Brewers' Research and Education Fund - Final report

Project from July 2021 to July 2024

Project title: Molecular marker development for 21st Century hop breeding – Fast tracking resistance breeding

Submitted by: Dr Klara Hajdu on behalf of Wye Hops Limited

Wye Hops Limited, China Farm Office, Upper Harbledown, Canterbury, Kent CT2 9AR.

Tel: 07474 294941 Email: klara.hajdu@wyehops.co.uk

Project Summary

The aim of this project was to improve the current UK hop breeding practices and speed up the development of agronomically and commercially stable British hop varieties through the development of modern breeding strategies. The UK hop acreage is in steady decline and English aroma and bittering varieties currently farmed in the UK are not able to meet the demand dictated by recent brewing trends. Together with foreign hops infiltrating the UK's beer industry environmental challenges such as recent extreme heat and drought periods and pathogen epidemies are also putting a strain on less resilient UK hop varieties creating increased commercial competition for domestic hop producers against imported hops.

The British Hop Association (BHA) has identified a clear need for new, locally adapted, UK-bred hops with strong agronomic characteristics and desirable aromas and an aim to transition from classical breeding practices to marker-assisted breeding (MAB) in the Wye Hops breeding programme. Ultimately, by improving breeding practices, we can enhance the competitiveness of British-bred hops against imported varieties currently favoured by national breweries. Consequently, we are safeguarding British hop production, and the future of the UK hop industry.

This current BREF project is a continuation of a previous BREF-funded Wye Hops project titled "British Hops: Genetic Marker Population - Maintenance of Plant Material." As part of the preceding project, four large bi-parental mapping populations were established at the Wye Hops breeding plot at China Farm. The goal was to provide the necessary germplasm (living plant) resources for future investigations into molecular marker-assisted hop breeding. The mapping populations were designed to segregate for important breeding qualities, making them suitable for trait mapping purposes. Key breeding traits include resistance to aphids, downy mildew (DM), powdery mildew (PM), and Verticillium wilt (VW), as well as dwarfism, agronomy, and cone characteristics (Figure 1).

One of the mapping populations, '50/2015,' derived from the female genotype 'Pilgrim' (which carries resistance to VW, PM, and DM) and the disease-susceptible male breeding line '316/1/10,' was used for the work conducted in this BREF project. Another mapping population, '40/2015,' derived from the PM and VW resistant male '15/2001/1' and the PM and VW susceptible female genotype 'Fuggle,' was also phenotypically assessed in this project, further enriching our database for future genetic mapping studies. While invaluable genetic resources have been made available through the preceding BREF projects, the laboratory infrastructure and scientific support necessary for the successful delivery of the project objectives have been provided through the supporting BHA-funded PhD project and in collaboration with the University of Kent and Growing Kent and Medway.



Figure 1. Four large (300+ individuals) genetic mapping populations established for future marker assisted breeding works at the China Farm breeding plot in 2016 via preceding BREF project titled 'British Hops: Genetic Marker Population -Maintenance of Plant Material'. These semi-permanent families segregate for important breeding traits due to their parents carrying contrasting characteristics and thus are suitable for genetic marker discovery purposes.

Introduction

Selection based hop cultivation, and the application of genetic principles started in 1906 when Professor Ernest Salmon of Wye College, Kent created and planted the first biparental cross and subsequently initiated the national hop breeding programme, which was soon followed by the emergence of breeding programmes worldwide (Darby, 2005a). Salmon's breeding programme laid the foundations for 20th century hop breeding and has been the cornerstone of global hop breeding to date. In the 20th Century when hop breeding for specific priorities began, English hop varieties that were renowned for their high-quality brewing properties, and "good English aroma" varieties were used for crossing in international breeding programmes (Darby, 2005a; Dredge, 2021). Consequently, most modern hop varieties worldwide are direct descendants of English hop varieties such as 'Brewers' Gold,' 'Fuggle,' and 'Goldings.'

There are currently 34 established British hop varieties available on the market with landrace varieties Goldings and Fuggle being farmed on the largest scale followed by bittering hops Target and Challenger. These varieties are farmed by just over 40 hop growers in the country and majority of the resulting hops are distributed across UK breweries (Capper, 2014). The total area of UK hop cultivation was just under 1,000 hectares as of the beginning of 2022 which has seen further decline to just under 600 hectares as of today, largely explained by and the aftermath of the COVID-19 pandemic (Chris Daws, EHL, personal communication 2024). According to statistics published in 2024, hops produced in the UK represent less than 1% of global hop production while USA hops dominate the market with 40% of global market share followed by Germany (36%), Czech Republic (7%), China (5%), the rest of Europe (6%) and the rest of the world (4%) (Statista Search Department, 2024). Despite its small contribution to worldwide hop production, the UK plays an important role in the global hop breeding scene. The importance of UK hops is greatly attributed to the historical and scientific significance of the Wye breeding programme and the extensive germplasm collection that was curated over the past century which now form the national hop collection.

In recent decades, the development of new, high impact aroma varieties has been the main objective of the British Hop Association due to pressure applied by the craft beer industry favouring 'new world' aroma hops over traditional English hops. While the development of high impact aroma hops currently drives the UK and global brewing industries and consequently all hop breeding programmes, other breeding characteristics such as yield, habit, alpha acid content and resistance to pests and diseases are also majorly important in the successful development of a new commercial variety. Resistance breeding is broadly recognized as one of the main hop breeding objectives due to the high economic impact of disease on plant development and yield (Henning et al., 2017).

Pest and disease resistance genes exist in UK germplasm however resistance breeding is a lengthy process which is currently achieved through the characterisation of resistance in individual hop plants through labour and time-consuming infection assays. In current hop breeding, artificial infection assays must be used to screen for resistance to three major hop diseases: powdery mildew caused by the pathogen *Podosphera macularis*, downy mildew caused by *Pseudoperonospora humulii* and Verticillium wilt caused by *Verticillium nonalfalfae*. The usefulness of modern breeding techniques employing molecular markers have been recognised in hop breeding programmes especially in disease resistance breeding since it can decouple the need of laboursome disease screening assays from finding out about a plant's resistance to certain diseases (Cobb et al, 2019).

Genetic or molecular markers are single "letter" SNP (Single Nucleotide Polymorphism) variations in the code of the DNA that is made up of A, C, T and G nucleotides in each individual organism. SNPs usually have two forms or "alleles" for example A or C, T or G, A or G. These markers can be used as

flags near genes of interest. Depending on the allelic state of a SNP marker they can indicate a specific phenotype such as disease resistant or susceptible in an individual (Jiang, 2013) (Figure 2a.). Importantly, genetic markers are inheritable and thus, can be bred for. Genetic markers can be obtained via genotyping, which is the partial sequencing of the DNA. While genotyping is concerned about capturing ONLY the differences between individuals' DNA - the SNPs that are different between different individuals - another approach, whole genome sequencing captures the code of the entire genome. High quality whole genome sequences are referred to as 'Reference genomes' and are a useful tool for plant breeders and geneticists as they provide a frame or map to place SNP markers against and help breeders locate markers on specific chromosomes near interesting genes (Figure 2b.).

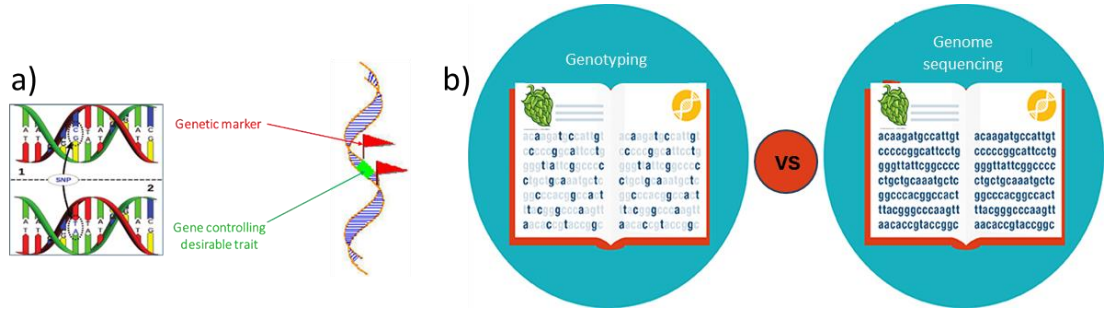


Figure 2. a) Single nucleotide variations (SNPs) in the DNA of individuals next to useful genes b) Genotyping (skimmed sequencing of DNA), used for finding markers vs Genome sequencing (total length of DNA sequenced) used as a map or reference

Both techniques are important for understanding the genomic composition of an organism and the identification of DNA regions that control important breeding traits (Anisimova, 2021). The use of molecular technologies has the potential to improve the efficiency of crop breeding since it allows for pre-selection of useful individuals earlier in the breeding process. With DNA sequencing becoming increasingly cheaper and more available for breeding programmes, the use of marker assisted breeding strategies in UK hop breeding is now an achievable goal.

Currently the bottleneck of hop breeding is the extensive phenotyping of disease resistance traits. Tolerance to Verticillium wilt is a prerequisite in new varieties, and breeding lines with susceptibility to powdery mildew disease are typically not progressed further in the breeding process. With no molecular marker tools currently available to UK hops, breeders are left with little to no choice but to continue with the cost and labour-intensive artificial infection assays to obtain information on the disease resistance status of breeding lines.

Since the early 1900s to date British hop varieties were bred solely through classical breeding approaches taking 10-15 years for a new variety to be commercially released. With the rapidly declining UK hop acreage and constant pressure from the brewing industry and environment this time window is too long. Due to recent years' technological advances new hop varieties can now be developed more efficiently and much more quickly than in the past.

Marker assisted breeding (MAB) offers a competitive advantage for hop variety development. To safeguard the British hop industry modern approaches must be incorporated in our breeding practices. This project has helped laying the foundations for the successful implementation of molecular breeding techniques in a 21st Century UK hop breeding programme.

Objectives

The overall aim of this project was to build on the germplasm resources generated in the preceding 2017 BREF project and make further steps towards the implementation of MAB in UK hop breeding with an initial focus on fast tracking disease resistance breeding.

The successful implementation of MAB has two prerequisites: **1) The availability of reliable phenotypic (trait expression) data**, for example for yield, resin quantities, disease resistance/susceptibility. **2) High quality genotypic data (DNA sequence) data** such as single nucleotide polymorphisms (SNPs).

Once phenotypic and genotypic data are available, the DNA regions associated with the expressed trait phenotypes can be deduced using statistical tools. Both phenotype and genotype data must be obtained for a large number of individuals to ensure statistical robustness of the investigations.

The objectives set out to be achieved in the 36 months duration of this project were divided into four work packages and aimed to generate the above described resources. The body of work undertaken in this project was based on the 50/2015 marker population which was selected for its female parent 'Pilgrim' which is a highly significant Wye Hops breeding line with multiple resistances (VW,PM,DM) and interesting breeding traits making it an ideal candidate for model studies. The 50/2015 family segregates for VW, PM and DM resistances due to the male parental line '316/1/10' being susceptible for all three diseases making the population suitable for resistance genetic mapping.

The main deliverables of the four work packages were as follows:

WP1: Phenotyping

In WP1 we generated high quality phenotypic data on two hop PM resistances. This work package included the propagation and PM screening of the PM R2 resistance segregating '50/2015' family and the propagation and powdery mildew screening of the PM R4 resistance segregating '40/2015' family.

WP2: Genotyping and DNA marker assays

Work package 2 included leaf samplings and DNA extractions of family 50/2015 individuals and the generation of genome wide SNP markers using DArTseq genotyping technology (Diversity Technology Arrays, Australia). We also generated more detailed DNA sequencing data for the two parental genotypes 'Pilgrim' and '316/1/10'. In this WP we also sampled and extracted DNA from 71 further hop genotypes unrelated family 50/2015 for the validation of PM markers.

WP3: Bioinformatics and low-cost molecular testing based on PCR (Polymerase Chain Reaction) technology (hop genotyping pipeline development)

Although not strictly a prerequisite for MAB, the availability of tailor made statistical and bioinformatics pipelines and suitable biotechnology platform(s) for the downstream application of genetic markers in breeding programmes are highly useful. This work package included the building of hop-specific DNA processing programmes and the development of KASP genotyping markers for the R2 powdery mildew gene.

WP4: Data analysis and dissemination of results

The final work package included the analysis and interpretation of the data generated in this project specifically the identification of powdery mildew resistance R2 gene, the genomic analysis of 'Pilgrim' and the evaluation of the usefulness of our in-house processing pipelines. Report writing for BREF and

the BHA, dissemination of results via seminars, conference talks and industry outreach events and manuscript writing of the findings of this project also fell under WP4.

Deliverable achieved during the 36 months period of this BREF project are found in Table 1 below:

Reporting period	Time period		WP	Task No.	Task	Deliverable
1st	2021	May	WP1	1.1.	Cuttings taken for propagation for PM tests from Pilgrim mapping population	500+ plants for phenotypic data collection
		May-	WP1	1.2.	Propagation of plants for PM testing	
		June	WP2	2.1.	Collecting leaf material for DNA extractions from seedlings on field	Leaf samples for genotyping
	2022	Jan-Mar	WP1	1.3.	Ordering and bulking up PM isolate	PM resistance phenotypic data for the Pilgrim mapping family segregating for PM R2 resistance
		Mar-Apr	WP1	1.4.	Powdery mildew pathogenicity tests	
		May	WP1	1.5.	Documenting PM assay results	
		Jun-Jul	WP2	2.2.	DNA extractions from 200+ seedlings from validation families	DNA extractions from the Pilgrim family for genotyping
		Aug-Oct	WP2	2.3.	Developing marker assays	Identifying candidate markers for PM resistance
2nd	2023	May	WP1	new	Cuttings taken for propagation for PM tests from Pilgrim mapping population	500+ plants for phenotypic data collection
		May-	WP1	new	Propagation of plants for PM testing	
		Aug-Oct	WP3	3.1	Hop genotyping - bioinformatics pipeline development	Highly useful and relevant resources for UK hop genetic research
		Oct-Dec	WP3	3.2.	Hop genotyping - PCR probe development	Identification of the R2 resistance gene
Final	2024	Jan	WP3	3.3.	Hop genotyping - sequencing	Validating candidate markers for PM resistance
		May	WP1	new	Powdery mildew pathogenicity tests	PM resistance phenotypic data for the Fuggle mapping family segregating for PM R4 resistance
		May	WP1	new	Documenting PM assay results	
		Jan-July	WP4	4.1.	Data analysis and interpretation	
		Oct	WP4	4.2.	Report writing	Final report

Procedures

Phenotyping

The R2 gene is one of the 7 recognised hop PM resistance (R) genes among Rb, R1, R2, R3, R4, R5 and R6 and an important powdery mildew resistance source in UK hop. These genes are effective against different strains of the pathogen *Podosphera macularis* that causes powdery mildew in hops. In our PM screening assays we used the *P. macularis* isolate vBv1v3 which is effective against the R1, Rb and R3 resistances but is defeated by the R2 gene in Pilgrim and the R4 gene in 15/2001/1.

We evaluated powdery mildew resistance conferred by the R2 gene in the 'Pilgrim' family in two experiments using the methods established by Darby (2005b). The first experiment was conducted in 2016 as part of the preceding BREF project. In 2016 301 individuals of Family 50/2015 and parents were assessed as individual seedlings. In 2022 we repeated the experiment on 107 individuals in clonal replicates. Propagation of replicates was done via cuttings taken from the original seedlings at China Farm. The cultivars 'Zenith' and 'Target' were included in both experiments with the expectation of 'Zenith' (not having the R2 gene) being susceptible and 'Target' (having the R2 gene) being fully resistant to the *P. macularis* vbv1v3 inoculum.

P. macularis requires the presence of a living hop plant to grow and cannot be maintained on sterile media. We used the susceptible genotype Northern brewer (NB) for the maintenance and bulking up PM inoculum (Figure 3.). The PM isolate was first applied on a single NB plant and then transferred onto more NB potted plants over a course of two months.



Figure 3. Powdery mildew 'towers' containing Northern Brewer plants covered in *Podosphera macularis* vbv1v3 spores. NB plants are used as 'living inoculum' for powdery mildew screening at Wye Hops.

Inoculation in both years took place in the Wye Hops Ltd glasshouse (Petham, Kent) by evenly covering approximately 30 cm tall potted plants with *P. macularis* spores shaken from Northern Brewer inoculator plants. Plants were kept at 20ºC with a 14h light period for 16-20 days before symptom assessment. The 'Zenith' isolate causes varying reactions in R2 gene families (Darby, 2013) and as such seedling reactions were ranked on a 1-5 scale: 1 for fully resistant, 2 for resistant, 3 for weak resistant/query resistant, 4 for partially susceptible, and 5 for fully susceptible. PM symptoms were scored 15 days after inoculation, with additional checks for plants scored as weak resistant or partially susceptible five days later. Phenotypic evaluation of powdery mildew resistance in the 40/2015 (Fuggle) family was also conducted in individual seedling stage in 2016 (by Darby) and as clonally replicated experiment in 2023 using the same methods as described above.

Statistical analysis of phenotypic data

We tested the observed segregation ratios of powdery mildew resistance linked to the R2 gene in the 50/2015 (Pilgrim) family using chi-squared analysis. In a two-way ANOVA we also assessed how genotype, experiment year, and the position of the replicated pots affected powdery mildew resistance. Overall resistance scores for each genotype were derived from a mixed linear model. We also calculated the heritability (H^2) of PM resistance in the 'Pilgrim' family.

Genotypic data collection

We submitted 188 DNA samples to DArT for genotyping, including multiple replicates of selected genotypes. Briefly genotyping of the DNA samples was done using enzymes that cut the DNA at certain known sequence sites. These enzymes cut out small, ~60 nucleotide (the A,C,T,Gs) long fragments from the DNA from active, genic regions as opposed to inactive "junk" regions which do not contain useful information. The short, informative DNA fragments resulting from the enzyme cut sites were then sequenced on an Illumina sequencing platform and the raw sequencing data was sent back to us.

Whole genome sequencing of parental genotypes

High quality DNA from the parents were also extracted and sequenced. This method did not use fragmentation enzymes, but instead the whole DNA was sequenced on a more powerful Illumina sequencer, aiming for a deeper sequencing coverage and more parts of the genome sequenced compared to genotyping. Our parental sequences provided a useful tool for the fine mapping of the SNPs identified through DArT genotyping.

Quality processing and SNP variant identification in the 188 hop samples

This step was done using an in-house pipeline developed specifically for our purposes. First, we assessed the quality of the raw sequencing fragments and removed artificially attached short sequences that labelled the different fragments and helped the sequencing machine distinguish between them. These sequences were no longer needed. Next, we filtered low-quality fragments which were shorter or longer than expected or had other anomalies. Good quality, trimmed fragments were then aligned to the 'Cascade' reference genome (Padgitt-Cobb et al., 2023). The 'Cascade' genome contains the complete set of contiguous sequences found the hop DNA and is the currently the most advanced hop reference genome available for hop genetics studies. Alignments were done by piecing every short DArT fragment against the contiguous reference sequences ultimately mapping their positions on the hop genome. Next, we compared fragments that mapped to the same position in Cascade across the 188 samples and looked for single nucleotides (SNPs) that appeared to have two different forms in the fragments. We then filtered these SNPs to retain only high-quality true variants and removed variants resulting from sequencing errors. The final set of SNPs were then annotated for their functions using gene information from the 'Cascade' genome reference.

Identification of SNPs that are linked with powdery mildew resistance

Significant associations between powdery mildew resistance and SNPs were identified using the Kruskal-Wallis test. The Kruskal-Wallis test is a statistical method used to compare multiple groups - in our case SNP genotypes - to see if there are differences in their medians against powdery mildew scores. We calculated the coefficient of determination (R^2) to assess how much of the PM resistance phenotypes were explained by genetic variation.

Investigation of genes related to powdery mildew resistance

Once significant PM resistance linked SNPs were identified we furthered our investigation into which genes might be responsible for the R2 type resistance found in 'Pilgrim'. For this, we examined genes previously found in the 'Cascade' reference genome near the region where our powdery mildew resistance was found. Reference genomes usually provide a high resolution, highly accurate framework for a specific species for molecular investigations. Although 'Cascade' is a different variety from 'Pilgrim' they represent the same species and share majority of their DNA codes. With some limitations, genes found in 'Cascade' can also be used to search for genes in 'Pilgrim'. We focussed on 'Cascade' genes that were associated with "defence responses" based on known biological functions.

KASP marker assays

KASP (Kompetitive Allele Specific PCR) is a method used to create specific primers for detecting SNPs. KASP markers are simple laboratory use probes that are designed to differentiate between the allelic states of SNPs across individuals. This is achieved with specific a set of two PCR primers that each only attach to one of the SNP alleles during PCR amplification and fluorescent probes attached to the primers that allow visual determination of which SNP allele was present in the samples (Figure 4.).

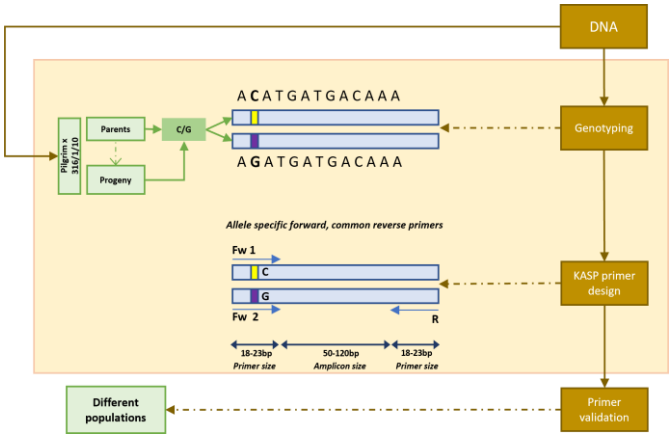


Figure 4. The process of designing KASP PCR primers.

We converted our significant PM resistance associated SNPs into KASP genotyping markers and selected further SNPs from the 'Pilgrim' whole genome sequences near candidate PM resistance genes to increase our chances of developing a highly functional KASP probe that is very close to our target gene. We designed these allele-specific primers and performed KASP assays on a panel of progeny and unrelated individuals to validate the consistency of the markers (i.e. whether the same SNP alleles always confirm the same phenotype in every individual).

Results

Powdery mildew phenotypic data

According to Darby (2001), the R2 gene for powdery mildew resistance is a single dominant gene and thus the segregation rate of susceptible and resistant plants in the 'Pilgrim' x 316/1/10 family was expected to be 1:1 – i.e. individuals either inherited the R gene or not. We tested this segregation using chi-squared analysis. The results showed a 1:1 ratio between resistant (scores 1-3) and susceptible (score 5) plants in both 2016 and 2022, indicating that R2 is linked to a single resistance locus ($X^2 = 0.29$, $p = 0.59$ for 2016; $X^2 = 0.35$, $p = 0.56$ for 2022).

There were no significant differences in phenotype scores between experimental replicates meaning that environment had little to no effect on inheritance of the R2 gene. This was expressed in a heritability (H^2) score of 0.99 which indicates a strong genetic influence on resistance (99% of variation explained by genetic factors).

The distribution of disease phenotypes was similar in 2016 and 2022, with four genotypes consistently showing no symptoms (PM score of 1) and 55 fully susceptible plants (PM score of 5) maintaining their scores across years. However, in the resistant category, there was variability: more genotypes were rated as "very poor resistant" (3) in 2016 and fewer as "poor resistant" (2) compared to 2022, likely due to higher infection severity in 2016. One genotype shifted from "query susceptible" (4) in 2016 to "poor resistant" (3) in 2022.

Genetic mapping of the hop powdery mildew resistance R2 gene

The KW analysis found 12 SNPs significantly associated ($p<0.05$) with powdery mildew resistance in each assessment year and in the combined analysis using both years' data. All significant markers were located on chromosome 6 of the hop genome within a 1.4 Mb region (309.9 Mb to 313.3 Mb). Ten of these markers were informative, as they segregated in the resistant female parent. The most significant (focal) SNP was located at 311,711,438 bp on chromosome 6 and explained 55% of the variation in PM resistance across our samples. The higher the percentage variation is explained by a genetic marker the closer it tends to be to the target gene with 100% indicating that the SNP is in the target gene.

Association of SNP alleles with resistance and susceptibility

We tested the association of marker alleles of our focal SNP 311,711,438 with PM resistant and susceptible phenotypes. Statistically significant differences were found among genotype groups at this locus ($F(3, 102) = [44.331]$, $p < 0.00001$). Since hops are diploid meaning they have two sets of chromosomes, one inherited from each of their parents (just like humans), SNPs found in hop have two alleles. In 'Pilgrim' the focal SNP 311,711,438 was represented with C/T alleles, while '316/1/10' had missing alleles -/- meaning the focal SNP was not present in the susceptible male. Individuals which inherited the 'C' allele from 'Pilgrim' showed resistance, averaging a phenotype score of 2.35, while those inheriting the 'T' allele from 'Pilgrim' were susceptible, with a score of 4.55 (Figure 5).

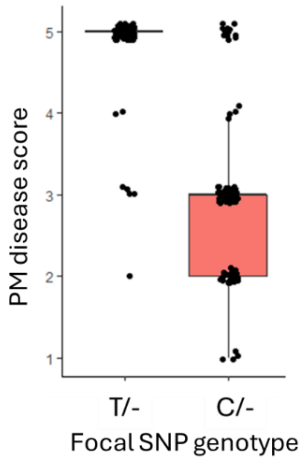


Figure 5. The powdery mildew disease score for each genotype associated with the 311711437 focal SNP. Genotypes containing the SNP allele C were resistant and genotypes containing the T allele were susceptible.

Investigation of disease resistance genes on chromosome 6 near powdery mildew markers

Chromosome 6 is 316,519,611 bp long and contains 9,387 genes (Padgitt-Cobb et al. 2022). We investigated potential powdery mildew resistance genes near the 12 significant SNPs that we identified in the 309M-312Mb window on Chr 6. This target region contained 426 genes from which 31 were putative disease resistance genes based on their involvement in processes like "defence response," "hypersensitive response," and "defence response to fungus". Among the defence-related genes near the PM resistance region, we found a cluster of three RPM1 genes (Figure 6.).

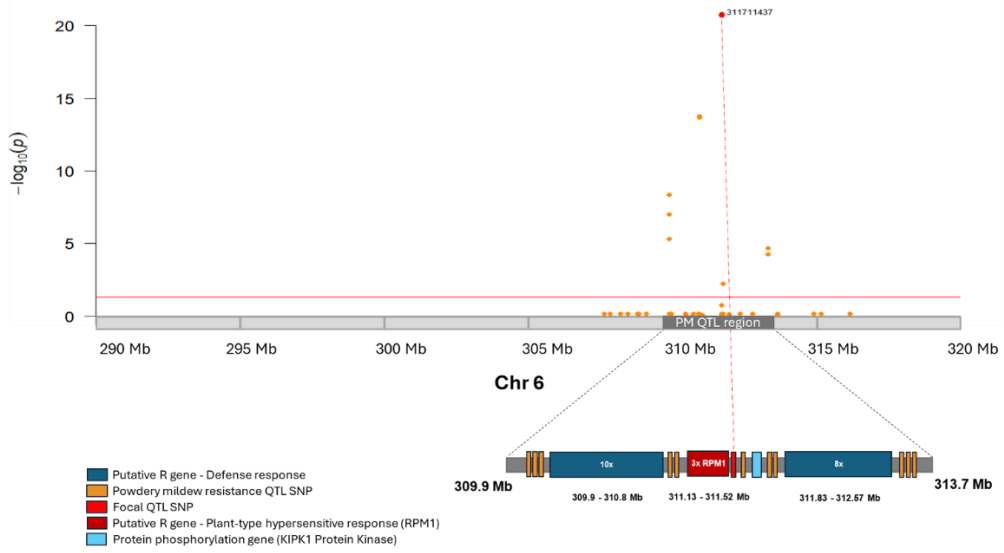


Figure 6. Manhattan plot showing significant PM resistance associated SNPs (orange dots) across Chromosome 6 of the hop genome. The horizontal red line shows the significance threshold of $-\log_{10} p$=0.05 values. Individual SNPs with significance scores to powdery mildew phenotype are marked at each position in the hop genome in Mb. The figure shows putative resistance genes including a cluster of RPM1 genes within the region containing PM resistance SNPs.

RPM1 genes are strong candidates for disease resistance. RPM1 operates through a powerful resistance mechanism like the response seen in R2 resistant hops. It detects pathogen presence and triggers a hypersensitive response (HR) within five hours of infection, which leads to plant cells to collapse and prevent the spread of the pathogen (Grant et al. 2000). RPM1 genes have been extensively studied and homologues – genes that have the same structure and function in different organisms - have been found in various other plant species such as Arabidopsis and wheat (Dangle et al. 1992). RPM1 has also been linked to wheat's resistance to powdery mildew (Nie & Ji 2019; Simeone et al. 2020). Given their role in powdery mildew resistance, these three RPM1 genes are promising candidates for conferring R2 resistance against *Podosphaera macularis* in hops.

KASP marker assessment

As part of this work, we developed a series of 16 KASP markers from SNPs that lied within the PM resistance region near our putative RPM1 genes. Once genetic markers show a strong association with the studied trait as confirmed via genetic mapping, they can be converted into simpler PCR assays that provides ease of use in applied breeding programmes where hundreds to thousands of new breeding lines need to be tested quickly and simply. To test whether a KASP marker is transferrable i.e. not only showing association between marker alleles and resistance status in the population it was first identified in (in our case in Family 50 individuals derived from 'Pilgrim') it is important to screen unrelated genotypes also. We tested our KASP probes on a panel of 27 unrelated hop genotypes with

various PM resistance background and a further 27 individuals from Family 50 with both resistant and susceptible phenotypes to test the consistency of our KASP markers in predicting PM resistance/susceptibility (Figure 7).

Validation panel (wider germplasm) with various PM resistance background	
PM resistance source	**Genotype**
Susceptible	Santiam
Susceptible	Saaz
Susceptible	Northern brewer
Susceptible	Fuggle
Susceptible	Ernest
Susceptible	Admiral
R1	Zenith
R2	Target
R2	Pilgrim
R2/Rq	Endeavour
R2/Rsov	Sovereign
R2/Rsov	38/2009/12 (sov s.)
R4	Serebrianka
R6	Nugget
Rq	Cascade
Rq/R4/R2	FZ03
Rq/R4/R2	FY04
Rq/R4/R2	FY03
Rq/R4/R2	FY02
Rq/R4/R2	FX91
Rq/R4/R2	FX902
Rq/R4/R2	FX90
Rq/R4/R2	FW22
Rq/R4/R2	FW21
Rq/R4/R2	FW20
Susceptible	Early Choice
Susceptible	Challenger

Further individuals from family 50/2015 (Pilgrim x 316/1/10)	
PM resistance source	**Genotype**
R2	50/2015/8
R2	50/2015/6
R2	50/2015/4
R2	50/2015/2
R2	50/2015/102
R2	50/2015/1
R2	50/2015/37
R2	50/2015/33
R2	50/2015/32
R2	50/2015/26
R2	50/2015/20
R2	50/2015/19
R2	50/2015/14
Susceptible	50/2015/279
Susceptible	50/2015/266
Susceptible	50/2015/265
Susceptible	50/2015/263
Susceptible	50/2015/251
Susceptible	50/2015/250
Susceptible	50/2015/236
Susceptible	50/2015/234
Susceptible	50/2015/221
Susceptible	50/2015/213
Susceptible	50/2015/188
Susceptible	50/2015/187
Susceptible	50/2015/185
Susceptible	50/2015/184

Figure 7. Validation panel consisting of unrelated hop genotypes and further family 50/2015 individuals tested with KASP markers.

Validation across a panel of these hops showed us that on of the KASP markers 'FM4' positioned at at 311,712,429Mbp on Chromosome 6 had good concordance across the population and the wider germplasm and thus was selected for further evaluations and possible use in the breeding programme to screen for R2 resistance in new breeding materials.

Discussion

Traits controlled by major dominant genes with high heritability (H2) such as powdery mildew resistance, are good candidates for MAB because they are easier to discover linked markers for. The R2 gene is an important powdery mildew resistance source in UK hop, and historically it has been the main powdery mildew resistance source used in the past four decades in the Wye Hops breeding programme. Thus, being able to detect the R2 allele in future and existing breeding lines would be highly advantageous in commercial hop breeding practices. The pyramiding of multiple powdery mildew resistance sources is one of the ongoing endeavours of the Wye Hops breeding programme. However, evaluation is currently solely based on pedigree information and on the observed phenotypic expression and segregation ratios in crosses, confirming the presence of different R genes, which requires inoculation with multiple different P. macularis sources. The use of genotyping markers discriminating between multiple powdery mildew R genes would speed up the selection of new breeding lines with multiple powdery mildew resistance sources and reduce the cost associated with disease screening, as well as the extensive year-round maintenance of inoculator plants and PM isolates.

Through this project, we found the R2 powdery mildew resistance location between specific DNA coordinates on chromosome 6, with three RPM1 candidate genes within the PM resistance region. We developed a KASP marker (FM4) linked to this region, which shows strong consistency across different hop varieties. The deployment of the FM4 KASP marker would be the first step towards the molecular breeding of hop cultivars with durable PM resistance and the first step towards marker-assisted resistance breeding in UK hops. KASP probes can be readily used by breeding programmes provided there is availability of the necessary laboratory infrastructure to carry out the tests on young seedlings

Summary of what has been achieved

In the preceding 2017 BREF project, four large hop mapping populations were established and maintained at the Wye Hops breeding plot, China Farm, with the purpose of providing future genetic and genomic evaluation studies with necessary germplasm material. These plants were planted in 2016 and maintained following the farm's standard procedures until the commencement of this project. One of the four populations, derived from the highly significant Wye Hops genotype 'Pilgrim,' that carries multiple favourable genes, including resistances to powdery mildew, downy mildew, and V. wilt, was selected and used for the studies undertaken in this project. We also selected the mapping population derived from the Wye Hops male line '15/2001/1,' which is highly resistant to wilt and carries the powdery mildew R4 gene which is an effective source of PM resistance in the UK and the landrace English genotype 'Fuggle,' which is highly susceptible to wilt and PM.

We clonally propagated the 'Pilgrim' and 'Fuggle' families via soft cuttings in two consecutive springs and artificially infected 1-year-old pot plants with the powdery mildew pathogen, allowing the recording of PM resistance in two large mapping populations, providing invaluable phenotypic data for MAB.

We also sampled and extracted the DNA of 188 individual plants from the 'Pilgrim' family in 2021 and sent the DNA samples to Diversity Arrays Technology (DArT) Inc., Australia, for sequencing. Sequencing data was received back from DArT and processed in-house using a custom-built bioinformatics pipeline, which formats and filters the sequencing reads, aligns them against a reference genome ('Cascade'), and identifies SNP polymorphisms. We found over 100,000 molecular markers across the 'Pilgrim' progeny individuals, which were used for the mapping of PM resistance. Moreover, we generated further high-resolution DNA sequences for the two parental genotypes 'Pilgrim' and '316/1/10' to further help our investigations.

In summary, through this BREF grant, we were able to conduct a comprehensive genetic analysis of disease resistance in UK hop. This BREF funding allowed the necessary groundwork to be undertaken for the in-depth analysis of the powdery mildew resistance R2 gene found in 'Pilgrim.' Furthermore, within this project, we were able to propagate plant material for a mapping population segregating for the powdery mildew R4 gene derived from the resistant male '15/2001/1.' The outcome of this project includes the generation of phenotypic and genotypic data for the 50/2015 (Pilgrim) family, the phenotypic data generation for the 40/2015 (Fuggle) population, and the development of a hop-specific bioinformatics pipeline that includes genome alignment, SNP calling, SNP filtering, and data manipulation/processing steps, which are transferable to future and ongoing hop genetics/genomics projects, and the development of further genetic markers for UK hop breeding.

Finally, the funding enabled the development of allele-specific PCR assays that have the potential to confirm powdery mildew R2 resistance in future UK hop breeding lines. The deployment of the FM4 R2 KASP markers is the first step towards applied molecular breeding of UK hop cultivars.

Follow on work

The results achieved in this project will continue to be built upon via various ongoing and new research initiatives. Through this project, we successfully mapped several significant SNPs on chromosome 6 of the hop genome, pointing towards a cluster of RPM1 proteins, which are candidate disease resistance genes. Confirming that one of the RPM genes corresponds to the R2 PM resistance gene is currently ongoing in collaboration with the University of Kent biotechnology hub.

The initial phenotypic characterisation of the four large marker populations was conducted under the preceding 2017 BREF project, and the genetic profiling of family 50/2015 was completed in this BREF project. Family 40/2015 was screened for PM resistance in a replicated experiment under this project as well. To complete the mapping of the R4 gene, individuals of this family will be genotyped. The remaining two marker families will also be genotyped, and genetic mapping work using existing and newly generated phenotypic data on disease resistance, agronomy, and cone chemistry will be conducted on them.

Generation of useful genomic resources for the Pilgrim family will be extended to the entire Wye Hops germplasm, including historic and modern breeding lines with available pedigree and trait information. The sampling, DNA extractions, and genotyping of ~900 individuals have been completed as part of a follow-on BREF project and an Innovate UK grant. Analysis of the data is currently ongoing in collaboration with Kent University and NIAB (National Institute of Agricultural Botany). The results generated here will not only aid in the confirmation of the R2 gene but will allow the mapping and in-depth investigations of Verticillium wilt resistance, other PM and DM resistance sources, and a multitude of agronomy and cone-related traits.

Building on the genotype data generated for the Wye Hops germplasm, we won Growing Kent and Medway funding to conduct a series of small-scale brew trials, sensory and chemical analyses on 50 hop varieties with various aroma backgrounds. This project, in collaboration with Ramsgate Brewery and the University of Kent, is the first attempt to combine brewing, sensory, chemistry, and genotype data for UK hop and investigate the genetic components related to hop aromas.

Outputs

The results generated through this BREF project allowed additional funding to be obtained for follow on research including a Growing Kent and Medway grant that provided laboratory infrastructure for the KASP genotyping work, a follow on BREF grant on wilt resistance genetic mapping and an Innovate UK grant on advanced genetic assisted breeding strategies both utilising the techniques and bioinformatics pipelines developed through this BREF project. Two further grants for hop and beer sensory investigations were also won with the Growing Kent and Medway grant scheme which will utilise the power of genetics in the prediction of aroma compounds in future hops. The importance and impact of this BREF project on the future of British hops was widely communicated through various outreach events to industry and the general public. Progress towards using genetic markers in the national breeding programme was made through the collaboration established with the University of Kent where standard laboratory procedures for MAB can be carried out in the future and by bringing together a consortium of research institutes, biotechnology companies and industry partners forming a long-term partnership with Wye Hops supporting UK hop research. The findings of this project have been disseminated through reports and meetings including industry committees such as the Hop Industry Committee of the IBD, participation in hop producer-group meetings and seminars, as well as lectures to students and visits to universities and hop research centres throughout the world.

Report to the R&D committee of the BHA, meeting held at Wye Hops, China Farm, August 2021.
Presentation titled 'Molecular Markers Project Update' given at the British Hop Association Technical Day held on zoom, November 2021.
Report to the R&D committee of the BHA, meeting held on zoom, November 2021.
Poster titled 'QTL mapping of the hop powdery mildew resistance R2 genetic locus' submitted at the British Society of Plant Pathology annual conference held in Birmingham, December 2021.
Report to the R&D committee of the BHA, meeting held at Thame, Reading, April 2022.
Presentation titled 'Steps towards marker assisted resistance breeding in UK hop' given at the Scientific Commission of the International Hop Growers Convention, Lugo, Spain, July 2022
Hop Breeding *session chaired* at the Scientific Commission of the International Hop Growers Convention, Lugo, Spain, July 2022
Report to the R&D committee of the BHA, meeting held at Wye Hops, China Farm, August 2022.
Growing Kent and Medway *grant application*, £15,000 won for Wye Hops for project titled 'Identifying Powdery Mildew Resistant Hop Genes', September 2022
Report to the R&D committee of the BHA, meeting held at Thame, Reading, November 2022.
Presentation titled 'Marker assisted breeding in UK hop' given at the University of Kent Fire Bio seminar series, Canterbury, February 2023.
Successful *defending of PhD viva*, Reading University, March 2023
Report to the R&D committee of the BHA, meeting held at Thame, Reading, March 2023.
Talk titled 'Disease Resistant Hops' given at the 'Pint of Science' science festival, Canterbury May 2023
Invited speaker on the topic 'UK hop breeding' at the Uradalmi Brewery Hop Harvest Day, Val, Hungary, July 2023
Innovate UK *grant application*, £575,000 won for project titled 'Genetic Informed Breeding of Climate Resilient UK Hop varieties', July 2023
Report to the R&D committee of the BHA, meeting held at Wye Hops, China Farm, August 2023.
Presentation titled 'Disease resistance breeding in UK hop' given at the University of Greenwich biotechnology seminar series, Gillingham, Kent, October 2023
Report to the R&D committee of the BHA, meeting held at Thames, Reading, November 2023.
Growing Kent and Medway *grant application*, £15,000 won for joint project with Ramsgate Brewery titled ''Enhancing the uptake of UK hops by national breweries through comprehensive sensory and chemical assessments", February 2024
Report to the R&D committee of the BHA, meeting held at Thames, Reading, April 2024.
Guest speaker Charles Faram Spreading Hoppiness Podcast series ''Beyond the bine: Inside the hop development programme". April 2024.
Hop Breeding *session chaired* at the Scientific Commission of the International Hop Growers Convention, Ljubljana, Slovenia July 2023
Presentation titled 'Marker assisted breeding in the UK hop breeding programme' given at the Plant and Animal Genome Conference Hop Breeding and Genetics workshop, San Diego, USA, January 2024
Presentation titled 'Hop Breeding in the 21st Century' given at the IBD Great Northern Section seminar series, Virtual/zoom, January 2024
Presentation titled 'Current hop Breeding at Wye Hops' given at the Deal Community Hop Growers' AGM, Canterbury, January 2024
Presentation titled 'Wye Hops recent directions' given at the British Hop Association/Kent university Hop Technical Day, Canterbury February 2024
Organiser of the British Hop Association/Kent university Hop Technical Day, Canterbury February 2024
Presentation titled 'Hop breeding advances in the UK' and 'Hop growing practices in the UK' given at the Minnesota Hop Growers' AGM, Chisago City, USA March 2024
*Host*ed Belgian Hop Growers and Hop Researchers delegation and talk given at Wye Hops breeding plot, Canterbury, August 2024
Report to the R&D committee of the BHA, meeting held at Wye Hops, China Farm, August 2024.

Presentation titled 'Genetic informed breeding of climate resilient UK hop' given at the Brewing Engineers' Association AGM, Burton Upon Trent October 2024.
Honorary Academic status awarded to KH by the University of Kent, School of Biotechnology. October 2024
Guest speaker Charles Faram Spreading Hoppiness Podcast series ''Hop breeding 101". October 2024.
Publication 2024, Hajdu K., Connell J., Armitage A.D., Darby P., Ainslie A., Wagstaff C., Cockerton H.M. 'Genetic mapping of the powdery mildew resistance R2 locus and sex phenotype in hop' - manuscript in preparation

Expenditure

The project ran from July 2021 to July 2024, for a total of 36 months. This period encompassed two full and two partial growing seasons, which was reflected in the contribution requested from BREF for the maintenance of plant material. The cost of genotyping was indirectly paid through the PhD project that was funded by the BHA. The cost of laboratory use was indirectly paid through a match-funded project between the University of Kent and Wye Hops, where Wye Hops contributed £10,000 to the total project costs, covering science staff, laboratory use, and KASP marker development. The detailed breakdown of actual costs associated with the resources required for this project is as follows: costs have been taken from the audited accounts for Wye Hops Ltd for the financial years 2021-22, 2022-23, and 2023-24.

	Forecast	**Actual**				
Original description		2021/22	2022/23	2023/24		**Description of costs**
Genotyping platform development	£9,300	£4,800.00	£5,040.00	£0.00	£9,840.00	Proportionate cost of PhD project funded by BHA
Phenotyping assays	£10,600	£1,328.00	£1,356.90	£0.00	£2,684.90	All year-round maintenance of powdery mildew isolates including lab equipment, isolate transfer materials and energy use.
Consultant and Technical personnel	£24,000	£7,700.00	£9,335.00	£8,489.00	£25,524.00	K Hajdu, M Baldock labour, Peter Darby consultancy
Consumables and services (lab use, extraction kits, media, pots etc.)	£7,400	£140.00	£565.00	£10,000.00	£10,705.00	Consumables and laboratory infrastructure provided by the University of Kent Wye Hops match funded Growing Kent and Medway project
Field and glass costs	£4,500	£3,194.00	£3,304.00	£3,703.00	£10,201.00	Annual field maintenance cost of mapping population. Glass house cost for the year-round maintenance of powdery mildew inoculator plants and the housing of PM infection assays.
Propagation	£7,400	£0.00	£3,375.00	£0.00	£3,375.00	Propagation of large powdery mildew infection assay material.
Direct overheads	£4,500	£1,540.00	£1,867.00	£1,697.80	£5,104.80	20% of labour
Other costs e.g., equipment, fees and repairs	£2,000	£856.00	£300.00	£950.00	£2,106.00	Repairs and laptop purchase after termination of PhD project.
Travel	£500	£240.00	£120.00	£120.00	£480.00	KH travels, dissemination of BREF project results, visiting Uni of Kent lab/staff.
TOTAL	**£70,200**				**£70,020.70**	

Total contribution requested from BREF **£35,100**

References

Darby, P. (2001). Single Gene Traits in Hop Breeding. Proceedings of the of the Scientific Commission

Darby, P. (2005a). Brewery History - The Journal of the Brewery History Society Online (p. Brew. Hist., 121, pp. 94–112).

Darby, P. (2005b). THE ASSESSEMENT OF RESISTANCE TO DISEASES IN THE UK BREEDING PROGRAMME. Proceedings of the Scientific-Technical Commissions - International Hop Growers` Convention.

Darby, P. (2013). Could there be a dominant gene for susceptibility to hop powdery mildew? Proceedings of the Scientific-Technical Commission of the International Hop Growers Convention.

Dredge, M. (2021). Respect Your Elders — How Fuggle and Golding Hops Changed Modern Beer

Capper, A. (2014). A Nuffield Farming Scholarships Trust Report The Export of Great British Hops

Cobb, J. N., Biswas, P. S., & Platten, J. D. (2019). Back to the future: revisiting MAS as a tool for modern plant breeding. Theoretical and Applied Genetics, 132(3), 647–667.

Jiang, G. L. (2013). Molecular Markers and Marker-Assisted Breeding in Plants. In Plant Breeding from Laboratories to Fields. https://doi.org/10.5772/52583

Anisimova Editor, M. (2021). Evolutionary Genomics Statistical and Computational Methods Second Edition Methods in Molecular Biology 1910. http://www.springer.com/series/7651 Gent, D. H., Claassen, B. J., Twomey, M. C., & Wolfenbarger, S. N. (2018). Identification of resistance to powdery mildew in publicly available male hop germplasm. Plant Health Progress, 19(3), 258–264. https://doi.org/10.1094/PHP-05-18-0027-RS

Wolfenbarger, S. N., Massie, S. T., Ocamb, C., Eck, E. B., Grove, G. G., Nelson, M. E., Probst, C., Twomey, M. C., & Gent, D. H. (2016). Distribution and characterization of podosphaera

Padgitt-Cobb, L. K., Kingan, S. B., & Henning, (2020). Genomic analysis of powdery mildew resistance in a hop (Humulus lupulus L.) bi-parental population segregating for “R6-locus.” Euphytica, 216(1), 1–15. https://doi.org/10.1007/s10681-019-2543-x

Padgitt-Cobb L.K., Pitra N.J., Matthews P.D., Henning J.A., Hendrix D.A. An improved assembly of the "Cascade" hop (*Humulus lupulus*) genome uncovers signatures of molecular evolution and refines time of divergence estimates for the Cannabaceae family. Hortic Res. 2022 Dec 7;10(2):uhac281. doi: 10.1093/hr/uhac281. PMID: 36818366; PMCID: PMC9930403.

Havill J.S., Richardson B.J., Rohwer C.L., Gent D.H., Henning J.A., Muehlbauer G.J. Identification of quantitative trait loci associated with R1-mediated resistance to powdery mildew and sex determination in hop (Humulus lupulus L.). Theor Appl Genet. 2023 Jun 15;136(7):154. doi: 10.1007/s00122-023-04399-7. PMID: 37318664.

Henning, J. A., Gent, D. H., Townsend, M. S., Woods, J. L., Hill, S. T., & Hendrix, D. (2017). QTL analysis of resistance to powdery mildew in hop (Humulus lupulus L.). Euphytica, 213(4), 1–13. https://doi.org/10.1007/s10681-017-1849-