Identification, molecular characterization and analysis of the role of MORC gene family in disease resistance mechanisms to biotrophic and necrotrophic fungi in barley

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1. Introduction

1.1 Barley

Cultivated barley (*Hordeum vulgare*) is one of the earliest domesticated crops known in human history. It is an annual herbaceous self-pollinating cereal crop belonging to the grass family. Barley is a diploid species with a large haploid genome size of around 5.1 gigabases (Gb). The crop species is derived from its wild relative *Hordeum vulgare* ssp. *spontaneum* found abundantly around western Asia and north east Africa, a region known as the Fertile Crescent. The success of barley as a domesticated crop species is owing to the fact that it is an adaptable plant cultivated across different agro-climatic conditions. It is grown as a winter crop in tropical regions and summer crop in temperate regions. Barley is moderately drought tolerant and can tolerate salt stress more than its close relative wheat (Nevo, E. *et al.*, 2012). Barley was ranked fourth among the cereal crops in terms of production and area under cultivation after maize, rice and wheat (http://faostat.fao.org). About three quarters of barley produced around the world is used as animal feed, around a fifth is used in malting of alcoholic and non-alcoholic beverages and the rest is used in various food items (Blake et al., 2011). The self-breeding nature of barley, along with being a diploid with low chromosome number, short breeding time and adaptability to different regions have made this crop a model for cereals and an excellent candidate for genetic studies (Saisho and Takeda. 2011). Lack of a reference genome has been a major impediment in using the large collection of available germplasm for fundamental and breeding science. This challenge has been partially overcome by the work of the International Barley Genome Sequencing Consortium which provides a structural and genetic framework for majority of barley genes along with comparative sequence and transcriptome data.

Just like most other plant species, barley is also infected by a wide range of plant pathogens like fungi, bacteria, nematodes, viruses and phytoplasma. The most common and devastating diseases however are of fungal origin. Fungal diseases of barley can be divided into leaf and stem diseases like blotches, stem and leaf rusts, powdery and downy mildews; diseases of head and seed such as head blights, ergots, smuts and finally diseases of the root. Diseases like head blight are particularly dangerous because of mycotoxins produced by the fungus. Presence of mycotoxins in the grain makes it unfit for human or animal consumption thus resulting in a huge crop loss to the producer. The other main barley diseases are leaf blight caused by bacteria and barley yellow dwarf which is the most widely distributed viral disease of cereals.

Diseases may be a result of genetic makeup of the cultivar, presence of causative agent, environmental conditions, abiotic stresses like nutrient deficiencies or a combination of these factors. Disease diagnosis is very important as it can help prevent significant crop losses. Common disease control strategies include use of resistant cultivars, crop rotation with non-host species like wheat or legumes; avoid sowing in off season to prevent building up of inoculum and use of chemical control agents like fungicides. Very few varieties have resistance to the wide range of pathogens that infect barley and disease control often involves a combination of the above mentioned methods.

Two major barley diseases in Europe; powdery mildew caused by the biotrophic pathogen *Blumeria graminis f.sp. hordei* and *Fusarium graminearum* head blight will be discussed in detail in the following sections. As a result of the increased population pressure of the pathogens to mutate arising from the widespread cultivation of crop plants across geographical locations, there is a very high demand for understanding mechanisms of plant resistance and susceptibility factors. This study focuses on barley MORC gene family and the role it plays in disease development to fungal pathogens with completely different colonization strategies.

1.2 Barley- powdery mildew interaction

Powdery mildews are among the world's most widespread plant pathogens. The name powdery mildew is derived from the fine white mass of mycelia formed by the fungi on leaf surfaces of diseased plants. They infect leaves, stems, flowers and fruits of a large number of flowering plants including economically important plants like grapes, cereals, fruit trees and ornamentals (Belanger et al., 2002). A large amount of money is spent annually for the control of powdery mildew epidemics worldwide. As it is an important and well characterized pathogen, powdery mildew is often used as a model to study plant pathogen interactions using cytological and molecular biology approaches. Being an obligate biotroph, researchers have not been able to cultivate the fungus on artificial medium, even though they are widely grown on detached leaves of their host plants. Cells and spores of powdery mildew are similar in structure to other Ascomycetes. They form cell walls and contain nuclei, vacuoles and other organelles (Akai et al., 1968). Spores of the fungi are pleomorphic or exhibiting morphologically different shapes. It is among the first fungi for which pleomorphism was described. Life cycle has either or both sexual phase (teleomorph) and asexual phase (anamorph).

The barley powdery mildew fungus *Blumeria graminis* formae specialis *hordei* is phylogenetically different from other powdery mildews. *Blumeria graminis* belongs to a distinct clade within the Erysiphales, infecting only members of Poaceae and produces unique conidia from which a primary germ tube and digitate appressoria are formed (Inuma et al., 2007). Despite these differences, much of the powdery mildew- plant interactions have been studied using this species.

As soon as an ascospore or conidium lands on the surface of a susceptible host, an infection process is initiated by the formation of germ tube which then elongates to form a hypha containing appressoria, penetration peg and a haustoria. Appressoria are short, lateral hyphal structures that produce penetration pegs, which breakdown physical barriers in plants through mechanical pressure and enzymatic activity (Green et al., 2002). Haustoria are specialized feeding structures which help in maintaining obligate biotrophy and help mobilize nutrients from plant to the growing fungus (Green et al., 2002). Leaves infected with powdery mildew appear senescent and chlorotic with intermittent patches of green tissues known as green islands (Coghlan et al., 1990). If the fungus manages to penetrate successfully, it then starts to colonize and reproduce in the plant. The hyphae start elongating and branching forming colonies which become visible macroscopically as white pustules on the leaf surface. Reproductive structures or conidiophores are eventually produced by the growing hyphae. Conidiophores are formed perpendicular to the host surface, with each conidium stacked successively on top of a newly formed conidium (Moriura et al., 2006). Conidial dispersion usually takes place by wind over short distances and is negatively correlated with high relative humidity(Grove 1998). Additionally, the airborne spore density follows a diurnal cycle with high spore concentrations in a period from morning to early afternoon (Grove 1998). Wind dispersed asexual conidia are responsible for the powdery mildew epidemic. Cleistothecia producing sexual spores are formed on ageing leaves. The fungus over-winters or survives harsh weather conditions like high temperature or drought in Cleistothecia which remain dormant in adverse environmental conditions (Zhang et al., 2006). Successful penetration, colonization, reproduction and dispersal of the powdery mildew fungus constitutes a compatible interaction which is illustrated on the following page.

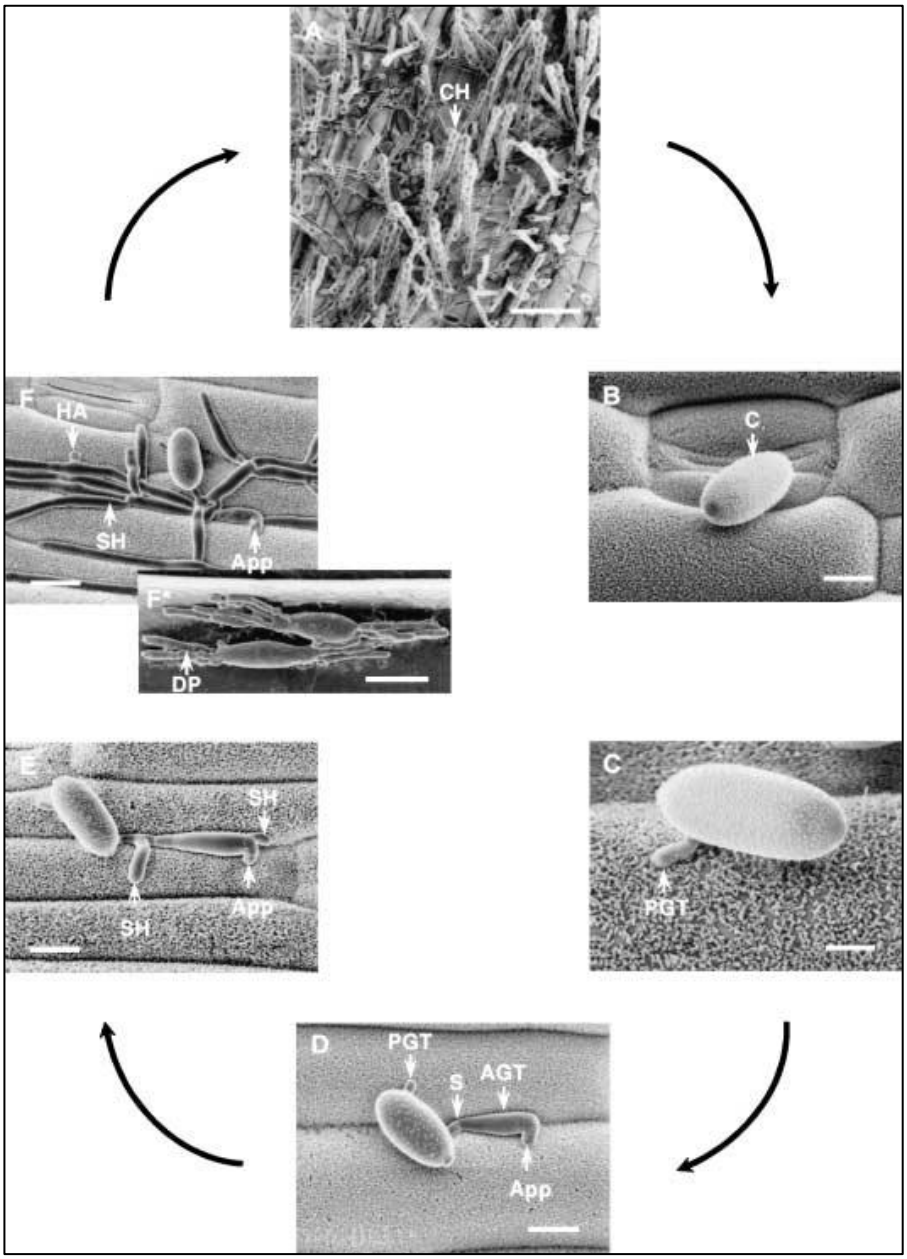


Figure1. Asexual life cycle of Barley Powdery Mildew fungus; A) Mature colony containing chains of conidia on a conidiophore. Each conidiophore contains several thousand conidia, which are wind dispersed to cause the epidemic. B) Conidium on surface of the leaf C) Conidium germinates and forms the primary germ tube after about 2 hours D) Formation of appressorial germ tube, penetration peg and a hooked appressorium E) Fungus starts producing secondary hypha soon after the formation of digitate haustoria F) Spread of fungus to neighbouring cells with formation of additional digitate haustoria. At any given time, a mature haustorium may have upto 10 digitate processes. (Adapted from Zhang et al., 2006)

Over the years a lot of research has been done concerning disease resistance of plants especially cereals wheat and barley to powdery mildews. Plant resistance to powdery mildews is a multistep process starting with penetration resistance. Mildew Locus O gene (MLO) supports effective host cell penetration of powdery mildew in barley. Recessive loss of function mutation (*mlo*) confers broad spectrum resistance to powdery mildew and arrests pathogenesis prior to cell invasion (Büschges et al., 1997). Typically, *mlo* resistance is characterized by cell wall appositions or reinforcements at the site of fungal entry, often directly below the penetration peg. Speed of cell wall reinforcement and composition of materials involved in papilla formation are crucial. Susceptibility occurs due to failure of cells to form papilla or formation of ineffective papilla (Aist and Bushnell 1991).MLO genes code for plant specific integral membrane proteins with a C-terminal calmodulin binding site and confer calcium dependent calmodulin binding (Devoto et al., 1999; Bhat et al., 2005). Fluorescence Resonance Energy Transfer experiments (Bhat et al., 2005) demonstrated that calmodulin binding to Barley MLO increased around the time of switch from surface to invasive growth. The protein de-regulates MAMP triggered immunity and *mlo*-resistance uses the same molecular pathway as MAMP triggered immunity (Humphry et al.,2006).Barley MLO was found to be part of a pathway negatively regulating plant immunity suggesting mlo based resistance is not a pleotropic effect, rather a consequence of the negative regulatory role of barley MLO protein (Humphry et al.,2010).

The other important resistance mechanism in barley against powdery mildew involves resistance genes that specifically detect pathogen avirulent factors and mount an immune response. This form of resistance results in the production of reactive oxygen species that leads to cell death and is known as the hypersensitive response (HR). Cell death by HR is a common defence mechanism against biotrophic fungi as it cuts off essential nutrient supply without which the fungus can't grow. The R-gene mediated resistance is race specific or effective only against specific isolates of *Blumeria graminis* formae specialis *hordei*. With just over 30 isolate specific variants, Mildew locus a (*Mla-1 to Mla-32*) is the most common resistance gene locus in Barley against *B. graminis* f. sp. *hordei* isolates (Jørgensen 1994). Most of the *Mla* specificities have been introduced into cultivated barley by plant breeding. Like most other R-proteins that confer resistance, they are proteins with an N-terminal nucleotide-binding (NB) site and C-terminal leucine-rich repeats (LRRs) where pathogen recognition is thought to be through sequence variable LRR region (Elliset al., 1999; Wei et al., 1999). Mla mediated resistance may or may not require two independent proteins Rar1

and Rar2 which implies the presence of more than one independent race-specific resistance signalling pathways (Jørgensen 1996; Freialdenhoven et al., 1994).

The Mla protein resides in the cytoplasm and interacts with the fungal molecules (Avr proteins) transported across the plant plasma membrane. During Mla-mediated resistance, recognition of the avirulence factors initiates cell death program within 24 hours of infection in the epidermal cells which later on spreads to the underlying mesophyll cells. Initially hydrogen peroxide accumulates below the penetration peg followed by a second wave of H_2O_2 burst where the whole infected epidermal cell is flooded with reactive oxygen species (Thordal-Christensen et al.,1997; Huckelhoven et al., 1999). H_2O_2 functions as a signalling molecule in plant defence at low concentration and aids in cell wall reinforcements, but is cytotoxic and directly kills the pathogen at high concentrations (Lamb and Dixon 1997). Host-cell suicide and pathogen resistance seems to be tightly linked as Mla, Rar1 and Rar2 mutants each lose the ability to activate the cell-death response (Freialdenhoven et al., 1994).The formation of cell wall appositions and execution of H_2O_2 mediated cell death are among the most common mechanisms of effective defence against colonization by biotrophic powdery mildew fungi.

1.3 *Fusarium graminearum*

Fusarium graminearum (teleomorph *Gibberella zeae*) an ascomycetes fungus belonging to the order hypocreales is a major global pathogen of cereal crops causing Fusarium head blight (FHB) in wheat and barley and responsible for ear rot of maize. Head blight is accompanied by blights or root rots and in areas of extensive maize cultivation, this disease renders cultivation of wheat and barley unfeasible. Fusarium head blight has the ability to destroy seemingly lush traits of crop land overnight and the disease severity is exacerbated by hot and humid conditions, intermittent rainfall during periods of grain-fill and flower development (McMullen et al., 1997).The infection cycle of *F. graminearum* starts with overwintering macroconidia of the fungus in soil or on plant debris. Growing mycelia give rise to fruiting bodies, which produce ascospores. The ascospores or sexual spores are primary source of inoculum (Markell and Francl, 2003).The ascospores produced by perithecia are forcibly discharged from these perithecia which upon landing on susceptible plant parts germinate within six hours to infect the respective cereal host plant (Beyer and Verreet2005). Fungal entry into the plant takes place through natural openings such as stomata, and needs soft tissue to start infecting the plant (Jansen et al., 2005). Infected

spikelets appear water soaked initially and later turn straw coloured due to chlorophyll loss. Under favourable climatic conditions, pinkish-red mycelium and conidia are formed in the infected spikelets, and the infection spreads to adjacent spikelets or in worst cases may affect the entire head. Kernels infected become shriveled and discoloured in appearance as a result of the mycelial outgrowths from the pericarp (Agrios 2005). Once infection is established, macroconidia are produced by asexual reproduction after a cycle of infection by ascospores, thus making the Fusarium head blight disease monocyclic (Beyer et al., 2004). Overwintering of these structures in the soil or in plant debris on the field gives rise to the mycelium and thus fresh inoculum in the next season. A recent increase in FHB occurrence can be attributed to widespread adoption of no-till practices and stubble retention worldwide. In addition to severe crop damage, infected kernels also contain mycotoxins such as deoxynivalenol (vomitoxin) that are toxic to humans, hogs, and other animals. Deoxynivalenol is known to cause vomiting and feed refusal posing a threat under high exposure levels thus making the grain unsuitable for human and animal consumption (Snijders 1990). The disease can account for upto 50% yield losses in the most severe cases and is more intense in taller cereal varieties.

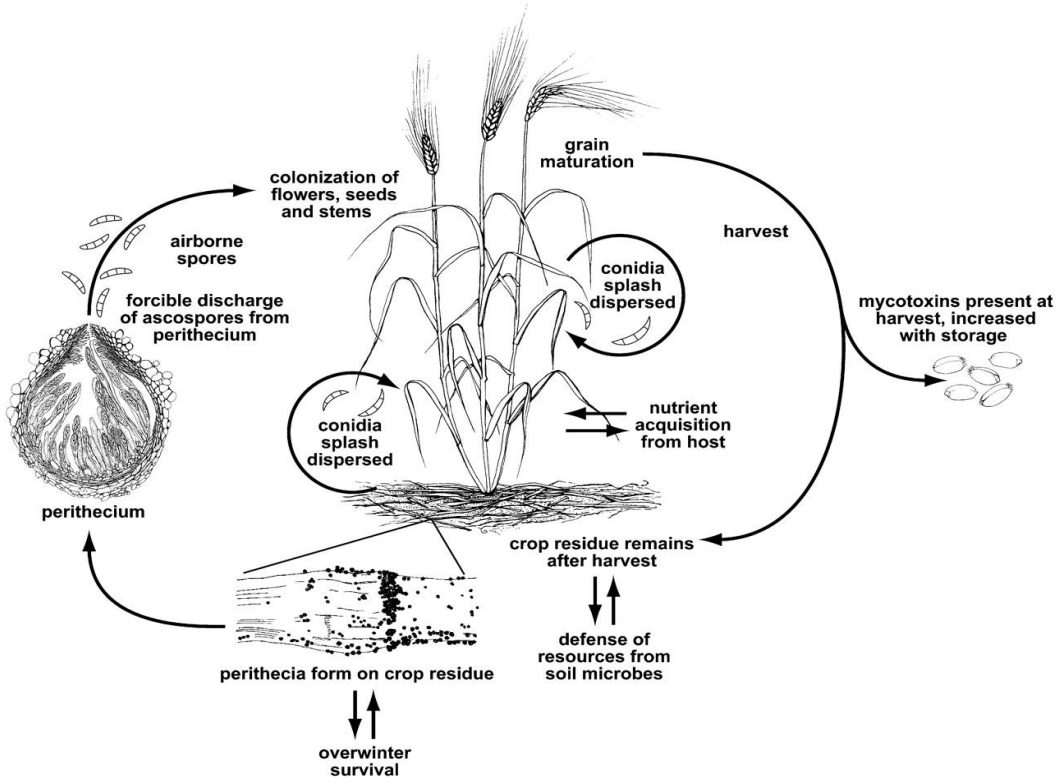


Figure2. The life cycle of *Fusarium graminearum* , causal agent of *Fusarium* head blight on wheat and barley. (Adapted from Frances Trail 2009)

For many years the debatable topic has been the colonization strategy of *Fusarium graminearum.* According to a study by Brown et al., in 2010 there were no indications of necrotrophy in the initial stages of colonization wherein the fungal hyphae remained in intercellular spaces of wheat cells followed by a subsequent increase in fungal biomass, cell death and necrosis. The study characterized the colonization as a special case of hemi-biotrophy, even though the lack of intracellular growth is not in accordance with the traditional view of biotrophy (Jansen et al., 2005).Specialized hyphal structures, called 'subcuticular hyphae' and 'bulbous infection hyphae', were observed in a study by Rittenour and Harris (2010) on infected wheat glumes. Because the development of the bulbous infection hyphae was dependent on the fungal GPMK1 gene (Rittenour and Harris,2010) encoding a mitogen-activated protein kinase (MAPK) and previously shown to be involved in *F. graminearum* pathogenicity (Jenczmionka et al., 2003; Urban et al., 2003) it was thought to be necessary for the infection process.*tri5* mutant deficient in trichothecene production is not impaired in the formation of these hyphal structures (Boenisch and Schäfer,2011), supporting the idea that toxins are not necessary for the initial colonization of wheat heads. Bulbous hyphae or any other specialized structures were not observed during infection of wheat roots by *F. culmorum* (Stephens et al., 2008), and Beccari et al. (2011) suggesting tissue specific variations in colonizing strategies by the fungus.TRI5 involved in toxin deoxynivalenol (DON) biosynthesis, was activated in inoculated wheat heads but not in anthers which are the initial targets of the pathogen during floral infection; showing a tissue-specific manner of toxin production. However, when the pathogen spreads to uninoculated spikelets (4–7 dpi), TRI5 expression was detected in the rachis node (Ilgenet al., 2009). In this case, DON biosynthesis is required to overcome the rachis which constitutes a formidable barrier to the spread of *F. graminearum* (Jansen et al., 2005).

Forward and reverse genetic analysis using mutants revealed eight mutants, named 'disease-attenuated *F. graminearum*' or 'daf', with reduced virulence (Baldwinet al., 2010) among which was daf10 a mutant that did not produce DON and, as expected, showed reduced virulence towards wheat in inoculation assays (Baldwin et al., 2010). DON is known to be an inhibitor of protein synthesis in eukaryotes (Pestka, 2010), yet its role as a virulence factor on plant cells remains poorly understood. Exogenous DON application triggers programmed cell death and strong defence gene expression in wheat and Arabidopsis (Desmond et al., 2008; Nishiuchi et al., 2006). The DON-mediated immune response activation in animals and plants is dependent on MAPK signalling pathways (Nishiuchi et al., 2006; Pestka, 2010). DON may

thus be considered as an *F. graminearum* 'effector' with a cross-kingdom action. These studies once again validate the importance of toxin producing genes among many others in successful host colonization and virulence.

So far, there have been several studies in both wheat and barley, investigating disease resistance to *Fusarium* and studying host genes induced during infection. Increased accumulation of Methyl jasmonate (MeJA) a metabolite belonging to the fatty acid pathway along with JA precursors linolenic and linolenic acids following *F. graminearum* challenge in the resistant barley genotype has led to the hypothesis that the JA pathway is the predominant defence signalling pathway operating in barley against *F. graminearum* (Kumaraswamy et al., 2011).In Arabidopsis however, the Salicylic acidpathway appears to be required for resistance and SA signalling mutants, *npr1* and *eds11*, as well as the SA-deficient mutant *sid1* displayed increased susceptibility to leaf infection by *F. graminearum* (Makandar et al., 2010).Conversely, the JA pathway appears to mediate disease susceptibility wherein the receptor mutant coi1 shows increased disease resistance (Makandar et al.,2010).Defence pathway induction of Wheat was found to be similar to in an experiment using resistant and susceptible wheat varieties (Ding et al., 2011). Additionally, the Ethylene signalling pathway is exploited by *F. graminearum* to cause enhanced disease susceptibility in Arabidopsis and wheat (Chen et al., 2009).

The pyramiding of multiple transgenes with different modes of action such as an antifungal plant defensin (AFP) (Li et al., 2011) and polygalacturonase inhibiting protein (Ferrari et al., 2011) which directly inhibit fungal growth could be used as an alternative for stronger and more durable resistance. Zealexin, a new class of sesquiterpenoid phytoalexins identified in maize has been shown to possess inhibitory activities against *F. graminearum* and in a purified form inhibited *F. graminearum* growth in physiologically active concentrations (Huffakeret al., 2011).

Another promising approach to reduce *Fusarium* disease incidence is the use of novel compounds that are applied externally and have an effect on pathogen growth. CNI-1493, a compound that inhibits fungal deoxyhypusine synthase (DHS) activity was used recently for complete prevention of disease development in both wheat and maize by external application without affecting grain development (Woriedh et al., 2011).

Bio control organisms such as bacterial strains *Bacillus* spp. and *Pseudomonas* spp.offer environmentally friendly disease control compared with chemical treatments. Henkes et al.

(2011) used as root inoculation system with carbon tracer elements to demonstrate *Pseudomonas* mediated disease protection system for *F. graminearum* where barley plants primed with *Pseudomonas fluorescens* before inoculation did not show distorted distribution of carbon allocation and were also less affected developmentally in response to infection. In Barley, expression patterns of large numbers of genes in response to *Fusarium culmorum*, was altered upon the application of biocontrol bacteria; including genes encoding lipid transfer proteins and protease inhibitors. This identified JA pathway as a modulator of *P. fluorescens*-mediated priming against *F. culmorum* infection in barley (Petti et al., 2010). From these studies, it can be inferred that biological control agents may stimulate host resistance mechanisms rather than having a direct inhibitory effect on the pathogen.

Resistant cultivars which confer disease resistance or tolerance to the toxin are not available currently and disease control through fungicide application is not cost efficient. Additionally efficient fungicide application to cereal heads is difficult and factors that influence disease development is incomplete or not well understood (McMullen et al.,1997; Pirgozliev et al., 2003). In view of these challenges biological control practices, resistant cultivars or genetic material that might help foster resistance to *Fusarium* are vital for crop protection industry and agriculture as a whole.

1.4 The plant immune system

Plants are infected by pathogens with different lifestyles such as biotrophs, hemi-biotrophs and necrotrophs (Agrios, 2005). Biotrophs are specialized pathogens that survive on living plant tissues, by developing an intimate relationship with their host plant and cannot be cultured on synthetic media. They are often adapted to a specific line or race of a given plant species and thus have a limited host range. Many biotrophs produce specialized feeding structures called haustoria by invagination of host cell plasma membrane, enabling them to create a specific environment for taking up nutrients (Voegele and Mendgen2003). Some biotrophs occasionally live in the intercellular space between leaf mesophyll cells. Necrotrophic pathogens are less specialized or are much less dependent on their host plants for survival. Most necrotrophs can easily be cultured on synthetic media as well as grow outside their hosts as saprophytes. They often produce toxins to kill host tissue before colonization or grow on plant tissues that are wounded and senescent (Agrios, 2005).

Plant pathogens devise different life strategies to colonize and infect a host plant. Pathogenic bacteria enter through natural openings (stomata and hydathodes), or gain access via wounds

and proliferate in intercellular spaces (the apoplast). Fungi also enter plant epidermal cells through natural openings or by mechanical pressure after which they extend hyphae on top of, or in between plant cells (Agrios, 2005). Additionally, effector molecules or virulence factors are delivered by these pathogens into the plant cell to enhance microbial fitness.

Plants employ a diverse mixture of local and systemic responses to fight invading pathogens. An important mechanism of plant resistance is innate immunity on which the plants rely heavily as a first line of defence. Innate immunity comes in two different variants (Jones and Dangl 2006; Chisholm et al., 2006) known as basal or horizontal disease resistance which includes non-host resistance and PAMP-triggered immunity (PTI) and resistance (R) gene-based or vertical disease resistance; popularly known as effector triggered immunity (ETI).These different forms are discussed more in detail in the following sections.

1.4.1 Non host resistance

Non host resistance is the mechanism by which an entire plant species is resistant to a specific parasite or pathogen, known to be pathogenic to other plant species. It is the most common and durable form of resistance to plant pathogens (Heath 2000). Plant cytoskeleton plays a significant role in non-host resistance and a loss of actin cytoskeletal function severely compromises non-host resistance in Arabidopsis against wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*) [Yun et al., 2003]. Secondary metabolites like saponins produced constitutively by plants also aid in defence against microorganisms. Lack of avenacin; a class of root-specific triterpene saponin, makes *Avena strigosa*, susceptible to non host fungal pathogens *G. graminis var. tritici* and *Fusarium culmorum* (Papadopoulou et al., 1999). Phytoalexins are antimicrobial compounds which are synthesized in response to pathogen attacks. One of the Arabidopsis phytoalexin-deficient (pad) mutants,*pad3-1*, is compromised in non host resistance against *Alternaria brassicicola* (Thomma et al.,1999).An invading pathogen also has to bypass several plant signalling components involved in the induction of plant defence. Ethylene perception is often required for basal resistance and an ethylene-insensitive tobacco mutant lacked non host resistance against several soil-borne fungi leading to development of spontaneous stem necrosis during soil growth (Knoester et al.,1998).Salicylic acid a key signalling molecule that activates plant defense responses was shown to playa role in non host resistance.*sid2* mutant of Arabidopsis defective in an enzyme that synthesizes salicylic acid, was shown to be susceptible to cowpea rust fungus (*Uromycesvignae*) generally not a pathogen of *Arabidopsis thaliana*. Additionally

Arabidopsis NahG plants (expressing salicylate hydroxylase, an enzyme that degrades salicylicacid) also supports growth of cowpea rust fungus (Mellersh and Heath 2003). Silencing of Wound-induced protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK); two signalling components of defense reactions in *Nicotiana benthamiana* allows multiplication and growth of *Pseudomonas cichorii* thus compromising nonhost resistance. Silencing of WIPK and SIPK however does not affect INF1 mediated HR (*Phytophthora infestans* elicitor that induces HR when inoculated on wild type *N. benthamiana*, a non host for P. infestans) on *N. benthamiana* (Sharma et al., 2003). Several nonhost disease resistance genes resistance against certain non host pathogens have now been identified. Among them is the Arabidopsis non host resistance gene, NHO1, that encodes a glycerolkinase and is required for non-host resistance against *Botrytis cinerea* and *Pseudomonas syringae* isolates from bean or tobacco which don't normally infect Arabidopsis (Kang et al., 2003).*P. syringae pv. tomato* DC3000, a virulent pathogen of Arabidopsis suppresses expression of NHO1 (Kang et al., 2003) suggesting a key role NHO1 plays in nonhost resistance against some pathogens and as a target for successful pathogens. Quite often, non host resistance against fungal pathogens is associated with the penetration resistance. *pen* (penetration) mutants in Arabidopsis showed increased penetration of the non host fungal pathogen Blumeria graminis f. sp. *hordei* (barley powdery mildew) [Collins et al., 2003] PEN encodes a syntaxin; a member of the SNARE super family of proteins that mediate membrane-fusion events and play a crucial role in papilla-related vesicle trafficking in the plasma membrane (Collins et al., 2003). PEN1 and PEN2 mutations reduced the ability of the plants to arrest conidia of *B. graminis f. sp. hordei* to , 20% of that of wild-type plants (Thordal-Christensen, H. 2003) as pen2 mutant shows alteration of cell-wall-related structure. Similar experiments identified two mutants, *ror1* and *ror2* (required for MLO-specified resistance and functional homologs of PEN1 gene), which enhance penetration of *B. graminis* f. sp. *hordei* on the host plant barley (Freialdenhoven et al., 1996; Collins et al., 2003) ; demonstrating a link between non-host and basal penetration resistance. Despite the progress in plant science and its importance in plant immunity, non host resistance remains poorly understood.

1.4.2 MAMP triggered immunity

MAMP triggered immunity (MTI), achieved through a set of defined receptors known as pattern recognition receptors (PRRs), is among the first line of defense in plants. The plant PRRs recognize conserved microbe-associated molecular patterns or MAMPs (Nürnberger et al., 2004). MAMP recognition leads to the activation of primary immune responses like alteration or reinforcement of cell wall material, callose deposition and the accumulation of defense-related proteins like chitinases, glucanases and proteases, which retard or inhibit colonization by invading pathogens (Van Loon et al., 2006). The most common MAMPs identified so far are bacterial molecules like flagellin, lipopolysaccharide (LPS) and elongation factor Tu (EF-Tu) along with chitin and β-glucans from fungi and oomycetes (Nürnberger et al., 2004). A particular domain of MAMP molecule possessing structural or enzymatic functions crucial for a microbe or pathogen is the target for recognition by pattern recognition receptors. In plants, FLS2 and ERF recognize the MAMPs flagellin (flg22) and bacterial elongation factor Tu (elf18) epitopes respectively (Gomez-Gomez and Boller2000; Zipfel et al., 2006). FLS2 has a cytoplasmic serine/threonine kinase domain and an extracellular leucine-rich repeat (LRRs) which is a form of Receptor like kinase (Rlk) [Gomez-Gomez and Boller 2000]. *Pseudomonas syringae* pv. *tomato* flagellin recognition by FLS2 restricts bacterial growth in the plant, whereas *fls2* mutants are more susceptible to this bacterial pathogen (Zipfel et al., 2004). Likewise, in *Arabidopsis thaliana* carrying ERF the MAMP elf18 triggers a primary defense response, similar to that induced by FLS2 (Zipfel et al., 2006). Even though, flg22 andelf18 are recognized by different RLKs, the primary defense responses induced upon their recognition are largely similar conferring an evolutionary advantage (Zipfel et al., 2006).So far, over 400 RLKs involved in both plant development and defense, have been identified in *A. thaliana* and rice. Additionally, LysM receptor kinase that recognizes fungal chitin (Kaku et al., 2006)and other receptors that recognize oomycete β-glucans have been identified in some plants (Gaulin et al., 2006). Plants also have LRR-containing receptor-like proteins (RLPs) recognizing fungal xylanase thus representing another class of PRRs that structurally resemble RLKs but lack the cytoplasmic kinase domain (Kruijt et al., 2005).Highly evolved and adaptive pathogens have devised a way to get past basal resistance in plants. This involves the secretion of effector molecules that suppress or compromise MAMP triggered immunity by modulating important proteins/genes in the basal resistance pathway. This leads to effector triggered susceptibility. To overcome this, plants have evolved resistance genes that produce R-proteins which

directly or indirectly recognize the effectors and lead to plant resistance. Plant pathogenic bacteria contain type III secretion system (TTSS) which is used by these pathogens to inject effectors that suppress primary defense responses of plants. The mechanism of deliveryof fungal effectors into plant cells is unclear; although most oomycete effectors carry an RXLR motif that has been suggested to facilitate effector up take into the plant cell.

1.4.3 Effector triggered immunity

Effector triggered immunityor ETI formerly called R-gene-based resistance that directly or indirectly recognize pathogen effectors through R-proteins constitutes a second layer of defense against invading pathogens. The term vertical resistance is also often used to imply the specific nature of interactions according to the gene-for-gene theory. This recognition event is characterized by strong defense reaction called the hypersensitive response (HR), that involves rapid apoptotic cell death and local necrosis (Martin et al., 2003). Most of the resistance genes encode cytoplasmic proteins with an N-terminal nucleotide-binding site (NBS) and C-terminal leucine-rich repeats (LRRs) where pathogen recognition is thought to be through sequence variable LRR region (Ellis et al., 1999; Wei et al., 1999).The R-gene mediated resistance is race specific or effective only against specific isolates of pathogen. Thus, detection of specific avirulence proteins or host targets perturbed by resistance proteins is specific, whereas the HR induction is non-specific and is generally effective against multiple plant pathogens. An example for indirect recognition of effector-induced perturbations of host targets is the *Arabidopsis thaliana* RIN4 protein, which is targeted by three different TTSS-dependent bacterial effectors (AvrRpm1, AvrB and AvrRpt2). The change in conformation or structure of RIN4 caused by effectors is monitored or recognized by two different resistance proteins (RPM1 and RPS2)[Axtell et al., 2003; Mackey et al., 2002]. Indirect recognition of effectors by RPs that induce secondary defense responses is known as the guard model (Van der Hoorn et al., 2002) and is observed more frequently than direct recognition. Direct recognition of effectors by RPs also occurs in a few cases and has been reported for the effectors of *Magnaportha grisea*, which causes blast disease in rice (Jia et al., 2000).

1.4.4 The Zig-Zag model of plant immunity

Based on the information available on the plant immune system Jones& Dangl in 2006 proposed the four phased 'zigzag' model (Figure 3). In the first phase, recognition of MAMPs by pattern recognition receptors, results in MAMP-triggered immunity (MTI) that retard or inhibit colonization by invading pathogens. In phase 2, effector molecules which act as virulence factors are deployed by successful pathogens to overcome MTI. The process gives rise to effector-triggered susceptibility (ETS). In the next phase, specific recognition of pathogen effector through one of the host NB-LRR proteins results in effector-triggered immunity (ETI). Direct or indirect recognition of an effector by one of the R-proteins results in an accelerated and amplified response, characterized by hypersensitive cell death response (HR) at the infection site and local necrosis thus leading to disease resistance. In the final phase, selection pressure forces pathogens to evolve and develop strategies to avoid ETI such as modification or mutation of the recognized effector gene and generating additional effectors that suppress ETI. Natural selection in plants helps them evolve simultaneously, which result in new R specificities that can trigger ETI in response to the new effector molecule.

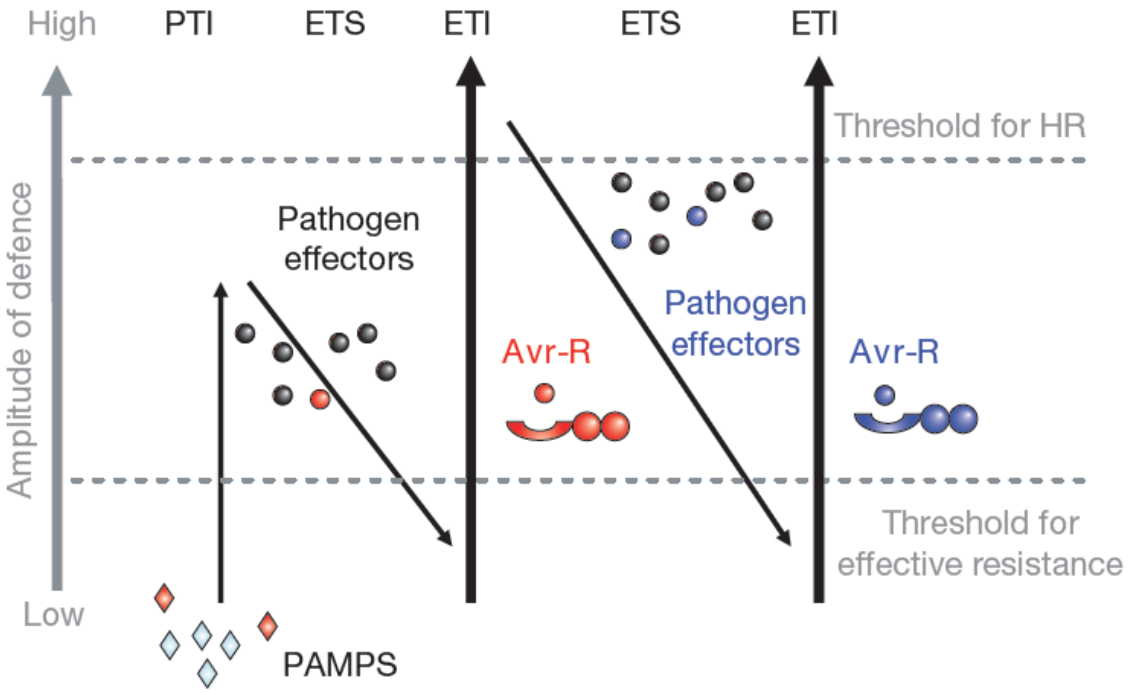


Figure3.A zigzag model illustrates the quantitative output of the plant immune system.(Adapted from Jones & Dangl 2006).

1.4.5 Systemic resistance in plants: SAR and ISR

Plants are also protected by systemic resistance mechanisms called Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR) which occur at sites distant from site of primary infection and prime the plants for subsequent pathogen attacks. Work dating back to 1960s, showed tobacco plants challenged with tobacco mosaic virus (TMV) subsequently developed resistance to secondary TMV infection in distal tissues (Ross 1961).The term systemic acquired resistance (SAR) was then used to refer to spread of resistance throughout the plant's tissues. The SAR effect is long-lasting and effective against a broad-spectrum of pathogens that includes viruses, bacteria, fungi and oomycetes (Ryals et al., 1996; Sticher et al., 1997). Molecular feature of SAR is the increased expression of a large number of pathogenesis-related genes (PR genes), in both local and systemic tissues and serve as molecular markers for the onset of SAR. Treatment of tobacco plants with salicylic acid (SA), aspirin (acetyl SA), or benzoic acid led to accumulation of PR protein and conferred resistance to TMV infection (White 1979).Additional proof for the involvement of SA in SAR was provided in 1990 by Malamy et al. who demonstrated rise in local and systemic SA concentration correlating with PR gene induction upon TMV infection of tobacco and Metraux et al. who showed increased SA levels in phloem sap of cucumber plants infected with either *Colletotrichum lagenarium* or tobacco necrosis virus (TNV). 2,6-dichloroisonicotinic acid (INA) an analogue of SA, and benzothiadiazole S-methyl ester(BTH) were found to induce the same set of PR genes as seen in SA induced systemic response. The compounds however were less cytotoxic than SA (Dincher et al., 1991; Görlachet al., 1996).Transgenic tobacco and Arabidopsis expressing NahG, a bacterial gene , encoding salicylate hydroxylase, which removes SA by conversion to catechol (Gaffney et al., 1993) accumulate very little SA upon pathogen infection, do not express PR genes and are impaired in SAR (Gaffney et al., 1993). *Arabidopsis thaliana* genetic analyses and mutant screens have identified a number of mutations in the gene, NPR1/NIM1 (NON-EXPRESSER OF PR GENES1/NONINDUCIBLE IMMUNITY1) (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996) which made these plants nonresponsive to SA. This led to elucidation of components downstream of SA in the SAR pathway. To summarize, SAR in most cases is triggered by local infection, which then provides long-term resistance to subsequent pathogen attack even in systemic tissues, requires the involvement of salicylic acid and is characterized by activation of PR genes.

ISR is the result of root colonization by plant growth-promoting rhizobacteria (PGPR), of which the best characterized are strains within several species of Pseudomonas (Van Loon et al., 1998). Unlike SAR, ISR does not involve the accumulation of pathogenesis-related proteins or salicylic acid (Pieterse et al., 1996), but instead, requires components of the jasmonicacid (JA) signaling pathway followed by the ethylene signaling pathway (Knoester et al., 1999; Pieterse et al., 1998). *Trichoderma asperellum* root colonization induces resistance to *Pseudomonas syringae* pv. *lachrymans* (Psl) in cucumber foliage (Shoresh et al., 2005). Trichoderma interaction with the plant, does not alter SA content, but reduces biocontrol activity of the organism when treated with diethyldithiocarbamicacid (DIECA), an inhibitor of JA production, or silver thiosulfate (STS), an inhibitor of ethyleneactivity ;suggesting role of both JA and ethylene in ISR mediated biocontrol activity of the fungi(Shoresh et al., 2005).In roots inoculated with *T. asperellum*, there was an upregulation of Lox1 that encodes a lipoxygenase involved in jasmonate synthesis (Shoresh et al., 2005). The induction of Lox1 takes place as early as 1 hour post *Trichoderma* inoculation followed by a second peak around 24 hours post inoculation, suggesting an activation of the octadecanoic pathway and the synthesis of JA. Another genePal1, coding for phenylalanine ammonia-lyase (PAL) was found to be upregulated by Trichoderma inoculation (Shoresh et al., 2005, 2008). Pal1catalyses the first step of phenylpropanoid pathway, which leads to production of phytoalexins and is thought to be activated by JA/ethylene signaling during the plant defense response. The transient activation of this gene by *Trichoderma* could contribute to the accumulation of phytoalexins, leading further to a better defense of the plants against Psl infection. In rhizobacteria-mediated ISR, ethylene response is thought to be downstream of JA response. Regulators of ethylene response pathway ETR1 and CTR1 are targeted in leaves of Trichoderma root-inoculated plants, and their expression is altered which enhances ethylene sensitivity in the leaves, leading to higher defense response to subsequent pathogen challenges (Shoresh et al., 2005).The PR proteins induced by SAR such as chitinase, β-1,3-glucanase, and peroxidase, were not induced by Trichoderma mediated ISR, even though they were upregulated on encountering a pathogen. Even though the PR genes are not constitutively expressed, priming of the systemic resistance system, leads to a much stronger and/or rapid response to a subsequent pathogen attack making the plant more resistant. (Pieterse et al., 2000, 2001; Waller et al., 2008).Induction of systemic responses by pathogens or beneficial organisms that infect leaves or roots of plants occurs simultaneously with local primary and secondary immune responses (Grantand Lamb2006). Induced resistance activated by biotrophic and necrotrophic pathogens differ as they are regulated by different

hormones. The level and effectiveness of both local and systemic resistance responses are thus dependent on hormones involved and by the type of plant pathogen that activates the response (Van Oosten et al., 2005; Glazebrook, 2005).

1.5 The MORC gene family and its role in plant-pathogen interactions

The carmovirus TCV or Turnip Crinkle Virus is an infectious agent that affects most Arabidopsis ecotypes. Resistance to the virus is mediated by an Arabidopsis R protein, HRT (HR to TCV) that induces defence gene expression, accumulation of salicylic acid and triggers the hypersensitive response (Kachroo et al., 2000). HRT is required for resistance to turnip crinkle virus (TCV) and plants lacking this R-gene allow systemic spread of the virus and die due to the failure in activation of HR and systemic responses (Kachroo et al., 2000). MORC1 formerly known as CRT1 (compromised for recognition of TCV) was identified in a genetic screen for mutants which despite carrying the R-gene HRT were compromised in the recognition of TCV's avr factor (Kang et al., 2008). MORC1 is an ATPase carrying a GHKL ATPase motif (Dutta and Inouye, 2000) and mutation causes premature termination of the ATPase protein. Arabidopsis genome analysis led to identification of two close ($\geq$70% a.a. identity) and four distant ($\leq$50% a.a. identity) homologues of MORC1. RNAi-mediated silencing of its two closest homologues,MORC2 (MORC1 Homologue 1) and MORC3, led to a greater disease susceptibility to TCV than that displayed by *morc1*, suggesting functional redundancy of MORC1 and their role in ETI against TCV(Kang et al., 2008).Additionally morc1 was also impaired in cell death induced by ssi4, a constitutively active R protein, and by avirulent bacterial pathogen *Pseudomonas syringae* carrying avrRpt2.The MORC1 protein was shown to physically interact with HRT, SSI4, and two other R proteins, RPS2 and Rx thus mediating defence signalling by R proteins belonging to distinct classes(Kang et al., 2008).Stable transgenic *morc1 morc2* double knockout (dKO) plants produced in the Col-0 background, lacking MORC1 and its closest homologue, displayed compromised resistance to avirulent bacterial pathogen *Pseudomonas syringae* pathovar *tomato* (Pst) and oomycete *Hyaloperonospora arabidopsidis* (Kang et al., 2010). Homozygous *morc3* mutant was lethal and hence triple KO was not tested. MORC1 was also found to be one of the only 11 genes identified whose knockout led to severe susceptibility to both virulent and avirulent forms of *H. arabidopsidis* (Wang et al.,2011). MORC1 sequence analysis revealed the presence ofa 'GHKL'(Gyrase, Hsp90, Histidine Kinase, MutL) ATPase motif (Dutta and Inouye, 2000) and an S5-fold domain (383aa–458aa) (Iyer et al., 2008; Kang et al., 2010; Langen et al., 2014). These domains are found typically in a class of proteins widely distributed in

eukaryotes and commonly found in prokaryotes known as the MORC (Microchidia) proteins or MORC family. They are a subset of the GHKL ATPase superfamily (Iyer et al., 2008). The first MORC protein to be isolated was Mouse MORC1, shown to be required for meiotic nuclear division (Watson et al., 1998). Prokaryotic MORC protein MutL, is a key enzyme involved in mismatch repair system functional during DNA replication (Iyer et al., 2006). Prokaryotic operons containing MORC-encoding genes are involved in restriction modification systems, the ancient self/non self-recognition system (Iyer et al., 2008). In addition to being a modulator of ETI Kang et al., in 2012 demonstrated the role of CRT1 and its closest homologue, CRH1, in PTI, basal resistance, non-host resistance and SAR. An Arabidopsis double knockout mutant, *morc1-2 morc2-1*, lacking MORC1 and its closest homolog MORC2 was compromised in PTI to virulent *Pseudomonas syringae*, suppressed basal resistance and/or systemic acquired resistance to TCV and compromised non host resistance to *Phytophthora infestans* (Kang et al., in 2012). Binding of MORC1 to PRR FLS2 was demonstrated in planta by co-immunoprecipitation (Co-IP) assays and the interaction was not affected by flg22-induced activation of FLS2. Using subcellular fractionation and transmission electron microscopy a subpopulation of CRT1 was found in the nucleus, which increased upon activation of ETI and, to a lesser degree, PTI (Kang et al., 2012). Arabidopsis MORC1 possesses DNA/RNA binding capacity and endonuclease activity in vitro, and mutations in MORC1 and its closest homologue enhance tolerance to the DNA-damaging agent mitomycin C, suggesting a potential role of this protein in the nucleus, possibly associated with DNA recombination and repair (R/R) and/or remodelling of chromatin superstructure (Kang et al., in 2012). Epigenetic gene silencing is achieved by methylation DNA and histone methylation of Transposable elements (TEs) and DNA repeats. Moissiard et al., 2012 identified mutations in two *Arabidopsis* genes, *morc1 and morc6* that caused derepression of methylated genes and TEs without losses of DNA or histone methylation. The pericentromeric heterochromatin of the two mutants was decondensed, which increased the interaction of these regions with the rest of the genome. RNAi-mediated silencing of *Caenorhabditis elegans* MORC homolog impaired transgene silencing (Moissiard et al., 2012). These mutant screens identified Arabidopsis MORC1 and its homologue MORC6 as factors required in epigenetic signal regulated alterations in DNA/chromosome superstructure.

1.6 CRISPR-Cas9 system: A novel technique for plant genome editing

Targeted genome engineering is one of the alternatives to classical breeding and generation of transgenic plants. Even though mechanisms like RNAi-mediated gene silencing are used widely to study gene functions, they have limitations like variation in knock down levels and reduction in knock down efficiency in successive generations. In view of this, several alternatives have been developed to obtain complete gene silencing (knock out). Zinc finger nucleases (ZFNs) and Transcription Activator Like effector nucleases (TALENs) can be used for targeted mutagenesis of genomes at specific loci. The major drawback of these systems is the laborious target site selection and design procedures leading to development of alternative approaches.

One such new technology is the type II clustered, regularly interspaced, short palindromic repeats (CRISPR) interference system; part of adaptive immunity in bacteria and archaea (Sorek et al., 2013). It is a naturally occurring microbial nuclease system protecting the bacteria against invading phages. The CRISPR locus contains a combination of CRISPR-associated genes that encode a bacterial endonuclease Cas9 and two short non-coding RNA elements known as CRISPR RNA (crRNA) and trans activating crRNA (tracrRNA).The non-coding pre-crRNA consist of an array of palindromic sequences (direct repeats) interspaced by short stretches of non-repetitive spacers (Sorek et al., 2013; Jinek et al., 2012; Cong et al., 2013).The Cas9 protein is a large monomeric DNA nuclease containing RuvC and HNH homologous nuclease domains. These two domains cleave the non-complementary and complementary strands respectively to generate a blunt cut in the target DNA (Jinek et al., 2012).The double stranded breaks (DSBs) disrupt gene function by forming premature stop codons or through mutations inserted by non-homologous end joining (NHEJ) pathway, or homology directed repair (Cong et al., 2013).The cleavage of target DNA takes place in 4 sequential steps. In the first step, transcription of the two non-coding RNAs, the pre-crRNA array and tracrRNA takes place. This is followed by hybridization of tracrRNA to the direct repeat or palindromic region of the pre-crRNA followed by processing of pre-crRNA into mature crRNAs containing individual spacer sequences. In the next step, Cas9 endonuclease is directed to the specific target sequence by the mature crRNA:tracrRNA complex via Watson-Crick base pairing between the spacer on the crRNA and the protospacer on the target DNA next to the protospacer adjacent motif (PAM), an additional requirement for target recognition. Finally,Cas9 endonuclease recognizes and create a double-stranded break within the protospacer region of the target DNA molecule (e.g., in a bacteriophage

genome)[Cong et al., 2013].Functional portions of crRNA and tracrRNA can be combined to give rise to a chimeric single guide RNA or SgRNA which along with Cas9 forms a targeted RNA-guided endonuclease (Mussolino and Cathomen 2013; Jinek et al., 2012).The Cas9 endonuclease could be easily redirected to different target sites by modifying the sequence of a chimeric single guide RNA (sgRNA) complexed with the enzyme (Jinek et al., 2012).Multiplexing can be achieved by combining Cas9 expression with multiple guide-RNAs targeting different loci in the target genome (Cong et al., 2013) thereby reducing the costs and speeding up generation of organisms with multiple, targeted mutations. Thus, RNA-guided endonuclease seem to combine the efficiency of ZFNs and TALENs with a much simpler design process, as target site selection is determined solely by base-complementarity to the guide RNA, and the protein does not require reengineering for each new target site.

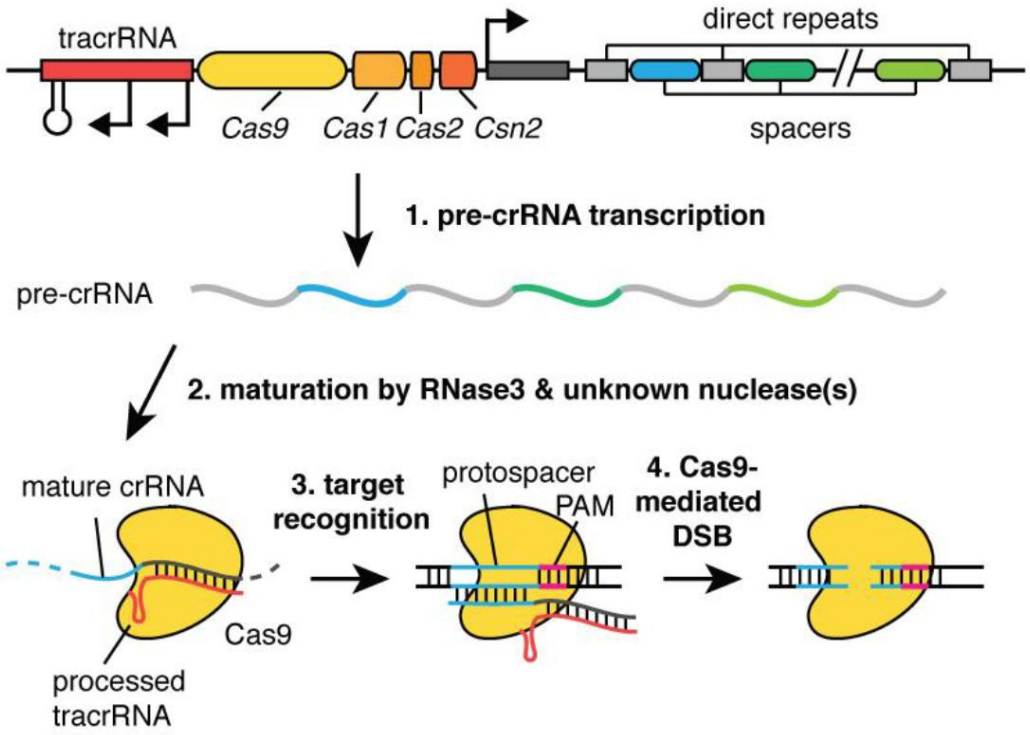


Figure4. Bacterial adaptive immunity through type II clustered, regularly interspaced, short palindromic repeats (CRISPR) interference system.(Adapted from CRISPR resources, Zhang lab: http://www.genome-engineering.org/crispr/?page_id=27)

For targeted mutagenesis in plants using CRISPR, plant codon-optimized version of Cas9 from the bacterium *Streptococcus pyogenes* was used (Shan et al., 2013; Miao et al., 2013). The second important component, a synthetic RNA chimera created by fusing crRNA with

tracrRNA known as single guide RNA (sgRNA) is required to form a complex with Cas9 nuclease for target recognition. The guide sequence located at the 5′ end of SgRNA determines DNA target specificity. The guide sequence is usually about 20 bp long (Jinek et al., 2012).The corresponding DNA target is also 20bp long followed by PAM sequence (NGG). Contrary to mammalian systems, plant guide sequences are of lengths varying from $(N)_{19\text{-}22}$NGG as against the stringent $(N)_{20}$NGG existing in mammalian systems (Shan et al., 2013; Miao et al., 2013; Feng et al., 2013). The plant sgRNAs are driven by type III RNA polymerase promoters, such as wheat U6 and rice U3. They have stringent requirements for transcription start sites to be "G" or "A", for U6 or U3 promoters, respectively. Therefore, the guide sequences follow the consensus G$(N)_{19–22}$NGG for the U6 promoter and A$(N)_{19–22}$NGG for the U3 promoter, where the first G or A may or may not pair up with the target DNA sequence (Shan et al., 2013; Miao et al., 2013; Feng et al., 2013). Transient assays help in rapid screening and optimization of a method. In plants, protoplast transformation and leaf tissue transformation using the agroinfiltration method have been used to test targeted mutagenesis by CRISPR/Cas9 system. Protoplast assay is good for achieving gene co-expression from separate plasmids, even though the protoplast isolation may be time consuming and prone to contamination. Mutations with efficiency of 15% were detected18 h after protoplast cultivation. Target mutation efficiencies were estimated by band intensities(Li et al., 2013). The induced mutations may be detected by PCR -restriction enzyme digestion assay. Cas9 nuclease usually cuts the target DNA about 3 bp away from the PAM and can be used to identify mutation in the target region which has a restriction site adjacent to the PAM motif. Repair of a DSB in protospacer region by the error-prone NHEJ pathway results in mutations that disrupt the restriction site. These, mutations are detected by PCR amplification of genomic DNA using primers specific for the target region and digesting resulting amplicons with the restriction enzyme (Li et al., 2013).Cloning and sequencing of these uncut bands revealed indels in the targeted gene. SgRNA with a length of 20 nucleotides of sequence complementarity to the OsPDS had the highest frequency and mutation efficiency (Li et al., 2013). Rice Phytoene desaturase gene (OsPDS) was knocked out using Cas9 plasmid and sgRNA expression plasmids bombarded into rice calli resulting in biallelic mutations and some homozygous mutations carrying the same one-nucleotide insertion. Albino and dwarf phenotype confirmed disruption of OsPDS (Li et al., 2013). CRISPR/Cas system application in plant cells, was demonstrated by DGU.US reporter assay, where DSB generated is repaired through Single Strand Annealing, thus restoring the GUS activity that led to strong GUS staining spots in rice calli (Miao et al., 2013). Endogenous

genes in rice CHLOROPHYLL A OXYGENASE 1 (CAO1) gene and LAZY1 gene were knocked out selectively using the CRISPR/Cas technology. Loss-of-function mutant cao1defective synthesis of Chlorophyll b (Chl b) showed a pale green phenotype and loss-of-function mutant of LAZY1 gene, exhibited a tiller-spreading phenotype which was observed after tillering stage. Sequencing analysis on these lines using gene-specific primersshowed mutations in specific regions confirming the disruption of the respective genes (Miao et al., 2013). From these studies, it is fairly conclusive that CRISPR-Cas technology can be used for gene silencing in a variety of plant systems with varying efficiency using simple and straightforward approaches for vector design and testing for transgenic plants carrying mutations.

1.7 Objectives of study

Barley (*Hordeum vulgare*) is one of the most important cereal crops, ranking fourth in the world in terms of production and area under cultivation. Barley and barley products are used as food and feed throughout the world. Barley like all other plant species is susceptible to a large number of plant pathogens ranging from viruses to bacteria, oomycetes and fungi that cause diseases in crop plants. Catastrophic plant disease aggravates the current food deficit in which at least 800 million people are inadequately fed and food security is seriously compromised. Plant pathogens are difficult to control because their populations are variable in time, space, and genotype. Disease spread may be minimized by the reduction of the pathogen's inoculum, inhibition of its virulence mechanisms, and promotion of genetic diversity in the crop. To avoid losses in yield there is also the possibility of transgenic modification of the agronomically relevant plants with genes that confer resistance. To do this, a basic understanding of plant defense mechanisms and plant-pathogen interactions is necessary.

Studies in *Arabidopsis thaliana* demonstrated that MORC gene family (formerly known as CRT) was involved in multiple disease resistance mechanisms in this dicotyledonous model plant. In Arabidopsis, the gene family is involved in resistance to wide range of pathogens such as viruses, bacteria and oomycetes. Studies also showed their interaction with plant resistance proteins and role in remodelling of chromosome superstructure. Despite the significance of this gene family in plant immunity, there is little information available on its role in monocotyledonous plants. This study aims to investigate the function of MORC gene family in the context of monocot model plant barley. Sequence analyses revealed highly

conserved homologs throughout several plant species including barley, suggesting that proteins belonging to the MORC-family might be involved in general disease resistance mechanisms. As a preparation to my studies, members of *HvMORC* gene family were identified and functionally characterised in transiently transformed plants. Further investigations were carried out using; stable transgenic lines bearing plasmids for either over expression or silencing by RNAi of CRT family members. The investigation addressed the response of MORC transgenic lines to Barley powdery mildew (*Blumeria graminis f.sp. hordei*) and *Fusarium graminearum*; two economically important pathogens of barley in temperate regions. The stably transformed plants were characterized for selection of transgenic lines and expression profiles of the transgenic lines were studied to identify if there was a co-relation between the phenotype observed and MORC expression. Finally, to biochemically characterize the MORCs recombinant HvMORC1 was used in enzymatic assays in an effort to try and explain the apparent similarities/ differences observed between the *Arabidopsis* MORCs and barley MORCs during the course of this study. The study finally addresses some important concerns - What is the role of barley MORC gene family in plant-pathogen interactions? What are the effects of MORC knockdown and overexpression in barley? Are the results comparable to those in *Arabidopsis* or are they contrary to the data in *Arabidopsis*?

2 Materials and Methods

2.1. Isolation of MORC genes and production of stable transgenic plants

Full-length sequences of HvMORC1 (accession no. HG316119), HvMORC2 (HG316120), were obtained from complementary DNA of barley 'Golden Promise'. PCR amplicons were ligated into pGEMt-easy (Promega) and verified by sequencing. HvMORC1 was cut from pGEMt-easy and cloned into SmaI and HindIII sites of plasmid p35S-Nos (for nopaline synthase terminator; DNA Cloning service) and HvMORC2 using EcoRI. For stable barley transformation, the HvMORC2 fragment in plasmid pAB-35S-RNAi ZeBaTA was cloned together with flanking terminators into theSacI/SpeI sites of p7i-Ubi-RNAi plasmid (DNA Cloning Service), replacing the GUS fragment. Expression cassettes from plasmids p35S::HvMORC1, p35S:: HvMORC2were cloned into SfiI sites of binary plasmid pLH6000 (AY234328, DNA Cloning Service), which was also used to produce the transgenic control plants designated as 'Empty vector'. Plasmids were electroporated (Gene Pluser, Biometra) into *Agrobacterium tumefaciens* strain AGL1 (Lazo et al., 1991) and used to transform spring barley 'Golden Promise' as described (Schultheiss et al., 2005; Imani et al., 2011).

2.2. Powdery mildew detached leaf assay

Barley powdery mildew (*Blumeria graminis f.sp. hordei*) race A6 was maintained in a climate cabinet and propagated on young seedlings of the susceptible barley cultivar 'Golden Promise' at 22°C/18°C (day/night cycle) with 60% relative humidity and a photoperiod of 16 hours with 240µmol m^2 s^{-1} photon flux density.

Evaluation of powdery mildew resistance of the HvMORC2 knockdown and HvMORC1 overexpressor lines was performed using a detached leaf assay. Cultivar 'Golden Promise' and/or transgenic barley cv. Golden Promise, containing the empty vector (pLH6000) were used as control. Seeds of transgenic lines and controls were surface sterilized using 6% sodium hypochlorite with vigorous shaking for 2 hours followed by several washing steps of 10 minutes each using tap water. The seeds were germinated on moist filter paper for 2 days in the dark and then transplanted in soil (Frühstorfer Erde Type T). The plants were maintained in a climate chamber at 22°C/18°C (day/night cycle) with 60% relative humidity and a photoperiod of 16 hours with 240µmol m^2 s^{-1} photon flux density.

After 12-14 days or the emergence of the secondary leaf (whichever was earlier), the second leaf was cut and placed in 0.8% water agar medium (w/v) containing 40 mg/L benzimidazole

in a square (10 x 10 cm) petri dish with the adaxial side of the leaf facing upwards. Each petri plate accommodated about 6 leaf segments (control and transgenic lines). Secondary leaves were used for this assay because first true leaves were small and the growth was non-uniform especially in the transgenic lines.

To use freshly produced conidia for inoculation, old conidia spores from the heavily infected Golden Promise seedlings were removed by gentle shaking of the plants 2 days prior to inoculation. A settling tower was used for inoculations. During inoculation, petri dishes containing the leaf segments were placed inside the tower and conidia from powdery mildew colonized seedlings were blown into the inoculation tower and allowed to settle down for 10 minutes. The density of inoculation was monitored by a haemocytometer and was adjusted to 2-5 conidia/mm² for macroscopic evaluation. The petri plates were then transferred to a growth chamber (Percival), for a period of 5 days and maintained at 22°C/18°C (day/night cycle) with 60% relative humidity, photoperiod of 16 hours and 60µmol m^{-2} s^{-1} lux light intensity.5 days post inoculation, the leaf segments were scored by counting the number of powdery mildew pustules per 3.5 mm² leaf area under a stereo microscope. The data was recorded and used for further analysis. For microscopic evaluation of cellular host response to *Bgh* infection, a higher inoculation density (15-20 conidia/mm²) is preferred.

2.3. *Fusarium graminearum* root rot using stable transgenic plants and STARTs root material

To test the resistance of transgenic barley to *Fusarium graminearum* root rot, wild type Golden Promise, HvMORC2 knockdown and overexpressor lines were surface sterilized in 6% sodium hypochlorite as described above. The husks of individual seeds were removed using a forceps and the seeds were laid out on distilled water soaked filter paper for germination. Fungal material (*Fusarium graminearum* WT 1003) for inoculation was obtained from 7- 10 day old SNA plates maintained in an incubator at 22°C. Conidial suspension was scratched from 1-week-old plates by using sterile water and filtered through a sterile mira-cloth (Calbiochem, http://www.merck-chemicals.de) prior to the adjustment of conidia concentrations to a density of 50,000 ml^{-1} macroconidia in 0.1% (v/v) Tween 20 and surface-sterilized 3d-old barley seedlings were dip inoculated for 2h by gentle shaking at room temperature.

Subsequently, inoculated seedlings were transferred into 6-cm-diameter pots filled with a substrate of sand and Oil-Dri (expanded clay; Damolin) 3:1 and grown at 22°C/18°C

(day/night cycle) with 60% relative humidity and a photoperiod of 16 hours but at 125 µmol photons m^{-2} s^{-1}.Plants were harvested at 10 d after inoculation (dai), root and shoot lengths were measured and disease symptoms were assessed. The disease symptoms were scored on a five point scale with "0" being the most resistant and "4" most susceptible. The parameters chosen for scoring were root, coleoptile and leaf necrosis. In addition, the plants were photographed and the organ lengths were measured by ImageJ (National Institute of Health, available at http://rsb.info.nih.gov/ij/). The plant material was then frozen and later used for DNA extraction to study relative fungal colonization by qPCR.

Root material produced by Stable Root Transformation System (Imani et al., 2011) was used to test and characterize the effects of *HvMORC2* knockdown in disease resistance to the necrotrophic fungus *Fusarium graminearum*. Barley immature embryos transformed with the knockdown plasmid- #474 pLH6000 Ubi::*MORC2*-RNAi was used in the production of roots where only *HvMORC1* was silenced. In addition, to the single knockdowns, a double knockdown for the two closest homologs *MORC1* and *MORC2* was studied using transformants carrying the double knockdown plasmid: #626 pLH6000 Ubi::*MORC1MORC2*-RNAi (provided by M. Claar). As a control, root material silenced for GUS was used. The plasmid used for transformation of this material was #621 pLH6000 Ubi::GUS-RNAi (provided by M. Claar).

STARTs (Imani et al., 2011) root material generated by tissue culture was obtained about 6weeks after transformation of the immature embryos. The root material along with the callus was isolated from the growth medium and treated with spore solution of *Fusarium graminearum* wild type strain1003. STARTS-generated roots were inoculated with 1.2 x 10^4 spores/ml) in 0.02% Tween 20 (v/v) for 2 h. The isolated root material was treated with *F. graminearum* spore solution for 2-3 hours with shaking at room temperature. After this incubation time, the roots were transferred to freshly prepared 0.8% water agar plates and subsequently maintained in the plant tissue culture room till they were ready for harvest. Root material was harvested 2dai and 5dai where the roots were washed with distilled water to remove any mycelia on the outer surface of the roots. The material was frozen, homogenized and DNA extracted to study relative fungal colonization by quantitative-PCR.

The fungal colonization of roots was studied by quantitative PCR using plant and fungal specific primers: HvUbi and FgTubulin (Appendix 2) specific for Barley ubiquitin (genebank M60175.1) and *Fusarium graminearum* tubulin (genebank DQ459633.1) respectively.

2.3.1 SNA (synthetic nutrient poor agar) plates for maintenance of fungal culture

Composition: All components listed below were obtained from Carl- Roth, Germany.

0.1% KH_2PO_4,

0.1% KNO_3,

0.1% $MgSO_4.7H_2O$,

0.05% KCL,

0.02% glucose,

0.02% sucrose,

and 2% agar.

Take 1 ml of *Fusarium graminearum* glycerol stock suspension and distribute it among 10 SNA plates

2.4 Isolation of DNA for quantitative PCR

DNA was isolated by CTAB method according to a modified protocol of Doyle and Doyle (1987). Plant material was ground into a fine powder by vigorous shaking for 30 seconds using a tissue lyser (Qiagen, Germany) in a 2 ml microcentrifuge tube with a metallic bead. The tubes containing ground material were immediately transferred to liquid nitrogen. Add 10µl of β-mercaptoethanol (Carl- Roth, Germany) to 5ml CTAB buffer and heat in a water bath at 65°C for 10 minutes. Add 700µl of pre-heated CTAB buffer to each of the tubes and incubate in a water bath at 60°C for 25 minutes. 700µl of CIA- Chloroform: Isoamyl alcohol (24:1) was added and mixed by inversion for about 5 minutes. The samples were centrifuged at 10,000 rpm for 15 minutes at room temperature. The clear aqueous supernatant was transferred to a new microcentrifuge tube containing 600µl of CIA, mixed by inversion for a few minutes and centrifuged at room temperature for 15minutes at 10,000 rpm. The supernatant was mixed thoroughly with 500µl isopropanol and placed on ice for 15-30 minutes. DNA yield can be increased by overnight precipitation in isopropanol at 4°C. After a centrifugation step DNA settles down at the bottom of the microcentrifuge tube as a thick pellet. The pellet was washed with 70% ethanol/10mM NH_4OAc. Finally, the dry pellet was resuspended in 30µl ddH_2O. The DNA concentration was measured by Nanodrop spectrophotometer (Peqlab, Biotechnologie GmbH)

CTAB extraction buffer:

2% CTAB (20g cetyltrimethylammonium bromide)

20mM EDTA (40ml EDTA stock (0.5M))

100mM Tris-Cl pH 8.0 (100ml Tris-Cl stock (1M))

1.4M NaCl (280ml NaCl stock (5M))

make up to 1 Liter with MilliQwater, pH 7.5 - 8.0, and autoclave

+ 0.2% Mercaptoethanol (add just prior use)

Wash Buffer:

76% Ethanol

10mM NH_4OAc

2.5 RNA extraction and cDNA synthesis

RNA extraction was performed by phenol-chloroform extraction method using the TRIZOL® Reagent (Life technologies, Germany) which is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). During sample homogenization or lysis, TRIZOL® Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. In the presence of TRIZOL® Reagent, RNA is protected from RNase contamination.

Plant material was ground into a fine powder by vigorous shaking using a tissue lyser in a 2 ml microcentrifuge tube for 30 seconds. The tubes containing ground material (about 250µl) were immediately transferred to liquid nitrogen. 1 ml TRIzol RNA-extraction buffer was added to the ground plant material and vortexed vigorously. The homogenized samples were incubated at room temperature for 5 minutes to permit the complete dissociation of nucleoprotein complexes. 200 µl of chloroform (Carl- Roth, Germany) was added. Tubes were vigorously shaken by hand for 15 seconds and incubated at RT for 2 to 3 minutes. The samples were centrifuged at 13,500 rpm for 20 minutes at 4°C.Addition of chloroform followed by centrifugation separated the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. The aqueous phase was transferred to a fresh tube. Precipitation of the RNA from the aqueous phase was achieved by mixing with 500 µl isopropanol (Carl- Roth, Germany). After incubation of samples on ice for 1 hour they were

centrifuged at 13,500 rpm for 15 minutes at 4°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube. The supernatant was removed and pellet washed with 900 µl 75% ethanol. After vortexing, the sample was centrifuged for 5-10 min at 4°C and 13.500 rpm. The ethanol washing solution was carefully removed and pellet dried by leaving the vial open (keep vials under a laminar hood). The pellet was dissolved by adding 30 µl H_2O DEPC and pipetting up and down. The RNA concentration was measured by Nanodrop spectrophotometer. The extracted RNA samples were then run on a 1.2% RNA-MOPS gel to check purity and integrity of RNA.

MOPS Buffer (3-N-morpholino propane sulfonic acid)

0.2M MOPS (41.86g/l)

0.05M Sodium Acetate (4.102g/l)

0.01M EDTA (3.722g/l)

Distilled water 1000ml

The contents were mixed well and pH adjusted to 7. 0.1% DEPC was added to the contents and mixed well on a magnetic stirrer by incubation at room temperature overnight.

Gel electrophoresis to test RNA integrity

1.2% Agarose	2.4g
1X MOPS buffer	190 ml
5% Formaldehyde	10 ml

A final volume of 10µl including 5µl 2X RNA loading buffer (Life technologies, Germany), 2µg RNA sample and DEPC water where necessary was prepared to be loaded on the agarose gel. The samples were heated at 94°C for 5 minutes to denature RNA and break the secondary structures. The samples were loaded on the gel and separated at 120 Volts for 1 hour.

2.5.1 DNAseI Treatment

Total RNA extracted from plant material usually contains accompanying DNA contamination. Prior to cDNA synthesis, it is imperative to remove the contaminating DNA molecule which is done by DNAseI treatment. RNA adjusted to a final concentration of 2µg was used as the starting material for DNAseI treatment followed by cDNA synthesis.

2µg RNA samples

2µl 10X DNAseI buffer (Fermentas GmbH, St. Leon-Rot, Germany)

2µl RiboLock™ RNAse inhibitor (1 U/µl; Fermentas GmbH, St. Leon-Rot, Germany)

2µl DNAseI (1 U/µl; Fermentas GmbH, St. Leon-Rot, Germany)

Make up the volume to 20 µl with DEPC-treated Water. Incubate at 37 °C for 30 min. Add 1 µl 50 mM EDTA and incubate at 65 °C for 10 min. RNA hydrolyses during heating with divalent cations in the absence of a chelating agent. Use the prepared RNA as a template for reverse transcriptase. Not more than 1 ul of DNase I, RNase-free was used per 1 µg of RNA.

2.5.2 cDNA synthesis

DNAseI digested RNA from the previous step was used for cDNA synthesis

Mix1:

10µl DNAseI treated RNA

1µl Oligo $(dT)_{18}$ primer (10µM)

1µl Random Hexamer primer (10µM)

If RNA template is GC rich or is known to contain secondary structures, mix gently, centrifuge briefly and incubate at 65°C for 5 min, chill on ice, briefly centrifuge and place on ice.

Mix 2:

4µl 5X reaction buffer (Fermentas GmbH, St. Leon-Rot, Germany)

0.5µl RiboLock™ RNAse inhibitor(40 U/µl; Fermentas GmbH, St. Leon-Rot, Germany)

2µl 10mM dNTPs

1.5µl RevertAid Reverse Transcriptase (200 U/µl; Fermentas GmbH, St. Leon-Rot, Germany)

Make up the final volume to 20µl by adding Mix 2 to Mix1.Mix gently and centrifuge briefly.

If oligo $(dT)_{18}$ primer or a gene-specific primer is used, incubate 60 min at 42°C.If random hexamer primer is used, incubate 10 min at 25°C followed by 60 min at 42°C.For transcription of GC rich RNA reaction temperature can be increased to 45°C.Terminate the reaction by heating at 70°C for 10 min. Do not heat-inactivate enzyme prior to analysis of long cDNA to avoid cleavage. After these steps, the reaction mixture was placed on ice. 80µl nuclease free water was added to the samples to give cDNA with a final concentration of 10ng/µl. The reverse transcription reaction product can be directly used in PCR or stored at -20°C.Use 2 µl of the reaction mix to perform PCR in 25 µl volume.

2.5.3 PCR to check cDNA synthesis

After cDNA synthesis, a standard semi-quantitative PCR was performed using primers amplifying the barley housekeeping gene Ubiquitin to confirm the success of cDNA synthesis and to show that the cDNA could be used for downstream applications like quantitative PCR. A 25µl PCR reaction consisted of the following components

2.5µl 10X BD Buffer (DNA Cloning Service, Hamburg, Germany)

2.5µl 2mM dNTPs

1.5µl 25mM $MgCl_2$ (DNA Cloning Service, Hamburg, Germany)

0.6µl Ubi-deg 60 forward primer

0.6µl Ubi-deg 60 reverse primer

0.2µl DCS Taq Polymerase (5U/µl; DNA cloning services, Hamburg, Germany)

Add 1µl template cDNA, make up the final volume to 25µl with nuclease free water and set up the PCR reaction. Separate the PCR products in a 1% agarose gel at 120V for 1 hour.

Temperature (°C)	**Time**	
Initial denaturation 95	5 min	
Denaturation 95	30 sec	**35 Cycles**
Annealing 60	30 sec	
Elongation 72	30 sec	
Final Elongation 72	5 min	

2.6 Fungal biomass quantification and gene expression analysis by quantitative PCR

Analysis of expression of MORC genes in the knockdown and overexpressor lines as well as the quantification of fungal colonization in these transgenic lines was done by relative quantification, where expression of a target gene relative to a housekeeping gene was quantified. For gene expression analysis, total RNA was extracted as described in section 2.5 and reverse transcribed using RevertAid Reverse Transcriptase kit (Fermentas GmbH, St. Leon-Rot, Germany). 10ng of cDNA thus obtained was used for quantitative real time PCR. Doyle and Doyle method described in section 2.4 was used for genomic DNA isolation from fungal infected plant roots. This genomic DNA was used to determine the amount of fungal DNA in infected plants by quantitative real-time PCR.

In the quantitative real-time PCR, the expression levels of target genes *HvMORC1*, *HvMORC2*, *Fusarium graminearum* Tubulin (genebank DQ459633.1) was quantified relative to the reference gene *Hordeum vulgare* Ubiquitin (genebank M60175.1) using the $2^{\Delta Ct}$ method (Shmittgen and Livak 2008). Amplifications were performed using 7.5µl of 2X Sybr green Jumpstart Taq Ready mix (Sigma-Aldrich, Munich, Germany) in a 15µl reaction containing 0.7µl each of forward and reverse primers(Appendix 2) and 10ng template cDNA. The 7500 fast qPCR machine (Applied biosystems, Foster City, USA) was used for the real time PCR reaction. The following PCR program was used for all reactions

1.Holding stage: 95°C, 5'	
2.Cycling stage:	
95°C- 15"	40X
58°C- 30"	
72°C- 30"	
3.Melt curve stage	
95°C- 15"	
65°C- 1'	
95°C- 30"	
29°C- 15"	

Three fluorescent readings were monitored at 72°C during each cycle. Melting curves were determined at the end of cycling to ensure specific amplification. Threshold values were set up manually where necessary, using Ct values (Cycles to threshold) determined and processed using the 7500 fast software from Applied biosystems. For comparison of expression level, ΔCt values were obtained by deducting the raw Ct values of target genes from respective raw Ct values of reference gene barley ubiquitin (Accession Nr., M60175)

2.7 Characterization of transgenic plants by REDExtract-N-Amp™ Plant PCR Kit

2.7.1 Extraction of DNA from plant samples

The REDExtract-N-Amp Plant PCR Kit contains all the reagents needed to rapidly extract and amplify genomic DNA from plant leaves. Since a large number of transgenic plants had to be tested and characterized, the REDExtract-N-Amp Plant PCR Kit (Sigma Aldrich, Germany) was used as an easier and faster alternative to traditional DNA extraction followed by PCR. Transgenic plants of the T1 generation overexpressing *HvMORC1* and *HvMORC2* as well as the lines silenced for *HvMORC2* were tested for the segregation of hygromycinphosphotransferase gene using primers (Appendix 2) specific for the 35S promoter driving the expression of hygromycinphosphotransferase (pGY1fwd2) and the hygromycinphosphotransferase gene (JI-Hyg-Rev) itself. Provided the T0 plants were screened and not tested to be chimeric, the T1 segregating population of the lines are expected to show a 3:1 ratio of transgenics: azygous in accordance with the Mendelian genetics. For DNA extraction, leaf strips of about 1-2 cm were cut out using a pair of scissors and collected in 1.5 ml microcentrifuge tubes. The tube was immediately placed on ice. To each tube containing a leaf segment, 75µl of extraction solution (Sigma Aldrich, Germany) was added, vortexed briefly and incubated at 95°C for 10 minutes. It was made sure that the leaf piece was covered by the extraction solution. After 10 minutes of incubation, 75µl of dilution solution (Sigma Aldrich, Germany) was added and mixed. The extracted DNA was used immediately for PCR or stored at 4°C to be used within 4-6 weeks.

2.7.2 PCR for the characterization of transgenic plants

A semi quantitative PCR was carried out using the DNA extracted using REDExtract-N-Amp Plant PCR Kit to identify and characterize transgenic plants and check for segregation in accordance with Mendelian genetics. The REDExtract-N-Amp PCR ReadyMix contains JumpStart Taq antibody for specific hot start amplification. The amplified DNA can be loaded directly onto an agarose gel after the PCR is completed. It is not necessary to add a separate loading buffer/tracking dye.

7µl REDExtract-N-Amp PCR ready mix

1µl Forward primer pGY1fwd2 (10µM)

1µl Reverse Primer JI-Hyg-Rev (10µM)

4µl Leaf extract

7µl Nuclease free water

Temperature (°C)	**Time**	
Initial denaturation 95	5 min	
Denaturation 95	30 sec	**38 Cycles**
Annealing 60	30 sec	
Elongation 72	1min 30 sec	
Final Elongation 72	5 min	

The PCR product was loaded on a 1% agarose gel and separated at 120V for 1 hour for the identification of a product of around 1200 base pairs.

2.8 Recombinant production of HvMORC1 protein and endonuclease assay

2.8.1 Expression and purification of recombinant HvMORC1

Recombinant HvMORC1 protein was expressed in *Escherichia coli* using pET28a-HvMORC1 plasmids and purified by affinity chromatography followed by size exclusion chromatography and Ion exchange chromatography.

The bacterial clone containing HvCRT1 construct was first verified for the rate of protein production and solubility of the recombinant protein using a small scale protein induction. Large scale protein production was performed using *E. coli* strain BL21 cells carrying the respective plasmids grown in 1 litre Luria- Bertani medium (LB) at 37 °C to OD_{600}= 0.6. Expression of CRT1 was induced by addition of 0.5mM Isopropyl β-D-1-thiogalactopyranoside (*IPTG*) for 20 h at 18°C. Bacterial cells were harvested by centrifugation at 5500rpm for 10 minutes at 4°C. The flask containing cell pellet was transferred to -80°C for 10 minutes and thawed on ice for a short while. This aids in cell lysis. The harvested cells were resuspended in lysis buffer, buffer A (50mM Tris/acetate, pH 7.5, 0.5M NaCl, 2mM dithiothreitol (DTT), 10% glycerol) containing 20mM imidazole and phenylmethylsulphonyl fluoride. After sonication (3 times, 30 seconds) and centrifugation (30min, 16,000 rpm, 4°C), the soluble His-tagged MORC1 protein was purified by affinity chromatography using Ni-NTA agarose resin (Novagen); following washing the CRT1-bound resin with buffer A containing 20mM imidazole, MORC1 was eluted in buffer A containing 300mM imidazole. The eluted MORC1 was subjected to gel filtration chromatography on a HiLoad 16/60 Superdex 200 column (GE Healthcare) equilibrated in

buffer B (50mM Tris–HCl/7.5, 300mM NaCl, 2mM DTT, 10% glycerol). The fraction containing highest amount of purified MORC1 was further purified using anion exchange chromatography on a SOURCE 15Q 4.6/100 PE column (GE Healthcare) equilibrated in buffer C (25mM Tris–HCl/8.0, 2mM DTT, 10% glycerol). After washing with buffer C containing 125mM NaCl, CRT1 was eluted with buffer C containing 500mM NaCl.

Protein concentration was estimated by Bradford assay kit (Bio-Rad, USA). Different concentrations of Bovine Serum Albumin were prepared for the estimation of standard curve. Purity and integrity of the recombinant protein was determined by separating protein aliquots using Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE) after each step of protein purification. After electrophoresis, gel was fixed by fixation solution for 30 minutes. Eventually, the gel was visualized using Coomassie blue staining solution. Staining solution was added to the gel and incubated with gentle shaking at room temperature overnight. To minimize background noise due to excessive staining, the gel was destained for 30 minutes using the destaining solution.

2.8.2 Endonuclease assay

10µl of reaction mixture contained 500nM of purified recombinant proteins, 200 ng arbitrary supercoiled plasmid DNA, pER8-HA12, in 50Mm Tris-HCl/pH8.0, 1mMDTT and 2mM metal salt ($MnCl_2$) as co-factor. Where applicable, ATP, Radicicol (Sigma-Aldrich, USA) or a combination of both were added to a final concentration of 1mM. Reactions were carried out at 37 °C for 8 hours, terminated by addition of an equal volume of 2xstop buffer (2% SDS, 100mM EDTA, 20% glycerol and 0.2% bromophenol blue), and then separated by electrophoresis on a 1% agarose gel at 75Volts using 1xTAE running buffer.

Luria-Bertani Liquid medium (LB-medium)

1% Tryptone/ Peptone (Sigma-Aldrich, USA)

0.5% Yeast extract (Sigma-Aldrich, USA)

0.5% NaCl (Sigma-Aldrich, USA)

Add the required amount of distilled water and autoclave.

Fixation Solution

10% Acetic acid (Fisher scientific, USA)

30% Isopropanol (Fisher scientific, USA)

60% Distilled water

Coomassie Brilliant Blue Staining Solution

20% (v/v) Coomassie solution (Fluka laboratories, USA)

20% (v/v) Methanol (Fisher scientific, USA)

60% (v/v) Distilled water

Destaining Solution

10% Glacial acetic acid (Fisher scientific, USA)

40% Methanol (Fisher scientific, USA)

50% Distilled water

2.9 Molecular cloning and plasmid construction for CRISPR mediated gene silencing

Primer design was mainly performed using the online tool primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Restriction sites were introduced in the primers or oligos when necessary. When the restriction sites were introduced at the ends for the primers to facilitate cloning, 2-4 bp overhangs were inserted to improve digestion efficiency of PCR products. All primers and guide sequence oligos used in this study were ordered from Eurofins MWG Operon and are listed in appendix 2. The freeware pDRAW32 (http://www.acaclone.com/) was used for vector designing, vector manipulations and information management and various steps of *in silico* cloning. In some of the cloning steps, restriction digestion was performed with enzymes from Fermentas (St. Leon-Rot, Germany). Selection of suitable reaction buffer for double digests was assisted by the online tool DoubleDigest(http://www.thermoscientificbio.com/webtools/doubledigest/)

CRISPR or Clustered Regularly Interspaced Short Palindromic Repeats; a bacterial adaptive immune system based genome editing mechanism was used to target barley phytoene desaturase (*HvPDS*) and *HvMORC1* to introduce mutations in these genes to achieve total gene silencing. Barley phytoene desaturase was identified by BLAST search using sequence of rice phytoene desaturase gene (LOC_Os03g08570) against the Barley whole genome shotgun (WGS) sequence database at NCBI. The contig CAJX010854629.1from cultivar bowman of the barley WGS project gave the best hit and was identified as the barley homolog of the rice PDS gene. Protein coding regions or exons of the gene were predicted using the based online protein predictor software from Softberry FGENESH+ (http://linux1.softberry.com/berry.phtml?topic=fgenes_plus&group=help&subgroup=gfs).

Guide sequences targeting 3 different positions in the coding region of *HvPDS* gene were designed. The publicly available sequence (GenBank: HG316119.1), was used to design guide sequences for the *HvMORC1* gene. Guide sequence is a 22-26 nucleotide long oligomer starting with either an 'A' or a 'G' which is the requirement for TypeIII promoters (rRNA promoters U3 and U6) and contains a restriction site; preferably at the 3' end (Appendix 2). Restriction site in the guide sequence helps in identification of Cas9 induced mutations by PCR and restriction digestion assay.

Gateway® recombination cloning technology (Life technologies) was used to create CRISPR/Cas9 compatible entry and destination vectors for barley transformation. The vectors (pEntry-OsU3SgRNA, pH-Ubi-cas9-7) obtained from the group of Li-JiaQu at the National Plant Gene Research Center, Beijing (Miao et al.,2013) and vectors (pUC18 Tau6-gRNA, pJIT163-CAS9) received from Qi Zhou at Chinese Academy of Sciences, Beijing, China (Li et al.,2013), were modified as mentioned below. The original and modified vectors are listed in appendix 3. The pEntry-OsU3SgRNA (Miao et al.,2013) was chosen as the donor entry vector into which other sequences could be cloned and pH-Ubi-cas9-7 was the designated recipient destination vector. The choice of entry and destination vectors was based on the presence of Gateway® recombination compatible attL and attrR sites which would enable easy transfer of non-coding CRISPR-RNA sequences into the destination vector. pEntry-OsU3SgRNA was used as it is without any modifications. The wheat U6 promoter was transferred from pUC18 Tau6-gRNA (Li et al.,2013) to entry vector pEntry-OsU3SgRNA by amplification of the TaU6 promoter region by #40Ascl TaU6-F and #41Sacll TaU6-R primers (Appendix 2), followed by digestion of entry vector and PCR product using *AscI* and *SacII* and ligation of PCR product to the cut open plasmid. HvU3 promoter sequence was identified by blasting TaU6 sequence against barley WGS database. The best hit obtained (GenBank: CAJX011995286.1) was identified as the putative HvU3 gene and a synthetic gene containing HvU3 promoter sequence, *BsaI* site and SgRNA (Appendix 1) cloned into pUC57 was obtained from a company specializing in oligo nucleotide synthesis (Genewiz, USA).HvU3 sequence was transferred to the entry vector using *AscI* and *SacII* digestion of the HvU3-pUC57 plasmid and ligation to the entry plasmid cut open using the same restriction enzymes. Sense and antisense oligonucleotides for guide sequences (Appendix 2) were hybridized by denaturing at 95°C for 5 minutes followed by gradual annealing at 4°C for about 30 minutes creating double stranded oligonucleotides with sticky ends compatible for further cloning steps. Appropriate double stranded guide

sequences were inserted into HvU3 and OsU3b containing plasmids by *BsaI* digestion and into TaU6 containing plasmid by *BbsI* digestion. Prior to *BbsI* digestion, an additional *BbsI* site in the entry vector was removed by site specific mutagenesis by PCR using primers #46 EntryBbslfwd2 and #47 EntryBbslRev2 (Appendix 2).The cassette for promoter driving the expression of non-coding CRISPR-RNA was transferred to the destination vector pH-Ubi-cas9-7 by LR reaction using Gateway® LR Clonase™ II Enzyme Mix (Life technologies, Invitrogen, Germany).

LR reaction

1. The following components were added to a 1.5 ml tube at room temperature and mixed:
   Entry clone (50-150 ng) 1-7 µl
   Destination vector (150 ng/µl) 1 µl
   TE buffer, pH 8.0 to 8 µl
2. The LR Clonase ™ II enzyme mix was thawed on ice for about 2 minutes and vortexed twice briefly (2 seconds each time).
3. 2 µl of LR Clonase ™II enzyme mix was added to each sample and mixed well by vortexing briefly twice. The samples were spun down, and incubated at room temperature overnight. 1 µl of the Proteinase K solution was added to each sample to terminate the reaction. The samples vortexed briefly and incubated at 37°C for 10 minutes. 1-2µl of the LR reaction was used in transformation of competent cells.

2.9.1. Transformation

The recombinant entry vectors and destination vectors (Appendix 3) were transformed into *Escherichia coli* (DH5α) cells by adding 80µl of defrosted chemically competent cells to 7µl of ligation reaction (1-2µl in case of LR reaction). The mixture was incubated on ice for 30minutes so that the DNA could accumulate to the cells. This was followed by a heat shock treatment in a water bath for 90 seconds at 42°C so that the cells could ingest the circular DNA. Soon after, the mixture was cooled down on ice for 3 minutes mixed with 220 µl of Luria-Bertoni (LB) medium without antibiotics and incubated for 1 hour at 37°C with shaking. Selection took place on LB plates with the appropriate antibiotic. The plates were incubated overnight at 37°C and on the next day, bacterial colonies were selected for colony PCR. Colony PCR was performed using specific primers (Appendix 2) which confirmed the presence of insert in the vector. 2 positive clones for each fragment were selected and grown overnight at 37°C in 6ml LB medium with the respective antibiotic.

2.9.2. Plasmid preparation

Positive clones confirmed with colony PCR were used to prepare an overnight culture. After 12hours, 2 ml of overnight culture was directly transferred to a reaction tube and centrifuged for 2minutes at 13,000 rpm. The plasmids were isolated with Pure Yield Plasmid mini-prep kit (PromegaGmbH, Germany) according to the instruction of the suppliers. Where applicable, midi-prep was performed using the NucleoBond Plasmid Purification Kit (Macherey-Nagel GmbH, Düren, Germany) according to the manufacturer's instructions. The plasmid concentration was measured with the help of a NanoDrop spectrophotometer. The extracted plasmids were stored at -20°Cuntil further use.

2.9.3. Protoplast Isolation and Transformation

Barley seedlings were grown in 16h light/8h dark conditions for 1-2 weeks. Care was taken in growing the plants as abiotic stress like change in temperature; flooding and drought conditions could affect transformation efficiency (Yoo et al., 2013). Healthy, fresh leaves were cut into fine strips (0.5–1-mm) and vacuum infiltrated for 30 minutes at 15-20 (in Hg) with an enzyme solution followed by incubation in the dark to digest cell wall. After 6-7 hour digestion, the enzyme solution was checked for the release of protoplasts. The enzyme solution turned green after digestion, which indicated the release of protoplasts. The enzyme/protoplast solution was diluted with an equal volume of W5 solution before filtration to remove undigested leaf tissues. Protoplasts were collected by centrifugation at 100g for 3 minutes and re-suspended in W5 solution to a concentration of 2 X 10^5 protoplasts ml^{-1} after counting cells under a stereo microscope using a hemacytometer. After washing with W5 solution, the protoplasts were transferred to ice for 30 minutes. W5 solution was removed as much as possible without touching the protoplast pellet. Protoplasts were finally re-suspended to a final concentration of 2 X 10^5 ml^{-1}in MMG solution.

Protoplast transformation was carried out in PEG solution. Transformation mixtures (10µg pH-Ubi-cas9 carrying either HvU3-guide-SgRNA or OsU3-guide-SgRNA or TaU6-guide-SgRNA mixed with 100µl protoplasts in 100µl PEG solution) were agitated gently by tapping on the sides of the tube. After 30minutes of incubation in the dark at Room temperature, protoplasts were washed with 400µl W5 solution by gently rocking or inverting the tube to stop the transfection process. They were then centrifuged at 100g for 2 min at room temperature using a bench-top centrifuge and resuspended in 1ml W5 solution and cultured in the dark at Room temperature in 6 well tissue culture plates(Greiner-Bio one,

Solingen, Germany) usually for 48hours. DNA extraction from protoplasts was done by CTAB method described above and the isolated DNA was used in PCR-RE assay (Shan et al., 2013)

Enzyme solution:

1.5% Cellulase R10 (Duchefa, Netherlands)

0.75% Macerozyme R10 (Duchefa, Netherlands)

0.6 M Mannitol (Carl- Roth, Germany)

10mM MES pH 5.7(Sigma-Aldrich, Germany)

10mM $CaCl_2$ (Carl- Roth, Germany)

0.1% BSA(Sigma-Aldrich, Germany)

W5 solution:

154 mMNaCl (Carl- Roth, Germany)

125 mM $CaCl_2$ (Carl- Roth, Germany)

5mM KCl and (Carl- Roth, Germany)

2mM MES pH 5.7

MMG solution:

0.4 M Mannitol

15 mM $MgCl_2$ (Carl- Roth, Germany)

4mM MES pH 5.7

PEG solution:

40% w/v PEG 4000 (Fluka, Germany)

0.2M Mannitol

0.1M $CaCl_2$

3 Results

3.1. Basal resistance to barley powdery mildew

3.1.1 Knockdown of *MORC2* increases basal resistance to barley powdery mildew

Stable transgenic plants overexpressing or silenced for one of the MORC family members was tested for basal resistance to powdery mildew using detached leaf assay. Secondary leaf from 10-12 day old barley plants was detached, placed on water agar plates and inoculated with barley powdery mildew *Blumeria graminis f.sp. hordei* race A6.In the first set of experiments, 5 independent lines or transformation events (out of a total 76 lines produced) knocked down for HvMORC2 gene; labelled KD-*hvmorc2* L5, L11, L21, L30 and L55 (Table1, Fig.5) were screened for powdery mildew resistance. The lines were selected on the basis of seed count (lines with at least 200 seeds were preferred over lines with lesser seed count) and presence of transgene (lines containing knockdown construct was tested in T0 generation by Rajkumar Vutukuri in his mater thesis work).Golden Promise, containing the empty vector (pLH6000) was used as control. Resistance mechanism tested here is basal resistance as the transgenic lines produced in Golden Promise background do not have any R-gene against *Blumeria graminis f.sp. hordei* race A6 and result in a compatible interaction.

Previous studies in Arabidopsis showed that knockout of MORC1 and its closest homolog MORC2 leads to increased susceptibility to bacterial and oomycete pathogens (Kang et al., 2008, 2010). Five days after inoculation of barley knockdown lines with powdery mildew, colonies growing on the leaf surface were counted and disease symptoms compared. Results indicated that KD-*hvmorc2* lines 11 and 55 (knockdown efficiency, refer figure 12) developed far fewer colonies compared to the control (Table1, Fig.5). The differences observed were found to be statistically significant (Fig.5). KD-*hvmorc2* L5 and L31 also had a similar tendency albeit without any statistical significance (Table1, Fig.5). KD-*hvmorc2* L21 behaved like the control and had a similar level of disease development.

Table 1: HvMORC2 Knockdown Lines tested and effects on basal resistance to Barley powdery mildew

	Control (E.V)	KD-*hvmorc2* L5	KD-*hvmorc2* L11	KD-*hvmorc2* L21	KD-*hvmorc2* L31	KD-*hvmorc2* L55
Mean Colony count	100	92	57	103	88	70
Standard error	6.9	5.9	5.3	9.3	8.8	5.8

Sample size	17	16	21	11	16	15

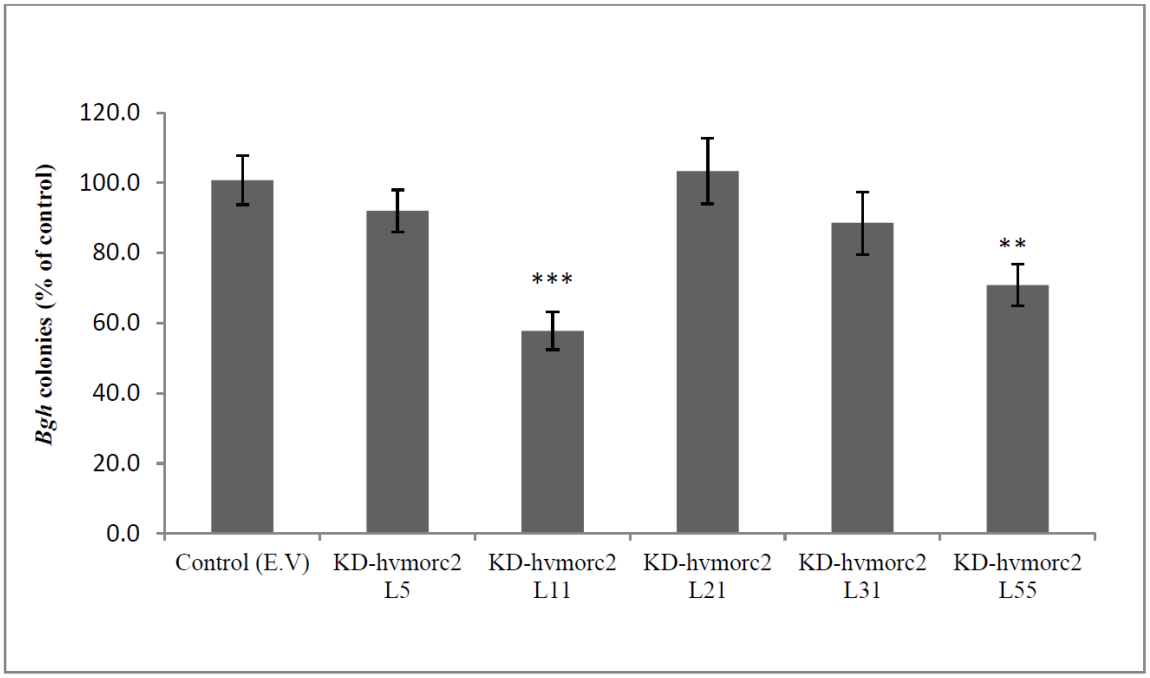


Figure5.Silencing of MORC2 results in enhanced basal resistance to powdery mildew. Detached second leaves of 12-day-old L5, L11, L21, L30 and L55 seedlings or control (e.v.) were inoculated with three to five conidia per mm^2 of *Bgh*A6. The colonies growing on the leaf surface were counted 5 days post inoculation (dpi). Two different matrices were used to count the leaf area as the leaves were not of uniform size. The bigger matrix had an area of 3.5cm^2 and the smaller matrix 1.2cm^2. The colony count values were adjusted to the bigger matrix to reduce experimental variation. The number of powdery mildew colonies on control (e.v.) is displayed as 100% and the other values were normalized relative to the control. Presented are the means $\pm$ standard error of at least 11 plants. Significant differences are marked: **P , 0.01, ***P , 0.001 (Student's t test)

The results obtained in the initial screening were in total contrast to the results in Arabidopsis. In barley, MORC2 knocked down lines (knockdown efficiency refer figure 12) were more resistant and in Arabidopsis it was the opposite. This applied not just to basal resistance, but also to R-gene mediated resistance and multiple layers of plant immunity (Kang et al., 2008, 2010, 2012). Due to the contradictory nature of the findings in barley and to identify additional lines having a resistance phenotype, powdery mildew detached leaf assay was repeated with 4 other independent lines KD-*hvmorc2* L9, L29, L32, L40 which were not tested in the first experiment.

Table 2: Additional Knockdown Lines tested and effects on basal resistance to Barley powdery mildew

	Control (E.V)	KD-*hvmorc2* L9	KD-*hvmorc2* L21	KD-*hvmorc2* L29	KD-*hvmorc2* L32	KD-*hvmorc2* L40
Mean Colony count	100	101	87	106	90	47
Standard deviation	55.8	35.9	26.0	36.5	31.7	30.4
Standard error	11.1	7.1	5.1	7.8	6.1	6.1
Sample size	25	26	26	22	27	25

Just as in the initial screening, one of the knockdown lines KD-*hvmorc2* L40 (knockdown efficiency refer figure 12) showed reduced colonization by the fungus compared to control and the difference was found to be statistically significant (Table2, Fig.6). The other transgenic lines were either slightly resistant (KD-*hvmorc2* L32) or had the same number of colonies as the control (KD-*hvmorc2* L9 and L29)

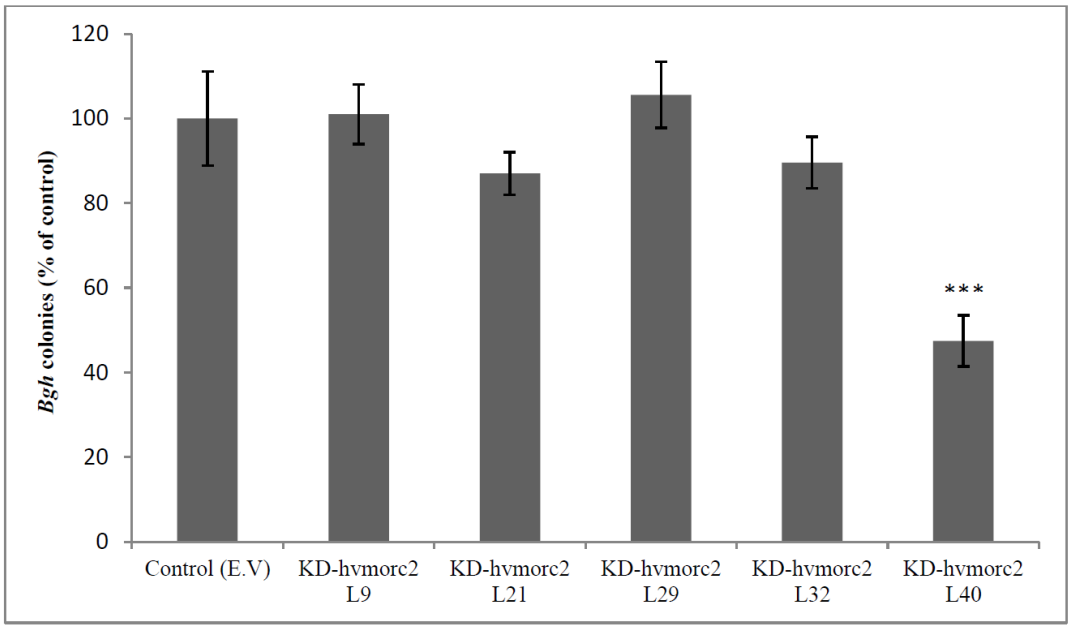
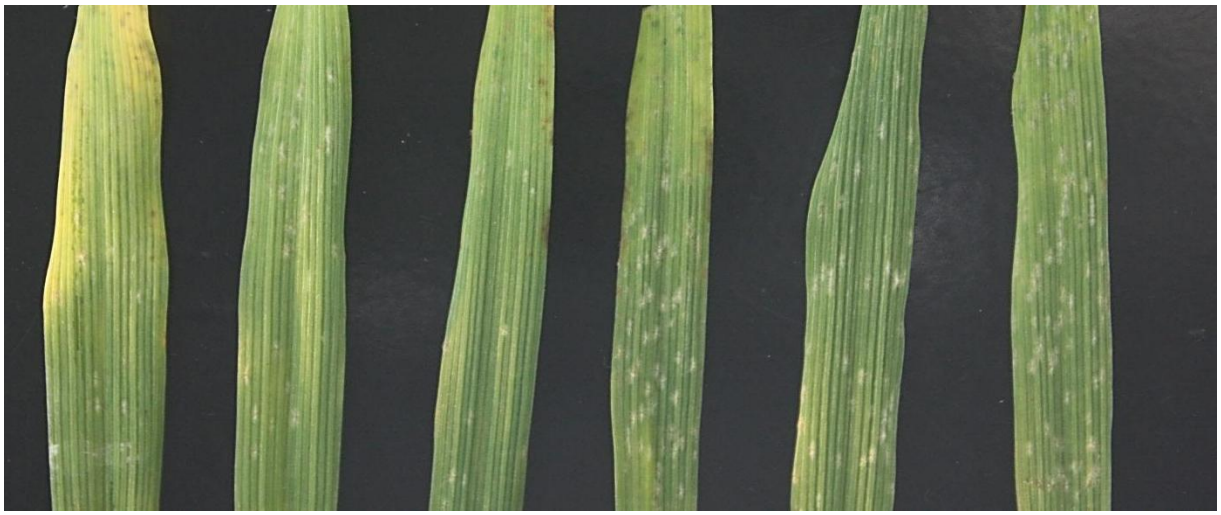


Figure6. Pathogen responsiveness of HvMORC2 knockdown lines to powdery mildew. The number of powdery mildew colonies on control (e.v.) is displayed as 100% and the other values were normalized relative to the control. Presented are the means ± standard error of at least 22 plants. Significant differences are marked: ***P, 0.001 (Student's t test)

3.1.2 Overexpression of *MORC1* increases susceptibility to barley powdery mildew

Detached leaf assay with powdery mildew demonstrated that knockdown of *HvMORC2* led to increased basal resistance to this fungal pathogen (Table1& 2, Fig. 5&6). To test and confirm if the opposite was also true i.e., if over expression of MORC led to increased susceptibility to powdery mildew, three independent lines over expressing HvMORC1 (HvMORC1 OEx L5, L8 and L13) were tested for resistance to *Blumeria graminis f.sp. hordei* race A6 by detached leaf assay. Susceptible cultivar Golden Promise, empty vector (golden promise containing pLH6000) and a transgenic line overexpressing GFP were used as controls. The pathogen assay was conducted as described for MORC2 knockdown lines and pustules counted 5 days after inoculation.



Golden promise	Empty Vector	35S:: GFP	HvMORC1 OEx L5	HvMORC1 OEx L8	HvMORC1 OEx L13

Figure7. Representative figure showing infected leaf segments 5 days post inoculation (5dpi). Leaf segments were removed from water agar plate, laid on a flat surface and photographed. Phenotypic comparison shows the transgenic lines to be more susceptible than the controls.

Pustules were counted five days after inoculation, and disease symptoms analysed. Results indicated that HvMORC1 OEx L5, L8 and L13 developed far more colonies compared to the control Golden Promise (Table3, Fig.7 & Fig.8). The differences observed were found to be statistically significant (Fig.8).

Table 3: HvMORC1 overexpressor Lines tested and effects on basal resistance to barley powdery mildew

	Golden promise	Empty vector	Control 35S::GFP	HvMORC1 OEx L5	HvMORC1 OEx L8	HvMORC1 OEx L13
Mean Colony count	100	89	79	220	147	130
Standard deviation	54.9	54.9	47.5	137.7	91.7	84.3
Standard error	10.3	10.3	9.3	27.0	17.2	16.2
Sample size	29	27	26	26	28	27

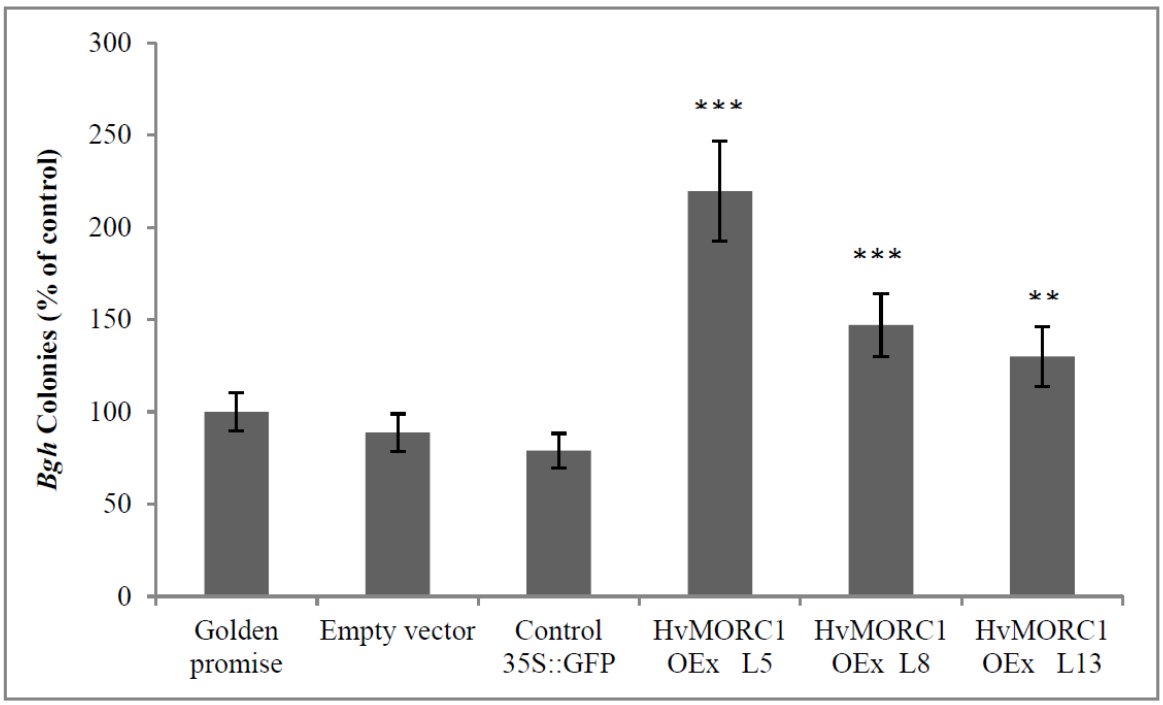


Figure8.Overexpression of MORC1leads to enhanced basal resistance against powdery mildew. Detached secondary leaves of 12-day-old L5, L8, and L13 seedlings and control were inoculated with three to five conidia per mm^2 of *Bgh*A6. The colonies growing on the leaf surface were counted 5days post inoculation (dpi). Two different matrices were used to count the leaf area as the leaves were not of uniform size. The bigger matrix had an area of $3.5cm^2$ and the smaller matrix $1.2cm^2$. The colony count values were adjusted to the bigger matrix. The number of powdery mildew colonies on control (Golden Promise) is displayed as 100% and the other values were normalized relative to the control. Presented are the means ± standard error of at least 26 plants. Significant differences are marked: **P , 0.01, ***P , 0.001 (Student's t test)

The results obtained in the initial screening were in consensus with the expected results in barley where knockdown of MORC resulted in increased resistance. Here, overexpression of HvMORC1 (for expression data refer figure 13) as expected led to enhanced susceptibility. Just as with the knockdowns, powdery mildew detached leaf assay was repeated with 2 new overexpressor lines HvMORC1 OEx L2 and L6 which were not tested in the first experiment. This was done to identify additional lines having similar susceptibility phenotype and confirm the findings of the first study with the overexpressor lines.

Golden Promise	HvMORC1 OEx L2	HvMORC1 OEx L5	HvMORC1 OEx L6

Figure9. Representative figure showing infected leaf segments 5 days post inoculation (5dpi). Leaf segments were removed from water agar plate laid on a dark background and photographed. Phenotypic comparison shows the transgenic lines to be more susceptible than the Golden Promise.

In this independent biological experiment with two new MORC1 overexpressor lines, both new overexpressor lines HvMORC1 OEx L2 and L6 in addition to previously tested HvMORC1 OEx L5 showed increased colonization by the fungus compared to control and the difference was found to be statistically significant (Table4, Fig.10). This experiment served as additional proof for the observation that overexpression of HvMORC1 (for expression data refer figure 13) leads to higher powdery mildew susceptibility.

Table 4: Additional HvMORC1 overexpressor Lines tested and effects on basal resistance to barley powdery mildew

	Golden promise	HvMORC1 OEx L2	HvMORC1 OEx L5	HvMORC1 OEx L6
Mean Colony count	100	121	127	142
Standard deviation	27.6	36.2	52.1	44.1
Standard error	5.3	7.5	10.9	8.6
Sample size	27	23	23	26

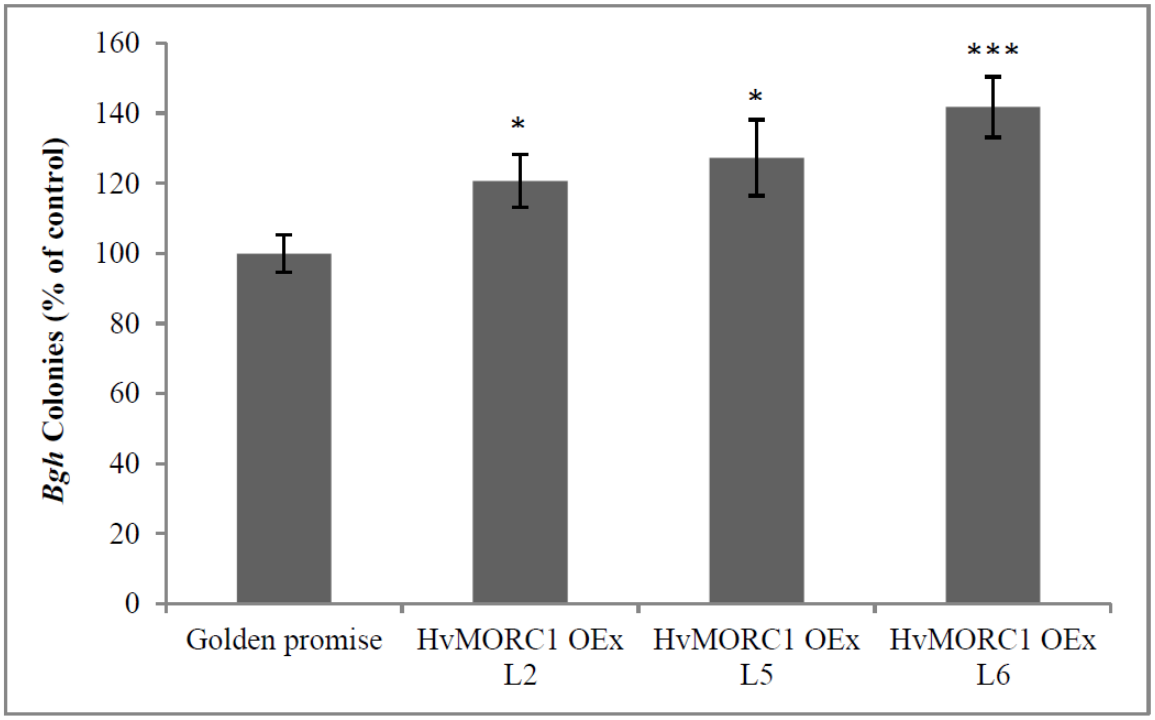


Figure10.Overexpression of MORC1 leads to enhanced basal resistance against powdery mildew. Detached secondary leaves of 12-day-old L2, L5, and L6 seedlings and Control were inoculated with three to five conidia per mm^2 of BghA6. The colonies growing on the leaf surface were counted 5 d post inoculation (dpi). The number of powdery mildew colonies on control (Golden Promise) is displayed as 100% and the other values were normalized relative to the control. Presented are the means of at least 26 plants. Significant differences are marked: *P , 0.05, ***P , 0.001 (Student's t test)

3.2 Characterization of T1 generation of transgenic plants

The HvMORC1 overexpressor lines and HvMORC2 knock down lines tested for powdery mildew resistance belonged to the T1 generation of transgenic plants. The T1 generation or the first generation of transgenic plants produced by tissue culture (comparable to the F2 generation in a Mendelian cross) represents a segregating population. A segregating population segregates 3:1 in accordance with laws of Mendelian genetics; with 3 plants carrying the insert/Transgene and 1 azygous plant lacking the transgene.

Since the plants tested belonged to a segregating population, it was necessary to identify plants carrying the transgene and those lacking the transgene. Characterization between transgenic and non-transgenic plants is important in understanding the biological function of desired genes. Additionally, a 3:1 segregation pattern would indicate a single copy insertion in the different lines tested. Only the lines with a powdery mildew phenotype were tested for segregation of transgene.

Transgenic plants of the T1 generation overexpressing *HvMORC1* and the lines silenced for *HvMORC2* were tested for the segregation of hygromycin resistance gene using primers (Appendix 2) specific for the 35S promoter driving the expression of Hygromycin resistance gene (pGY1fwd2) and the Hygromycin phosphotransferase gene itself (JI-Hyg-Rev).PCR amplification gives a product of around 1050 base pairs with these primers. Only the transgenic plants contain this gene and not the azygous plants. The plants tested positive are to be used for biological assays to test effects of transgene and propagation, while the azygous plants are the best controls to be included in the same experiments with the positively tested plants.

Individual plants of HvMORC2 knock down lines L11, L40 and L55 along with HvMORC1 overexpressor lines L5, L8 and L13 were characterized for the presence/absence of transgene. All three knockdown lines KD-*hvmorc2* L11, L40 and L55 showed nearly a 3:1 segregation pattern for the transgene. In KD-*hvmorc2*L11 14 out of 19 plants tested were transgenic. 22 out of 29 L55 plants also contained the transgene. For KD-*hvmorc2* L40 (Fig.11) 22 out of 30 plants tested were transgenic. In case of HvMORC1 overexpressor lines, HvMORC1 OEx L5 and L8 segregated 3:1 (19/27 and 22/28 respectively), while L13 segregated in an unusual pattern of 1:1 with 14 transgenic plants and 13 azygous plants. The positively tested plants along with 2-3 azygous plants were chosen for propagation.

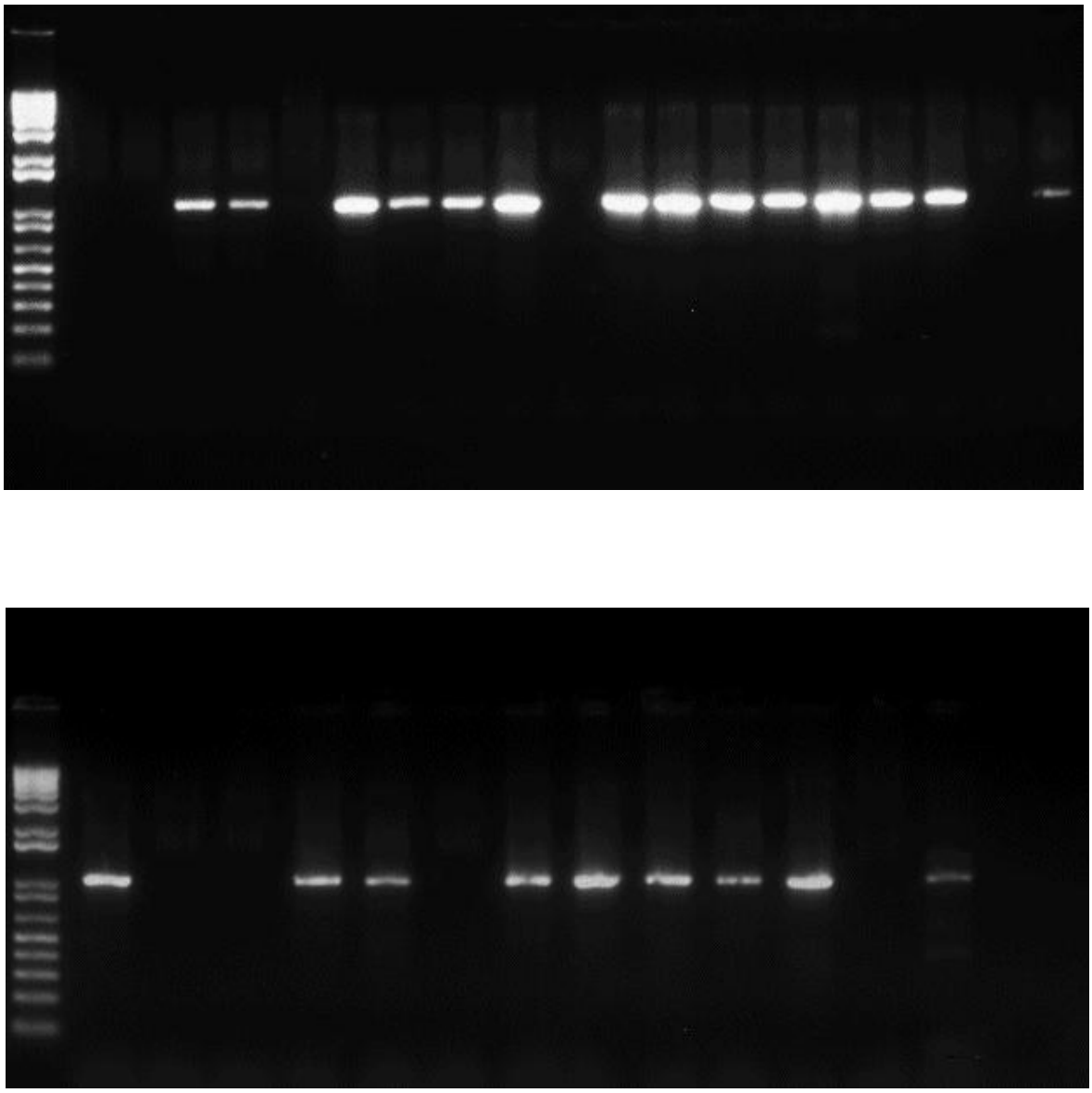


Figure11. Representative figure of KD-*hvmorc2* L40 for identification of transgenic and non-transgenic plants. PCR products of 1055 base pairs were separated on a 1% agarose gel at 120V for 1 hour and visualized using a UV Transilluminator. 22 out of 30 plants tested contained the transgene. This was in consensus with the expected segregation pattern of 3:1 according to the laws of Mendelian genetics. The azygous plants were tested once again to confirm that they were not false negatives. The other lines were tested using a similar procedure (data not shown).

3.3 Relative Quantification of MORC transcripts in Knockdown and Overexpressor lines

Following the pathogen assay with barley powdery mildew and characterization of transgenic plants, the next step was to identify knockdown efficiency in the RNAi silenced plants. Analysis of expression of MORC genes in the knockdown lines was done by relative quantification, where expression of a target gene (HvMORC2) relative to a housekeeping gene (HvUbi) was quantified (refer appendix 2 for primers used).In KD-*hvmorc2* L40 which had the best resistance phenotype against powdery mildew, expression of HvMORC2 was reduced by 50% (Fig.12) compared to empty vector control. In the other two lines KD-*hvmorc2* L11 and L55 the transcript levels were reduced by 43% and 42% respectively (Fig.12). This confirms the finding that knockdown leads to increased resistance to powdery mildew (Table1 &2, Fig. 5&6).

	Empty vector	KD-*hvmorc2* L11	KD-*hvmorc2* L40	KD-*hvmorc2* L55
Relative expression	100	57	50	58
Standard error	5.2884358	9.1564366	7.5387734	4.3096019

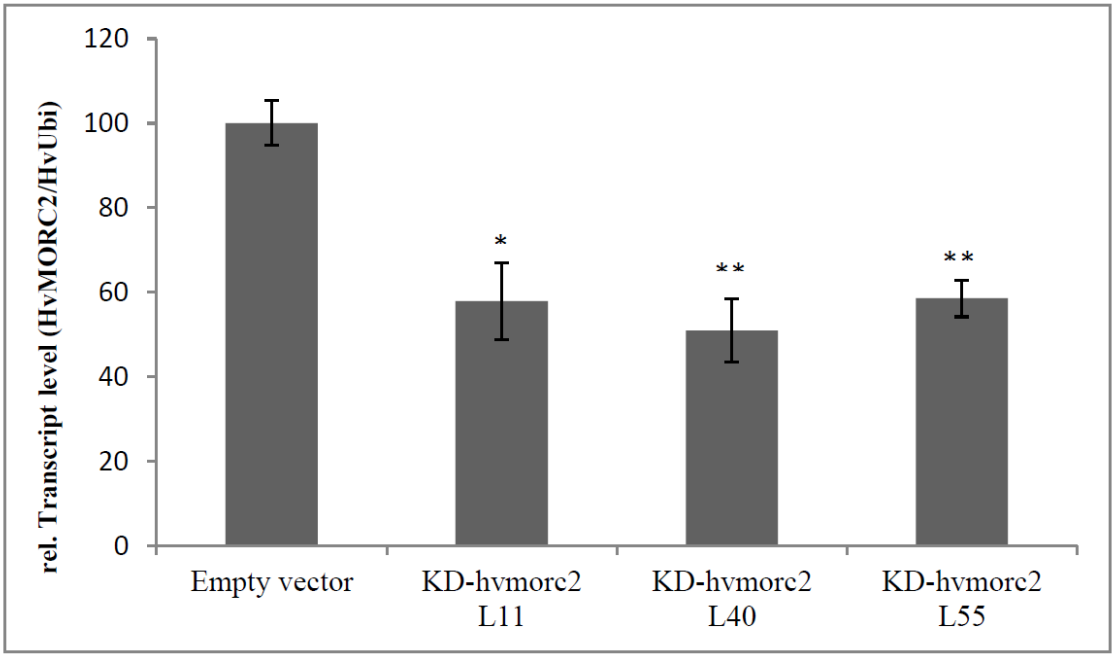


Figure12. Average transcript levels of HvMORC2 in RNAi lines L11, L40, and L55 as quantified by quantitative -PCR with normalization to barley ubiquitin and comparison to the

empty vector control. Values are calculated from at least 10 positively tested T1 plants per line (Student's t-test $p < 0.05$ *, $p < 0.01$ **)

As in case of RNAi lines, the transcript level in lines constitutively overexpressing MORC was analysed by Quantitative PCR. Expression of HvMORC1 gene relative to Ubiquitin (HvUbi) was quantified (refer appendix 2 for primers used). A comparison was made to the control plants. All three overexpressor lines tested HvMORC1 OEx L5, L8 and L13 showed a 10 fold increase in transcript levels (Fig.13) compared to the control plants. Expression levels were found to be in agreement with the hypothesis that an increased expression of HvMORC1 led to an increased susceptibility to barley powdery mildew (Figure 8, 10).

	Control	HvMORC1-OEx L5	HvMORC1-OEx L8	HvMORC1-OEx L13
Relative expression	100	1097	1092	1022
Standard error	4.084543	203.4696	163.1321	103.5668

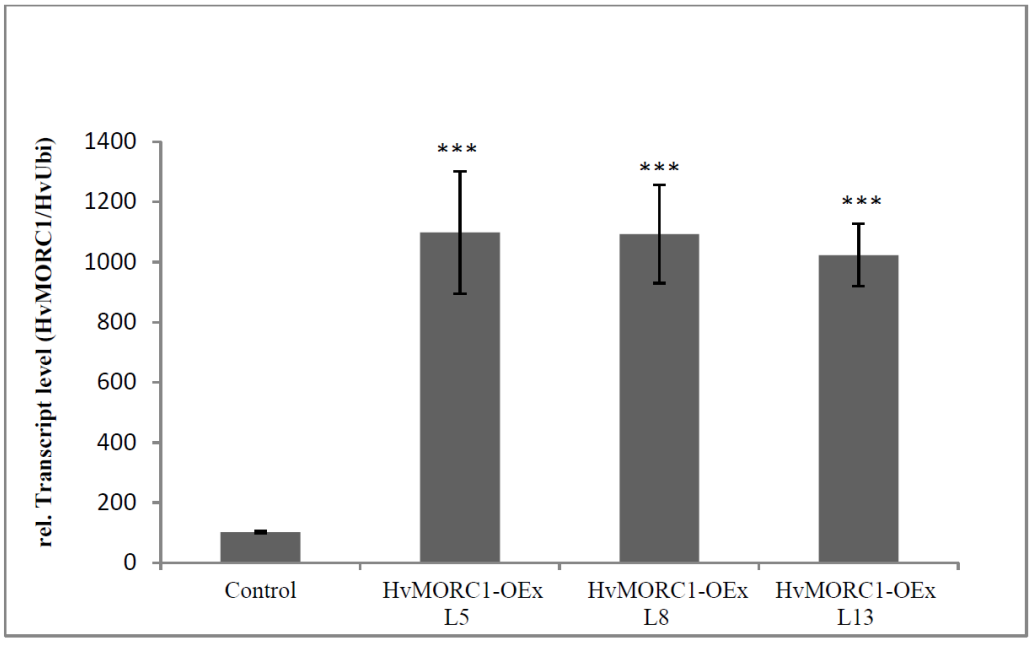


Figure13. Average transcript levels of HvMORC1 in overexpressor lines L5, L8, and L13 (constitutively overexpressing HvMORC1) as measured by quantitative -PCR with normalization to barley ubiquitin and comparison to the empty vector control. Values are

calculated from at least 10 positively tested T1 plants per line. (Student's t-test $p < 0.001$ ***)

3.4Knockdown of *MORC2* increases resistance and overexpression of *MORC2* enhances susceptibility to *Fusarium graminearum*

Knockdown of MORC2 led to an increased basal resistance to barley powdery mildew, which is a biotrophic pathogen. To find out if MORC gene also modulates resistance to pathogens with different lifestyles, MORC2 knockdowns and MORC2 overexpressor lines were tested for disease resistance to the necrotrophic cereal pathogen *Fusarium graminearum*. By this time, T2 generation of knockdown lines (Langen et al., 2014) were available for testing. Additionally, T1 lines constitutively overexpressing the same gene-MORC2 (Langen et al., 2014) were included in this experiment. This experiment aimed to compare effects of both overexpression and knockdown of the same gene- MORC2. Surface sterilized seeds of RNAi lines KD-*hvmorc2* L11.15 and KD-*hvmorc2* L40.17 as well as overexpressor lines HvMORC2 OEx L27 and HvMORC2 OEx L30 along with control cultivar Golden Promise were germinated on moist filter paper. 3 days later, the seedlings were inoculated with *Fg* macroconidia (50,000/ml), transferred to sand and Oil-Dri substrate and 10 days later evaluated for infections. Control plants, like HvMORC1 overexpressors, showed symptoms of heavy root rot infections, while KD-hvmorc2 plants retained a healthy appearance comparable to mock treated plants (Fig.14).

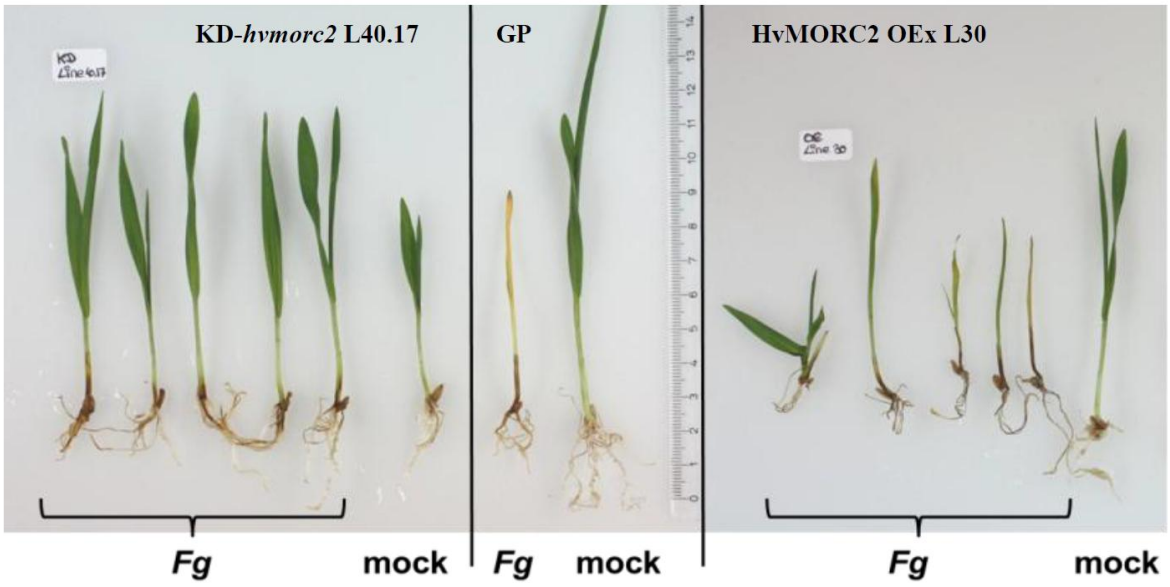


Figure14. Representative figure showing the disease symptoms in KD-*hvmorc2* L40.17 (left panel), control Golden Promise (middle panel) and HvMORC2 OEx L30 (right panel) to

Fusarium graminearum treatment. Knockdown line shows resistance to Fusarium treatment and despite browning of roots retains a healthy appearance. Overexpressor and control plants show root rotting and tissue necrosis.

The disease symptoms were grouped into three categories- root necrosis, coleoptile necrosis and leaf necrosis. The three disease categories were scored on a scale ranging from 0 to 4, with 0 being the most resistant and 4 being the most susceptible. Scoring confirmed results discussed in figure 14 with Golden Promise and overexpressor lines showing high values for leaf, coleoptile and root necrosis (Fig.15). In comparison, the knockdown lines were less necrotic and had lower scores in the evaluation (Fig.15).

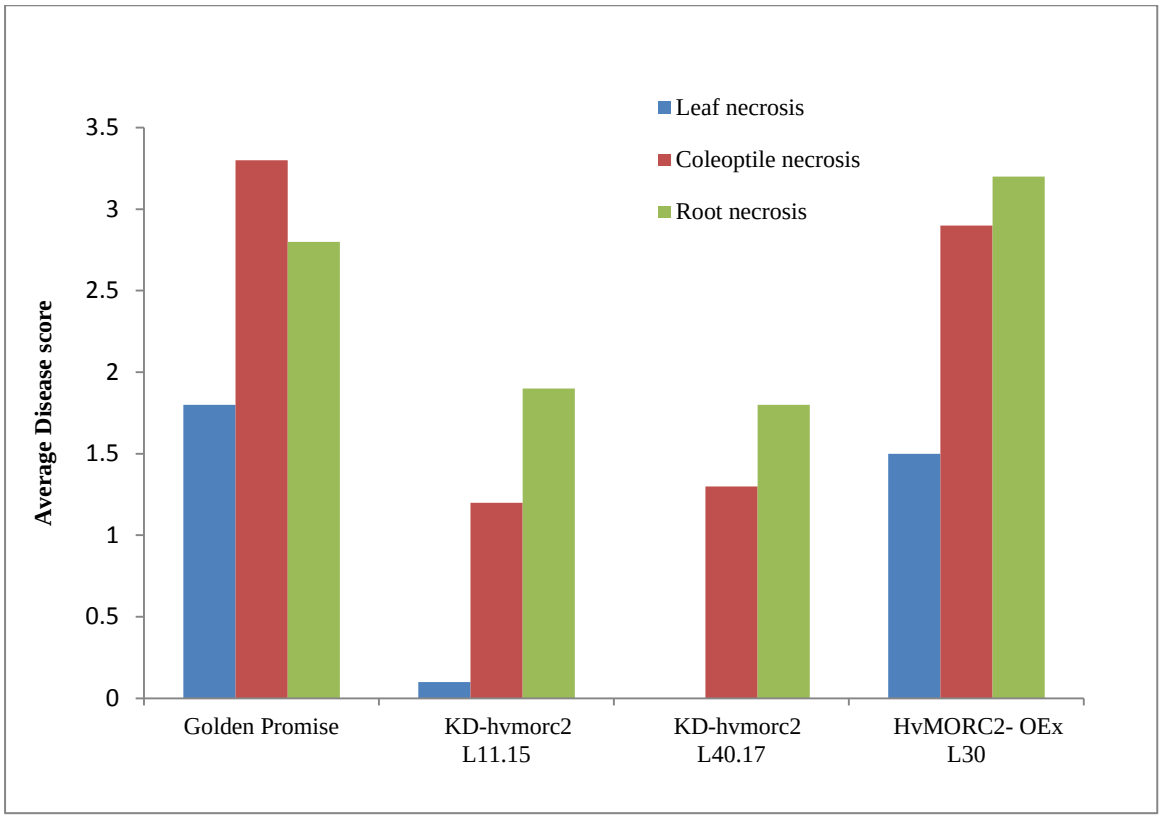


Figure15. Evaluation of disease symptoms in Golden Promise, HvMORC2 RNAi lines L11.15 and 40.17 as well as HvMORC2 overexpressor line L30 in response to *Fusarium graminearum* treatment. Plants were scored for leaf necrosis, coleoptile necrosis and root necrosis on a scale of 0 (most resistant) to 4 (most susceptible). Presented is a mean value of 12 plants for each line. The experiment was repeated twice with similar results.

The organ lengths were measured using the program ImageJ to understand effects of *Fusarium graminearum* treatment on plant growth and development. Shoot and root lengths measured were significantly greater in KD-*hvmorc2* lines L11 and L40 compared with either control or HvMORC1 overexpressing lines L27 and L30 (Table5, Fig.16).

Table5. Effects of *Fusarium graminearum* treatment on organ lengths in various genotypes

Genotype	Shoot length (cm)	Root length (cm)
Golden Promise	6.16 ± 0.72	1.77 ± 0.23
KD-*hvmorc2* L11.15	8.24 ± 0.47	2.42 ± 0.15
KD-*hvmorc2* L40.17	7.83 ± 0.30	2.49 ± 0.20
HvMORC2 OEx L27	5.98 ± 0.73	1.83 ± 0.27
HvMORC2 OEx L30	5.58 ± 0.49	1.64 ± 0.22

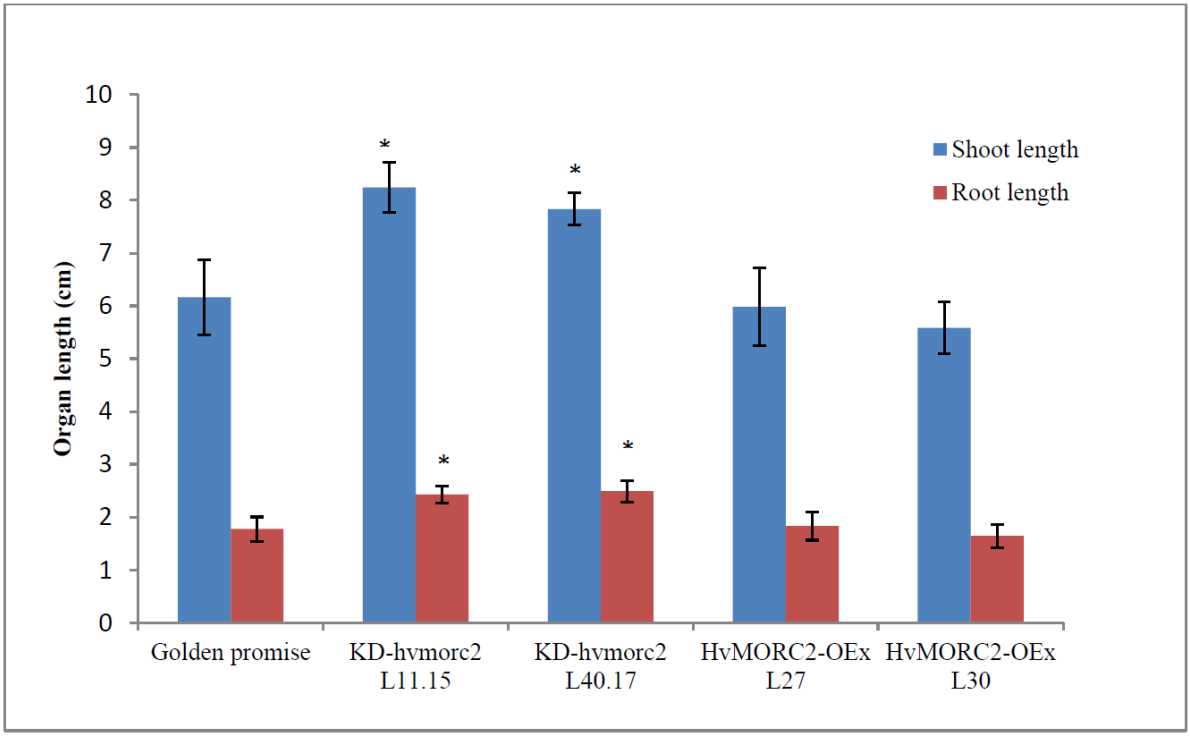


Figure16. Effect of *Fusarium* treatment on organ length of transgenic plants compared to Golden Promise. Presented is a mean value of 12 plants for each line. The experiment was repeated twice with similar results. (Student's t-test $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***)

As an additional proof, differences seen in transgenic lines to Fusarium infection were confirmed using quantitative PCR. Amount of fungal genomic DNA in the infected root samples was identified by relative quantification, where levels of a fungal gene Tubulin (FgTub) relative to plant gene (HvUbi) was quantified (refer appendix 2 for primers used).Consistent with phenotypic evidence, quantification of fungal DNA in KD-*hvmorc2* (L11.15 and L40.17) roots by quantitative PCR analysis revealed up to 60% reduced fungal colonization (Fig.17) compared with control plants and HvMORC1 overexpressors.

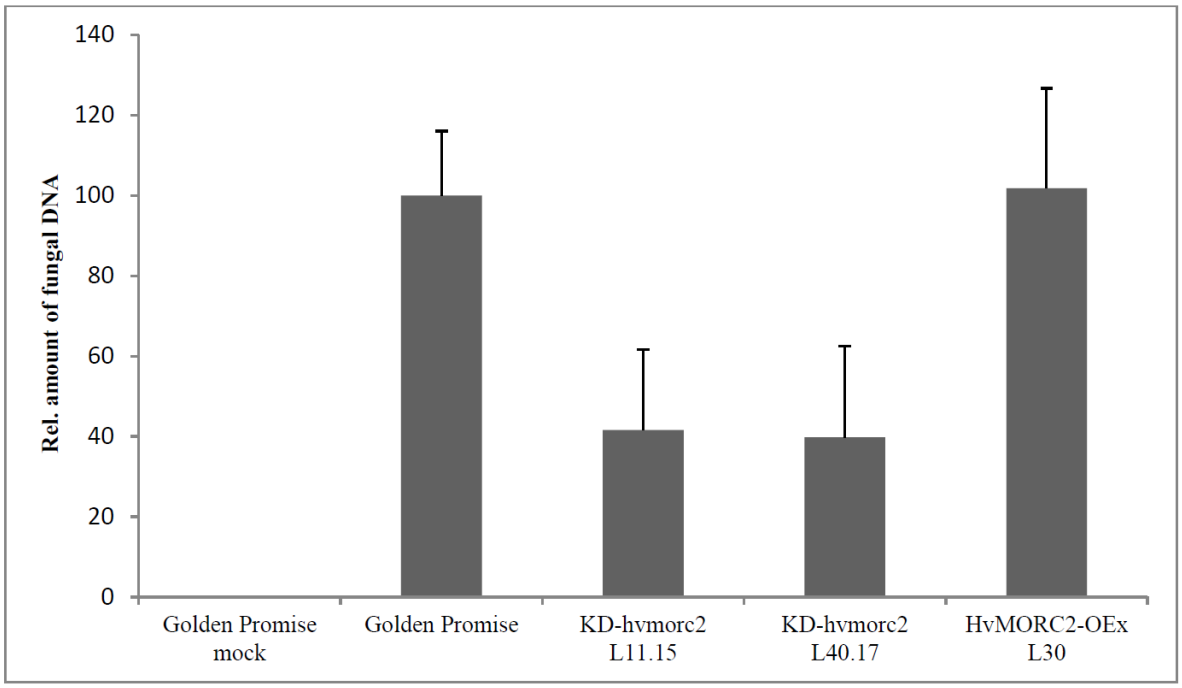


Figure17.*Fusarium* colonization was determined in barley roots 10dai by quantitative real-time PCR using primers (Appendix 2) specific for *F. graminearum* TUBULIN (FgTUB) and for barley HvUBIQUITIN (HvUBI). Displayed are means with standard errors of two independent biological experiments.

3.4.2 Knockdown of *MORC2* in STARTs roots enhances resistance to *Fusarium graminearum*

Root material produced by Stable Root Transformation System (Imani et al., 2011) was used additionally to test and characterize the effects of *MORC2* knockdown in disease resistance to the necrotrophic fungus *Fusarium graminearum*. STARTS-generated roots were inoculated with 1.2 x 10^4 spores/ml) and subsequently maintained on water agar plates in the plant tissue culture room till they were ready for harvest. Root material was harvested 5dai (phenotypically indistinguishable) and washed with distilled water to remove any mycelia on the outer surface of the roots. The fungal colonization of roots was studied by quantitative PCR using plant and fungal specific primers: HvUbi and FgTubulin (Appendix 2) specific for barley ubiquitin and *Fusarium graminearum* tubulin respectively. STARTs root material produced from barley immature embryos transformed with the knockdown plasmid- #474 pLH6000 Ubi:: *MORC2*-RNAi were less colonized by Fusarium than roots transformed with control plasmid,#621 pLH6000 Ubi::GUS-RNAi (Fig.18). These results confirm use of STARTs as a fast and efficient system that allows assessment of gene function in root tissues.

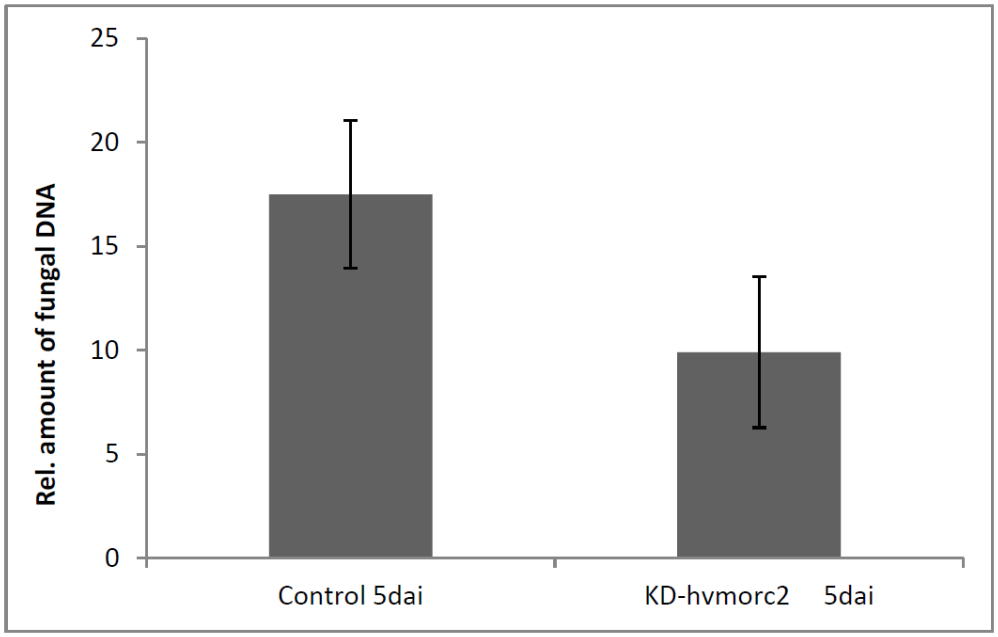


Figure18. Fusarium colonization of STARTs roots was tested 10 dai by quantitative real-time PCR using primers (Appendix 2) specific for *F. graminearum* TUBULIN (FgTUB) and for barley HvUBIQUITIN (HvUBI). Displayed are means with standard errors of two independent biological experiments.

3.5 HvMORC1 Possesses Endonuclease Activity

Studies in Arabidopsis showed that *AtMORC1* has endonuclease activity (Kang et al.,2012). Even though the ATPase domain of the MORCs is conserved and well characterized, not much is known about the endonuclease domain. To understand if the contrasting functions in Arabidopsis and barley could be explained through differences in the enzymatic activities of these two proteins, endonuclease assay was performed using recombinant HvMORC1 (Materials and Methods 2.8.1). The protein was purified using three step purification and tested for its ability to convert supercoiled plasmid DNA to relaxed DNA by producing a single stranded break in the substrate DNA molecule (Fig.15). Endonuclease activity of HvMORC1 was compared to a commercially available restriction endonuclease. Consistent with the results in Arabidopsis (Kang et al., 2012), HvMORC1 exhibited a Co-factor (Mn^{2+}) dependent endonuclease activity (Fig. 19). This result shows that differences in Arabidopsis versus Barley are not due to differences in enzymatic activities as thought before. However, further validation of this preliminary finding may be necessary to completely rule out the role of these proteins in contrasting biological functions.

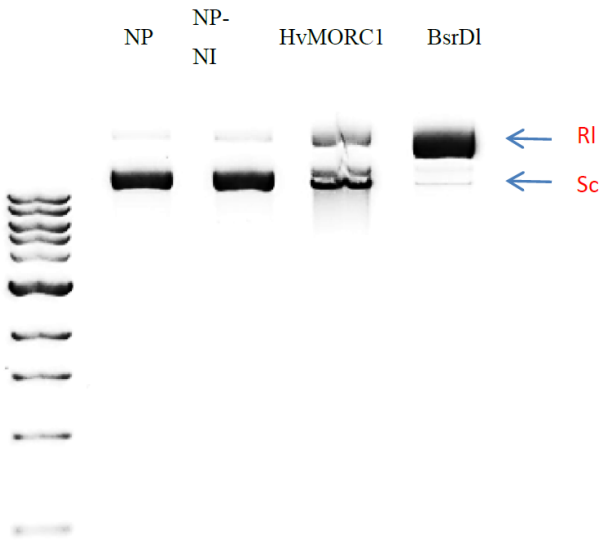


Figure 19. Agarose gel electrophoresis showing endonuclease activity of HvMORC1. Recombinant Proteins (500 nM) was incubated with 200 ng of pER8 supercoiled plasmid DNA for 8 h at 37°C in presence of 2 mM Mn^{2+}cation as the co-factor. The commercially available endonuclease BsrDl was used as a positive control. Endonucleolytic cleavage results in accumulation of relaxed and linearized DNA. The experiment was repeated two times using different protein preparations with similar results. sc, Supercoiled; rel, relaxed DNA. NP, no protein; NP-NI, no protein no incubation

3.6 Plasmid construction for CRISPR-Cas9 mediated gene silencing

Clustered Regularly Interspaced Short Palindromic Repeats or CRISPR system was used to selectively target and silence barley phytoene desaturase gene. HvPDS gene silencing results in photo bleaching that can be used as a proof of concept to test efficiency of CRISPR constructs. For this, gateway recombination compatible entry and destination constructs were produced. Entry vectors were constructed for three different promoter systems- rice and barley U3 promoter and wheat U6 promoter. Rice U3 promoter and gateway recombination site containing entry plasmid (Fig.20) was obtained from the group of Li-JiaQu (The National Plant Gene Research Center (Beijing). This was modified to create entry vectors with barley U3 or wheat U6 promoters that replaced the rice U3 promoter.

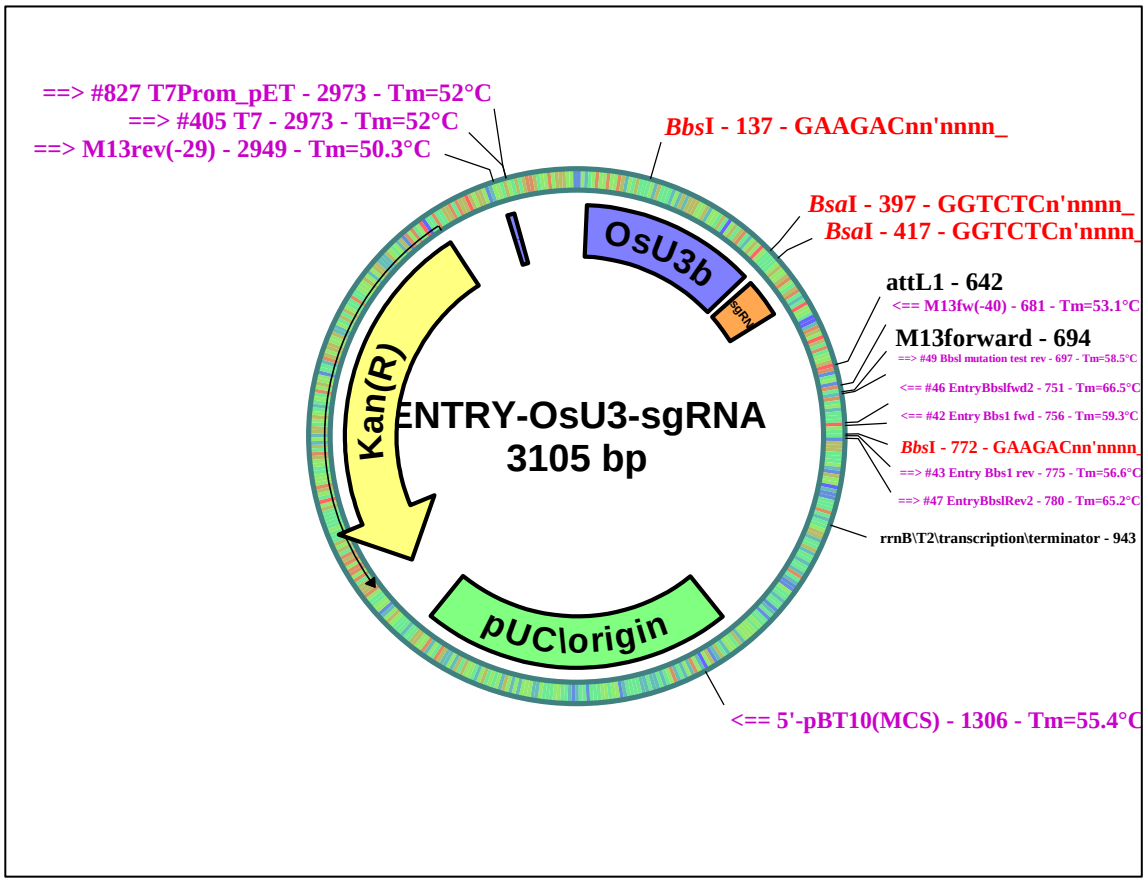


Figure20. Vector map of the entry vector pEntry-OsU3SgRNA

Kan-R: Kanamycin resistance for bacterial selection, SgRNA: Single guide RNA, a chimera of CRISPR-RNA and tracrRNA, pUC: Origin of replication of the plasmid, OsU3b: rice U3 promoter.

The wheat U6 promoter was transferred from pUC18 Tau6-gRNA (Qi Zhou, Chinese Academy of Sciences) to entry vector (pEntry-OsU3 SgRNA) by amplification of the TaU6 promoter region by AscI TaU6-F and SacII TaU6-R primers (Appendix 2), followed by digestion of entry vector and PCR product using AscI and SacII and ligation of PCR product to the cut open plasmid (Fig.21 and Fig.22).

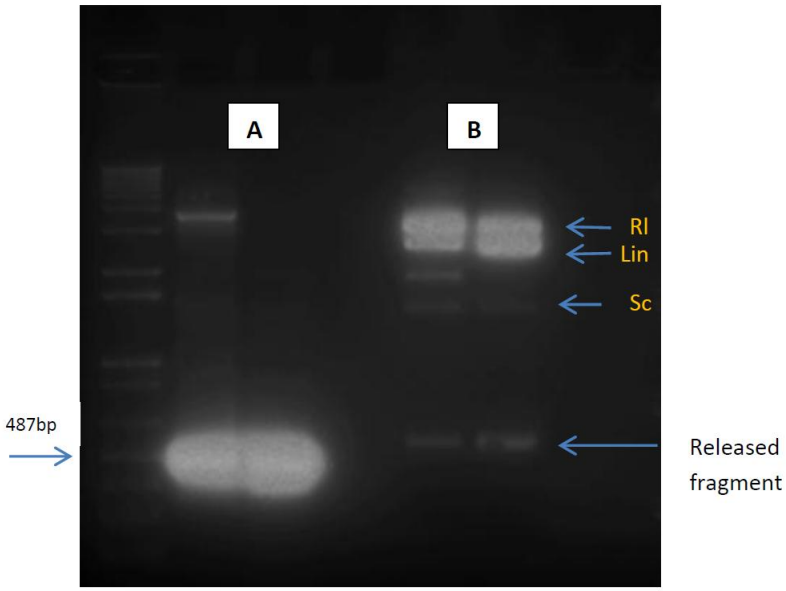


Figure21. A) PCR amplification of pUC18 Tau6-gRNA using specific primers gives a product of 487 base pairs. B) Restriction digestion of pEntry-OsU3 SgRNA using AscI and SacII results in the following- uncleaved supercoiled (Sc) DNA (1500bp), linearized (Lin) DNA (~2500bp) and relaxed (Rl) DNA (~3000bp). Additionally, a 547bp fragment is released. PCR product (487bp) is ligated to cut open linearized DNA (2545bp) to give the final entry vector containing the TaU6 promoter (3032bp).

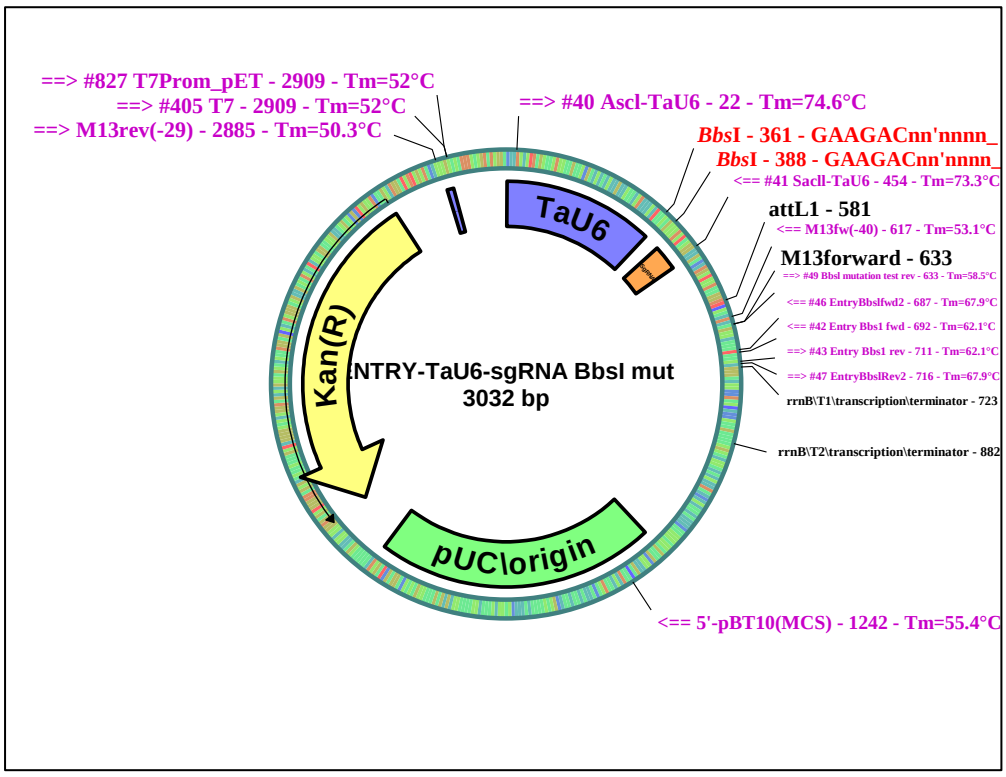


Figure22. Vector map of the entry vector pEntry-TaU6 SgRNA

HvU3 sequence was transferred to the entry vector using AscI and SacII digestion of the HvU3-pUC57(Genewiz, USA) plasmid and ligation to the entry plasmid (pEntry-OsU3SgRNA) cut open using the same restriction enzymes (Fig.23 and Fig.24).

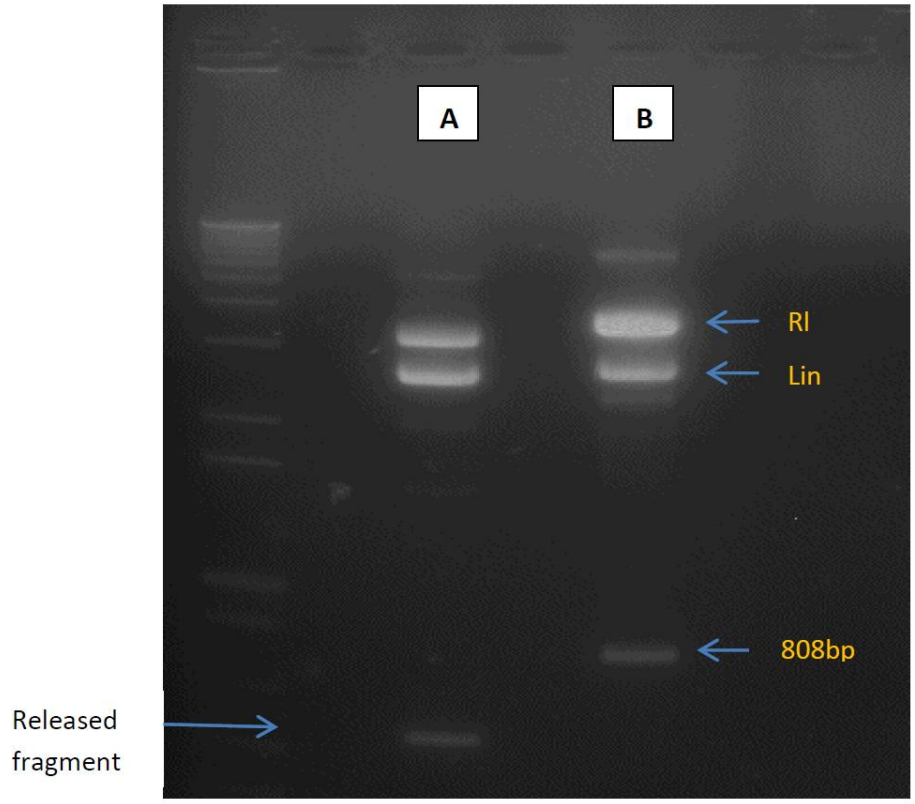


Figure23. A) Restriction digestion of pEntry-OsU3 SgRNA using AscI and SacII yields - linearized (Lin) DNA (~2500bp) and relaxed (Rl) DNA (~3000bp). Additionally, a 547bp fragment is released. B) AscI and SacII digestion ofHvU3-pUC57 produces- linearized (Lin) DNA (~2600bp) and relaxed (Rl) DNA (~3400bp). An 808bp fragment containing HvU3 is also released. HvU3 containing fragment (808bp) released from HvU3-pUC57is ligated to cut open linearized DNA (2549bp) to give the final entry vector containing the HvU3 promoter (3357bp). Vector map of the complete entry vector is given on the following page.

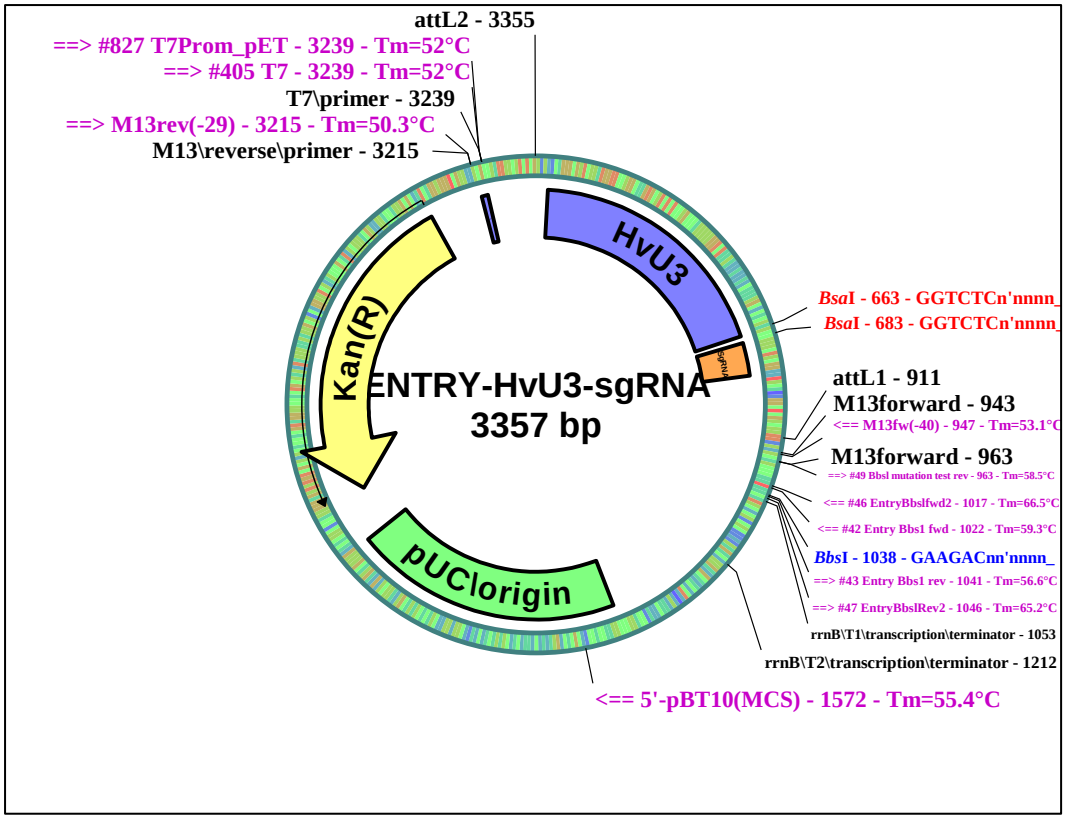


Figure24. Vector map of the entry vector pEntry-HvU3 SgRNA

4. Discussion

4.1 The MORC family

The MORC gene family has been shown to play an important role in developmental processes, immunity, chromatin superstructure remodelling among others in a wide range of eukaryotic organisms including plants. The MORC family is also widely found in prokaryotes, even though the distribution is sporadic (Iyer et al., 2008). The earliest eukaryotic studies on MORC was conducted in mice, where the protein was identified to express specifically in male germ cells and a mutation caused aberrations in spermatogenesis and led to male sterility (Watson et al., 1998; Inoue et al., 1999). The mouse MORC contains a nuclear localization. This and the similarity of the *morc* mutant phenotype to other characterized mouse knock-out mutations indicate a role in transcriptional regulation, cell division, DNA repair and chromatin rearrangement (Inoue et al., 1999). It also contains a coiled-coil domain leading to speculations of protein-protein interactions (Inoue et al., 1999). MORCs belong to a distinct eukaryotic gene superfamily with phylogenetically diverse members having largely unrelated functions. The GHKL ATPase superfamily is made up of protein families as diverse as DNA topoisomerase II (introduces negative supercoil during DNA replication), molecular chaperones HSP90 (assists in proper folding of proteins, conversion of proteins to active forms), DNA-mismatch-repair enzymes MutL (replaces mismatched nucleotides on the newly synthesized DNA strand) and histidine kinases (Dutta and Inouye, 2000). These functionally different proteins except HSP90 are united by the presence of DNA binding and ATPase domains (Ban and Yang, 1998a; Wang 1996; Obermann et al., 1998; Panaretou, B. et al., 1998). The energy of ATP-binding or ATP hydrolysis, or both, is utilized by these proteins to perform their various cellular functions (Dutta and Inouye, 2000). Prokaryotic MORC functions in restriction modification systems along with DNA helicases and endonucleases (Iyer et al., 2008). They seemed to have evolved from a structural reorganization of protein complexes by the action of common ancestors like MutL and topoisomerase ATPase modules. These studies also suggest that the eukaryotic counterparts of the prokaryotic MORCs may also function in chromatin remodelling in response to epigenetic signals such as histone and DNA methylation (Iyer et al., 2008).

The first such study in plants revealed 7 members of MORC family in Arabidopsis which consists of AtMORC1 and its 6 homologs (Kang et al., 2008). AtMORC2 and AtMORC3 have 81% and 70% identity respectively at the aa level with AtMORC1. The other members

were a little further off and less related to AtMORC1 (≤ 50% aa identity). Much like other MORCs of the GHKL ATPase superfamily, AtMORC1 was found to have a putative ATPase domain between aa 105–197 designated ‘‘HATPase_C’’ in the NCBI domain database and an S5-fold domain (383aa–458aa) [Iyer et al., 2008; Kang et al., 2008]. AtMORC1 was also demonstrated to have ATPase activity (Kang et al., 2008). However, the AtMORC1 does not have any sequence similarity with any of the known GHKL proteins other than in the ATPase region.

Kang et al., 2008 showed that *atmorc1* mutants were compromised in their resistance to turnip crinkle virus (TCV). AtMORC1 was demonstrated to interact with HRT and other NB-LRR proteins thus signifying its role in R-gene mediated resistance. AtMORC1 is additionally involved in modulating hypersensitive response mediated cell death induced by the constitutively active R-protein ssi4 and mutant versions of *atmorc1* are impaired in ssi4 mediated cell death (Kang et al., 2008). MORC1 knockout delayed HR in response to avirulent *Pst* carrying avrRpt2 (Kang et al., 2008). Therefore, AtMORC1 is involved in signaling pathways of diverse, important R-genes and is required for HR development and disease resistance. This study elucidated the role of AtMORC1 and other close homologs in R-gene mediated resistance in Arabidopsis.

A subsequent study showed interaction of AtMORC1 with the chaperone HSP90 of the GHKL family in addition to an already established interaction with a variety of R-proteins (Kang et al., 2010); leading to the speculation that AtMORC1 may act as a co-chaperone along with HSP90. MORC1 does not interact with auto activated R proteins ssi4, RPM1, RPS2, and RCY1 suggesting a role of MORC1 in R protein activation (Kang et al., 2010). ATPase activity of AtMORC1 might be necessary for this activation as truncated morc1 protein lacking ATPase region did not demonstrate HRT mediated cell death and was susceptible to TCV infection (Kang et al., 2010). R Gene–mediated resistance to bacterial pathogen *P. syringae* (RPS2 and RPM2 resistance against avirulent avrRPT2 and avrRpm1) and oomycete *Hyaloperonospora arabidopsidis* (RPP8 resistance) was compromised in *atmorc1 atmorc2* double mutant suggesting a possible involvement in defense against other pathogen types and that AtMORC1 mediated defense is not restricted to viral pathogens (Kang et al., 2010). Silencing of AtMORC1 homologs inhibited Pto or RPM1 triggered cell death in *N.benthamiana* proving its involvement with R-proteins of both major groups CC-NB-LRR and TIR-NB-LRR and a confirmation that MORC1 interaction with resistance proteins and role in plant immunity doesn’t seem to be limited to *Arabidopsis thaliana*.

Additionally, AtMORCs were shown to be involved in basal resistance to TCV and virulent *Pseudomonas syringae*. A double knockout mutant *atmorc1-1 atmorc2-1* (dKO) produced in a background lacking HRT was more susceptible to TCV infection than wild type control plants. Disease susceptibility phenotype observed was confirmed by immunoblot (Kang et al., 2012). Pre-treatment with flagellin epitope flg22 failed to prime immune responses in dKO plants as compared to the wild type against *P.syringae* infection. The dKO plants were also impaired in ROS production and callose deposition suggesting that knockout of AtMORC1 and AtMORC2 compromised basal resistance to the bacterial pathogen. MORC1 also physically interacts with flagellin receptor FLS2 when expressed at physiological levels, and this is not disrupted by FLS2 activation. (Kang et al., 2012). Knockdown of AtMORC1 and AtMORC2 renders non-host Arabidopsis susceptible to *P. infestans* infection as indicated by higher levels of disease severity, penetration efficiency and chlorotic cell death as well as lower callose deposition in dKO compared to WT plants arguing that the AtMORC family also has a role in non-host resistance (Kang et al., 2012). The same study also found reduced salicylic acid accumulation in systemic leaves of dKO plants upon pathogen inoculation suggesting a role of MORCs in full Systemic Acquired Resistance (SAR) development. Using subcellular fractionation and transmission electron microscopy a subpopulation of MORC1 was found in the nucleus, which increased upon activation of ETI and, to a lesser degree, PTI (Kang et al., in 2012). Arabidopsis MORC1 possesses DNA/RNA binding capacity and endonuclease activity in vitro, and mutations in MORC1 and its closest homologue enhance tolerance to the DNA-damaging agent mitomycin C, suggesting a potential role of this protein in the nucleus, possibly associated with DNA recombination and repair (R/R) and/or remodelling of chromatin structure (Kang et al., in 2012).

An independent study by Moissiard et al., 2012 showed the involvement of Arabidopsis MORCs in chromosome remodelling and manipulation of chromatin superstructure. Knockout mutants of *atmorc1* and *atmorc6* led to derepression of transposable elements and decondensation of pericentromeric region leading to changes in transcriptional profiles of silenced genes and interaction of pericentromeric region with other parts of the genome. Knockdown of *Caenorhabditis elegans* MORC homolog also led to inhibition of transgene silencing. The results indicate an involvement of MORCs as regulators in eukaryotic gene silencing (Moissiard et al., 2012). In a follow up study, AtMORC6 was shown to physically interact with AtMORC1 and AtMORC2 in two mutually exclusive protein complexes

(Moissiard et al., 2014). RNA-sequencing analyses indicated that AtMORC1 and AtMORC2 repress a common set of genes. AtMORC6 and MOM1 (Morpheus Molecule 1); an Arabidopsis protein causing changes to chromatin structure without changing methylation patterns, were found to regulate very similar set of genes further fuelling speculations that MORCs in Arabidopsis are involved in chromatin rearrangement and gene silencing (Moissiard et al., 2014).

4.2 Identification of MORCs in Barley

Genome wide analysis based on barley genomic and cDNA sequence data led to the identification of MORC homologs in barley. Out of the five genes identified, HvMORC1 and HvMORC2 had the highest amino acid (aa) sequence identity to Arabidopsis AtMORC1; at 47% and 48% respectively. They were also found to be closely related to each other with 90% similarity at aa level. The other three HvMORC6a, HvMORC6b and HvMORC7 were <40% identical on the aa level to Arabidopsis AtMORC1. HvMORC6a and HvMORC6b however are much closely related to AtMORC6 than to HvMORC1 (Langen et al., 2014). Barley MORCs also contain the GHKL ATPase domain and S5 fold domains as in case of Arabidopsis MORC1 (Kang et al., 2008); a defining feature of the GHKL superfamily (Iyer et al., 2008). The CRT-like MORC subfamily additionally contains a C-terminal putative basic leucine zipper (bZIP) domain predicted to be involved in protein-protein interactions (Langen et al., 2014).

4.3 Characterization and functional studies with barley MORCs

After identification of members of MORC family in barley, they were cloned using specific primers (materials and methods 2.1, Appendix 2) and the constructs were used in transient transformation for functional biological assays after Bgh infection and for transformation of scutellar tissue of barley immature embryos for production of stable transgenic plants or STARTs roots (materials and methods 2.1, 2.3). Several lines of stable transgenic plants overexpressing or silenced for different MORC genes were produced. These were then tested for disease resistance against barley powdery mildew; a biotrophic pathogen and *F.graminearum*; a devastating necrotrophic pathogen of cereals. These were then further characterized to confirm presence of transgene and the relative transcript levels in the overexpressor and knockdown lines were quantified by real time PCR.

4.3 .1 Barley MORCs play a role in basal resistance to barley powdery mildew fungi

Several transgenic RNAi lines with reduced expression of HvMORC2 were tested for resistance/susceptibility to powdery mildew fungi. Initial screening identified two lines; #62 KD-*hvmorc2* L11 and L55 (table 1, figure 5) to be more resistant to barley powdery mildew infection. These two lines were less colonized by the fungus compared to the controls as indicated by reduction in pustule counts, which were reduced by 43% and 30% in L11 and L55 respectively (table 1). Additionally, a third knockdown line #62 KD-*hvmorc2* L40 also showed a similar phenotype with 53% reduction (table 2, figure 6) in pustules compared to wild type/ empty vector controls. Transcript levels in the three lines #62 KD-*hvmorc2* L11, L40 and L55 were quantified by qPCR and showed a 43%, 50% and 42% decrease in relative abundance in these knockdown lines compared to the controls (Figure 12). As results from three independent transformation/insertion events, they provide a solid confirmation for the phenotype observed that RNAi mediated knockdown of HvMORC2 increased resistance to *Blumeria graminis* f.sp *hordei*. The results are in consensus with effects observed in transient transformation assays with powdery mildew, where detached leaves co-bombarded with GFP and HvMORC2 or HvMORC1 knockdown constructs showed lesser fungal penetration compared to the leaves bombarded with empty vector constructs (data not shown). To test if the opposite was true and overexpression of HvMORCs led to increased susceptibility, three independent transformants HvMORC1 OEx L5, L8 and L13 were tested for powdery mildew resistance and found to be significantly more susceptible than control plants as indicated by mean colony counts (Table 3, Figure 8).The barley results are conclusive but contradictory to the effects of MORCs seen in Arabidopsis. Studies so far in Arabidopsis (Kang et al., 2008, 2010, 2012) demonstrate MORC1 and MORC2 to be factors necessary for plant resistance. Knock out of AtMORC1 and its close homolog MORC2 leads to susceptibility to a wide range of pathogens including viruses, bacteria and oomycetes in addition to affecting multiple layers of plant immunity such as basal resistance, R-gene mediated resistance and non- host resistance. But in barley, the initial studies point to the fact that HvMORCs might function as susceptibility factors or negative regulators of plant immunity by interacting with specific targets in plant defense mechanism. Since the stable transgenic plants were produced in Golden Promise background, which does not contain any R-genes against Bgh A6, it is fairly conclusive that barley MORCs play at least a role in modulating basal resistance. As the resistant transgenic lines exhibited lesser colony counts than the controls, one might speculate that the knock down lines might be reinforced in penetration resistance which prevents or

retards fungal entry into the cell by formation of an effective papilla (Aist and Bushnell 1991). Another possibility could be that MORC1 in its active form and fully functional physiological levels might interact with negative regulators of basal resistance like Mildew Locus O (MLO) protein, which supports penetration by powdery mildew fungus. Barley MLO was found to be part of a pathway negatively regulating plant immunity suggesting *mlo* based resistance is not a pleotropic effect, rather a consequence of the negative regulatory role of barley MLO protein (Humphry et al., 2010). In the absence or reduced levels of MLO or MORC or both, the fungus may not be able to get entry into the cell. As a consequence, it is unable to grow and reproduce resulting in lesser colony counts compared to the wild type controls. There is very little hard evidence to prove this theory, but it can't be completely overruled at this point. Further analysis showed that transient over expression of HvMORC1 and other MORC homologs (clade III HvMORC6a) and clade II HvMORC7) in barley leaf epidermal cells of resistant cultivar Sultan5 compromised MLA12 mediated resistance to powdery mildew fungus (Langen et al., 2014). Leaves transformed with different MORC over expression constructs had significantly lower amounts of epidermal HR and mesophyll HR as well as higher levels of fungal elongated secondary hyphae compared to leaves transformed with empty vector constructs (Langen et al., 2014). These results are a clear indication of a role of barley MORCs not just in basal resistance, but in R-gene mediated resistance or effector triggered immunity as well. Just like in case of basal resistance, MORCs might negatively regulate R-gene mediated defense responses. Co-immunoprecipitation experiments reveal a weak interaction between HvMORC1 and HvMLA12 (Langen et al., 2014) further fuelling speculation that MORCs interact directly or indirectly with plant resistance factors including R-proteins to modulate disease resistance at different levels. AtMORC1 was in earlier studies found to interact with a variety of resistance proteins and to play a significant role in defense signalling mechanisms (Kang et al., 2008, 2010). Mla mediated resistance may or may not require other independent proteins like Rar1 and Rar2 which implies the presence of more than one independent race-specific resistance signalling pathways (Jørgensen 1996; Freialdenhoven et al., 1994). It is still not clear where MORCs fit in the signalling cascade, but from the phenotypic effects and biochemical assays its involvement in plant immunity is fairly obvious, even though a detailed investigation of these initial findings is necessary to explain a negative regulatory the barley MORCs might have in plant immunity.

4.3.2 Barley MORC2 knockdown enhances resistance to cereal pathogen *Fusarium graminearum*

Fusarium graminearum is a devastating pathogen of cereal crops leading to large worldwide crop losses and affecting grain quality by producing mycotoxins. Even though it is a widespread pathogen accounting for large economic losses, resistance mechanisms described so far have been ineffectual. A robust resistance mechanism against this pathogen is yet to be described. Resistant cultivars which confer disease resistance or tolerance to the toxin are not available currently and disease control through fungicide application is not cost efficient because the fungus develops resistance to the fungicide and new compounds with novel modes of actions have to be developed periodically. Additionally efficient fungicide application to cereal heads is difficult and factors that influence disease development is incomplete or not well understood (McMullen et al., 1997; Pirgozliev et al., 2003).

To test if the resistance mechanism conferred by barley MORCs is also effective against this head blight, ear and root rot causing necrotrophic pathogen, stable transgenic MORC lines knocked down for barley MORC2 #62 KD-*hvmorc2* L11.15 and L40.17 along with stable transgenic lines over expressing HvMORC2 #67 HvMORC2 OEx L27 and L30 were infected with *F.graminearum* and disease symptoms were analysed. Results showed reduced levels of Fusarium growth in the knockdown lines as observed visually and from organ measurements compared to wild type cultivar Golden Promise (table5, figures 14, 15 and 16). The overexpressor lines in contrast showed similar levels of disease symptoms or slightly more susceptibility compared to control (table5, figures 14, 15, and 16). Fungal root colonization demonstrated by quantitative PCR supported the biological effects observed, thus providing an additional proof for increased disease resistance to *F.graminearum* in HvMORC2 knockdown lines (figure 17). These results further substantiate the evidence that knockdown of MORCs in barley leads to increased disease resistance and that the effects observed in powdery mildew were not a one off finding. This further strengthens the view that while Arabidopsis MORC is a plant resistance factor, its homologs in barley might play a negative regulatory role. The exact mechanism of its function is still debatable. AtMORC1 was however shown to be involved in SAR and found to be necessary for systemic accumulation of salicylic acid and complete SAR development. Furthermore, in Arabidopsis, the salicylic acid pathway appears to be required for resistance and Arabidopsis SA signalling mutants, *npr1* and *eds11,* as well as the SA-deficient mutant *sid1* displayed increased susceptibility to leaf infection by *F. graminearum* (Makandar et al., 2010). This fits the

theory that Arabidopsis MORC mutants fail to accumulate systemic SA, are impaired in SAR and have increased disease susceptibility (Kang et al., 2014) even though disease resistance to Fusarium has not been studied yet. An accumulation of salicylic acid would mean suppression of jasmonic acid and ethylene pathways which are in turn required for resistance to necrotrophic pathogens in barley. Increased accumulation of methyl jasmonate (MeJA) an oxylipin belonging to the fatty acid pathway along with JA precursors linolenic and linolenic acids following *F. graminearum* challenge in the resistant barley genotype has led to the hypothesis that the JA pathway is the predominant defence signalling pathway operating in barley against *F. graminearum* (Kumaraswamy et al., 2011). If barley MORC has a role in SA accumulation, this could possibly explain increased resistance observed to Fusarium in HvMORC2 knockdown lines which might fail to accumulate SA. Then again, lack of SA would affect resistance to biotrophs such as the one observed against powdery mildew unless it is mediated through one of the resistance pathways independent of SA such as penetration resistance or papilla mediated resistance involving mlo proteins. Due to the lack of solid proof supporting this theory, we can only speculate the pathways MORCs might be involved in and the roles it might have in plant defense. It is also very unusual for a protein to be involved in resistance mechanisms to pathogens with completely different lifestyles such as necrotrophy and biotrophy. This might suggest that barley MORCs like its Arabidopsis counterparts might be a general factors in plant defense and might target overlapping mechanisms or pathways triggering plant defense reactions even though unlike Arabidopsis; barley MORCs might be negative regulators of plant defense.

4.3.3 Contrasting functions of barley and Arabidopsis MORCs

The factors contributing to contrasting function of barley and Arabidopsis MORCs still remain unclear. One possible reason could be that Arabidopsis is a dicotyledonous plant and barley is monocotyledonous and that MORCs might have contrasting functions and characteristics in these two broad sub-divisions within the plant kingdom. Recent results in the group of Daniel Klessig at Boyce Thompson Institute for Plant Research, Ithaca NY however contradict this hypothesis as MORCs from solanaceous crops potato and tomato both belonging to the dicotyledonous group of plants have contrasting biological phenotypes just as in barley and Arabidopsis (unpublished data, personal communication). A more probable explanation could be the structure of the protein itself or the cell environment in which the protein is expressed including its interaction partners required for proper protein folding and protein stability. This possibility was analysed by overexpressing HvMORC1 and

AtMORC1 in Arabidopsis dKO mutant knocked out for atmorc1 and atmorc2 and checking disease resistance/ susceptibility in these modified systems. The *atmorc1-1 atmorc2-1* dKO plants were shown to be more susceptible to avirulent *Pseudomonas syringae* pathovar *tomato* containing the *AvrRpt2* gene and that overexpression of AtMORC1 in dKO could complement the loss of function mutation and restore disease resistance to wild type levels (Kang et al., 2010). Overexpression of HvMORC1 however did not complement the susceptibility phenotype and the HvMORC1 expressing lines if anything were more susceptible to infection by avirulent pseudomonas (Langen et al., 2014). Additionally, initial studies indicate transient overexpression of AtMORC1 in #62 KD-*hvmorc2* lines enhances an already elevated resistance phenotype in these barley transgenic lines (data not shown). These results are a strong indication that the contrasting effects in barley and Arabidopsis are due to the respective proteins themselves as they seem to retain their properties (Arabidopsis MORC1 overexpression leads to resistance and barley MORC1 overexpression leads to susceptibility) regardless of the system in which they are expressed due to which they fail to restore each other's function. A detailed molecular and biochemical investigation to the protein properties and function may provide the key to the question of contrasting function of these proteins.

4.3.4 Barley MORC1 has DNA binding and endonuclease activities

Barley MORC1 is member of the GHKL ATPase superfamily along with the Arabidopsis MORCs. As expected for the members of GHKL ATPase superfamily and consistent with results of AtMORC1 and AtMORC6 (Kang et al., 2008, 2010) HvMORC1 exhibits ATPase activity (Langen et al., 2014). An additional functional domain identified as the putative endonuclease domain within the S5 fold (Kang et al., 2010; Langen et al., 2014) is less conserved in this group and a described feature of only some proteins like the bacterial MORC prototype MutL. AtMORC1 was previously shown to have endonuclease activity (Kang et al., 2012). A functional difference in this domain by means of its enzymatic properties could address the issue of contrasting function of MORCs in barely and Arabidopsis. Recombinantly produced HvMORC1 however exhibited Mn^{2+} dependent endonuclease activity as evidenced by conversion of supercoiled DNA to relaxed and linearized forms (Figure 19; Langen et al., 2014). The evidence suggests that enzymatic properties of HvMORC1 are not different from AtMORC1. However, it has to be mentioned here that the protein purification step, although done in three steps (materials and methods 2.8) did not result in highly pure proteins as few, faint additional bands were still detectable

after SDS-PAGE (data not shown). In simple terms, presence of *E.coli* protein contaminants co-purified cannot be excluded. That would suggest endonuclease activity might be rather a by-product of co-purified contaminants rather than HvMORC1 itself. An independent experiment where an empty plasmid was recombinantly expressed in *E.coli* and purified using the same protocol resulted in weak endonuclease activity (data not shown). To summarise, these results in biochemical assays suggest that the contrasting function of barley versus Arabidopsis MORC1 is not due to differences in enzyme activities, although contaminations and factors like purity of proteins influencing endonuclease activity cannot be completely overruled at the moment.

HvMORC1 like its Arabidopsis counterpart was also shown to bind DNA through surface exposed aromatic amino acids, excluding the possibility that DNA binding and the changes caused thereafter were responsible for the contrasting biological phenotypes observed in the two species (Langen et al., 2014). Subcellular localization, which might be crucial for determining the fate and biological functions of proteins, was studied by transmission electron microscopy and HvMORC1 like AtMORC1 seemed to localize in the nucleus and in the cytoplasm may be in the endosomes (Kang et al., 2010). The sub-population of HvMORC1 in the nucleus increased upon exposure to pathogen stimulus like treatment with the PAMP flg22 (Langen et al., 2014) consistent with involvement of MORCs in basal resistance mechanisms in barley. From all these results, we could speculate that MORCs are nuclear residing proteins which use their DNA binding ability and energy released by ATP hydrolysis to manipulate chromatin structure and to interact with other nuclear proteins either as monomers or as protein complexes. These interactions might be synergistic or mutually exclusive and might lead to biological effects observed.

4.4 CRISPR-Cas system for gene knockout

Biological studies with RNAi transgenic plants are highly reliant on the stability of knockdown construct and constantly high silencing efficiency of RNAi lines in successive generations. Contrary to conventional mutagenesis techniques where a gene function is completely abolished and irreversible in mutants, RNAi techniques have highly variable efficacies depending on the gene silenced, regions within specific genes and even within plants carrying identical constructs (Wang et al., 2005). Additionally, even after silencing using RNAi system, some amount of transcript is left over that might influence biological function or in some cases reduced transcript levels might not be enough to generate a

phenotype (Small 2007). Besides, the knockdown efficiency with identical constructs may range from anything between 0%-90% reduction in transcript levels that might make comparison of biological assays and data analysis difficult. From our own experience, T3 generation of stable transgenic plants silenced for HvMORC2 gene showed loss of silencing effect as observed in qPCR results (data not shown). The same lines were shown to have upto 45% reduction in relative transcript levels in T1 generation. The loss in silencing might be a concern if experiments are planned with this later generation of transgenic plants and might affect production, identification and use of homozygous lines altogether. A reason could be that HvMORCs might be necessary for gene silencing as in case of *C.elegans* where silencing of a MORC homolog resulted in loss of transgene silencing in this nematode worm. From our observations and using the current knowledge available from various literary sources, it is imperative that a more robust system for gene knockout is necessary to analyse gene functions and prevent trans-generational losses in silencing efficiency.

Keeping this in mind, the prokaryotic Type II CRISPR-Cas system was introduced as an alternative system for producing stable transgenic plants knocked out for desired genes. Another advantage of this technique is multiplexing or knocking out more than one gene simultaneously (Cong et al., 2013). Recent studies have shown the use of CRISPR-Cas system as an effective tool to silence genes in many eukaryotes, including plants (Shan et al., 2013; Miao et al., 2013 and Feng et al., 2013). Miao et al., (2013) and Shan et al., (2013) demonstrated an effective knock out of rice and wheat genes using CRISPR system but studies in barley are lacking. To deploy this system in barley, we cloned different constructs specifically targeting HvPDS and HvMORC1 genes in barley. CRISPR knockout constructs with three different TypeIII or rRNA polymerase promoters (rice U3 promoter, wheat U6 promoter and the *de novo* predicted barley U3 promoter) were produced (Materials and methods 2.9, Figure 20, 22 and 24) to compare the efficiencies of the three in generating knock outs in barley. The plasmids produced together with the best promoter system will be used in future studies to establish the CRISPR system for barley. The constructs produced might be used readily in transient transformation experiments using protoplasts followed by PCR and RE assay as described by Shan et al., 2013 to test the efficiency of these constructs and as a proof of concept that the system could be effectively used for gene knockout in barley. Constructs with highest mutation efficiency could then be used directly for producing stable transgenic plants as the cloned binary vector systems are ready for use in Agrobacterium mediated stable plant transformations. Alternatively, they could also be used

for biological assays using STARTs method, which allows assessment of gene function in transgenic root tissues within a short period of time (Imani et al., 2011). Either way, the system should be first tested using simple, straightforward approaches like protoplast transformations in transient systems and when found successful be established as an effective alternative to RNAi for gene silencing. If studies so far are any indication to go by, the future for this technology looks very promising. Despite the advancements and spur in studies using this technology published recently, some key aspects like off target effects still remain unclear. It is expected that more such studies would shed light on this topic and help establish CRISPR-Cas system as a technology for the future.

Summary

World agriculture today faces many challenges owing to global climate change and aberrant weather phenomenon. A concomitant result of this abiotic change has been the spread and increase of plant diseases and associated disease causing agents that has put world food security under serious threat, especially in developing countries. Under these challenging circumstances, alternatives to conventional crop protection strategies have gained worldwide attention in recent years. Our study here highlights one such strategy that depends on MORC gene family which is widely distributed throughout eukaryotes and in many plant species. Altered expression of MORCs affected plant resistance to pathogens wherein overexpression of HvMORC1 increased susceptibility to barley powdery mildew and RNAi-mediated silencing of HvMORC2 resulted in enhanced resistance to this biotrophic pathogen. Additionally, HvMORC2 silencing also confers basal resistance to necrotrophic pathogen *Fusarium graminearum*; a finding which might have interesting agricultural applications as it is considered to be a devastating cereal pathogen and robust broad spectrum resistance against Fusarium diseases are yet to be identified. The HvMORC protein is described to reside in the nucleus and is shown to have interesting biochemical properties such as DNA binding and endonuclease activity further fuelling speculation that MORCs have an important nuclear role such as chromatin remodelling that might contribute to different phenotypes observed. Finally, barley MORCs have contrasting function to their Arabidopsis counterparts. Even though they are closely related proteins with very similar structural domains and enzymatic properties, the Arabidopsis MORC seems to act as a positive regulator of plant defense mechanisms while barley MORC has a negative regulatory role in plant immunity. What is even more interesting, they failed to complement each other's function when expressed in reciprocal systems and retained their functions despite change in biological

system and their biochemical environment. These results let us speculate that the contrasting effects observed are species specific and might be the properties of the proteins themselves. A further, detailed molecular and biochemical analysis of these genes might offer a brighter insight into this exciting topic. Nevertheless, the MORC gene family has tremendous potential for agricultural applications as they have also been identified in other important cereal crops like rice, wheat (*Triticum aestivum*) and maize (*Zea mays*) a staple for about three quarter of world's population. Studies in some of the other plant systems would add valuable information to our current understanding of MORCs and help engineer resistance in economically important crop plants.

6. Zusammenfassung

Weltlandwirtschaft heute steht vor vielen Herausforderungen durch den globalen Klimawandel und anomale Wetterphänomen. Eine gleichzeitige Ergebnis dieser abiotischen Veränderung war die Ausbreitung und Zunahme von Pflanzenkrankheiten und die damit verbundenen Krankheitserreger, die Welternährungssicherheit ernsthaft bedroht gesetzt hat, vor allem in Entwicklungsländern. Unter diesen schwierigen Umständen haben Alternativen zu herkömmlichen Pflanzenschutzstrategien weltweite Aufmerksamkeit in den letzten Jahren an Bedeutung gewonnen. Unsere Studie unterstreicht hier eine solche Strategie, die auf MORC Gen-Familie, die überall in Eukaryonten und in vielen Pflanzenarten verteilt wird, hängt. Veränderte Expression von MORCs betroffen Pflanze Resistenz gegen Krankheitserreger, bei Überexpression von HvMORC1 erhöhte Anfälligkeit für Gerstenmehltau und RNAi-vermittelte Silencing HvMORC2 zu einer verstärkten Widerstand gegen diese Erreger biotrophe. Darüber hinaus räumt HvMORC2 Silencing auch basalen Resistenz gegen Fusarium graminearum nekrotrophe Erreger; eine Feststellung, die interessante Anwendungen in der Landwirtschaft haben könnte, da sie als zu einer verheerenden Getreide Erreger und robuste breites Spektrum Widerstand gegen Fusarium Krankheiten werden noch identifiziert werden können. Die HvMORC Protein beschrieben im Kern befinden und dargestellt interessante biochemische Eigenschaften, wie die DNA-Bindung und Endonuclease-Aktivität weiter zu Spekulationen, die MORCs eine wichtige Rolle Kern wie Chromatin-Remodeling, die zu verschiedenen beobachteten Phänotypen beitragen könnten. Schließlich, Gerste MORCs haben kontras Funktion, ihre Arabidopsis Kollegen. Auch wenn sie eng verwandten Proteinen mit sehr ähnlichen strukturellen Domänen und enzymatische Eigenschaften scheint die Arabidopsis MORC als positiver Regulator der pflanzlichen Abwehrmechanismen handeln, während Gerste MORC hat eine negative regulatorische Rolle in der Pflanzen Immunität. Was noch interessanter ist, konnten sie sich gegenseitig ergänzen, wenn Funktion im reziproken Systemen exprimiert und trotz Veränderung der biologischen Systems und ihrer biochemischen Umwelt behielten ihre Funktionen. Diese Ergebnisse lassen vermuten wir, dass die beobachteten Effekte kontras artspezifisch sind und möglicherweise die Eigenschaften der Proteine selbst zu sein. Eine weitere, detaillierte molekulare und biochemische Analyse dieser Gene könnte bieten eine bessere Einsicht in dieses spannende Thema. Dennoch hat die MORC-Gen-Familie ein enormes Potenzial für Anwendungen in der Landwirtschaft, wie sie auch in anderen wichtigen Getreide wie Reis, Weizen (Triticum aestivum) und Mais identifiziert worden (Zea

Mays) ein Grundnahrungsmittel für rund drei Viertel der Weltbevölkerung. Studien in einige der anderen Anlagensystemen würde wertvolle Informationen für unser gegenwärtiges Verständnis der MORCs hinzufügen und helfen Ingenieur Widerstand in wirtschaftlich bedeutenden Kulturpflanzen.

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8. Supplementary data

Appendix 1- Sequence information

HvMORC1 gene

>gi|571265242|emb|HG316119.1| Hordeum vulgare mRNA for CRT1-like GHKL ATPase (MORC1 gene), cultivar Golden Promise

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HvMORC2 Gene

>gi|571265244|emb|HG316120.1| Hordeum vulgare mRNA for CRT1-like
GHKL ATPase (MORC2 gene), cultivar Golden Promise
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Rice Phytoene desaturase

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Single guide RNA (SgRNA sequence)

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HvU3 promoter sequence (underlined)

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Appendix 2- Primer and CRISPR oligonucleotide sequences

Oligo name	Sequence (5'-->3')	Comments
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#7-SP HvUBir	TGTGCTTGTGCTTTTGCTTC	
#555 gr-HvCRT1b-2	TGGTCACTGAGCCAGCAACAACGTTACA	Test HvMORC2-kd
#484 gr-HvCRT2nr	GGAGCAACCATAGCATCCAT	
#24 FgTub-F	GGTCTCGACAGCAATGGTGTT	Fg colonization
#25 FgTub-R	GCTTGTGTTTTTCGTGGCAGT	
#26 JI_Hyg-F	TATCGGCACTTTGCATCGCG	Characterization of transgenic plants
#27 JI_Hyg-R	GATCGGACGATTGCGTCGCA	
pGY1fwd2	CGTTCCAACCACGTCTTCAA	
#40Ascl-TaU6	GGCGCGCCGACCAAGCCCGTTATTCTGA	CRISPR cloning
#41Sacll-TaU6	CCGCGGCCGCAAAAAAAGCACCGACTCG	
#703v Ubi deg 60-F	ACCCTCGCCGACTACAACAT	cDNA quality check
Ubi deg 60-R	CAGTAGTGGCGGTCGAAGTG	
#42Entry Bbs1 fwd	GCCCAGTCTTtCGACTGAGC	Point mutation of BbsI site in vector backbone for CRISPR cloning
#43Entry Bbs1 rev	GCTCAGTCGAAAGACTGGGC	
#46 EntryBbslfwd2	GAAAGGCCCAGTCTTTCGACTGAGCCTTTC	
#47 EntryBbslRev2	GAAAGGCTCAGTCGAAAGACTGGGCCTTTC	
#422 gr-T35s_F	GAGATTTTATTGAGAGCAGTAT	Colony PCR
#421 gr-T35s_R	GTGTGCTGATAAATACAAATAC	Colony PCR
#827 T7Prom_pET	TAATACGACTCACTATAGGG	Colony PCR
#828 T7Term_pET	GCTAGTTATTGCTCAGCGG	Colony PCR
#775v Ubi-intron_fwd2	TTTAGCCCTGCCTTCATACG	Colony PCR
C121-r	GTTGGGCGATCAGATTCTC	Colony PCR
C126-f	tcgtgaagaagaccgaggtt	Colony PCR
#516V nosT	ATTGCCAAATGTTTGAACGA	Colony PCR

Oligo name	Sequence (5'-->3')	Comments
#50OsU3b-seed1-f	ggcACAGTTAACTATTTGGAAGCTGG	Target- HvPDS
#51OsU3b-seed1-r	aaaCCCAGCTTCCAAATAGTTAACTG	Target- HvPDS
#52OsU3b-seed2-f	ggcAGCAAACAAATTCTGTACATTGG	Target- HvPDS
#53OsU3b-seed2-r	aaaCCCAATGTACAGAATTTGTTTGC	Target- HvPDS
#54OsU3b-seed3-f	ggcACTTTACCGGCGCCCTTGAATGG	Target- HvPDS
#55OsU3b-seed3-r	aaaCCCATTCAAGGGCGCCGGTAAAG	Target- HvPDS
#56HvU3-seed1-f	agcACAGTTAACTATTTGGAAGCTGG	Target- HvPDS
#57HvU3-seed2-f	agcAGCAAACAAATTCTGTACATTGG	Target- HvPDS

#58HvU3-seed3-f	agcACTTTACCGGCGCCCTTGAATGG	Target- HvPDS
#59TaU6-seed1-f	cttGCCGTGGGCTTCGTGCTCTGCAGG	Target- HvPDS
#60TaU6-seed1-r	aaacCCTGCAGAGCACGAAGCCCACGG	Target- HvPDS
#61TaU6-seed2-f	cttGAAAACACAGTTAACTATTTGG	Target- HvPDS
#62TaU6-seed2-r	aaacCCAAATAGTTAACTGTGTTTT	Target- HvPDS
#63TaU6-seed3-f	cttGACATGTCTTTAGCATGCAAGG	Target- HvPDS
#64TaU6-seed3-r	aaacCCTTGCATGCTAAAGACATGT	Target- HvPDS
#70 Os:MORC1seed1-f	ggcACAGGGGACTTCGACCGCGCGC GG	Target- HvMORC1
#71 Os:MORC1seed1-r	aaaCCCGCGCGCGGTCGAAGTCCCCTG	Target- HvMORC1
#72 Os:MORC1seed2-f	ggcATGGAGGAGGAATGGATCC TGAAGG	Target- HvMORC1
#73 Os:MORC1seed2-r	aaaCCCTTCAGGATCCATTCCTCCTCCA	Target- HvMORC1
#74 Os:MORC1seed3-f	ggcAGACAAATTATCATTGAGTACT GG	Target- HvMORC1
#75 Os:MORC1seed3-r	aaaCCCAGTACTCAATGATAATTTGTC	Target- HvMORC1
#76Hv:MORC1seed1-f	agcACAGGGGACTTCGACCGCGCGCGG	Target- HvMORC1
#77Hv:MORC1seed2-f	agcATGGAGGAGGAATGGATCCTGAAGG	Target- HvMORC1
#78Hv:MORC1seed3-f	agcAGACAAATTATCATTGAGTACTGG	Target- HvMORC1
#79Ta:MORC1seed1-f	cttGACAGGGGACTTCGACCGCGCGC GG	Target- HvMORC1
#80Ta:MORC1seed1-r	aaaCCCGCGCGCGGTCGAAGTCCCCTGT	Target- HvMORC1
#81Ta:MORC1seed2-f	cttGTTCAAGGATATTGAGAGCCATGG	Target- HvMORC1
#82Ta:MORC1seed2-r	aaaCCCATGGCTCTCAATATCCTTGAA	Target- HvMORC1
#83Ta:MORC1seed3-f	cttGACAAATTATCATTGAGTACTGG	Target- HvMORC1
#84Ta:MORC1seed3-r	aaaCCCAGTACTCAATGATAATTTGT	Target- HvMORC1

Appendix 3-Vector maps

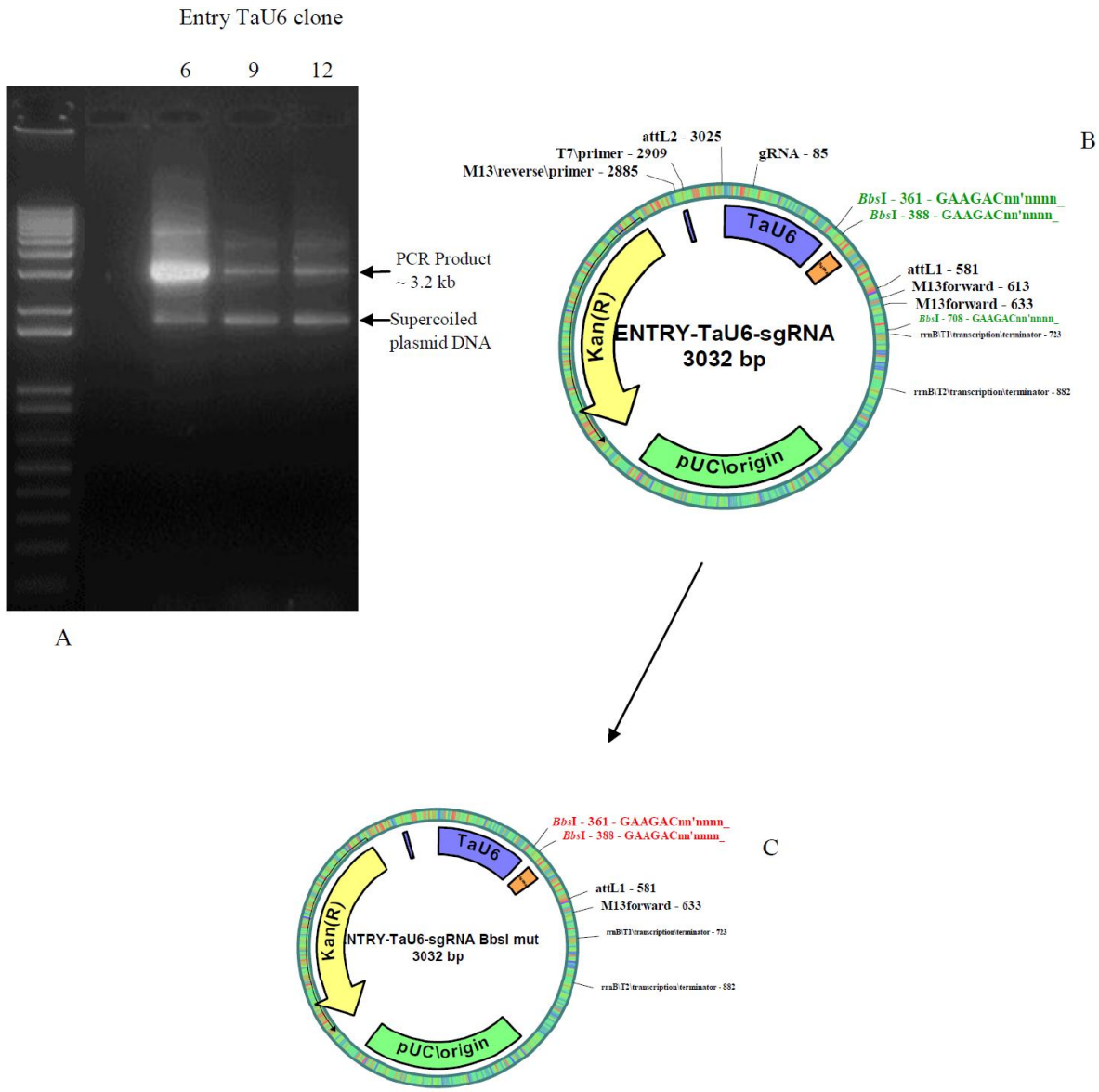
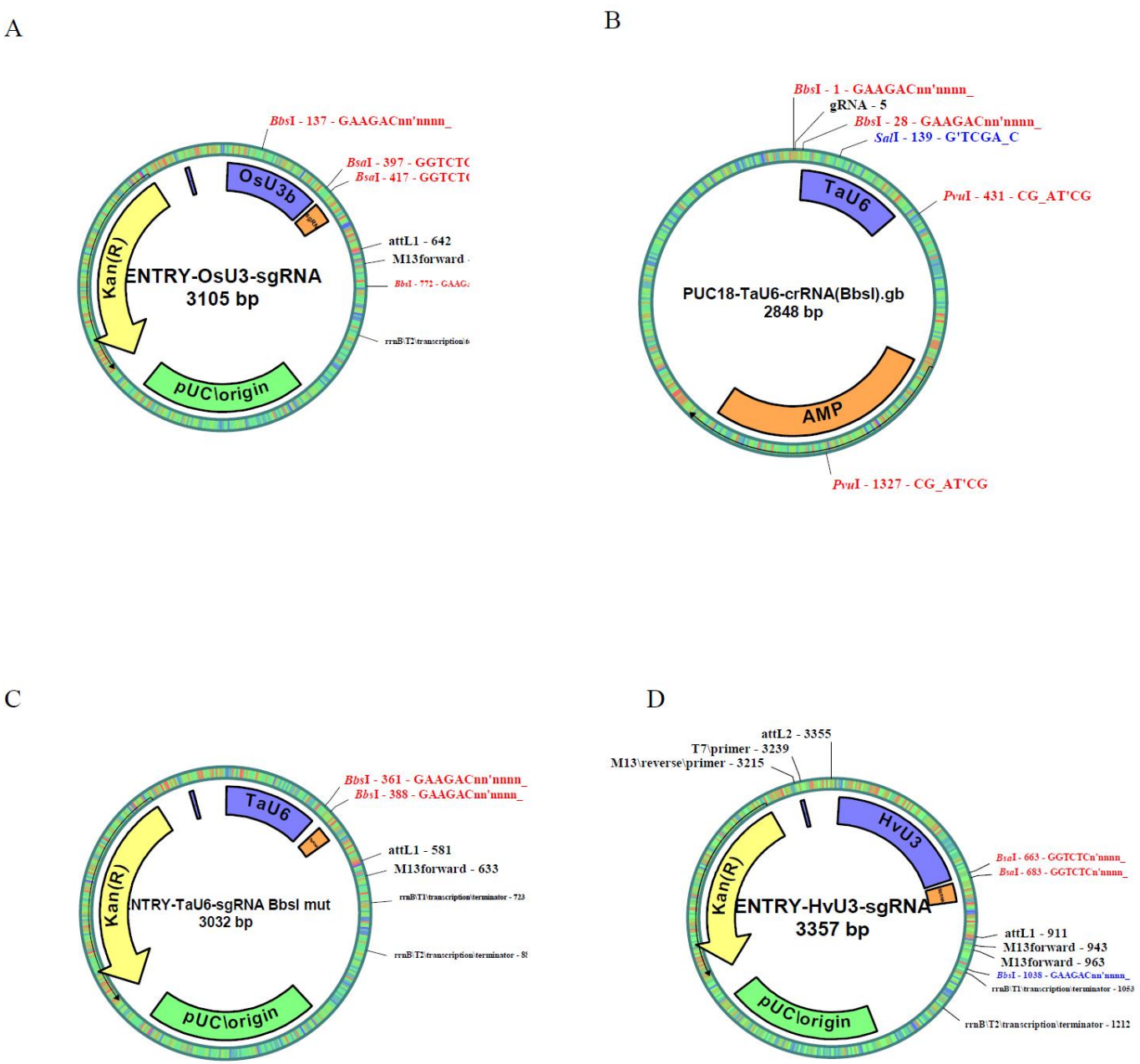


Figure1. PCR to point mutate BbsI site in the entry vector pEntry-TaU6-SgRNA. Primers flanking the BbsI site (#46 EntryBbslfwd2 and #47 EntryBbslRev2) were used to create the point mutation by a PCR reaction which gives a product of ~3 kb (A). The PCR product is digested using DpnI which specifically digests only methylated sequences and removes the template



plasmid (B). The PCR product is then transformed into competent cells, plasmids extracted and sequenced to confirm the point mutation.

Figure.2 Entry vectors used in CRISPR cloning. A. pEntry-OsU3SgRNA was used without any modifications and served as the entry vector. B. Wheat U6 promoter was cloned from pUC18 Tau6-gRNA using primers #40Ascl-TaU6 and #41Sacll-TaU6 into entry vector to give rise to Entry-TaU6-SgRNA (C). Additional BbsI sites were removed from this vector backbone using the method described in figure1 D. HvU3 sequence was transferred to the entry vector using AscI and SacII digestion of the HvU3-pUC57 plasmid and ligation to the entry plasmid cut open using the same restriction enzymes.

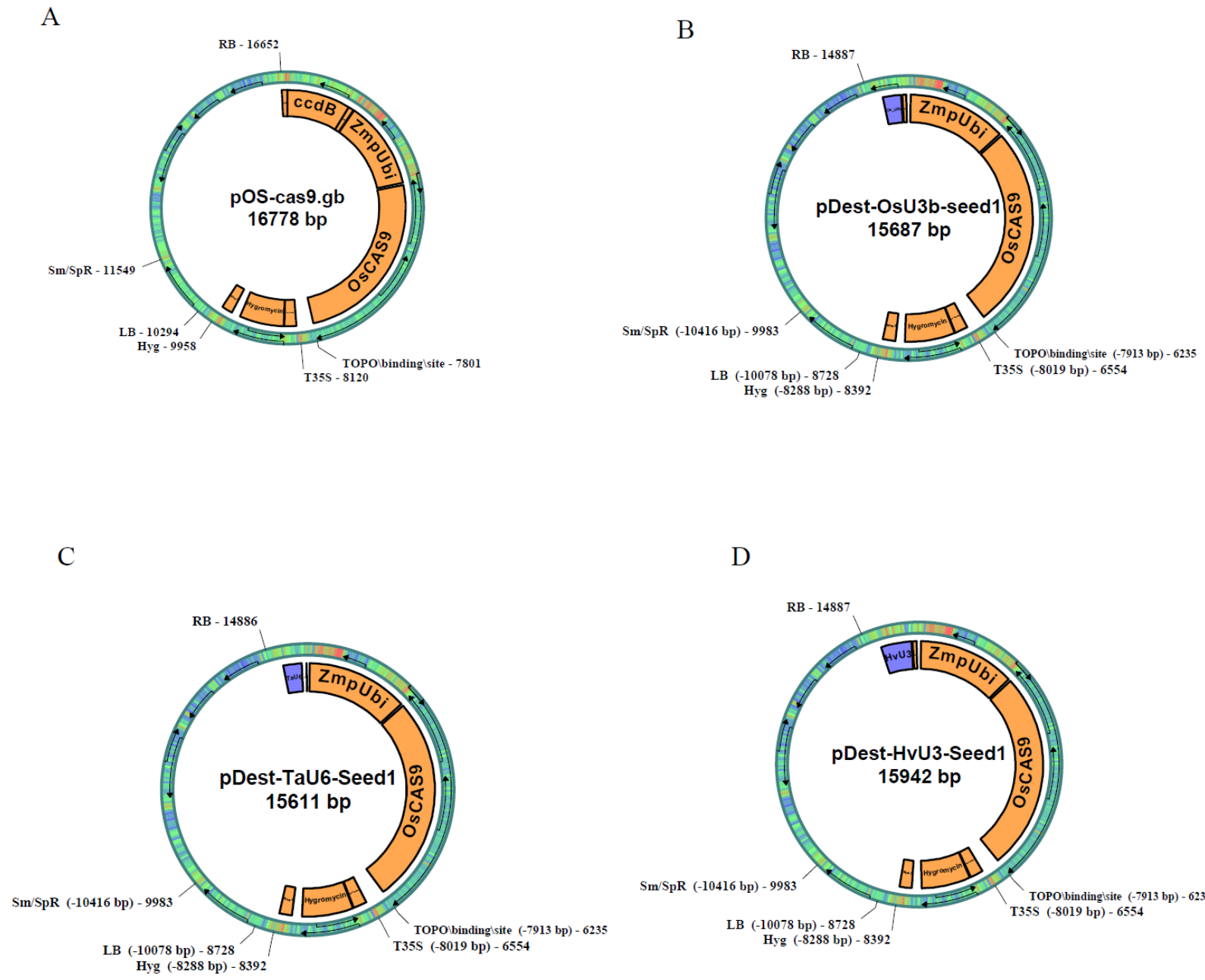


Figure3. Destination vectors A. Original destination vector pH-Ubi-cas9-7. The non-coding CRISPR-RNA + Promoter cassette was transferred to the destination vector by LR reaction using Gateway® LR Clonase™ II Enzyme Mix to give rise to either pDest-OsU3 (B), pDest-TaU6 (C) and pDest-OsU3 (D).

Appendix 4- Seed usage and characterization

Hygromycin test (+ve/-ve)	#62 MORC2 kd	seed count
	L1	500
	L2	400
	L3	150
	L4	250
+	L5	450
+	L6	200
+	L7	none
	L8	280
+	L9	400
+	L10	40
+	L11	450
+	L12	50
	L13	150
	L14	none
	L15	300
	L16	250
	L17	400
	L18	250
	L19	100
	L20	none
-	L21	450
	L22	50
-	L23	30
	L24	100
	L25	300
	L26	300
+	L27	150 + 200
-	L28	300
+	L29	350
-	L30	170
+	L31	500
+	L32	350
	L33	none
	L34	50

#64 MORC1 OE	seed count
1	120
2	180
5	250
6	220
7	30
8	150
9	100
11	100
12	100
13	200

Segregation in T1 generation

Line	Azygous	Transgenic
#62 MORC2kd L11	5	14
#62 MORC2kd L40	8	22
#62 MORC2kd L55	7	22
#64 MORC1OE L5	8	19
#64 MORC1OE L8	6	22
#64 MORC1OE L13	13	14

Lines tested in biological assays

	L35	150
+	L36	300
	L37	50
+	L38	150
	L39	250
+	L40	450
	L41	none
	L42	400
	L43	30
+	L44	30
	L45	350
+	L46	100
+	L47	200
	L48	300
	L49	400
+	L50	50
+	L51	150
+	L52	120
	L53	30
	L54	30
+	L55	450
	L56	300
	L57	200 + 250
	L58	250
	L59	100
+	L60	120
	L61	250
	L62	250 + 400
	L63	280
	L64	250
	L65	150
	L66	100
	L68	20
	L69	100
+	L70	250
	L72	150
	L73	150
	L74	250
-	L75	100
	L76	450

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

Declaration

I declare that the dissertation and the points shared here are completely my own work, written without any illegitimate or immoral third party help and entirely using the materials as stated in my thesis.

I have indicated in the text the sources of external material taken from published articles and books, be it word for word or in substance and where I have made statements based on personal communication with concerned people or oral information given to me.

Throughout the duration of my work as described in the dissertation, I have adhered to good scientific practices stipulated in the “statutes of the Justus Liebig University Giessen for the safe guarding of Good Scientific Practice”

Signature:

Date:

Acknowledgements

Most people say that it is the intellect which makes a great scientist. They are wrong: it is character. These are the words of the greatest scientist mankind has ever seen- Albert Einstein. Keeping this in mind I started on my character building journey which was riddled with hardships and innumerable experiences- good and bad. Throughout this journey, there have been few people who have played a key role in my life whom I would like to thank here.

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To conclude, thanks everyone for being part of my wonderful journey and I hope at least few of you'll be around till the final destination!!