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Philosophiae Doctor (PhD), Thesis 2016:32

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Microdochium nivale in perennial grasses: Snow mould resistance, pathogenicity and genetic diversity

Microdochium nivale i flerårig gras: Resistens mot snømugg, patogenitet og genetisk diversitet

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List of papers

I. Abdelhalim M., Rognli O.A., Hofgaard I.S., Østrem L. and Tronsmo A.M. **Snow mould resistance under controlled conditions and winter survival in the field in populations of perennial ryegrass, meadow fescue and Festulolium are partly dependent on ploidy level and degree of northern adaptation**. 2016. Canadian Journal of Plant Science. (In press, accepted on 23/12/2015).

II. Abdelhalim M., Brurberg M.B, Hofgaard I.S. and Tronsmo A.M. **Pathogenicity, host specificity and genetic diversity in isolates of *Microdochium nivale* and *M. majus***. (Manuscript)

III. Kovi M.R., Abdelhalim M., Ergon Å., Kunapareddy A., Asp T., Tronsmo A.M. and Rognli O.A. Global transcriptome changes in perennial ryegrass during early infection by pink snow mould. (Manuscript)

Summary

Forage production of perennial grass species in the northern hemisphere is limited by their winter survival ability. The predicted future changes in the environmental conditions constitute both opportunities and challenges for grass production. Overwintering diseases caused by snow mould fungi such as *Microdochium nivale* are one of the limiting factors for grass production in Scandinavia. Therefore, to reduce the risk of snow mould attack under future climates, with longer and warmer autumns, the role of cold hardening independent resistance to snow mould needs to be well understood. Snow mould resistance in non-hardened plants of cultivars and breeding populations of *Festulolium*, inoculated under controlled conditions with an aggressive *M. nivale* isolate, was associated with the ploidy level of the entries. Tetraploid entries of *Festulolium* had a significantly better resistance to snow mould than diploid entries in three out of four experiments. In addition, winter survival of cultivars and breeding populations of *Festulolium* and *Lolium perenne* in inoculated field experiments was associated with the degree of northern adaptation of the entries. Hence, cultivars and breeding populations that had been exposed to natural selection in northern Norway (above 65° N) showed good levels of winter survival.

Host specialization within *M. nivale* isolates should be taken into consideration when screening to identify resistant plant materials is performed. Therefore, it will be beneficial to improve our understanding of the genetic and phenotypic (pathogenicity) differences between *M. nivale* isolates for future snow mould resistance screening. For that reason, a pathogenicity test of 15 *M. nivale* isolates and two *M. majus* isolates was conducted on four different grass cultivars of *Lolium perenne* and *Festulolium* hybrids. Large variation in pathogenicity was detected between the *M. nivale* isolates, and isolates from grasses were more pathogenic than isolates from cereals. The genetic diversity of nearly 40 *M. nivale* and *M. majus* isolates was studied by sequencing four different genetic regions; Elongation factor-1alpha (*EF-1α*), Beta-tubulin, RNA polymerase II (*RPB2)* and Internal transcribed spacer (ITS). Sequence variation was found between *M. nivale* isolates from different host plants (grasses vs. cereals) and between isolates from different geographic regions (Norway and UK vs. North America). Sequence results from the *RPB2* and β-tubulin genomic regions were more informative than those from the ITS and *EF-1α* genes.

In order to improve the process of screening for resistant plant materials there is a need for fast and reliable methods. Visual scoring of snow mould symptoms (based on the proportion of dead tillers to the green tillers) was correlated with the amount of *M. nivale* DNA in plant tissue. However, genotypes with severe symptoms and high content of *M. nivale* DNA still had good regrowth.

Understanding the nature of resistance to snow mould that is independent of cold acclimation will help to improve and develop resistant cultivars with improved adaptation to the predicted future climate. The global transcriptome analysis of two *Lolium perenne* genotypes (one snow mould resistant and one susceptible) during early stage of infection (four days after inoculation) showed that non-cold hardened plants are able to initiate the defence pathway against snow mould pathogen via regulation of defence response genes. The resistant genotype showed higher expression levels of genes coding for several pathogen related proteins such as PR-1, PR-2, PR-3, PR-5, PR-13 and PR-14. In addition, the snow mould resistant genotype showed activation of the PAMP trigger immunity (PTI) pathway, especially by the up-regulation of the expression levels of calcium-dependent protein kinase *CDPK*, respiratory burst oxidase *Rboh* and calcium-binding protein CML *CaM/CML*. Furthermore, the resistant genotype showed high transcription levels of several WRKY genes such as WRKY 70 and WRKY 75. Therefore, it is expected that the up-regulation of these genes will lead to the activation of the salicylic acid pathway. The candidate genes identified in the transcriptome study constitute potential molecular marker resources for breeding perennial ryegrass cultivars with improved resistance to pink snow mould.

Key words:

Festuca pratensis, Festulolium, Lolium perenne, Microdochium majus, Microdochium nivale, quantitative polymerase chain reaction (qPCR), pathogenicity, genetic diversity, transcriptome, pink snow mould resistance**.**

Sammendrag

På den nordlige halvkule er fôrproduksjonen av flerårige eng- og beitevekster begrenset av overvintringsevnen til plantene. De predikerte endringene i framtidige klima kan medføre både muligheter og utfordringer i grasproduksjonen. Overvintringssjukdommer forårsaka av sopper som *Microdochium nivale* er en av de begrensende faktorene for grasproduksjonen. For å redusere risikoen for overvintringssjukdommer i det predikerte framtidige klimaet, med lengre og varmere høster, er det viktig å forstå betydningen av kuldeherdings-uavhengig resistens mot disse sykdommene. Resistens mot overvintringssopp i ulike populasjoner av uherda *Festulolium* under kontrollerte betingelser var assosiert med ploidinivå. Tetraploide *Festulolium* hadde signifikant bedre resistens enn diploide populasjoner i tre av fire forsøk. Vinteroverlevelse av ulike populasjoner av *Festulolium* og *Lolium perenne* i felt var assosiert med graden av nordlig tilpasning hos populasjonene. Populasjoner som hadde vært utsatt for naturlig seleksjon i Nord-Norge (over 65° N) hadde god overvintring.

Det bør tas hensyn til vert-spesialisering blant *M. nivale*-isolater når man tester plantemateriale for resistens. Det vil derfor være nyttig å forbedre vår forståelse av genetiske og fenotypiske (patogenitet) forskjeller mellom *M. nivale*-isolater for framtidig testing av plantemateriale. Av den grunn ble 15 *M. nivale*-isolater og to *M. majus*-isolater testet for patogenitet på fire ulike sorter av *Lolium perenne* og *Festulolium.* Det var stor variasjon mellom *M. nivale*-isolatene. *M. nivale*-isolater fra gras var mer patogene enn *M. nivale*-isolater fra korn. I tillegg ble den genetiske diversiteten blant nesten 40 *M. majus* og *M. nivale*-isolater studert ved å sekvensere fire ulike genetiske områder, elongation factor-1alpha (*EF-1α*), β-tubulin, RNA polymerase II (*RPB2)* og internal transcribed space (ITS). Det var sekvensforskjeller forskjeller mellom *M. nivale*-isolater fra ulike vertplanter (gras vs. korn) og ulike geografiske områder (Norge and Storbritannia vs. Nord-Amerika). Sekvensene fra *RPB2*- og β-tubulin var mer informative enn de fra ITS og *EF-1α.*

For å forbedre prosessen med å velge ut resistent plantemateriale er det behov for raskere og mer presise metoder. Visuell scoring av symptomer på overvintringssopp (basert på andel døde skudd i forhold til grønne skudd) var korrelert med mengde *M. nivale* DNA i plantevevet. Likevel var det noen genotyper som hadde store symptomer og mye *M. nivale* DNA samtidig som de hadde god gjenvekst.

En forståelse av den type resistens mot overvintringssopp som er uavhengig av kulde-herding vil bidra til å forbedre og utvikle resistente sorter tilpasset de forventede klimaendringene. Transkriptom-analyse av to *Lolium perenne*-genotyper (en resistent og en mottakelig) på et tidlig infeksjonsstadium (fire dager etter inokulering) viste at uherda planter kan sette i gang forsvarssystemet mot overvintringssopp. Den resistente genotypen hadde høyere ekspresjon av gener for flere patogenese-relaterte proteiner som for eksempel PR-1, PR-2, PR-3, PR-5, PR-13 og PR-14. Hos den resistente genotypen var signalveien for "PAMP Triggered Immunity" (PTI) aktivert, spesielt var "Calcium-Dependent Protein Kinase" (*CDPK*), "Respiratory burst oxidase" (*Rboh*) og "Calcium-binding protein CML" (*CaM/CML*) oppregulert. Den resistente genotypen hadde også høyere ekspresjon av flere WRKY-gener, som for eksempel WRKY 70 og WRKY 75. Det er antatt at oppregulering av disse genene vil føre til aktivering av salisylsyre-signalveien. Kandidatgenene som er identifisert i transkriptomstudiet utgjør en potensiell ressurs for å utvikle molekylære markører for foredling av sorter av flerårig raigras med forbedret resistens overfor snømugg..

Nøkkelord:

Festuca pratensis, Festulolium, Lolium perenne, Microdochium majus, Microdochium nivale, kvantitativ PCR (qPCR), patogenitet, genetisk diversitet, transkriptom, snømugg

1. Introduction

1.1 Forage production and climate change

Grasslands are considered as one of the largest ecosystems in the world (Reheul et al. 2010), covering 40.5 % of the world's terrestrial area (excluding Greenland and Antarctica). Ryegrasses, such as perennial ryegrass (*Lolium perenne* L.) and Italian ryegrass (*Lolium multiflorum* Lam.), are the most widely used grass species in the temperate zone (Humphreys et al. 2010), especially in Europe (cover 23% of the grassland). The genus *Lolium* is a member of the grass family *Poaceae*, and can be divided into two main groups based on pollination behaviour (Humphreys et al. 2010). One group is the inbreeding species, *L. temulentum* and *L. remotum*, which are mainly considered weeds in cereal crops. The other group represents the outbreeding species and the most economically important such as *L. multiflorum* (Italian and Westerwolths ryegrasses) and *L. perenne* (perennial ryegrass).

Perennial ryegrass *L. perenne* is a naturally cross-pollinated species with high degree of self-incompatibility (Humphreys et al. 2010). Perennial ryegrass is diploid with 7 pairs of chromosomes (2n=2x=14) with a relatively large genome (2,068 Mb) with 76% of the genome estimated to be repetitive sequences (Byrne et al. 2015). The Mediterranean region is likely the origin of perennial ryegrass, and probably it was spread across Europe and North Africa as a weed of cereal crops by farmer migrations (Kellogg 2001).

Other forage grass species, such as fescues, are important as well, and are important components of intensively managed grasslands. Fescues are mostly cultivated in the temperate regions, especially in the northern hemisphere (Rognli et al. 2010). Meadow fescue (*Festuca pratensis* Huds.) and tall fescue (*Festuca arundinacea* Schreb.) are the most common broad leaved fescues used in forage production (Rognli et al. 2010). Meadow fescue is a diploid outbreeding species (2n=2x=14), while tall fescue is an allohexaploid species (2n= 2x=42) (Rognli et al. 2010). Meadow fescue is more suitable for intensive grazing system; besides it is more prevalent at higher latitude than tall fescue (Rognli 2013). Based on the geographic origin, tall fescue cultivars can be divided to two main groups, Continental (originating from Central and Northern Europe, more winter-hardy) and Mediterranean (originating from Southern Europe and North Africa, less winter hardy and show summer dormancy) (Rognli et al. 2010). Furthermore, the advantages of using meadow fescue cultivars are to increase tolerance to abiotic and biotic stress, and adaptation

to grazing and frequent cutting (Rognli 2013). Meadow fescue and tall fescue have been used extensively during the last 30 years to develop *Festulolium*, i.e. hybrids between ryegrasses and fescues (Rognli et al. 2010). The aim has been to combine the high productivity and forage quality of ryegrasses with the high level of adaptation to environmental stresses in fescues. *Festulolium* hybrids have been developed and are being grown (Pociecha et al. 2009).

Cultivated grasslands for mowing and pasture are one of the most important sectors in Norwegian agricultural production, covering 65% of Norway's cultivated land (Fig. 1. Source: Statistics Norway, http://www.ssb.no/a/english/kortnavn/jordbruksareal_en/tab-2012-11-26-03-en.html , accessed 02 September 2015). The total area of productive of grassland in Norway increased by 16% from 2013 to 2014 (Statistics Norway, https://www.ssb.no/en/jord-skog-jakt-og-fiskeri/statistikker/jordbruksavling/aar/2015-02-09#content, accessed 02 September 2015).

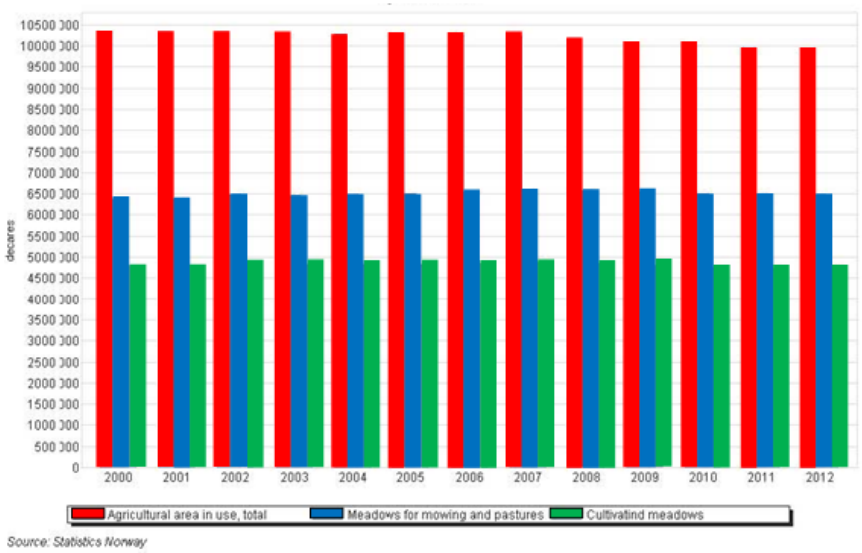

Fig.1. Development of the total agricultural area in use in Norway (decares), the area of meadows for mowing and pastures, and cultivated meadows from 2000 to 2012.

Overwintering plants face several challenging conditions during winter and early spring such as sub-zero temperature exposure, flooding, ice encasement, snow mould attack and soil heaving (Gusta et al. 2009). Moreover, plant adaptation to winter conditions is largely dependent on regional environmental conditions, therefore winter adapted plants in one region may not be winter adapted in another region (Larsen 1994). Winter survival of grasses is a complex trait, it combines tolerance to several stress factors, such as freezing, ice encasement, hypoxia and low temperature fungi (Bertrand et al. 2009; Ergon et al. 1998; Humphreys 1989). One of the major challenges for grass production in Norway is winter survival, due to its effect on yield and to the high cost of re-establishing meadows after winter kill (Thorsen & Höglind 2010).

Using different scenarios of greenhouse gas emissions, the Intergovernmental Panel on Climate Change (IPCC) reported that global temperature will be increasing during the next 100 years by 1.4 to 5.8 °C and about 4 °C in the northern hemisphere (IPCC 2007). Therefore it is expected that the temperate zone may move northwards by 500 to 800 km (IPCC 2007; IPCC 2012). The effects of climate change on agricultural production in the Nordic region can be characterised by an earlier start of the growing season in the spring and prolonged growth during autumn, with shorter suitable time for cold acclimation (Carter

2008). Hence, it is expected that the length of the growing season (with mean daily temperature of 5 to 7 °C) will increase by one to two months (Peltonen-Sainio et al. 2008; Roos et al. 2011). Prediction models based on Norwegian conditions show that temperature will be increased during all seasons (Thorsen & Höglind 2010); also the number of growing degree days (the annual sum of degree days above 5 °C) are expected to increase by 100 to 200 ° (Hanssen-Bauer et al. 2003). Forage grass cultivars used today at higher latitudes are adapted to long day light conditions. Even if the temperature might increase under future climate, cultivar productivity might not change since light conditions will remain the same (Rapacz et al. 2014; Uleberg et al. 2014).

In general, grass plants under Norwegian conditions start to cold acclimate at around 10 °C during early autumn and through winter increase their winter hardiness (Larsen 1994). Under future climates, increased temperature and short day length in autumn, combined with low light intensity, can reduce the hardening period up to 20 days, and that will reduce the plant's ability to cold harden (Thorsen & Höglind 2010; Uleberg et al. 2014). The effect of increased autumn temperature on cold acclimation can vary between forage crop species; timothy and perennial ryegrass plants are more sensitive than red clover (Rapacz et al. 2014). Moreover, insufficient cold hardening will increase the risk of spring frost injuries in the coastal regions of Norway, also the risk of ice encasement injury will increase in the regions with rapid decline during the period of snow cover, such as in Tromsø (69°40′ N, 18°56′ E) in Northern Norway (Thorsen & Höglind 2010). Drought can also affect grass production in Norway, especially if evaporation during summer increases, resulting in reduced growth of early cultivars (Olesen & Bindi 2002; Uleberg et al. 2014). Although climate changes may have positive effects on agricultural production in the northern hemisphere due to the increased precipitation and higher temperatures (Murray & Gaudet 2013; Olesen & Bindi 2002; Uleberg et al. 2014); new challenges may face the currently used cultivars in relation to their degree of adaptation to the new climatic conditions.

1.2 Biotic stress and winter survival.

Forage grasses growing in temperate and sub-arctic regions face harsh winter climates and frequent exposure to lethal low temperatures (Gaudet et al. 1999). However, deep snow cover acts as an insulation layer protecting the plants from winter injury by maintaining soil temperature between 0 and -10° C (Bruehl 1982). Snow cover creates special microclimatic conditions (dark, cold and humid), which reduce photosynthesis and plant metabolism. Moreover, snow cover provides a suitable environment for low temperature fungal pathogens. Psychrophilic or low temperature fungi can cause severe damages to plants under snow cover. Snow mould is one of the main diseases occurring under such nival environment. Several fungal spices cause snow mould under different winter conditions (Tronsmo 2013).

The northern hemisphere weather conditions during winter play an important role in defining the main snow mould pathogen (Gaudet et al. 1999; Tronsmo 2013). In regions with snow cover lasting longer than 200 days on frozen soil, the main snow mould pathogen is *Sclerotinia borealis* (Bubak and Vleugel), *Typhula ishikariensis* Imai, and low temperature basidiomycetes (LTB) (Gaudet et al. 1999; Nissinen 1996). In maritime regions, where snow cover last around 70 days on non-frozen soil, other snow mould pathogen such as *Microdochium nivale* (Fr.) Samuels and Hallet, *Typhula incarnata* Lasch ex Fr., and *Pythium iwayami* S. Ito. Hirane are more common (Årsvoll 1975; Bruehl 1982; Gaudet et al. 1999; Tronsmo 2013). The presence of snow cover gives better conditions for snow mould pathogens compared to other pathogens, which remain dormant under snow. Årsvoll (1975) identified more than 30 fungal species on grasses after snow melt, but most of these fungi were mainly inactive under snow cover.

Cold hardening is one of the most complicated physiological changes in plants (Gaudet et al. 1999). During cold hardening different plant species shows several physiological changes, which are mainly characterized by reduction in growth, height, leaf surface area and relative water content, and by increase in cytoplasmic content (Gaudet et al. 1999; Krol et al. 1984; Yoshida et al. 1998). Under cold hardening conditions, resistance to snow mould is achieved rapidly, while freezing tolerance requires longer time and is achieved gradually (Gaudet et al. 1999) . Snow mould resistance in winter wheat could be achieved after one or two weeks under cold hardening conditions (Nakajima & Abe 1996),

while high level of freezing tolerance requires more than 6 weeks of cold hardening (Gaudet 1994). Moreover, cold harden plants can lose freezing tolerance faster than snow mould resistance when they are exposed to warm temperatures (Tronsmo 1984). In addition, light is considered to be an important factor in order to achieve snow mould resistance during cold acclimation, but it is not necessary for achieving freezing tolerance (Årsvoll 1977; Gaudet et al. 1999). The relation between freezing tolerance and snow mould resistance is still unclear, in order to understand this relation, especially in forage grasses, further research is needed.

1.3 Biotic stress and climate change.

Due to the high level of complexity of the plant-pathogen interactions, it is difficult to estimate the effect of climate changes on disease development. Hence, one stress factor can eliminate the effect of another factor, such as high temperature and high ozone level on barley powdery mildew (Roos et al. 2011). The predictions of the changes in the future environmental conditions in the Nordic region show that the growing season will be prolonged, therefore some diseases may find more favourable conditions (Fågelfors et al. 2009; Peltonen-Sainio et al. 2008; Roos et al. 2011). Diseases such as brown and yellow rust on wheat are expected to increase. However, other diseases such as powdery mildew and septoria leaf blotch, are expected to decrease due to the expected drier summers (Roos et al. 2011). Also the predicted increase in precipitation combined with increased temperature can augment the damage caused by soilborne pathogens, such as *Verticillium* wilt, take-all and club root diseases (Evans et al. 2009; Roos et al. 2011). Other diseases are expected to spread over new areas, such as potato late blight which is expected to spread towards the north (above 65° N) as the spring becomes warmer and more humid (Fågelfors et al. 2009; Widmark et al. 2007).

1.4 Snow mould caused by *Microdochium nivale*.

1.4.1 Symptoms and disease cycle

M. nivale mainly attacks the plants under snow cover during winter or under humid and cold conditions during autumn and early spring. When the snow cover melt, disease symptoms appears as patches of pinkish-white mycelium covering the infected plants (Fig. 2), and after drying the dead leaves form a compressed paper-like layer (Hofgaard 2003; Tronsmo et al. 2001). During the growing season, infections by *M. nivale* can vary from water soaked leaves with pinkish mycelium to leaf lesions with light brown centre and dark brown margins (Tronsmo 2013). The fungus can cause other diseases, such as *Fusarium* patch on turf grasses, and on cereals it can cause stem rot, leaf blotch and *Fusarium* Head Blight (FHB) (Simpson et al. 2000; Tronsmo et al. 2001; Tronsmo 2013). The dispersal of *M. nivale* occurs by conidial spores, mycelium and ascospores (Tronsmo 2013); inoculum can also spread by seeds or soil (Fig. 3).

Fig. 2. Attack of *M. nivale* on *Festulolium* breeding populations in late April at Bodø, Northern Norway (67° 17′ N). (Photos by A. Larsen)

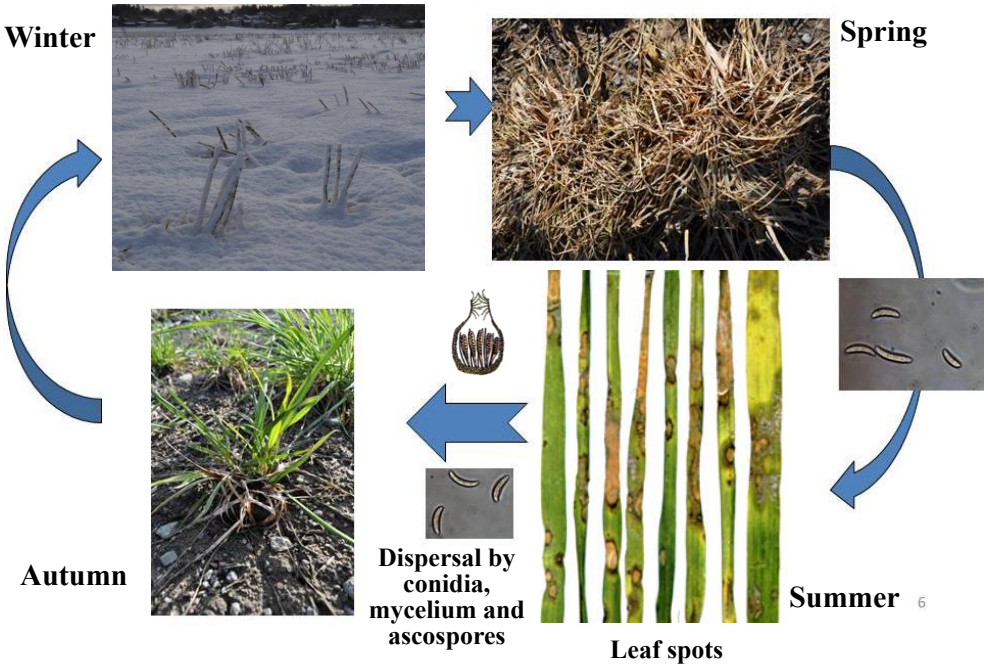

Fig. 3. The life cycle of *M. nivale* on grasses (Photos by M. Abdelhalim, adapted from (Tronsmo 2013), leaf spot photo was a courtesy of J. Drew Smith)

1.4.2 The pathogen (host preference and genetic diversity).

The snow mould pathogen was first described by Fries (1825) under the name *Lanosa nivalis*. The fungus was later classified under the genus *Fusarium* as *F. nivale* Ces. ex Berlese & Voglino (1886). Wollenweber and Reinking (1935) divided the species in two varieties, *F. nivale* var. *majus* and *F. nivale* var. *nivale*. Gams and Müller (1980) re-classified the fungus as *Gerlachia nivale* due to the absence of the conidial foot cell. Later Samuels and Hallett (1983) synonymised the genus *Gerlachia* with the genus *Microdochium*. Based on the DNA sequences of the elongation factor 1, Glynn et al. (2005) suggested that var. *majus* and var. *nivale* should be considered as separate species. Until now, fungal conidial morphology has been used as the main method of identification in most studies of *M. nivale* and *M. majus* (Glynn et al. 2005; Hofgaard et al. 2006; Jewell & Hsiang 2013). The differentiation between *M. majus* and *M. nivale* has mainly been based on conidial size (Fig. 4). The average size of *M. majus* conidial spore is 4.2 - 6 µm width and 13 - 33 µm length, while the width of *M. nivale* conidial spores are less than 3.8 µm with lengths 8 - 27 µm with 1 to 3 septa (Glynn et al. 2005). In addition, some studies use molecular identification using PCR (Polymerase Chain Reaction) techniques as an additional tool confirming the results (Hayashi et al. 2014; Jewell & Hsiang 2013; Nielsen et al. 2013)

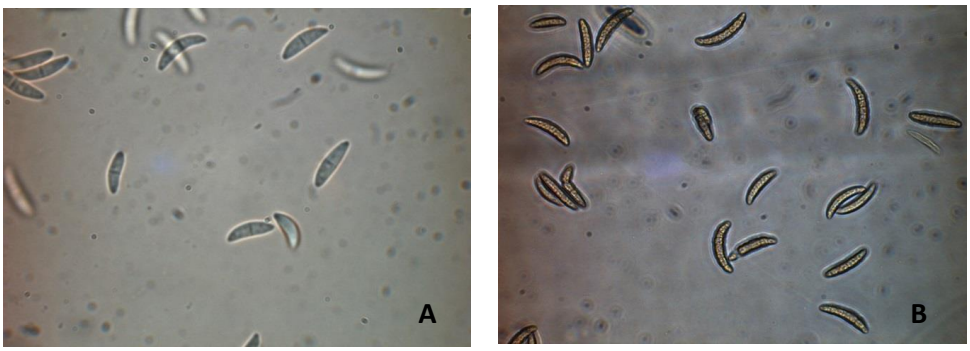

Fig. 4. Conidial spores of *M. nivale* (A), and *M. majus* (B). (Photos by M. Abdelhalim)

The sexual stage of the fungus (teleomorphs) was classified first as *Calonectria nivalis* Schaffnit (Tronsmo et al. 2001). The genus was later reclassified as *Griphosphaeria* and then to *Micronectriella* by Booth (1971) due to the production of darkly-pigmented perithecia. Lately, the recognized teleomorph names for *M. nivale* and *M. majus* were

Monographella nivalis (Gams & Müller 1980). Most of the recent publications use the asexual stage name (anamorph) *M. nivale* and *M. majus* when referring to the two species. Production of ascospores has not been observed under natural conditions; however, Litschko and Burpee (1987) found that the production of ascospores of *M. majus* was possible under laboratory conditions.

The difference between var. *majus* and var. *nivale* (*M. nivale* and *M. majus*) has been observed by several researchers, and has been documented for several aspects such as host preferences (Diamond & Cooke 1997; Hofgaard et al. 2006; Lees et al. 1995; Mahuku et al. 1998; Simpson et al. 2000), morphology (Litschko & Burpee 1987), production of sexual spores (Smith 1983), and recently as molecular and genetic differences (Glynn et al. 2005; Hayashi et al. 2014; Jewell & Hsiang 2013; Maurin et al. 1995; Nicholson et al. 1996; Parry et al. 1995).

Differences between *M. nivale* and *M. majus* in host preferences were reported in several studies. *M. nivale* is more pathogenic on winter rye *Secale cereale* than *M. majus* (Simpson et al. 2000), the same is the case with perennial ryegrass (Hofgaard et al. 2006) ; while *M. majus* was more pathogenic on winter wheat (Diamond & Cooke 1997; Simpson et al. 2000). Furthermore, variation in pathogenicity between isolates of *M. nivale* was found on grasses (Hofgaard et al. 2006; Holmes 1976) and on cereals (Diamond & Cooke 1999; Maurin et al. 1995). According to Hofgaard et al. (2006) highly pathogenic isolates grow faster at 2 °C on PDA media (potato dextrose agar), and have higher activity of the cell wall-degrading enzymes β-glucosidases.

Genetic diversity between *M. nivale* and *M. majus* isolates has been studied using various molecular techniques. Lees et al. (1995) used restriction fragment length polymorphism (RFLP) of internal transcribed spacer (ITS) of genomic regions encoding ribosomal RNA to differentiate between the two species. Moreover, Nicholson et al. (1996) used the random amplified polymorphic DNA (RAPD) technique to identify genomic regions specific to each species. Later, Glynn et al. (2005) suggested that *M. nivale* and *M. majus* should be considered as separate species, based on the differences in elongation factor 1 genome sequences. Recently, Jewell and Hsiang (2013) have used β-tubulin gene sequences and RNA polymerase II subunit genes, as well as internal transcribed spacer (ITS) and the elongation factor 1 alpha gene, to differentiate between *M. nivale* and *M.*

majus isolates from different host plants and different geographic regions. In this study, isolates from different geographic origin (Europe vs. North America) and different host plants (grasses vs. cereals) were different based on RNA polymerase II and β-tubulin genes sequences.

1.4.3 Pink snow mould and climate change.

The overall changes in the future climate will make it possible to grow perennial ryegrass in areas where it could not grow before, but the risk of fungal diseases such as snow mould has to be taken into consideration (Thorsen & Höglind 2010). The snow mould pathogen *M. nivale* is a very adaptive fungus. The fungus has two phases. Firstly, a psychrophilic phase that allows the fungus to grow and attack the plants under cold conditions (until - 6 °C). Secondly, a mesophilic phase that gives the fungus the ability to grow in warm weather up to 28 °C (Årsvoll 1975; Tronsmo 2013). Therefore, the ability of *M. nivale* to grow at warmer conditions can increase the occurrence of snow mould attacked plants in areas which have not been severally infested before (Rapacz et al. 2014). Moreover it is difficult to predict the effect of climate changes on winter pathogens, since these changes will affect the pathogens as well as the plants (Rapacz et al. 2014). Some studies predict that climate changes will lead to shorter winters and less snow cover, thus fewer snow mould attacks on cereals and grasses should be expected in the future (Boland et al. 2004; Rapacz et al. 2014; Roos et al. 2011). On the other hand, extreme weather incidents will probably be more frequent (Stocker et al. 2013), which could lead to severe snow mould attacks in some years (Murray & Gaudet 2013; Rapacz et al. 2014). In addition, insufficient environmental conditions for cold acclimation will reduce snow mould resistance, which is enhanced by cold hardening. Therefore, grass breeders need to identify genotypes that combine sufficient adaptation to the future weather conditions with snow mould resistance that is independent of cold hardening (Rapacz et al. 2014; Tronsmo 2013).

1.5 Screening for snow mould resistance.

Snow mould disease development is a complicated process, which requires suitable environmental conditions. The presence of snow cover is the most crucial factor for fungal establishment. Laboratory methods were developed in order to mimic microclimatic conditions under snow cover and identify plant material resistant to snow mould (Årsvoll 1977; Hofgaard 2003; Miedaner et al. 1993; Nakajima & Abe 1990). Most of these methods are labour and time consuming, so in order to overcome this problem other methods have been developed, such as the detached leaf assay. This method has been used to assess snow mould resistance of winter rye (Hömmö 1994), winter wheat (Diamond & Cooke 1999; Ergon & Tronsmo 2006) and barley (Browne & Cooke 2005). A molecular technique based on polymerase chain reaction (PCR) for identification and quantification of the fungal DNA has been developed (Glynn et al. 2007; Hayashi et al. 2014; Nicholson et al. 1998; Nielsen et al. 2013) in order to study the correlation between the amount of fungal DNA and disease development, and also to monitor the distribution of the fungal population.

1.6 Defence response towards snow mould infection.

Snow mould resistance is regarded as a quantitative trait, and resistant cultivars are mainly characterized by being high yielding with good stress tolerance. However, under conditions of severe attacks by snow mould, most resistant cultivars will be infested (Ergon & Tronsmo 2006; Gaudet & Kozub 1991; Gaudet et al. 1999). The plant developmental stage at the time of infection plays an important role in snow mould development. Older plants are generally more resistant to snow mould than younger plants (Årsvoll 1977; Bruehl 1982; Gaudet et al. 1999). Therefore, snow mould resistance can be divided into two types according to the plant developmental stage. The first is the pre-tillering resistance, in which, according to Gaudet and Kozub (1991), winter wheat plants express good levels of resistance to snow mould. The same is the case for winter barley plants at the pre-tilling stage (Cavelier 1986). The second is the plant age related resistance, which can be related to increased carbohydrate accumulation. This type of resistance takes place in all cultivars, but it occurs at a higher rate in resistant cultivars (Gaudet et al. 1999). Moreover, plant size is an important factor in snow mould resistance. Large plants are more resistant, possibly because they can relocate their carbohydrate reserves towards the crown tissue more rapidly (Bruehl

& Cunfer 1971; Gaudet 1994).

The mechanism by which cold acclimation is enhancing snow mould resistance is not completely understood, as well as how it also enhances resistance to other diseases (Gaudet et al. 2011). Therefore it is generally described as nonspecific defence reactions triggered by cold stress (Dubas et al. 2011). Several studies have documented the relation between snow mould resistance and accumulation of soluble carbohydrates during cold acclimation (Gaudet 1994; Gaudet et al. 1999; Iriki et al. 2005; Mohammad et al. 1997; Østrem et al. 2011; Pociecha et al. 2008; Pociecha et al. 2010; Rapacz et al. 2014; Yoshida et al. 1998). Snow mould resistant cultivars were characterized by enhanced accumulation of polysaccharides, especially fructans in crown tissues during autumn and early winter, followed by a slow consumption rate of the polysaccharides during winter and early spring (Gaudet et al. 1999; Yoshida et al. 1998). Furthermore, the increase in the degree of the polymerization of the polysaccharides could reduce the consumption of the components by *M. nivale* during infection (Gaudet et al. 1999).

Under snow cover, the plants start to degrade the stored polymerized polysaccharides such as fructans to simple sugars. This process activates the hexokinase signal transduction pathway, which triggers gene expression of several genes related to pathogen defence responses, such as peroxidase, pathogen related (PR) proteins and thaumatin like proteins (Herbers et al. 1996; Tattersall et al. 1997). Furthermore, plant exposure to low temperature induces the expression of several forms of anti-freeze proteins, which could also have non-specific anti-fungal activity (Gaudet et al. 1999). Hon et al. (1995) found that anti-freeze proteins from winter rye were very similar to the PR proteins chitinase and β-glucosidase. Furthermore, according to Kuwabara et al. (2002), cold-induced thaumatin-like proteins revealed also antifungal activity in winter wheat.

Plant responses to snow mould infection is the result of the coordinated interaction between carbohydrate catabolism and hormone activity (Pociecha et al. 2013). During early response (6 days after inoculation) pathogen related proteins, such as chitinases (PR3 and PR4), β-1,3-glucanase (PR2) and thaumatin-like (PR5), were activated in resistant genotypes of winter wheat (Gaudet et al. 2011). Resistant genotypes of *Festulolium* were characterized by high peroxidase activity, intense lignification, and high concentrations of salicylic acid during early infection (within 6 days after inoculation) (Pociecha et al. 2008;

Pociecha et al. 2009). The activation of salicylic acid during early stage of infection is important for regulation of defence responses in plants, mainly to biotrophic plant pathogens, by activating systemic acquired resistance (SAR) and by producing PR proteins (Pociecha et al. 2010; Szechyńska-Hebda et al. 2013). Therefore, the progression of *M. nivale* infection is usually influenced by the physical and the chemical conditions of the plant tissue. Hence, the fungus behaves as biotroph when the plants are cold acclimated with fortified cell walls, and the plant defence system is induced. However, when the plants are susceptible, the fungus behaves as necrotroph (Szechyńska-Hebda et al. 2015).

Identifying the mechanisms involved in snow mould resistance will permit researchers to develop suitable markers in order to select more resistance cultivars. Moreover, selection for resistance to one snow mould pathogen could increase the resistance level to other pathogens as well, since according to several reports, resistance to snow mould is not specific to a certain pathogen (Bruehl 1967; Gaudet & Kozub 1991; Gaudet et al. 1999; Gaudet et al. 2011). Besides that, the heritability of snow mould resistance is considered high in winter wheat and grasses and breeding for improved snow mould resistance should therefore be possible (Amano & Osanai 1983; Gaudet 1994; Tronsmo 1993).

1.6.1 The use of next generation sequencing in understanding plant defence systems.

During recent years, different methods for genome sequencing have been developed and used to answer several biological questions (Metzker 2010; Nowrousian 2010; Shendure & Ji 2008). Next-generation sequencing (NGS) allows the sequencing of millions of bases in one round at low cost (Egan et al. 2012). Furthermore, the costs and capabilities of these methods have continuously improved during time. NGS technologies produce a large amount of data, raising many challenges to computational biologists, bioinformaticians, and researchers, who are striving to assemble and analyse NGS data (Egan et al. 2012; Nowrousian 2010). The main common feature of all NGS equipment is the high degree of parallelization, in which millions of sequencing reactions occurs at the same time within small reaction volumes (Nowrousian 2010; Schuster 2007).

The use of NGS for obtaining transcriptome sequences is known as RNA deep sequencing or RNA-seq (Wang et al. 2009). The first studies using RNA-seq were published in 2008 for the yeasts *Saccharomyces cerevisiae* (Nagalakshmi et al. 2008) and *Arabidopsis thaliana* (Lister et al. 2008). Since then, a large number of studies in other organisms have used RNA-seq (Marguerat & Bähler 2010). Compared to other methods, such as EST (Expressed Sequence Tags) sequencing and microarray, the use of RNA-seq technology provides large amount of data that allow not only the quantification of gene expression, but the identification of the transcripts at single-nucleotide level without depending on prior annotation or prior knowledge regarding transcribed regions (Marguerat & Bähler 2010). The major challenge working with RNA-seq data is the sequence assembly and statistical model used for identifying differently expressed genes. But due to the high usage demands on this technology, the data analysis methods are in continuous development (Egan et al. 2012). Therefore, several methods for detecting and analysing differentially expressed genes are available. Hence, analytical packages for these data are available to be evaluated by several researchers (Marguerat & Bähler 2010). The two main methods for RNA-seq transcriptome assembly are; reference based assembly, which is depending on a reference genome, and *de-novo* assembly. Ward et al. (2012) compared the two methods, and concluded that the methods provide comparable results. Although the reference based assembly provides a statistical outline for distinction expression analysis and assumption initiation, while the de-novo assembly is more competent for discovering unique sequences and for providing more possible querying for the transcripts and the expression levels.

RNA-seq has been used in several recent studies to understand the complexity of plant pathogen interactions. Understanding the resistance mechanism involved at different stages of infection is always challenging, especially for hemibiotrophic pathogens. However, the use of RNA-seq technology can help us to gain more insight into this complex interaction. Recently Zuluaga et al. (2015) studied the transcriptome of tomato (*Solanum lycopersicum*) during three infection stages of *Phytophthora infestans* : biotrophic, the shift from the biotrophic to the necrotrophic stage, and the necrotrophic stage. This study revealed the major restructuring of plant metabolism during the different infection stages, including the major changes in secondary metabolic pathways. Additionally, more than 100 putative resistance genes were induced, and both JA and SA levels showed variable changes during the infection stages. Also during the biotrophic phase, induction of both PTI (pathogen-triggered immunity) and ETI (effector-triggered immunity) were observed.

Plant pathogens can behave differently during the infection of a compatible host compared to an incompatible host. Kellner et al. (2014) used RNA-seq technology to study the transcription profile of *Zymoseptoria tritici* at the early stages of infection of a compatible host (wheat) and an incompatible host (*Brachypodium distachyon*). The study found that the infection regulatory pathways were common in both hosts, and that genes induced during infection of wheat were in two large clusters, which may represent candidate pathogenicity islands. Furthermore, in the response to reactive oxygen species produced by wheat defence mechanism, detoxification genes were up-regulated during plant infection. These findings indicate that these genes might have a role in determining determine the host range of this pathogen.

Recently several researchers use RNA-seq to identify the defence responses to pathogen infection. Lin et al. (2014) used this technique for a comparative transcriptome study of the molecular responses to the pathogen *Phytophthora sojae* among ten soybean near isogenic lines. The study characterized genes of regulatory networks associated with resistance to *P. sojae* involved in ethylene (ET), jasmonic acid (JA), reactive oxygen species (ROS), and MAPK (MAP-kinase) signalling. These findings showed the significant role of these signalling pathways in molecular defence responses. In another study by Muñoz-Bodnar et al. (2014), RNA-seq based analysis of cassava plants inoculated with *Xanthomonas axonopodis* allowed the identification of host genes and pathways leading to plant resistance. These genes were involved in several biological processes such as photosynthesis, jasmonate metabolism and phenylpropanoid biosynthesis.

2. The thesis.

2.1 Project background

Forage production of perennial grass species at the northern hemisphere is limited by the winter survival ability of these species. Scenarios of the predicted future climate show that average temperature and precipitation will increase in the Nordic region (IPCC 2007; Uleberg et al. 2014). The period of the growing season will increase; however, the period for optimal cold acclimation will decrease (Thorsen & Höglind 2010; Uleberg et al. 2014). The predicted changes in environmental conditions constitute an opportunity and at the same time, a challenge for forage production based on perennial grasses. Therefore, improved adaptation should be the breeding strategy to counteract the negative effects of climate change, as well as to make good use of the positive effect (Olesen et al. 2011)

Overwintering diseases caused by snow mould fungi such as *M. nivale* are one of the limiting factors for grass production in Scandinavia (Rapacz et al. 2014; Tronsmo 2013). In order to reduce the risk of snow mould attack under future climates, with inferior conditions for cold acclimation in the autumn due to higher temperatures, the role of cold hardening independent resistance to snow mould need to be better understood. Therefore, it is necessary to identify resistant plant material under non-cold hardening conditions, and to investigate such materials in order to identify resistance genes using.

In order to improve the screening methods for identification of resistant plant material, there is a need for faster and more reliable methods. In addition, host specialization within *M. nivale* isolates should be taken into consideration when screening for resistance performed. Therefore, it will be beneficial to improve our understanding of the genotypic and phenotypic (pathogenicity) differences between *M. nivale* isolates for future snow mould resistance screening.

2.2 Main hypothesis and objectives

A warmer climate will allow forage production based on perennial ryegrass and ryegrass-like *Festulolium* in most parts of Norway. Current cultivars suffer severe injuries caused by low-temperature fungi. Identification of germplasm with inherent and cold-induced snow mould resistance will facilitate breeding of robust cultivars adapted for climatic change. In order to achieve progress in breeding for improved snow mould resistance, better methods for screening plant materials and characterization of the pathogen population are needed.

Hypothesis 1: There is genetic variation in germplasm of perennial ryegrass and *Festulolium* for snow mould resistance independent of cold acclimation.

Objective 1: Identify sources of snow mould resistance, which are independent of cold hardening in different grass populations under controlled conditions, and study the relationship between resistance under controlled conditions and winter survival in the field.

Hypothesis 2: There is genetic and phenotypic (pathogenicity) variation between and among isolates of *M. nivale* and *M. majus* from different host plants.

Objective 2: Test pathogenicity of a range of isolates of *M. nivale* and *M. majus* and perform a phylogenetic study to reveal relationships between the isolates based on sequencing of specific regions of genes.

Hypothesis 3: The amount of fungal DNA in infected plants is correlated with disease severity and level of resistance, and can be quantified by real-time PCR.

Objective 3: Perform real-time PCR using specific primers designed to amplify the elongation factor 1-α gene on DNA extracted at different time-points from a defined set of genotypes of a *L. perenne* cultivar inoculated with a single *M. nivale* isolate.

Hypothesis 4: Studying differentially expressed genes after inoculation with *M. nivale* of plants with different levels of snow mould resistance will make it possible to identify

candidate resistance genes and develop molecular markers to be used in selecting for resistance.

Objective 4: Perform a global transcriptome investigation using RNA sequencing on RNA sampled from genotypes with differential snow mould resistance inoculated with *M. nivale* in order to identify candidate genes associated with defence responses and host-pathogen interactions.

2.3 Methods and results.

2.3.1 Screening for snow mould resistance under field and controlled conditions (Paper I)

For controlled conditions experiments, seeds of 23 cultivars and populations of *L. perenne*, *Festulolium* and *F. pratensis* (listed in Paper I, Table 1) were germinated in a greenhouse. Seedlings were transplanted after 2 weeks into new pots. The plants grew further in the greenhouse for 4 weeks at temperature 18-20°C and 16 h photoperiod. The experiment was performed twice; during autumn 2011 (exp. A) and during spring 2012 (exp. B). Two isolates of *M. nivale* (isolate 201050 from *Festulolium* and isolate 200231 from *L. perenne*) were used separately as inoculum source during the experiment. In order to select these isolates, the pathogenicity of 17 isolates was tested (as described in paper II). Inoculum was prepared from each isolate using spore suspensions containing 1×10^6 conidia ml^{-1}. The plants were inoculated by spraying each pot containing five plants with 10 ml spore suspension. Inoculated plants were covered with moistened cellulose wadding and black plastic sheets and then incubated at 2 °C in darkness for eight and twelve weeks. After incubation, the plants were cut and allowed to regrow for 2 weeks at 22 °C and 16 h light. Resistance to *M. nivale* was calculated as relative regrowth, i.e. dry weight (g) of inoculated plants divided by dry weight of non-inoculated plants within the same population. Orthogonal contrasts were used to test differences between species and species/ploidy levels in relative regrowth using PROC GLM in SAS version 9.2 (SAS Institute Inc., Cary, NC, USA).

The results of this experiments showed that the two *M. nivale* isolates used in this study were not significantly different as regards pathogenicity, except in experiment A after 8 weeks of incubation where the difference in pathogenicity between isolates was barely significant (Table 2, paper I). Orthogonal contrasts revealed that *F. pratensis* entries were on average significantly less resistant than other entries (except in experiment A after 12 weeks of incubation), whereas there was no significant differences in resistance between *L. perenne* and *Festulolium* entries (Table 2, paper I). Furthermore, significant differences in snow mould resistance were found between amphiploid *Festulolium* (tetraploid) entries and diploid entries in most of the controlled experiments, with tetraploid entries such as

FuRs0463 and FuRs0465 being the most resistant (Fig. 5). Entries adapted to south Norwegian conditions, such as FuRs0465, and continental central European conditions, such as Felopa (LmFp/T) and Picaro (Lp/D), displayed relatively good levels of snow mould resistance (Fig. 5)

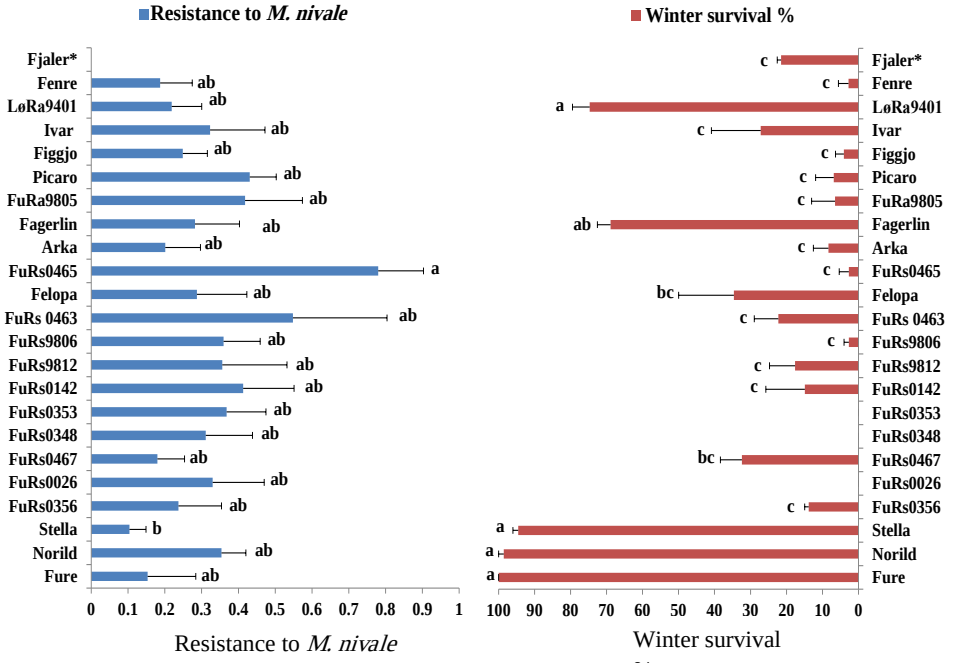

Fig. 5. Variation in resistance to *M. nivale* and winter survival in cultivars and breeding populations of *L. perenne*, *F. pratensis* and *Festulolium* (Table 1, paper I). Resistance was measured as relative regrowth (dry weight of inoculated plants divided by dry weight of non-inoculated plants) after 12 weeks (experiment A and B) incubation under artificial snow cover followed by 2 weeks of regrowth. Winter survival is the percentage of plants on the plots alive in the spring of 2013. Error bars indicate standard errors of the mean. Bars marked with different letters indicate significant differences (using Tukey's honest significance test at $P \leq 0.05$). * Cultivar only used in the field experiments.

In the field experiment, seedlings were kept for 3 weeks in the greenhouse at 22 °C and natural light conditions. In mid-September 2011, the plants were transplanted into the field. The experimental design was a randomized block design with 4 replicates each with 23 plots (one plot per entry) at Ås, Norway (59°39′37″N, 10°47′1″E). *M. nivale* isolate 200231 was chosen for inoculation (the same isolate used in the controlled conditions experiments). Inoculum was prepared on wheat grains and spread between the plants in mid-November 2011 and mid-November 2012, while one replicate was kept non-inoculated. The visual appearance of the tillers of each plant was scored in May 2012 and 2013 using the following scale: 0 = no green tillers, 1 = some green tillers, 2 = less than 50% green tillers, 3 = more than 50% green tillers, and 4 = all tillers green (Fig. 6). The plants were cut three times during the 2012 growing season, and dry matter yield (DMY) was determined. In spring 2013, winter survival was recorded as the number of surviving plants divided by the total number of plants per plot.

Fig. 6. Scale for the visual assessment of snow mould disease severity during field experiment in May 2012. The scale was as follows: 0 = no green tillers, 1 = some green tillers, 2 = less than 50% green tillers, 3 = more than 50% green tillers, and 4 = all tillers green (Photos by M. Abdelhalim)

Visual assessment of winter injury after the winter of 2011-2012 showed that the *Festulolium* entries FuRs0348 and FuRs0353 (diploid and adapted to south Norwegian conditions) had lowest survival (visual scorings around 2). The other entries showed almost the same degree of winter injury. The *Festulolium* cv. Felopa had the highest total yield, while the *L. perenne* entry FuRa9805 was lowest yielding. Diploid entries of *Festulolium*

showed the highest yield, significantly different from tetraploid *Festulolium* entries, and analysis of variance based on the proportion of plants surviving the last winter (recorded in July 2013) showed significant differences between entries (Fig. 5). Among *Festulolium* and *L. perenne* entries, northern adapted cultivars of *L. perenne* (Fagerlin and LøRa9401) had the highest winter survival (70 to 80 % of the plants survived). Amphiploid *Festulolium* (tetraploid) hybrids, such as Felopa and FuRs0467, had higher winter survival than introgression hybrids (diploid). *F. pratensis* cultivars had the highest winter survival (Fig. 5).

Correlation analysis showed that resistances observed under controlled conditions and observed in the field were not correlated. Northern adapted cultivars of *L. perenne*, such as Fagerlin and LøRa9401, showed good winter survival in the field but had only a moderate snow mould resistance in the test under controlled climate conditions.

2.3.2 Pathogenicity test (Paper II)

A pathogenicity test was performed using four grass cultivars from two grass species; *Lolium perenne* cv. Figgjo and Ivar, and *Festulolium* hybrids FuRs9812 and FuRs0463. The experiment was performed twice. In the first experiment, plants were incubated for 8 weeks and in the second experiment for 9 weeks after inoculation. Isolates of *M. nivale* (15 isolates) and *M. majus* (two isolates) were used in this experiment. The isolates were kept at -80 °C as a part of the fungal culture collection at NIBIO, Plant Health and Biotechnology Division; Ås, Norway (listed in Table 1/ Paper II). The isolates were previously identified based on colony and conidial morphology; also asporogenic isolates were classified using PCR methods by Hofgaard, et al. (2006). Plant materials were propagated under controlled conditions as described in Paper II. Inoculum was prepared using conidial suspensions of the different isolates. Conidial spores were produced by incubating the fungus on PDA plates at 20 °C for 7 days under 12 hours cycle of near-ultraviolet (NUV) and white light for sporulation. Conidial suspensions were prepared by washing the fungal culture with 10 ml sterile distilled water containing 0.2 % TWEEN 20. Spore suspensions were adjusted to 1×10^6 conidia ml^{-1}. The plants were inoculated by spraying 1 ml inoculum per plant. Incubation and re-growth assessment of plants were performed under controlled conditions as described in Paper II. The pathogenicity of isolates was calculated as 1- relative regrowth as descripted by Hofgaard, et al. (2006).

The results from these experiments showed that *M. nivale* isolates 200231 (isolated on *L. perenne*), 201050 (isolated on Festulolium), 200272 (isolated on *F. pratensis*) and 201053 (isolated on *L. perenne*) were the most pathogenic isolates, while isolates 200114 (isolated on *D. glomerata*) and 200136 (isolated on *L. perenne*) were least pathogenic (Fig. 7). The two *M. majus* isolates showed very low pathogenicity on all cultivars (Fig. 7). *M. nivale* isolates from grasses were more pathogenic than isolates from cereals (Fig. 8). The isolate x cultivar interaction was significant only in the 8 week experiment, and was caused by a differential reaction of the cultivars to the cereal isolates (Table 2, paper II).

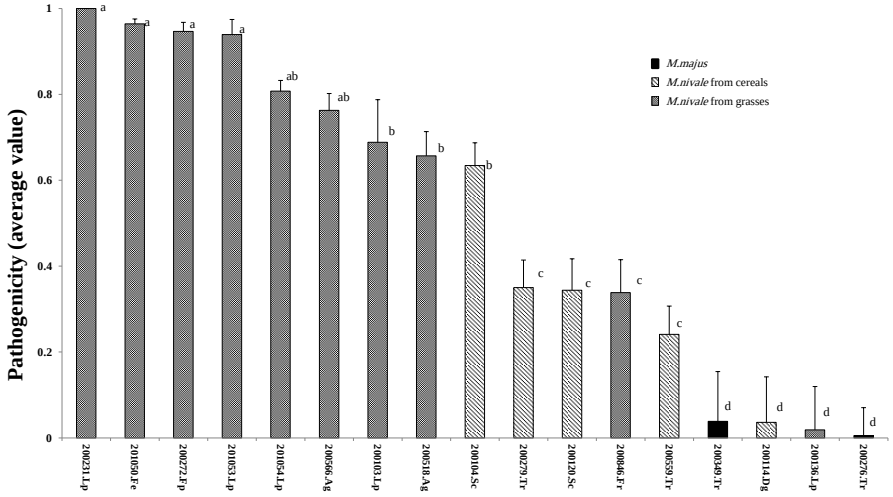

Fig. 7. . Pathogenicity (0 = min; 1= max) of seventeen *Microdochium* sp. isolates (fifteen *M. nivale* and two *M. majus*) on four different grass cultivars (Figgjo, Ivar, FuRs9812 and FuRs0463). Pathogenicity was measured as 1- relative regrowth (dry weight of inoculated plants divided by dry weight of non-inoculated plants after incubation for eight and nine weeks (experiments A and B respectively) under artificial snow cover, followed by two weeks of regrowth (average value of four cultivars). Isolate number are labelled with host origin (Lp for *L. perenne*, Fe for *Festulolium*, Fp for *Festuca pratensis*, Ag for *Agrostis stolonifera*, Sc for *Secale cereale*, Tr for *Triticum* sp, Fr for *Festuca rubra* and Dg for *Dactylis glomerata*). Error bars indicate standard errors of the mean of the two experiments. Bars with the same letter are not significantly different (P value <0.05).

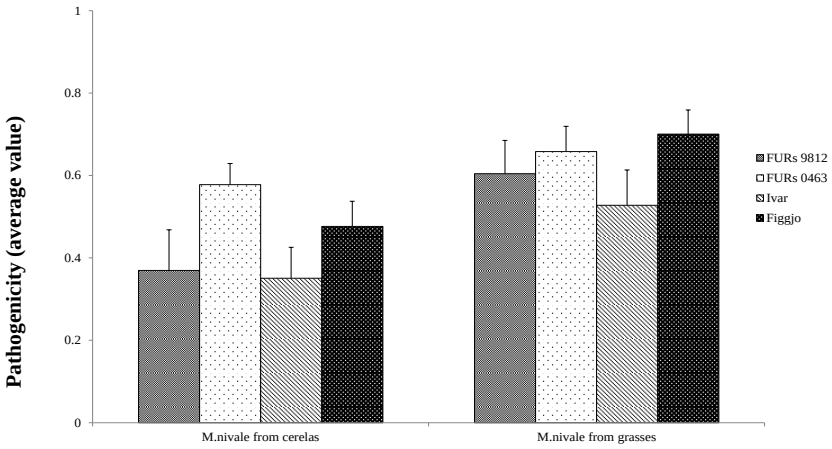

Fig. 8. Pathogenicity (0 = min; 1= max) of *M. nivale* isolates from cereals (mean values of five isolates) and grasses (mean values of ten isolates) on four different grass cultivars (Figgjo, Ivar, FuRs9812 and FuRs0463). Pathogenicity was measured as 1- relative regrowth (dry weight of inoculated plants divided by dry weight of non-inoculated plants at eight weeks (experiment A) and nine weeks (experiment B) after incubation under artificial snow cover followed by two weeks of regrowth. The values are averaged across experiments.

2.3.3 DNA sequencing of M. nivale and M. majus isolates (Paper II)

Fungal isolates (as listed in Table 1/ Paper II) were inoculated on PDA plates and incubated for seven days at 20° C in darkness. Mycelium was harvested by carefully scraping it off the media surface using a clean razor blade, and then frozen quickly in liquid N_2. Later the samples were ground using mortar and pestle. DNA was extracted from100 mg ground mycelium using DNeasy Plant Mini Kit (Qiagen Inc., Germany), according to the manufacturers' protocol. PCR reactions were performed using specific primers for four different genomic regions: the Internal transcribed spacer (ITS), Elongation factor 1-alpha (*EF1a*), RNA polymerase II subunit (*RPB2*) and *β-tubulin*. The PCR products were sequenced in both directions at GATC Biotech (Germany). DNA sequences were analysed using the Lasergene Seqman software (DNAStar Inc.). Previously deposited *RPB2* and *β-tubulin* sequences of *M. nivale* and *M. majus* from the NCBI database were also included. Neighbour-joining (NJ) phylogenetic trees were constructed for each gene by the CLC Main Workbench version 6.9.1 (CLC Inc., Aarhus, Denmark) software using the maximum likelihood (ML) algorithm with 1000 bootstrap replicates.

The sequences of the four genomic regions revealed slightly different phylogenetic structures. For the β-tubulin gene, *M. nivale* isolates formed two clades; the first represents isolates from grasses in Norway and North America (with the exception of isolate 200108). The second *M. nivale* clade represents only isolates from cereals from Norway, United Kingdom and North America. For the *RPB2* gene, *M. majus* isolates grouped together in one cluster, which was divided into two subclades, one represents isolates from Norway and United Kingdom and the other represents isolates from North America. *M. nivale* isolates from Norway and United Kingdom were grouped in one sub-cluster. Isolates from North America were all in one cluster, which was divided into two sub-clusters corresponding to the host origin (cereals vs. grasses). The phylogenetic tree based on the *EF-1α* gene sequences only showed two major clusters, one represents isolates of *M. nivale* and the other represents isolates of *M. majus*. The ITS sequence analysis revealed low genetic diversity, but still two major clusters were formed for isolates of *M. nivale* and *M. majus*.

2.3.4 *Quantification of Microdochium nivale by real-time PCR (Paper III)*

The experiment conducted in this study was part of a snow mould resistance test for selecting resistant and susceptible genotypes for the global transcriptome analysis. In this experiment, eight genotypes (termed A, B, C, D, E, F, M and K) were chosen randomly from *L. perenne* cv. Fagerlin and divided into tillers to obtain sufficient plant materials for the snow mould resistance test and fungal DNA quantification. Inoculum was prepared using homogenized mycelium and final inoculum was adjusted based on optical destiny of 0.5 at 430 nm. The plants were inoculated by spraying (1 ml inoculum per plant). Snow mould resistance test and the visual assessment of the disease symptoms were performed as described in Paper III.

For the DNA extraction leaves and stems above 5 cm from the soil level were collected from each genotype at different time points (6 and 8 weeks after inoculation) and at 1 and 4 days after inoculation from two of the genotypes (genotype F and M). Samples were kept at -80 °C until DNA was extracted.

Real-time PCR primers specific for *M. nivale* were designed based on the elongation factor 1-α gene. Specific real time PCR primers for the plant housekeeping gene LpGAPDH (Petersen et al. 2004) were used as an internal control for plant DNA. The amount of fungal and plant DNA in the sample were quantified by a standard curve algorithm based on cycle threshold value (Ct) using a 10 fold dilution series of known amount of DNA. The amount of fungal DNA was calculated as pg fungal DNA per µg plant DNA for each sample.

Quantification of *M. nivale* DNA showed that genotypes with severe symptoms of injury contained the highest amount of fungal DNA. Therefore, a significant correlation was found between disease severity and the amount of *M. nivale* DNA. However, the correlation between the amount of *M. nivale* DNA and relative regrowth was not significant. Both at one day and at 4 days after inoculation, no significant differences in the amount of *M. nivale* DNA was found between genotypes F and M.

2.3.5 Transcriptional analysis during early infection by M. nivale (Paper III)

Based on the snow mould resistance test, and the quantification of *M. nivale* DNA (as described in paper III), one resistant genotype (M) and one susceptible genotype (F) of the perennial ryegrass cv. Fagerlin were chosen for transcriptome analyses (Fig. 9). There were three groups of plants according to the different treatments: 1) control (non-inoculated and non-incubated plants), 2) non-inoculated (non-inoculated plants sampled after 4 days of incubation), and 3) inoculated (inoculated plants sampled after 4 days of incubation). Each treatment was represented by two biological replicates. Leaf samples were collected for RNA extraction (a total number of 12 samples) then immediately placed in liquid nitrogen and stored at -80° C.

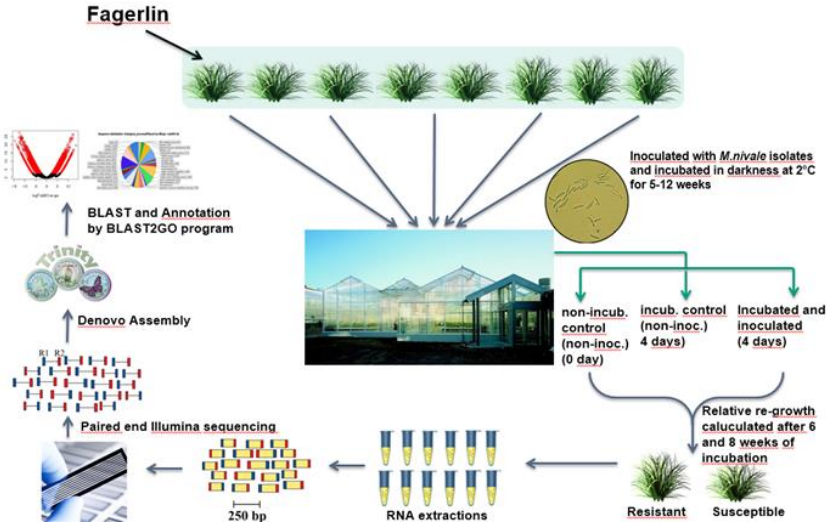

Fig. 9. The experimental design for the global transcriptome analysis during early infection by *M. nivale*.

Extraction of total RNA was performed using the Pure Link RNA Mini Kit (Life technologies, USA) plus plant RNA Isolation Aid (Life technologies, USA). After the concentration and quality had been checked, the twelve RNA samples with RIN values above 7 were used separately to construct cDNA libraries with fragment lengths of 200 bp (±25 bp). Then, paired-end sequencing was performed using the Illumina sequencing platform (HiSeq™ 2000) at GATC Biotech Ltd., Germany.

Real time analysis (RTA) output was analysed using the CASAVA software (version 1.6, Illumina) generating FastQ files with paired-end reads with a length of 100 bp.

After trimming adapter sequences and filtering low quality reads, we followed the bioinformatics pipeline outlined in Fig. 10 for *de novo* assembly and further detection of Differentially Expressed Genes (DEG). Gene expression levels were measured as expected number of fragments per kilo base of transcript sequence per millions base pairs sequenced (FPKM). Pairwise comparisons were carried out between all the selected time points by fitting normalized count data with a Generalized Linear Model (GLM) estimating a negative binomial distribution to the calculated mean values of the two biologically independent samples. The DEGs were annotated using Blast2GO® software

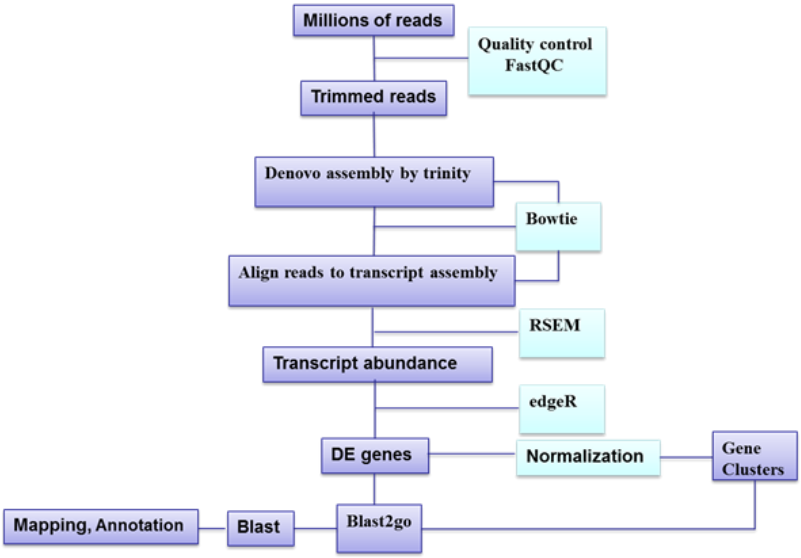

Fig. 10. Work flow of RNA sequence data analysis.

A total number of 178 and 165 million reads of 100 bp were generated for the susceptible (F) and resistant (M) genotype, respectively. The *de novo* assembly yielded 261,978 contigs for the susceptible genotype, with N50 of 1,784 bp, and 188,355 contigs for the resistant genotype with N50 of 1,672 bp. Using the genotype specific assemblies, we identified 2,354 and 3,748 differentially expressed transcripts (with false discovery rate (FDR) < 0.05) between incubated-noninoculated and incubated-inoculated plants in the susceptible and resistant genotype, respectively, with several up-, down- and contra-regulated transcripts (Figure 2. Paper III). When using the reference-based assembly mapping, we identified 95 and 210 differentially expressed transcripts between incubated-noninoculated and incubated-inoculated plants in the susceptible and the resistance genotype, respectively (Figure 2. Paper III). Nearly 75% of the differentially expressed transcripts had blast hits to the extracted NCBI database. Among the transcripts with blast hits, 40–52% of the differentially expressed transcripts were annotated using Blast2GO. Seven GO terms were enriched when comparing the differentially expressed transcript sets from incubated-noninoculated vs incubated-inoculated plants of the two genotypes (Fig.6, Paper III). Interestingly, five GO terms were overrepresented in the resistant genotype, in terms related to cell wall cellulose metabolic process, cell wall pectin metabolic process, cell morphogenesis, actin nucleation and organelle epidermal cell differentiation.

The results of this study showed that several genes involved in the initiation of pathogen-associated molecular pattern (PAMP) immunity were detected, like cysteine-rich receptor-like protein kinase (CRK), calcium-dependent protein kinase (CDPK), calcium-binding protein CML (CaM/CML), and NADPH oxidase. Pathogen related genes like PR-1, β-1,3-Glucanase (PR-2), chitinase II/V (PR-3), thaumatin-like (PR-5), and lipid-transfer protein (PR-14) were up-regulated in the resistant genotype under incubation and inoculation conditions (Table 3, Paper III). Several potential candidate genes for pathogen resistance were identified in this study such as *chitinase 5*, lipid transfer protein, serine-glyoxylate aminotransferase and *WRKY 75*. A hypothetical model for gene regulation in the plant-pathogen interaction pathway after four days of incubation with the pink snow mould pathogen, based on the pathogen related DEGs identified in this study, is presented in Fig. 6, Paper IV.

2.4 General discussion.

Pathogenesis test was conducted at first, in order to select suitable isolates for further experiments (paper II). In this study, *M. nivale* isolates from grasses were significantly more pathogenic than isolates from cereals when tested on the four grass cultivars, confirming the results of Hofgaard, et al. (2006). They found that *M. nivale* isolates from grasses were highly pathogenic towards perennial ryegrass, that highly pathogenic isolates grew faster on PDA (potato dextrose agar) media at 2 °C, and had higher activity of β-glucosidases cell wall-degrading enzymes. According to the results presented in paper II, isolates 200231 (host origin *L. perenne*) and 201050 (host origin *Festulolium*) were the most pathogenic isolates when tested on *L. perenne* and *Festulolium* cultivars. Thus, these two isolates were chosen for screening grass populations for snow mould resistance under controlled conditions (paper I). Isolate 200231 was used as inoculum for the transcriptome analysis following snow mould infection in controlled conditions (paper III), and artificial inoculation of the field experiment (paper I). The difference in pathogenicity between isolates 200231 and 201050 used in snow mould resistance tests under controlled conditions (paper I) were not significant in most of the experiments. The interaction between isolates and cultivars was only significant in one out of four experiments presented in paper 1. This interaction was probably due to the longer incubation period (12 weeks), which largely reduced the plant re-growth and several cultivares were killed.

In the study presented in paper I, snow mould resistance was evaluated in non-cold acclimated plants under controlled conditions in order to simulate the circumstances of warmer autumns and shorter cold acclimation periods expected in the future. The results from this study showed that the degree of adaptation and the genetic background of the entries had significant influences on their responses to snow mould infection. Hence, entries adapted to south Norwegian conditions, such as FuRs0465, and continental central European conditions, such as Felopa (LmFp/T) and Picaro (Lp/D), had an inherently good level of snow mould resistance independent of cold acclimation. Therefore, these entries may be considered as good sources for snow mould resistance under future climatic conditions.

Resistance to snow mould is considered to be a quantitative trait, and under field conditions it can be masked by the winter survival ability of the plant (Ergon et al. 2003; Gaudet 1994; Hofgaard, et al. 2006). We found that artificial inoculation in the field experiment was

most efficient when the weather conditions were favourable for *M. nivale*, i.e. during the winter of 2012-2013 when the snow cover lasted quite long, from the beginning of January until the end of April (Fig. 11). Winter survival under field conditions was largely determined by the degree of northern adaptation of each entry. Moreover, the selection for only two winters in Bodø (67° 17′ N) increased winter survival of FuRs0463 (30% of plants survived) in comparison to Fabel (3 % of plants survived), a southern adapted cultivar from which FuRs0463 was selected. In addition to that, entries of *L. perenne* adapted to North Norwegian conditions, such as Fagerlin and LøRa9401, showed good winter survival. Therefore, these differences reflect the higher inherent potential for adaptation in the plant breeding material adapted to North Norwegian conditions compared with conditions that are more southern. Therefore, plant material adapted to North Norwegian conditions, such as the *L. perenne* cultivar Fagerlin, was chosen for further studies to identify the genetic sources of snow mould resistance using global transcriptome analysis (RNA-seq) as presented in paper III.

Fig. 11. Field trail for snow mould resistance assessment at Ås, Norway in May 2012 and 2013. (Photos by M. Abdelhalim)

The screening method for snow mould resistance represented in paper I, showed several limitations. This method is labour and time consuming, as well as requiring large and expensive facilities such as greenhouses and controlled climate chambers. Another technique for assessing fungal biomass in the host tissue was developed using quantitative polymerase chain reaction (qPCR). This method was based on monitoring the accumulation of the amplicons from elongation factor 1-α gene using fluorophores that associate with double-stranded DNA, such as SYBR Green (as presented in paper III). The results from this experiment showed significant correlation between the amount of *M. nivale* DNA and disease severity (based on the visual assessment of disease symptoms). However, no significant correlation was found between the amount of *M. nivale* DNA and snow mould resistance (calculated as relative regrowth). Despite this fact, the method was useful for selecting suitable genotypes for the transcriptome study (paper III). For instance, genotype F had a high content of fungal DNA and a relatively low relative regrowth; and genotype M had a relative low amount of fungal DNA and a high relative regrowth. Consequently, both these genotypes were selected for studying global transcriptome changes in the plant genotypes during early stages of snow mould infection.

In order to explore the variation in pathogenicity between isolates on the genetic level, the nucleotide sequence of four different genomic regions (as listed in paper II) were used to study the genetic variation between approximately 40 isolates including *M. nivale* and *M. majus.* The results from this study showed larger genetic variation between *M. nivale* isolates than between *M. majus* isolates, which could be due to the existence of host preferences between *M. nivale* populations in comparison to *M. majus*. Furthermore, these findings agreed with the previous studies by Maurin et al. (1995), Glynn et al. (2005) and Mahuku et al. (1998). Sequence analysis of the RNA polymerase II gene revealed differences between isolates according to their geographic origins. Hence, all North-American isolates of *M. nivale* were placed in one cluster, and *M. majus* isolates from Norway grouped in one cluster. This finding agrees with a recent study by Jewell and Hsiang (2013), showing that isolates of different geographic origin (Europe vs. North America) were different based on RNA polymerase II gene sequences. On the other hand, β-tubulin, Internal transcribed spacer (ITS), and Elongation factor 1-alpha (*EF1α*) gene sequences did not reveal clear differences between isolates reflecting geographic origin. Moreover, the RNA polymerase II gene sequences showed differences between Norwegian isolates from cereals and grasses, which support the assumption of the existence of host

specialization among *M. nivale* isolates. This assumption was also supported by the differences between isolates in pathogenicity. This result is considered very important for grass breeders as well as plant pathologist because fungal populations with high levels of genetic diversity, such as *M. nivale*, are more likely to have a wider host range, to faster overcome host resistance or to develop resistance to fungicides, than those with low genetic diversity (McDonald & McDermott 1993). Therefore understanding plant pathogen population structure may have a large impact on snow mould management and grass production.

Management of diseases caused by *M. nivale* relies on resistance breeding, preventive application of fungicides in field, and seed treatment (Tronsmo et al. 2001). A sustainable crop production in future calls for reduced use of fungicides and integrated pest management (Bonos et al. 2006). Both for the purpose of resistance breeding as well as for precise and knowledge-based fungicide application, the current methods for disease assessment need to be improved (Tronsmo 2013). There is a need to understand better the response to snow mould infection at the genetic and molecular level, and implement molecular techniques in resistance testing. This will help to select suitable plant material for future breeding programs. In the study presented in paper III, we have taken advantage of the high throughput RNA sequencing technologies to study transcriptome changes in one resistant and one susceptible genotype of the Norwegian perennial ryegrass cultivar 'Fagerlin' during early infection by pink snow mould. However, even if the quantification of *M. nivale* DNA at this early stage of infection (one and four days after inoculation) showed no significant difference between the resistant and the susceptible genotype, differences in transcript levels as a response to *M. nivale* inoculation was found. Plants of the resistant genotype had higher expression levels of PR-1a, PR-2, PR-5, and PR-14 four days after inoculation with *M. nivale* than the susceptible genotype. The higher expression levels of these PR-proteins are often used as markers for activation of the SA (salicylic acid) signaling pathway (Gaudet et al. 2003; Gaudet et al. 2011; Pociecha et al. 2009; Sels et al. 2008). These results agree with a previous study by Gaudet et al. (2011) in winter wheat, the authors describe the early response to snow mould infection in resistant plants (6 days after inoculation) by the activation of pathogen related proteins such as PR-3, PR-4, PR-2 and PR-5. In the present study, the transcriptome analysis of the snow mould resistant genotype showed activation of the PAMP trigger immunity (PTI) pathway, particularly by the upregulation of the expression level of calcium-dependent protein kinase *CDPK*,

respiratory burst oxidase *Rboh* and calcium-binding protein CML *CaM/CML*. Furthermore, during this stage, the activation of pre-invasive penetration defence such as stomatal closure take place, as well as the activation of early post-invasive defence such as cell wall fortification by callose formation (Pociecha et al. 2013; Ton et al. 2009). Several important transcription factors that belong to the WRKY and NBS-LRR gene family were highly expressed in resistant plants. The high expression level of these transcription factors was also found in snow mould resistant winter wheat plants (Gaudet et al. 2011).

The results from this study can be used in further research, since it demonstrate the role of the pathogenesis related proteins during snow mould infection. Our global transcriptome analysis shows that non-cold hardened plants are able to integrate the defence pathway against the snow mould pathogen via the regulation of defence response genes.

2.5 Main conclusions and future perspectives.

In order to breed grass cultivars that are adapted to future climates, it is important to maintain high levels of genetic variation. Therefore, more knowledge about the phenotypic and genotypic variation in the current germplasm is required to increase productivity of the cultivars and maintain good levels of genetic variation. Identification of plant materials with good snow mould resistance that is independent from cold acclimation will help the breeders to develop new cultivars that are able to adapt to the future climates with longer and milder autumns. The study described in paper I showed that screening for snow mould resistance under controlled conditions could help in distinguishing the most resistant and the most susceptible genotypes and to separate snow mould resistance from other winter stress factors. Furthermore, winter survival under field conditions was largely determined by the degree of northern adaptation. Therefore, to improve winter survival of non-adapted materials, natural selection for several generations might be necessary. The sources of resistance to snow mould identified in this study, especially from Norwegian adapted cultivars and populations, should be investigated further. Cultivars and populations adapted to central European conditions could be considered used as source of snow mould resistance under future climates.

The use of quantitative PCR as a method to evaluate the severity of snow mould infection (as described in paper III) revealed that this method could be used to assist the

selection of snow mould resistant plant materials. However, the method can be more useful if the samples are collected from the suitable tissue at the appropriate time point. The method needs to be improved in order to be able to detect differences in fungal DNA from different isolates.

The results of paper II showed that isolates from grasses were significantly more pathogenic on the four grass cultivars than isolates from cereals. Therefore, it is important to perform pathogenicity test in order to define the host preference of *M. nivale* isolates ahead of selecting isolates for future snow mould resistance screening. Moreover, differences between isolates based on the geographic origin and host plants were found using the gene sequences of *RPB2* and β-tubulin. Despite the fact that the results from this study were based on genomic sequences of 4 genes using nearly 40 isolates, further investigations using more candidate genes and a larger number of isolates will be necessary.

Transcriptome analysis of one susceptible and one resistant genotype of *Lolium perenne* revealed that expression levels of plant defence-related genes such as PR-1a, PR-2, PR-5, and PR-14 provide a possible mechanism for the non-cold induced snow mould resistance. Therefore, these results will permit the researchers to develop suitable markers to select resistant cultivars. Moreover, identifying several candidate genes associated with defence response might provide a scientific basis for further investigations to attain more in–depth understanding of host-pathogen interactions. The hypothetical model for plant-pathogen interaction pathway after four days of incubation with *M. nivale* (presented in paper III) can be used as a base for future research to investigate this pathway at different stages of snow mould infection.

The work presented in this thesis provides increased knowledge for the grass breeders, particularly on the effect of the cultivar background (adaptation and ploidy level) on snow mould resistance. In addition, it provides genetic evidence for plant responses to snow mould infection. Therefore, the use of this genetic information as markers for resistance, beside the implementation of the fungal DNA quantification, can be used for screening large numbers of genotypes for snow mould resistance. Moreover, the studies performed in this thesis, in relation to the snow mould pathogen *M. nivale*, provide strong evidence for the existence of differences in host preferences between the fungal isolates.

This knowledge should be considered in the selection of suitable isolates for screening different grass species, breeding populations and genotypes for snow mould resistance.

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Paper I

Abdelhalim M., Rognli O.A., Hofgaard I.S., Østrem L. and Tronsmo A.M.

Snow mould resistance under controlled conditions and winter survival in the field in populations of perennial ryegrass, meadow fescue and *Festulolium* are partly dependent on ploidy level and degree of northern adaptation.

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ABSTRACT

Pink snow mould caused by *Microdochium nivale* is a serious cereal and grass disease in several temperate regions. In this study, resistance to snow mould was evaluated under controlled conditions in nine promising breeding populations and two cultivars of *Festulolium*, three cultivars of *Festuca pratensis*, six cultivars and two breeding populations of *Lolium perenne* and one cultivar of hybrid ryegrass using non-hardened plants. In addition, winter survival was evaluated in field plots inoculated with *M. nivale*. Under controlled conditions, tetraploid entries of *Festulolium* had significantly better resistance to snow mould than diploid entries in three out of four tests. Diploid and tetraploid entries of *L. perenne* showed similar levels of resistance under controlled conditions. In the field trial, entries of both *L. perenne* and *Festulolium* that had been exposed to natural selection in northern Norway (above 65° N) showed good levels of winter survival. In general, under controlled conditions snow mould resistance of *Festulolium* entries was associated with ploidy level, whereas under field conditions winter survival of *L. perenne* entries was associated with their degree of northern adaptation. However, resistance to snow mould in non-hardened plants tested under controlled conditions was not correlated with winter survival in the field.

Key words: Pink **s**now mould; *Microdochium nivale*; *Festuca pratensis*; *Festulolium*; *Lolium perenne*; resistance.

INTRODUCTION

Microdochium nivale (Fr.) Samuels & Hallett (teleomorph *Monographella nivalis* (Schafnitt)) (Smiley et al. 1992) is the most widespread and opportunistic pathogen on grasses, and it is a serious pathogen in cold, temperate regions of the northern hemisphere (Tronsmo et al. 2001). *M. nivale* thrives in a wide range of environmental conditions, and causes pink snow mould on turf, forage grasses and winter cereals. In synthetic media (culture) it can grow at temperatures from -6°C to 28°C (Årsvoll 1977) and in the field at temperatures below 0°C (Larsen 1994). In Norway, snow mould caused by *M. nivale* is considered as the most prevalent snow mould on both cereals and grasses (Årsvoll 1973; Ergon et al. 2003; Larsen 1994). Unlike other snow mould fungi, *M. nivale* do not require snow cover to be able to cause damage and for that reason pink snow mould is common in the coastal regions of Norway (Larsen 1994). Injuries by *M. nivale* in forage grasses are most severe under snow covering unfrozen soil at temperatures between 0 °C and 5 °C (Årsvoll 1973; Smith 1981). *M. nivale* infects different parts of the plant both above and under the soil surface. Microclimatic conditions, especially ambient air temperature and humidity, highly influences the infection process (Smith 1981).

Snow mould resistance is ensued from a combination of the genotype and the environmental conditions. Low temperature leads to cold acclimation (hardening) and enhanced resistance, and sub-lethal low temperature is thus the most important environmental factor affecting snow mould resistance (Ergon et al. 2003). Most studies of snow mould resistance have been conducted using cold acclimation as a pre-treatment; therefore association between frost tolerance and snow mould resistance was found in cold-hardened plants (Tronsmo 1984b). However, no such correlation was found in unhardened plants (Hofgaard et al. 2003; Tronsmo 1984b), and variation in snow mould resistance in unhardened plants of different grass species is sparsely documented by experiments in controlled climate. However, there are large differences between cultivars of different origin, as demonstrated for cultivars of timothy from northern and southern Norway, Denmark and the Netherlands (Tronsmo 1994). Testing of snow mould resistance in the field is complicated due to the difficulty of separating injuries due to snow mould from other winter damage caused by e.g. ice encasement, carbohydrate depletion and low photosynthetic efficiency (Ergon et al. 1998). According to Gaudet (1994) there is no direct

correlation between snow mould resistance and frost tolerance, but cultivars with high frost tolerance often show higher resistance to snow mould. Similarly, no strong correlation was found between snow mould resistance and frost tolerance in perennial ryegrass (Hofgaard et al. 2003). Snow mould resistance in grasses increases after cold acclimation during autumn, while the resistance decreases during late spring (April) (Årsvoll 1977; Prończuk and Zagdańska 1993; Tronsmo 1984a). In mild winters, resistance to snow mould is the decisive factor for winter survival (Prończuk and Zagdańska 1993).

Due to climate change, particular stressors affecting environmentally sensitive traits in plants will escalate in certain regions and decline in others (Murray and Gaudet 2013). According to the fifth assessment report of the intergovernmental panel on climate change (IPCC), different scenarios of the future climate indicate that the average global temperature will increase by 1 °C to 3.7 °C by 2100, particularly during winter and especially at higher latitudes (Stocker et al. 2013). Shorter periods of snow cover will be expected, and the severity of snow mould may decrease significantly (Rapacz et al. 2014). On the other hand, extreme weather incidents will probably be more frequent (Stocker et al. 2013), which could lead to severe attack by snow mould in some years (Rapacz et al. 2014). For northern Scandinavia, higher temperatures and more precipitation in autumn are projected (Uleberg et al. 2014), and this will counteract cold acclimation of perennial plants. Robust cultivars of perennial grasses that can survive winters either with or without cold acclimation will be needed in the future. Breeding for disease resistance is considered to be the main strategy to increase snow mould resistance in winter cereals and grasses (Gossen et al. 2001). New cultivars of grasses should be selected based on tolerance to freezing, ice-encasement and resistance to low temperature fungi (Larsen 1994). Breeding programs have been designed to transfer genes responsible for cold (Østrem et al. 2010) and drought tolerance (Humphreys et al. 1997) from *Festuca* into *Lolium*. *F. pratensis* is an important source for introgression of genes for cold tolerance into ryegrass (Humphreys et al. 2003). The first *Festulolium* hybrids, Prior and Elmet, were registered 30 years ago in Europe, and currently more than 30 *Festulolium* hybrids have been registered worldwide (Kopecky et al. 2005). The main goal of *Festulolium* breeding is to combine the stress tolerance of the fescues (*Festuca* spp.) with the early growth, high yield and high nutritional value of the ryegrasses (*Lolium* spp.) (Breese et al. 1981). *F. pratensis* is the most freezing tolerant among the *Festuca*

spp. and the species best adapted at the northern hemisphere. Identification of traits of importance for winter survival will help the breeders to map and select for the major genes responsible for both biotic and abiotic stress responses and to integrate those genes into new cultivars (Humphreys et al. 2003). In addition to that, winter survival is controlled by many environmental factors that lead to unreliable results, making the selection procedure difficult under such variable conditions (Prończuk and Zagdańska 1993). Experiments need several replications during different seasons in order to give reliable results (Prończuk and Zagdańska 1993). Earlier studies have demonstrated that cold hardening has a major effect on augmenting resistance to snow mould (Årsvoll 1977; Gaudet et al. 2011). Furthermore understanding the nature of snow mould resistance, independent of cold acclimation, will help to develop resistant cultivars with improved adaptation to the predicted future climate.

The aim of the present study was to identify plant material of *Festulolium* and *Lolium perenne* with good adaptation to non-cold hardening conditions. The level of resistance against *M. nivale* was evaluated in diploid and tetraploid *Festulolium* and *L. perenne* populations and cultivars, and *Festuca pratensis* cultivars, under controlled climatic conditions. These results were further compared with the degree of winter survival of *M. nivale*-inoculated plants of the same cultivars and populations under field conditions**.**

MATERIALS AND METHODS

1. Plant Material

Basic information on the 23 entries and populations of *L. perenne*, *Festulolium* and *F. pratensis* is presented in Table 1. The plant material consisted of diploid *Festulolium* introgression lines (*L. perenne* x *F. pratensis* and *L. multiflorum* x *F. arundinaceae*), amphidiploid (tetraploid) *Festuloliums* (*L. perenne* x *F. pratensis*, *L. multiflorum* x *F. arundinaceae* and *L. multiflorum* x *F. pratensis*) and three diploid *F. pratensis* cultivars of Nordic origin. Entries of *L. perenne* were divided according to their ploidy level: five diploid entries (two Norwegian entries and three central European) and three Norwegian tetraploid entries, also one hybrid ryegrass cultivar (*L. multiflorum* x *L. perenne*).

2. Testing Snow Mould Resistance under Controlled Conditions

2.1 Growing Conditions

Seeds were germinated at 18–22°C in a greenhouse, after 2 weeks, seedlings were transplanted into 10 cm pots (five seedlings per pot) with three replicates for inoculation and three non-inoculated replicates as control for each incubation period. The plants grew further in the greenhouse for 4 weeks at temperature 18-20°C and 16 h photoperiod in pots with a fertilized soil mixture (Gartner jord, TJERBO). The greenhouse was supplemented with a light source (Constant Color CMH™ Lamps 400W) at about 250 µmol. The plants were fertilized weekly with a mixture of 80 g/L Kristalon™ fertilizer 06-12-36 and 60 g/L of YaraLiva® Calcinit 15.5-0-0 (Yara International ASA, Oslo, Norway). The experiment was performed twice; experiment A was conducted during autumn 2011 and repeated in spring 2012 (experiment B).

2.2 Inoculation with M. nivale

The plants were inoculated with two isolates of *M. nivale* separately (not mixed) in the experiment. Isolate 201050 was isolated from *Festulolium* at Bjørke Experimental Farm (Graminor AS), Norway (60°48'4" N, 11°7'49" E) and isolate 200231 was isolated from *L. perenne* at Ås, Norway (59°39′37″ N, 10°47′1″ E). Isolates were identified according to Hofgaard et al. (2006a).. Isolates were obtained from the fungal culture collection at NIBIO, Plant Health and Biotechnology Division, Ås, Norway. In order to select these isolates, the

aggressiveness of 20 isolates was tested (data not shown). Inoculum was prepared (as described by Browne and Cooke 2004) by cultivating the fungal isolates on potato dextrose agar (PDA) plates incubated at 20°C for 7 days under 12 hours cycles of near-ultraviolet (NUV) and white light. Conidial suspensions were prepared by adding 10 ml sterile distilled water to each plate and scraping the agar with a glass objective slide. Spore suspensions of each isolate were adjusted with distilled water containing 0.2 % TWEEN 20 to 1×10^6 conidia ml^{-1}. The plants were inoculated 6 weeks after sowing by spraying each pot containing five plants with 10 ml spore suspension. Distilled water was used to mock inoculate control plants. Inoculated plants (three pots per entry per isolate) were randomly distributed among separate trolleys, while non-inoculated plants were placed in separate trolleys. Immediately after inoculation, the plants were covered with moistened cellulose wadding and black plastic sheets to maintain high humidity during incubation at 2°C in darkness. Non-inoculated plants were treated the same way as inoculated plants. The plants were incubated for 8 and 12 weeks in both experiments.

2.3 Evaluation of Snow Mould Resistance

After incubation for 8 and 12 weeks, the plants were cut to five cm above the soil surface and allowed to regrow in a greenhouse at 22°C and 16 h supplemental light as described above. After 2 weeks of regrowth, the plants were harvested (all parts above soil surface) and dried at 60°C for 3 days in order to measure dry weight (g DW pot^{-1}). Resistance to *M. nivale* was calculated as relative regrowth, i.e. dry weight (g) of inoculated plants divided by dry weight of non-inoculated plants within the same population after regrowth. Relative regrowth values approaching 1 indicate high resistance as described by Hofgaard et al. (2006a).

3. Assessment of Winter Survival in Field

3.1 Growing Conditions

Seeds were germinated at 18-20°C in a greenhouse at the end of July 2011. After 2 weeks, the seedlings were transplanted into 5.5 cm plastic pots (one plant per pot) and kept in the greenhouse for 3 weeks at 22°C under natural light conditions before they were moved outside for adaptation to natural conditions for 1 week. The plants were then transplanted into the field in mid-September 2011 in a completely randomized block experiment with four replicates each with 23 plots (one plot per entry) at an experimental field at the Norwegian University of Life

Sciences, Ås, Norway (59°39′37″N, 10°47′1″E). Each plot consisted of 24 single plants planted in a 4x6 row/column design with 15 cm between the plants in each direction. Weather data during the two following winter seasons were obtained from the nearest weather station (http://lmt-cloud.bioforsk.no/agrometbase/getweatherdata.php). Snow depth, air and soil temperature were observed from October until May each year (Fig. 2), and resistance to winter injury was scored visually in the spring (end of May) each year.

3.2 Inoculation with M. nivale

A highly aggressive isolate of *M. nivale* (isolate 200231, originally isolated from *L. perenne*) was chosen for inoculation, based on a preliminary screening (data not shown). Three replicates were inoculated in mid-November 2011 and mid-November 2012, while one replicate was kept non-inoculated. Inoculum of *M. nivale* was prepared on wheat grains according to Miedaner et al. (1993). Sterilized wheat grains were inoculated, and then incubated at 15°C for 10 days. Field inoculation was performed by adding two gram of infested grains (30- 35 grains) to each plant. Non-inoculated plants were sprayed twice with the fungicide Stratego EC 250 (Bayer Crop Science AG Monheim, Germany) before planting into the field and before inoculation.

3.3 Visual Assessment of Injuries

The degree of winter injuries was recorded in May 2012 and 2013, following the start of spring growth. The visual appearance of the tillers of each plant was scored using the following scale: 0 = no green tillers, 1 = some green tillers, 2 = less than 50% green tillers , 3 = more than 50% green tillers , and 4 = all tillers green (Hofgaard et al. 2003). The plants were cut three times during the 2012 growing season, dry matter yield (kg per plot) and percentage of the first cut to the total yield were determined. In spring 2013, winter survival was recorded as the number of surviving plants divided by the total number of plants per plot.

4. Statistical Analyses

Statistical analyses were performed using PROC GLM in SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). Orthogonal contrasts were used to test for differences between species as follows: *F. pratensis* vs. *L. perenne* and *Festulolium*, *L. perenne* vs. Festulolium, diploid vs.

tetraploid *L. perenne*, and diploid vs. tetraploid *Festulolium*. Pearson correlation coefficients between greenhouse and field data (except *F. pratensis* entries) were estimated using Minitab statistical software version 16 (Minitab Inc., State College, PA) *F. pratensis* entries were excluded from the correlation analysis due to the difference between *F. pratensis* and the other species in developmental stage at the time of inoculation during the experiments in controlled conditions. Differences between entries in snow mould resistance and winter survival were tested using Tukey's honest significance test, and differences between entries in dry matter yield and the degree of winter injury were tested using Fisher's Least Significant Difference (LSD) test, both tests performed using Minitab statistical software (version 16). The Ryan-Einot-Gabriel-Welsch (REGWQ) multiple range test in SAS was used to test for differences between species and species/ploidy levels in relative regrowth under controlled climate conditions, and dry weight of the first cut in the field following inoculation.

RESULTS

1. Snow Mould Resistance under Controlled Conditions

We found that the *M. nivale* isolates used in this study (200231 and 201050) was not significantly different as regards pathogenicity, except in experiment A after 8 weeks of incubation where the difference in pathogenicity between isolates was barely significant (Table 2; $P \leq 0.045$).. Differences in snow mould resistance between species were more significant in the tests performed during spring (experiment B, F-values of 12.19^{***} and 8.45^{***}) than in the tests performed in the autumn (experiment A, F-values of 2.52^{*} and 2.86^{*}). Orthogonal contrasts revealed that *F. pratensis* entries were on average significantly less resistant than other entries (except in experiment A after 12 weeks of incubation), whereas there was no significant differences in resistance between *L. perenne* and *Festulolium* entries (Table 2). Significant differences in resistance were also found between diploid and tetraploid *Festulolium* entries (F-values of 7.14^{**}, 5.67^{*} and 17.22^{***}), except for experiment A after 8 weeks of incubation; with tetraploid *Festulolium* being most resistant. Conversely, no statistically significant differences were found between diploid and tetraploid *L. perenne* entries (except for experiment B after 8 weeks of incubation, $F=17.20^{***}$); although diploid *L. perenne* entries in general seem to be most resistant. Interactions between isolates and entries and between isolates and species were not significant (except between isolate and entries in experiment A after 12 weeks of incubation, $F=2.41^{*}$). Relative regrowth measured 2 weeks after incubation for 12 weeks under artificial snow cover (experiment A and B) showed that tetraploid *Festulolium* entries, such as FuRs0465 and FuRs0463, were most resistant (Fig. 1). Entries that were generated through natural selection in Northern Norway (above 65° N), such as FuRs0463, also showed good resistance levels. In general, tetraploid *Festulolium* entries showed highest resistance levels in both experiments, and *F. pratensis* cultivars were the most susceptible (Table 3).

2. Field Experiment

Weather data (duration and depth of the snow cover, as well as soil and air temperatures) from October to May 2011-2012 and 2012-2013 are presented in Fig. 2. The climate in the two years was very different. The 2011 late autumn was very mild compared to the late autumn of

2012, resulting in a deeper and longer lasting soil frost in 2012. Also the snow cover was very shallow and transient in the winter 2011-2012 compared with the following winter.

After the winter of 2011-2012, visual assessment of winter injury showed that the *Festulolium* entries FuRs0348 and FuRs0353, both diploid and adapted to south Norwegian conditions, had lowest survival (visual scorings around 2). The other entries showed almost the same degree of winter injury, with a visual scorings around 3 (Table 4). Total dry matter (DM) yield was recorded by harvesting three times (week 25, 30 and 36) during the growing season of 2012 (Table 4). The *Festulolium* cv. Felopa had the highest total yield equivalent to 6.54 t ha^{-1}, while the *L. perenne* entry FuRa9805 was lowest yielding (2.94 t ha^{-1}). Only the dry weight of the first cut showed significant differences between species and entries. *F. pratensis* was significantly lower yielding compared to other species, while diploid entries of *Festulolium* showed the highest yield, significantly different from tetraploid *Festulolium* entries (Table 3 and 5).

After the winter of 2012-2013, the visual assessment of winter injury (in May 2013) did not reveal clear differences between entries (Table 4). However, analysis of variance based on the proportion of plants surviving the last winter (recorded in July 2013) showed significant differences between entries (Fig. 1). *F. pratensis* cultivars had the highest winter survival. Among *Festulolium* and *L. perenne* entries, northern adapted cultivars of *L. perenne* (such as Fagerlin and LøRa9401) had the highest winter survival, almost 70 to 80 % of the plants survived. Moreover, amphiploid *Festulolium* (tetraploid) hybrids such as Felopa and FuRs0467 had higher winter survival than introgression hybrids (diploid); no plants of FuRs0026, FuRs0348 and FuRs0353 survived (Fig. 1).

3. Correlation Analysis.

Correlation analysis based on the data from controlled conditions and the field experiments showed no significant correlations between snow mould resistance under controlled conditions and the degree of winter injury under field conditions (Table 6).

Tetraploid *Festulolium* hybrids (such as FuRs0465 and FuRs0463) showed high snow mould resistance under controlled conditions but their winter survival in the field was low. Northern adapted cultivars of *L. perenne*, such as Fagerlin and LøRa9401, showed high winter

survival in the field but had only a moderate snow mould resistance in the test under controlled climate conditions. A significant positive correlation was found only between the visual assessment of winter injury and the percentage of plants surviving the winter 2012-2013.

DISCUSSION

1. Snow mould resistance under controlled conditions

The main objective of the present study was to identify plant materials of *L. perenne* and *Festulolium* with good adaptation to the extended growth season caused by climate warming at higher latitudes. Warmer autumns may give inferior conditions for cold acclimation. Cold hardening is essential for development of maximal resistance to snow moulds in grasses (Tronsmo 1984b; Tronsmo 1994), and current cultivars of perennial grasses may be more prone to snow mould injuries in the future climate. Thus, we need forage grass cultivars with adequate levels of winter survival and snow mould resistance adapted to the new climate. Little is known about the variation in snow mould resistance of cultivars and breeding populations of *L. perenne* and *Festulolium* developed for northern regions. In this study, we wanted to evaluate snow mould resistance in non-cold acclimated plants in order to mimic the expected future conditions during warmer autumns, and compare this with winter survival of the same plant materials in the field.

Two experiments (A and B) were conducted (once during autumn and once during spring) and the plants were incubated for 8 and 12 weeks after inoculation. We used different lengths of the incubation period because in our experience, a longer incubation period is needed when inoculating with fungal spores compared to inoculation with a mycelial suspension. Moreover, the perennial ryegrass and *Festulolium* plants at 6 weeks age were quite well developed at the time of inoculation. Significant differences in snow mould resistance between species were detected in both test A and B, but highly significant differences ($P \leq 0.001$) were only found in the experiment performed during spring. Årsvoll (1977) also found that snow mould resistance under controlled conditions depends upon the growth conditions before inoculation. In both our tests, differences in resistance between entries were less distinct (lower F-values) after a long incubation period (12 weeks). Therefore, it is difficult to detect differences in resistance if the plants are propagated at higher temperatures or at longer photoperiods also if the plants are incubated for longer periods. Similar results were found by Årsvoll (1977). In the current study no significant interactions were found between species and isolates. Two *M. nivale* isolates from different host plants were used to reveal host preferences between isolates since plant materials used in this study consisted of different grass species. However, differences in

pathogenicity between the isolates were not-significant in most of our experiments. The interaction between isolates and entries was only significant in experiment A after 12 weeks of incubation. However, no significant difference was found between isolates in this experiment. The significant isolate x entry interaction was most likely due to the longer incubation period generally reducing plant regrowth and several entries died, especially entries inoculated with isolate 200231 which was more pathogenic than isolates 201050.

F. pratensis is generally known to have better winter survival than both *L. perenne* and *Festulolium* (Pociecha et al. 2008). Nonetheless, in the present study entries of *F. pratensis* had the lowest resistance to snow mould, measured as relative regrowth. The reason may be the plants were at a much younger developmental stage at the time of inoculation due to the slower establishment of *F. pratensis*. Thus, this result may reflect that the test method used in this study, is unsuitable for comparing grass species with very different rates of germination and growth. In a study of injury caused by *M. nivale* or *Sclerotinia borealis* in cultivars of *Phleum pratense* and *F. pratensis*, the variation in plant development did not appear as a confounding factor (Tronsmo 1984a). However, both *P. pratense* and *F. pratensis* have slower development than *L. perenne* and *Festulolium.* An alternative approach for comparing different grass species is to test older plants that have been cut once or twice.

In the present study, we found significant differences in snow mould resistance between amphiploid *Festulolium* (tetraploid) entries and diploid entries in most of the controlled experiments, with tetraploid entries such as FuRs0463 and FuRs0465 being the most resistant ones. Amphiploid hybrids demonstrated better winter survival than diploid introgression hybrids under Nordic conditions (Østrem et al. 2013). Also the entries degree of northern adaptation had significant influence on their responses to snow mould infection. However, we found that entries adapted to south Norwegian conditions, such as FuRs0465, and continental central European conditions, such as Felopa (LmFp/T) and Picaro (Lp/D), were able to achieve a good level of snow mould resistance. Furthermore, this might reflect the genetic background of the material and not only the area of adaptation.

Due to global warming, future weather conditions during autumn may not be sufficient for cold acclimation of most of the current cultivars. Regional climate change projections conclude that the largest warming is projected for northern Europe, and that the duration of the snow season will very likely be shorter (Christensen et al. 2007). Under future climate,

occurrence of snow mould attack may be less frequent, but more serious outbreaks can be expected in some years (Murray and Gaudet 2013; Rapacz et al. 2014). Thus, southern adapted materials may be considered as good sources for snow mould resistance under future climatic conditions with shorter cold acclimation periods.

2. Field experiment

In the present study, *F. pratensis* cultivars survived well during both winters, which can be explained by these cultivars being highly adapted to harsh winter conditions. In addition to the different weather conditions during the two years, a reduced winter survival from first to the second (and third) year for the non-native species (*L. perenne* and *Festulolium*) is as expected. Furthermore we found that artificial inoculation in the field experiment was most efficient when the weather conditions were favourable for *M. nivale*, i.e. during the winter of 2012-2013 when the snow cover lasted quite long, from the beginning of January until the end of April. On the contrary, the winter conditions during the winter of 2011-2012 were not suitable for snow mould development, most entries survived well and very little variation in winter survival was revealed (Table 4). Therefore, a non-significant correlation was found between the degree of winter injury and the percentage of the first cut to the total yield. Resistance to snow mould is considered to be a quantitative trait, and under field conditions it can be masked by the winter survival ability of the plant (Ergon et al. 2003; Gaudet 1994; Hofgaard et al. 2006b).

In the present study, winter survival under field conditions was largely determined by the degree of northern adaptation of each entry, except for *F. pratensis* entries, which survived equally good irrespective of origin. A southern adapted diploid introgression hybrid (FuRs0467) showed better winter survival (32% of plants survived) than hybrid FuRs0348 developed from surviving plant materials of a population from United Kingdom (0% survival). Although both being bred by introgression method and selected under Norwegian conditions these differences reflect the higher inherent potential for adaptation in the plant breeding material originating from Nordic conditions compared with more southern conditions. Furthermore, the *Festulolium* entry FuRs0463, which was selected from the southern adapted cultivar Fabel (FuRs9806) under Northern Norwegian conditions, showed good winter survival. Hence, the selection for only two winters in Bodø (67° 17′ N) increased winter survival of FuRs0463 (30% of plants survived) in comparison to Fabel (3 % of plants survived). In order to improve winter survival of non-adapted

materials, natural selection for several generations will be necessary since adaptation processes takes a long time. Natural selection of *Festulolium* has proved successful (Casler et al. 2002) and therefore, such material could be useful in breeding program for future climates. The adaptation type of *L. perenne* entries also had considerable impact on their response towards snow mould infection. Entries adapted to North Norwegian conditions, such as Fagerlin and LøRa9401, showed good winter survival and they showed moderate resistance towards snow mould in the controlled condition experiments. Winter survival depends on many factors and traits; one of them is snow mould resistance. Previous studies have shown that *P. pratense* and *F. pratensis* populations originating from Northern Norway were more resistant to snow mould fungi and had less winter injury than southern adapted populations (Årsvoll 1977; Jamalainen 1974). Climatic conditions in Scandinavia provide great potential for utilizing *Festulolium* hybrids (Østrem et al. 2013), which combine the high productivity of *Lolium* with the high winter survival of *Festuca* (Rapacz et al. 2005). The major problems facing *Festulolium* breeding programs are low seed production and genetic instability, mainly because the domination of the ryegrass genome over the fescue genome (Kopecky et al. 2005; Thomas et al. 2003). The risk of losing *Festuca* genes responsible for winter survival and disease resistance during the backcrossing is considerable (Østrem et al. 2013). Identification of the different traits responsible for winter survival and disease resistance will help breeders to select and map the major genes responsible and integrate those genes into new hybrids (Humphreys et al. 2003). According to Østrem et al. (2013) natural selection under Nordic conditions generally should result in better winter survival ability of the *Festulolium* hybrids.

Our results did not reveal significant correlations between snow mould resistance of non-hardened plants under controlled conditions and winter survival under field conditions. This may indicate that cold acclimation exerts a major influence on the expression of snow mould resistance. However, snow mould resistance testing under controlled conditions could help in identifying the most resistant and the most susceptible genotypes or populations, and to separate snow mould resistance from other winter stress factors. Moreover, the sources of resistance to snow mould identified in this study, especially from Norwegian adapted entries, should be exploited in further breeding programs, taking into consideration that also entries adapted to central European conditions could be good sources of snow mould resistance under future climates.

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Table1. Species origin and basic characteristics of the plant materials used in greenhouse and field experiments.

Species	Abbreviation / Ploidy level	Entry [a]	Country of origin [b]	Description of genetic origin / adaptation level (regions) [c]
Meadow fescue *F. pratensis*	Fp / D	Fure	NO	Southern adapted.
		Norild	NO	Northern adapted.
		Stella	DK	Southern adapted (DLF Trifolium®, DK)
Festulolium	Lp^{Fp} / D	FuRs0356	NO	Southern adapted, UK origin
		FuRs0026	NO	Southern adapted, UK origin and selected in NO (61°N).
		FuRs0467	NO	Southern adapted, Nordic material selected in mid-Norway (63°N).
	Lm^{Fa} / D	FuRs0348	NO	Southern adapted, UK origin and selected in NO (Fureneset).
	LmFp / D	FuRs0353	NO	Southern adapted. Dihaploid plants, mainly from cv. Felopa (PL).
	LpFp / T	FuRs0142	NO	Southern adapted, UK x Nordic material.
		FuRs9812	NO	Southern adapted, UK origin with several cycles of natural selections in mid-Norway (63°N).
		FuRs9806 (Fabel)	NO	Southern adapted. UK origin, natural selection of single plants in NO (61°N).
		FuRs0463	NO	Southern adapted. Selection in Fabel after two winters in northern Norway (67°N).
	LmFp / T	Felopa	PL	Southern adapted. (DANKO®, PL).
	LmFa / T	FuRs0465	NO	Southern adapted. CZ (Hladke Zivotice®) x commonly used Lm cultivars
Perennial ryegrass *L. perenne*	Lp / D	Arka	PL	Southern adapted (Central European/continental conditions, PL).
		Fagerlin	NO	Northern adapted.
		FuRa9805	NO	Southern adapted.
		Picaro	NL	Southern adapted (Eurograss)
	Lp / T	Figgjo	NO	Southern adapted.

		Ivar	NO	Northern adapted.
		LøRa9401	NO	Northern adapted; surviving plants at high altitude.
		Fjaler [d]	NO	Southern (mid-Norway) adapted.
Hybrid ryegrass	LmLp / T	Fenre	NO	Southern adapted.

The entries are designated according to species and for the *Festulolium* entries to their hybrid origin or introgression line composition; Lp = *L. perenne*, Lm = *L. multiflorum*, Fa = *F. arundinacea* and Fp = *F. pratensis*, with the abbreviation given as a superscript, e.g. ^{Fp} when the entry consists of segments of *F. pratensis* in the *L. perenne* genome (introgression line). Ploidy level is indicated as diploid (D) and tetraploid (T).

[a] All entries designated "FuRs" are candivars, i.e. promising breeding populations; the remaining entries are commercial cultivars

[b] Country of origin (country codes, as defined by International Organization for Standardization, ISO 3166 code. NO for Norway, DK for Denmark, PL for Poland and NL for Netherlands)

[c] Southern: adapted to south Norwegian conditions (from 57° to 65° N); northern: adapted to North Norwegian conditions (above 65° N).

[d] Cultivar only used in the field experiments

Table 2. Analysis of variance of resistance against two *Microdochium nivale* isolates among 22 cultivars and candivars of *L. perenne*, *F. pratensis* and Festulolium under controlled climate conditions during autumn (exp. A) and spring (exp. B). The plants were inoculated 6 weeks after sowing and incubated under artificial snow cover for 8 and 12 weeks. Resistance was measured as relative regrowth, i.e. dry weight of inoculated plants divided by dry weight of non-inoculated control plants.

Experiment		A				B			
Incubation period		8 weeks		12 weeks		8 weeks		12 weeks	
Source	DF	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F
Isolate	1	5.15	0.0458	0.22	0.6401	1.63	0.2060	0.00	0.9793
Species	4	2.52	0.0500	2.86	0.0350	12.19	<.0001	8.45	<.0001
Entries (species)	16	5.13	<.0001	3.20	0.0013	4.88	<.0001	2.41	0.0053
Fp vs. all	1	5.44	0.0229	0.01	0.9135	21.68	<.0001	10.25	0.0020
Lp vs. Fe	1	3.39	0.0701	2.79	0.1024	3.83	0.0537	2.51	0.1169
Fe / D vs. Fe / T	1	0.8	0.3737	7.14	0.0107	5.67	0.0196	17.22	<.0001
Lp/ D vs. Lp / T	1	0.81	0.3715	1.24	0.2714	17.20	<.0001	2.00	0.1607
Isolate*species	4	2.18	0.0812	0.36	0.8347	1.63	0.1753	1.08	0.3733
Isolate* Ent.(species)	16	1.08	0.3931	2.41	0.0114	1.15	0.3273	1.41	0.1587

Fp=*Festuca pratensis;* Fe= Festulolium; Lp=*Lolium perenne;* Ploidy level is indicated as diploid (D) and tetraploid (T).

Table 3. Relative regrowth (resistance [a]) under controlled climate conditions and dry weight of the first cut in the field following inoculation averaged within species and species/ploidy levels.

Controlled climate conditions experiments [a]					
Experiments	A		B		Dry weight of first cut [b]
Incubation period	8 weeks	12 weeks	8 weeks	12 weeks	
Festuca pratensis	0.53*b*	0.1*ab*	0.61*b*	0.35*c*	0.68*d*
Festulolium / D	0.56*ab*	0.08*ab*	0.83*a*	0.44*bc*	0.96*a*
Festulolium / T	0.61*ab*	0.06*b*	0.75*a*	0.63*a*	0.84*b*
Lolium perenne /D	0.69*a*	0.11*ab*	0.83*a*	0.51*ab*	0.78*bc*
Lolium perenne /T	0.65*ab*	0.18*a*	0.65*b*	0.44*bc*	0.87*ab*

[a] Resistance towards two *Microdochium nivale* isolates in 22 cultivars and candivars of *L. perenne*, *F. pratensis* and *Festulolium* under controlled climate conditions during autumn (exp. A) and spring (exp. B). Resistance was measured as relative regrowth, i.e. dry weight of inoculated plants divided by dry weight of non-inoculated control plants.

[b] Dry weight (Kg) of inoculated plots (3 replications, 24 plants in each) during the 2012 growing season.

Ploidy level is indicated as diploid (D) and tetraploid (T). Different letters indicate significant differences based on Ryan-Einot-Gabriel-Welsch (REGWQ) multiple range test ($P \leq 0.05$).

Table 4. Dry matter yield (DMY t ha^{-1}) and the degree of winter injury in the spring of 2012 and 2013.

Entry	Species	DMY 2012 [a] (t ha^{-1})	Winter injury [b] (2012)	Winter injury [b] (2013)
Fure	*F. pratensis*	3.97*cd*	2.6*abc*	2.4*a*
Norild	*F. pratensis*	3.95*cd*	2.5*cd*	2.4*a*
Stella	*F. pratensis*	4.24*bcd*	2.6*abc*	2.0*a*
FuRs0356	*Festulolium*	5.02*abcd*	2.8*abc*	0.1*b*
FuRs0026	*Festulolium*	4.73*abcd*	2.8*abc*	0.0*b*
FuRs0467	*Festulolium*	6.21*ab*	2.9*ab*	0.2*b*
FuRs0348	*Festulolium*	5.99*abc*	1.9*e*	0.0*b*
FuRs0353	*Festulolium*	6.42*a*	2.1*de*	0.0*b*
FuRs0142	*Festulolium*	4.96*abcd*	2.7*abc*	0.1*b*
FuRs9812	*Festulolium*	4.4*abcd*	2.6*abc*	0.1*b*
FuRs9806	*Festulolium*	4.2*bcd*	2.5*abc*	0.0*b*
FuRs0463	*Festulolium*	4.59*abcd*	2.7*abc*	0.1*b*
Felopa	*Festulolium*	6.54*a*	2.6*abc*	0.2*b*
FuRs0465	*Festulolium*	4.88*abcd*	2.5*abc*	0.0*b*
Arka	*L. perenne*	5.31*abc*	2.8*abc*	0.0*b*
Fagerlin	*L. perenne*	4.75*abcd*	2.8*abc*	0.4*b*
FuRa9805	*L. perenne*	2.94*d*	2.8*abc*	0.0*b*
Picaro	*L. perenne*	5.33*abc*	2.9*a*	0.0*b*
Figgjo	*L. perenne*	6.05*abc*	2.7*abc*	0.0*b*
Ivar	*L. perenne*	4.61*abcd*	2.7*abc*	0.1*b*
LøRa9401	*L. perenne*	3.91*cd*	2.8*abc*	0.6*b*
Fjaler	*L. perenne*	4.47*abcd*	2.7*abc*	0.1*b*
Fenre	*L. perenne*	5.21*abc*	2.6*abc*	0.0*b*

[a] Total dry mater yield (tonne per hectare) over 3 cuts during the growing season of 2012.

[b] The degree of winter injury was measured by visual scoring during the growing season of 2012 (May 2012) and 2013 (May 2013). Visual assessment was done according to the

following scale: 0 = no green tillers, 1 = some green tillers visible, 2 = green tillers found in less than half of the total plant area, 3 = green tillers found in more than half of the plant area, and 4 = green tillers observed in the whole plant area. Different letters indicate significant differences based on Fisher's Least Significant Difference (LSD) test ($P < 0.05$).

Table 5. Analysis of variance of the dry matter yield (kg/plot[a]) of the first cut during the growing season of 2012.

Source	DF	F Value	Pr > F
Rep	2	2.66	0.0814
Species	4	5.0	0.0050
Entries (species[b])	17	3.99	<.0001
Fp vs. all	1	10.23	0.0018
Lp vs. Fe	1	2.42	0.2188
Fe / D vs. Fe / T	1	4.45	0.0408
Lp/ D vs. Lp / T	1	2.98	0.5742

[a] Dry weight (kg) of infected plots (3 replications with 24 plants in each replicate).

[b] Fp=*Festuca pratensis*, Fe= Festulolium, Lp=*Lolium perenne.* Ploidy level is indicated as diploid (D) and tetraploid (T).

Table 6. Correlation between resistance to snow mould under controlled conditions, measured as relative re-growth, and winter survival and yield observed in a field trial over 2 years.

Traits	The degree of winter injury (2012)[b]	The degree of winter injury (2013)[b]	% first cut to the total yield (2012)	% Winter survival (2013)[c]
Resistance to snow mould[a]	- 0.131^{ns}	- 0.236^{ns}	- 0.191^{ns}	- 0.241^{ns}
The degree of winter injury (2012)		0.229^{ns}	0.049^{ns}	0.256*
The degree of winter injury (2013)			-0.007^{ns}	0.982*
% first cut to the total yield (2012)				-0.013^{ns}

* Significant at the 0.05 level, ^{ns} non-significant.

a Resistance was measured as relative regrowth (dry weight of inoculated plants divided by dry weight of non-inoculated plants) after twelve weeks (exp. A and B) incubation under artificial snow cover followed by two weeks of regrowth.

b The degree of winter injury was measured by visual scoring during the growing season of 2012 (May 2012) and 2013 (May 2013). Visual assessment was done according to the following scale: 0 = no green tillers, 1 = some green tillers visible, 2 = green tillers found in less than half of the total plant area, 3 = green tillers found in more than half of the plant area, and 4 = green tillers observed in the whole plant area.

c The percentage of plants on the plots alive in the spring of 2013.

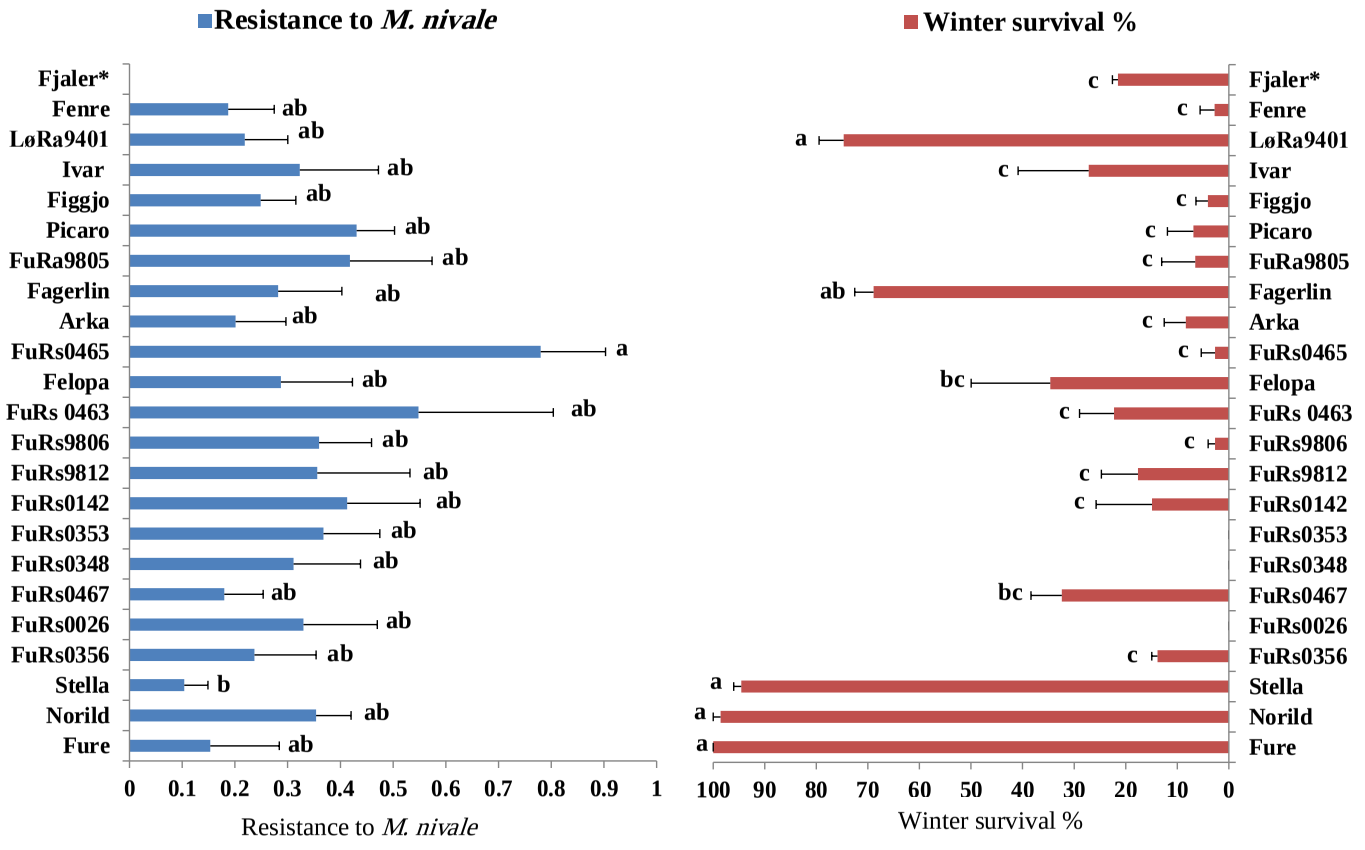
Resistance to M. nivale
Fjaler* ab
Fenre ab
LøRa9401 ab
Ivar ab
Figgjo ab
Picaro ab
FuRa9805 ab
Fagerlin ab
Arka ab
FuRs0465 a
Felopa ab
FuRs 0463 ab
FuRs9806 ab
FuRs9812 ab
FuRs0142 ab
FuRs0353 ab
FuRs0348 ab
FuRs0467 ab
FuRs0026 ab
FuRs0356 ab
Stella b
Norild ab
Fure ab
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1
Resistance to M. nivale
Winter survival %
c Fjaler*
c Fenre
a LøRa9401
c Ivar
c Figgjo
c Picaro
c FuRa9805
ab Fagerlin
c Arka
c FuRs0465
bc Felopa
c FuRs 0463
c FuRs9806
c FuRs9812
c FuRs0142
FuRs0353
FuRs0348
bc FuRs0467
FuRs0026
c FuRs0356
a Stella
a Norild
a Fure
100 90 80 70 60 50 40 30 20 10 0
Winter survival %

Fig.1. Variation in resistance to *M. nivale* and winter survival in 23 grass entries (Table 1). Resistance was measured as relative regrowth (dry weight of inoculated plants divided by dry weight of non-inoculated plants) after 12 weeks (experiment A and B) incubation under artificial snow cover followed by 2 weeks of regrowth. Winter survival is the percentage of plants on the plots alive in the spring of 2013. Error bars indicate standard errors of the mean. Bars marked with different letters indicate significant differences (using Tukey's honest significance test at $P \leq 0.05$). * Cultivar only used in the field experiments.

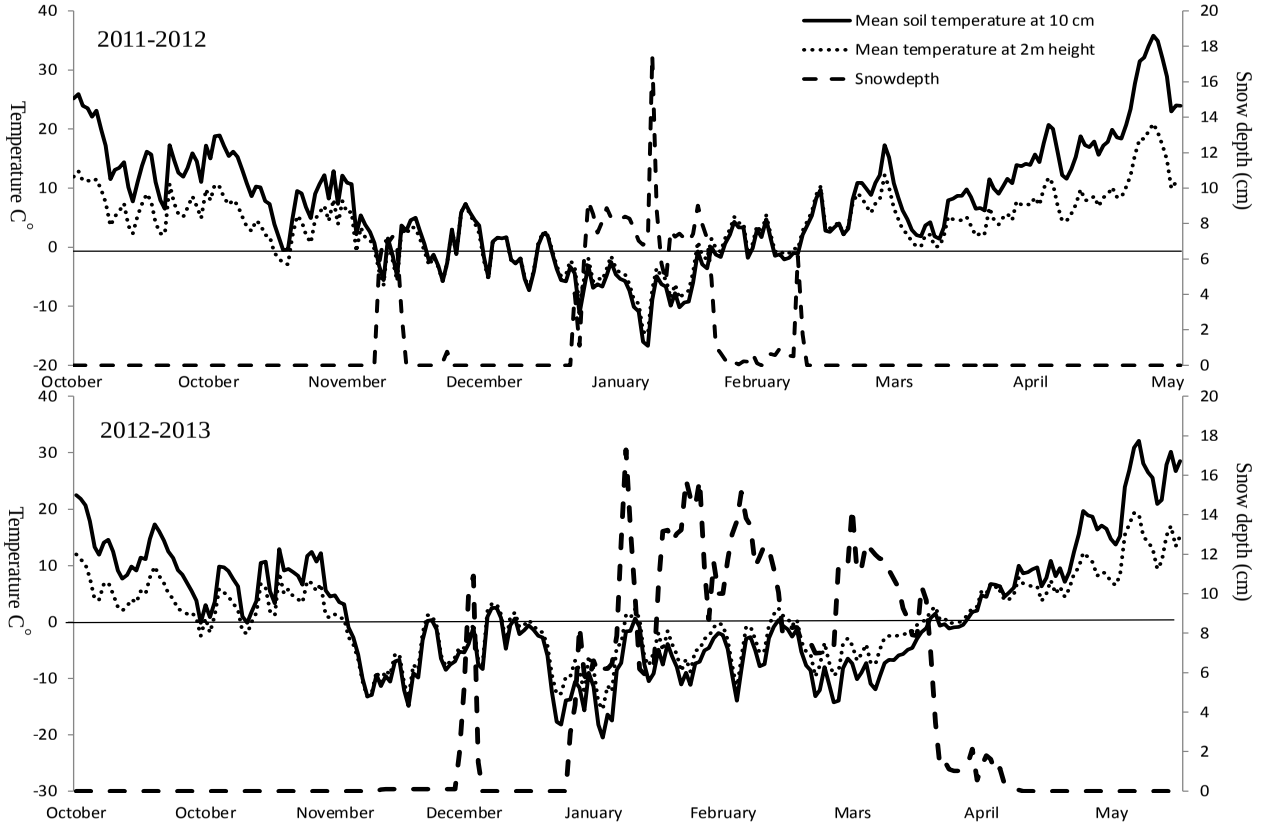

Fig. 2. Soil and air temperature (C°), and snow depth (cm) recorded during autumns and winters in the field.

Paper II

Abdelhalim M., Brurberg M.B., Hofgaard I.S. and Tronsmo A.M.

Pathogenicity, host specificity and genetic diversity in isolates of *Microdochium nivale* and *M. majus*

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Abstract

Microdochium majus and *Microdochium nivale* cause important diseases in grasses and cereal crops in the temperate regions. Both fungi can infect the plants during winter as well as under cool humid conditions during spring and fall. In the present study, the relation between pathogenicity and genetic diversity of different strains of both species were investigated. A pathogenicity test of 14 *M. nivale* isolates and two *M. majus* isolates was conducted on four different grass cultivars of *Lolium perenne* and *Festulolium* hybrids. A large variation in pathogenicity was found between the *M. nivale* isolates. Furthermore, *M. nivale* strains originally isolated from grasses were more pathogenic than isolates from cereals. The genetic diversity of *M. nivale* and *M. majus* isolates was studied by sequencing four different genetic regions; Elongation factor-1alpha (*EF-1α*), Beta-tubulin, RNA polymerase II (*RPB2)* and Internal transcribed space (ITS). Phylogenetic trees based on the sequence of the four genes, resolved *M. nivale* and *M. majus* isolates into separate clades. Higher genetic diversity was found among *M. nivale* isolates than among *M. majus* isolates. Furthermore, genetic differences were found between *M. nivale* isolates from different host plants (grasses vs. cereals) and different geographic regions (Norway and UK vs. North-America). Sequence results from the *RPB2* and β-tubulin genomic regions were more informative than those from the ITS and *EF-1α* genes. The genetic and phenotypic differences detected between Norwegian *M. nivale* isolates from cereals and grasses support the assumption of the existence of host specialization within *M. nivale* isolates.

Key words:

Microdochium majus, *Microdochium nivale*, pink snow mould, genetic diversity and pathogenicity

Introduction

Microdochium nivale (Fr.) Samuels and I. C. Hallett is the most prevalent low temperature pathogen in cereals and grasses in Norway (Årsvoll 1973; Ergon et al. 2003; Larsen 1994). The fungus was first described by the Swedish mycologist E.M. Fries (1825) under the name *Lanosa nivalis*. Fries characterized the fungus by its ability to attack wheat and grass plants under snow cover (Noble & Montgomerie 1956). Due to its similarity with *Fusarium* species, it was given the name *Fusarium nivale* (Tronsmo et al. 2001). The fungus has been reclassified several times (Booth 1971; Gams 1989; Glynn et al. 2005; Samuels & Hallett 1983). The fungus produce conidia of two different sizes and with different number of septa and was according to this divided into two varieties, var. *majus* and var. *nivale*, by Wollenweber (1930). Gams and Müller (1980) reclassified the fungus as *Gerlachia nivale* due to the absence of conidial foot cell. Later Samuels and Hallett (1983) showed that the fungus rather belong to the genus *Microdochium*.

Molecular genotyping techniques have been applied to investigate genetic differences between isolates of *Microdchium* var. *majus* and *Microdchium* var. *nivale*. Parry et al. (1995) were able to differentiate between the two varieties using restriction enzyme analysis of the Internal Transcribed Spacer (ITS) region. Lees et al. (1995) confirmed the distinction between the two varieties by using RAPD (Random Amplified Polymorphic DNA) genotyping. Lees et al also detected higher levels of diversity in var. *nivale* isolates than in var. *majus* isolates. Later Glynn et al. (2005) suggested that var. *majus* and var. *nivale* should be two different species, *Microdochium majus* and *Microdochium nivale*, based on the elongation factor 1 alpha gene sequences. Several studies have used constitutive genes such as RNA polymerase II (*RPB2*) and the β-tubulin gene to study fungal population structure in relation to host or geographic origin (Einax & Voigt 2003; Myllys et al. 2001; Pavlic et al. 2009). Recently Jewell and Hsiang (2013) used β-tubulin and *RPB2*, elongation factor 1 alpha (*EF-1α*) gene sequences, in addition to the ITS region to differentiate between *M. nivale* and *M. majus* isolates from different host plants and different geographic regions. In that study, isolates from different geographic origin (Europe vs. North America) and different host plants (grasses vs. cereals) were different based on *RPB2* and β-tubulin genes sequence.

Variation in pathogenicity between *M. majus* and *M. nivale* has been reported in several studies on grasses (Hofgaard et al. 2006; Holmes 1976) and on cereals (Diamond &

Cooke 1999; Maurin et al. 1995). Isolates of *M. nivale* were more pathogenic on winter rye than *M. majus* isolates (Simpson et al. 2000), as well as on perennial ryegrass (Hofgaard et al. 2006), while *M. majus* isolates were more pathogenic on winter wheat (Diamond & Cooke 1997; Simpson et al. 2000). Furthermore, differences in pathogenicity was found among isolates of *M. nivale* from grasses and from cereals (Årsvoll 1973; Hofgaard et al. 2006; Litschko & Burpee 1987; Smith 1983). Host specialization within the groups of *M. nivale* isolates has been studied by molecular genotyping techniques such as RAPD and IGS-RFLP (the intergenic spacer restriction fragment length polymorphisms) (Mahuku et al. 1998), and also by DNA sequencing (Jewell & Hsiang 2013). Moreover, both studies indicate the existence of host specialization especially within *M. nivale* isolates. In the study by Mahuku et al. (1998) isolates from different grass species (100 isolates) were grouped in four clusters according to their host using IGS-RFLP analysis .

An effective strategy for disease control should be based on knowledge of the population structure of the plant pathogen, and how the level of genetic variation between the pathogen populations reflects the interaction between the pathogen and the host plant (Allard 1990; McDonald & McDermott 1993). Therefore, the aim of our study was to characterize genetic and phenotypic (pathogenicity) diversity of different Norwegian isolates of *M. nivale* and *M. majus* by testing pathogenicity of 17 isolates (15 isolates of *M. nivale* and two isolates of *M. majus*) and sequencing the genomic regions of four genes; Elongation factor-1 alpha (*EF-1α*), Beta-tubulin, RNA polymerase II (*RPB2)* and Internal transcribed space (ITS).

Materials and methods

1. Fungal isolates, identification and cultivation

The isolates used in this study were mainly collected in the period from 1975 to 2010, *M. nivale* isolates were mainly isolated from leaves of both cereals and grasses displaying snow mould symptoms, whereas most of the *M. majus* isolates were isolated from symptomatic leaves or seeds of wheat (Table 1). *M. nivale* isolates NG26 and NG42, and *M. majus* isolates NG3 and NG36 were kindly provided by Simon Edwards, Harper Adams University, UK. One isolate of *M. majus* (OP2A) was kindly provided by Roy Browne, University College Dublin, Ireland. The isolates were preserved at – 80 °C as mycelium on 5 mm PDA (potato dextrose agar) plugs in 1.5 ml microfuge tubes in the fungal culture collection at NIBIO, Plant Health Division, Ås, Norway (Table 1). Fungal isolates were identified to species based on colony and conidial morphology according to Gerlach and Nirenberg (1982). Asporogenic isolates (7 isolates listed in Table 1) were classified using polymerase chain reaction (PCR) according to Glynn et al. (2005).

2. Pathogenicity test

A pathogenicity test was performed on the grass species *Lolium perenne* cvs. Figgjo and Ivar, and *Festulolium* hybrids FuRs9812 and FuRs0463. Seeds were germinated in a greenhouse at (18 to 22) °C and 16 h photoperiod. The greenhouse was supplemented with a light source (Constant Color CMH™ Lamps 400W) of about 250 µmol photosynthetic active radiation (PAR) m^{-2} s^{-1}. After two weeks, the seedlings were transplanted to 10 cm pots containing fertilized soil mixture (Gartner jord, Tjerbo); 5 plants per pot and 6 pots per cultivar (3 for inoculation and 3 as controls). The plants were fertilized weekly with a mixture of 80 g/L Kristalon™ fertilizer 06-12-36 and 60 g/L of YaraLiva® Calcinit 15.5-0-0 (Yara International ASA, Oslo, Norway).

Isolates of *M. nivale* (15 isolates) and *M. majus* (two isolates) were selected for this study (as listed in table 1). To recover the isolates after storage, they were inoculated onto 9 cm PDA plates and incubated for 10 days at 9 °C in darkness. The isolates were then transferred to new plates and incubated at 20 °C for 7 days under 12 hours cycles of near-ultraviolet and white light for sporulation. Conidial suspensions were prepared by washing the agar with 10 ml sterile distilled water containing 0.2 % Tween 20, and adjusted to 1×10^6 conidia ml^{-1}.

Each of the 17 isolates was inoculated on each of the four cultivars (3 pots per isolate) by spraying 10 ml of the spore suspensions per pot, as described by Hofgaard et al. (2006). Controls were sprayed with distilled water containing 0.2 % Tween 20. Inoculated plants were randomly distributed on four trolleys, while non-inoculated plants were placed on two separate trolleys. To maintain high humidity during incubation at 2 °C in darkness, all plants (including controls) were covered with moist cellulose wadding and black plastic sheets immediately after inoculation. The experiment was conducted twice, the first time (experiment A) plants were incubated for 8 weeks after inoculation and the second time (experiment B) plants were incubated for 9 weeks after inoculation.

After incubation, the plants were cut to 5 cm above the soil surface and allowed to regrow in the greenhouse under the same conditions as described above. After two weeks of regrowth, all plant material above the ground were harvested and dry weights (g /pot) were determined. Pathogenicity was calculated as 1- relative regrowth (as described by Hofgaard et al. 2006).

Analysis of variance in pathogenicity between isolates was performed using PROC GLM in SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). Significant differences between isolates ($P < 0.05$) in pathogenicity were calculated by Ryan-Einot-Gabriel-Welsch (REGWQ) multiple range test in SAS.

3. DNA extraction

Isolates of *M. nivale* (30 isolates) and *M. majus* (14 isolate) were selected for DNA sequencing. Isolates were a part of the same collection as mention above (isolate description was listed in table 1). Fungal isolates were inoculated on PDA plates and incubated for one week at 20 °C in darkness. Mycelium was harvested by carefully scraping it off the agar surface using a clean razor blade, and then frozen quickly in liquid nitrogen and grinded using a mortar and a pestle. DNA was extracted from100 mg grinded mycelium using DNeasy Plant Mini Kit (Qiagen Inc., Germany), according to the manufacturer's protocol. DNA quality was verified by agarose gel electrophoresis. DNA was stored at – 20 °C prior to PCR amplification.

4. PCR amplification and sequencing

PCR amplification was performed in a 25 µl volume containing 2.5 µl of Taq polymerase buffer (10 x GeneAmp PCR buffer contain 15 mM $MgCl_2$), 10 pmol each of forward and reverse primer (Invitrogen Ltd, UK), 200 µM dNTP, 1.0 unit Taq DNA polymerase (AmpliTaq - Applied Biosystems, Foster City, CA) and 2 µl of fungal DNA. For ITS amplification the cycling protocol was performed according to White et al. (1990). For the *EF-1α* gene amplification the protocol was according to Glynn et al. (2005). For the *RPB2* gene and the β-tubulin gene the amplification protocols were as described by Jewell et al.(2013) . The PCR products were visualized using gel electrophoresis and a UV-transilluminator (GelDoc 1000 gel documentation system, BioRad). The PCR products were purified and sequenced in both directions at GATC Biotech (Germany).

5. Sequence data analysis

DNA sequence data were assembled and analysed using the Lasergene Seqman software (DNAStar Inc.). Genomic sequences used in further analyses have been deposited in the NCBI GenBank® database (accession numbers: KT736151 - KT736180 for *EF-1α*, KT736181 - KT736220 for ITS, KT736221 - KT736256 for β-tubulin and KT736257 - KT736288 for *RBP2*). Fragments of 723, 647, 404, and 399 bp were used to produce multiple sequence alignments for *RBP2*, β-tubulin, *EF-1α* and ITS respectively. Available sequence of *RPB2* and β-tubulin for isolates from North America were obtained from the NCBI GenBank® database (supplementary Table 1). The sequence alignments were performed using the CLC Main Workbench version 6.9.1 (CLC Inc. Aarhus, Denmark) with default parameters. Neighbour-joining (NJ) phylogenetic trees were constructed for each gene by the CLC software using maximum likelihood (ML) algorithm with 1000 bootstrap replicates. Pairwise comparison was computed based on aligned sequence using CLC Genomic Workbench version 6.9.1 (CLC Inc. Aarhus, Denmark) to estimate the percentage of identity (percentage of identical residues in alignment position to overlapping alignment between sequences), as well as the pairwise distance based on the Jukes-Cantor distance. The sequences of *Microdochium bolleyi* were used as out-group for *RPB2* and β-tubulin genes. The branch tips of each tree were labelled with isolate number, species (M or N for *M. majus* or *M. nivale*, respectively), host origin (C or G for cereals or grasses, respectively), and geographic origin (NA for North America, NO for Norway, UK for United Kingdom and IR for Ireland). .

Results

1. Pathogenicity and host specialization

Significant and large differences in pathogenicity were found among *M. nivale* and *M. majus* isolates (Fig. 1). Plants infected with *M. nivale* isolates 200231, 201050, 200272 and 201053 had the lowest relative regrowth (lower than 0.1), while plants inoculated with isolates 200114 and 200136 had the highest relative regrowth (higher than 0.9) (Fig. 1). *M. majus* isolates (two isolates) showed very low pathogenicity on all four grass cultivars (Fig. 1). *M. nivale* isolates from grasses were more pathogenic (mean value of the relative regrowth was 0.4) than isolates from cereals (mean value of the relative regrowth was 0.6), when inoculated on the four grass cultivars (Fig. 2). Moreover, the interaction between isolates and cultivars was significant only in experiment A (Table 2).

2. Sequence variation between isolates

The sequence of the four different genomic regions *RPB2*, ITS, *EF-1α* and β-tubulin revealed slightly different phylogenetic structures. However, the *RPB2*and the β-tubulin separated *M. nivale* and *M. majus* isolates clearer than the other two did. For the β-tubulin gene, all isolates of *M. majus* formed a single clade with a bootstrap value of 99 % and 99.6 % sequence identity, while the *M. nivale* isolates formed two clades with bootstrap values of 100 % and pairwise distance of 0.05 (Fig. 3a). The first *M. nivale* clade represents isolates from grasses (17 isolates) in Norway and North-America with 99.6 % sequence identity, with the exception of isolate 200108 that was isolated from wheat in Norway. The second *M. nivale* clade represents only isolates from cereals (10 isolates), originating from Norway, United Kingdom and North-America with 99.4 % sequence identity. For the *RPB2*, also all *M. majus* isolates grouped together in one cluster, which was divided into subclades, all with bootstrap values of 100 % and pairwise distance of 0.07 (Fig. 3b). One clade represents isolates from Norway and United Kingdom (8 isolates from Norway and 1 isolate from UK) with 99.4 % sequence identity; the other represents isolates from North-America (3 isolates) with 97.2 % sequence identity. Furthermore all *M. nivale* isolates from Norway and United Kingdom (21 isolates from Norway and two isolates from UK) were grouped in one sub-cluster with a bootstrap value of 100 % and 96.4% sequence identity. Isolates from North-America (6 isolates) were all in one cluster, which was divided into two sub-clusters corresponding to the host origin (cereals vs. grasses).

The phylogenetic analysis based on the *EF-1α* gene sequence (Fig. 4a) only showed two major clusters with pairwise distance of 0.08, one represents isolates of *M. nivale* (20 isolates with 99.7 % sequence identity) and the other represents isolates of *M. majus* (10 isolates with 96.1 % sequence identity). No clear sub-clusters were formed and we found no clear difference between isolates based on the origin of their host. The ITS sequence analysis (Fig. 4b) revealed low genetic diversity, but still two major clusters were formed for isolates of *M. nivale* and *M. majus* with pairwise distance of 0.02 (28 isolates with 99.2 % and 12 isolates with 99.8 % sequence identity respectively).

Discussion

The main aim of this study was to investigate pathogenicity of *M. nivale* isolates from different host plants and the genetic variation among isolates of *M. nivale* and *M. majus.* Therefore, a pathogenicity tests was conducted with 15 isolates of *M. nivale* and two isolates of *M. majus* on four different grass cultivars. The nucleotide sequences of four different genomic regions were investigated in approximately 40 isolates including *M. nivale* and *M. majus.*

We detected large differences in pathogenicity between the 17 *M. nivale* isolates. Since *M. nivale* isolates are regarded as more pathogenic on grasses than *M. majus* (Hofgaard et al. 2006), only a few *M. majus* isolates were used in this study. This result confirmed previous studies using different species of grasses (Hofgaard et al. 2006; Holmes 1976) and cereals (Diamond & Cooke 1999; Maurin et al. 1995). According to Hofgaard et al. (2006) highly pathogenic isolates had faster growth on PDA at 2 °C, as well as higher activity of the cell wall-degrading enzyme β-glucosidases than isolates with low pathogenicity. This may indicate that pathogenicity and host specialization of an isolate may be related to cell wall composition of the host and the isolate's ability to produce specific cell wall degrading enzymes. The present study showed that isolates from grasses were significantly more pathogenic on the four grass cultivars than isolates from cereals. This result agrees with a previous study by Hofgaard et al. (2006), who investigated the differences in pathogenicity between *M. nivale* isolates and found that isolates from grasses were highly pathogenic towards perennial ryegrass. Significant interaction between isolates and cultivars was only found in experiment A, the reason for this could be that cultivars from different species show different resistances level during this experiment, whereas significant difference was found between *Festulolium* and *Lolium perenne* cultivars (*Festulolium* cultivars were more resistance).

In order to study the genetic variation between isolates, the nucleotide sequence of four genomic regions (*RPB2*, *EF-1α* and β-tubulin as well as the ITS) were sequenced in almost 40 isolates of *M. nivale* and *M. majus* from Norway, United Kingdom and Ireland. Clear genetic differences between the two fungal species were detected in all four genomic regions. This results agrees with previous studies by Glynn et al. (2005) and by Jewell and Hsiang (2013), while in the latter study the ITS sequences could not differentiate between the two species. In the present study the differences between isolates based on the

geographic origin and host plants were found using the gene sequences of *RPB2* and β-tubulin. Moreover, our results do support the proposition by Glynn et al. (2005) that *M. nivale* and *M. majus* should be considered as two separate species.

Furthermore larger genetic variation was found among *M. nivale* isolates in comparison to *M. majus*. This result agrees with the previous studies by Maurin et al. (1995), Glynn et al. (2005) and Mahuku et al. (1998). Fungal populations with high level of genetic diversity such as *M. nivale* are more likely to have a wider host range, and to develop resistance to fungicides than those with low genetic diversity (McDonald & McDermott 1993; Walker et al. 2009). Therefore understanding plant pathogen population structure may have a large impact on snow mould management and grass production.

In order to study the association between geographic origin of the isolates and sequences variation, the gene sequences of *RPB2* and β-tubulin from six North-American isolates were obtained from NCBI GenBank®. Only one of the studied genes (*RPB2*) clustered *M. nivale* isolates according to their geographic origins; North-American isolates were placed in one cluster with a bootstrap value of 100 %, while isolates from Norway and UK formed another cluster. The β-tubulin gene sequences revealed less clear difference between isolates connected to their geographic origin. This result agrees with a recent study by Jewell and Hsiang (2013) who found that isolates from different geographic origin (Europe vs. North America) were genetically different based on the *RPB2* gene sequence, whereas the β-tubulin gene sequence did not reveal that difference.

The β-tubulin gene sequences, revealed differences between *M. nivale* isolates connected to the host plants they were isolated from (cereals vs. grasses). *M. nivale* isolates from cereals formed one cluster with a bootstrap value of 54 %, while isolates from grasses (except isolate 200108 from cereals) formed another cluster with a bootstrap value of 50%. *M.nivale* has a wide host range, but different isolates show different host preferences. Therefore, more investigation is required to understand the relation between host preference and genetic variation in *M. nivale* and *M. majus* populations.

In conclusion, the result from this study supports the classification of *M. nivale* and *M. majus* as two different fungal species. The genotypic and phenotypic (pathogenicity) differences detected between Norwegian *M. nivale* isolates from cereals and grasses in our study, support the assumption of the existence of host specialization within *M. nivale* isolates. Further studies may reveal that the "pathotypes" of this species should be regarded

as separate species or subspecies. In conducting screening tests for snow mould resistant cultivars of different grass species, it is therefore important to select isolates of the right "pathotype" of the fungus.

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Table 1. Geographic and host-plant origin of *Microdochium nivale* and *M. majus* isolates, and the different genomic regions sequenced from each isolate.

Isolate ID	Original isolate name	Species	Host plant	Geographic origin	Genomic regions sequenced			
					ITS[b]	*EF-1*[c]	β-tubulin	*RPB2*[d]
200101[(2)]	3920	*M. majus*	*Hordeum vulgare*	Norway	+[e]	-[f]	+	-
200105[(2)]	4896	*M. majus*	*Triticum* sp.	Norway	+	+	+	+
200106	4897	*M. majus*	*Triticum* sp.	Norway	+	+	+	-
200107[(2)]	4898	*M. majus*	*Triticum* sp.	Norway	+	-	+	+
200109	4900	*M. majus*	*Triticum* sp.	Norway	+	+	+	+
200112	4925	*M. majus*	*Triticum* sp.	Norway	-	-	+	+
200130	4/91	*M. majus*	*Poa annua*	Norway	-	-	+	+
200132	13/91	*M. majus*	*Triticum* sp.	Norway	+	+	+	+
200276[a]	NG3	*M. majus*	*Triticum* sp.	England	+	+	+	-
200278	NG36	*M. majus*	*Triticum* sp.	England	+	+	+	+
200284	OP2A	*M. majus*	*Triticum* sp.	Ireland	+	-	+	-
200349[a]	67/03	*M. majus*	*Triticum* sp.	Norway	+	+	+	-
200404	122/03	*M. majus*	*Triticum* sp.	Norway	+	+	+	+
200434	12/04	*M. majus*	*Triticum* sp.	Norway	+	+	+	+
200103[a]	4222	*M. nivale*	*Lolium perenne*	Norway	+	+	+	+
200104[a]	4223	*M. nivale*	*Secale cereale*	Norway	+	-	+	+
200108[(2)]	4899	*M. nivale*	*Triticum* sp.	Norway	-	+	+	+
200111	4902	*M. nivale*	*Triticum* sp.	Norway	-	+	+	+
200113	1/77	*M. nivale*	*Phleum pratense*	Norway	+	+	-	+
200114[a]	4/83	*M. nivale*	*Dactylis glomerata*	Norway	+	-	-	+
200116	39/83	*M. nivale*	*Festuca pratensis*	Norway	+	-	-	-
200118[(2)]	3/86	*M. nivale*	*Lolium perenne*	Norway	+	+	+	-
200119	4/86	*M. nivale*	*Lolium perenne*	Norway	+	+	+	+
200120[a]	19/87	*M. nivale*	*Secale cereale*	Norway	+	+	+	+
200122[(2)]	21/87	*M. nivale*	*Lolium perenne*	Norway	+	+	+	+
200124	28/87	*M. nivale*	*Lolium perenne*	Norway	+	+	+	+
200131	6/91	*M. nivale*	*Festuca pratensis*	Norway	+	-	+	-
200136[a]	5/93	*M. nivale*	*Lolium perenne*	Norway	+	+	+	+
200231[a]	3/98	*M. nivale*	*Lolium perenne*	Norway	+	+	+	+
200258[(2)]	30/98	*M. nivale*	*Lolium perenne*	Norway	+	+	+	+
200272[a]	1/99	*M. nivale*	*Festuca pratensis*	Norway	+	+	-	-
200277	NG26	*M. nivale*	*Triticum* sp.	England	+	+	+	+
200279[a]	NG42	*M. nivale*	*Triticum* sp.	England	+	+	+	+
200444	22/04	*M. nivale*	*Secale cereale*	Norway	+	+	+	+
200518[a]	02/06	*M. nivale*	*Agrostis stolonifera*	Norway	+	-	+	+
200555	39/06	*M. nivale*	*Festuca rubra*	Norway	+	+	+	+
200559[a]	43/06	*M. nivale*	*Triticum* sp.	Norway	+	+	+	+
200566[a]	50/06	*M. nivale*	*Agrostis stolonifera*	Norway	+	+	+	-
200846[a]	148/08	*M. nivale*	*Festuca rubra*	Norway	+	+	-	-
201049	49/10	*M. nivale*	*Festulolium*	Norway	+	-	-	-
201050[a]	50/10	*M. nivale*	*Festulolium*	Norway	+	+	+	+
201052	52/10	*M. nivale*	*Lolium perenne*	Norway	+	-	-	+
201053[a]	53/10	*M. nivale*	*Lolium perenne*	Norway	+	-	-	+
201054[a]	54/10	*M. nivale*	*Lolium perenne*	Norway	+	-	+	+

[(2)] Asporogenic isolates were classified using PCR according to Glynn et al. (2005); + The amplicon was sequenced and analysed in this study; - The amplicon did not give a good quality sequence. [a] Isolates used in the pathogenicity test. [b] Internal Transcribed Spacer region. [c] Elongation factor-1α. [d] RNA polymerase II.

Table 2. Analysis of variance (based on the general linear model) in the pathogenicity of 15 isolates of *M. nivale* on four different grass cultivars (Figgjo, Ivar, FuRs9812 and FuRs0463). Pathogenicity was measured after eight weeks of incubation in experiment A and nine weeks in experiment B followed by two weeks of regrowth.

		Experiment A				Experiment B			
Source	df	Type III Sum of Squares	Mean Square	F [a]	Sig. [b]	Type III Sum of Squares	Mean Square	F [a]	Sig. [b]
M. nivale isolates host origin (cereals vs. grasses)	1	1.05	1.05	44.08	<.0001	0.62	0.62	12.87	0.001
Isolates (host origin)	13	10.79	0.83	34.76	<.0001	21.95	1.68	34.94	<.0001
Cultivars species (Fe vs. Lp)	1	0.10	0.10	4.44	0.039	0.003	0.003	0.08	0.779
Cultivars (species)	2	0.31	0.15	6.67	0.002	0.73	0.36	7.65	0.001
Isolates* Cultivars (species)	42	1.72	0.04	1.72	0.026	2.72	0.06	1.34	0.145
Error	60	1.43	0.02			2.9	0.04		

[a] F value for differences between levels.
[b] p value for differences between levels.

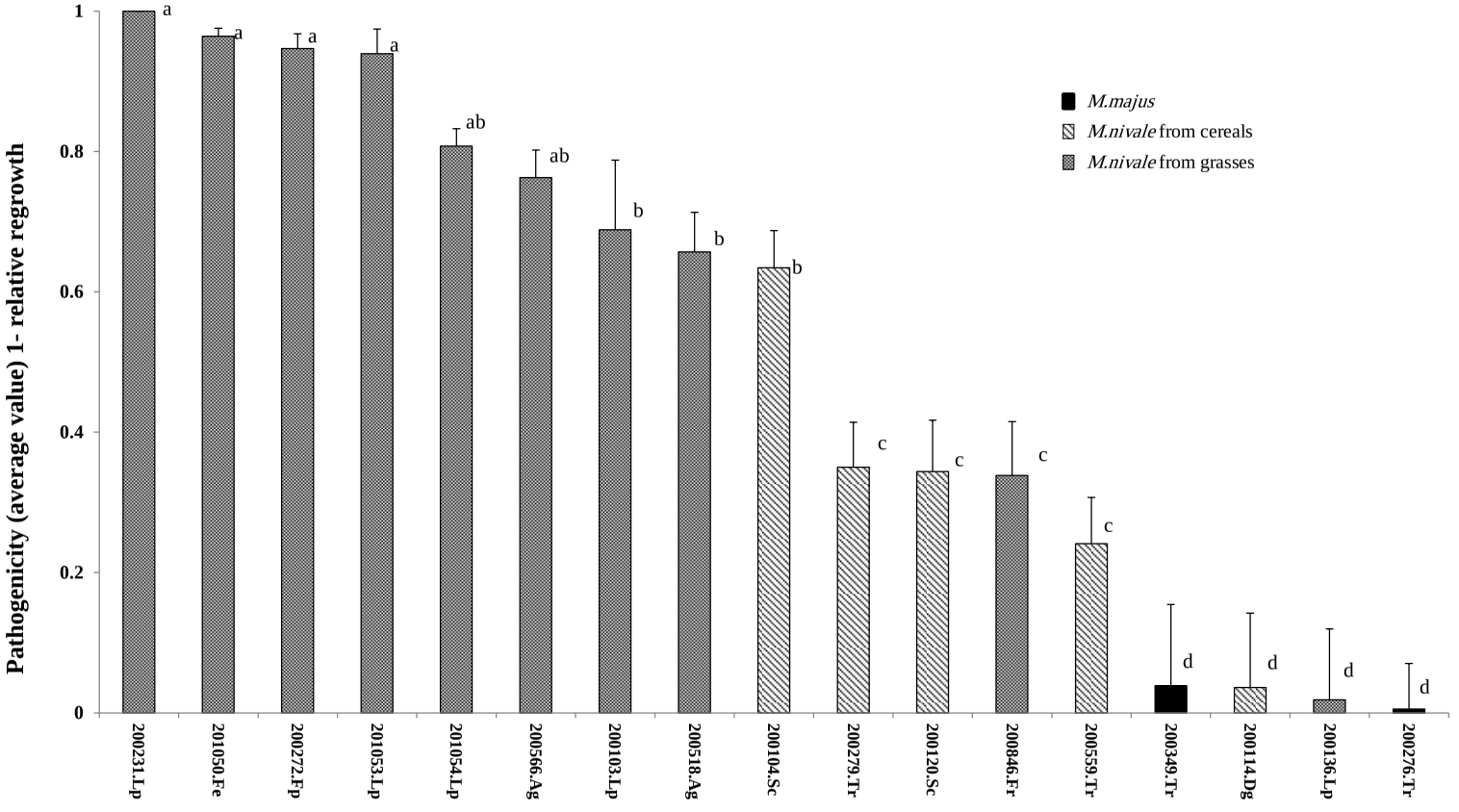
Pathogenicity (average value) 1- relative regrowth
1
0.8
0.6
0.4
0.2
0
M.majus
M.nivale from cereals
M.nivale from grasses
a
a
a
a
ab
ab
b
b
b
c
c
c
c
d
d
d
d
200231.Lp
201050.Fe
200272.Fp
201053.Lp
201054.Lp
200566.Ag
200103.Lp
200518.Ag
200104.Sc
200279.Tr
200120.Sc
200846.Fr
200559.Tr
200349.Tr
200114.Dg
200136.Lp
200276.Tr

Fig. 1. Pathogenicity (0 = min; 1= max) of seventeen *Microdochium* sp. isolates (fifteen *M. nivale* and two *M. majus*) on four different grass cultivars (Figgjo, Ivar, FuRs9812 and FuRs0463). Pathogenicity was measured as 1- relative regrowth (dry weight of inoculated plants divided by dry weight of non-inoculated plants after incubation for eight and nine weeks (experiments A and B respectively) under artificial snow cover, followed by two weeks of regrowth (average value of four cultivars). Isolates ID is labelled with host origin (Lp for *L. perenne*, Fe for *Festulolium*, Fp for *F. pratensis*, Ag for *A. stolonifera*, Sc for *S. cereal*, Tr for *Triticum* sp, Fr for *F. rubra* and Dg for *D. glomerata*). Error bars indicate standard errors of the mean of the two experiments. Bars with the same letter are not significantly different (P value <0.05).

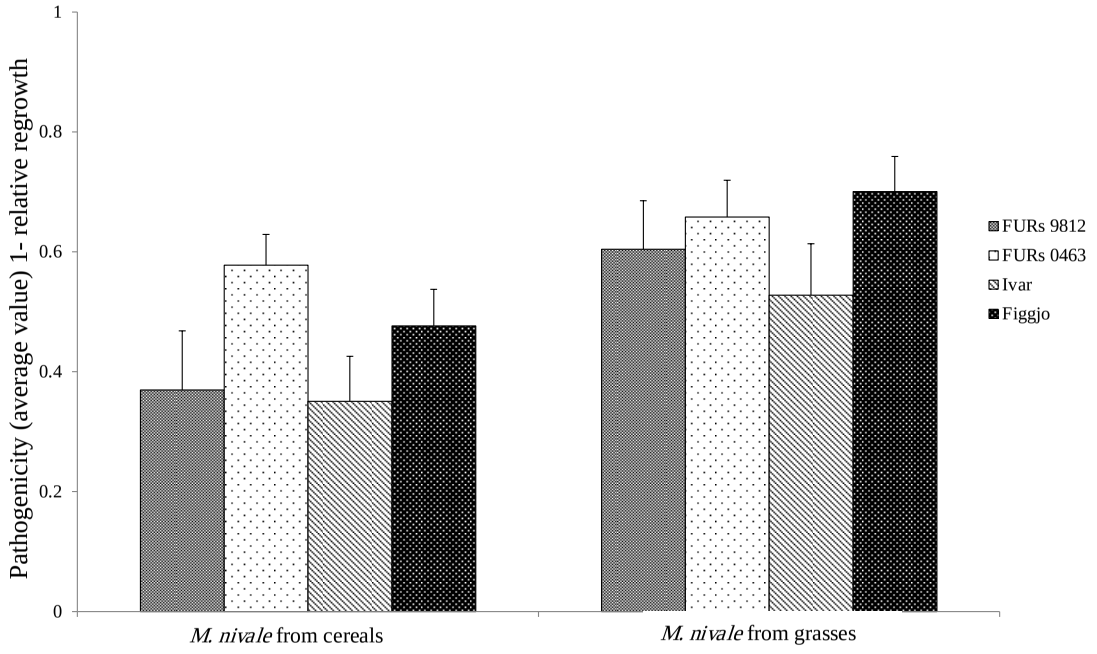

Fig. 2. Pathogenicity (0 = min; 1= max) of *M. nivale* isolates from cereals (average value of five isolates) and grasses (average value of ten isolates) on four different grass cultivars (Figgjo, Ivar, FuRs9812 and FuRs0463). Pathogenicity was measured as 1- relative regrowth (dry weight of inoculated plants divided by dry weight of non-inoculated plants at eight weeks (experiment A) and nine weeks (experiment B) after incubation under artificial snow cover followed by two weeks of regrowth. Error bars indicate standard errors of the mean of the two experiments.

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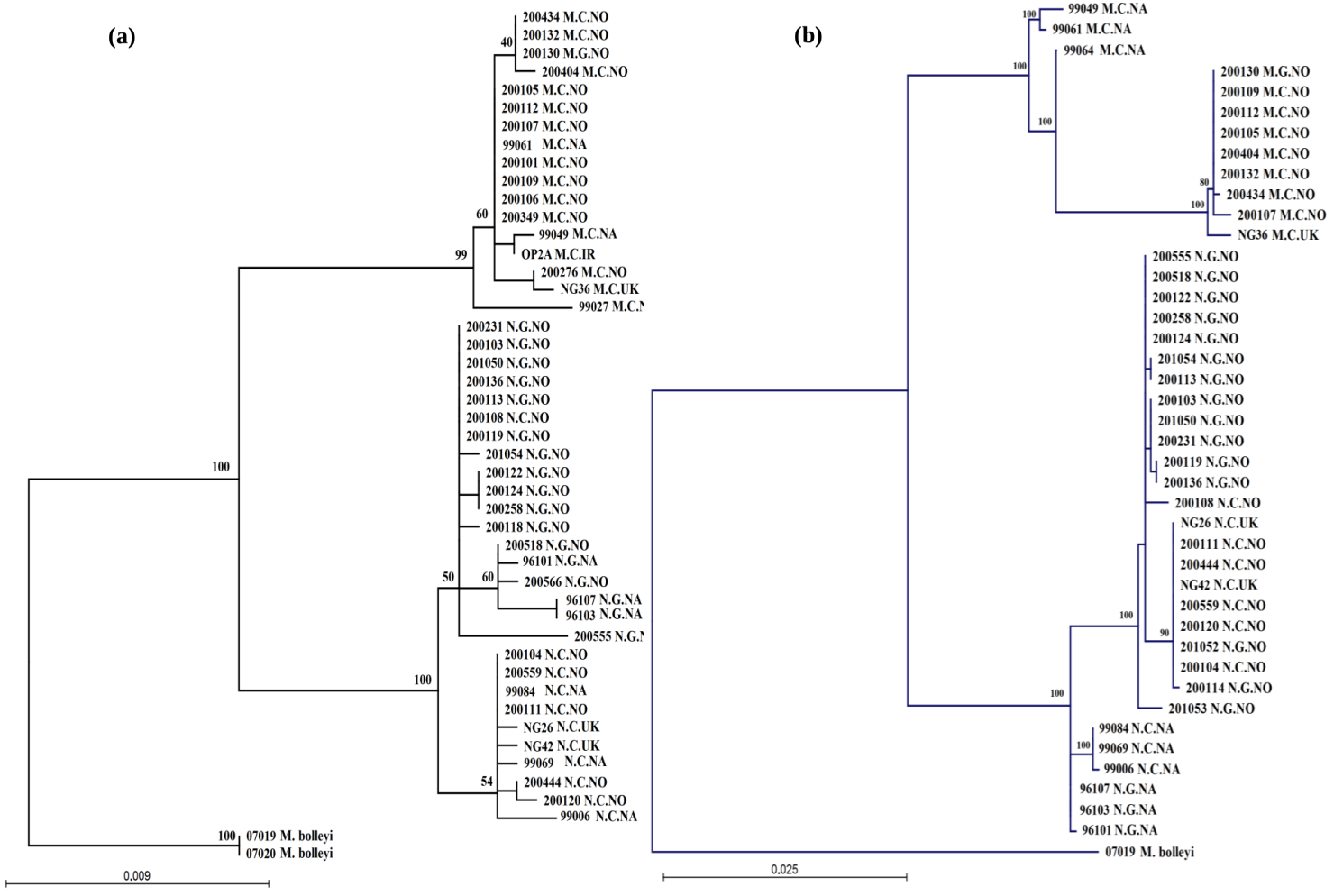
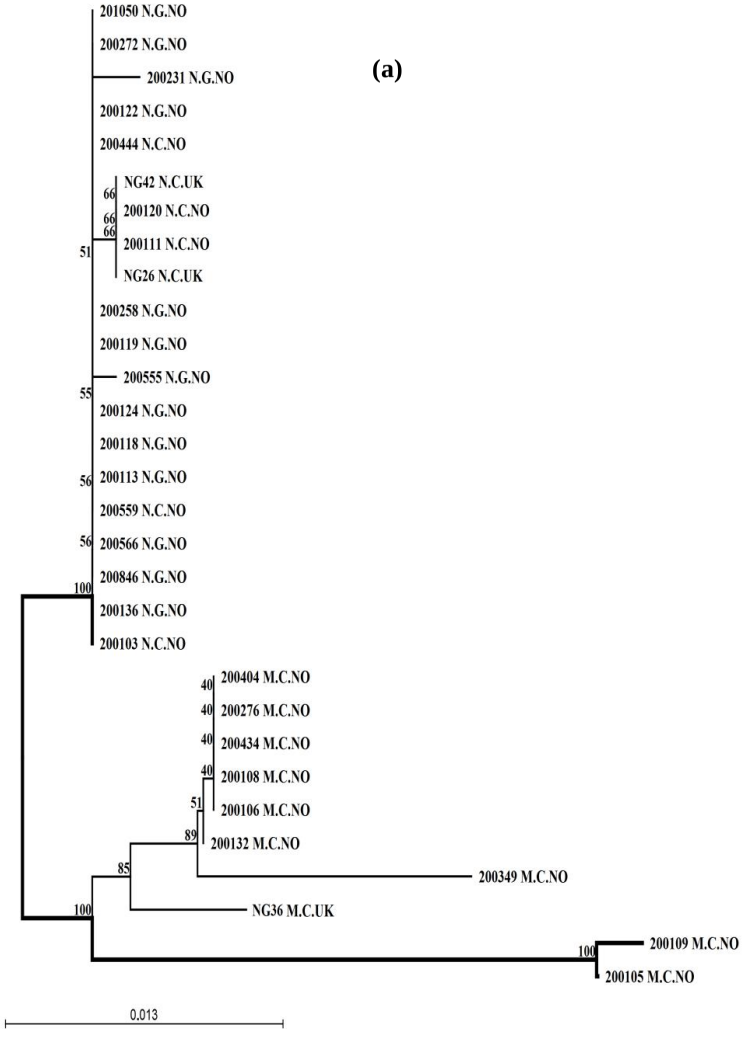

(a)
200434 M.C.NO
200132 M.C.NO
200130 M.G.NO
200404 M.C.NO
200105 M.C.NO
200112 M.C.NO
200107 M.C.NO
99061 M.C.NA
200101 M.C.NO
200109 M.C.NO
200106 M.C.NO
200349 M.C.NO
99049 M.C.NA
OP2A M.C.IR
200276 M.C.NO
NG36 M.C.UK
99027 M.C.N
200231 N.G.NO
200103 N.G.NO
201050 N.G.NO
200136 N.G.NO
200113 N.G.NO
200108 N.C.NO
200119 N.G.NO
201054 N.G.NO
200122 N.G.NO
200124 N.G.NO
200258 N.G.NO
200118 N.G.NO
200518 N.G.NO
96101 N.G.NA
200566 N.G.NO
96107 N.G.NA
96103 N.G.NA
200555 N.G.N
200104 N.C.NO
200559 N.C.NO
99084 N.C.NA
200111 N.C.NO
NG26 N.C.UK
NG42 N.C.UK
99069 N.C.NA
200444 N.C.NO
200120 N.C.NO
99006 N.C.NA
07019 M. bolleyi
07020 M. bolleyi
0.009
(b)
99049 M.C.NA
99061 M.C.NA
99064 M.C.NA
200130 M.G.NO
200109 M.C.NO
200112 M.C.NO
200105 M.C.NO
200404 M.C.NO
200132 M.C.NO
200434 M.C.NO
200107 M.C.NO
NG36 M.C.UK
200555 N.G.NO
200518 N.G.NO
200122 N.G.NO
200258 N.G.NO
200124 N.G.NO
201054 N.G.NO
200113 N.G.NO
200103 N.G.NO
201050 N.G.NO
200231 N.G.NO
200119 N.G.NO
200136 N.G.NO
200108 N.C.NO
NG26 N.C.UK
200111 N.C.NO
200444 N.C.NO
NG42 N.C.UK
200559 N.C.NO
200120 N.C.NO
201052 N.G.NO
200104 N.C.NO
200114 N.G.NO
201053 N.G.NO
99084 N.C.NA
99069 N.C.NA
99006 N.C.NA
96107 N.G.NA
96103 N.G.NA
96101 N.G.NA
07019 M. bolleyi
0.025

Fig. 3. Neighbour-Joining phylogenetic trees obtained using maximum likelihood (ML) algorithm with 1000 bootstrap replicates. The phylogenetic trees were constructed based on (a) the β-tubulin gene sequence; (b) the RNA polymerase II gene sequence. The tips of the tree are labelled with isolate number, species (M or N for *M. majus* or *M. nivale*, respectively), host origin (C or G for cereals or grasses, respectively), and geographic origin (NA for North America, NO for Norway, UK for United Kingdom and IR for Ireland)..


(a)
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200272 N.G.NO
200231 N.G.NO
200122 N.G.NO
200444 N.C.NO
NG42 N.C.UK
200120 N.C.NO
200111 N.C.NO
NG26 N.C.UK
200258 N.G.NO
200119 N.G.NO
200555 N.G.NO
200124 N.G.NO
200118 N.G.NO
200113 N.G.NO
200559 N.C.NO
200566 N.G.NO
200846 N.G.NO
200136 N.G.NO
200103 N.C.NO
200404 M.C.NO
200276 M.C.NO
200434 M.C.NO
200108 M.C.NO
200106 M.C.NO
200132 M.C.NO
200349 M.C.NO
NG36 M.C.UK
200109 M.C.NO
200105 M.C.NO
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55
56
56
100
40
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51
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85
100
100
0.013

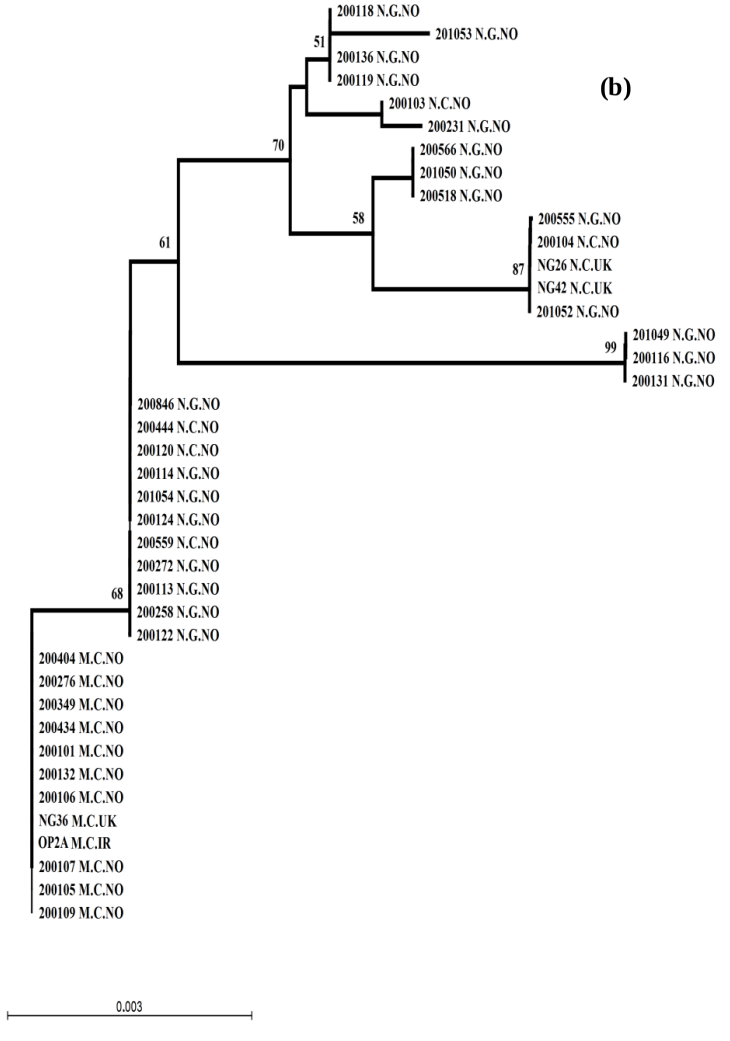
(b)
200118 N.G.NO
201053 N.G.NO
200136 N.G.NO
200119 N.G.NO
200103 N.C.NO
200231 N.G.NO
200566 N.G.NO
201050 N.G.NO
200518 N.G.NO
200555 N.G.NO
200104 N.C.NO
NG26 N.C.UK
NG42 N.C.UK
201052 N.G.NO
201049 N.G.NO
200116 N.G.NO
200131 N.G.NO
200846 N.G.NO
200444 N.C.NO
200120 N.C.NO
200114 N.G.NO
201054 N.G.NO
200124 N.G.NO
200559 N.C.NO
200272 N.G.NO
200113 N.G.NO
200258 N.G.NO
200122 N.G.NO
200404 M.C.NO
200276 M.C.NO
200349 M.C.NO
200434 M.C.NO
200101 M.C.NO
200132 M.C.NO
200106 M.C.NO
NG36 M.C.UK
OP2A M.C.IR
200107 M.C.NO
200105 M.C.NO
200109 M.C.NO
51
70
58
87
99
61
68
0.003

Fig. 4. Neighbour-Joining phylogenetic trees obtained using maximum likelihood (ML) algorithm with 1000 bootstrap replicates. The phylogenetic tree was constructed based on (a) the elongation factor 1-alpha (*EF-1α*) gene sequence and (b) the internal transcribed spacer (ITS) gene sequence. The tips of the trees are labelled with isolate number, species (M or N for *M. majus* or *M. nivale*, respectively), host origin (C or G for cereals or grasses, respectively), and geographic origin (NA for North America, NO for Norway, UK for United Kingdom and IR for Ireland).

Supplementary tables

Table 1. Isolate IDs and accession numbers of *Microdochium nivale* and *M. majus* gene sequences obtained from the NCBI GenBank®.

Isolate ID	Species	Host plants	Accession number	Gene sequence
99061	*M. majus*	*Triticum* sp.	JX280568	β-tubulin
			JX280555	*RPB2*
99049	*M. majus*	*Triticum* sp.	JX280567	β-tubulin
			JX280554	*RPB2*
99027	*M. majus*	*Triticum* sp.	JX280566	β-tubulin
99064	*M. majus*	*Triticum* sp.	JX280553	*RPB2*
96101	*M. nivale*	*Agrostis palustris*	JX280571	β-tubulin
			JX280552	*RPB2*
96103	*M. nivale*	*Agrostis palustris*	JX280572	β-tubulin
			JX280550	*RPB2*
96107	*M. nivale*	*Agrostis palustris*	JX280570	β-tubulin
			JX280551	*RPB2*
99069	*M. nivale*	*Triticum* sp.	JX280581	β-tubulin
			JX280544	*RPB2*
99084	*M. nivale*	*Triticum* sp.	JX280569	β-tubulin
			JX280545	*RPB2*
99006	*M. nivale*	*Triticum* sp.	JX280580	β-tubulin
			JX280546	*RPB2*

Paper III

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Global transcriptome changes in perennial ryegrass during early infection by pink snow mould

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Summary

Lack of resistance to pink snow mould (*Microdochium nivale*) is seen as a major constraint for adaptation of perennial ryegrass (*Lolium perenne* L.) at higher latitudes. Plants generally become more resistant to snow moulds after cold acclimation, and almost all investigations of genetic variation in resistance have been performed using cold acclimated plants. However, there may be variation in resistance mechanisms that are functioning independently of cold acclimation. In this study our aim was to identify candidate genes involved in such resistance mechanisms. We first characterized variation in resistance to *M. nivale* among non-acclimated plants of eight genotypes from the Norwegian cultivar 'Fagerlin' and selected one resistant and one susceptible genotype for transcriptome analysis. Total RNA was extracted from leaf blade tissue of plants exposed to three different treatments: non-inoculated and non-incubated plants, non-inoculated plants after four days of incubation, and inoculated plants after four days of incubation. cDNA libraries were prepared and paired-end sequencing performed using Illumina Hiseq 2000. Transcriptome profiles, GO enrichment and KEGG pathway analysis indicate that defence response related genes are differentially expressed between plants incubated at non-inoculated and inoculated conditions, within both resistant and susceptible genotypes. A significant up-regulation of defence related genes as well as genes involved in cell wall cellulose metabolic processes and aryl-alcohol dehydrogenase (NADP+) activity was observed in the resistant genotype. The candidate genes identified in this study might be potential molecular marker resources for breeding perennial ryegrass cultivars with improved resistance to pink snow mould.

Keywords: *Lolium perenne*, *Microdochium nivale*, RNA sequencing, *de novo* assembly, disease resistance, plant-pathogen interaction.

Introduction

Perennial ryegrass (*Lolium perenne* L.,) belongs to the Poaceae family. It is a diploid species (2n=2x=14) native to Europe, Asia and Northern Africa (Humphreys et al. 2010). It is the an important forage grass in the temperate regions of the world because of its high forage quality and yield. Out of 52 million ha of grasslands in Europe, 23% is cultivated with *Lolium* species, with perennial ryegrass being the most widespread species. Perennial ryegrass has low resistance against pink snow mould, however, tetraploid cultivars have better resistance than diploid and turf cultivars (Humphreys et al. 2010).

Winter injury is regarded as a serious constraint for the production of winter cereals and grasses at northern latitudes (Årsvoll 1975; Nakajima & Abe 1996). The fungus *Microdochium nivale* (Fr.) Samuels & Hallet is considered to be the most widespread cause of biotic winter injury of these crops (Tronsmo et al. 2001). It is an opportunistic species causing pink snow mould on winter cereals, turf and forage grasses at low temperatures, with or without a snow cover. High humidity, constant temperatures at or below 0° C under snow cover is highly favourable to snow mould disease development (Årsvoll 1973; Smith 1981). During the growing season, this fungus also causes leaf blotch in oat, foot rot, seedling blight and head blight in cereals (Parry et al. 1993) and microdochium patch disease on golf courses (Gange and Case 2003).

Resistance to snow mould is enhanced by cold acclimation (Årsvoll 1977; Tronsmo 1984, Nakajima and Abe 1996). During this process the plant undergoes numerous physiological and bio-chemical changes which are essential for winter survival (Sandve et al. 2011). Some of these changes are also thought to increase resistance to diseases, e.g. cellular dehydration and accumulation of defence-related proteins and fructans (Kuwabara and Imai 2009). Genetic variation in cold-induced resistance to pink snow mould in triticale have been shown to be associated with changes in physical and chemical properties of the leaf surface and cell walls (Szechynska-Hebda et al. 2013), and with photosynthetic acclimation and peroxidase activity (Szechynska-Hebda et al. 2015).

Previous studies on snow mould resistance have almost exclusively been performed on cold acclimated plants. However, some genetic variation in resistance is also present in non-acclimated winter wheat (Ergon and Tronsmo 2006), and this resistance may be masked when testing cold acclimated plants. Cold acclimation-independent resistance may be more specific to *M. nivale*, and is likely to be important to prevent diseases caused by *M. nivale*

during the growing season, such as microdochium patch. It may also be increasingly important with the predicited climate change, involving milder winters and more frequent deacclimateion events (Rapacz et al. 2014).

The use of molecular techniques for precise quantification of the fungal biomass in infected plants can facilitate the selection process for disease resistant genotypes (Heid et al. 1996; Nishio et al. 2005). Usage of real-time PCR in quantification of plant pathogens infestation have increased in the last two decades. It is quicker, more specific and sensitive compared to traditional methods based on symptom assessment or plant dry weight (Gachon et al. 2004). Therefore, Elongation factor 1a (*EF-1a*) gene was used in our study for accurate quantification of *M. nivale* DNA during snow mould infestation. The elongation factor 1a (*EF-1a*) gene has formerly been used to recognize *M. nivale* and *M. majus* as separate species (Glynn et al. 2005). The elongation factor 1a (*EF-1a*) has also been used to study the genetic variation among isolates (Jewell & Hsiang 2013). Moreover, Nielsen et al. (2013) developed a real time PCR assay based on this gene sequence using SYBR Green in order to investigate the population of *M. nivale* and *M. majus* over time in Danish cereals. Additionally, competitive PCR methods have also been developed for *M. nivale* and *M. majus* quantification in infected tissues (Nicholson et al. 1996).

As plant responses are complex and include various physiological processes, transcriptome analysis is an important tool to understand them (Martin et al. 2013). Next-generation sequencing (NGS) technologies have become revolutionary tools creating outstanding possibilities for understanding the complex transcriptomes (Shendure & Ji 2008). The use of whole transcriptome sequencing (RNA-seq) provides more comprehensive understanding of transcription initiation sites, improved detection of alternative splicing events and the detection of gene fusion transcripts (Shendure & Ji, 2008; Lu et al. 2012). These technologies are able to handle *de novo* sequencing of large genomes, revealing individual genome differences within the same species and quantify gene expression (Tarazona et al. 2011; Xu et al. 2011). In particular, it now enables global transcriptome studies to be performed in non-model species that have lacked many of the array based assays that are successfully used to study gene expression in the model species.

In the present work, we have taken advantage of the high throughput RNA sequencing technologies to study the global transcriptome changes in perennial ryegrass during early infection by pink snow mould (Supplementary Fig. 1). Also we performed a

quantitative PCR test specific for *M. nivale* that could be used to evaluate different grass genotypes for snow mould resistance. The aim of this study was to identify genes involved in snow mould resistance independent of cold acclimation in perennial ryegrass.

Materials and Methods

Plant materials and growth conditions

Mother plants of eight randomly selected genotypes of perennial ryegrass, cultivar Fagerlin, were divided into multiple ramets and planted in a fertilized soil mixture (Gartnerjord, TJERBO) and grown in the greenhouse at 20-22°C (day/night) and 18 hours light period with light intensity (Constant Color CMH™ Lamps 400W) at about 200-250 µmol m^{-2} s^{-1}. The plants were fertilized weekly with a mixture of 80 g/L Kristalon™ fertilizer 9-5-25 (N-P-K) and 60 g/L of YaraLiva® Calcinit 15.5-0-0 (Yara International ASA, Oslo, Norway), diluted to a conductivity of 2 mS/cm.

Snow mould resistance test

Isolate 200231 of *M. nivale* (isolated from *L. perenne* at Ås, Norway (59°N)) was obtained from the fungal culture collection at the Norwegian Institute of Bioeconomy Research (NIBIO), Ås, Norway. The inoculum was prepared according to Tronsmo (1993) and Hofgaard et al. (2006). Briefly, the fungus was incubated at 9 °C, in darkness, on potato dextrose agar (PDA) for two weeks. Erlenmeyer flask containing 100 ml potato dextrose broth (PDB) was then inoculated with four plugs (5mm diameter) of fungal mycelium and incubated at 15 °C in darkness. Fungal mycelium was harvested after 10 days by filtering through cheesecloth. The mycelium was homogenized in distilled water containing 0.01% TWEEN® 20 (SIGMA) using an Ultra Turrax. The inoculum was diluted to an optical destiny of 0.5 at 430 nm. Plants were inoculated by spraying (1 ml inoculum per plant on average) and the control plants were sprayed with distilled water. After inoculation, the plants were covered with moist cellulose wadding and black plastic sheets to simulate snow cover and then incubated at 2 °C in darkness for six and eight weeks. Each week during incubation, the plants were repositioned in the room.

After incubation, the plants were moved to a greenhouse at 20-22 °C and 18 hours of light for recovery. The plants were cut at five cm above the soil surface and allowed to regrow for two weeks. The regrown plants were harvested (all parts above soil surface) and dried at 60 °C for three days in order to measure dry weight (g DW $plant^{-1}$). Relative regrowth was calculated for each inoculated plant as the dry weight divided by the average

dry weight of non-inoculated plants within the same genotype. Relative regrowth values approaching 1 represents resistant plants. Disease severity was visually scored two days later according to the following scale: 0 = no green tillers, 1 = some green tillers visible, 2 = green tillers found in less than half of the total plant area, 3 = green tillers found in more than half of the plant area, and 4 = green tillers observed in the whole plant area. After visual assessment of the symptoms, plant leaves and stems were harvested (5 cm above soil surface) and kept at -20 °C for DNA extraction for fungal quantification.

Real-time PCR for fungal quantification

Plant materials (leaves and stems above five cm from soil) were collected from the eight genotypes at two different time points (6 and 8 weeks after inoculation), also from genotype F(S) and M(R) at 1 and 4 days after inoculation. For the samples collected after 6 and 8 week after inoculation, plant materials were stored at -20 °C until DNA was extracted. For samples collected after 1 and 4 days, the same plant materials treated for gene expression analysis (see below section) were utilized for fungal DNA quantification. For DNA extractions, samples were frozen quickly in liquid N_2 and grinded using mortar and pestle. DNA was extracted from the grinded plant tissue using DNeasy® Plant Minikit (QIAGEN) according to manufacturer's protocol (Qiagen Inc., Germany). The quality of the extracts was measured using a NanoDrop® ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and visualized by electrophoresis through 1.5% agarose gels.

Real-time PCR primers specific for *M. nivale* were designed based on the elongation factor 1-α gene sequence by Glynn et al. (2005) using Primer Express software version 2 (Applied Biosystems®, Foster City, USA) based on the following parameters; amplicon Length of 50 to 150 bases (for optimum PCR efficiency), primer Length of 20 bases, melting temperature (Tm) of 58 °C to 60 °C (Optimal 59 °C), G + C content being between 30 and 80 %; and the last five nucleotides at the 3′ end do not contain more than two G + C residues. The following primer set was chosen based on the regions of identity within the elongation factor 1-α gene sequence between isolates, forward primer EF1-F: 5´-GGTCTTGGCTTGCACAAACA-3´ and reverse primer EF1-R: 5´-AGCACAACAGGCGTGGATAAG-3´.

Quantification of plant and fungal DNA was carried out by real time PCR in a total volume of 25 µl, using 2x SYBR® Green PCR master mix (Applied Biosystems), 300 nM of each primers (Invitrogen Ltd, UK) and 2 µl of 10x diluted template DNA. Specific real time PCR primers for the plant housekeeping gene LpGAPDH (Petersen et al. 2004) were used as internal control for plant DNA. PCR was performed on Applied Biosystems 7900HT instrument with a standard 96-well block (Applied Biosystems). For all the PCR reactions the following cycling parameters were used: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min followed by dissociation curve analysis at 60°C-95°C. The data was analysed using Sequence Detection Software (SDS) Version 2.2.1 (Applied Biosystems). The amount of fungal and plant DNA in the samples were quantified by a standard curve algorithm based on cycle threshold value (Ct) using a 10-fold dilution series of known amount of DNA, starting with 5 ng for fungal DNA and 100 ng for plant DNA and three technical replicates. Samples were tested in two technical replicates. The amount of fungal DNA was calculated as pg fungal DNA per µg plant DNA for each sample.

Plant treatment, tissue sampling and RNA extraction for gene expression analysis

Leaf samples were collected from genotype F, a susceptible genotype (hereafter termed S) and genotype M, a resistance genotype (actually less susceptible, here after termed R), which had been exposed to three different treatments: non-incubated and non-inoculated plants (NI-NI), incubated and non-inoculated plants (I-NI) after four days of incubation, and incubated and inoculated plants (I-I) after four days of incubation, with two biological replicates, a total number of 12 samples. The collected samples were immediately placed in liquid nitrogen and stored at -80°C until used for RNA extraction. The frozen leaf samples were crushed with a pestle and mortar and total RNA was extracted using the Pure Link RNA Mini Kit (Life technologies, USA) and Plant RNA Isolation Aid (Life technologies, USA). On-Column DNAse kit was used to remove the DNA contamination. The concentration and quality was checked using the Nanodrop (Nanodrop Technologies, Wilmington, DE, USA) and Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) equipment.

cDNA library construction and Illumina sequencing

Twelve RNA samples with RIN (RNA Integrity Number) values above seven were used to construct separate cDNA libraries with fragment lengths of 200 bp (±25 bp). Then, paired-end sequencing was performed by GATC Biotech Ltd., Germany (http://www.gatc-biotech.com/en/index.html) using the Illumina sequencing platform (HiSeq™ 2000). Real time analysis (RTA) output was analysed using the CASAVA software (version 1.6, Illumina), generating pass filtering FastQ files with Qphred +64 quality values. Paired-end reads with a length of 100 bp were generated. The quality of the reads was analysed using FastQC (http://www.bioinformatics.babraham. ac.uk/projects/fastqc/)

De novo and reference based transcriptome analysis

After trimming adapter sequences and filtering low quality reads using the sickle program (https://github.com/najoshi/sickle/blob/master/README.md), the bioinformatics pipeline (Supplementary Fig. 2) was followed for *de novo* assembly and further detection of differentially expressed genes. Briefly, the clean reads derived from the two genotypes susceptible (S) and resistant (R) were used to construct separate *de novo* assemblies for each genotype using the Trinity assembler (release 2013-02-25) (Grabherr et al. 2011) with the following settings; Trinity.pl --seqType fq --JM 20G --left F_1_Lolium.fq-QT.gz --right F_2_Lolium.fq-QT.gz --CPU 16 -min_contig_length 200 --SS_lib_type FR --full_cleanup --min_kmer_cov 2 --output Trinity_201 2>&1 > logfile.lolium-F. The *de novo* assembled transcripts were then used as a reference to map back the individual reads by Bowtie. Further, we estimated transcript abundances in each genotype and treatment combination using RSEM version 1.1.11 (Li and Dewey 2011). A maximum of one mismatch (–bowtie-n 1) was allowed in the seed region of the reads. In another approach, to facilitate comparison of the two genotypes, we aligned all clean reads from each genotype and treatment combination to a reference transcriptome of an inbred *L. perenne* genotype, generated from a combination of root, stem, leaf sheath, leaf and meristem samples (Farrell et al. 2014), and estimated transcript abundance as described above.

De novo assembly validation by CEGMA

CEGMA software (version 2.4) (Paraa et al. 2007) was used to assess the completeness of the S and R transcriptome assembly datasets. This program assesses the presence and coverage of a set of 248 extremely conserved core eukaryotic genes (CEGs). It is routinely used for evaluating genomic assemblies, however, it has also been used for

evaluating transcriptome assemblies (Faino et al. 2012; Nakasugi et al. 2013).The software was run with default parameters with the included reference dataset of 248 ultra-conserved Core Eukaryotic Genes (CEGs).

Identifying differentially expressed genes, BLAST and functional annotation

Gene expression levels were measured as expected number of fragments per kilobase of transcript sequence per millions mapped reads (FPKM) (Trapnell et al. 2010). The transcript matrix files derived from RSEM program were processed with the edgeR program (Robinson et al. 2010) using perl script (DE_analysis.pl) in the Trinity pipeline for detecting differentially expressed genes determined with a False Discovery Rate (FDR) of 0.05. Briefly, pairwise comparisons were carried out between all the selected time points and edgeR analysis was performed by fitting normalized count data with a generalized linear model (GLM) estimating a negative binomial distribution to the calculated mean values of the two biologically independent samples. For each gene, fold changes and P values (pval) as well as P values adjusted (padj) for multiple testing with the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995), were used to control FDR. The sequence with padj of less than 0.05 was deemed to be significantly differentially expressed genes (DEGs). The variance stabilized data obtained from edgeR was used as input for clustering, and for constructing multidimensional scaling plots using R integrated in the Trinity pipeline. The transcripts showing differential expression at any time point during snow mould infection were clustered using a K-means clustering algorithm.

The DEGs were annotated using Blast2GO (Conesa et al. 2005; Conesa and Götz, 2008). An E-value threshold of 10^{e-06} was used for the BLASTx search, and 10^{e-10} for the annotation, with a cut-off value of 55 and a GO weight Hsp-hit value of 20. The enrichment analysis for the differential gene ontology term distribution was performed with a p-value significance cut-off value of 0.01. Gene ontology classifications of differentially expressed genes in the resistant (R) and susceptible (S) genotypes were generated using the web histogram tool WEGO (Ye et al. 2006). Pathway analysis was performed using the KEGG function implemented in the Blast2GO tool.

Validation of RNAseq expression profiles by qRT-PCR

Expression patterns of five defence related genes (Chitinase 2, Chitinase 5, WRKY transcription factor, Thaumatin-like PR3, Pathogenesis-related protein 1 and 5) differentially expressed between I-NI and I-I samples identified in this transcriptome studies

were analysed using qRT-PCR. The same RNA used for sequencing was used for validating the genes by qRT-PCR. Based on the transcriptome sequences of the five genes, primers (Supplementary Table 3) were designed using primer express software version 2 (Applied Biosystems®, Foster City, USA). Efficiency test of the primers was performed on different samples for normalization of the expression level. The EXPRESS two-Step qRT-PCR kit, which includes the SuperScript VILO cDNA Synthesis kit, was used for generating the single-stranded cDNA that was later used for quantifying the amount of specific gene expression using forward and reverse primers, following the manufacturer's instructions. cDNA synthesis was done using up to 2.5 µg of the total RNA in 20 µl reaction. Five µl of cDNA (5x diluted) was used in each well of Fast Optical 96 well plate along with other components making the total volume 20 µl. The fast cycling program was then set at 95°C for 20 sec, 40 cycles of 95°C for three sec (denaturation) and 60°C for 35 sec (annealing). Then each plate (with samples) for each gene with a bar code was placed in ABI7500 qRT-PCR machine. The SYBR® Green dye was used to detect the amplified products. The expressions of the specific genes were normalized by using LpGAPDH (EC 1.2.1.12) as the reference gene. The expression of the target gene relative to the reference gene at 4 days after inoculation using the $2^{-\Delta\Delta CT}$ method where the $\Delta\Delta CT$ = (CT of target – CT of reference) 4 days after inoculation – (CT of target – CT of reference) before inoculation, which gives the mean relative expression of target genes at this time point (Livak & Schmittgen 2001).

Results

Snow mould resistance test

The eight genotypes of *L. perenne* cv. Fagerlin varied in resistance towards snow mould. Based on the relative regrowth, these differences were significant. Genotype F had the lowest relative regrowth, while genotype M had the highest relative regrowth (Fig. 1). Thus, we selected two genotypes based on their responses to snow mould infection for transcriptome analysis, genotype F as a susceptible genotype (hereafter termed S) and genotype M as a resistance genotype (here after termed R).

Quantification of M. nivale DNA

Quantification of *M. nivale* DNA in leaf and stem tissue of the eight genotypes showed that genotypes with severe symptoms of injury (such as genotype F) contained the highest amount of fungal DNA. Genotypes that had the lowest amount of fungal DNA (such as genotype C) also had the lowest scoring of visual disease severity (Fig 2). There was a significant correlation between disease severity and the amount of *M. nivale* DNA. The correlation was negative due to the ranking system of the visual symptoms (Supplementary Table 1). However, the correlation between the amount of *M. nivale* DNA and relative regrowth was not significant. Samples collected from a resistant genotype (M) and a susceptible genotype (F) after 1 and 4 days from inoculation showed no significant difference in the amount of *M. nivale* DNA. However, more fungal DNA was detected after 4 days in the susceptible genotype (Supplementary Fig. 3).

De novo based susceptible (S) and resistant (R) transcriptome assemblies

A total of 178 million reads and 165 million reads of 100 bp were generated for the S and R genotypes, respectively (Table 1). Separate transcriptome assemblies were generated for each genotype using all their respective reads. The *de novo* assembly yielded 261,978 contigs for the S genotype, with N50 of 1,784 bp, and 188,355 contigs for the R genotype with N50 of 1,672 bp (Table 1). The longest assembled contigs in the S and R genotype were 17,632 and 12,882 bp, respectively. To estimate the quality of the assemblies, we compared them to the *Brachypodium distachyon* coding sequence consisting of 31,029 entries. There were 27,135 *B. distachyon* sequences (87.45%) that had a significant hit in the S transcriptome assembly and 27,399 (88.30%) that had a significant hit in the R transcriptome assembly. Further, we used the CEGMA pipeline (Paraa et al.

2007) to evaluate the completeness of our assemblies. The percentage of complete CEGs in R and S assemblies are 82.66 and 93.95, respectively, and the percentage of partially complete CEGs ranged from 90.73 to 98.79 (Table 2). The average number of orthologs per CEG in the R and S assemblies is 3.72 and 3.83, respectively, and the percentage of detected CEGs that had more than one ortholog was 96.1 and 97.0, respectively.

Differentially expressed transcripts detected by de novo and reference based methods

In order to estimate the expression levels of each assembled transcript at the different time points, the reads from each sample were mapped onto their respective genotype specific *de novo* assemblies and also to the reference inbred *L. perenne* transcriptome (Farrell et al. 2014). In the case of each sample, more than 83-90% of the reads mapped onto the assembled transcripts. Using the genotype specific assemblies in a series of pairwise comparisons between samples, 2,354 and 3,748 differentially expressed transcripts were identified with false discovery rate (FDR) < 0.05 between NI-NI and I-I samples; 1,602 and 3,080 between NI-NI and I-NI samples; and 83 and 275 between I-NI and I-I samples in the S and the R genotype, respectively, with several up-, down- and contra-regulated transcripts (Figure 3A1; Figure 3B1). When using the reference based assembly mapping, 880 and 1,391 differentially expressed transcripts were identified between NI-NI and I-I samples; 755 and 1,050 between NI-NI and I-NI samples; and 95 and 210 between I-NI and I-I samples in the S and the R genotype, respectively, with several up-, down- and contra-regulated transcripts (Figure 3A2 and Figure 3B2).

In addition, heat maps were generated for each genotype based on the differential expression data from edgeR in order to determine the sample relationships (Fig. 4). A clear separation was seen between non-incubated (NI) and incubated (I) samples in both genotypes, where incubated inoculated (I-I) and incubated non-inoculated (I-NI) grouped together (Fig. 4). Even the expression data generated from reference based mapping clearly differentiated between incubated and non-incubated samples. Both S and R incubated grouped together and were separated from non-incubated samples (Fig. 4).

Annotation and GO of differentially expressed transcripts

Approximately 75% of the differentially expressed transcripts had blast hits to the Viridiplantae database extracted from NCBI. The top hit species are *Brachypodium distachyon* followed by *Hordeum vulgare*, which are most closely related *L. perenne*.

Among the transcripts with blast hits, 40–52% of the differentially expressed transcripts were annotated using Blast2GO. Putative descriptions and functions were assigned to the transcripts predominantly based on annotations from *H. vulgare* and *B. distachyon* and *Arabidopsis thaliana*. Gene Ontology classifications of DEGs at I-NI vs. I-I conditions in genotypes R and S were generated using WEGO (Ye et al. 2006). The results are summarized in three main GO categories: cellular component, molecular function and biological process (Fig. 5). Comparisons of the functional categories of genotype R with those of genotype S reveal differences in terms of the biological processes. DEGs responses to stress, biotic stimulus were highly represented in the R genotype, while DEGs response to death is only seen in S genotype

Fisher's exact test from Blast2GO was used for GO enrichment analysis between R and S to determine if any gene ontology (GO) terms were over- or under-represented in the various sets of differentially expressed transcripts. A total of seven GO terms were enriched when comparing the differentially expressed transcript sets from I-NI vs. I-I conditions of the two genotypes (Fig. 6). Out of these, five were overrepresented in the R genotype, in terms related to cell wall cellulose metabolic process, cell wall pectin metabolic process, cell morphogenesis, actin nucleation and organelle epidermal cell differentiation. Transcripts assigned to aryl-alcohol dehydrogenase (NADP+) activity and phycobilisome were present only in R genotype.

In addition, several genes involved in the initiation of pathogen-associated molecular pattern (PAMP) immunity, like cysteine-rich receptor-like protein kinase (CRK), cyclic nucleotide gated channel (CNGC), calcium-dependent protein kinase (CDPK), respiratory burst oxidase (Rboh), calcium-binding protein CML (CaM/CML), and NADPH oxidase were detected in these studies. Several pathogen related genes like PR-1, β-1,3-Glucanase (PR-2), chitinase II/V (PR-3), thaumatin-like (PR-5), and lipid-transfer protein (PR-14) are upregulated in genotype R compared with genotype S under I-I conditions (Table 3). We also found several potential pathogen resistance candidate genes like *chitinase 5*, lipid transfer protein, serine-glyoxylate aminotransferase and *WRKY 75* highly upregulated in the R genotype under I-NI treatment compared with the I-I treatment (Table 3). All potential candidate genes involved in the response of *L. perenne* to inoculation with *M. nivale* are listed in Table 3 with homologues in *A. thaliana* and *B. distachyon*. A hypothetical model for gene regulation in the plant-pathogen interaction pathway after four days of incubation

with the pink snow mould pathogen, based on the pathogen related DEGs identified in this study, is presented in Fig. 7.

Furthermore, the Kyoto encyclopedia of genes and genomes (KEGG) database was used to detect different pathways in response to *M. nivale* in the S and R genotypes. Blast to the KEGG database showed that 5009 DEGs were involved in 135 pathways (Supplementary Table 2). Pathways with highest representation among the genes were involved in purine metabolism (5.19%, 260 genes), biosynthesis of antibiotics (5.09%, 255 genes), thiamine metabolism (4.25 %, 213 genes), starch and sucrose metabolism (2.91%, 146 genes) and aminobenzoate degradation (2.61%, 131 genes).

Validation of transcripts by Real-time PCR

In order to validate the expression profiling by Illumina sequencing, the expression levels of six genes, including two chitinase genes, three PR-related genes and one WRKY family gene were further analysed by qRT-PCR. The results showed that all the genes showed differential expression levels between S and R genotypes (Supplementary Fig.5). The expression patterns of five genes (*chitinase 5*, *chitinase 2*, *PR-1*, *PR-3* and *WRKY*) showed similar expression patterns as detected in RNA seq analysis, thus 83% of qPCR data was correlated with the RNAseq data.

Discussion

Snow mould resistance test and quantification of M. nivale DNA.

With the aim of facilitating the selection process for plant genotypes with a high resistance to snow mould. Real-time PCR was used for a precise quantification of *M. nivale* DNA in 8 genotypes of *L. perenne*. Fungal DNA was quantified in plants directly after incubation at 2 °C for 6 and 8 weeks. No correlation was found between the amount of *M. nivale* DNA and snow mould resistance calculated as relative regrowth. Nevertheless, some of the genotypes had a high content of fungal DNA and a relatively low relative regrowth (genotype F); whereas others had a relative low amount of fungal DNA and a high relative regrowth (genotype M). Therefore these genotypes (genotype F as susceptible (S) genotype and genotype M as resistant (R) genotype) were selected for gene expression analysis. However, this method could not detect significant difference in the amount of *M. nivale* DNA between resistant and susceptible genotype after one and four days from inoculation. Higher expression of disease related genes was detected in the resistant than in the susceptible genotype. These findings underline the role of defence response during this early stage of infection and show that this response can be independent from the level of the fungal infestation.

Significant correlation was found between the amount of *M. nivale* DNA and visual assessment of disease severity. However, after 8 weeks of incubation, disease severity was similar across genotypes, while significant differences in the quantity of *M. nivale* DNA were detected. The different results from the two methods could be due to the high sensitivity of the real-time PCR method. Furthermore, the plants incubated for a longer period (8 weeks) had a higher amount of fungal DNA than the plants incubated for 6 weeks, which is in agreement with other studies reporting that longer incubation period increases snow mould infestation even in resistant genotypes (Gaudet et al. 2011; Tronsmo 2013). In general, neither visual assessment of disease severity, nor the amount of *M. nivale* DNA in leaves is good indicators of snow mould resistance in *L. perenne*. In the present study, plant regrowth after inoculation and incubation for several weeks with *M. nivale* was not correlated with fungal biomass or disease severity. Some genotypes such as M and K showed severe symptoms on their leaf tissues, but still had good regrowth, possibly because the lower stem was not infected. On the other hand, genotype C had a poor regrowth despite limited symptoms and fungal DNA detected in the leaves.

The use of quantitative PCR assay could be considered a useful method for breeders to utilize in the selection of snow mould resistant materials. However, if the amount of fungal DNA shall be used as a screening method for resistance, it has to be based on infestation of lower stem tissue of the plants. Moreover, the application of quantitative real-time PCR will facilitate the understanding of the environmental effect in disease development (Schaad & Frederick 2002; Schena et al. 2004).

Comparisons between de novo and reference based assembly mapping

High throughput sequencing capabilities have made the process of assembling a transcriptome easier, even for the non-model organisms without a reference genome. But the quality of a transcriptome assembly must be good enough to capture the most comprehensive catalogue of transcripts and their variations, and to carry out further transcriptomic experiments (Marchant et al. 2015). The CEGMA analysis (Table 2) showed high coverage of ultra-conserved CEGs in the assemblies of the S and R genotypes, demonstrating their completeness in terms of gene content. However, a common question is whether reference based assembly gives better results than a *de novo* based assembly.

In this study, we compared both *de novo* and reference based assembly mapping (Farrel et al. 2014), and detected a larger number of differentially expressed transcripts in a pair wise comparisons between NI-NI and I-I; NI-NI and I-NI conditions, than the pair-wise comparison between I-NI and I-I condition (Fig. 3). It was expected that there would be a larger number of transcripts differentially expressed when plants were transferred from growth (non-incubation) conditions (at 20-22°C and 18 hours of light) to incubation conditions (2°C and darkness without light) due to the significant changes in temperature and light. This was seen both for the susceptible (S) and the resistant (R) genotypes as several differentially expressed transcripts may be involved in a rapid response to cold stress and photoperiod, in addition to abiotic stress related genes. On the other hand, very few differentially expressed transcripts were observed between I-NI and I-I (2°C and darkness without light) conditions in both S and R genotypes. The annotation results of the detected transcripts between I-NI and I-I conditions in both *de novo* and reference based mapping identified similar genes involved in biotic stress, immune response, cell death. Thus showing the potential of *de novo* method in capturing the essential transcripts even in the absence of reference genome, which also demonstrated in raspberry studies by Ward et al (2012).

Potential candidate genes in response to snow mould infection

To our knowledge, this is the first transcriptome study using RNA-seq to understand the response of *L. perenne* to the early infection of pink snow mould (*M. nivale*). Several of the differentially regulated genes such as disease related proteins, calmodulin binding proteins, lipid transfer proteins, and flavonoid biosynthesis (Table 3) detected in R and S genotypes between the I-NI and I-I conditions are involved in different defence-response mechanisms. The R genotype showed higher expression levels of several pathogenis related proteins such as PR-1, PR-2, PR-3, PR-5, PR-13 and PR-14. These results are similar to Gaudet et al. (2003) where winter wheat snow mould resistance was associated with the accumulation of PR-1a, PR-2, PR-5, and PR-14. The higher expression levels of these PR-proteins are often considered as markers for activation of the salicylic acid (SA) signalling pathway (Gaudet et al. 2003; Sels et al. 2008; Pociecha et al. 2009; Gaudet et al. 2011). Pociecha et al. (2009) also demonstrated that resistant genotypes of *Festulolium* are characterized by high SA concentrations during snow mould infection. PR proteins seem to be part of a larger set of SA and jasmonic acid (JA)-dependent defence responses in which each PR protein may contribute differently to the snow mould infection. For instance the *A. thaliana* mutant *ein2*, which is defective in ET/JA signalling, showed low expression level of PR-12, PR-3 and PR-4 and high susceptibility to *B. cinerea* (necrotrophic pathogens). Conversely, the salicylic acid induction–deficient mutants of Arabidopsis expressed PR-2 and PR-5 and accumulated high levels of camalexin after pathogen inoculation (Nawrath and Metraux, 1999).

WRKY proteins, another important defence related group of proteins, constitutes a superfamily of transcription factors, involved in the regulation of different physiological platforms in plants, including pathogen defence, trichome development and senescence. In this study, *WRKY65*, *WRKY70* and *WRKY75* were upregulated after inoculation with snow mould (Table 3). It is also reported that WRKY transcription factors contributed to the defence against *Pseudomonas syringae* in tomato and play a partially conserved role in basal defence in tomato and *Arabidopsis* (Bhattarai et al. 2010).

Plant-pathogen interaction pathway

The plant immunity system consists of two main levels (Jones & Dangl 2006). The first level is based on the perception of pathogen-associated molecular patterns (PAMPs), which activates the PAMP-triggered immunity pathway (PTI). The second level is the recognition of pathogen effectors, which activates pathogen related PR genes in a process

called effector-triggered immunity (ETI). In the present study, the transcriptome analysis of the snow mould resistant genotype showed that the PAMP trigger immunity (PTI) pathway was activated (Fig. 7), particularly by the up-regulation of the expression level of calcium-dependent protein kinase *CDPK*, respiratory burst oxidase *Rboh* and calcium-binding protein CML *CaM/CML*. Therefore, the activation of the PTI inhibits the snow mould pathogen from colonizing the plant tissues by increasing the production of reactive oxygen species and cell wall reinforcement. These results are similar to the studies in *Festulolium*, where resistant genotypes are characterized by high peroxidase activity, intensive lignification, callus formation and high concentrations of reactive oxygen species during the stage of early infection (within 6 days from inoculation) (Pociecha et al. 2008, 2009).

Transcription factors, such as WRKY, play important role in defence responses towards several plant pathogens (Seo & Choi 2015). Moreover, the transcription level of WRKY genes are up-regulated by several stress factors, in particular pathogen infection (Ishihama & Yoshioka 2012). In *Arabidopsis*, 49 out of 72 WRKY genes tested responded to bacterial infection or salicylic acid (Dong et al. 2003), and 8 *Arabidopsis* WRKY genes (WRKY18, WRKY 38, WRKY 53, WRKY 54, WRKY 58, WRKY 59, WRKY 66, and WRKY 70) were characterized as direct targets of *NPR1*, a key regulator of SA signalling (Wang et al. 2006). In the present study, the resistant genotype showed high transcription levels of several WRKY genes such as WRKY 70 and WRKY 75. Therefore, it is expected that the up-regulation of these genes will lead to the activation of the salicylic acid pathway (Dong et al. 2003; Chen et al. 2013). Furthermore, our results also showed down-regulation of WRKY 18 and WRKY 33, which are responsible for the activation of the JA pathway and the deactivation of the SA pathway (Eulgem & Somssich 2007; Ishihama & Yoshioka 2012). Moreover, in a study by Gaudet et al. (2011), the expression levels of WRKY 34 and WRKY 16 were up-regulated in snow mould resistant genotypes of winter wheat, which led to the activation of the JA pathway. However, other studies showed that *M. nivale* infection is usually influenced by the physical and the chemical conditions of the plant tissue, thus the fungus behaves as a biotroph when the plant defence system is induced and the SA pathway is activated (Dubas et al. 2011; Szechyńska-Hebda et al. 2013; Szechyńska-Hebda et al. 2015).

Differences in gene ontologies between R and S genotypes

GO enrichment analysis by Fischer's exact test revealed that several GO terms are over- expressed in the R genotype (Fig. 6). *Arabidopsis thaliana* defence-related protein ELI3 is an aromatic aryl-alcohol dehydrogenase (NADP+) (Somssich et al. 1996). Expression of this gene was shown to be rapidly and transiently stimulated in cultured parsley cells upon treatment with a cell wall preparation (elicitor) from the phytopathogenic oomycete *Phytophthora sojae*, and histochemical studies revealed local and rapid accumulation of ELI3 mRNA around infection sites in parsley leaves (Schmelzer et al. 1989). The presence of this gene in other plant species, such as potato (*Solanum tuberosum*), alfalfa (*Medicago sativa*), and *A. thaliana*, was also demonstrated (Trezzini et al. 1993). Plant-type cell wall cellulose metabolic process, cell wall pectin metabolic process, cell morphogenesis and epidermal cell differentiation plays a crucial role in plant-pathogen interactions (Bellincampi et al. 2014). These GO terms are significantly overexpressed in R genotype. Plants have developed a system for sensing pathogens and monitoring the cell wall integrity, upon which they activate defence responses that lead to a dynamic cell wall remodelling required to prevent disease (Bellincampi et al. 2014). Significant enrichment of these GO terms in the R genotype show that these gene systems are involved in defence responses to pink snow mould infection in perennial ryegrass.

Conclusions

In this study, we have conducted transcriptome analysis of a susceptible (S) and a resistant (R) genotype of *L. perenne* in response to early infection of snow mould disease. Here we report for the first time the genes that are differentially expressed between a susceptible and a resistant genotype using RNA sequencing. Our studies showed that many pathogen related genes are upregulated during snow mould infection in the resistant genotype. Further GO enrichment analysis confirmed that specific GO terms related to plant defence are over-represented in the resistant genotype. A list of putative candidate defence associated genes identified in this study might provide a scientific basis for further investigations to attain more in-depth understanding of host-pathogen interactions and development of resistant cultivars by marker-assisted breeding.

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Tables

Table 1. Characteristics of the *de novo* transcriptome assemblies.

	Susceptible genotype (S)	Resistant genotype (R)
Min. contig length (bp)	201	201
N50 (bp)	1,784	1,672
Max. contig length (bp)	17,632	12,882
Total no. of contigs	261,978	188,355
Sum of the reads	178,000,000	165,000,000

Table 2. Results of CEGMA analysis for *de-novo* assembly validation.

Out of 248 CEGs[1]	Resistant genotype (R)	Susceptible genotype (S)
% of fully represented	82.66	93.95
% of at least partially represented	90.73	98.79
Average number of orthologs per CEG	3.72	3.83
% of detected CEGs with more than 1 ortholog	96.10	97.00

[1] CEGs: Core Eukaryotic Genes

Table 3. List of differentially expressed genes that can be considered as potential candidate genes involved in response to *M. nivale* in two Lolium perenne , cv Fagerlin genotypes, R (resistant genotype), and S (susceptible genotype).

Sequence ID	*Arabidopsis thaliana* homologue	*Brachypodium distachyon* homologue	Description	Log FC[(a)] I-NI (S/R)	Log FC[(b)] I-I (S/R)
comp10786_c0_seq2		Bradi1g36400.2	26s protease	8.78	-11.8
comp11656_c0_seq1	AT3G08550.1	Bradi2g07890.3	Abscisic acid insensitive protein	-2.94	11.58
comp11730_c0_seq1	AT4G16830.3	Bradi5g10027.1	Abscisic acid protein	10.98	-0.36
comp12003_c0_seq2	AT4G16830.3	Bradi5g10027.1	Abscisic acid protein	-11.65	-1.36
comp13445_c0_seq1	AT2G27730.1	Bradi4g38600.2	Atpase inhibitor protein	10.06	-11.4
comp13446_c0_seq1	AT5G62000.4	Bradi2g59480.1	Auxin response factor 2	-0.05	11.57
comp14875_c0_seq1	AT5G62000.4	Bradi2g59480.1	Auxin response factor 2	11.15	11.57
comp15074_c0_seq2	AT1G56220.4	Bradi4g31110.2	Auxin-repressed protein	-4.60	11.96
comp15317_c0_seq1	AT2G41140.1	Bradi1g61637.1	Calcium -dependent protein kinase 1	-0.51	7.07
comp15765_c0_seq1	AT5G23580.1	Bradi4g24390.1	Calcium-dependent protein kinase sk5	1.11	10.83
comp16692_c0_seq1	AT1G35670.1	Bradi4g24390.1	Calcium-dependent protein kinase sk5	8.36	9.74
comp17044_c0_seq1	AT5G57580.1	Bradi3g05760.2	Calmodulin binding protein	0.37	-10.5
comp17054_c0_seq1	AT3G49050.1	Bradi2g00831.1	Calmodulin-binding heat shock protein	0.48	-9.68
comp17565_c0_seq2	AT3G16920.1	Bradi4g34040.1	Chitinase 2	-9.07	9.47
comp22298_c0_seq1	AT3G54420.1	Bradi5g14430.1	Chitinase 5	3.42	3.16
comp23317_c0_seq2	AT3G12500.1	Bradi3g32340.1	Class II chitinase	-0.71	3.26
comp23443_c0_seq2	AT2G02120.1	Bradi3g49380.1	Defensin precursor	-9.00	0.40
comp23963_c0_seq1	AT3G14470.1	Bradi1g29560.2	Disease resistance protein	1.59	11.71
comp24194_c0_seq2	AT1G64160.1	Bradi1g20185.1	Disease resistance protein	1.38	3.17
comp24580_c0_seq2	AT3G46730.1	Bradi1g51961.2	Disease resistance protein 3	7.97	3.57

comp24988_c1_seq1	AT1G72540.1	Bradi1g51961.2	Disease resistance protein 3	6.42	-2.26
comp25199_c0_seq1	AT3G14460.1	Bradi2g25327.1	Disease resistance protein rga3	5.05	-2.01
comp26954_c0_seq8	AT3G46730.1	Bradi3g15593.1	Disease resistance protein rpm1	9.76	8.47
comp27012_c0_seq1	AT3G46730.1	Bradi4g24887.1	Disease resistance protein rpm1	1.10	9.53
comp27236_c0_seq2	AT1G59780.1	Bradi3g15593.2	Disease resistance protein rpm1	12.59	6.93
comp27390_c0_seq1	AT3G07040.1	Bradi4g35317.1	Disease resistance protein rpm1	9.81	1.00
comp27751_c0_seq17	AT3G07040.1	Bradi4g35317.1	Disease resistance protein rpm1	11.93	2.13
comp28444_c0_seq2	AT1G58602.1	Bradi4g21950.2	Disease resistance protein rpp13	-1.17	10.66
comp28535_c0_seq2	AT3G20770.1	Bradi1g63780.1	Ethylene signal transcription factor	9.53	2.16
comp28907_c0_seq3	AT2G27050.1	Bradi1g63780.1	Ethylene signal transcription factor	3.70	-11.2
comp28998_c0_seq2	AT5G03280.1	Bradi4g08380.1	Ethylene-insensitive protein 2-like	6.49	10.66
comp29029_c0_seq9	AT1G53910.3	Bradi1g46690.3	Ethylene-responsive transcription factor 1	0.25	2.03
comp29851_c0_seq7	AT3G14230.3	Bradi2g02100.1	Ethylene-responsive transcription factor crf4	-9.85	0.94
comp30083_c0_seq2	AT1G53910.3	Bradi1g46690.3	Ethylene-responsive transcription factor rap2	2.42	-0.74
comp30409_c0_seq3	AT1G55270.1	Bradi3g01360.3	F-box kelch-repeat protein	0.85	12.01
comp30635_c0_seq3		Bradi3g31520.1	F-box protein	2.16	-11.8
comp30748_c0_seq3	AT2G42620.1	Bradi1g49120.2	F-box protein ore9-like	0.54	9.56
comp30853_c0_seq1	AT2G24270.4	Bradi3g36930.1	Glyceraldehyde-3-phosphate dehydrogenase	12.60	-12.8
comp30959_c0_seq10	AT3G25530.1	Bradi3g46080.1	Glyoxylate reductase 1	4.57	11.89
comp31014_c0_seq1	AT5G02500.1	Bradi1g03720.1	Heat shock protein 70	5.10	13.11
comp31072_c0_seq5	AT5G02500.1	Bradi1g03720.1	Heat shock protein 70	4.02	12.15
comp31301_c0_seq19	AT5G63890.1	Bradi1g17340.1	Histidinol dehydrogenase	1.37	-12.7
comp31318_c0_seq3	AT4G14420.1	Bradi1g75100.2	HR-like lesion-inducing protein	4.10	-12.7
comp31337_c1_seq2	AT1G15690.2	Bradi1g30550.1	Inorganic H pyrophosphatase protein	-4.93	13.84
comp31380_c0_seq1	AT2G38540.1	Bradi4g25750.1	Lipid transfer protein	-1.76	3.91
comp31450_c0_seq51	AT2G38540.1	Bradi4g25750.1	Lipid transfer protein	-2.82	1.86
comp31553_c0_seq3	AT2G42880.1	Bradi2g45870.1	MAP kinase protein	12.53	-0.05

comp31649_c0_seq29	AT3G55270.1	Bradi2g37450.2	MAP kinase phosphatase	13.85	-0.09
comp31684_c0_seq5	AT5G56580.1	Bradi1g75150.1	MAP kinase protein	12.57	-3.52
comp31934_c0_seq20	AT1G53570.5	Bradi3g45790.1	MAPkkk protein kinase	0.47	0.57
comp31966_c0_seq4	AT1G53570.2	Bradi5g10670.2	MAPkkk protein kinase	10.90	0.28
comp31978_c0_seq6	AT1G07180.1	Bradi2g53970.1	NAD(P)H dehydrogenase 1	0.06	12.56
comp32014_c0_seq2	AT3G14470.1	Bradi1g29560.2	NB-ARC disease resistance protein	2.33	-7.97
comp32363_c0_seq37	AT4G26090.1	Bradi5g15560.1	NB-ARC disease resistance protein	8.92	1.35
comp33360_c0_seq1	AT2G26040.1	Bradi1g64920.1	Pathogenesis-related protein 1	-1.44	12.21
comp33451_c0_seq1	AT4G25780.1	Bradi1g57540.1	Pathogenesis-related protein 1	1.75	4.48
comp33568_c0_seq1	AT3G04720.1	Bradi4g14930.1	Pathogenesis-related protein 4	-3.31	1.58
comp34254_c0_seq1	AT1G75050.1	Bradi4g05440.1	Pathogenesis-related protein 5	0.50	5.60
comp34926_c0_seq1	AT1G78780.2	Bradi2g08707.1	Pathogen-related protein	-8.18	0.37
comp35213_c0_seq1	AT1G09570.2	Bradi1g10520.2	Phytochrome A	-5.79	-10.4
comp35885_c0_seq1	AT4G35470.1	Bradi3g33990.1	Plant intracellular ras group-related LRR 4	9.20	12.03
comp35990_c0_seq1	AT1G64060.1	Bradi2g19090.5	Respiratory burst oxidase protein 2	-0.48	9.35
comp36198_c0_seq1	AT2G39840.1	Bradi3g55614.3	Serine threonine protein phosphatase pp1	3.01	-12.06
comp36434_c0_seq1	AT2G39840.1	Bradi3g55614.3	Serine threonine protein phosphatase pp1	3.03	-11.4
comp37190_c0_seq1	AT4G33950.1	Bradi1g07620.1	Serine threonine-protein kinase	1.24	-0.32
comp38150_c0_seq1	AT3G13380.1	Bradi4g27440.1	Serine threonine-protein kinase	-0.28	0.37
comp41496_c0_seq1	AT4G33080.1	Bradi2g33530.2	Serine threonine-protein kinase cbk1	-1.22	-8.39
comp42382_c0_seq1	AT4G33080.2	Bradi2g33530.1	Serine threonine-protein kinase cbk1	-1.84	-8.13
comp43183_c0_seq1	AT5G02800.1	Bradi1g76362.2	Serine threonine-protein kinase pbs1	0.06	-9.37
comp44101_c0_seq1	AT5G22840.1	Bradi1g08660.2	Serine threonine-protein kinase srpk2	10.60	-1.25
comp45052_c0_seq1	AT2G13360.1	Bradi3g39750.2	Serine-glyoxylate aminotransferase	-0.71	1.41
comp46601_c0_seq1	AT3G15610.1	Bradi1g36840.1	Serine-threonine kinase receptor-associated	1.95	-4.15
comp48325_c0_seq1	AT2G45950.1	Bradi1g62007.1	SKP1-like protein 21	-1.25	8.57
comp49870_c0_seq1	AT4G11650.1	Bradi4g05440.1	Thaumatin domain family protein	-9.07	12.16

comp5056_c0_seq1	AT4G11650.1	Bradi3g07960.1	Thaumatin pathogenesis-related protein 3	-9.96	3.14
comp64771_c0_seq1	AT2G02760.1	Bradi2g05400.2	Ubiquiting-conjugating enzyme 2	-1.97	13.67
comp6936_c0_seq1	AT4G31800.2	Bradi1g30870.1	WRKY DNA-binding protein 18	-9.91	-2.84
comp73317_c0_seq1	AT4G31800.2	Bradi3g06070.1	WRKY DNA-binding protein 18	1.95	-1.39
comp7627_c0_seq1	AT5G56270.1	Bradi4g33370.1	WRKY DNA-binding protein 2	-10.43	10.95
comp76423_c0_seq1	AT2G38470.1	Bradi2g00280.1	WRKY DNA-binding protein 33	2.32	-5.45
comp7959_c0_seq1	AT5G64810.1	Bradi2g18530.1	WRKY DNA-binding protein 51	4.05	0.34
comp8111_c0_seq1	AT1G29280.1	Bradi2g49906.1	WRKY DNA-binding protein 65	-8.24	0.05
comp8129_c0_seq1	AT2G46400.1	Bradi1g17660.1	WRKY DNA-binding protein 70	-0.47	1.84
comp8631_c0_seq2	AT5G13080.1	Bradi4g19060.1	WRKY DNA-binding protein 75	0.53	1.49
comp8708_c0_seq1	AT3G55980.1	Bradi4g05990.2	Zinc finger protein 33	-0.97	9.28
comp8930_c0_seq1	AT2G16485.1	Bradi4g35977.2	Zinc finger protein 44	-2.49	0.13
comp9122_c0_seq1	AT2G27580.1	Bradi1g06036.1	Zinc finger stress-associated protein 6	0.39	9.21
comp9340_c0_seq1	AT1G07360.1	Bradi1g48140.1	Zinc finger protein 40	0.20	-9.68
comp9759_c0_seq1	AT3G12630.1	Bradi3g39850.1	Zinc finger stress-associated protein 5	-10.85	-9.95

[(a)] The $\log_2$ of the fold change between the resistant (R) and susceptible (S) genotype under incubated and non-inoculated (I-NI) conditions after 4 days of incubation. [(b)] The $\log_2$ of the fold change between the resistant (R) and susceptible (S) genotype under incubated and inoculated (I-I) conditions after 4 days of incubation.

Supplementary Table 1. : Correlations between relative regrowth (g dry weight), visual scoring of symptoms and the amount of fungal DNA in plant tissue of 8 genotypes of *L. perenne* cv. Fagerlin, after inoculation with *M. nivale* and incubation for 6 and 8 weeks at +2 °C.

Variables	(1)	(2)	(3)
(1) Relative regrowth[(a)]	-		
(2) Visual scoring of symptoms[(b)]	-0.106 [ns]	-	
(3) The amount of fungal DNA[(c)]	0.023 [ns]	-0.497 [*]	-

[*] Correlation is significant at the 0.01 level; [ns] Correlation is not significant.

[(a)] Relative regrowth (dry weight of inoculated plants divided by dry weight of non-inoculated plants) after incubation under artificial snow cover followed by two weeks of regrowth.

[(b)] Visual assessment was done according to the following scale: 0 = no green tillers, 1 = some green tillers visible, 2 = green tillers found in less than half of the total plant area, 3 = green tillers found in more than half of the plant area, and 4 = green tillers observed in the whole plant area.

[(c)] The amounts of fungal DNA (pg. fungal DNA/ng plant DNA).

Supplementary Table 2. List of pathway-enriched differentially expressed genes in the resistant (R) and susceptible (S) genotype under incubated and inoculated (I-I) conditions after 4 days of incubation.

Pathway	Differentially expressed genes (No./%)	Pathway ID
Purine metabolism	260 (5.19%)	map00230
Biosynthesis of antibiotics	255 (5.09%)	map01130
Thiamine metabolism	213 (4.25%)	map00730
Starch and sucrose metabolism	146 (2.91%)	map00500
Aminobenzoate degradation	131 (2.61%)	map00627
Glycolysis / Gluconeogenesis	90 (1.80%)	map00010
T cell receptor signaling pathway	77 (1.54%)	map04660
Galactose metabolism	70 (1.40%)	map00052
Methane metabolism	69 (1.38%)	map00680
Glyoxylate and dicarboxylate metabolism	67 (1.34%)	map00630
Carbon fixation in photosynthetic organisms	65 (1.30%)	map00710
Phenylpropanoid biosynthesis	59 (1.18%)	map00940
Pyruvate metabolism	57 (1.14%)	map00620
Glycerolipid metabolism	52 (1.04%)	map00561
Amino sugar and nucleotide sugar metabolism	51 (1.02%)	map00520
Fructose and mannose metabolism	51 (1.02%)	map00051
Glycine, serine and threonine metabolism	50 (1.0%)	map00260
Pentose phosphate pathway	49 (0.98%)	map00030
Drug metabolism - other enzymes	46 (0.92%)	map00983
Pyrimidine metabolism	43 (0.86%)	map00240
Carbon fixation pathways in prokaryotes	36 (0.72%)	map00720
Valine, leucine and isoleucine degradation	35 (0.70%)	map00280
Glutathione metabolism	34 (0.68%)	map00480
Cyanoamino acid metabolism	34 (0.68%)	map00460
Tryptophan metabolism	30 (0.60%)	map00380
Glycerophospholipid metabolism	30 (0.60%)	map00564
Fatty acid degradation	29 (0.58%)	map00071
alpha-Linolenic acid metabolism	27 (0.54%)	map00592
Metabolism of xenobiotics by cytochrome P450	27 (0.54%)	map00980
Phenylalanine metabolism	27 (0.54%)	map00360
beta-Alanine metabolism	26 (0.52%)	map00410
Phosphatidylinositol signaling system	26 (0.52%)	map04070
Oxidative phosphorylation	25 (0.50%)	map00190
Pentose and glucuronate interconversions	25 (0.50%)	map00040
Ubiquinone and other terpenoid-quinone biosynthesis	24 (0.48%)	map00130
Arginine and proline metabolism	23 (0.46%)	map00330
Nitrogen metabolism	23 (0.46%)	map00910
Terpenoid backbone biosynthesis	23 (0.46%)	map00900
Cysteine and methionine metabolism	23 (0.46%)	map00270
Alanine, aspartate and glutamate metabolism	23 (0.46%)	map00250

Propanoate metabolism	23 (0.46%)	map00640
Ascorbate and aldarate metabolism	23 (0.46%)	map00053
Citrate cycle (TCA cycle)	22 (0.44%)	map00020
Butanoate metabolism	22 (0.44%)	map00650
Lysine degradation	21 (0.42%)	map00310
Sphingolipid metabolism	21 (0.42%)	map00600
Tyrosine metabolism	21 (0.42%)	map00350
Linoleic acid metabolism	20 (0.40%)	map00591
Inositol phosphate metabolism	20 (0.40%)	map00562
Porphyrin and chlorophyll metabolism	19 (0.38%)	map00860
Sulfur metabolism	18 (0.36%)	map00920
Retinol metabolism	18 (0.36%)	map00830
Pantothenate and CoA biosynthesis	17 (0.34%)	map00770
Phenylalanine, tyrosine and tryptophan biosynthesis	17 (0.34%)	map00400
Chloroalkane and chloroalkene degradation	16 (0.32%)	map00625
Other glycan degradation	16 (0.32%)	map00511
Arachidonic acid metabolism	15 (0.30%)	map00590
Aminoacyl-tRNA biosynthesis	15 (0.30%)	map00970
Arginine biosynthesis	13 (0.26%)	map00220
Caprolactam degradation	13 (0.26%)	map00930
Histidine metabolism	13 (0.26%)	map00340
Tropane, piperidine and pyridine alkaloid biosynthesis	12 (0.24%)	map00960
Ether lipid metabolism	12 (0.24%)	map00565
Steroid hormone biosynthesis	12 (0.24%)	map00140
One carbon pool by folate	11 (0.22%)	map00670
Limonene and pinene degradation	11 (0.22%)	map00903
Biosynthesis of unsaturated fatty acids	10 (0.20%)	map01040
Nicotinate and nicotinamide metabolism	10 (0.20%)	map00760
Selenocompound metabolism	10 (0.20%)	map00450
Valine, leucine and isoleucine biosynthesis	9 (0.18%)	map00290
Streptomycin biosynthesis	9 (0.18%)	map00521
Lysine biosynthesis	9 (0.18%)	map00300
Isoquinoline alkaloid biosynthesis	9 (0.18%)	map00950
Fatty acid elongation	9 (0.18%)	map00062
Plant-pathogen interaction	8 (0.16%)	map04626
Geraniol degradation	8 (0.16%)	map00281
Glycosphingolipid biosynthesis - globo series	8 (0.16%)	map00603
Benzoate degradation	8 (0.16%)	map00362
Vitamin B6 metabolism	8 (0.16%)	map00750
Steroid biosynthesis	8 (0.16%)	map00100
Flavonoid biosynthesis	8 (0.16%)	map00941
Steroid degradation	7 (0.14%)	map00984
Caffeine metabolism	7 (0.14%)	map00232
Toluene degradation	7 (0.14%)	map00623

Photosynthesis	7 (0.14%)	map00195
N-Glycan biosynthesis	7 (0.14%)	map00510
Cutin, suberine and wax biosynthesis	7 (0.14%)	map00073
Novobiocin biosynthesis	7 (0.14%)	map00401
Various types of N-glycan biosynthesis	6 (0.12%)	map00513
Carotenoid biosynthesis	6 (0.12%)	map00906
Chlorocyclohexane and chlorobenzene degradation	6 (0.12%)	map00361
Monobactam biosynthesis	6 (0.12%)	map00261
Monobactam biosynthesis	6 (0.12%)	map00261
Styrene degradation	6 (0.12%)	map00643
Glycosaminoglycan biosynthesis - heparan sulfate / heparin	6 (0.12%)	map00534
Glycosaminoglycan degradation	6 (0.12%)	map00531
Xylene degradation	5 (0.10%)	map00622
Glucosinolate biosynthesis	5 (0.10%)	map00966
Glycosphingolipid biosynthesis - ganglio series	5 (0.10%)	map00604
Synthesis and degradation of ketone bodies	5 (0.10%)	map00072
Taurine and hypotaurine metabolism	4 (0.08%)	map00430
Biotin metabolism	4 (0.08%)	map00780
Butirosin and neomycin biosynthesis	4 (0.08%)	map00524
C5-Branched dibasic acid metabolism	4 (0.08%)	map00660
Carbapenem biosynthesis	3 (0.06%)	map00332
Naphthalene degradation	3 (0.06%)	map00626
Zeatin biosynthesis	3 (0.06%)	map00908
Indole alkaloid biosynthesis	3 (0.06%)	map00901
Benzoxazinoid biosynthesis	3 (0.06%)	map00402
Isoflavonoid biosynthesis	3 (0.06%)	map00943
Riboflavin metabolism	3 (0.06%)	map00740
Ethylbenzene degradation	3 (0.06%)	map00642
Folate biosynthesis	3 (0.06%)	map00790
Insect hormone biosynthesis	2 (0.04%)	map00981
Lipoic acid metabolism	2 (0.04%)	map00785
Primary bile acid biosynthesis	2 (0.04%)	map00120
Sesquiterpenoid and triterpenoid biosynthesis	2 (0.04%)	map00909
Diterpenoid biosynthesis	2 (0.04%)	map00904
Fluorobenzoate degradation	2 (0.04%)	map00364
Stilbenoid, diarylheptanoid and gingerol biosynthesis	2 (0.04%)	map00945
Biosynthesis of terpenoids and steroids	2 (0.04%)	map01062
Aflatoxin biosynthesis	2 (0.04%)	map00254
Tetracycline biosynthesis	2 (0.04%)	map00253
Phosphonate and phosphinate metabolism	2 (0.04%)	map00440
mTOR signaling pathway	2 (0.04%)	map04150
Polyketide sugar unit biosynthesis	1 (0.02%)	map00523
Other types of O-glycan biosynthesis	1 (0.02%)	map00514
D-Alanine metabolism	1 (0.02%)	map00473

D-Glutamine and D-glutamate metabolism	1 (0.02%)	map00471
Peptidoglycan biosynthesis	1 (0.02%)	map00550
Flavone and flavonol biosynthesis	1 (0.02%)	map00944
Biosynthesis of vancomycin group antibiotics	1 (0.02%)	map01055
Biosynthesis of ansamycins	1 (0.02%)	map01051
Atrazine degradation	1 (0.02%)	map00791

Supplementary Table 3. Real-time PCR Primers for quantification of gene expression.

Target gene	Primer name	sequence (5´-3´)	Reference
PR-5	PR-5F	GCAGCTGAACAGCGGCGAGACGTGGAAC	Zhang et al. (2011)
	PR-5R	GCCGGTGCTGCAGGAGAAGCCCATGC	
PR-1	PR1-F2	AGCACAAGGCTGCAGTCGTA	This study
	PR1-R2	CTTGCAGTCGCCGATCCT	
Thaumatin-like PR3	Thi-F2	AACTGCCCGGATGCCTATC	This study
	Thi-R2	CGTTGCAGCCGTGTGTTTT	
Chitinase 5	Chi5-F1	CCAGTGGTGGCGTTCAAGA	This study
	Chi5-R1	CACCCCATGCACGTTGGT	
Chitinase 2	Chi2-F2	CCCGATGTTGAACGACTTCTG	This study
	Chi2-R2	TCGGTTTCTACAAGCGCTACTG	
WRKY	WRKY-F1	CGCTCCACCCTCTTCTTCAC	This study
	WRKY-R1	TGTGCTGTGTGCAGGAACTACTAC	
LpGAPDH	LpGAPDH-F	CATCACCATTGTCTCCAACG	Petersen et al. (2004)
	LpGAPDH-R	AACCTTCAACGATGCCAAAC	

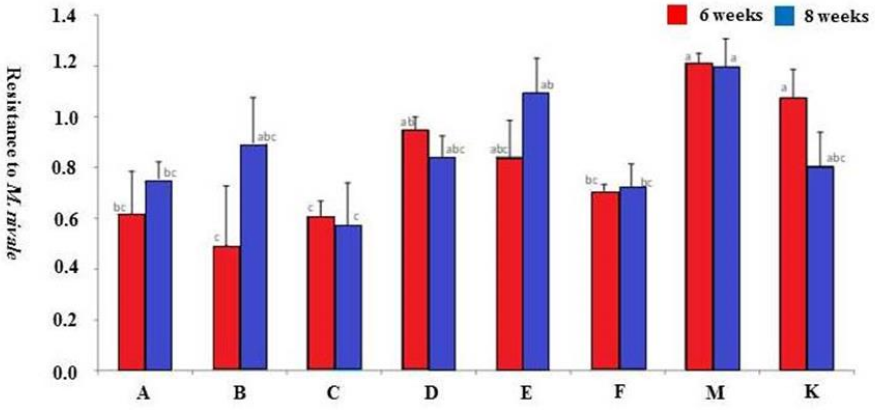


Figure 1. Resistance to *M. nivale* in 8 genotypes, measured as relative regrowth (dry weight of inoculated plants divided by dry weight of non-inoculated plants) after 6 and 8 weeks incubation under artificial snow cover followed by two weeks of regrowth. Error bars indicate standard errors of the mean, and bars marks with different letters indicate significant differences ($P<0.05$).

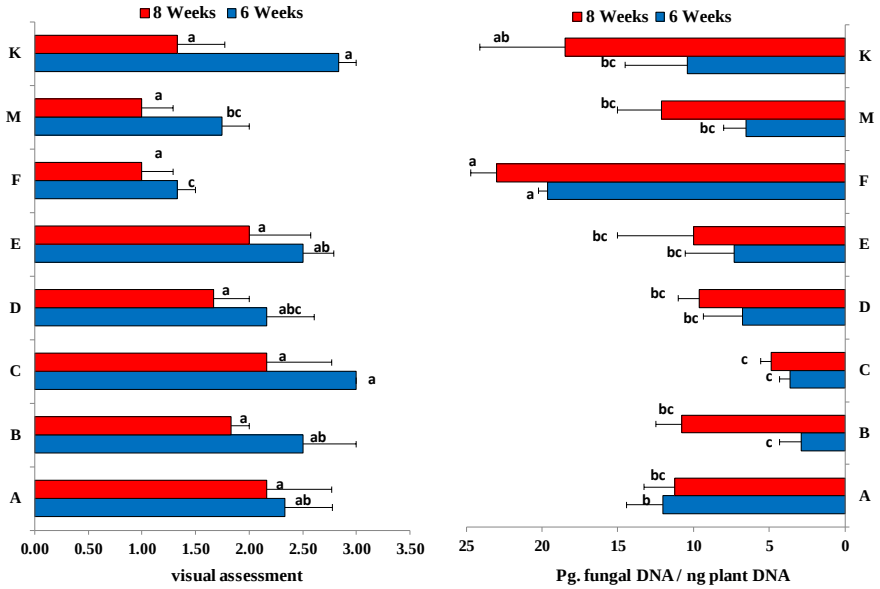


Figure 2. Symptoms visual assessment and the mount of *M. nivale* (isolate 200231) DNA (pg. fungal DNA/ng plant DNA), in 8 genotypes of *L. perenne* cv. Fagerlin after 6 and 8 weeks from inoculation. Error bars indicate standard errors of the mean, and bars marks with different letters indicate significant differences ($P<0.05$).

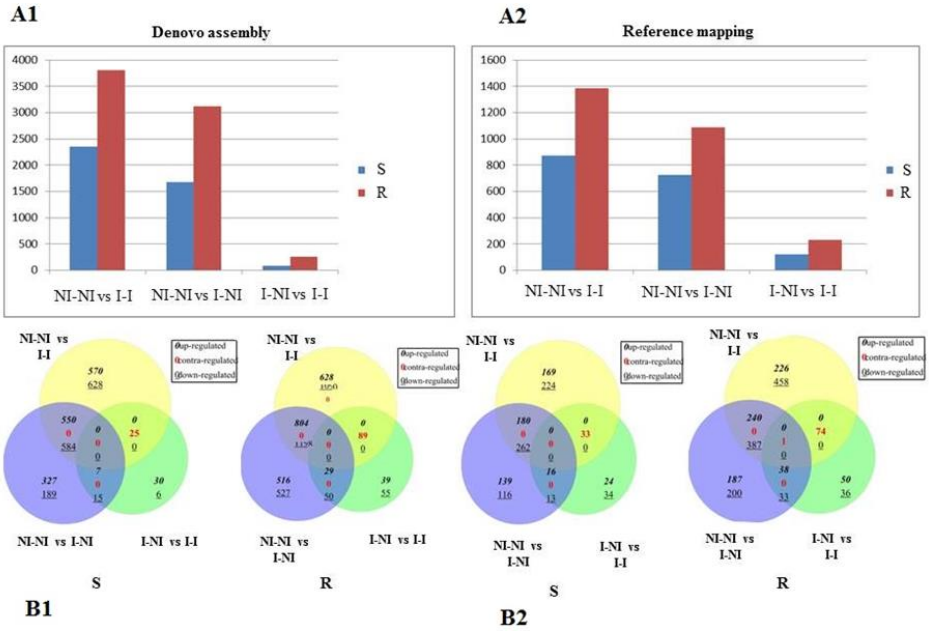


Figure 3. The number of differentially expressed transcripts identified using *de novo* assembly method (A1) and the reference (inbred *L. perenne* transcriptome) based assembly method (A2) with FDR<0.05. Venn diagrams showing the number of up-, down- and contra-regulated transcripts that were common and specific for the pairwise comparisons using *de novo* assembly (B1) and the reference based assembly (B2). R; resistant genotype, S; susceptible genotypes. NI-NI; non- incubated and non-inoculated plants, I-I; incubated and inoculated plants after 4 days of incubation, I-NI; incubated and non-inoculated plants after 4 days of incubation.

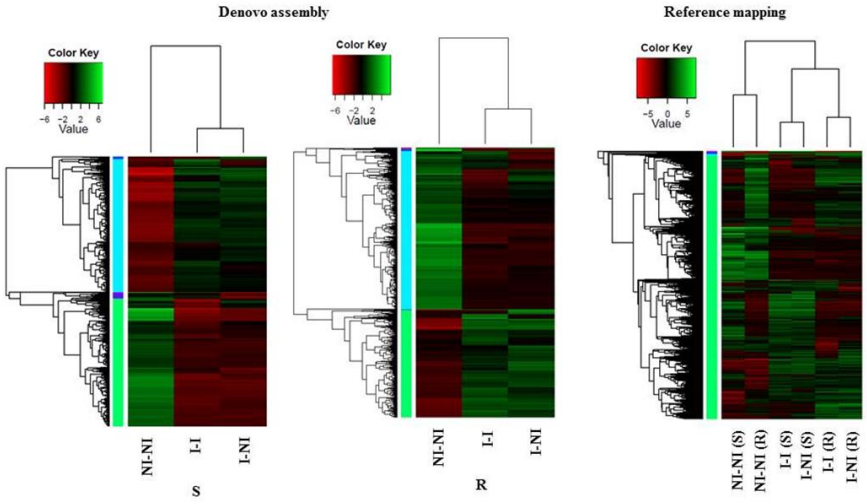


Figure 4. Heat maps of differentially expressed genes detected using de novo assemblies and reference based assembly for each genotype and grouped according to their expression patterns. X-axis represents the experimental conditions. R; resistant genotype, S; susceptible genotype. NI-NI; non- incubated and non- inoculated plants, I-I; incubated and inoculated plants after 4 days of incubation, I-NI; incubated and non-inoculated plants after 4 days of incubation.

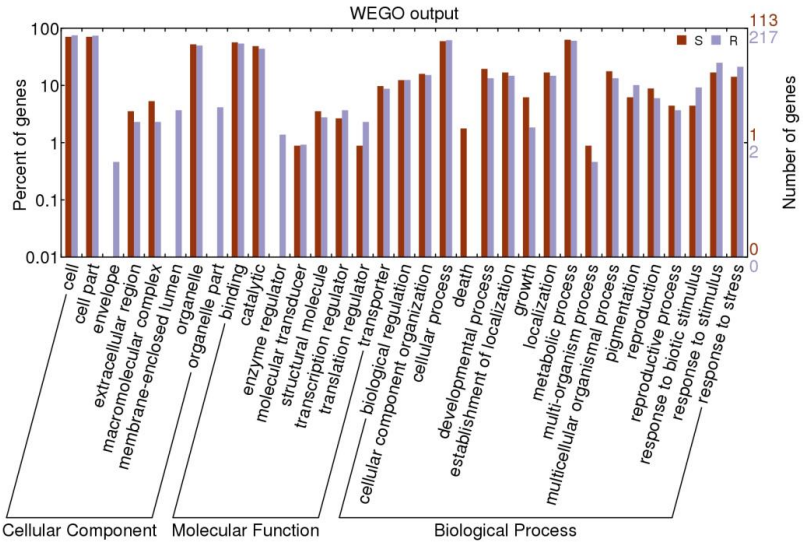


Figure 5. Gene ontology classifications of differentially expressed genes observed during pairwise comparisons of incubated non-inoculated (I-NI) and incubated inoculated (I-I) in resistant (R) and susceptible (S) genotypes generated by WEGO tool (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) generated automatically by the web histogram tool WEGO (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) using the newest GO archive provided. The results are summarized in three main GO categories: cellular component, molecular function and biological process. The right y-axis indicates the number of genes in a category. The left y-axis indicates the percentage of a specific category of genes in that main category.

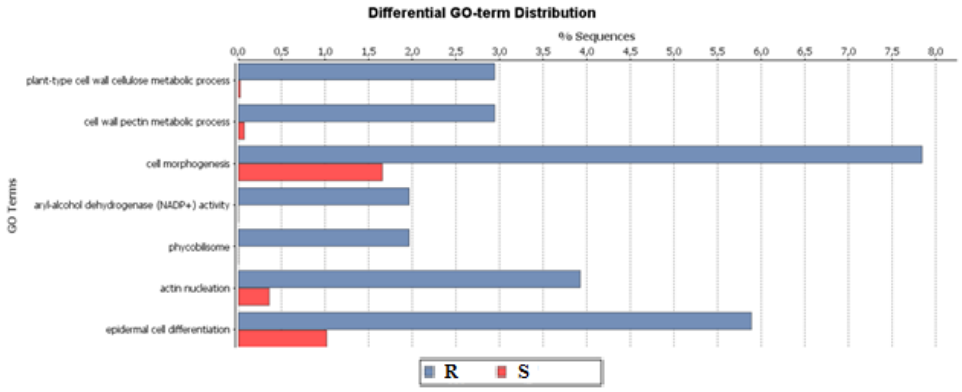


Figure 6. Annotation differences between resistant (R) and susceptible (S) genotypes detected by Fischer's exact test.

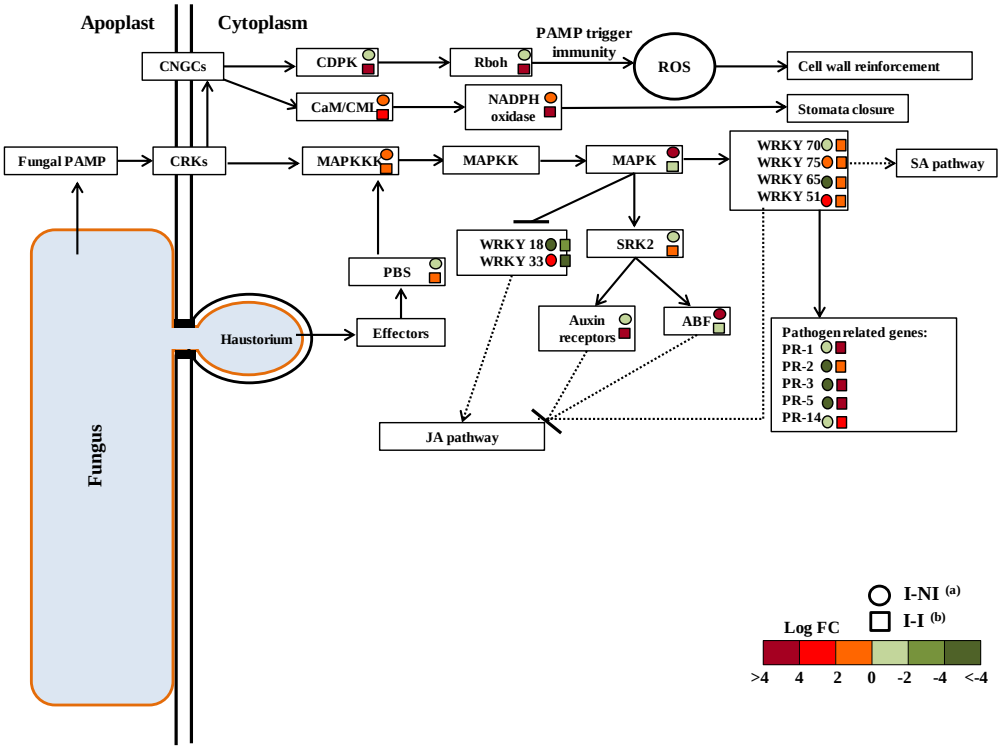
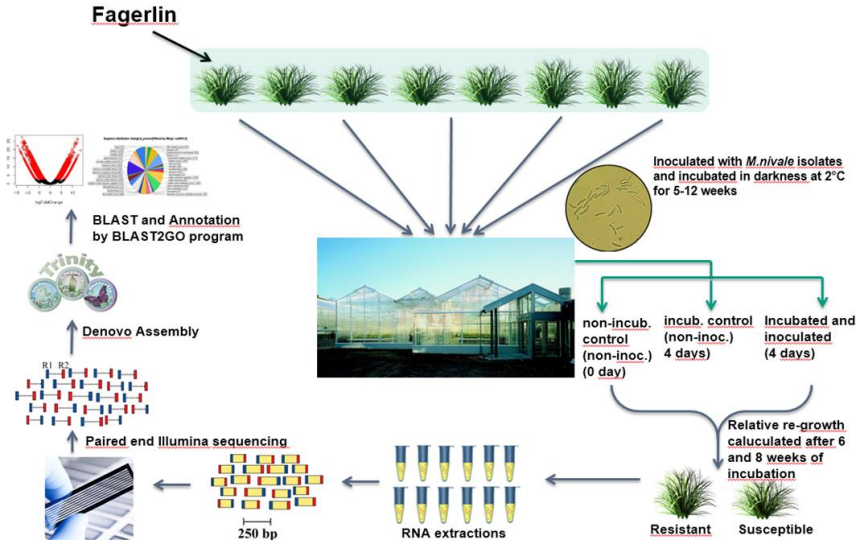
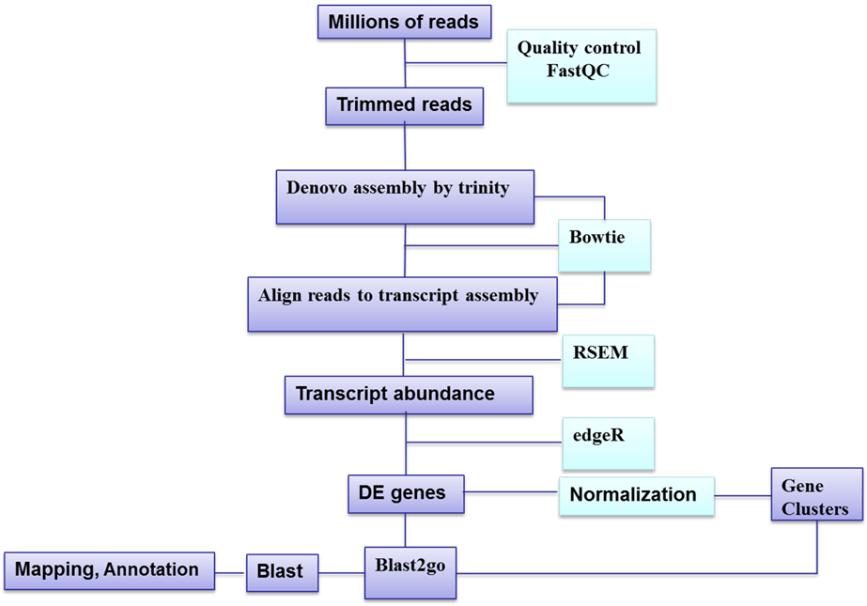


Figure 7. Hypothetical modules for plant-pathogen interaction after 4 days of incubation with snow mould pathogen *M. nivale* derived by KEGG plant-pathogen interaction pathway (http://www.genome.jp/kegg/) and network of WRKY transcription factors (Eulgem & Somssich (2007) . The recognition of pathogen-associated molecular pattern (PAMP) initiate PAMP trigger immunity via the activation of cysteine-rich receptor-like protein kinase (CRK), cyclic nucleotide gated channel (CNGC), calcium-dependent protein kinase (CDPK), respiratory burst oxidase (Rboh), calcium-binding protein CML (CaM/CML), and NADPH oxidase. The activation of PAMP trigger immunity initiate the production of reactive oxygen species (ROS), which might activate the plant hypersensitive response (HR) , cell wall reinforcement, as well as stomata closure. Defence responses are also instigated upon recognition of the fungal effectors in the host cell by serine/threonine-protein kinase PBS (PBS) and the activation of MAP kinase cascades such as mitogen-activated protein kinase kinase kinase (MAPKKK), mitogen-activated protein kinase kinase (MAPKK), and mitogen-activated protein kinase (MAPK). Effectors triggered immunity (ETI) initiate the production of several pathogen related proteins such as PR-1, β-1,3-glucanase (PR-2), chitinase II/V (PR-3), thaumatin-like (PR-5), and lipid-transfer protein (PR-14). Both PAMP triggered immunity and effectors triggered immunity alternate the production of salicylic acid (SA) and jasmonic acid (JA) by the

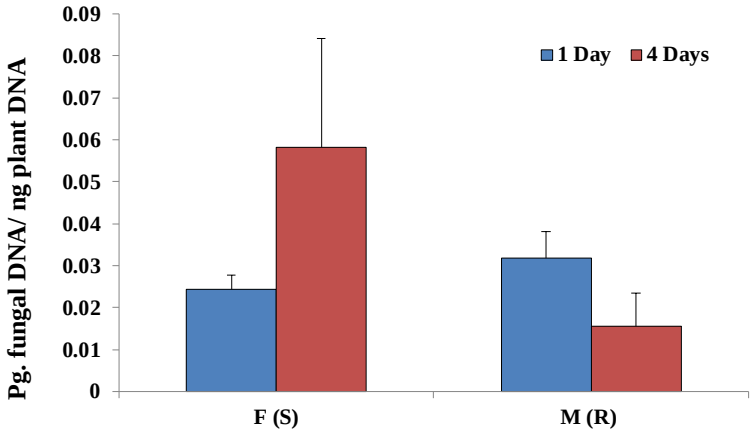
action of distinct transcription factors WRKY such as WRKY 75, WRKY 70, WRKY 18, and WRKY 33. Pathogen-triggered SA signaling also by the activation of serine/threonine-protein kinase2 (SRK2), auxin receptors, and abscisic acid responsive element binding factor (ABF).



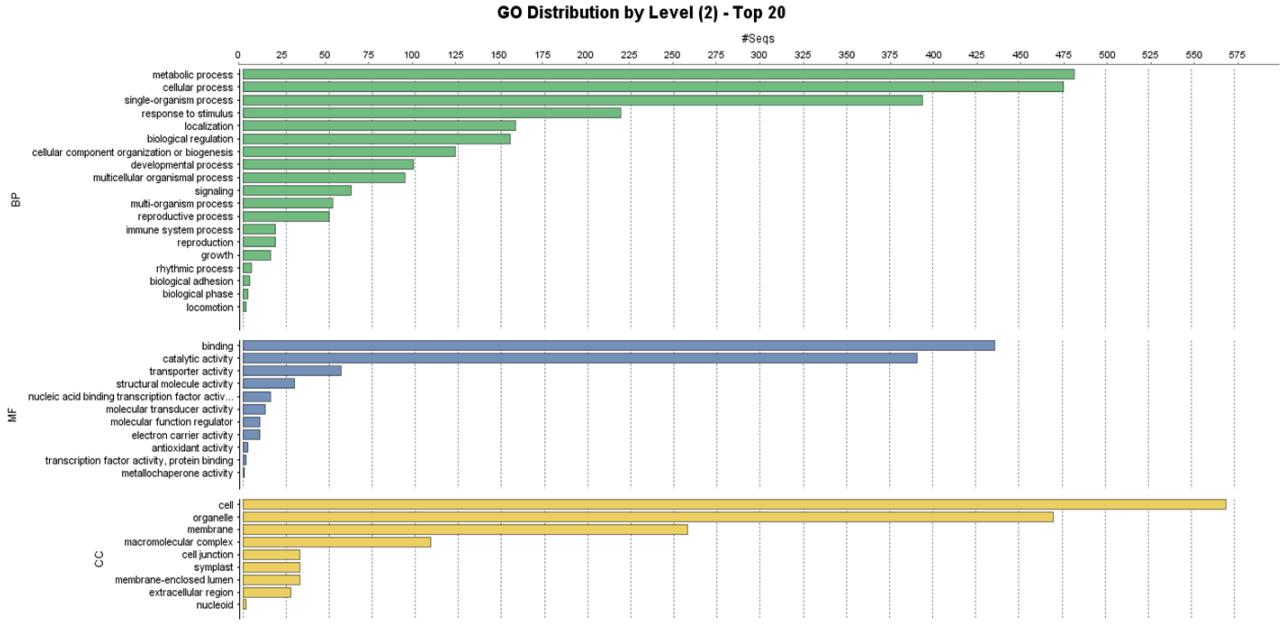
Supplementary figure 1. Overview of experimental design.



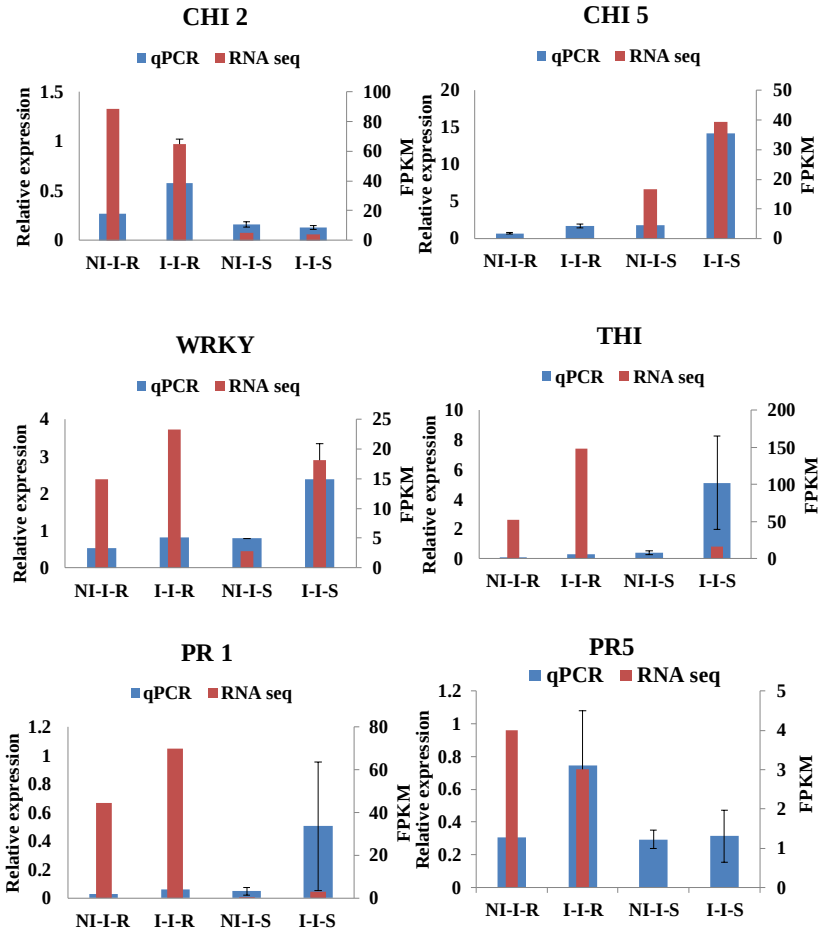
Supplementary figure 2. Work flow of RNA sequence data analysis.



Supplementary figure 3. Amount of fungal DNA (pg. fungal DNA/ng plant DNA) in two genotypes of *L. perenne* cv. Fagerlin (resistant genotype M (R) and susceptible genotype F (S) inoculated with *M. nivale* (isolate 200231). Samples were collected 1 and 4 days after inoculation. Error bars indicate standard errors of the mean.



Supplementary figure 4. The highest 20 Gene Ontology GO groups based on the differentially expressed genes between resistant (R) and susceptible (S) genotypes under incubated-inoculation conditions.



Supplementary figure 5. Validation of gene expression of 6 selected genes by qRT-PCR. CHI2; Chitinase 2, CHI5; Chitinase 5, WRKY; WRKY transcription factor, THI; Thaumatin-like PR3, PR1 ; pathogenesis-related protein 1, PR5; pathogenesis-related protein 5. Expression level was calculated based on $2^{-\Delta\Delta Ct}$ method. R; resistant genotype, S; susceptible genotypes. NI-NI; non- incubated and non- inoculated plants, I-I; incubated and inoculated plants after 4 days of incubation, I-NI; incubated and non-inoculated plants after 4 days of incubation.