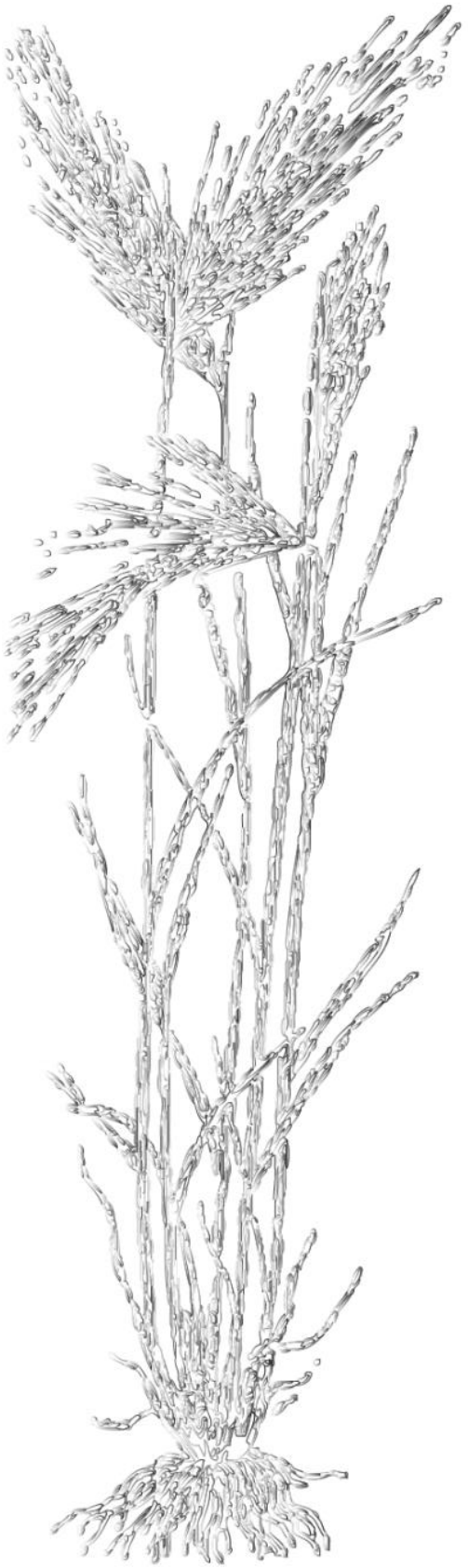
Polymorphism in the *Rphq2* region of barley (*Hordeum vulgare* L.)



Master thesis

Author

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Date

05-05-2015

Place

Wageningen University, Wageningen

Polymorphism in the *Rphq2* region of barley (*Hordeum vulgare* L.)

Haplotype determination of Rphq2 region and latency period to leaf rust (*P.hordei*) evaluation of barley cultivars and wild barley accessions (*Hordeum vulgare* L. *Hordeum vulgare* subsp. *spontaneum* and *H. agriocrithon*).

Master thesis

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Abstract

The QTL *Rphq2* has a substantial effect to leaf rust in Barley. It explains on average 35.5% of the phenotypic variation in the seedling stage. Vada and SusPtrit have two different haplotypes of the *Rphq2* region; PAA and APP respectively. Because this lack of homology, no further recombination is expected in the segment and further fine-mapping is impossible. The relation between *Rphq2* haplotype, partial resistance and origin of the wild barley accessions (*H. spontaneum* and *H. agriocrithon*) was investigated to determine if the PAA-APP polymorphism is also present in wild barley accessions. Wild barley accessions from North Africa, the Near-East, Caucasus, Middle-East and Central Asia were used. The two major haplotype groups in *H. spontaneum* accessions were PAA (68.5%) and APP (20.1%). In addition to these two haplotypes, haplotypes AAA, APA, PPA and PPP were found. The largest diversity in *Rphq2* was found in the Near East with haplotypes APA, PPA and PPP. The dominant presence of haplotypes PAA and APP in barley cultivars and the examined *H. spontaneum* accessions as well as the presence of haplotypes APA, PPA and PPP in *H. spontaneum* suggests the split in the *Rphq2* region to have occurred in the ancestor of *H. spontaneum*. North-African accessions have a significantly higher relative latency period of *P. hordei* than accessions from the Caucasus, Near East, Middle East and Central Asia. Since North African accessions originate from coastal places with higher rainfall and humidity, the environmental conditions could play a role in the disease development of *P. hordei*. Consequently natural selection towards partially resistant *H. spontaneum* to *P. hordei* in this region could have taken place. Sequencing of the *V.Perox-3* (PAA specific) and *V.Kin*-1 (common between PAA and APP) genes for the cultivars and *H. spontaneum* accessions revealed 0.024 base substitution per site in the *V.Perox-3* gene compared to 0.101 in the *V.Kin-1* gene. This indicates that the *V.Perox-3* gene is more conserved than the *V.Kin-1* gene. Cultivars with *H. laevigatum* as one of the ancestors are more related to the *H. spontaneum* accessions in the *V.Perox-3* gene. No relation was found between the ancestors of the cultivars and *H. spontaneum* accessions in the *V.Kin-1* gene. The development of *P. hordei* was scored in inoculated barley lines which were susceptible and partial resistant to *P. hordei* to establish the biological point at which the development of *P.hordei* is hindered in partial resistant lines. The development of *P. hordei* showed no difference up to 7.5 hai. The development of *P. hordei* 10 hai is slower in L94*Rphq2* compared to L94 in the transition from substomal vesicle (SSV) to haustorial mother cell (HMC). It is possible that high thymol concentration in L94*Rphq2* hampers the transition from SSV to HMC.

Key words: *Hordeum vulgare, Puccinia hordei, Rphq2*, polymorphism, partial resistance.

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1 Introduction

1.1 Partial resistance in barley to leaf rust

Leaf rust in barley (*Hordeum vulgare*) is caused by the pathogen *Puccinia hordei*. Due to reduced photosynthetic capacity of the plant and metabolic competition between the plant and the pathogen susceptible barley cultivars can have yield reduction up to 30% (Feuerstein et al. 1990; Arnst et al. 1979). Despite a compatible infection of the pathogen partially resistant cultivars were found to have lower infection frequency and sporulation rate of the pathogen and a longer latency period (Niks and Marcel 2009). The hindered development of the pathogen is probably due to a pre-haustorially acting mechanism not associated with hypersensitivity (Niks 1986; Marcel et al. 2007). Genes responsible for the hypersensitivity response are, unlike genes associated with partial resistance, well studied (Chelkowski et al. 2003). The hypersensitivity response is the result of a single *R*-gene while multiple genes have an effect in partial resistance. It is essential pinpoint the genes responsible to partial resistance in barley in order to obtain more knowledge about the partial resistance response.

1.2 Major effect QTL *Rphq2*

Several quantitative trait loci (QTLs) for partial resistance of barley to barley leaf rust have been mapped in recombinant inbred lines (RILs) of barley. Among these were RILs derived from a cross between the partially resistant parent Vada and the susceptible parent L94 and RILs derived from a cross between Vada and the susceptible parent SusPtrit (Qi et al. 1998; Jafary et al. 2006). The Dutch cultivar Vada has been developed from a cross between *H. Laevigatum* and the Swedish landrace Gold by Dros (1957) and the susceptible line L94 originates from an Ethiopian landrace (Parlevliet 1975). SusPtrit is an experimental line developed for high susceptibility to non-adapted rust fungi (Atienza et al. 2004). One particular QTL found to have a substantial effect to leaf rust was *Rphq2* (Qi et al. 1998). It explains on average 35.5% of the phenotypic variation for partial resistance to leaf rust in the seedling stage (until the fourth leaf stadium of the plant) (Wang et al. 2010). *Rphq2* is located at the end of the long arm of chromosome 2. In Vada *Rphq2* was donated by *H. Laevigatum* (Giese et al. 1993; Arru et al. 2003). The effect of *Rphq2* was confirmed with the development of near isogenic lines (NILs) of Vada and L94. An introgressed fragment of 4.6 centiMorgans (cM) containing the *Rphq2* segment from Vada was incorporated in L94 and an introgressed fragment of 5.2 cM containing the *rphq2* segment from L94 was incorporated in Vada by Marcel et al. (2007). *Rphq2* prolonged the latency period of *P. hordei* in NIL L94*Rphq2* by 28 hours and shortened the latency period of *P. hordei* in NIL Vada*rphq2* by 23 hours (Marcel et al. 2007; Marcel et al. 2008). *Rphq2* was subsequently fine mapped in subNILs of Vada-*rphq2* to an interval of 0.11 cM (Marcel et al. 2007).

1.3 *Rphq2* linkage block in barley cultivars

To identify the gene in *Rphq2* responsible for the partial resistance to *P. hordei* bacterial artificial chromosome (BAC) libraries were constructed from Vada and SusPtrit by Yeo et al (2014). BAC clones containing the *Rphq2* segment were used to construct a contig covering the *Rphq2* segment in Vada. No complete coverage of the *rphq2* segment was achieved in SusPtrit and therefore the contig was incomplete. Sequencing and subsequent gene annotation of both contigs revealed 12 genes predicted in Vada and 12 genes predicted in SusPtrit between EST markers WBE114 and WBE115 (Figure 1). Only five of the predicted genes for Vada and SusPtrit are present in both lines. Because of the lack of homology in *Rphq2* between Vada and Susptrit, no further recombination is expected in the segment and further fine-mapping is impossible using these two cultivars (Yeo et al. 2014).

In order to test if the lack of homology in the *Rphq2* region was only between Vada and SusPtrit, barley cultivars were scored for presence (P) and absence (A) of the *V.Perox-3*, *S.UF* and *S.Kin-1* genes by Wang (2014, unpublished). The *V.Perox-3* gene is Vada specific and *S.UF* and *S.Kin-1* are SusPtrit specific genes. This makes the Vada having the haplotype "PAA" (*V.Perox-3*: Present, *S.UF*: Absent and *S.Kin-1*: Absent) and SusPtrit having the haplotype "APP". Scoring 194 barley cultivars resulted in 57% of the cultivars having the Vada like PAA haplotype and 43% of the cultivars having the SusPtrit like APP haplotype (Wang, 2014 unpublished). None of the cultivars examined had a combination of Vada and SusPtrit genes. The distribution of haplotypes PAA and APP suggests that recombination is suppressed in the *Rphq2* region and an early and complete split in haplotypes could have occurred (Yeo et al. , 2014).

A possible explanation for the lack of homology in the *Rphq2* region is that the accessions with the PAA haplotype originate from a different domestication centre than the accessions with the APP haplotype. The *Rphq2* region could have differentiated for these domestication centres and pairing of the *Rphq2* region was disabled. A similar observation was made in the *bz* region in maize by Fu and Dooner (2002). The common assumption that barley has a single centre of domestication is replaced with the hypothesis that multiple domestication origins were involved in forming the modern cultivated barley (Arnst et al. 1979; Morrell and Clegg 2007; Pourkheirandish and Komatsuda 2007; Igartua et al. 2013; Ren et al. 2013; Jakob et al. 2014; Zeng et al. 2015).

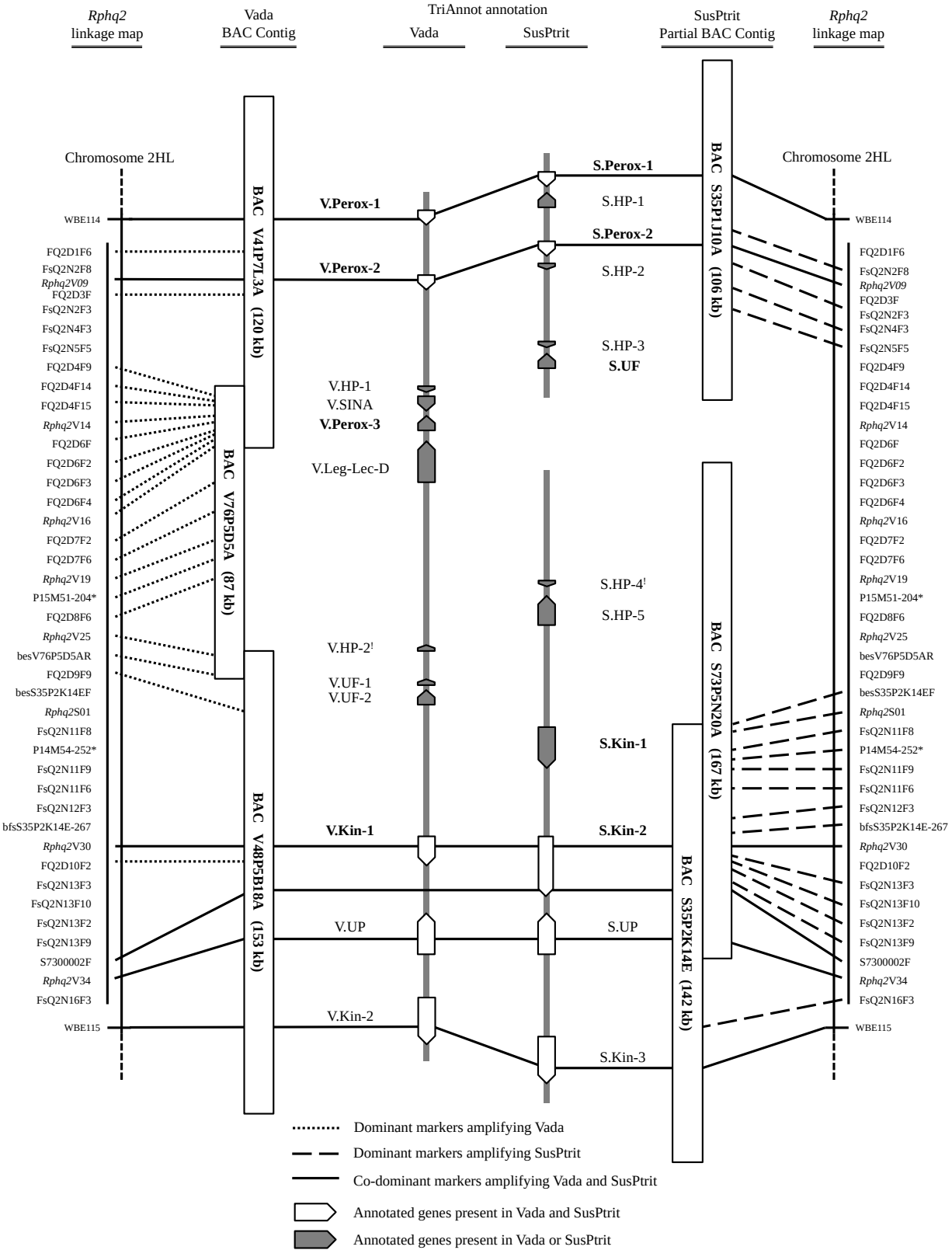

Figure 1. Representation of the 0.1 cM marker interval of the *Rphq2* region: including the linkage map, BAC contigs of Vada and SusPtrit and genes annotated. Abbreviations: HP = hypothetical protein; Kin = kinase; Leg-Lec-D = legume lectin domain; Perox = peroxidase; S = SusPtrit; SINA = Seven in absentia protein; UF = unknown function; UP = uncharacterized protein; V = Vada. Figure duplicated from Yeo et al. (2014).

1.4 Research objectives

The main objective was to study the relation between *Rphq2* haplotype, partial resistance and origin of the wild accessions to determine if the PAA-APP polymorphism found in modern barley cultivars is also present in wild barley accessions. In order to achieve this objective wild barley (*H. spontaneum* and *H. agriocrithon*) accessions from North Africa, the Near-East, Caucasus, Middle-East and Central Asia were used to investigate their haplotype and latency period of *P. hordei*.

The second objective was to move towards further fine mapping of the *Rphq2* region. Because of the lack of homology in the *Rphq2* segment between the PAA and APP haplotypes, no further recombination is expect in the segment and further fine-mapping is hampered using these two haplotypes. Since the partial resistance *to P. hordei* is most likely due to a resistance gene in the PAA haplotype, in Vada donated by *H. laevigatum*, we searched for a cultivar or accession susceptible to *P. hordei* at seedling stage with the PAA haplotype. By crossing this susceptible accession with a partial resistant PAA haplotype, the *Rphq2* segment can be further fine-mapped in the future. In addition to the wild barley accessions, barley cultivars known to have the PAA haplotype were scored for their latency period of *P. hordei* at seedling stage.

In addition to the previous objectives, the variation in the *V.Perox-3* gene specific for PAA haplotypes was compared with variation in the *V.Kin-1* gene common between the PAA and APP haplotypes. Comparison of the variation between these genes gives insight in the conservation of the genes in the *Rphq2* region. Last of all, the development of P. hordei was scored in inoculated barley lines which were susceptible and partial resistant to *P. hordei* to establish the biological point at which the development of *P.hordei* is hindered in partial resistant lines. Simultaneously leaf samples were taken for a future gene expression test by qPCR. The combination of these two tests can give an insight on the gene which is responsible for the partial resistance in the *Rphq2* segment.

2 Material and methods

2.1 Polymorphism of *Rphq2* in wild barley accessions

In order to obtain more knowledge about the split in haplotypes found in the Rphq2 region, wild barley accessions, barley land races and barley cultivars were scored for their presence of *V.Perox-3, S.UF* and *S.Kin-1* genes.

2.1.1 Plant material

In this research the haplotype of 345 *H. spontaneum* accessions, ten *H. agriocrithon* accessions and five Spanish barley land races was scored. The *H. spontaneum* consisted of a collection of 318 georeferenced accessions obtained from professor Steffenson (University of Minnesota) and 27 accessions from North-Africa obtained from the centre for genetic resources (CGN) the Netherlands and the Spanish national research council (CSIC) (Table 1). Ten *H. agriocrithon* accessions were obtained from the leibniz-institut für pflanzengenetic (IPK) Germany and originated from Tibet. Five barley land races originated from Spain and were obtained from CSIC.

Table 1. Origin of *H. spontaneum* accessions used in this research to score the *Rphq2* haplotype and relative latency period to P. hordei. The accessions were grouped based on their geographical origin.

Group	Origin
North Africa	Morocco, Tunisia, Algeria, Libya and Egypt.
Near East	Lebanon, Syria, Iraq, Jordan, Palestine and Cyprus
Caucasus	Armenia, Azerbaijan, Turkey and Russia
Middle East	Afghanistan, Pakistan and Iran
Central Asia	Khazarstan, Tajikistan, Turkmenistan and Uzbekistan

2.1.2 DNA isolation and amplification

In order to score the prementioned accessions for haplotype of the three genes in the *Rphq2* region, DNA was isolated and the three genes were amplified. Five seeds per accessions were sown in a plastic trays. Cultivars Vada, Golden Promise, L94*Rphq2* and SusPtrit were added as references. The seedlings were nursed in the greenhouse with approximately 12 hours light and 20°C during the day and 12 hours darkness and 14°C during night time. After 21 days 1.5 cm leaf sample was taken from the tip of the primary leaf of one plant per accession. The leaf samples were collected in micronic (costar) 2 ml tubes which contained two metal beads and 125 µl of CTAB extraction buffer (Table 2) and immediately placed in liquid nitrogen after sampling to prevent degradation of DNA.

Table 2. The CTAB extraction buffer stock for fixing DNA during isolation. (EDTA: Ethylenediaminetetraacetic acid).

Quantity	Component
0.5 gr	Alkyltrimethyl-ammonium bromide (Sigma M7635)
36.5 ml	MQ (H_2O)
5.0 ml	1 M Tris (pH 8.0)
7.0 ml	5 M NaCl
1.0 ml	0.5 M EDTA (pH 8.0)
0.5 ml	14 M ß-mercapto ethanol

Leaf samples were crushed using the Qiagen TissueLyser II© at 25 Hz for three minutes. The crushed leaf samples were centrifuged for 30 seconds at 1500 rpm and placed in boiling water for five minutes to lyse the cells. After boiling, the samples were centrifuged for ten minutes at 4000 rpm to separate the lysate from the residue. 20 µl lysate of each sample was pipetted in a polymerase chain reaction (PCR) plate and 60 µl MQ (H_20) was added to dilute the samples. 0.7 µl of each sample template, 9.3 µl PCR mastermix and 10 µl mineral oil was placed in a designated well on the PCR plate. The mineral oil was added to prevent evaporation of the sample and PCR mastermix during the PCR procedure. The primers used for amplification of *V.Perox-3* , *S.UF* and *S.Kin-1* genes and Actin were developed with Bioinformatics™ and validated with Premierbiosoft™ (Supplemental Table 2). All PCRs were pefromed on the S1000™ Thermal Cycler form Biorad©. The initialization was at 95°C for four minutes, denaturation at 98° for 10 seconds, annealing at 59°C for 30 seconds, elongation at 72°C for 35 seconds and the final elongation at 72°C for 60 seconds. The denaturation, annealing and elongation steps were repeated 35 times. 3 µl fluorescence loading was added to the PCR product to stain the DNA. The samples were pipetted into 2% TBE agarose gel and run for approximately 20 minutes at 120 volt. The agarose gel was checked with the Octopus II© machine. In case of no amplification of the three genes of interest, the sample was tested with Actin; a household gene which should show amplification if the DNA is of good quality. If the Actin did not give an amplification in the samples, new leaf samples were taken and tested again with the three genes.

2.2 Partial resistance to *Puccinia hordei*

Next to the haplotype the partial resistance of barley accessions and cultivars was qualified. In order to qualify the partial resistance the LP of *P.hordei* of accessions and cultivars was determined. To quantify the partial resistance, LP the most effective factor since it has the smallest experimental random error (Parlevliet 1976; Parlevliet 1979).

2.2.1 Plant material

All 345 *H. vulgare* subsp. *spontaneum* accessions, ten *H. agriocrithon* accessions and five barley land races described in the previous chapter were used to qualify latency period of *P. hordei*. In addition to the fore mentioned accessions 85 barley cultivars obtained from the CGN were used. The cultivars were found to have the PAA haplotype by Wang (2014, unpublished) in previous research (Supplemental Table 3). The partial resistance of the cultivars was first estimated in a screening experiment. Cultivars found to have a low level of partial resistance were used to qualify the exact latency period of *P. hordei*.

2.2.2 Pathogen isolate

In this experiment the standard isolate 1.2.1 of *P. hordei* was used. The isolate was multiplied and maintained in the greenhouse on the susceptible line L94. During sporulation of the pathogen the inoculum was collected from L94 and stored at room temperature before inoculation. Freshly collected inoculum was used for the inoculation of the plant material.

2.2.3 Inoculation and incubation

Per 37 x 39 cm flat, 18 accessions or cultivars were sown in two parallel rows. In each flat L94 and L94*Rphq2* were added as references. Per accession 4 seeds were sown. After 16 days two seedlings per accession were pinned adaxial side upward to the ground. All additional seedlings were removed. The seedlings were inoculated with 1 mg *P. hordei* urediospores in a setting tower as described by Niks (1982). To achieve even distribution of the inoculum, the urediospores were mixed with 49 mg lycopodium spores. After inoculation the flats were transferred to an incubation room with a relative humidity at saturation point. The next day the pins were removed, and the plants were transferred to a greenhouse compartment.

2.2.4 Latency period observation

The number of mature pustules were counted once on the 6^{th} day after inoculation, twice on the 7^{th} and 8^{th} day after inoculation and once on the 9^{th} day after inoculation as described by Parlevliet (1975). Final count of the mature pustules took place 10 days after inoculation. The LP was calculated according to the equation LP_{50}= A + (B/C) X D (A = time from inoculation until last counting before 50% pustules were mature; B = time between the counting before and after 50% of pustules were mature; C = increase in number of pustules during period B; D = 50% count minus number of pustules

at the start of period B) (Niks et al. 2011). Subsequently the relative LP on seedlings (RLP), where the LP on L94 seedlings was set at 100, was calculated as described by Parlevliet (1975). Hypersensitive cultivars and accessions were assessed according to the infection type of *P. hordei* at the final count. The infection type indicates the level of resistance based on hypersensitivity ranged from 0 to 9, indicating resistant and susceptible respectively (Niks et al. 2011)(Table 3).

Table 3. Infection types in barley accessions and cultivars infected by *Puccinia hordei*. [a]R (infection types 0,1,2,3 and 4), MR (5,6 and 7) and S (8 and 9) indicate resistant, moderately resistant and susceptible, respectively. Table duplicated from Niks et al. (2011).

Infection type	Description	Level of resistance [a]
0	No symptoms	R
1	Small necrotic of chlorotic flecks	
2	Flecks womewhat larger	
3	Minute uredosori surrounded by necrotic or chlorotic tissue	
4		
5	Small uredosori surrounded by some necrotic or chlorotic tissue	MR
6		
7	Larger Uredosori surrounded by some chlorotic tissue	
8	Uredosori surrounded by very faint chlorosis	S
9	Well-developed uredosori, no chlorosis or necrosis. There is often a pale halo around the uredosori	

2.3 Crossing of "PAA" haplotype plants

In order to further fine-map the *Rphq2* segment it is essential to obtain a accession or cultivar which has the PAA haplotype. Cultivars found to have the PAA haplotype and more susceptible to *P. hordei* than L94*Rphq2* were crossed with L94*Rphq2*. The susceptible PAA plants served as mother in the cross and L94*Rphq2* as father. Mother plants were emasculated when the tip of the awn appeared above the flag leaf. Emasculation was performed by opening roughly ⅔ of the flower and removing the anthers. The emasculated spikes were protected against cross fertilization by a semi-transparent paper bag. Three days after emasculation, fertilization was done by dispersing pollen of cultivar L94-*Rphq2* on the emasculated flower.

2.4 Sequence comparison between accessions

The variation in the *V.Perox-3* gene specific for PAA haplotypes was compared with variation in the *V.Kin-1* gene common between the PAA and APP haplotypes. Comparison of the variation between these genes gives insight in which of the two genes is more conserved.

2.4.1 Plant material

A total of 17 cultivars and four *H. vulgare* supsp. *Spontaneum* accessions, all with the PAA haplotype were used in this experiment. The *H. vulgare* subsp. *spontaneum* accessions consisted of number 28 originating from Palestine and a RLP of 126, number 45 originating from Jordan and a RLP of 107 and number 80 originating from Jordan and having a high level of partial resistance (infection type two).

2.4.2 Peroxidase and kinase genes sequencing

The plant material was sown in plastic pots (14 cm Ø) and grown in the greenhouse with approximately 12 hours light and 20°C during the day and 12 hours darkness and 14°C during night time. After 21 days 4 cm leaf sample of one plant per accession was taken from the tip of the primary leaf. The leaf samples were collected in micronic (costar) 2 ml tubes which contained two metal beads and immediately placed in liquid nitrogen to prevent degradation of DNA. DNA of the accessions was isolated with the use of the DNeasy Plant Mini Kit™ from Qiagen©. Primers for the *V. Perox-3* and *V.Kin-1* genes were developed with Bioinformatics™ and validated with Premierbiosoft™. Both genes were amplified by a nest PCR since they are too large for sequencing. The *V.Perox-3* and V.Kin-1 genes have a size of 1035 and 2310 base pair (bp) respectively (Wang, 2014 unpublished). The nest PCR results in a small template by which sequencing is improved. The first PCR amplification was done using primers *V.Perox-3(1)* and *V .Kin-1(1)* and *V.Perox-3(2)* and *V.Kin-1(2)* and *V.Kin-1(3)* were used in the second amplification. PCRs were performed on the S1000™ Thermal Cycler from Biorad©. The initialization was at 95°C for four minutes, denaturation at 98° for 10 seconds, annealing at 60°C for 30 seconds, elongation at 72°C for 35 seconds and the final elongation at 72°C for two minutes. The denaturation, annealing and elongation steps were repeated 35 times. The PCR products were cleaned with the illustra™ Microspin™ G-50 columns. The PCR products were sequenced by GATC Biotech©.

2.4.3 Phylogeny tree construction

Sequences of the *V.Perox-1* and *V.Kin-1* genes were aligned separately for all accessions and compared using MEGA 5™ based on the small nucleotide polymorphisms (SNPs). For both genes similarity trees were constructed using the neighbour-joining (NJ) method. The NJ method is a fast method which tends to have a high probability of constructing the correct tree (Saitou and Nei 1987).

2.5 Expression of genes in the *Rphq2* region and pathogen development

The development of *P. hordei* was scored in inoculated barley lines which were susceptible and partial resistant to P. hordei to establish the biological point at which the development of *P.hordei* is hindered in partial resistant lines.

2.5.1 Plant material

The gene expression analysis was performed on lines L94, L94*Rphq2*, SxG114 and 182*Q20*. Line SxG114 is derived from a cross between SusPtrit and Golden Promise and susceptible to *P.hordei* at seedling stage and line 182*Q20* is derived from a cross between *H. bulbosum* and Golden Promise. Line 182*Q20* has a very high level of resistance to *P. hordei* at seedling stage which is not based on a hypersensitive reaction (Johnston et al. 2013b).

2.5.2 Pathogen isolate

In this experiment the standard isolate 1.2.1 of *P. hordei* was used. The isolate was multiplied and maintained in the greenhouse on the susceptible line L94. During sporulation of the pathogen the inoculum was collected and stored at room temperature for inoculation. Freshly collected inoculum was used for the inoculation of the plant material.

2.5.3 Inoculation and incubation

Two experiments were performed in order to quantify the gene expression and qualify the pathogen development; one with early sampling (3.5-8 hours after inoculation (hai)) and one with later sampling (10-111 hai). Both experiments had the same experimental set up but differed in sampling time of the plants. Two lines were sown in one 37 x 39 cm flat in parallel rows. The lines were sown in two flats resulting in 4 flats containing all four cultivars in duplicate. After 16 days the flats were prepared for inoculation. Two flats containing seedlings of the four cultivars were inoculated with 46 mg lycopodium spores and 4 mg of *P. hordei* urediospores (inoculated treatment) and two flats containing seedlings of the four cultivars were only inoculated with 46 mg lycopodium spores (mock-inoculated treatment). The inoculation was executed in a setting tower as described by Niks et al. (2011). After inoculation the flats were transferred to an incubation room with a relative humidity at saturation point.

2.5.4 RNA isolation and gene expression

Leaf samples of approximately 75 mg were taken at 3.5, 5.5 and 8 hai in the early experiment and at 10, 15, 39, 63, 87, 111 and 135 hai in the later experiment. Per accession two leaf samples were taken every time point in the first experiment and three leaf samples were taken per accession in the second experiment. The leaf samples were immediately placed in liquid nitrogen and stored at -80°C to prevent degradation of the RNA. RNA was isolated according to the Ambion© RNA TRIzol© reagent protocol (Supplemental Figure 1). The isolated RNA was synthesised into coding DNA (cDNA) with use of the iScript™ cDNA Synthesis Kit from Biorad©.

2.5.5 Microscopic observations

Plant material from the gene expression experiment was also sampled for microscopic analysis. The development of the pathogen could be paired with the results of the gene expression test by this set-up. Middle segments of approximately 6 cm^2 of inoculated leaves were collected at the moment as in the gene expression test. The collected leaves were prepared for fluorescence microscopy as described by Niks (1986). Evaluation of the leaves took place on a Zeiss Axiophot photo microscope with an aniline blue filter. On every leaf sample 50 sporelings were evaluated and classified according to their developmental stage. On leaf samples from the first experiment the developmental stages of spores which at least developed a germination tube were scored and classified. In the second experiment the spores at least developing a germination tube and an appressorium were scored.

3 Results

3.1 Haplotype scoring of wild barley accessions

Of the 345 *H. spontaneum* accessions, 333 were scored for the presence of *V.Perox-3*, *S.UF* and *S.Kin-1* genes. Seed germination of 12 accessions was insufficient to collect DNA. Most common haplotypes of the *Rphq2* segment were PAA (68.5%) and APP (20.1%), but haplotypes AAA, APA, PPA and PPP were also found (Table 4). Of the ten *H. agriocrithon* accession eight had the APP haplotype and two the PAA haplotype. Of the five Spanish land races one had the APP haplotype, three the PAA haplotype and one the AAA haplotype.

Table 4. *Rphq2* haplotypes found *H. spontaneum*. Haplotypes based on amplification of *V.Perox-3*, *S.UF* and *S.Kin-1* genes. (P: presence of gene, A: absence of gene).

Haplotype	Number of accessions	Percentage of accessions
AAA	27	8.1
APA	1	0.3
APP	67	20.1
PAA	228	68.5
PPA	5	1.5
PPP	5	1.5
Total	**333**	**100**

The *H. spontaneum* accessions were clustered in five groups based on their geographical origin. In North Africa the APP haplotype was dominant while the PAA haplotype was dominant in eastern regions (Figure 2). The largest differences were found in Central Asia where 87% of the *H. spontaneum* accessions were PAA while only 3% were APP (Table 5). Accessions with the AAA haplotype were found in all regions ranging in presence from 6% to 11%. Haplotypes APA, PPA and PPP were found in North Africa, the Near East and the Caucasus. The haplotype of *H. spontaneum* accessions is related with the origin of the accession (Chi-square p>0.000).

Table 5. Presence of *H. spontaneum* haplotypes of the *Rphq2* region at geographical origin. (Other: haplotypes APA, PPA and PPP).

Origin	PAA	APP	AAA	Other
North-Africa	36%	55%	6%	3%
Near-East	72%	16%	8%	5%
Caucasus	52%	38%	7%	3%
Middle-East	79%	11%	11%	0%
Central-Asia	87%	3%	11%	0%

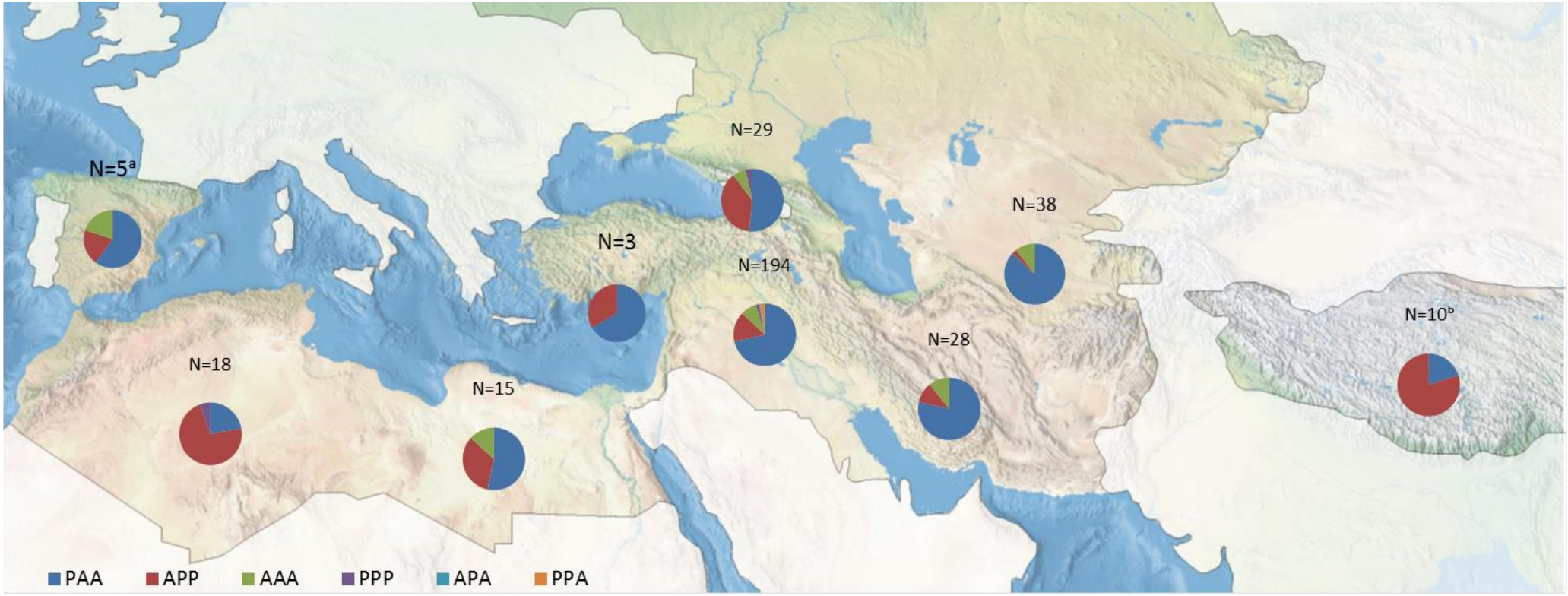

Figure 2. The haplotypes in the *Rphq2* region of sampled *H. spontaneum* accessions*, H. agriocrithon* accessions and Spanish land races based geographical distribution. The distribution of haplotypes found in the accessions is shown in the pie charts. Coloured areas on the chart indicate countries of origin of sampled accessions (N: Number of accessions [a]: Spanish land races [b]: *Hordeum agriocrithon* accessions).

3.2 Partial resistance of wild barley accession

Of the 345 *H. spontaneum* accession the relative latency period (RLP) of *P. hordei* at seedling stage on 286 accessions was determined (Supplemental Table 1). Seed germination and plant growth of 32 accessions was insufficient to determine the RLP. A hypersensitivity response to *P. hordei* was observed and scored in 27 *H. spontaneum* accessions. The infection type of the hypersensitive accessions ranged from 0 (no symptoms) to 7 (large uredosori surrounded by some chlorotic tissue) and the most common infection type of these accessions was 3 (minute uredosori surrounded by necrotic or chlorotic tissue). The average RLP to *P. hordei* of 286 *H. spontaneum* accessions was 110 and ranged from 94 to 136 (Figure 3). One *H. agriocrithon* accession was hypersensitive and had an infection type of 7. The RLP to *P. hordei* of nine *H. agriocrithon* accessions was 105 on average ranging from 100 to 116. The RLP to *P. hordei* of Spanish land races was 119 on average ranging from 99 to 128. In previous research of Wang (2014, unpublished), barley cultivars were scored for their haplotype in the *Rphq2* region. The 85 cultivars with the PAA haplotype were screened for their partial resistance to *P. hordei*. Of these 85 cultivars, the latency period of *P. hordei* on 15 cultivars was similar to L94 and L94*Rphq2*. Determining the precise latency period of these 15 cultivars resulted in finding two extremely susceptible cultivars: Un Ae 3.1 and Senor with RLP of 105 and 102 respectively.

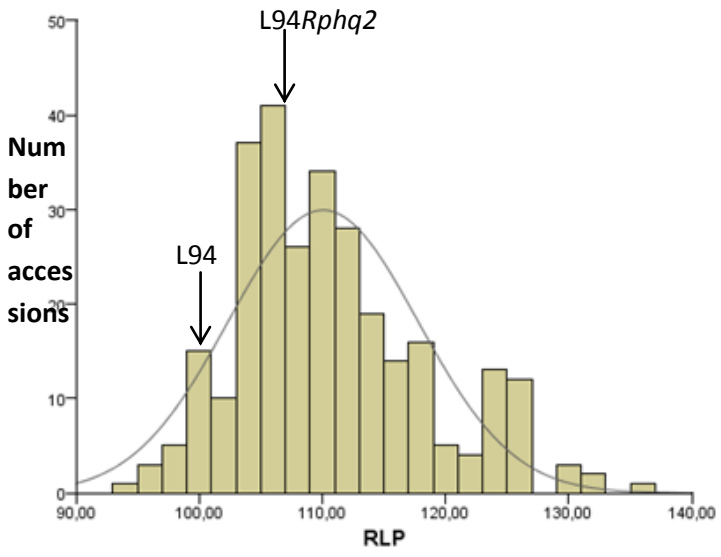

Figure 3. Frequency distribution of the relative latency period (RLP) for 286 *H. spontaneum* accessions. Values of L94 (100) and L94*Rphq2* (107) are indicated by arrows.

The RLP of *P. hordei* on accessions from North Africa was significantly longer than the accessions from eastern regions (Anova p=0.012). Between groups Near East, Caucasus, Middle East and Central Asia, no significant differences were found in relative latency periods (Table 6).

Table 6. Mean and standard deviation of the relative latency period of *H. spontaneum* accessions.

Origin	Mean	St. dev.
North Africa	114.2[a]	8.99
Near East	109[b]	8.93
Caucasus	109[b]	6.44
Middle East	110[b]	6.11
Central Asia	110[b]	5.82

No significant relation is found between haplotypes of the accession and RLP (Anova P=0.258). The average RLP of the PAA and APP accessions are 109 and 111 respectively. However, extreme susceptible haplotypes (RLP 94, 95 and 96) were all PAA and the extreme resistant (RLP: 136) accession had the APP haplotype (Figure 4).

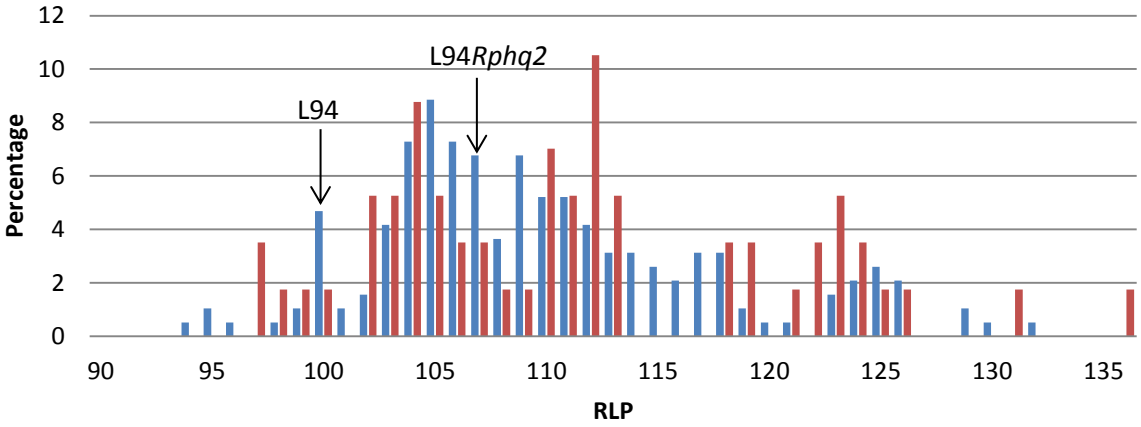

Figure 4. Percentage distribution of the RLP of the dominant haplotypes PAA (blue bars) and APP (red bars). Values of L94 (100) and L94*Rphq2* (107) are indicated by arrows.

3.3 Crossing susceptible PAA with partial resistant PAA

Two cultivars Senor and Un. Ae. 3.1 and three extremely susceptible *H. spontaneum* accessions with the PAA haplotype were selected for the crossing with L94*Rphq2*. The selected *H. spontaneum* accessions were 50, 103 and 128 and had a RLP of 94, 95 and 98 respectively. Accessions 50 and 128 originated from Syria and accession 103 originated from Jordan. Multiple crosses between Senor x L94*Rphq2* and Un. Ae. 3.1 x L94*Rphq2* resulted in approximately 80 F1 seeds for both crosses. The crosses between extremely susceptible *H. spontaneum* accessions with the PAA haplotype and L94*Rphq2* resulted in approximately 20 F1 seeds per cross.

3.4 Comparing sequences

The *V.Perox-3* and *V.Kin-1* sequences of 18 barley cultivars and four *H. spontaneum* accessions were compared. In the *V.Perox-3* gene phylogeny tree, three clades with large similarities were observed (Figure 5). One strong clade with almost no substitution between cultivars consisted of Annabell, Bereta, Britta, Goldie, Krona, Linus, Mentor and Meltan. The cultivars in this clade have no common ancestor but are all developed without *H. laevigatum* as ancestor. One clade with more substitutions between cultivars consisted of Delita, Barke, Cecilia and Nigrinudum. As well as the first clade, these cultivars have no common ancestor but are all developed without *H. laevigatum* as ancestor. Nigrinudum was found to be the most distinct within the clade. All four *H. spontaneum* accessions are found in the same clade. Next to these accessions, cultivars Golden Promise, Gei, Princesse and Vada are ranked in the same clade. Princesse is derived from a cross between Universe and Ricardo, and Universe is derived from a cross between Abed 3371 and Vada. The pedigree of Gei is not known. Both Vada and Princesse have *H. laevigatum* as one of the ancestors. The cultivar Steptoe has no association in substitutions between any of the used cultivars or *H. spontaneum* accessions.

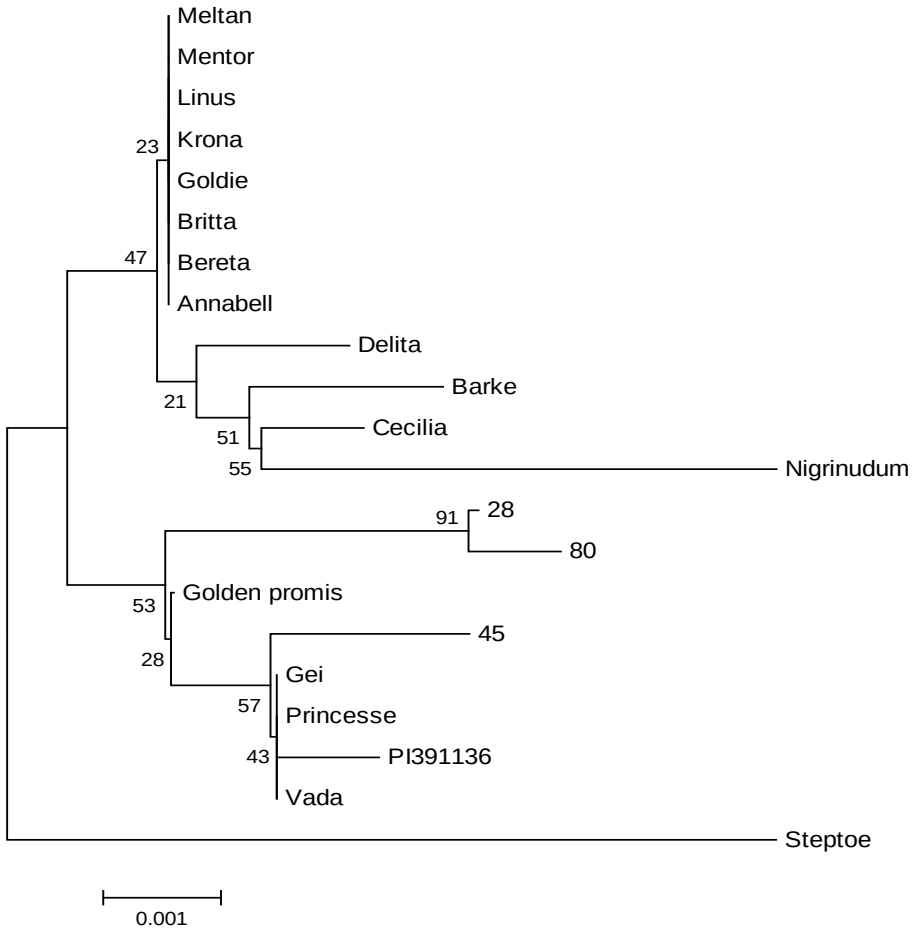

Figure 5. Phylogeny tree of 18 barley cultivars and 4 *H. spontaneum* accessions based on single nuclear polymorphisms (SNPs) in the *V.Perox-3* gene. Tree was constructed using the neighbour-joining method, with distances calculated based on number of substitutions. Strength tree was calculated by bootstrapping 1000 times indicated at each branch.

In the *V.Kin-1* gene more substitutions were found than in the *V.Perox-3* gene. In the *V.Perox-3* gene on average 0.101 substitutions per base pair were found while in the *V.Kin-1* gene on average 0.024 substitution per base pair were found. The phylogeny tree of the *V.Kin-1* gene showed no clear grouping and most clades consisted of two cultivars or accessions indicating less similarity within the phylogenetic tree (Figure 6). These cultivars have no direct common ancestor but are all formed without *H. laevigatum* as ancestor. No clear relationship or common ancestor could be found for the additional cultivars and *H. spontaneum* accessions.

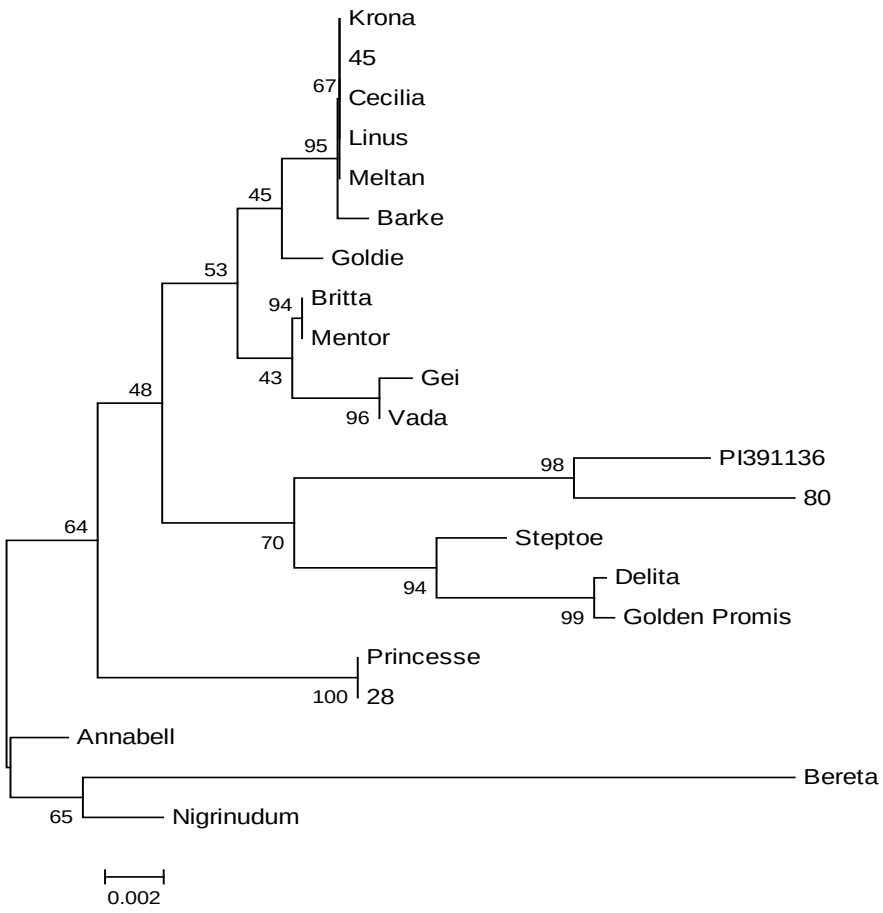

Figure 6. Phylogeny tree of 18 barley cultivars and 4 *H. spontaneum* accessions based on single nuclear polymorphisms (SNPs) in the *V.Kin-1* gene. Tree was constructed using the neighbour-joining method, with distances calculated based on number of substitutions. Strength tree was calculated by bootstrapping 1000 times indicated at each branch.

3.5 Expression of genes in the *Rphq2* region and pathogen development

The gene expression analysis was performed on lines L94, L94*Rphq2*, SxG114 and 182*Q20*. Due to time shortage, the gene expression experiment could not be completed. The total RNA was successfully isolated for all leaf samples taken. The isolated RNA of 3.5, 5.5, and 8 HAI of lines L94 and SxG114 was synthesised into cDNA. Execution of the gene expression experiment was not possible since the Biorad© quantative PCR (qPCR) machine was not available. In addition to this problem, the reference gene *UBC* appeared to have multiple melting points making it not suitable as a reference. New primers for additional reference genes were ordered and reference gene *PP2A* showed single amplification in constructed cDNA making it suitable as reference in qPCR (Supplemental Figure 2). The first round of developed primers for all genes in the *Rphq2* region resulted in three primer combinations with single amplification (Table 7). The remaining nine primer combinations for genes in the *Rphq2* region had multiple amplification products which made the primers not usable for qPCR (Table 8). New primers have been developed and ordered for these genes, but still need to be tested for specificity (Table 9).

Table 7. Primers tested for the qPCR of genes *Perox-1*, *HP-2!* and *UF-1*. The target genes were amplified using these primer pairs making them useable for qPCR at future use (F: forward, R: reverse).

Primer name	Sequence
Perox-1(Q1) F	CAGCCGTAGTACACTGGTTCTG
Perox-1(Q1) R	GCCTCTGCCCTTGAGGTACA
HP-2!(Q8)F	GAACTCTTTTCCGTGATGAG
HP-2!(Q8)R	GTTGTTGTTGGAGGAGGTC
UF-1(Q9)F	GTGTACCCGAGTGTTGTTCT
UF-1(Q9)R	CCGTTCACTTGACCTTATGT

Table 8. Primer combinations previously tested for genes in the *Rphq2* region. These primers showed multiple amplification bands and are therefore not usable in the qPCR procedure (F: forward, R: reverse).

Primer name	Sequence	Primer name	Sequence
Perox-2(Q3) F	GTTCGCCAGGTCCATGGAGA	***SINA(Q5)R***	TGAACCAGGAGAAGCTGA
Perox-2(Q3) R	GAAGCCCTCCTCCTCCACAC	***Leg-Lec-D(Q7)F***	GTCTGGCTCGGGAATGATAA
Perox-3(Q6) F	CACCTCCGACATGGCACTCG	***Leg-Lec-D(Q7)R***	TTGACGGAGAGGATTTTGCT
Perox-3(Q6) R	ACGGCAGTCCCGATGACGA	***UF-2(Q10)F***	TACGACTGGAGGGAGATGGT
HP-1(Q4) *F*	CTCCTAGCATGGCTCTACCG	***UF-2(Q10)R***	CTCTCAATGCACACGTCGAG
HP-1(Q4) *R*	CCGATAGGCAAACCTGTCAT	***UP(Q12)F***	TCGAAGAAGTCTCCAAAGAG
SINA(Q5)*F*	CACCTACCAAGACGAAGAAG	***UP(Q12)R***	CTGATTCTGGAGTACCGTTG

Table 9. Primers for qPCR of the genes in the *Rphq2* region. The primer combinations have to be tested in for their melting temperature and specificity before use in the qPCR (F: forward, R: reverse).

Primer name	Sequence	Primer name	Sequence
V.HP-1(G4)-F2	ACAGGTTTGCCTATCGGATG	***V.UF-2(G10)-F2***	GCTCGACGTGTGCATTGAG
V.HP-1(G4)-R2	CGGATGATGGAGGATCTTTG	***V.UF-2(G10)-R2***	CAAGCCCAACCAAACTTGC
V.SINA(G5)-F2	GAAGAAGGAGGTTATGGACTGC	***V.Kin-1(G11)-F2***	ACATCCATCTCCACAGCAAG
V.SINA(G5)-R2	CACACGCCTTCTTCTTACTCC	***V.Kin-1(G11)-R2***	TGATATCCCGGTGCAACAC
V.SINA(G5)-F3	CGTGAAGAAGGAGGTTATGGAC	***V.Kin-1(G11)-F3***	TCTACGATCTCATGCCGAAC
V.SINA(G5)-R3	TTACTCCCCCGGAGCTTG	***V.Kin-1(G11)-F4***	TAGAGCAGTGCAGACCCAAG
V.Leg-Lec(G7)-F2	GCACACAAAGAGCAAGCAAG	***V.Kin-2(G13)-F2***	CATTTCACCAGGGTCATTCC
V.Leg-Lec(G7)-R2	CGATGTGGTCGTAGGTATCG	***V.Kin-2(G13)-R2***	GTTCCAAAACCTCCCTTTCC
V.Leg-Lec(G7)-F3	GACGAGGGAGGAACTTTGC	***V.Kin-2(G13)-F3***	GCACATGCATCAAGAACTGG
V.Leg-Lec(G7)-R3	ATGACGCAGTTGCCGTAGTAG	***V.Kin-2(G13)-R3***	ATGTCGCTCTTCTCCGTCAG
V.HP-2(G8)-F2	TTCAAATCCTCTGCGAACG	***Actin (F)***	GGAATCCACGAGACGACCTACA
V.HP-2(G8)-R2	ACGACCCTTGTTGTTGTTGG	***Actin (R)***	CTTGCTCATACGGTCAGCGATA
V.HP-2(G8)-F3	ATTCAAATCCTCTGCGAACG	***PP2A (F)***	CTGTGGGAATATGGCATCAATC
V.HP-2(G8)-R3	GACGACCCTTGTTGTTGTTG	***PP2A (R)***	AATAGTCTGGCGTTCTACGAGTT

3.6 Development of *Puccinia hordei* sporelings

Observations of *P. hordei* isolate 1.2.1 on infected leaf samples of L94, L94*Rphq2*, SxG114 and 182*Q20* at different hours after inoculation (hai) revealed differences in development of the sporelings on the examined lines. In the first observation (3,5 hai) the spores formed germtubes (GT), appressoria (APP) and even some substomal vesicles (SSV)(Figure 7). Later observations (5,5, 7,5, 10 and 16 HAI) revealed sporelings further developing with increasing numbers of SSV and haustorium mother cells (HMC). At time points 3.5, 5.5 and 8 hai no clear differences were found in the development of *P. hordei* on the four lines. Large differences in development of *P. hordei* was observed at 10 and 16 HAI between the lines. In susceptible line L94 over 40% of the spores had developed into the SSV and almost 60% of the spores had developed into HMC with the majority producing haustoria at 10 HAI. In the partial resistant line L94*Rphq2* the development of *P. hordei* is slower than in L94. At 10 HAI, 60% of the spores had developed SSV and only 30% had developed HMC with the minority of them producing haustoria. The development of HMC on L94*Rphq2* is slower than on L94. The development of *P. hordei* on SxG114 is faster than on L94 at 10 hai. On SxG114 the majority of the spores (90%) had developed HMC and more than 60% of those had developed one or two haustoria. In contrast to the faster development of *P. hordei* on SxG114 compared to L94, the development of *P. hordei* on 182*Q20* seems hampered at the formation of haustoria. At 10 and 16 hai, over 90% of the spores had developed HMC but only 20% of those have succeeded in developing haustoria.

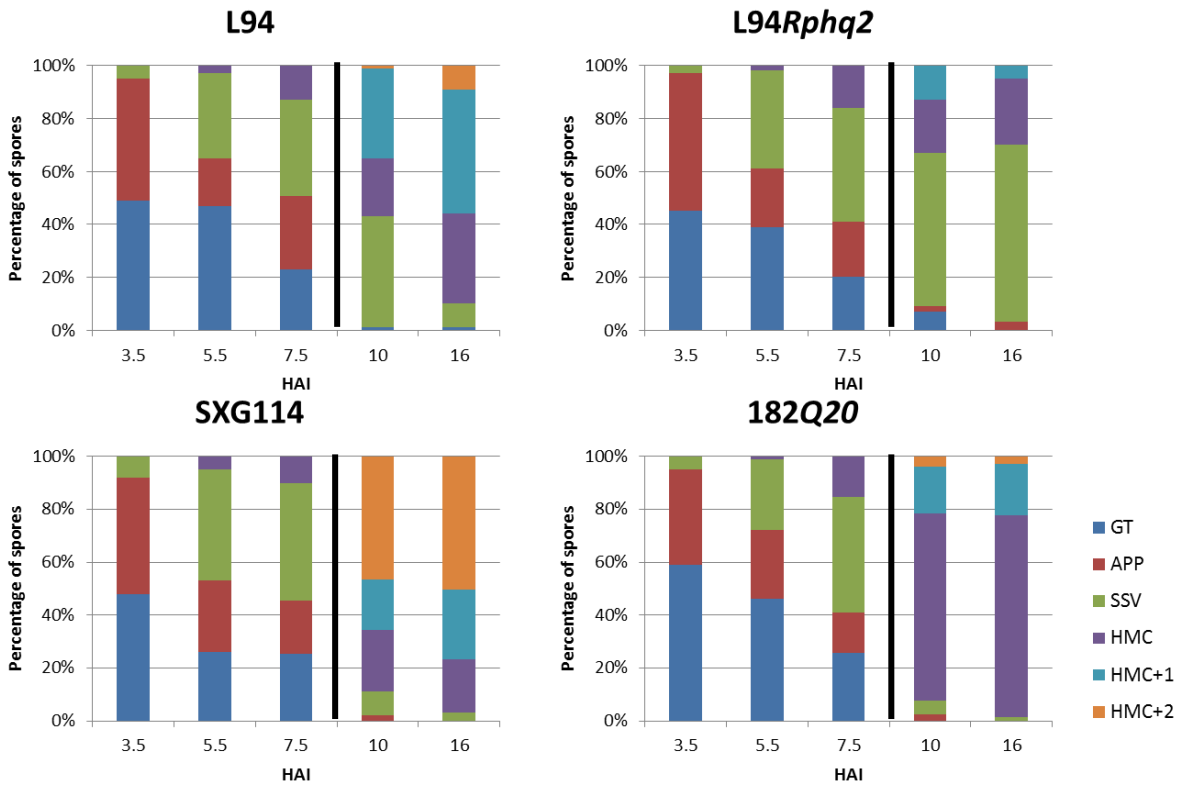

Figure 7. Development of *Puccinia hordei* on L94, L94*Rphq2*, SxG114 and 182*Q20* (HAI: hours after inoculation). Observations of 3.5, 5.5 and 7.5 HAI are from experiment one, and observations of 10 and 16 HAI from experiment 2 (spit indicated by black bar). (GT: germination tube, APP: appresoria, SSV: substomal vesicle, HMC: haustorium mother cell, HMC+1: haustorial mother cell with one haustorium, HMC+2: haustorial mother cell with two haustoria).

4 Discussion and conclusions

4.1 Polymorphism of *Rphq2* in wild barley accessions

Of the 333 *H. spontaneum* accessions, 27 were scored as having the AAA haplotype. The lack of amplification of the *V.Perox-3*, *S.UF* and *S.Kin-1* genes should be due to the absence of these genes in the examined accessions. An alternative explanation might be that the developed primers for the genes of interest were not specific in these accessions. The non-specificity of one or multiple primers could have been caused by SNP in the binding sequence of the primer. For this reason, each gene was tested with two primer pairs with different binding sequences to ensure the gene of interest was actually missing and lack of amplification was not due to non-specificity of the used primers. On the other hand, the amplification product could originate from a similar sequence region in the genome which allows binding of the primers. These unwanted amplification products are not likely to have the same size of the target sequence and can be identified on electrophoresis gel as a larger or shorter amplification product than the target sequence.

The examined cultivars by Wang (2014 unpublished) were found to have either the PAA or the APP haplotype which suggests an ancient linkage block in the *Rphq2* region (Yeo et al., 2014). The two major haplotype groups in *H. spontaneum* accessions in this research were PAA (68.5%) and APP (20.1%). In addition to these two haplotypes, haplotypes AAA, APA, PPA and PPP were found in the *H. spontaneum* material (Table 4). The finding of these additional haplotypes compared to the PAA and APP haplotypes found in the examined cultivars ascertain that more diversity is found in the *Rphq2* region in *H. spontaneum* than in modern cultivars. The largest diversity in *Rphq2* was found in the Near-East with haplotypes APA, PPA and PPP. The large diversity in *H. spontaneum* in the Near-East is also found in seven single copy loci by Jakob et al. (2014) and in three single copy loci by Morrell et al. (2003). These findings are both in line with the present opinion that the primary centre of origin of barley is in the Fertile Crescent, and secondary origin centres are present outside the Fertile Crescent (von Bothmer et al. 2003). The dominant presence of haplotypes PAA and APP in barley cultivars and the examined *H. spontaneum* accessions as well as the presence of haplotypes APA, PPA and PPP in *H. spontaneum* in the Near East suggests the split in the *Rphq2* region to have occurred not in *H. spontaneum* but in its ancestor. Scoring accessions from the secondary genepool (*H. bulbosum*) and tertiary genepool (other wild *Hordeum*) for their haplotype in the *Rphq2* region could provide valuable insight to the haplotype split in the *Rphq2* region. If *H. bulbosum* also has the linkage block in the *Rphq2* region as found *in H. spontaneum*, it is most likely that it originates from the common ancestor.

Another possibility is that the split in haplotypes in the *Rphq2* region has occurred in *H. spontaneum*. The haplotype of the examined *H. spontaneum* accessions was significantly related with the geographical origin of the accessions (Chi-squire P>0.000). The APP haplotype was found to be dominant (55%) in North African accessions while the PAA haplotype was found to be dominant (52-87%) in accessions in the Near East, Caucasus, Middle East and Central Asia (Table 5). Research of Morrell and Clegg (2007) proposed a possible second domestication of barley east of the Fertile crescent based on haplotype frequencies seven loci. The data provided in this research also suggests differential domestication centres for the accessions harbouring the PAA haplotype compared to accessions harbouring the APP haplotype, but the places at which this has happened remains unknown.

4.2 Partial resistance in wild barley

Determining the RLP of *H. spontaneum* accessions revealed a wide range of partial resistance. H. spontaneum accessions with higher susceptibility than L94 were found (i.e. RLP:94) as well as accessions with higher partial resistance than Vada (RLP: 123) were found (i.e. RLP: 136). North-African accessions were found to have a significantly higher RLP of *P. hordei* than accessions from the Caucasus, Near-East, Middle-East and Central-Asia (Table 6). The increased partial resistance in North African accessions could be due to a higher disease pressure of *P. hordei*. There is no evidence that the pathogen pressure for leaf rust was higher in North-Africa compared to Eastern regions, but Fetch et al. (2003) found that resistance to *P. hordei* was more often found in *H. spontaneum* from the mesic (humid) than in xeric (dry) areas. Since North African accessions mostly originate from coastal places with higher rainfall and humidity, the environmental conditions could play a role in the disease development. Consequently natural selection towards partially resistant *H. spontaneum* to *P. hordei* in this region could have taken place.

In addition to the significantly higher RLP of *P. hordei*, the majority of the North African accessions were scored as having the APP haplotype. Assuming that the APP haplotype lacks the major partial resistance gene in the *Rphq2* region, it is expected that the higher RLP to *P. hordei* in North African accessions is caused by one or several other partial resistance genes in the genome. To date, 20 QTLs are identified for partial resistance to leaf rust in barley cultivars with different effect sizes (Qi et al. 1998; Jafary et al. 2006; Marcel et al. 2008; van Berloo et al. 2001; Wang et al. 2010). Combinations of these 20 QTLs, or additional QTLs are likely to be present in the examined *H. spontaneum* accessions. The absence of significant relation between haplotype and latency period of *P. hordei* on *H. spontaneum* is not unexpected since these other genes present in the genome are likely to have a large contribution to the partial resistance.

4.3 Conservation of genes in the *Rphq2* region

The sequences of the *V.Perox-3* gene and *V.Kin-1* gene of 17 cultivars and four *H. spontaneum* accessions were compared. The *V. Perox-3* gene is only present in cultivars and accessions having the PAA haplotype while the *V.Kin-1* gene is present in accessions and cultivars with both the PAA and APP haplotype. Sequencing of the *V.Perox*-3 and *V.Kin*-1 gene for the cultivars and *H. spontaneum* accessions revealed on average 0.024 base substitution per site in the *V.Perox-3* gene compared to an average of 0.101 base substitutions per site in the *V.Kin-1* gene. This indicates that the *V.Perox-3* gene is more conserved than the *V.Kin-1* gene. The higher number of substitutions in the *V.Kin-1* gene is not unexpected since the gene is present in accessions with the PAA haplotype as well as in accessions with the APP haplotype. The *V.Kin-1* gene can pair between the PAA and APP haplotypes resulting in higher recombination and subsequently base substitutions in this gene. This is not the case in the *V.Perox-3* gene since it can only pair between PAA haplotypes.

In addition to the difference in base pair substitution, the observed variation in the cultivars and *H. spontaneum* accessions in the *V.Perox-3* gene is not associated with the variation observed in the *V.Kin-1* gene. There appears to be a relation between the constructed phylogeny tree of the *V.Perox-3* gene and the ancestor of the cultivars and *H. spontaneum* accessions. Cultivars with *H. laevigatum* as one of the ancestors are more related to the *H. spontaneum* accessions in the *V.Perox-3* gene (Figure 5). In contrast, this is not observed in the phylogenetic tree of the *V.Kin-1* gene. No relation was found between formation of clades and the ancestors of the cultivars and *H. spontaneum* accessions (Figure 6). This finding provides further evidence that the *V.Perox-3* gene is more conserved than the *V.Kin-1* gene..

4.4 Influence of *Rphq2* on the development of *P. hordei*

The development of *P. hordei* shows no difference on L94, L94*Rphq2*, SxG114 and 182*Q20* up to 7.5 hai (Figure 7). Until then, the spores develop germ tubes (GT), appressoria (APP) and, although in small frequencies, substomatal vesicles (SSV) and haustorial mother cells (HMC). The lack of difference in the development of the four lines up to 7.5 hai is expected since partial resistance is based on a defence mechanism causing failing of haustoria formation (Niks 1986). Before the haustoria are formed the development of *P. hordei* is expected to be equal on partial resistant lines and susceptible lines since partial resistant lines have a susceptible infection type (Niks 1982). The development of *P. hordei* 10 hai is reduced in L94*Rphq2* compared to L94: while 60% of the spores formed at least HMC in L94, only 30% of the spores did so in L94*Rphq2* (Figure 7). The difference in development is larger at 16 HAI: in L94 90% of the spores formed at least HMC compared to 30% in L94*Rphq2.* These results suggest that the hampered development of P. hordei on L94*Rphq2* is due to a prehaustorial acting mechanism but not by failure of haustoria formation as described by (Niks

1986). The development of P. hordei in L94*Rphq2* seems to be slowed down in the transition from SSV to HMC. This result is not expected since the partial resistance induced by *Rphq2* was assumed to be associated with the pathogen associated molecular patterns (PAMP)(Heath 2000). These are secreted by the pathogen as soon as the haustoria invades the host cell. The plant recognises these PAMPs and induces its defence. The finding in this research that the transition from SSV to HMC is hampered by *Rphq2* suggest that the pathogen is recognised at an earlier developmental stage. Thymol, a compound which can destroy the permeability of the pathogens membrane, is found to be more present in L94Rphq2 than in L94 (Qi et al. 2015). It could be that thymol hampers the transition from SSV to HMC.

Remarkably, the development of *P. hordei* on SxG114 is faster than in susceptible line L94 after 7.5 hai. At 10 HAI 60% of the spores on SxG114 have developed one or two haustoria compared to 35% in L94. At 16 HAI almost 80% of the spores on SxG114 have developed haustoria compared to 55% on L94. This is not expected since SxG114 has a slightly longer latency period (RLP: 103) than L94 (this thesis). The faster development of haustorial mother cells and subsequently haustoria could be caused by lower thymol concentrations in SxG114. After the formation of haustoria the PAMPs of *P. hordei* could be recognised by the plant inducing the defence mechanism. This defence mechanism could be stronger in SxG114 than in L94 resulting in a longer latency period.

At 10 and 16 HAI in 182*Q20* over 90% of the spores have formed HMC, and over 20% of these have formed one or two haustoria. In contrast to L94*Rphq2*, the development of *P. hordei* seems hampered in the transition from HMC to haustoria formation in 182*Q20*. Line 182*Q20* has a very high level of partial resistance that is not based on hypersensitivity (Johnston et al. 2013). The histology results of this research confirm that 182Q20 has a very high level of partial resistance, although the effect is different than in L94*Rphq2*. This finding is in line with the proposition of (Johnston et al. 2013a) that *Rphq2* present in L94*Rphq2* and *Rph22* present in 182*Q20* might encode allelic forms of the same gene with the presence of each allele resulting in a different level of response.

5 Future objectives

5.1 *Rphq2* haplotype scoring in *Hordeum*

Only the PAA haplotype and APP haplotype were found present in examined barley cultivars by Wang (2014, unpublished). In the examined *H. spontaneum* accessions, the PAA haplotype and APP haplotype was dominant. In addition to these two haplotypes, haplotypes AAA, APA, PPA and PPP was also found present in *H. spontaneum* accessions. Scoring accessions from the secondary genepool (*H. bulbosum*) and tertiary genepool (other wild *Hordeum*) for their haplotype in the *Rphq2* region could provide valuable insight to the haplotype split in the *Rphq2* region. *H. bulbosum* and *H. spontaneum* have the same common ancestor. If the PAA haplotype and APP haplotype are dominant in *H. bulbosum* it is probable that the split in haplotypes originates from their ancestor.

5.2 Towards fine-mapping the *Rphq2* region

Cultivars Senor and Un Ae 3.1, line SxG114 and *H. spontaneum* accessions 50, 103 and 128 were all found to have the PAA haplotype, but to be susceptible to *P. hordei* at seedling stage. The sequences of all genes in the *Rphq2* region of these cultivars, *H. spontaneum* accession and SxG line should be compared with the sequences of all genes in the *Rphq2* region in Vada. Vada has the PAA haplotype and has a gene in the *Rphq2* region which causes partial resistance to *P. hordei* at seedling stage. Comparing the sequences of the *Rphq2* region between the susceptible PAA haplotype and the partial resistant PAA haplotype could reveal differences in one or multiple genes in the *Rphq2* region. Genes which have no large differences in sequences are less likely candidate genes for the partially resistance while genes differing in sequence between the susceptible and partially resistant PAA haplotypes could be. It could also be possible that one or multiple genes in the partially resistance PAA haplotype are completely absent in the susceptible PAA haplotype. These genes are very likely to be candidate genes since they could transcribe for the partial resistance in the PAA haplotype. The comparison may therefore lead to demoting *Rphq2* candidate genes.

5.3 Gene expression experiment

Lines L94, L94*Rphq2*, SxG114 and 182*Q20* have been inoculated with *P.hordei* and mock inoculated at seedling stage. Leaf samples have been taken at 3.5, 5.5, 8, 10, 15, 39, 63, 87, 111 and 135 hai. The RNA has been isolated for all leaf samples and for lines L94*Rphq2* and SxG114 the cDNA was constructed of the isolated RNA. For the remaining samples of L94 and 182*Q20* the cDNA needs to be constructed. In order to evaluate the gene expression of the genes present in the *Rphq2* region, specific primers are required to amplify the 12 genes. For three genes, primer combinations have been developed and proven to be specific for qPCR. Primer combinations for the remaining nine genes have been ordered but have to be tested for specificity. It is recommended to use the household gene *PP2A* for the qPCR as a reference since it showed only one amplification band and is

therefore specific. Since the development of *P. hordei* in the histology experiment showed difference in the four used lines between 7.5, 10 and 16 hai, it is advisable to use the samples of these two time points to investigate the possible difference in expression of genes in the *Rphq2* region. Gene expression analysis in extra samples has lower priority and should be avoided since it is very laborious.

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8 Supplemental tables and figures

Table 1. Latency period, haplotype and origin of examined wild barley accessions. Hypersensitive accessions were not scored for latency period but infection type (LP: latency period. RLP: latency period relative to L94 with L94: 100. A: absent. P: present. ND: non determined.

Nr.	Avarage LP	RLP	Infection type	*V.Perox-3*	*S.UF*	*S.Kin-1*	Haplotype	Origin	Nr.	Avarage LP	RLP	Infection type	*V.Perox-3*	*S.UF*	*S.Kin-1*	Haplotype	Origin
1			7	A	A	A	AAA	SYR	**37**	252	125		P	A	A	PAA	PAL
2			4	A	P	P	APP	SYR	**38**			3	A	P	P	APP	PAL
4			4	P	A	A	PAA	SYR	**39**	215	125		P	A	A	PAA	JOR
5			5	P	A	A	PAA	JOR	**41**	203	104		P	A	A	PAA	PAL
6	173	105		A	A	A	AAA	JOR	**42**	208	107		P	A	A	PAA	PAL
7	172	104		P	A	A	PAA	JOR	**43**	219	112		P	A	A	PAA	PAL
8	228	117		P	A	A	PAA	JOR	**44**	204	118		A	P	P	APP	PAL
9	ND	ND		P	A	A	PAA	JOR	**45**	185	107		P	A	A	PAA	JOR
10	179	109		P	A	A	PAA	AFG	**46**	179	104		P	A	A	PAA	JOR
11	169	103		P	A	A	PAA	IRQ	**47**	202	101		A	P	A	APA	JOR
12	170	103		P	A	A	PAA	AFG	**48**	214	106		A	A	A	AAA	TUR
13	215	130		P	A	A	PAA	IRQ	**49**	ND	ND		P	A	A	PAA	TUR
14	184	112		P	A	A	PAA	AFG	**50**	170	94		P	A	A	PAA	SYR
15	171	104		P	A	A	PAA	AFG	**51**	215	114		P	A	A	PAA	SYR
16	188	114		A	A	A	AAA	IRN	**52**	194	113		P	P	A	PPA	JOR
17	185	112		A	P	P	APP	SYR	**53**	187	103		P	A	A	PAA	PAK
18	175	106		A	P	P	APP	AFG	**54**	190	95		P	A	A	PAA	SYR
19	217	126		A	P	P	APP	IRN	**55**	195	100		P	A	A	PAA	SYR
20	250	124		P	A	A	PAA	TUR	**56**	220	110		P	A	A	PAA	TUR
21			3	P	A	A	PAA	IRQ	**57**	210	108		P	A	A	PAA	SYR
22			3	P	A	A	PAA	TUR	**58**	215	107		P	A	A	PAA	CYP
23	213	106		P	A	A	PAA	IRN	**59**	203	104		P	A	A	PAA	CYP
24	214	110		P	A	A	PAA	IRN	**60**	200	100		A	P	P	APP	EGY
25	211	110		A	A	A	AAA	PAK	**61**	207	112		P	A	A	PAA	SYR
26	224	115		A	A	A	AAA	TJK	**62**			1	P	A	A	PAA	SYR
27	ND	ND		ND	ND	ND	ND	AZE	**63**	204	102		P	A	A	PAA	SYR
28	218	126		P	A	A	PAA	PAL	**64**	220	109		P	A	A	PAA	SYR
29	250	124		P	A	A	PAA	PAL	**65**	183	106		P	A	A	PAA	SYR
30	215	111		P	A	A	PAA	PAL	**66**	204	110		P	A	A	PAA	SYR
31	ND	ND		P	A	A	PAA	PAL	**67**	191	106		P	A	A	PAA	SYR
32	216	116		P	A	A	PAA	PAL	**68**	210	104		A	P	P	APP	SYR
33	185	98		A	P	P	APP	PAL	**69**	195	97		A	P	P	APP	SYR
34	179	105		P	A	A	PAA	PAL	**70**	181	105		A	P	P	APP	SYR
35	224	111		A	P	P	APP	PAL	**72**	202	108		P	A	A	PAA	LBY
36	179	104		P	A	A	PAA	AFG	**73**	199	107		P	A	A	PAA	LBY

Nr.	Avarage LP	RLP	Infection type	*V.Perox-3*	*S.UF*	*S.Kin-1*	Haplotype	Origin	Nr.	Avarage LP	RLP	Infection type	*V.Perox-3*	*S.UF*	*S.Kin-1*	Haplotype	Origin
74	187	104		A	A	A	AAA	LBY	**123**	212	113		P	A	A	PAA	IRN
75	217	126		P	A	A	PAA	LBY	**124**	ND	ND		P	A	A	PAA	IRN
78	177	113		A	P	P	APP	SYR	**125**	195	105		P	A	A	PAA	UZB
79	225	118		P	A	A	PAA	JOR	**126**	214	115		P	A	A	PAA	LBN
80			2	P	A	A	PAA	JOR	**127**	215	126		A	A	A	AAA	SYR
81	186	100		P	A	A	PAA	JOR	**128**	183	98		P	A	A	PAA	SYR
82	254	136		A	P	P	APP	JOR	**129**	179	104		P	A	A	PAA	SYR
83			6	P	A	A	PAA	JOR	**130**	195	100		P	A	A	PAA	SYR
85	216	115		A	A	A	AAA	JOR	**131**	187	104		P	A	A	PAA	SYR
89	205	111		A	P	P	APP	JOR	**132**	186	99		P	A	A	PAA	LBN
92	204	109		P	A	A	PAA	JOR	**133**	ND	ND		ND	ND	ND	ND	LBN
93	198	104		A	A	A	AAA	JOR	**134**	196	105		P	A	A	PAA	LBN
94	209	112		P	A	A	PAA	JOR	**135**	183	106		P	A	A	PAA	LBN
95	205	109		P	A	A	PAA	JOR	**136**	196	104		P	A	A	PAA	LBN
97			3	P	A	A	PAA	JOR	**137**	181	105		P	A	A	PAA	LBN
100	202	109		P	A	A	PAA	JOR	**138**	ND	ND		ND	ND	ND	ND	LBN
101	200	107		P	A	A	PAA	JOR	**139**	173	96		P	A	A	PAA	LBN
102	193	103		P	P	A	PPA	JOR	**140**	182	107		A	A	A	AAA	LBN
103	183	95		P	A	A	PAA	JOR	**141**	179	99		P	A	A	PAA	LBN
104	ND	ND		P	A	A	PAA	JOR	**142**	191	106		P	A	A	PAA	LBN
105	232	123		P	A	A	PAA	JOR	**143**	202	119		A	P	P	APP	LBN
106	198	106		P	A	A	PAA	SYR	**145**	196	114		ND	ND	ND	ND	LBN
107			4	P	A	A	PAA	SYR	**146**	215	116		P	A	A	PAA	IRN
108	199	107		A	A	A	AAA	SYR	**147**			3	P	A	A	PAA	IRN
109	174	102		P	A	A	PAA	SYR	**148**	207	112		P	A	A	PAA	IRN
110	196	105		A	A	A	AAA	SYR	**149**	222	120		P	A	A	PAA	IRN
111	224	121		P	A	A	PAA	SYR	**150**	215	115		P	A	A	PAA	IRN
112	197	105		P	A	A	PAA	SYR	**151**	225	122		A	P	P	APP	SYR
113	190	103		P	A	A	PAA	TKM	**152**	204	118		P	A	A	PAA	IRN
115	215	125		P	A	A	PAA	TKM	**153**	195	105		P	A	A	PAA	IRN
116	210	112		P	A	A	PAA	TKM	**154**	185	108		P	A	A	PAA	IRQ
117	206	111		P	A	A	PAA	TKM	**155**	210	112		P	A	A	PAA	IRQ
119	196	114		P	A	A	PAA	UZB	**156**	186	109		P	A	A	PAA	IRQ
120	204	118		P	A	A	PAA	TJK	**157**	179	104		P	A	A	PAA	IRQ
121	215	115		P	A	A	PAA	IRN	**158**	193	104		P	A	A	PAA	IRQ
122	185	108		P	A	A	PAA	IRN	**159**	188	100		P	A	A	PAA	SYR

Nr.	Avarage LP	RLP	Infection type	*V.Perox-3*	*S.UF*	*S.Kin-1*	Haplotype	Origin	Nr.	Avarage LP	RLP	Infection type	*V.Perox-3*	*S.UF*	*S.Kin-1*	Haplotype	Origin
160	199	106		P	A	A	PAA	SYR	199	188	110		A	P	P	APP	SYR
161	183	106		A	P	P	APP	SYR	200	ND	ND		P	A	A	PAA	SYR
164	181	105		P	A	A	PAA	SYR	201	184	107		P	A	A	PAA	SYR
165	204	110		A	A	A	AAA	SYR	202			2	P	A	A	PAA	SYR
166	182	107		P	A	A	PAA	SYR	203	205	109		P	A	A	PAA	SYR
167	186	109		P	A	A	PAA	SYR	204	195	114		P	A	A	PAA	TKM
168	185	104		P	A	A	PAA	LBN	205	178	104		P	A	A	PAA	RUS
169	187	105		P	A	A	PAA	LBN	206	ND	ND		P	A	A	PAA	SYR
170	ND	ND		A	P	P	APP	LBN	207	199	105		P	A	A	PAA	UZB
171	ND	ND		P	A	A	PAA	LBN	208	189	110		P	A	A	PAA	UZB
172	205	111		P	A	A	PAA	IRN	209	181	105		P	A	A	PAA	UZB
173	210	112		A	P	P	APP	IRN	210	188	107		ND	ND	ND	ND	UZB
174	172	97		A	A	A	AAA	IRN	211	173	99		A	P	P	APP	UZB
175	205	109		P	A	A	PAA	IRN	212	ND	ND		P	A	A	PAA	UZB
177	186	104		P	A	A	PAA	IRQ	213	188	107		P	A	A	PAA	UZB
178	191	102		A	P	P	APP	IRQ	214	185	105		P	A	A	PAA	UZB
179	198	103		A	P	P	APP	LBY	215	194	111		P	A	A	PAA	TKM
180	197	105		P	A	A	PAA	LBY	216	210	112		P	A	A	PAA	TKM
181	216	115		P	A	A	PAA	JOR	217	198	105		A	P	P	APP	ARM
182	199	106		P	A	A	PAA	JOR	218	ND	ND		ND	ND	ND	ND	KAZ
183	212	119		P	A	A	PAA	JOR	219	185	105		P	A	A	PAA	KAZ
184	210	112		P	A	A	PAA	LBY	220	ND	ND		A	A	A	AAA	KAZ
185	ND	ND		A	A	A	AAA	LBY	221	190	108		P	A	A	PAA	TJK
186	183	103		A	A	A	AAA	TUR	222	185	106		P	A	A	PAA	TJK
187	208	109		P	P	P	PPP	TUR	223	189	108		P	A	A	PAA	TJK
188	ND	ND		A	P	P	APP	TUR	224	192	110		P	A	A	PAA	TJK
189	177	100		ND	ND	ND	ND	TUR	**225**	199	106		P	A	A	PAA	TJK
190	197	103		A	P	P	APP	TUR	**227**	182	104		A	P	P	APP	AZE
191	199	104		A	P	P	APP	TUR	**228**	204	110		A	P	P	APP	AZE
192	215	114		P	A	A	PAA	TUR	**229**	197	112		A	P	P	APP	AZE
193	194	113		P	A	A	PAA	TUR	**230**	205	111		A	P	P	APP	AZE
194	196	105		P	A	A	PAA	TUR	**231**	197	104		A	P	P	APP	AZE
195	184	107		P	A	A	PAA	TUR	**232**	189	108		A	P	P	APP	AZE
196				P	A	A	PAA	TUR	**233**	208	111		P	A	A	PAA	AFG
197	219	117		P	A	A	PAA	SYR	**234**	201	107		A	P	P	APP	CYP
198	198	105		P	P	P	PPP	SYR	**235**	206	110		P	P	P	PPP	JOR

Nr.	Avarage LP	RLP	Infection type	*V.Perox-3*	*S.UF*	*S.Kin-1*	Haplotype	Origin	Nr.	Avarage LP	RLP	Infection type	*V.Perox-3*	*S.UF*	*S.Kin-1*	Haplotype	Origin
236	191	102		A	P	P	APP	JOR	**278**			3	ND	ND	ND	ND	PAL
237	187	100		P	A	A	PAA	JOR	**279**			4	P	A	A	PAA	PAL
238	208	111		P	A	A	PAA	JOR	**280**	ND	ND		P	A	A	PAA	PAL
240	198	103		P	A	A	PAA	JOR	**281**	232	119		P	A	A	PAA	PAL
241	180	103		P	A	A	PAA	JOR	**282**	212	113		P	A	A	PAA	PAL
242	208	109		P	A	A	PAA	JOR	**283**	ND	ND		P	P	A	PPA	PAL
243	215	112		A	P	P	APP	JOR	**284**	222	118		P	P	A	PPA	PAL
244	218	116		P	A	A	PAA	JOR	**285**	173	100		ND	ND	ND	ND	PAL
245	197	103		P	A	A	PAA	JOR	**286**	209	111		P	A	A	PAA	PAL
246	204	117		P	A	A	PAA	JOR	**287**	199	106		P	A	A	PAA	PAL
247	203	108		P	A	A	PAA	JOR	**288**	200	106		A	A	A	AAA	PAL
248	199	104		ND	ND	ND	ND	JOR	**289**			1	P	A	A	PAA	PAL
250			0	P	A	A	PAA	JOR	**290**	175	102		P	A	A	PAA	PAL
252	249	132		P	A	A	PAA	JOR	**291**	229	131		A	P	P	APP	PAL
253	219	117		P	A	A	PAA	JOR	**292**			4	A	P	P	APP	PAL
254	223	118		P	A	A	PAA	JOR	**293**			3	P	A	A	PAA	PAL
255	210	112		A	P	P	APP	JOR	**294**	215	123		A	P	P	APP	PAL
256	218	114		P	A	A	PAA	JOR	**295**	ND	ND		A	P	P	APP	SYR
257	208	110		P	A	A	PAA	JOR	**296**	192	111		P	A	A	PAA	SYR
258	177	101		P	A	A	PAA	JOR	**297**	175	100		P	A	A	PAA	SYR
259	222	118		P	A	A	PAA	JOR	**298**	193	101		P	A	A	PAA	SYR
260	199	106		P	A	A	PAA	JOR	**299**			1	A	P	P	APP	SYR
261	206	110		P	A	A	PAA	JOR	**300**	200	115		A	A	A	AAA	SYR
262	215	115		P	A	A	PAA	JOR	**302**	207	110		A	A	A	AAA	SYR
263	179	102		A	A	A	AAA	JOR	**303**	186	106		P	A	A	PAA	SYR
265	205	105		P	A	A	PAA	JOR	**304**	177	104		A	P	P	APP	SYR
266	208	107		P	A	A	PAA	JOR	**305**	175	103		P	P	P	PPP	SYR
267	188	107		P	A	A	PAA	JOR	**306**	181	106		P	A	A	PAA	SYR
268	182	105		P	A	A	PAA	JOR	**307**	ND	ND		P	A	A	PAA	SYR
269	ND	ND		P	A	A	PAA	LBN	**308**	192	113		A	A	A	AAA	SYR
270	225	129		P	A	A	PAA	PAL	**309**	194	113		P	A	A	PAA	SYR
271	ND	ND		P	A	A	PAA	PAL	**310**	209	109		P	A	A	PAA	SYR
274			3	P	P	A	PPA	PAL	**311**	191	112		A	P	P	APP	SYR
275	242	129		P	A	A	PAA	PAL	**312**	208	110		P	A	A	PAA	SYR
276			4	P	A	A	PAA	PAL	**314**			5	A	P	P	APP	SYR
277			4	A	A	A	AAA	PAL	**315**	217	116		A	A	A	AAA	SYR

Nr.	Avarage LP	RLP	Infection type	*V.Perox-3*	*S.UF*	*S.Kin-1*	Haplotype	Origin	Nr.	Avarage LP	RLP	Infection type	*V.Perox-3*	*S.UF*	*S.Kin-1*	Haplotype	Origin
316			2	A	P	P	APP	SYR	**361**	239	123		P	A	A	PAA	DZA
317	216	123		P	A	A	PAA	SYR	**362**	220	113		A	P	P	APP	TUN
318	ND	ND		P	A	A	PAA	SYR	**363**			0	A	P	P	APP	CZE
319	184	109		P	A	A	PAA	SYR	**364**			1	P	A	A	PAA	LBY
320	188	100		P	A	A	PAA	SYR	**365**	237	123		A	P	P	APP	LBY
323	212	113		P	A	A	PAA	TKM	**366**	228	118		A	P	P	APP	MAR
324	188	100		P	A	A	PAA	TKM	**367**	239	123		P	P	P	PPP	MAR
326	203	107		P	A	A	PAA	TKM	**368**	241	124		A	P	P	APP	MAR
329	208	111		P	A	A	PAA	TKM	**369**	197	102		A	P	P	APP	MAR
330	188	110		P	A	A	PAA	TKM	**370**	213	109		A	P	P	APP	MAR
331	193	113		P	A	A	PAA	TKM	**371**	239	122		A	P	P	APP	MAR
332	190	111		P	A	A	PAA	TKM	**372**	215	110		A	P	P	APP	MAR
333	205	110		P	A	A	PAA	TKM	**373**	191	97		A	P	P	APP	MAR
334	218	117		P	A	A	PAA	TKM	**374**	240	113		A	P	P	APP	MAR
335	179	105		P	A	A	PAA	TKM	**375**	244	125		A	P	P	APP	MAR
336	175	103		ND	ND	ND	ND	TKM	**376**	202	103		P	A	A	PAA	MAR
337	218	114		P	A	A	PAA	TUR	**377**	245	125		P	A	A	PAA	LBY
338	195	115		ND	ND	ND	ND	TUR	**378**	226	116		P	A	A	PAA	MAR
340	221	118		P	A	A	PAA	TUR	**379**	224	125		P	A	A	PAA	LBY
341	195	100		P	A	A	PAA	TUR	**380**	241	123		A	P	P	APP	LBY
342	213	109		P	A	A	PAA	TUR	**387**	213	110		A	P	P	APP	LBY
343	241	124		P	A	A	PAA	TUR									
344	180	103		A	P	P	APP	TUR									
345	246	126		P	A	A	PAA	UZB									
346	227	117		A	A	A	AAA	UZB									
347	215	110		A	A	A	AAA	UZB									
348	217	124		P	A	A	PAA	ND									
349	245	126		P	A	A	PAA	ND									
350	198	105		A	P	P	APP	ND									
354			6	P	A	A	PAA	ND									
355	201	107		A	P	P	APP	ND									
356	207	107		P	A	A	PAA	DZA									
357	ND	ND		P	A	A	PAA	NF									
358	235	121		A	P	P	APP	MAR									
359	241	124		A	P	P	APP	MAR									
360	231	119		A	P	P	APP	MAR									

Supplemental Table 2. The sequences of the primers used to amplify the V.Perox-3, S.UF and S.Kin-1 genes and actin, the household gene. The primers with the same number after the gene name are one pair. Two primer pairs were used for each gene.

Primer	Sequence (5' – 3')
V.Perox-3 (1) F	CGTATGGGTTTGTAGGTGTAGCA
V.Perox-3 (1) R	CAGTTCGCCAAGTCGATGACCA
V.Perox-3 (2) F	CCTCAAACTCACACACGAACAAC
V.Perox-3 (2) R	CTGTGCCTCTGCCTTAATGTATTTC
S.UF (1) F	CCGAGATCCTTGTTGCACTATTAC
S.UF (1) R	GGTATACCTGTCACTAACAAACACT
S.UF (2) F	GTGCTTAATTTGACTGACCTCCTTC
S.UF (2) R	TCTCCATCACCTCTGTCGACAAC
S.Kin-1 (1) F	CCGGTACAGTCCATGTTTTTCTC
S.Kin-1 (1) R	CTCAGTGCTTCAGATGTTGCTTAG
S.Kin-1 (2) F	GCTGCTCAGAGAGTGACGTGAT
S.Kin-1 (2) R	TTGCGAATCATGTAACAAGCCTTAC
Actin F	GGAATCCACGAGACGACCTACA
Actin R	CTTGCTCATACGGTCAGCGATA

Supplemental Table 3. Barley cultivars scored as having the PAA haplotype by Wang (2014, unpublished) (P: present. A: absent).

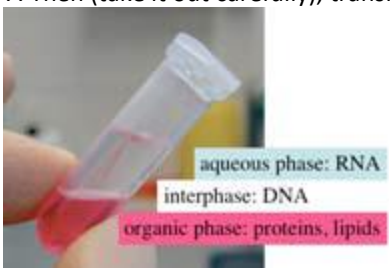
	Gene				Gene		
Cultivar	V.Perox-3	S.UF	S.Kin-1	**Cultivar**	V.Perox-3	S.UF	S.Kin-1
Abed4611	P	A	A	**Maud**	P	A	A
Abed50015	P	A	A	**Mazurka**	P	A	A
Abed5193	P	A	A	**Merete**	P	A	A
Alanis	P	A	A	**Midas**	P	A	A
Albright	P	A	A	**Mie**	P	A	A
Aravis	P	A	A	**Miralix**	P	A	A
Ariel	P	A	A	**Miranda**	P	A	A
Aspen	P	A	A	**Nevada**	P	A	A
Astoria	P	A	A	**Nigrinudum**	P	A	A
Atem	P	A	A	**NSL 94-4109**	P	A	A
Bond	P	A	A	**Optima**	P	A	A
Brenda	P	A	A	**Otira**	P	A	A
Brewster	P	A	A	**P1391136**	P	A	A
Britta	P	A	A	**Paloma**	P	A	A
Cadeau	P	A	A	**Pauline**	P	A	A
Canut	P	A	A	**Peel**	P	A	A

Caskant	P	A	A	**PF11011-52**	P	A	A
Cathrine	P	A	A	**PF11202-53**	P	A	A
Cecilia	P	A	A	**Pongo**	P	A	A
Century	P	A	A	**Potter**	P	A	A
Chamant	P	A	A	**Prestige**	P	A	A
Chariot	P	A	A	**Princesse**	P	A	A
Christian	P	A	A	**Prisma**	P	A	A
Cicero	P	A	A	**Prolog**	P	A	A
Decanter	P	A	A	**Punto**	P	A	A
Delibes	P	A	A	**Ricarda**	P	A	A
Delita	P	A	A	**Riga**	P	A	A
Etna	P	A	A	**Roxana**	P	A	A
Evelyn	P	A	A	**Scarlett**	P	A	A
Extract	P	A	A	**Senor**	P	A	A
Ferment	P	A	A	**Setpoe**	P	A	A
Fusion	P	A	A	**SJ5085**	P	A	A
Gant	P	A	A	**SJ5095**	P	A	A
Gei	P	A	A	**Steptoe**	P	A	A
Goldie	P	A	A	**Sultane**	P	A	A
Jill	P	A	A	**Texane**	P	A	A
Korinna	P	A	A	**Thuringia**	P	A	A
Krona	P	A	A	**Tirup**	P	A	A
Lenka	P	A	A	**Tofta**	P	A	A
Linus	P	A	A	**Trebon**	P	A	A
Lofa Abed	P	A	A	**Trianon**	P	A	A
Loma	P	A	A	**UN AE 3.1**	P	A	A
Lux	P	A	A	**Verona**	P	A	A
Lysimax	P	A	A	**Viskosa**	P	A	A
Mandolin	p	A	A	**Wren**	P	A	A

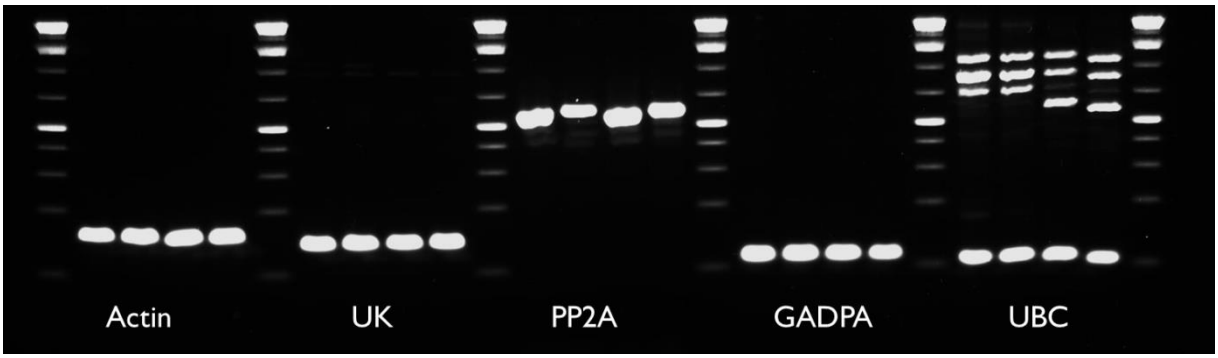
Supplemental Figure 1. RNA extraction protocol for barley using TRIzol reagent.

Materials:
- 1.5 ml and 2 ml Eppendorf tubes
- 75% ethanol (prepared with sterile MQ)
- MQ (autoclaved)
- Sterile pipette tips
- TBE 1X (autoclaved/fresh)
- Electrophoreses camber & gel tray & combs
(Clean by 0.5% SDS then MQ then 75% ethanol and finally dry with Kimberly tissues)
- Liquid nitrogen
- Isopropanol (2-propanol)
- Chloroform
- TRIzol®
- Eppendorf 1.5 ml tubes (sterile, take new bag)

Method
1. Let the centrifuge pre-run to get to 4°C (takes 30 minutes).
2. Put the approx. 100 mg of powder in a 2ml SCREW tubes.
3. Add 1 ml of TRIzol® Reagent (put the bottle on ice!) and mix thoroughly for 30 seconds
on a vortex, incubate for 5 min at room temperature.
4. Add 0.2 ml chloroform (per 1ml TRIzol® Reagent used), then cap the tube securely.
5. Shake tube vigorously by hand for 15 seconds (do not vortex!!), then incubate for 5 min at RT.
6. Centrifuge at 12000 *x g*/13000 rpm for 15 minutes at 4°C.
7. Then (take it out carefully), transfer 0.5 ml of the aqueous phase (upper, colourless) to a new 1.5 ml tube.



8. (if needed, add an 0.2 ml volume of chloroform to remove the excess of phenol, vortex 30 seconds and centrifuge 2 minutes 12000 *x g*, transfer the aqueous (0.4 ml) to a fresh tube)
9. Add 0.4 ml of isopropanol (cooled better) and mix by inversion twice (6-8 times) and incubate for 10 minutes at RT.
10. Centrifuge at 12000 *x g* for 10 minutes at 4°C.
11. Discard the supernatant.
12. Wash the pellet with 1 ml 75% ethanol.
13. Centrifuge at 7500 *x g* for 5 minutes at 4°C.
14. Wash one more time with 75% EtOH (to remove the salt!)
15. Discard the supernatant and air dry the pellet for 10 minutes by placing the tube upside-down on a Kim wipe. Do
not let the pellet dry over 15 minutes. (Meanwhile, clean the electrophoreses camber & gel tray & combs)
16. Add 50 µl of RNase free MQ (autoclaved) and flick the tube to dissolve the pellet (**pipetting up and down, heat
to 55-60°C for 10 min if not dissolve).
17. Determine the concentration and purity on the Nanodrop.
18. Run (2 µl RNA + 1 µl LD) on a RNase free 1% gel at 80 V for 1 hour (bands pattern, no smear).
19. Continue with cDNA synthesis or store at -80°C.



Supplemental Figure 2. Amplification products of household genes for the qPCR procedure. The household gene *UBC* is not usable since multiple amplification products are present. The household gene *PP2A* is the best option since it has single amplification products. The sizes of the amplification products of *Actin*, *UK* and *GADPA* are too small for qPCR.