chapter 6, the consequences of using inoculum concentrations of 10× and 100× lower than that commonly used were investigated. During this study it was observed that at 100× lower inoculum concentration the abnormal seedling phenotype was eliminated in susceptible lines, but persisted in resistant lines, which suggested a large fitness cost to the host associated with *Un8* resistance.

To investigate the possible fitness cost observed in Chapter 6 and to better understand the plant defense pathways associated with *Un8* resistance expression, expression analysis of key genes representing well-known phytohormone biosynthesis/signaling and metabolic pathways was undertaken in Chapter 7. The most significant changes in gene expression were observed for two genes involved in the cytokinin pathway, specifically in the oxidation of cytokinins (CKs), at time points that coincided with early seed germination and the seedling stage. Cytokinins have long been recognized as important regulators of plant growth and more recently have been shown to play roles in plant-pathogen interactions as mediators of disease resistance or susceptibility (Choi et al. 2010; Choi et al. 2011; Grosskinsky et al. 2011; Jiang et al. 2013; Siddique et al. 2015; Shanks et al. 2016).

Several pathogens have been shown to synthesize CKs which act as virulence factors. This has been observed in the rice blast pathogen *Magnaporthe oryzae* (*M. oryzae*) (Jiang et al. 2013; Chanclud et al. 2016), and of significance to this study, in the corn smut pathogen *U. maydis* (Bruce et al. 2011; Morrison et al. 2017). It is therefore feasible to hypothesize that CKs may also be produced by *U. nuda* and play an important role in facilitating infection of barley. Thus, it is possible that barley has evolved a defence strategy to *U. nuda* based on

recognition of *U. nuda*-produced CKs via the *Un8* gene, which triggers the over-expression of the host CK pathway, including *CKX1* and *CKX2.1*, which in turn will degrade CKs from both host and pathogen in resistant lines. This would not only inhibit further pathogen growth and achieve resistance, but would also disrupt the homeostasis of barley CKs resulting in the observed fitness cost in the form of stunted seedlings (Chapter 6). If this working hypothesis is ultimately shown to be an accurate description of barley's reaction to *U. nuda*, it would represent a novel mechanism of resistance that has not yet been described. While this reaction may appear to be an extreme manner of dealing with a pathogen, it is fundamentally similar to the hypersensitive response in which cells within a plant are sacrificed for the benefit of the entire plant. The difference in this case is that the entire plant is sacrificed for the benefit of the larger population of plants.

8.2 Future Directions

8.2.1 Confirming the *Un8* candidate gene

In barley, one method used to confirm the function of a candidate gene would be through *Agrobacterium*-mediated transformation to express the putative gene in a susceptible genotype. The most likely barley genotype for this purpose is the spring cultivar (cv.) 'Golden Promise' because of its high transformation efficiency (Hensel et al. 2008). The reaction of cv. 'Golden Promise' to *U. nuda* has been evaluated and it has been determined to be susceptible. Appropriate sequence information for the *Un8* candidate gene has been sent to IPK Gaterslaben, Germany, to transform the cv. 'Golden Promise' with the *Un8* candidate gene from a resistant accession. One concern about *in vivo* expression of the *Un8* candidate gene is that being a kinase, its overexpression by a strong promoter such as Ubi-1, may have negative pleiotropic effects mediated through the disruption of endogenous CK homeostasis resulting in poor seedling survival. Transgenic barley plants might therefore be generated only after supplementation of the regeneration medium with exogenous CKs, such as N^6-benzylaminopurine (Mrízová et al. 2013). Alternatively, transforming 'Golden Promise' with the *Un8* candidate gene under the control of its own promoter may avoid this problem.

The other approach to confirm that this is the *Un8* gene would be to silence the function of the gene through various reverse genetic strategies. Such approaches include Targeting Induced Local Lesions IN Genomes (TILLING), Zinc-finger nucleases, transcription activator-like effector nucleases, and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) methods (Sander and Joung 2014). The CRISPR-Cas9 system has been established in barley (Lawrenson et al. 2015) and with it, both the *Un8* candidate gene and the *CKX* genes (*CKX1* and *CKX2.1*) could be rendered unfunctional via mutation to confirm our hypotheses. However, one complication associated with this strategy is that we do not know which resistant genotype is amenable to gene transformation and regeneration through tissue culture. In addition, one must consider the possibility that other *CKX* genes could compensate for the loss of function of *CKX1* and *CKX2.1* during the loose smut resistance response. Alternatively, TILLING does not require plant transformation. After chemical mutagenesis, mutations could be confirmed in the *Un8* candidate gene and *CKX* genes using available primers followed by analysis of altered phenotype (Kurowska et al. 2011). This strategy has been applied in barley to investigate the functions of the centromeric histone H3 (Karimi-Ashtiyani et al. 2015).

In addition, because no resistance allele could be identified in wild barley accessions, it appears that the mutation(s) in the *Un8* candidate gene resulting in resistance may have arisen after domestication. However, whether these mutations occurred early after domestication in the Near East centre of origin, or after barley began to spread beyond the centre of origin to regions such as Azerbaijan, will require further investigation by surveying a wider array of germplasm from these regions.

8.2.2 Investigating the *Un8*-mediated late post-penetration resistance and protein function of the *Un8* candidate gene

During the early stages of *U. nuda* infection, nutrients are allocated to the growth of *U. nuda* which is particularly demanding on the host considering the importance of allocating nutrients for seed development. It was found that small and medium sized seeds always carry more loose smut than large seeds (McFadden et al. 1960; Chapter 6) as more nutrients are supposed to be allocated to the growth of *U. nuda* in the smaller sized seeds. As a result, one

key subject for future study of the *U. nuda*-barley pathosystem is to investigate the mechanisms underlying late post-penetration resistance, that is, are there specific host molecules, including peptides (Lee et al. 2011), which identify the presence of this pathogen and which are in turn monitored by the *Un8* gene. In addition, to test the importance of the CK pathway in *Un8*-conditioned loose smut resistance, one possible method is the exogenous application of CKs to the abnormal seedlings from resistant lines to check whether seedling growth can be restored.

It is also interesting to note the similarity among the predicted protein structure of the *Un8* candidate gene, the *Rpg1* gene, and the Janus kinase (JAK) protein family. The Rpg1 protein and JAK family identified in barley and mammals, respectively, also contain two kinase domains (Brueggeman et al. 2002; Yamaoka et al. 2004). However, only one kinase domain is functional within the Rpg1 protein and JAKs, while the other regulates activity (Yamaoka et al. 2004; Nirmala et al. 2006), so it would be interesting to determine if this was also the case for the predicted protein of the *Un8* candidate gene.

8.2.3 Sequencing and editing the *U. nuda* genome

One path to determining if the hypothesis in Chapter 7 has merit would be to obtain the genome sequence of *U. nuda* to determine if CK-synthesis genes exist. The genome *U. hordei* has been sequenced and may provide a helpful resource for identifying cytokinin genes in *U. nuda*. This was certainly the case when comparison of the two maize-infecting smut fungi (*U. maydis* and *S. reilianum*) genomes showed a remarkable degree of synteny. Access to the two barley smut fungi *U. nuda* and *U. hordei*, would also be valuable to understand more about their infection processes, and in turn, the different resistance mechanisms conferred by the same host. Moreover, targeted alterations in the genome by CRISPR-Cas9 genome editing technology would enable efficient investigation of the role of any cytokinin genes discovered for their contributions to the virulence of *U. nuda*. This technology has already been utilized in the rice blast fungus *M. oryzae* (Arazoe et al. 2015) and *U. maydis* (Schuster et al. 2016) to learn more about the virulence genes in these pathogens.

8.2.4 Evaluating the fitness cost of *Un8* in the absence of *U. nuda* infection

It is unclear whether there is also a fitness cost associated with the *Un8* gene in the absence of *U. nuda* infection, as has been observed with other resistance genes. There is no evidence of this from the CDC barley program (Beattie, personal communication), however this effect is known in some cases. For example, Tian et al. (2003) demonstrated a reduction in fitness of *Arabidopsis* plants which contain the *RPM1* gene, such as lower shoot biomass and reduced seed production. The *mlo* gene of barley, which is an excellent source of powdery mildew resistance, is linked with a yield penalty and necrotic spotting even in the absence of infection (Kjær et al. 1990; Brown 2002). Given this excellent source of powdery mildew resistance and the importance of this disease, continuous breeding efforts over a period of 40 years, especially in Europe, to compensate for the negative pleiotropic effect on yield finally resulted in the first commercial spring barley release in 1979 in the Netherlands (Jørgensen et al. 1992). Interestingly, not all *mlo* alleles behave the same way. A newly discovered *mlo* allele from an Ethiopian landrace does not display such pleiotropic effects and should be a valuable gene for future breeding efforts (Ge et al. 2016).

To determine the impact of the *Un8* gene in the absence of the pathogen one could investigate the performance of near isogenic lines differing only for *Un8*. This could alternatively be accomplished by mutating the *Un8* candidate allele in resistant lines using the CRISPR-Cas9 system.

8.3 Highlights from This Work

- The *Un8* candidate gene is predicted to encode a protein with two kinase domains;
- The barley landrace CN91953 was confirmed as the source for the *Un8* candidate gene by allele sequencing;
- A simple and reliable histological method was developed to diagnose infection of *U. nuda* in barley seeds;
- A more suitable inoculum concentration for screening barley loose smut resistance is 0.01 g spore/L;

- By early seedling development stage barley loose smut resistance, conditioned by *Un8*, is completed;
- The cytokinin pathway may be responsible for *Un8*-mediated loose smut resistance, as well as the associated plant fitness cost associated with resistance.

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APPENDICES

Appendix A Detailed information on markers identified in the *Un8* interval between Un8SNP1 and Un8SNP6 in the TR09398 × TR07728 population.

Name	Origin	Primer Sequences (5'-3')		Type	Unigene ID[a]	BAC ID[b]	Morex Contig
Un8SNP1[c]	HarvEST: Barley	F R Reporter 1 Reporter 2	CTTGTCAGTTGAATGCCAATCTCTT GTTCGACAATGATTGCATCTCACA TGCAGCTTGGTCTCAAT AGCCTGGCCTCAAT	TaqMan	4245	HVVMRX83KhA0023N07_c1	contig_158214
8487	HarvEST: Barley	F1 F2 R	AGTACCCTCACCCTTACAAATTC GTACCCTCACCCTTACAAATTG CACCTCTACATTTGGGTCCTTG	Allele-specific PCR	8487	HVVMRXALLhA0368G13_v37_c5	contig_1569595
48060	iSELECT Assay	F R1 R2	GTCCATTTCTCAGGCTCAGTG CCGACGGTCAAGGTCTCA CGACGGTCAAGGTCTCG	Allele-specific PCR	48060	HVVMRXALLhB0089C19	contig_38392
Un8SNP7	Morex BAC	F R Reporter 1 Reporter 2	GGCGAAATTCTCCTTGAAAACATGT CATGTTTGCCCCAATAAAAATGTCTAAC CACTTGTCTTGCCACTTC TTCACTTGTCTTACCACTTC	TaqMan	1406	HVVMRXALLhA0772N02_IPK_NODE_0001	contig_5603
HI1406	Morex BAC	F R	AAGTGCGACGACGGAATACA ATAGGACGCAAACCGACAAG	CAPS (cut by Msc I)	-	HVVMRXALLhA0772N02_IPK_NODE_0001	-
1406	HarvEST: Barley	F1 F2 R	TGTCCTTAGTTCACTTGTCCTG CATGTCCTTAGTTCACTTGTCCTA GCTACTACTGACTATCGCCACAT	Allele-specific PCR	1406	HVVMRXALLhA0772N02_IPK_NODE_0001	contig_5603
Un8SNP4[c]	HarvEST: Barley	F R Reporter 1 Reporter 2	CTACATCTGCTCCTGCGACTT TCAAAATCGAGCTTCCCATCACAAT CACAGTATATCGCACGAGAA TCACAGTATATCACACGAGAA	TaqMan	16527	HVVMRXALLeA0355N04	contig_171284

Appendix A. (continued).

Name	Origin	Primer Sequences (5'-3')		Type	Unigene ID[a]	BAC ID[b]	Morex Contig
0751D06 F6/R6	Morex BAC	F R	TCAGAGATGGCTGTGAGGATG CTGCGTTTACGAAGATGGATGT	Allele-specific PCR	-	HVVMRXALLmA0180J17_sc1	contig_93215
0498L15 F8/R8	Morex BAC	F R	TCGGTGTTCAGTCCCAAGTC TGACCCTGCTGGTAGGTAGAGT	Allele-specific PCR	-	HVVMRXALLhA0498L15_NODE_0053.1	-
0498L15 F3/R3	HarvEST: Barley	F R	TGGCTGCAATATCATGGTCAT AATCATTTGCCAGGTCAGAAG	Amplicon size	15283	HVVMRXALLeA0154F16_sc2	contig_46703
3602	HarvEST: Barley	F R	TTGCTGTTTGGTCTGGTCTTG GCACCTTCAGCAATCTCAATCT	CAPS (cut by Taq I)	3602	HVVMRXALLeA0217G19	contig_43658
10924	iSELECT Assay	F R1 R2	GCTTCTGTTCACGCCACTGT CGCTTACTCCATGGTGCTAAT CGCTTACTCCATGGTGCTAAG	Allele-specific PCR	10924	HVVMRXALLeA0217G19	contig_43658
17452	Synteny	F R	CCTGGTGGTGATGCAGAAGAT CACCTGATAGGCAGAGGAGTACAC	Amplicon size	17452	HVVMRX83KhA0046D01_c1	contig_162350
13742	HarvEST: Barley	F R	AAGACCATCACGCTGGAG CAATGACAAACGACAGGG	CAPS (cut by Sac II)	13742	HVVMRXALLhA0568K13_c1	contig_275666
21217	Synteny	F R	GGTCGGGAGTATGACTTAGGAAT CAGGAAGCAAGGAATACTGGAAT	Allele-specific PCR	21217	HVVMRXALLhA0568K13_v11_c1	contig_159925
Un8SNP6[c]	HarvEST: Barley	F R Reporter 1 Reporter 2	GGCAACCCACGGAAACAC CCACGCTGATCTTATCTATGGCTAA TGACGACCAAACGATACAT TGACGACCAAAATATAG	TaqMan	14722	HVVMRXALLhB0144C24	contig_39431

[a]Unigene ID determined from HarvEST:Barley v.1.83, assembly 35;
[b]The ‘sc’ or ’c’ suffix indicates the scaffold or contig, respectively, on which the predicted gene resides within the associated BAC;
[c]Previously developed by Eckstein.

Appendix B Detailed information for the genes used in the qRT-PCR experiment to study the barley-*U.nuda* interaction. Genes include the *Un8* reference gene, the *TUBA* internal expression reference control, genes related to the autophagy and target of rapamycin processes and genes associated with six phytohormone pathways and antimicrobial proteins.

Gene Symbol	Name	Function[a]	Primer Sequence (5' to 3')[b]	Accession Number
ATG5	Autophagy-related protein 5	Autophagy	F- GGACCGTTTGAGGAGGACTT R- CAATCACTGACAGGAATCGCA	AK362511
ATG8a	Autophagy-related protein 8a	Autophagy	F- GGAGGCTAACCGCATAAGAGAG R- CTTGATACGCTTACGAACCACG	MLOC_74964
ATG8c	Autophagy-related protein 8c	Autophagy	F- CGAGGAGTAACCTTCCAGAGATG R- GAATCATACACGCTGCCCAT	AK250515
TORa	Target of rapamycin a	TOR-related	F- TTGCTCCAGTGCTTGTTCGT R- CATTGCTTCCATCCAAGACGA	MLOC_13770
TORb-1	Target of rapamycin b-1	TOR-related	F- CAGCACAGGAGGTCGTTGATA R- CACAAAATGGCAACTCGTATCA	MLOC_14340
AFB2-1	Auxin signaling F-box 2-1	IAA-related	F- CTTCTTCGTAAGGCTCCACAAC R- GCAGGTAATCTGGAACAGCATC	AK355927
AFB2-2	Auxin signaling F-box 2-2	IAA-related	F- GGCGAACTGTCTTTGTGGGTA R- TCATCTGACACCACCATCCG	MLOC_56088.1
GH3.2	Gretchen Hagen 3.2	IAA-related	F- GGACAATAATGGGTTAGGCTGC R- TTGCGATGTAGGGCTGGAC	MLOC_60505.1
BIN2	Brassinosteroid-insensitive 1	BR-related	F- TTGGGATTGTCTTCCAGGCTA R- TGGTTGGCATTGCTGTAGTGT	AK364823
BZR1	Brassinazole resistant 1	BR-related	F- CTTGATTGGGTCTGGTGGATT R- CTCCATTTCCGCTGTGAACTC	AK359453
CKX1	Cytokinin oxidase/dehydrogenase 1	CK-related	F- CGACGCTCAACTACGACAAC R- GGTCCAGGAACTCCAGGTAG	MLOC_58639.1
CKX2.1	Cytokinin oxidase/dehydrogenase 2.1	CK-related	F- ATCAGGAGGGGCTCATTGG R- GCAGTAGCACATCCAGCCTC	MLOC_53923.1
CKX3	Cytokinin oxidase/dehydrogenase 3	CK-related	F- TCGCCATACAAGTTCATCCAG R- CCACTGTCACCTTGCTTAGAGA	MLOC_52357.1
CKX7	Cytokinin oxidase/dehydrogenase 7	CK-related	F- AGGTTGTGCTTCGGTCTGCT R- GGAGAAGTCCCACGGTGTAGA	MLOC_15141.1
CKX11	Cytokinin oxidase/dehydrogenase 11	CK-related	F- GAGAGGATGGGGGAGATGAT R- GAGACGAAGAGGTTGAGCCAC	AK355215

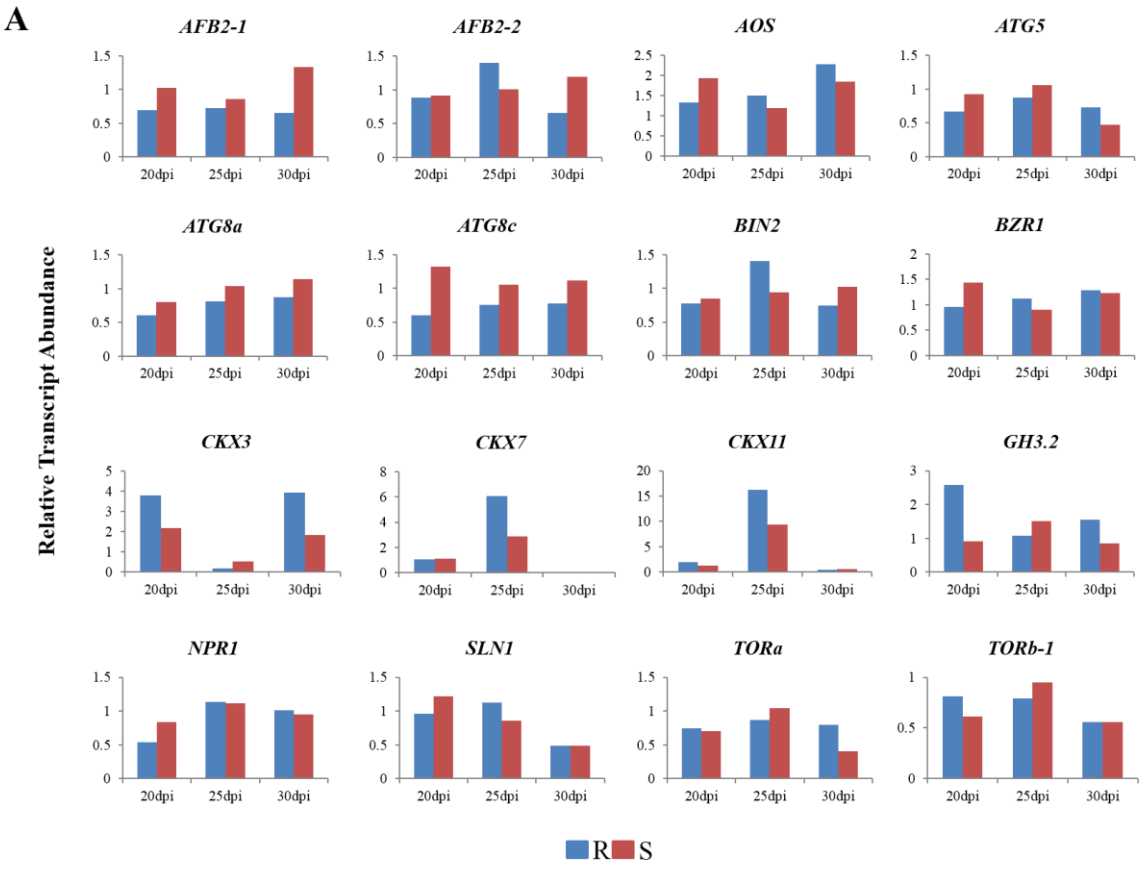
Appendix B. (continued).

Gene Symbol	Name	Function[a]	Primer Sequence (5' to 3')[b]	Accession Number
SLN1	Slender 1	GA-related	F- ATTCCTGGACCGCTTCACC R- ACCCTTCCTTCTCTTCCACCT	AK372064
NPR1	Non-expresser of pathogenesis-related gene1	SA-related	F- TGGCTTTGGCGAGGATAAT R- TCTCATCCGAGCCAAGTGTT	MLOC_64922.1
PR1b	Pathogenesis-related protein 1b	Antimicrobial protein	F- CTGGAGCACGAAGCTGCAG R- CGAGTGCTGGAGCTTGCAGT	X74940.1
PR2	Pathogenesis-related protein 2	Antimicrobial protein	F- TGTTCGCCATGTTCAACGA R- CCAAAGTGCTTCTCCGTGTCA	AY612193.1
PR5	Pathogenesis-related protein 5	Antimicrobial protein	F- CGCCGACCAACTATTCGAA R- GTCGTCCTTGGCATAGCTATAGG	AK355059
AOS	Allene oxide synthase	JA-related	F- GGAGGCGGTGCACAACAT R- GACGGGAACAGGATCTTCATG	AK366287
LOX2a	Lipoxygenase isozyme 2a	JA-related	F- CGGCAGACTCCCTCATCACTAAAG R- GGCAGCAACAGGTCGTGGTAG	MLOC_64972.1
LOX2b	Lipoxygenase isozyme 2b	JA-related	F- TCATTCCTCGTGTCGTCCAG R- CGAACTCCTCGTCTCTGAACC	AK370754.1
OPR	Oxophytodienoate reductase	JA-related	F- CGACAGGGATGATGGAAATAAAG R- GCGCCCATATGCTACCAAGT	AK250031.1
TuBA	Alpha tubulin	Endogenous control	F- TTCGCCCGTGGTCATTACA R- GCATTGAAGACAAGGAAGCCC	AK250165
Un8	*U. nuda* resistance gene 8	*Un8* candidate	F- AACCTCCACTTTGTCTTGTCCGTA R- GATCCTCGTGTACTCAGGTCTCAGGGATT	MLOC_38442.1

[a]TOR, target of rapamycin; IAA, indole-3-acetic acid; BR, brassinosteroid; CK, cytokinin; GA, giberelin acid; SA, salicylic acid; JA, jasmonic acid;
[b]Primers for *AOS*, *OPR*, *PR1b*, *PR2*, *PR5* are from Chauhan et al. 2015; Primers for TuBA is from Jarošová and Kundu 2010; Primers for *LOX2a* is from Walters et al. 2014. Primer pairs for other genes were designed in this study.

Appendix C

Summary of gene expression analysis in response to *U. nuda* infection during seed maturation and early seedling stage.



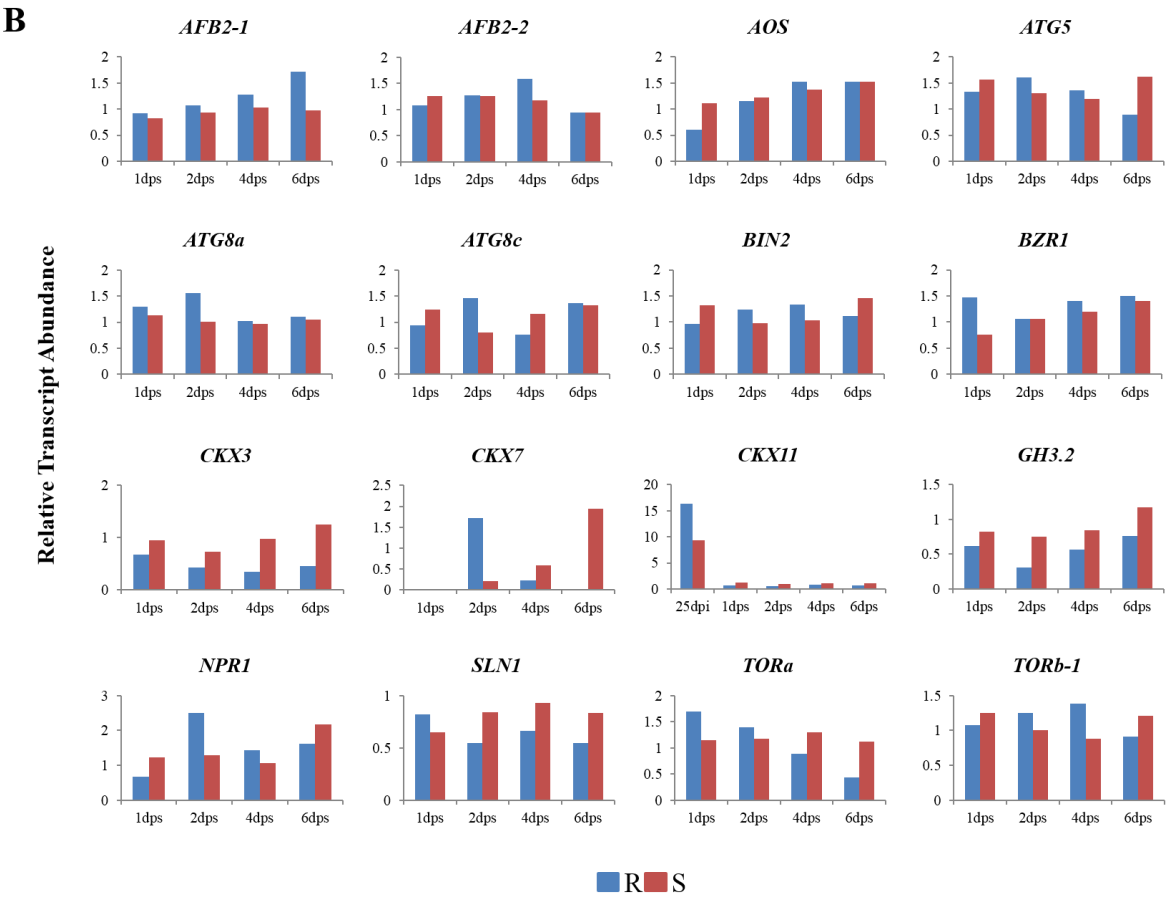


Fig. C Summary of gene expression analysis.

(A) Relative transcript abundance of selected barley genes in response to *U. nuda* infection during seed maturation.

(B) Relative transcript abundance of selected barley genes in response to *U. nuda* infection at seedling stage.

The barley *TuBA* gene was used as the internal control and expression values were normalized to the mock-inoculated samples in which expression was set to 1. Expression values for each gene are presented from one biological replicate with three technical replicates. IAA pathway: *AFB2-1*, *AFB2-2*, *GH3.2*; BR pathway: *BIN2*, *BZR1*; CK pathway: *CKX3*, *CKX7*, *CKX11*; GA pathway: *SLN1*; SA pathway: *NPR1*; JA pathway: *AOS*; Autophagy pathway: *ATG5*, *ATG8a*, *ATG8c*; TOR pathway: *TORa*, *TORb-1*. AFB, Auxin signaling F box; BIN, Brassinosteroid-insensitive; BZR, Brassinazole resistant; CKX, Cytokinin oxidase/dehydrogenase; SLN, Slender; NPR, Non-expresser of pathogenesis-related; AOS, Allene oxide synthase; ATG, Autophagy. Dpi, day post inoculation. Dps, day post sowing of inoculated seeds.