December 2023

Final Project Report 648

Yellowhammer:
A multi-locus strategy for durable rust resistance in wheat, in the face of a rapidly changing pathogen landscape

Camila Zanella[1], Joachim Nwezeobi[1], Sarah Holdgate[1], Lucy James[1], Charlotte Nellist[1], Tansy Chia[1], Keith Gardner[1], Consortium Partners[2, 3, 4, 5, 6, 7, 8, 9] and Lesley A. Boyd[1]

[1]NIAB, 93 Lawrence Weaver Road, Cambridge CB3 0LE UK
[2]AHDB, Stoneleigh Park, Kenilworth, Warwickshire CV8 2TL UK
[3]DSV UK Ltd, Top Dawkins Barn, Waddington, Banbury, Oxfordshire OX17 1FE UK
[4]KWS UK Ltd, 6 Church Street, Thriplow, Royston, Hertfordshire SG8 7RE UK
[5]Limagrain UK Ltd, Station Road, Docking, Kings Lynn, Norfolk, PE31 8LS UK
[6]RAGT Seeds Ltd, Grange Road, Ickleton, Essex CB10 1TA UK
[7]Syngenta UK Ltd, Hill Farm Road, Whittlesford, CB22 4QT UK
[8]Sejet Planteforædling, Nørremarksvej 67, 8700 Horsens Denmark
[9]Lantmännen Lantbruk, Onsjövägen 13, 26881 Svalöv Sweden

This is the final report of a 48-month (plus 12 no-cost extension) project (21120068) that started October 2018. The work was funded by BBSRC and a contract for £111,842 from AHDB.

CONTENTS

1. Abstract

The BBSRC LINK 'Yellowhammer' project was a collaboration between NIAB, seven breeding companies and AHDB. It assessed the genetic diversity underpinning yellow rust resistance in winter wheat varieties. In 2018, it established a panel of 427 winter wheat varieties that represented the genetic diversity deployed across the UK and northern Europe over the past 60 years. Between 2018 to 2022, this panel was grown across multiple field trial sites in the UK, France, Germany, Denmark and Sweden. In each trial, yellow rust resistance was assessed. The panel was screened for SNP variants using the Breeders 35K wheat array, which identified 19,703 SNPs that were used in a Genome Wide Association Study (GWAS) to assess the yellow rust resistance effective in each wheat variety across the multi-site-by-year field trials. These SNP markers were mapped against the Chinese Spring genome reference to identify the chromosomal location of each SNP. From these, multiple GWAS marker trait associations (MTAs) for yellow rust resistance were identified. Forty-five MTAs were identified using cut-off criteria. An MTA was called if (1) it had a significance score above threshold of > 3.0, or (2) had a significance score above threshold of > 1.5 in more than one season or location. Of these 45 MTAs, 29 were present in over 90% of the GWAS panel lines, suggesting that these yellow rust resistance loci have become fixed in UK and northern European winter wheat germplasm. MTAs that occurred at a low frequency (less than 20%) were 1A011 (18.5%), 2B153 (17.3%) and UN004 (6.25%). The MTA 3B004 was found in 21.7% of the GWAS panel lines.

MTAs were confirmed in the varieties Dickens, Gladiator, Crusoe, Mallet and Revelation using bi-parental mapping populations. The MTAs confirmed were 1A011, 1B545, 2A019, 2A087, 2B153, 2D999, 3A010, 3B004, 4B605, 5B677, 5A685 and 6A612 and UN004. The MTA 2A019 appears to be located within a translocation from *Aegilops ventricosa* located on the short arm of chromosome 2A. 2A019 was found in 41.86% of the GWAS panel. This *Ae. ventricosa* translocation is believed to be the translocation 2NS/2AS that contributed the *Yr17* gene and to which virulence was found in the variety Brigadier in 1996. Screening the panel with KASP markers diagnostic for *Yr17* confirmed the presence of the *Ae. ventricosa* translocation, all lines carrying the MTA 2A019 also testing positive for *Yr17*. This would suggest that the 2NS/2AS may carry additional yellow rust resistance genes effective in the UK and northern Europe. Published KASP markers were used to screen for cloned yellow rust resistance genes. However, only the markers for *Yr5, Yr15* and *Yr17* proved to be diagnostic, distinguishing between the control DNAs. *Yr5* was not detected in any of the Yellowhammer lines, while *Yr15* was found in the homozygous state in 2.3% of the lines, below the level at which an allele can be detected by GWAS analysis.

This GWAS has enabled us to identify those yellow rust resistance loci that have remained effective over the duration of this study and in which geographical locations (variation in effectiveness potentially due to variation in the *Pst* population at each location). It has also identified which yellow rust loci have been used less frequently in UK and northern European winter wheat breeding, and therefore provide a potential underexploited resource.

A microscopic examination of the development of *Puccinia striiformis* f. sp *tritici* (*Pst*), the fungus responsible for yellow rust, was undertaken in near-isogenic lines (NILs), with and without selected yellow rust resistance loci. Assessment of the MTA 2A019 indicated that this yellow rust resistance primarily stopped the development of *Pst* at the formation of runner hyphae. The fungus was able to germinate and enter the wheat leaf, forming a sub-stomatal vesicle within the stomatal cavity of the leaf and establish infection. However, in the lines carrying 2A019 the pathogen was less able to produce runner hyphae and spread through the leaf tissue. In lines containing the MTA 6A612 runner hyphae were able to develop, but the formation of pustules production was greatly reduced.

To identify candidate wheat genes responsible for the yellow rust resistance MTA, a *Pst* infection time course analysis of the NILs was undertaken. Flag leaves of NILs, inoculated with a single isolate of *Ps*t, NIAB 19/501, were sampled at 1, 2, 3, 7 and 14 days after inoculation (dai). RNA was extracted from these *Pst* inoculated flag leaves and assessed for wheat gene expression using RNAseq. As a proof-of-concept, NILs with and without the cloned yellow rust resistance gene *Yr15* were included in this RNAseq analysis. Comparing *Pst* inoculated samples to uninoculated flag leaves identified the *Yr15* gene, which was present in the top 10 wheat genes that were differentially expressed between these treatments. This provides compelling evidence for this approach as a way of identifying the wheat gene/s underlying yellow rust resistance loci. Identification of the gene/s responsible for the resistant phenotype enables the development of the ideal marker, selecting between resistant and susceptible alleles of the yellow rust resistance gene. Using this RNAseq approach we were also able to identify candidate genes for 2A019 and 6A612.

The Yellowhammer project has provided wheat breeders with a holistic view of the sources of yellow rust resistance they have available to them within their winter wheat breeding programmes. This includes knowledge of resistances that are principally fixed in their wheat materials, as well as resistance loci that are underutilised, with some sources of resistance only being present in 2.3% and 6.25% of the wheat varieties, i.e., *Yr15* and UN004, respectively. The partners have been able to take the SNP markers identifying these sources of yellow rust resistance to develop DNA markers that are diagnostic for each locus and use these in their breeding programmes to deliver new wheat varieties with effective yellow rust resistance. The microscopic development of the yellow rust pathogen has shown that different sources of yellow rust resistance interact with the pathogen in different ways, indicating potentially different resistance mechanisms. By combining resistances that function through different mechanisms it is believed that more durable resistance can be achieve.

2. Introduction

Wheat *(Triticum aestivum)* is the UK's most important arable crop, being grown across 1.8 million hectares. UK wheat production in 2022 reached 15 million tonnes and was worth £4.35 billion at farm-gate. Ensuring efficient wheat production is a key component underpinning sustainable intensification of UK food production and national food security.

Yellow rust (Yr) is a major threat to wheat production in temperate and maritime climates, as found in the UK and northern Europe, with infection significantly reducing yield. Yellow rust is caused by the biotrophic fungus *Puccinia striiformis* f. sp. *tritici* (*Pst*), an airborne pathogen that produces multiple cycles of infection during the wheat growing season. Integrated pest management (IPM) approaches are applied to limit disease. Disease prevention via growth of resistant varieties is a key component of this IPM scheme and represents an environmentally sustainable solution. Consequently, the effective deployment of existing and new sources of Yr resistance within wheat varieties is increasingly important.

New races of *Pst* are discovered almost every year in the UK, representing new virulence profiles, but also introducing new virulences, resulting in the loss of effective yellow rust resistance in many wheat varieties. This problem is exemplified by the discovery in 2011 of the ‘Warrior’ race. The ‘Warrior’ race demonstrated that *Pst* isolates could migrate into the UK from considerable distances (Hovmøller et al 2016). This ‘Warrior’ race subsequently replaced the pre-2011 *Pst* population, creating a new and genetically diverse *Pst* population within the UK.

The overall aim of the Yellowhammer project was to find those yellow rust resistance loci that were most effective under field conditions, thereby providing durable resistance against multiple *Pst* races. To achieve this, we developed a panel of winter wheat varieties that represented the wheat varieties grown within the UK and northern European over the past 60 years – Yellowhammer panel. The Yellowhammer panel was used in a Genome Wide Association Study (GWAS) to identify those sources of yellow rust resistance effective across multiple sites and years. The panel was tested at multiple sites in the UK, France, Germany, Denmark and Sweden, from 2018 to 2022. Yellow rust resistance loci identified through the GWAS were validated through the generation of bi-parental mapping populations between selected wheat varieties. Near-isogenic lines (NILs) were developed by Limagrain and RAGT to enable detailed examination of the yellow rust resistance phenotypes, as well as examine changes in wheat gene expression, following inoculation with *Pst*, as a way of identifying the candidate genes responsible for the yellow rust resistance. These NILs were assessed for yellow rust resistance in the field and used in a glasshouse, *Pst* inoculation trial, taking *Pst* inoculated flag leaves at 1,3,7 and 14 days after inoculation. The development of *Pst* was assessed microscopically at each time point to look for differences in *Pst* development that could be associated with the yellow rust resistance loci present in the NILs. Total RNA was extracted at the same time points to assess changes in wheat gene expression related to *Pst* developmental stage and the yellow rust resistance genes present.

3. Materials and methods

3.1. Genome Wide Association Study of yellow rust resistance

3.1.1. Phenotyping of GWAS panel

The Yellowhammer panel was sown at multiple locations and across multiple seasons, between 2018 and 2022 (**Table 1**). Each line was assessed for yellow rust infection at least once within a season. Yellow rust infection levels were either scored as a percentage of leaf area infected or on the scale 1 (fully resistant) to 9 (fully susceptible). Those data sets scored using the 1 to 9 scale were also converted to percentage infection scores using the conversion in Table 2. The percentage yellow rust infection scores were also transformed using a log10+1 scale (Table 2). This produced 3 data sets for each field trial by score date. In total, 237 yellow rust score data sets were produced and used in the GWAS analyses.

Company	Location	2018	2019	2020	2021	2022
KWS	Thriplow, Camb., UK	2 scores	1 score	3 scores	2 scores	2 scores
KWS	Wetze, Niedersachan, DE		2 scores	1 score	1 score	2 scores
DSV	Asendorf, Niedersachan, DE		1 score	2 scores	1 score	
DSV	Wardington, Oxford, UK		2 scores	2 scores	2 scores	1 score
Limagrain	Rothwell, Lincoln., UK	3 scores	2 scores	3 scores	1 score	1 score
	Osgodby, Lincoln., UK		2 scores			
	Woolpit, Suffolk, UK				2 scores	1 score
RAGT	Ickleton, Camb., UK	2 scores	1 score	2 scores	2 scores	2 scores
	Lille, FR		1 score	2 scores	2 scores	1 score
Syngenta	Whittlesford, Camb., UK		1 score	2 scores		2 scores
	Chartes, Centre-Val de Loire, FR			3 scores	2 scores	2 scores
Sejet	Midtjylland, DK		2 scores	2 scores	1 score	
	Syddanmark, DK		2 scores	2 scores	1 score	2 scores
Lantmannen	Svalöv and Bjertorp, SE		2 scores	2 scores	1 score	2 scores

Table 1. All field yellow rust assessment undertaken on Yellowhammer GWAS wheat panel

Score scale	Percentage of infection	Log10+1 scale
1	0	0.000
2	0.1	0.041
3	1	0.301
4	5	0.778
5	10	1.041
6	25	1.415
7	50	1.708
8	75	1.881
9	100	2.004

Table 2. Yellow rust field scores (conversion table)

3.1.2. Genotyping of GWAS panel

DNA was extracted from the Yellowhammer panel of 427 wheat varieties (Fulton et al. 1995). DNA from each line was sent to RAGT for genotyping on the breeders 35K single nucleotide polymorphism (SNP) array (Allen et al. 2017). The genotyping data were filtered, removing markers with > 20% missing values and minor allele frequencies (MAF) of < 5%. The remaining missing values were imputed using the R package missForest (Stekhoven and Buehlmann, 2012), which fits a random forest (Breiman, 2001) on the observed genotypic data to predict the missing SNP data. GWAS was performed using the R package GWASpoly (Rosyara et al. 2016), which identified marker-trait associations (MTAs) using the Mixed Linear Model (MLM). The GWAS accounted for population structure (PC=5) and kinship as fixed and random effects, respectively. The kinship matrix was determined using GWASpoly and a datamatrix of 3,167 SNPs, skimmed using thresholds of 0.60. The significance of MTAs was determined using the false discovery rate (FDR) (Benjamini & Hochberg, 1995) using a q-value cut-off of q=0.05. Markers in Manhattan plots were ordered according to physical position on the Chinese Spring reference genome RefSeq v1.0 (IWGSC, 2018). An MTA was considered a potentially genuine yellow rust resistance locus when (1) it had a significance score above threshold of > 3.0, or (2) had a significance score above threshold of > 1.5 in more than one season or location.

3.2. Screen of Yellowhammer panel for cloned yellow rust resistance genes

The Yellowhammer panel was screened with published KASP markers reported to detect yellow rust resistance genes using the PCR-based KASP genotyping assay as described by the manufacturer (https://www.lgcgroup.com). This included markers for *Yr17* and the cloned Yr genes *Yr5, Yr7, YrSp, Yr15, Yr18, Yr36 and Yr46* (Table 3). The KASP markers were first checked for functionality using control wheat lines with and without the target Yr gene. KASP primers that proved to differentiate

between lines with and without the target Yr gene were then used to screen all the lines of the Yellowhammer GWAS panel. Two different diagnostic markers for *Yr15* (Yr15_R5 and Yr15_R8) were used as technical reps. For Yr gene markers that failed in the initial test, alternative KASP primers were selected and tested similarly with controls. All markers, and their primer sequences, used in this study are given in Table 3.

Gene	Resistant Control	Susceptible Control	Primer name	Primer Sequence (5' to 3')	Reference
**Yr15*	Siskin and RAGT NIL 18_007	Torch and RAGT NIL 18_004	Yr15_R5_F1 Yr15_R5_F2 Yr15_R5_COM	GAAGGTCGGAGTCAACGGATTAGTCAACTTGGATTACACTGAAGTT GAAGGTGACCAAGTTCATGCTAGTCAACTTGGATTACACTGAAGTC AGATATCACACTGAACATACTGATGAG	Ramirez-Gonzalez et al., 2015
			Yr15_R8_F1 Yr15_R8_F2 Yr15_R8_COM	GAAGGTCGGAGTCAACGGATTCAGATCCCCGGTTCTCTCAAG GAAGGTGACCAAGTTCATGCTCAGATCCCCGGTTCTCTCAAA CCCCCAAATGATCGAGAATA	Ramirez-Gonzalez et al., 2015
**Yr17*	Evolution, Brigadier	Santana, Courtot	Yr17_F1 Yr17_F2 Yr17_COM	GAAGGTCGGAGTCAACGGATTCGCCGTTCCGAAYACGAGG GAAGGTGACCAAGTTCATGCTCGCCGTTCCGAAYACGAGA CCCTGGCTTGCACCTTCGACAA	Helguera et al., 2003
**Yr5*	Av**Yr5*	Avocet S	Yr5_F1 Yr5_F2 Yr5_COM	GAAGGTCGGAGTCAACGGATTGCGCCCCTTTTCGAAAAAATA GAAGGTGACCAAGTTCATGCTCTAGCATCAAACAAGCTAAATA ATGTCGAAATATTGCATAACATGG	Marchal et al., 2018
Yr7	Lee, Cadenza	Avocet S	Yr7-A_F1 Yr7-A_F2 Yr7-A_COM	GAAGGTCGGAGTCAACGGATTTTAGTCCTGCCCCATAAGCG GAAGGTGACCAAGTTCATGCTTTAGTCCAGCCCCATAAGCC CAGTGTTAAAACCAGGGAGGA	Marchal et al., 2018
			Yr7-B_F1 Yr7-B_F2 Yr7-B_COM	GAAGGTCGGAGTCAACGGATTTGGAGGTATCATCTGGTGAG GAAGGTGACCAAGTTCATGCTTGGAGGTATCATCGGGTGAA CATCAAAATCATCGCCTATGT	Marchal et al., 2018
			Yr7-D_F1 Yr7-D_F2 Yr7-D_COM	GAAGGTCGGAGTCAACGGATTGCTGGAAAGGCTTGACATCA GAAGGTGACCAAGTTCATGCTGCTGGAAAGGCTTGAGATCG AATGGCGTGGTAAGGACAGA	Marchal et al., 2018
			Cad0127_Yr7_F1 Cad0127_Yr7_F2 Cad0127_Yr7_COM	GAAGGTCGGAGTCAACGGATTAAGTGATGTCGGGAGGAGC GAAGGTGACCAAGTTCATGCTAAGTGATGTCGGGAGGAGT TGGAGAATGGAAGTTCTTTTGTGT	Marchal et al., 2018
			Cad0127_M1_F1 Cad0127_M1_F2 Cad0127_M1_COM	GAAGGTCGGAGTCAACGGATTACATATTCGTGGAGGCCGG GAAGGTGACCAAGTTCATGCTACATATTCGTGGAGGCCGA TGGTGAACTCTGATAGGAACTTC	Marchal et al., 2018

			Cad0127_M5_F1 Cad0127_M5_F2 Cad0127_M5_COM	GAAGGTCGGAGTCAACGGATTCATCATCCATTTTCCCTCTCGC GAAGGTGACCAAGTTCATGCTCATCATCCATTTTCCCTCTCGT AGCTTCTTTAGAACATGCCAAC	Marchal et al., 2018
			Yr7-Cad1551_F1 Yr7-Cad1551_F2 Yr7-Cad1551_COM	GAAGGTCGGAGTCAACGGATTCACAATCATCAAGATGAAGCG GAAGGTGACCAAGTTCATGCTCACAATCATCAAGATGAAGCA CCAACAATATCTCAGTTACCTCATTG	Marchal et al., 2018
			Yr7-Cad1978_F1 Yr7-Cad1978_F2 Yr7-Cad1978_COM	GAAGGTCGGAGTCAACGGATTTGCATCCTTCCAGGACAAATG GAAGGTGACCAAGTTCATGCTTGCATCCTTCCAGGACAAATA AACCAGGGAGGACGCTTATG	Marchal et al., 2018
			Yr7-Cad0127_M3_F1 Yr7-Cad0127_M3_F2 Yr7-Cad0127_M3_COM	GAAGGTCGGAGTCAACGGATTCGGAACCAATCACCTCGGG GAAGGTGACCAAGTTCATGCTCGGAACCAATCACCTCGGA ATGTTGTCCACGGCGATTAA	Marchal et al., 2018
			Yr7-Cad0127_M9_F1 Yr7-Cad0127_M9_F2 Yr7-Cad0127_M9_COM	GAAGGTCGGAGTCAACGGATTGCCTTTCTTCATCTGGCCTTTAGC GAAGGTGACCAAGTTCATGCTGCCTTTCTTCATCTGGCCTTTAGT TGTGGTACGAGTTGGCATACC	Marchal et al., 2018
			BS00016650_51_F1 BS00016650_51_F2 BS00016650_51_COM	GAAGGTCGGAGTCAACGGATTCTATTTCTTCTCCTACTTGTCCTGT GAAGGTCGGAGTCAACGGATTCTATTTCTTCTCCTACTTGTCCTGC CTGCTGTTCCAAGTAGAGAGG	Bouvet et al., 2022
YrSP	Spalding Prolific	AvocetS	YrSP_F1 YrSP_F2 YrSP_COM	GAAGGTCGGAGTCAACGGATTGAGAAAATCAGCAGGTGG GAAGGTGACCAAGTTCATGCTGAGAAAATCAGCAGGTGC AGCGAGTTGAGGACATTGGT	Marchal et al., 2018
			YrSP_M1_F1 YrSP_M1_F2 YrSP_M1_COM	GAAGGTCGGAGTCAACGGATTGTGCTATTATTAGTAGTACTAAAATTTTGACT GAAGGTGACCAAGTTCATGCTGTGCTATTATTAGTAGTACTAAAATTTTGACC GCATACGAGAATAATAATCTGCTGTCTGAA	Marchal et al., 2018
			YrSP_M2_F1 YrSP_M2_F2 YrSP_M2_COM	GAAGGTCGGAGTCAACGGATTATCCCCAAGCAGCTCTGGGTTA GAAGGTGACCAAGTTCATGCTCCCCAAGCAGCTCTGGGTTG CAGATTGTGCGCAAGAGGAATGTCAA	Marchal et al., 2018
			YrSP_M3_F1 YrSP_M3_F2 YrSP_M3_COM	GAAGGTCGGAGTCAACGGATTCAGTTTTTCAAGCATGCCTTGGCTT GAAGGTGACCAAGTTCATGCTAGTTTTTCAAGCATGCCTTGGCTC CACATCTTGTCGCCCTGGGGAA	Marchal et al., 2018

Yr18	Av*Yr18	Avocet S	Yr18-A (wMAS000003)_F1	GAAGGTCGGAGTCAACGGATTGGTATGCCATTTAACATAATCATGAT	http://www.cerealsdb.uk.net/
			Yr18-A (wMAS000003)_F2	GAAGGTGACCAAGTTCATGCTGGTATGCCATTTAACATAATCATGAA	
			Yr18-A (wMAS000003)_COM	TACTATATGGGAGCATTATTTTTTTCC	
			Yr18A new_F1	GAAGGTCGGAGTCAACGGATTCTGGTATGCCATTTAACATAATCATGAA	JIC source
			Yr18A new_F2	GAAGGTGACCAAGTTCATGCTCTGGTATGCCATTTAACATAATCATGAT	
			Yr18A new_COM	CGCATGACAATAAGTTTCACTCATGCAAA	
			Yr18-B (wMAS000004)_F1	GAAGGTCGGAGTCAACGGATTTGTAATGTATCGTGAGAGATTTGCAG	http://www.cerealsdb.uk.net/
			Yr18-B (wMAS000004)_F2	GAAGGTGACCAAGTTCATGCTATTGTAATGTATCGTGAGAGATTTGCAT	
			Yr18-B (wMAS000004)_COM	GATCATTATCTGACCTGTGCGAATGAATA	
			Yr18-B new_F1	GAAGGTCGGAGTCAACGGATTTGTAATGTATCGTGAGAGATTTGCAG	Fang et al., 2020
			Yr18-B new_F2	GAAGGTGACCAAGTTCATGCTATTGTAATGTATCGTGAGAGATTTGCAT	
			Yr18-B new_COM	AGGTGAATAAATATGAGCATCAGT	
			Lr34/Yr18_F1	GAAGGTCGGAGTCAACGGATTGGGAGCATTATTTTTTTCCATCA	Fang et al., 2020
			Lr34/Yr18_F2	GAAGGTGACCAAGTTCATGCTGGGAGCATTATTTTTTTCCATCT	
			Lr34/Yr18_COM	AGCGAATCCAGTATGGAAAT	
			JIC-Yr18-wMAS004_F1	GAAGGTCGGAGTCAACGGATTCCATCATTCAGTCACCTCGCAGT	JIC source
			JIC-Yr18-wMAS004_F2	GAAGGTGACCAAGTTCATGCTCATCATTCAGTCACCTCGCAGC	
			JIC-Yr18-wMAS004_COM	GTGTTTGGAAGTATGAAGCAATAAATCGAT	
Yr36	Paragon +Yr36 and UC1041 +Yr36	Paragon -Yr36 and UC1041 -Yr36	Yr36 (wMAS000017)_F1	GAAGGTCGGAGTCAACGGATTCAAGAGGGGAGAGACATGTTACTTA	http://www.cerealsdb.uk.net/
			Yr36 (wMAS000017)_F2	GAAGGTGACCAAGTTCATGCTCAAGAGGGGAGAGACATGTTACTTT	
			Yr36 (wMAS000017)_COM	GATTATGGGAGTAGGTTGGTGAGATAAAA	

			Yr36 (new)_F1	GAAGGTCGGAGTCAACGGATTCGATGCTTCTCTCAGAACGa	Fu et al., 2009
			Yr36 (new)_F2	GAAGGTGACCAAGTTCATGCTTTCGATGCTTCTTGTAGAACACA	
			Yr36 (new)_COM	GATTGGTTCTTGACGTATGTTTT	
Yr46	PI250413 +ve for Yr46/Lr67	PI250413 -ve for Yr46/Lr67	Yr46-A (csSNP856)_F1	GAAGGTCGGAGTCAACGGATTGCTACTACTATTGGTAGCCTA	Forrest et al., 2014
			Yr46-A (csSNP856)_F1	GAAGGTGACCAAGTTCATGCTGCTACTACTATTGGTAGCCTG	
			Yr46-A (csSNP856)_COM	CCAGTAGCTTATGGCACTCAAA	
			Yr46-B (TM4_67)_F1	GAAGGTCGGAGTCAACGGATTTCATCATCGGCAGGATCCTGCTTC	Moore et al., 2015
			Yr46-B (TM4_67)_F2	GAAGGTGACCAAGTTCATGCTTCATCATCGGCAGGATCCTGCTTG	
			Yr46-B (TM4_67)_COM	AACGTACGTAATCTTGCTTACTGA	
			csSNP723_F1	GAAGGTCGGAGTCAACGGATTGCTGCCCAGAGACGCTTGAGC	Forrest et al., 2014
			csSNP723_F2	GAAGGTGACCAAGTTCATGCTGCTGCCCAGAGACGCTTGAGT	
			csSNP723_COM	GTAGCTCCTCCCTGCGATG	
			csSNP754_F1	GAAGGTCGGAGTCAACGGATTAAATTATAGCAACTAGAATACCTGCATCAA	Forrest et al., 2014
			csSNP754_F2	GAAGGTGACCAAGTTCATGCTAAATTATAGCAACTAGAATACCTGCATCAT	
			csSNP754_COM	GTGCACTAAAGAATTGCACATGTGCATAA	
			csSNP856_F1	GAAGGTCGGAGTCAACGGATTGCTACTACTATTGGTAGCCTG	Forrest et al., 2014
			csSNP856_F2	GAAGGTGACCAAGTTCATGCTGCTACTACTATTGGTAGCCTA	
			csSNP856_COM	CCAGTAGCTTATGGCACTCAAA	
			csSNP275_F1	GAAGGTCGGAGTCAACGGATTATAAGAGTATGGTTCTCTGGCGACT	Forrest et al., 2014
			csSNP275_F2	GAAGGTGACCAAGTTCATGCTATAAGAGTATGGTTCTCTGGCGACA	
			csSNP275_COM	TTTGGTCTCTATAACCCGCCAGGAT	

Table 3. Published KASP markers for cloned yellow rust resistance genes *Yr5, Yr7, YrSP, Yr15, Yr18, Yr36* and *Yr46,* and the Yr gene *Yr17*, tested against established control wheat lines with and without target Yr gene

*Only in the case of *Yr5, Yr15* and *Yr17* did the primers prove diagnostic for the target Yr gene and were subsequently used to screen Yellowhammer GWAS panel.

3.3. Bi-parental population mapping

Bi-parental populations were developed by the partners to allow for validation of the yellow rust resistance MTAs identified in the Yellowhammer GWAS wheat panel. The crosses made, populations developed, and analyses undertaken are shown in Table 4.

3.3.1. Dickens x Reflection QTL mapping

The population Dickens x Reflection was screened using the Breeders 35k array. This enabled direct comparison of the location of identified QTL to the MTAs identified in the Yellowhammer GWAS panel. The population was assessed for yellow rust resistance in field trials by RAGT in 2018, 2019, 2021 and 2022. Genetic linkage maps were constructed using R/qtl (Broman et al., 2003). To construct the genetic map, all segregating markers that showed polymorphism in at least one parent were used. Ratios of marker segregation were calculated using Chi-square test. Markers that satisfied the expected Mendelian segregation ratio were included for mapping ($p = 0.01$). Markers were grouped with a minimum logarithm of odds (LOD) score of 5.0 and a recombination frequency of 0.25. A regression mapping algorithm was used to build the linkage map. Map distances were calculated in centiMorgans (cM) according to the Kosambi mapping function (Kosambi, 1943). QTL analysis was performed using R/qtl (Broman et al., 2003). Interval mapping (IM) was initially performed to identify potential QTL, followed by a multiple QTL model (MQM), using the stepwiseqtl function for forward and backward selections, with a check for interactions to identify multiple QTL. Significance thresholds were determined using 1,000 permutations and $\alpha = 0.05$. QTL mapping was undertaken by Ruth Bryant at RAGT and Camila Zanella at NIAB.

3.3.2. Mallet x Solstice QTL mapping

The population Mallet x Solstice was screened using the Breeders 35k array. This enabled direct comparison of the location of identified QTL to the MTAs identified in the Yellowhammer GWAS panel. The population was assessed for yellow rust resistance in field trials by DSV in 2021 and 2022. The genetic map construction and QTL analysis followed the same procedures above. QTL mapping was undertaken by Camila Zanella at NIAB.

3.3.3. Revelation x Solstice QTL mapping

The population Revelation x Solstice was screened using the Gatersleben 7k array. This did not allow a direct comparison of the location of the identified QTL with the MTAs identified in the Yellowhammer GWAS panel. The population was assessed for yellow rust resistance in field trials by Sejet in 2022. The genetic map construction and QTL analysis followed the same procedures above. QTL mapping was undertaken by Camila Zanella at NIAB.

3.3.4. Flanders x Solstice QTL mapping

The population Flanders x Solstice was screened using the Breeders 35k array, which enabled direct comparison of the location of identified QTL to the MTAs identified in the Yellowhammer GWAS panel. The population was assessed for yellow rust resistance in field trials by Syngenta in 2021. QTL mapping was undertaken by staff at Syngenta.

3.3.5. Graham x Solstice QTL mapping

The population Graham x Solstice was screened using the Breeders 35k array, which enabled direct comparison of the location of identified QTL to the MTAs identified in the Yellowhammer GWAS panel. The population was assessed for yellow rust resistance in field trials by Syngenta in 2021. QTL mapping was undertaken by staff at Syngenta.

3.3.6. Gladiator x Vuka KASP marker screen

It was decided not to create a full genetic map of the Gladiator x Vuka population, but to screen the population with KASP markers designed to MTAs identified and developed in the WAGTAIL project and believed to be present in Gladiator. These included KASP markers made to MTAs 2A019, 2B051, 2B752 and 3B004. Marker screens were undertaken by RAGT. The population was assessed for yellow rust resistance by DSV in field trials in 2021 and 2022.

3.3.7. Crusoe x Solstice KASP marker screen

It was decided not to create a full map of the Crusoe x Solstice population, but to screen the population with KASP markers designed to MTAs identified and developed in the WAGTAIL project and believed to be present in Crusoe. These included KASP markers made to MTAs UN004 and 2A019. Marker screens were undertaken by DSV. The population was assessed for yellow rust resistance by DSV in field trials in 2020, 2021 and 2022.

3.3.8. KWS QTL mapping

KWS developed mapping populations for the wheat varieties Stigg, Cougar and Illustrious all crossed to the yellow rust susceptible variety Solstice. Genetic mapping and QTL analyses of these lines was undertaken by KWS.

Parents	Pop. size and type	Mapping approach	Year	Partner
Dickens x Reflection	128 RILs made by SSD	35K breeders SNP array: 4652 SNPs retained	2018 2019 2021 2022	RAGT
Gladiator x Vuka	93 RILs made by SSD	It was decided not to create a full genetic map of this population but to screen with KASP markers for MTA believed to be in Gladiator.	2020 2021	RAGT
Mallet x Solstice	102 lines made by SSD	35K breeders SNP array	2021 2022	DSV
Crusoe x Solstice	96 lines	It was decided not to create a full genetic map of this population but to screen with KASP markers for MTA believed to be in Crusoe.	2020 2021 2022	DSV
Revelation x Solstice	124 lines	7K Gatersleben wheat array	2020 2021	Sejet
Graham x Solstice	106 lines made by SSD	Problems encountered with DNA and marker quality.	2021	Syngenta
Flanders x Solstice	144 lines made by SSD		2021	Syngenta
Stigg x Solstice	101 lines, mix of F3 & F4	Genotyped with KWS internal array. Analyses undertaken by KWS.		KWS
Cougar x Solstice	84 lines, mix of F3 & F4	Genotyped with KWS internal array. Analyses undertaken by KWS.		KWS
Illustrious x Solstice	101 lines, mix of F3 & F4	Genotyped with KWS internal array. Analyses undertaken by KWS.		KWS

Table 4. Bi-parental crosses and population development. The following crosses and populations were not pursued: Spotlight x Solstice (DSV); Yeoman x Solstice (Syngenta); Reflection x Evolution (Sejet); Villein x Solstice (Sejet); Extase x Solstice (Lantmannen); Elysee x Solstice (Lantmannen); NIAB Synthetic 112 x Solstice (Lantmannen); NIAB Synthetic x Robigus (Lantmannen); RIL – Recombinant Inbred Line; SSD – Single Seed Decent.

3.4. Time course analysis of *Puccinia striiformis* f. sp. *tritici* development in near-isogenic lines (NILs) containing selected yellow rust resistant Marker Trait Associations

A time course analysis of *Pst* development and differential wheat gene expression in NILs developed by Limagrain and RAGT (Table 5) was undertaken, examining flag leaves inoculated with the *Pst* isolate NIAB 19/501. *Pst* isolate 19/501 was collected from the wheat variety Bennington in 2019 and has been shown to be virulent on the yellow rust resistance genes *Yr1, 2, 3, 4, 6, 7, 9, 17, 25, 32*, and on the wheat varieties Sp – Spalding prolific; So – Solstice; Wa – Warrior; Ca – Cadenza, St – Sterling, in seedling tests. Plants of each NIL were grown to flag leaf emergence. The flag leaf on each of the first three tillers of each plant were inoculated with *Pst* isolate NIAB 19/501 and then placed at 8ºC, in total darkness, at 100% humidity for 24 hours. Segments of *Pst* inoculated flag leaves were taken at 1, 3, 7 and 14 days after inoculation (dai) for microscopic examination and RNAseq analysis. Yellow rust infection was allowed to develop on the third flag leaf and the level of yellow rust infection scored at 21 dai.

Target Yr gene	**NIL name**	**Parents**	**Company**
Yr15	18PD1040.003.007 – *Yr15* 18PD1040.003.004 - susceptible	Siskin x Torch (background)	RAGT
WAGTAIL HIT2.2	19PD1020.016.028 – [a]HIT2.2 19PD1020.016.050 – susceptible	Option x Torch (background)	RAGT
WAGTAIL HIT1, HIT4/5, HIT2.1	LGWU17-3213-018 RRR (HIT1;HIT4/5[b];HIT2.1) RRS (HIT1;HIT4/5[b]) SRR (HIT4/5;HIT2.1) [c]SRS (HIT4/5)	Belgrade x Evolution	Limagrain

Table 5. Near-isogenic lines (NILs) developed by Limagrain and RAGT. [a]The HIT2.2 in the RAGT NIL was found to be different from the HIT2.1 in the Limagrain NIL. [b]HIT4/5 was fixed in all the NIL made by Limagrain. NIL The NIL SRS was used as the susceptible for comparisons with NIL RRR, RRS and SRR. RRR contains HIT1;HIT4/5[b];HIT2.1; RRS contains HIT1;HIT4/5[b] and SRR contains HIT4/5.

3.4.1. Microscopic examination of *P. striiformis* f. sp. *tritici* development

3.4.1.1 Preparation of leaf samples for microscopy: The flag leaf samples were cut into 2 cm segments. The leaf segments were cleared (removal of chlorophyll) and fixed in a solution of ethanol: chloroform (3:1, v/v) and 0.15% (v/w) trichloroacetic acid for 72 hours. After 72hours the leaf segments were rinsed in this solution, washed twice in 50% ethanol

for 10 min, and then placed in 0.1M NaOH for 1.5 hours to ensure optimal clearing. The leaf segments were then washed twice with deionized water (DI). The samples were left in 0.1 M Tris-HCl buffer (pH 5.8) for 30 minutes before staining with 0.1% (w/v) Uvitex 2B (dissolved in 0.1 M Tris-HCl buffer) for 8 min. Four washes with DI water followed and one with 25% glycerol. The samples were left in DI water overnight to remove excess Uvitex B stain. Leaf segments were stored in 50% glycerol until ready to be mounted on microscope slides. The leaf segments were mounted in 75% glycerol on a microscope slide and covered with a cover slip which was sealed with nail polish. The microscope slides were covered to ensure light did not reach the Uvitex B-stained leaf segments before observation down the microscope.

3.4.1.2 Observation and scoring of Pst development: Pst development was observed using epifluorescence microscopy on a Leica DM 2500, applying filter A4 and magnifications x10 and x20. The number of spores observed at defined stages of *Pst* development (Table 6) were counted, recording, on average, 50 spores on each of three leaf segments.

***Pst* stage**	**Description of *Pst* development stage**
Pst1	Germinated spore
Pst2	Germ tube enters stomata and forms a Sub-Stomatal Vesicle (SSV)
Pst3	Formation of Infection Hyphae and Haustorial Mother Cells (HMC)
Pst4	Formation of first Runner Hypha (RH)
Pst5	Runner Hyphae elongate and infect additional host cells
Pst6	Formation of pustules

Table 6. *Pst* developmental stages recorded

3.4.2. Transcriptomic analysis of differential wheat gene expression

3.4.2.1. RNA extraction, RNAseq library preparation and analysis

Segments of *Pst* inoculated flag leaves were taken at 1, 2, 3, 7 and 14 days after inoculation and placed on RNAlater (Sigma-Aldrich). Segments of uninoculated flag leaves were sampled as a control. RNA was extracted from flag leaves using RNAeasy Plant Mini kit (Qiagen) with on-column DNase digestion (Qiagen). RNA integrity was confirmed on 1% agarose-bleach gel (Aranda et al., 2012) and quantified by NanoDrop 8000 (Thermo Scientific). RNA quality was also assessed using the 2100 Bioanalyser (Agilent). For each time-point three biological replicate were used to make libraries for RNAseq. The RNA samples were sent to Novogene, Cambridge, UK for library construction using poly A enrichment. Each library was sequenced using paired-end sequencing on

Illumina HiSeq 4000. Novogene undertook quality control checks on the RNA, the construction of 132 RNA libraries, data quality control, base calling and formatting, delivering approximately 150 bp paired-end reads.

Further quality checks on the paired-end reads were performed: Adapter sequences were trimmed using Trim Galore (https://github.com/FelixKrueger/TrimGalore). After trimming, reads of less than 50 bases were removed from the data set. To identify trends, clusters and outliers among the three biological replicate RNAseq libraries and among treatments, principal components analysis was performed using ggplot2 package (Wickham 2016).

Read count quantification was performed using kallisto (Bray et al 2016) and the wheat Chinese Spring IWGSC cDNA RefSeq v2.0 (International Wheat Genome Sequencing Consortium) transcriptome reference. Differential gene expression analysis between NILs were performed using DESeq2 R package (Love et al 2014). Parameters were set to Log2FC ± 1 and adjusted value of p <0.05 to detect differentially expressed genes (DEGs) between the NILs. Clustering analyses were performed using pheatmap package (https://CRAN.R-project.org/package=pheatmap) to plot a heatmap of the top 50 DEGs based on *p* adjusted value.

4. Results

4.1. Variation in yellow rust infection levels across locations

Considerable variation was seen in the levels of yellow rust infection within the Yellowhammer GWAS panel across the different field trial locations and years. This reflected the different environments, some locations being more conducive to yellow rust infection (Figure 1 data for 2022 shown). However, the differences in yellow rust infection may also be due to differences in the race composition of the populations of *Pst* present at each location.

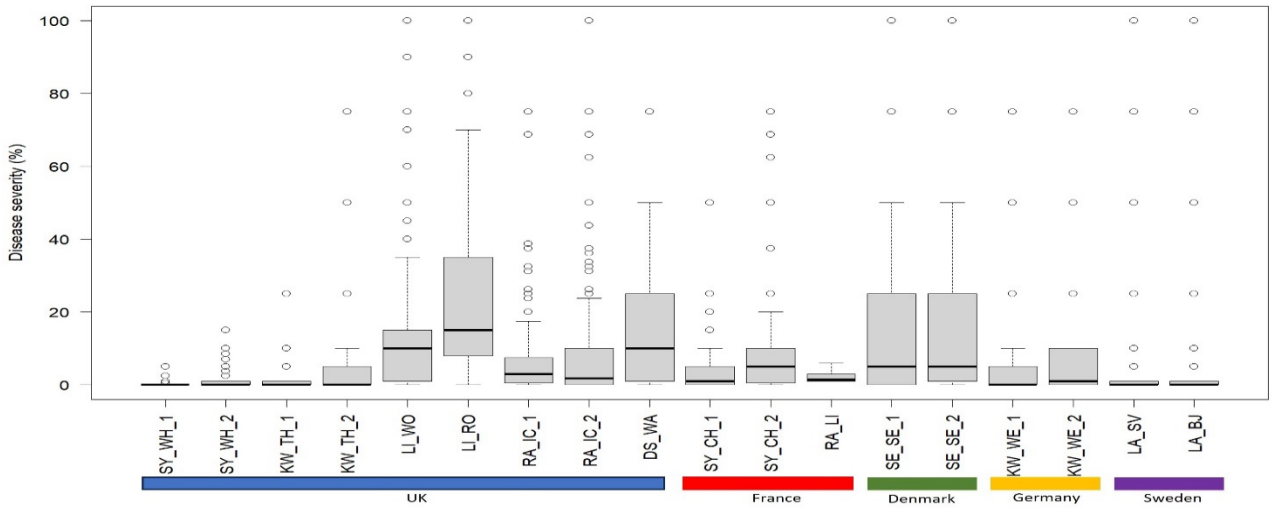


Figure 1. Yellow rust percentage infection levels seen across trial sites in 2022. SY_WH – Syngenta, Whittlesford; SY_CH – Syngenta, Centre-Val de Loire; KW_TH – KWS, Thriplow; KW_WE – KWS, Wietze, Niedersachan; LI_WO – Limagrain, Woolpit; LI_RO – Limagrain, Rothwell; RA_IC – RAGT, Ickleton; RA_LI – RAGT, Annoeullin; DS_WA – DSV, Waddington; SE_SE – Sejet, Syddanmark; LA_SV – Lantmannen, Svalov; LA_BJ – Lantmannen, Bjertorp.

4.2. GWAS analysis of yellow rust resistance in the Yellowhammer winter wheat panel

Assessment of yellow rust resistance, over multiple seasons and locations, in general identified the same yellow rust resistant marker trait associations (MTAs), although any given MTA was not always significantly effective across all locations and/or in all seasons. The most significant MTAs found across seasons and locations are presented in Table 7

Forty-two MTAs were identified using the cut off criteria: An MTA was called if (1) it had a significance score above threshold of > 3.0, or (2) had a significance score above threshold of > 1.5 in more than one season or location. Of these 42 MTAs, 29 were present in over 90% of the GWAS panel lines, suggesting that these yellow rust resistance loci have become fixed in UK and Northern European winter wheat germplasm. MTAs that occurred at a low frequency (less than 25%) were 1A011 (18.5%), 2B153 (17.4%), 3B004 (21.8%) and UN004 (6.25%). Other MTAs present in the Yellowhammer panel at lower frequencies, and therefore of potential value to winter wheat breeding, are 2A647 (43.8%), 2D999 (30.7%), 3A010 (57.4%), 3A241 (61.1%), 4B605 (38.4%) and 6A612 (43.1%). The **MTA 2A019**, while only present in 41.9% of the Yellowhammer panel, was considered to be ineffective towards yellow rust across Northern Europe. The **MTAs 4A713** and **5A020** were both present in 80.6% of the panel.

Yellow rust MTAs	**Score above threshold**	**Peak marker (position)**	**R allele frequency in panel**	**Year and location at which resistant allele was detected**					
				2018	**2019**	**2020**	**2021**	**2022**	**Country**
1A011	4.01	AX-94762508 (3.26)	18.5%	y	Y	y	n	y	UK/DE/DK
1A524	2.65	AX-95192108 (510.4)	95.4%	n	N	y	n	y	FR/DE
1B545	2.8	AX-94911435 (543.1)	96.1%	n	N	y	y	n	SE/UK
1D002	3.26	AX-94519856 (2.67)	93.8%	n	N	n	y	n	DE
2A019	22.78	AX-94988794 (10.6)	41.9%	y	Y	n	n	n	Only effective in UK
2A087	4.01	AX-94885471 (88.0)	95.8%	n	Y	y	y	n	FR/DK/UK/ DE
2A647	1.64	AX-94611897 (647.9)	43.8%	n	Y	y	n	n	DK/UK
2B051	6.92	AX-94671304 (48.0)	96.5%	n	N	y	y	y	SE/DE/UK/ FR

2B153	3.77	AX-95247247 (155.0)	17.4%	n	Y	y	n	y	FR/UK
2B236	10.36	AX-95243361 (250.0)	96.5%	n	Y	y	y	y	FR/UK
2B298	12.67	AX-94524231 (290.7)	96.8%	n	Y	y	y	y	FR/UK/DK/SE
2B559	5.48	AX-95116475 (560.0)	96.5%	n	N	y	n	y	DK/UK
2B592	5.32	AX-94475906 (592.5)	93.8%	n	Y	y	y	n	DK/FR/UK
2B657	5.37	AX-94386142 (654.5)	96.5%	n	n	y	y	n	DK/FR/UK
2B752	5.39	AX-94650643 (752.9)	90.7%	n	y	y	n	y	DK/FR/UK
2D619	3.20	AX-94485190 (644.1)	97.7%	n	n	n	n	y	FR
2D999	6.75	AX-94410941 (47.0)	30.7%	n	y	y	n	n	DK/UK
3A010[a]	2.22	AX-95236410 (7.8)	57.4%	n	n	y	n	n	UK
3A045	2.65	AX-95009752 (20.3)	95.1%	n	n	y	y	n	SE/FR
3A241[b]	2.92	AX-94671257_b (241.9)	61.1%	n	n	y	n	y	UK
3B004	3.78	AX-95129156 (4.2)	21.8%	n	y	y	y	y	UK/DK
3B050	6.07	AX-95138608 (49.9)	92.1%	n	y	y	n	n	UK/FR
3B561	4.43	AX-94607716 (576.0)	96.3%	n	y	y	y	y	FR/UK//SE
4A713	5.59	AX-94926827 (737.5)	80.6%	y	y	y	n	y	UK/FR/DK
4B605	2.39	AX-94601523 (605.2)	38.4%	n	y	n	n	y	UK/DK/DE/FR
4B672	8.41	AX-94486277 (673)	95.8%	n	y	n	n	n	SE

5A020	2.07	AX-94734779 (20.4)	80.6%	n	y	y	y	y	UK/FR/DK/DE
5A048	3.86	AX-94382505 (46.2)	96.3%	n	y	y	n	n	UK/DK
5A475	5.08	AX-94428048 (476.2)	93.1%	n	y	y	y	y	SE/FR/DK
5A607	10.31	AX-95127839 (617)	94.4%	n	n	y	y	y	SE/DE/UK
5A702	3.1	AX-94905515 (686)	93.8%	n	y	y	y	y	DK/FR/UK/DE
5B478	6.06	AX-95114472 (479)	96.8%	n	y	y	y	y	FR/SE/UK/DK/DE
5B697	1.71	AX-94522127 (703.3)	95.4%	n	y	y	y	y	UK/FR/DE
6A612	11.29	Position 596.77 to 617.66	43.1%	y	y	y	y	y	UK/FR/DE/DK
6B450	3.57	AX-94636603 (450.10)	96.9%	n	n	y	n	n	UK
6B718	2.18	AX-94951003 (714.6)	93.1%	y	n	y	y	y	DE/UK/SE/FR
7A076	2.08	AX-94735683_a (76.4)	96.8%	n	y	y	n	y	DE/UK
7A730	4.97	AX-94951226 (730.4)	96.5%	n	y	y	y	y	FR/UK/DE
7B033	2.02	AX-94638774 (356)	92.8%	n	n	n	y	n	FR/UK
7D548	5.27	AX-94427492 (548.9)	93.8%	n	y	y	y	y	UK/SE/DK/FR
UN004	8.64	AX-94543007 (263)	6.25%	n	y	y	y	y	UK/DE/DK
UN025	5.92	AX-94943212 (688)	93.3%	n	y	y	n	y	FR/UK

Table 7. An MTA was considered a potentially genuine yellow rust resistance locus when (1) it had a significance score above threshold of > 3.0, or (2) had a significance score above threshold of > 1.5 in more than one season or location. [a]The MTA 3A010 was included in the table despite not meeting the criteria for inclusion because it was subsequently detected in the bi-parental mapping population Reflection x Dickens. [b]The MTA 3A241 was included as the significance score above threshold (2.92) was close to the threshold value of 3.0. n – MTA was not detected in this year; y - the MTA was detected in this year.

4.2.1. MTAs found on wheat chromosomes group 1:

The **MTA 1A011** was primarily effective in the UK (scores above threshold: 2022 – 4.01; 2020 – 2.03 and 2018 – 1.71), although was significant in Germany (2.34) and Denmark (1.79) in 2019. In the WAGTAIL project a yellow rust resistance was identified in the same location – YR_RARESUS03. **MTA 1A524** was a small effect MTA that occurred in Germany (2.65) and France (2.2) in 2020, and again in France in 2022 (1.15). The **MTA 1B545** was seen in Sweden in 2020 (2.8) and in the UK in 2021 (1.33). This MTA may be located on a 1B/1R translocation. The **MTA 1D002** was only detected in Germany in 2021 (3.26).

4.2.2. MTA found on wheat chromosomes group 2:

The **MTA 2A019** was primarily effective in the UK in 2018 (9.38) and 2019 (22.78), not being significant in 2020, 2021 or 2022. MTA 2A019 was also detected in France in 2019 (5.52) and 2020 (1.02), and in Denmark in 2019 (4.11). 2A019 located to the same position as YR_HIT01 identified in the WAGTAIL project. The position of this MTA spanned a region from the end of the short arm of chromosome 2A up to 24.5cM and was considered to represent a translocation from the tertiary wheat gene pool, *Aegilops ventricosa.* The yellow rust resistance gene *Yr17* was transferred to hexaploid wheat from *A. ventricosa* as a translocation to the short arm of chromosome 2A: 2NS/2AS (Bariana and McIntosh 1993). The **MTA 2A087** also covered an extended region on chromosome 2A, from position 80.5 to 105.4, and therefore may also be on the translocation from *A. ventricosa.* This MTA was effective in Germany (4.01), Denmark (3.41) and the UK (2.88) in 2019. It then appeared in France in 2020 (1.97) and 2021 (3.51). The **MTA 2A647** was detected in Denmark in 2019 (1.64), and in the UK in 2019 (1.32) and 2020 (1.47).

As expected, considerably more MTAs were detected on chromosome 2B. Many race-specific yellow rust resistance genes have been reported on chromosome 2B. **MTA 2B051** was most effective in Sweden in 2021 (6.92) and 2022 (6.49). It was also detected in Germany in 2021 (3.94) and 2020 (2.63), in the UK in 2020 (4.17) and 2021 (2.0), and in France in 2020 (3.51). **MTA 2B153** was found in in the UK in 2022 (3.77) and in France in 2020 (3.06) and 2019 (1.75). The **MTA 2B236** was found in France and the UK. In France it was detected in 2020 (10.36) and 2021 (3.61). In the UK it was detected first in 2019 (5.25), then again in 2020 (4.2) and 2022 (1.53). **MTA 2B298** was

found principally in France (2019-6.51; 2020-12.67; 2021-5.36; 2022-2.63) and the UK (2019-6.83; 2020-5.17; 2021-5.15; 2022-4.02) but did appear in Denmark in 2019 (2.91) and Sweden in 2021 (2.0). The **MTA 2B559** was principally effective in Denmark in 2020 (5.48) and 2022 (1.48), with a low level of significance being detected at one location in the UK in 2022 (1.85). The **MTA 2B592** was most effective in 2020 in Denmark (5.32) and France (5.15). However, it was also detected in the UK in 2019 (2.54) and 2021 (3.80). The **MTA 2B657** was most significant in Denmark in 2020 (5.37) but was also detected in 2021 in France (2.4) and the UK (2.28). The **MTA 2B752** was the strongest in Denmark (5.39) and France (5.03) in 2019 but was also found in the UK in 2019 (3.53). MTA 2B752 was also found in Denmark in 2020 (2.22) and in the UK in 2022 (1.63).

Only two MTA was found on chromosome 2D, MTA 2D619 and 2D999. **MTA 2D619** was only found in France in 2022 (3.2). The **MTA 2D999** was found in Denmark in 2020 (6.75) and in the UK in 2019 (2.04). However, this MTA was unusual. Of the 2 alleles identified, allele “a” was effective in Denmark, while allele “b” was effective in the UK.

4.2.3. MTA found on wheat chromosomes group 3:

The **MTA 3A010** was only detected in the UK 2020 (2.22). While this MTA did meet the selection criteria for calling a MTA as significant (a MTA was considered a potentially genuine yellow rust resistance locus when (1) it had a significance score above threshold of > 3.0, or (2) had a significance score above threshold of > 1.5 in more than one season or location), we included 3A010 in Table 7 because this MTA was subsequently found in the bi-parental mapping population Reflection x Dickens. The **MTA 3A045** was detected in France in 2020 (2.09) and Sweden in 2021 (2.65). **MTA 3A241** was only detected in the UK, first in 2020 (2.92) and again in 2022 (1.95).

The **MTA 3B004** was detected in the UK and Denmark. In the UK it was detected in 2022 (3.78), 2021 (3.66) and 2020 (3.13), while in Denmark this MTA was detected in 2022 (2.46), 2021 (2.63) and 2019 (2.28). The **MTA 3B050** was detected in the UK in 2020 (6.07) and 2019 (1.7), and in France in 2020 (4.03). The **MTA 3B561** was found in France in 2020 (4.43) and 2021 (2.04), and in the UK in 2022 (3.0) and Sweden in 2019 (1.54).

4.2.4. MTA found on wheat chromosomes group 4:

MTA 4A713 was most effective in the UK in 2022. It was less effective in the UK in 2018, 2019, and 2021, but did not show as a significant MTA in 2020. 4A713 was effective in Denmark in 2022 (score above threshold 2.43) and in France in 2020 (score above threshold 1.82).

MTA 4B605 exhibited only low levels of significance above the threshold, appearing in the UK (1.95), Denmark (2.39) and Germany (2.03) in 2019, and again in the UK (2.18), Denmark (1.18) and now in France (1.41) in 2022. **MTA 4B672** was only detected in Sweden in 2019, having a score above threshold of 8.41.

4.2.5. MTA found on wheat chromosomes group 5:

The **MTA 5A020** was of small effect (2.07) but appeared in all years from 2019 until 2022. It was most prominent in the UK (2.07) and France (2.07), but also appeared in Denmark in 2019 (1.47) and Germany in 2020 (1.45). **MTA 5A048** was detected in the UK in 2019 (1.3) and 2020 (3.86), and in Denmark (1.7) in 2019. **MTA 5A475** was principally effective in Sweden in 2019 (5.08). A reduced effect was seen in Sweden in 2021 (2.15) and 2022 (1.28), and in France in 2019 (1.82) and 2020 (1.96), and in Denmark in 2019 (1.67). **MTA 5A607** was detected in Sweden in 2022 as a major effect association (10.31). 5A607 was also detected in Sweden in 2021 (3.3) but had a much lower level of significance. 5A607 was detected in Germany in 2020 (1.9), and in the UK in 2020 (1.73) and 2022 (2.72). The **MTA 5A702** was detected in France (2.59) and the UK (2.01) in 2019, in Denmark (3.1), France (1.53) and Germany (1.32) in 2020, only in the UK (2.66) in 2021, and then in Denmark (1.59), France (1.49) and the UK (1.47) in 2022.

The **MTA 5B478** was detected in all five countries, although was most effective in 2020 and in France (6.06); DK (3.61); UK (3.2); DE (2.33). In 2022 5B478 was most effective in Sweden (4.73) and in Denmark (2.03). It was also effective in the UK in 2019 (2.46) and 2021 (1.29). The **MTA 5B697** was detected across the UK, Germany and France from 2019 to 2022, but at very low levels of significance: 2019 and 2020, France (1.27 and 1.52); 2021 in Germany (1.44) and in 2022 in the UK (1.71).

4.2.6. MTA found on wheat chromosomes group 6:

The **MTA 6A612** was significant across a considerable length of the chromosome 6A. This may indicate that the yellow rust resistance is located on a translocation. 6A612 contributed a large yellow rust resistance, indicating a probably major, R-gene resistance. The resistance was effective across all years, showing a more significant effect in the UK, France and Germany. 6A612 was not effective in Sweden. Scores above threshold were 2022, FR-10.0; DE-8.93; UK-7.13; DK-5.0, 2021 UK-11.29; FR-10.01 and DK-5.1; 2020, UK-10.86, DE-9.68, FR-7.79 and DK-2.16, 2019, FR-8.87; UK-6.33; DK-5.16 and DE-3.44, 2018, UK-3.39. MTA 6A612 was found in 43.1% of the wheat lines within the Yellowhammer GWAS panel.

The **MTA 6B450** was only detected in the UK in 2020 (3.57). The **MTA 6B718** was detected in the UK in 2018 (2.14), 2020 (2.05), 2021 (2.02) and 2022 (1.83), in Germany in 2020 (2.45) and in 2021 (2.18), in France in 2022 (1.16) and Sweden in 2022 (1.75).

4.2.7. MTA found on wheat chromosomes group 7:

The **MTA 7A076** was found in the UK in 2019 (1.35) and 2022 (1.38) and in Denmark in 2020 (2.08). The **MTA 7A730** was found in all years from 2019 to 2022, in France 2019 (2.96), 2020 (3.05) and 2021 (4.97), in the UK in 2019 (1.94) and 2022 (3.28). 7A730 was also detected in Germany in 2021 (1.63).

The **MTA 7B033** was only detected in 2021, in France (2.02) and the UK (1.73). The **MTA 7D548** was primarily effective in the UK, being detected in 2019 (5.27), 2020 (1.47), 2021 (2.15) and 2022 (3.38). However, it was detected in Denmark in 2019 (1.42) and 2022 (1.32), Sweden in 2021 (1.48) and in France in 2022 (1.35).

The MTA classified as **UN004** was detected in the UK in 2020 (5.49), 2021 (6.74) and 2022 (8.64), in Germany in 2019 (5.65) and 2020 (5.38) and in Denmark in 2019 (3.4) and 2020 (3.87). The **MTA UN025** was found in France in 2019 (3.83) and 2020 (5.92), and in the UK in 2019 (5.63) and 2022 (3.41).

4.3. Validation of the Marker-Trait Associations identified in the Yellowhammer GWAS panel

To verify the MTAs for yellow rust resistance identified in the Yellowhammer GWAS panel several bi-parental populations were made by the partners (Table 4). MTAs have been confirmed in the varieties Dickens, Gladiator, Crusoe, Mallet and Revelation. The MTAs confirmed were 1A011, 1B545, 2A019, 2A087, 2B153, 2D999, 3A010, 3B004, 4B605, 5B677, 5A685, 6A612 and UN004.

4.3.1. RAGT populations:

RAGT developed two bi-parental mapping populations, Reflection x Dickens and Gladiator x Vuka. The wheat variety Reflection was not included in the Yellowhammer GWAS panel, while Dickens carried most of the yellow rust resistance MTAs identified in the GWAS. Therefore, we were not able to predict which MTAs we would expect to see segregating in the cross Reflection x Dickens. However, QTL mapping identified the MTAs 2B153, 3A010, 5B697 and 6A612, all predicted by the GWAS analysis to be present in the variety Dickens. The MTA 2A019 was also identified in Dickens with the 2018 and 2019 field trial yellow rust infection data. As expected, this MTA was not seen in 2021, corresponding to the loss of this MTA as an effective resistance in 2020. However, MTA 2A019 contributed to the yellow rust resistance in this population in 2022, suggesting that the resistance may have a residual effect. The MTA 2D999 was detected in the variety Reflection.

Screening with KASP markers designed to specific yellow rust MTAs thought to be present in Gladiator confirmed that MTAs 2A019 and 3B004 were segregating in the Gladiator x Vuka population. Although the MTA 2A019 was not significant in the GWAS analysis after 2019, it was detected in the Gladiator x Vuka population in 2021 and 2022. The MTA 3B004 was only detected in 2021 in the Gladiator x Vuka population.

4.3.2. DSV populations:

The partner DSV developed two bi-parental populations by crossing the yellow rust susceptible wheat variety Solstice to Mallet and Crusoe. The Mallet x Solstice population was predicted to segregate for the MTAs, 1A011, 5A020 and UN004, Mallet contributing the resistance allele for all

three MTAs. QTL mapping confirmed the presence of 1A011 and UN004 in Mallet, although the MTA 5A020 was not detected.

The wheat variety Crusoe should contain the yellow rust resistance MTAs 1A011, 2A019, 4B605, 5A020 and UN004, with Solstice carrying the susceptible alleles for these MTAs. In addition, Solstice contains the resistant alleles for MTAs 2B033, 2B051 and 4A713. KASP markers made to the principal SNP marker defining MTA UN004, i.e., AX-94543007, confirmed that UN004 was segregating in the Crusoe x Solstice population in 2020, 2021 and 2022.

4.3.3. Sejet populations:

Sejet developed and analysed one mapping population for the Yellowhammer project - Revelation x Solstice. The Revelation x Solstice population should segregate for 2A019, 2B033, 2D999, 3A010, 3A241, 3B050 and 4B605. The Revelation x Solstice population was mapped using the Gatersleben 7K array and not the breeders 35K array used in the GWAS. This meant that a direct comparison of QTL and MTAs could not be made. We were however able to identify QTL segregating within the Revelation x Solstice population, in both 2020 and 2021, that corresponded to the locations of the MTAs 2A019 and 3A010. We could confirm that MTAs 1B545, 2A087 and 5A685 were segregating in the Revelation x Solstice population in 2020, and MTA 4B605 in 2021.

4.3.4. Syngenta populations:

Problems were encountered with the quality of the marker data obtained on the two populations made by Syngenta: Flanders x Solstice and Graham x Solstice. Therefore, imputation of missing marker data was undertaken, replacing missing data points with estimated values based on existing marker information. As this approach can introduce bias into the analysis only the largest and most significant QTL were considered as viable results. The Graham x Solstice population should segregate for 1A011, 2D999, 3B004, 5A020 and 6A612. However, only one major QTL was identified with the 2021 yellow rust data set. This QTL came from Graham and lay on chromosome 6A, so could be the MTA 6A612. The Flanders x Solstice population should segregate for multiple MTAs, with Flanders carrying the resistant allele for MTAs 2B153, 4B605, 5A020 and 6A612, while Solstice contains the resistant alleles for 3A010, 3A241 and 5A702. However, only two QTLs were identified in the Flanders x Solstice population, one on chromosome 2B which was donated by Flanders and therefore could be 2B153, and another on 2D donated by Solstice. No MTA was identified on chromosome 2D in the GWAS analysis that would be segregating between Flanders and Solstice.

4.3.5. KWS populations:

KWS developed three mapping populations, crossing Solstice to Stigg, Illustrious and Cougar. The small size of the populations meant that only in the population Stigg x Solstice could a yellow rust resistance QTL be identified. This QTL corresponded to the Yr gene *Yr15*.

4.4. Identification of known yellow rust resistance genes within the Yellowhammer panel

The Yellowhammer GWAS panel was screened with KASP markers reported to be diagnostic for cloned yellow rust resistance genes *Yr5* and *Yr15,* as well as markers for *Yr17*. The *Yr5* marker was able to differentiate between the positive, *Yr5*-containing, control wheat line Av*6*Yr5* and the negative control AvocetS. However, *Yr5* was not detected in any of the wheat lines making up the Yellowhammer GWAS panel. *Yr15* appeared less frequently, occurring in the homozygous state in only 10 (2.3%) of the lines, and in 14 (3.3%%) lines in the heterozygous state. Because the frequency at which *Yr15* occurred in the GWAS panel was below 5% *Yr15* was not detected in the GWAS analysis. *Yr17* was detected in 160 of the 427 GWAS panel lines in the homozygous state (37.5%), and in the heterozygous state in another 23 lines (5.4%). Only 4 varieties were found where both *Yr15* and *Yr17* were confirmed to be present in the homozygous state: Ochre, Timaru, Crispin and Marston.

Six KASP markers reported for *Yr7* (Table 3) were screened using the *Yr7* wheat differential varieties Lee and Cadenza as the *Yr7* positive control, and DNA from AvocetS as the negative, non-*Yr7* containing, control. However, these KASP markers did not distinguish between the *Yr7* positive and negative DNA controls. The *YrSP* markers (Table 3) could not distinguish between the wheat varieties Spalding Prolific and AvocetS. None of the markers reported for *Yr18* could distinguish between the positive control line Av*Yr18* and the negative control line AvocetS. Similarly, the markers reported to be diagnostic for *Yr46* could not distinguish between the NIL PI250413 +ve and -ve for *Yr46*. The only marker reported to be diagnostic for *Yr36,* that distinguished between Paragon with and without *Yr36,* was the marker Yr36 (new) (Table 3). However, alleles in the GWAS panel lines did not cluster with either the Paragon+*Yr36* or the Paragon-*Yr36* control DNAs.

4.5. Microphenotyping and RNAseq analyses of Near-Isogenic Lines (NILs) carrying different yellow rust resistance MTAs

NILs were developed by Limagrain and RAGT that carry MTAs for yellow rust resistance identified in the WAGTAIL project. These MTAs were designated HITs1, 2 and 4/5 (Table 5). In the Limagrain NIL HIT4/5 was fixed, only HIT1 and HIT2 were segregating. HIT1 corresponds to the MTA 2A019 found in the Yellowhammer panel, and HIT2 to the MTA 6A612. HIT4/5 corresponds to the Yellowhammer MTA 2D999. Using KASP markers designed to the WAGTAIL marker: Kukri_c18149_581 we could confirm the R allele for HIT1 (2A019) in the lines RRR and RRS (Table 5). The R allele for HIT2 (6A612) in RRR and SRR was confirmed using KASP marker to AX-94634087. However, these markers indicated that the HIT2 in the RAGT NIL 19PD1020.016.028 is not the same yellow rust resistance locus as in the Limagrain NIL. Therefore, we designated the

Limagrain HIT2 and HIT2.1 and the RAGT HIT2 as HIT2.2, The cloned yellow rust resistance gene *Yr15* was confirmed to be present in 18PD1040.003.007 using published markers (Table 3).

The average yellow rust infection field scores for the Limagrain NIL are 1 for RRR (HIT 1; HIT 4/5; HIT 2.1), 4 for SRR ((HIT 4/5; HIT 2.1), 6 for RRS (HIT1; HIT 4/5) and 7 for line SRS (HIT 4/5) in 2022. For the RAGT NILs with and without HIT2.2 the yellow rust phenotypes in 2021 were recorded as 2 (NIL 19PD1020.016.028) and 5 (NIL 19PD1020.016.050). For the NIL with and without Yr15 the yellow rust phenotypes in 2021 were 1 (18PD1040.003.007) and 5.25 (18PD1040.003.004)

4.5.1. Glasshouse inoculation of NILs with and without target yellow rust resistance loci.

The NILs were used in a controlled *Pst* inoculation experiment undertaken in the glasshouse. Flag leaves were inoculated with the *Pst* isolate NIAB 19/501 collected from the wheat variety Bennington in 2019. NIAB 19/501 is virulent on the yellow rust seedling resistances *Yr1, 2, 3, 4, 6, 7, 9, 17, 25, 32*, and on the wheat varieties Spalding prolific; Solstice; Warrior; Cadenza and Sterling. Flag leaf material was collected from three plants before inoculation with NIAB 19/501 to provide a control for RNAseq analysis. Flag leaves were collected from *Pst* inoculated plants at 1, 3, 7 and 14 days after inoculation (dai) for both RNAseq analysis and microscopic observation of *Pst* development. At 21 dai yellow rust infection was scored on flag leaves. Differences in the levels of yellow rust infection could be seen between the NILs (Figure 2).

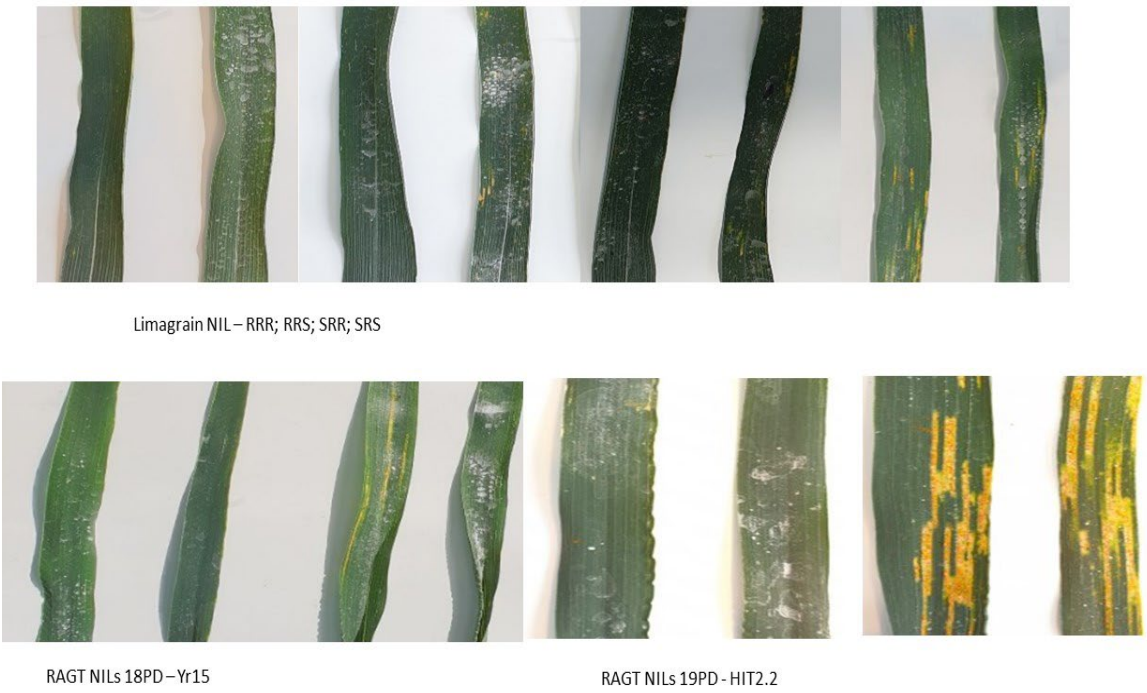


Figure 2. Yellow rust infection on near-isogenic lines

4.5.2. Microscopic analysis of *Pst* isolate NIAB 19/501 on flag leaves of NILs with and without target yellow rust resistance loci.

The development of *Pst* isolate NIAB 19/501 was followed microscopically (Figure 3) recording the number of spores that were present at each of the *Pst* development stages listed in Table 6.

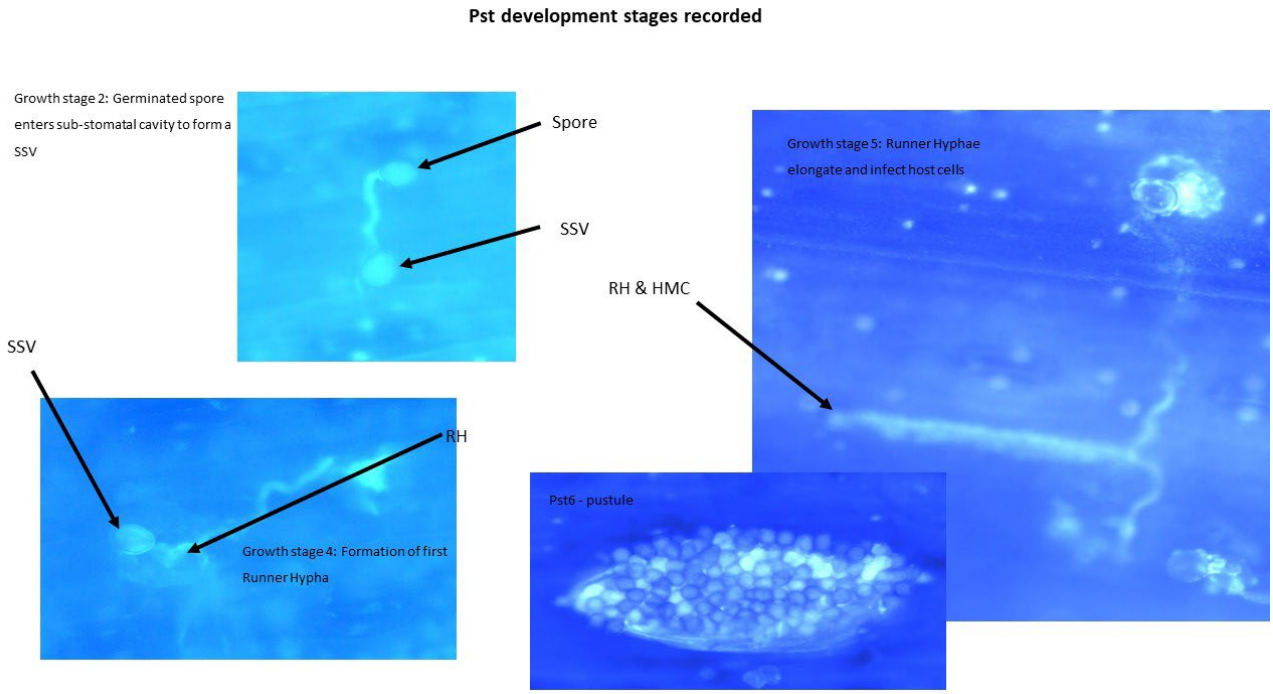


Figure 3. Developmental stages of *Puccinia striiformis* f. sp. *tritici* recorded. SSV - Sub-stomatal vesicle; HMC – haustorial mother cell; RH – runner hyphae.

Assessment of the NILs with (RRS) and without (SRS) HIT1 (corresponding to MTA 2A019), and that did not have HIT2.1 (corresponding to 6A612), but were fixed for WAGTAIL HIT4/5, indicated that HIT1 appeared to primarily stop *Pst* development at development stage Pst4, i.e., formation of runner hyphae. In line SRS more spores were found at later developmental stages, i.e., Pst5 and Pst6, than in the line RRS (Figure 4 A). Assessment of the NIL with (SRR) and without (SRS) HIT2.1 (corresponding to MTA 6A612), that did not have HIT1 (corresponding to 2A019) but was fixed for HIT4/5, indicated that HIT2.1 allowed runner hyphae elongation, but prevented pustule formation (Figure 4 B).

Assessment of the RAGT NILs with (19PD-028) and without (19PC-050) HIT2.2 did not differ in terms of the rate of *Pst* development (Figure 5A). Similar percentages of germinated spores had reached each of the subsequent developmental stages at 3, 7 and 14 dai in the two NILs. Similarly, the NILs with (18PD-007) and without (18PD-004) *Yr15* did not differ in terms of the rate of *Pst* development (Figure 5B). This would suggest that HIT2.2 and *Yr15* have little effect on the early stages of *Pst* development, conferring resistance by restricting pustule formation.

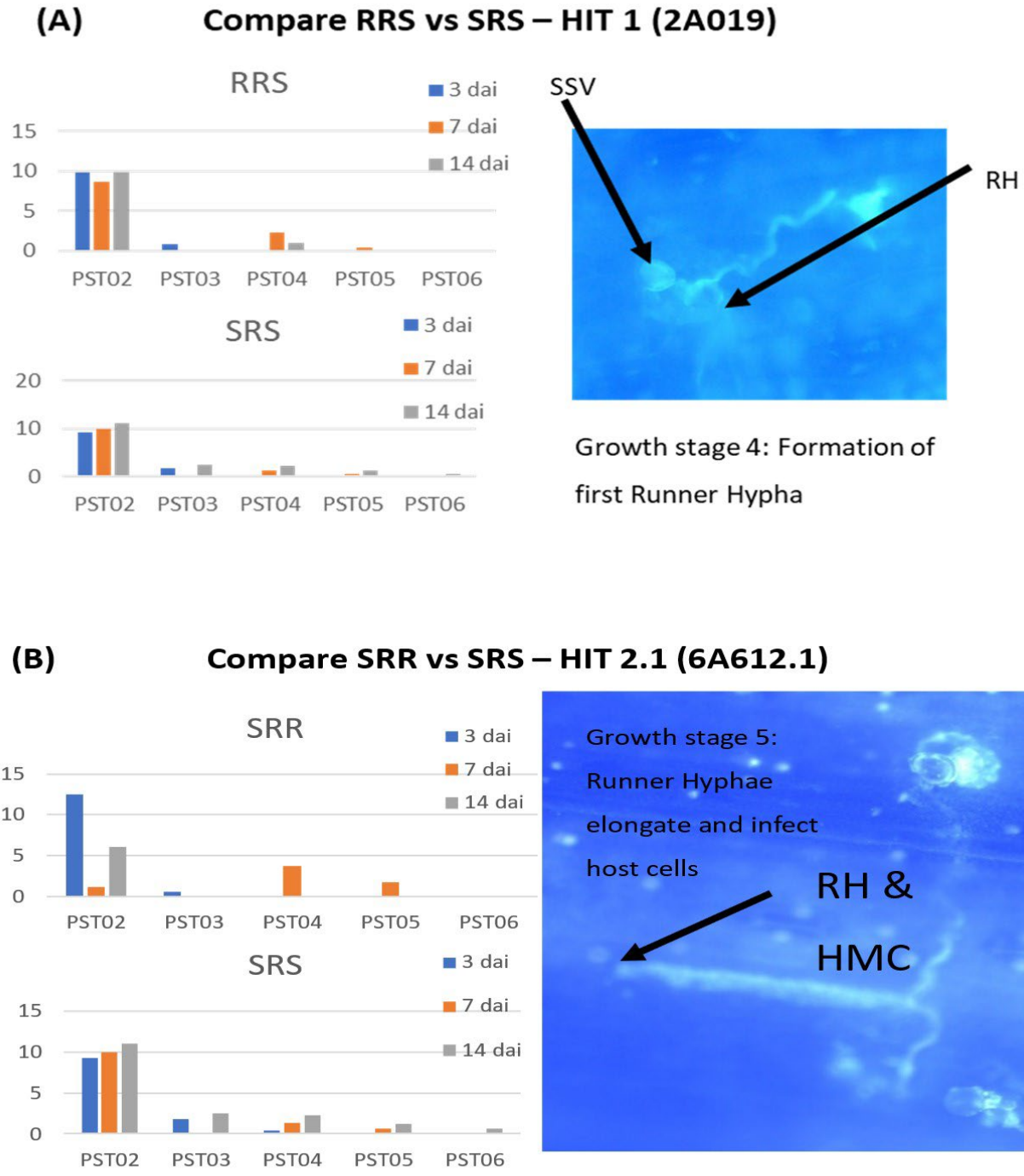


Figure 4. Comparison of NILs (A) with and without HIT 1 (2A019), and (B) with and without HIT 2.1 (6A612). The percentage of *Pst* developmental stages as a percentage of germinated spores.

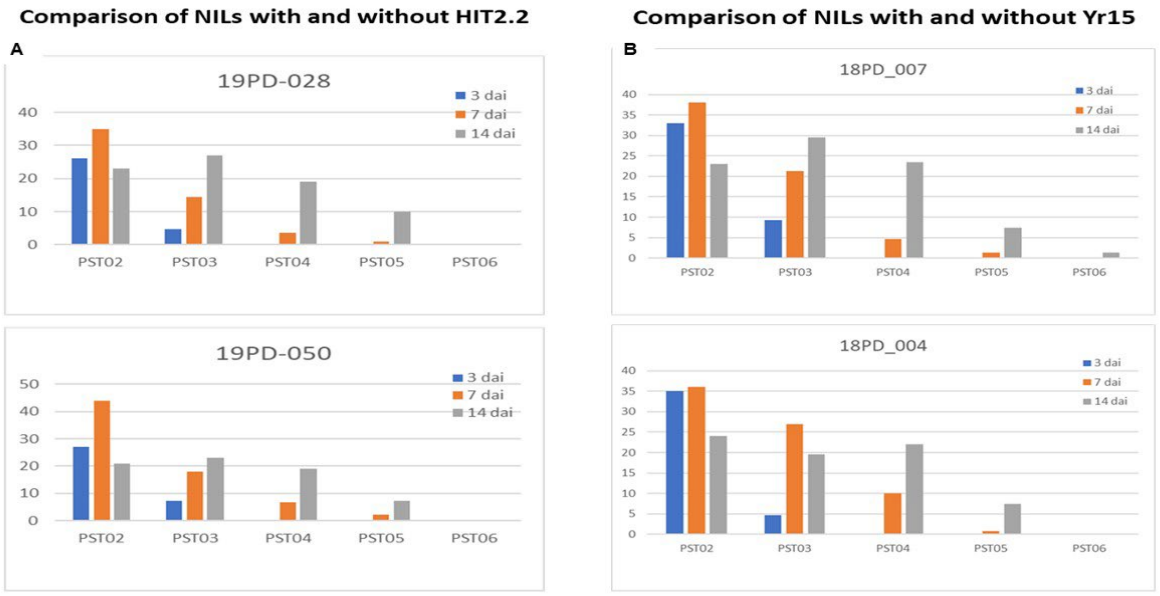
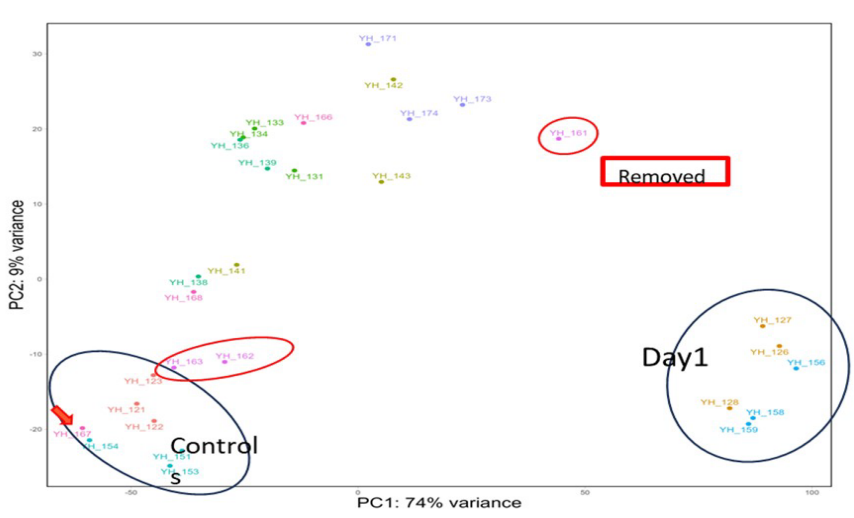


Figure 5. Comparison of (A) NILs with HIT2.2 (19PD-028) and without HIT2.2 (PD19-050 and (B) NILs with *Yr15* (18PD_007) and without *Yr15* (18PD_004). The percentage of *Pst* developmental stages are shown as a percentage of total germinated spores.

4.5.3. RNAseq analysis of NILs

4.5.3.1 RAGT lines 18PD1040.003.007 (Yr15+) and 18PD1040.003.004 (yr15)

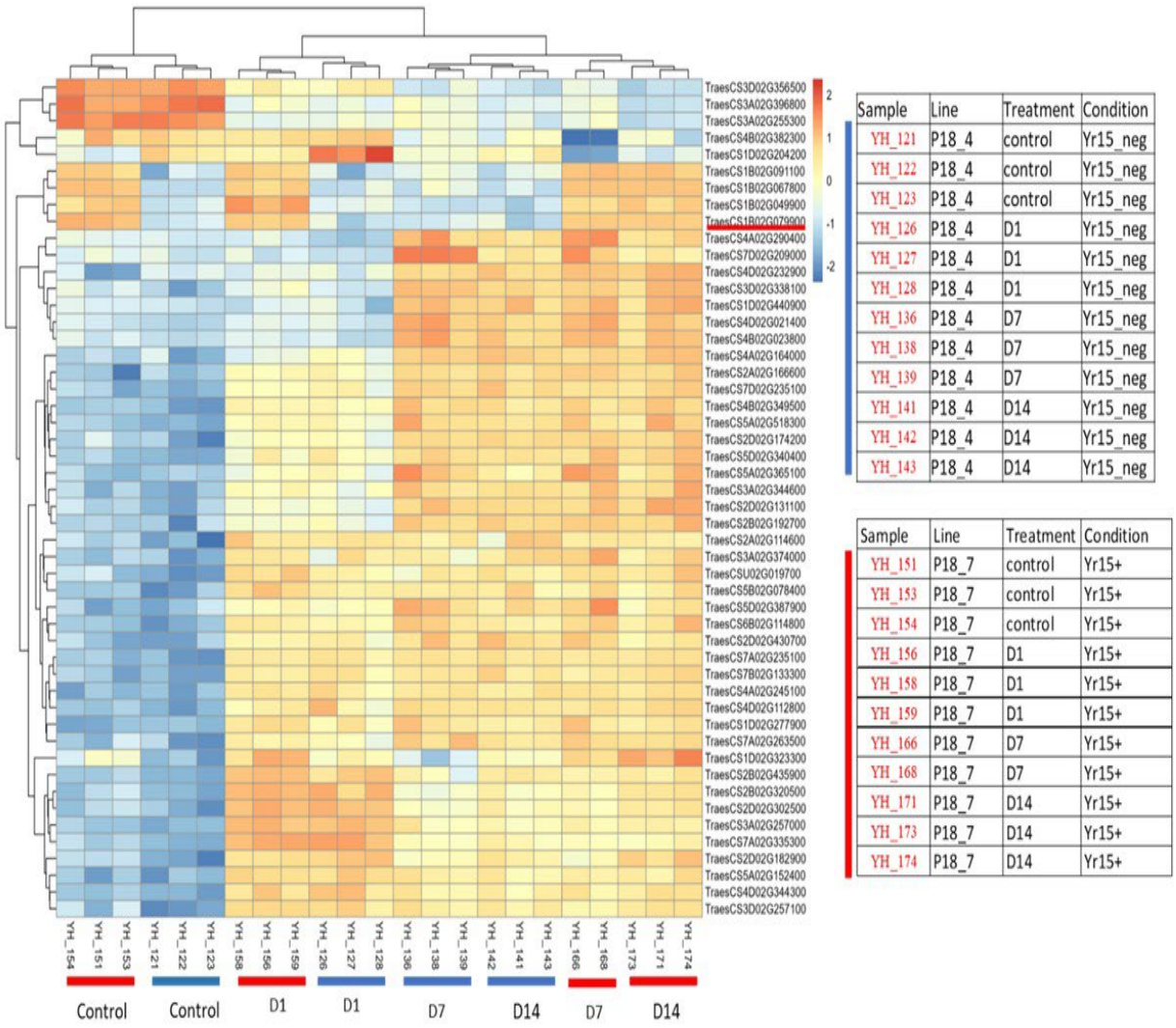
Segments of *Pst* inoculated flag leaves were sampled at 1, 3, 7 and 14 dai from three biological replicate samples from the NILs 18PD1040.003.007 (*Yr15*+) and 18PD1040.003.004 (*yr15*). Uninoculated flag leaves were sampled as a control. Paired-end reads were mapped against the Chinese Spring IWGSC cDNA RefSeq v2 reference. The libraries were normalized and a PCA analyse was performed, PC1 explained 74% and PC2 explained 9% of the variance (Figure 6).



Sample	Line	Treatment	Condition
YH_121	P18_4	control	Yr15_neg
YH_122	P18_4	control	Yr15_neg
YH_123	P18_4	control	Yr15_neg
YH_126	P18_4	D1	Yr15_neg
YH_127	P18_4	D1	Yr15_neg
YH_128	P18_4	D1	Yr15_neg
YH_131	P18_4	D3	Yr15_neg
YH_133	P18_4	D3	Yr15_neg
YH_134	P18_4	D3	Yr15_neg
YH_136	P18_4	D7	Yr15_neg
YH_138	P18_4	D7	Yr15_neg
YH_139	P18_4	D7	Yr15_neg
YH_141	P18_4	D14	Yr15_neg
YH_142	P18_4	D14	Yr15_neg
YH_143	P18_4	D14	Yr15_neg
YH_151	P18_7	control	Yr15+
YH_153	P18_7	control	Yr15+
YH_154	P18_7	control	Yr15+
YH_156	P18_7	D1	Yr15+
YH_158	P18_7	D1	Yr15+
YH_159	P18_7	D1	Yr15+
YH_161	P18_7	D3	Yr15+
YH_162	P18_7	D3	Yr15+
YH_163	P18_7	D3	Yr15+
YH_166	P18_7	D7	Yr15+
YH_167	P18_7	D7	Yr15+
YH_168	P18_7	D7	Yr15+
YH_171	P18_7	D14	Yr15+
YH_173	P18_7	D14	Yr15+
YH_174	P18_7	D14	Yr15+

Figure 6. Principal component analysis comparing the three biological replicates of the uninoculated control and *Pst* inoculated flag leaf samples 1 (D1), 3 (D3), 7 (D7) and 14 (14D) dai of 18PD1040.003.007 (*Yr15*+ - PD18_007) and 18PD1040.003.004 (yr15) (PD18_004) NILs.

Inconsistent results among the biological replicates for 3 dai were observed. Therefore the 3 dai RNAseq libraries were removed from the analysis. Library YH_167 (7 dai) was also removed from the analysis due to poor similarities with the other 2 biological replicates for 7 dai. Comparing all the remaining libraries together, i.e., the uninoculated flag leaf controls and the *Pst* inoculated flag leaves at 1, 7 and 14 dai, 98 wheat differentially expressed genes (DEGs) were identified (Figure 7). TraesCS1B02G079900 was identified among the top 10 DEGs. TraesCS1B02G079900 is the Chinese Spring non-functional wtk1 allele homolog of the *Yr15* gene, WKP1 (Klymiuk et al., 2019). Visual analysis of the raw data showed that very low or no expression of the TraesCS1B02G079900 RNA transcript was observed in the susceptible NIL in comparison with the NIL carrying *Yr15*.



Sample	Line	Treatment	Condition
YH_121	P18_4	control	Yr15_neg
YH_122	P18_4	control	Yr15_neg
YH_123	P18_4	control	Yr15_neg
YH_126	P18_4	D1	Yr15_neg
YH_127	P18_4	D1	Yr15_neg
YH_128	P18_4	D1	Yr15_neg
YH_136	P18_4	D7	Yr15_neg
YH_138	P18_4	D7	Yr15_neg
YH_139	P18_4	D7	Yr15_neg
YH_141	P18_4	D14	Yr15_neg
YH_142	P18_4	D14	Yr15_neg
YH_143	P18_4	D14	Yr15_neg

Sample	Line	Treatment	Condition
YH_151	P18_7	control	Yr15+
YH_153	P18_7	control	Yr15+
YH_154	P18_7	control	Yr15+
YH_156	P18_7	D1	Yr15+
YH_158	P18_7	D1	Yr15+
YH_159	P18_7	D1	Yr15+
YH_166	P18_7	D7	Yr15+
YH_168	P18_7	D7	Yr15+
YH_171	P18_7	D14	Yr15+
YH_173	P18_7	D14	Yr15+
YH_174	P18_7	D14	Yr15+

Figure 7. Top 50 genes different expressed between 18PD1040.003.007 (*Yr15*+ - P18_7) and 18PD1040.003.004 (yr15) (P18_4) comparing uninoculated controls and all *Pst* inoculated time-point in one analysis. The Chinese Spring gene TraesCS1B02G079900 (underlined in red) is the non-functional wtk1 allele homolog of *Yr15* (WKP1).

4.5.3.2. Limagrain NILs with and without HIT1 and HIT2.1

Segments of *Pst* inoculated flag leaves were sampled at 1, 2, 3, 7 and 14 dai from three biological replicate samples from the Limagrain NILs RRR, RRS, SRR and SRS (Table 8). Uninoculated flag leaves were sampled as a control. The libraries were normalized and a PCA analyse was performed (Figure 8). The outlying libraries YH48, YH57 and YH89 were removed from further analysis. Paired-end reads were mapped against the Chinese Spring IWGSC cDNA RefSeq v2 reference. Comparing the RRR libraries to the SRS libraries (Figure 9) identified 284 DEG within the genomic region (0 to 57 Mb) covering HIT1 (2A019) and 158 DEG within the genomic region (595 – 617 Mb) covering HIT2.1 (6A612) (Figure 9; Table 9). Of the 284 DEG located within HIT1 30 were annotated as NBS-LRR genes and 16 as kinases, while within the HIT2.1 genomic region 9 were annotated as NBS-LRR genes and 18 as kinases.

Table 8. RNAseq libraries of Limagrain NIL: RRR, RRS, SRR and SRS

Sample	Condition	Treatment
YH31	RRR	mock
YH32	RRR	mock
YH33	RRR	mock
YH36	RRR	day1
YH38	RRR	day1
YH39	RRR	day1
YH42	RRR	day2
YH43	RRR	day2
YH44	RRR	day2
YH46	RRR	day3
YH47	RRR	day3
YH48	RRR	day3
YH51	RRR	day7
YH52	RRR	day7
YH53	RRR	day7
YH56	RRR	day14
YH57	RRR	day14
YH58	RRR	day14
YH01	RRS	mock
YH02	RRS	mock
YH03	RRS	mock
YH06	RRS	day1
YH07	RRS	day1
YH08	RRS	day1
YH11	RRS	day2
YH12	RRS	day2
YH13	RRS	day2
YH16	RRS	day3
YH17	RRS	day3
YH18	RRS	day3
YH21	RRS	day7
YH22	RRS	day7
YH23	RRS	day7
YH26	RRS	day14
YH27	RRS	day14
YH28	RRS	day14

Sample	Condition	Treatment
YH91	SRR	mock
YH92	SRR	mock
YH_94	SRR	mock
YH96	SRR	day1
YH_99	SRR	day1
YH_100	SRR	day1
YH102	SRR	day2
YH103	SRR	day2
YH104	SRR	day2
YH108	SRR	day3
YH109	SRR	day3
YH_110	SRR	day3
YH111	SRR	day7
YH112	SRR	day7
YH_114	SRR	day7
YH116	SRR	day14
YH_117	SRR	day14
YH_120	SRR	day14
YH61	SRS	mock
YH62	SRS	mock
YH63	SRS	mock
YH66	SRS	day1
YH67	SRS	day1
YH68	SRS	day1
YH72	SRS	day2
YH73	SRS	day2
YH74	SRS	day2
YH76	SRS	day3
YH77	SRS	day3
YH78	SRS	day3
YH81	SRS	day7
YH82	SRS	day7
YH83	SRS	day7
YH87	SRS	day14
YH88	SRS	day14
YH_89	SRS	day14

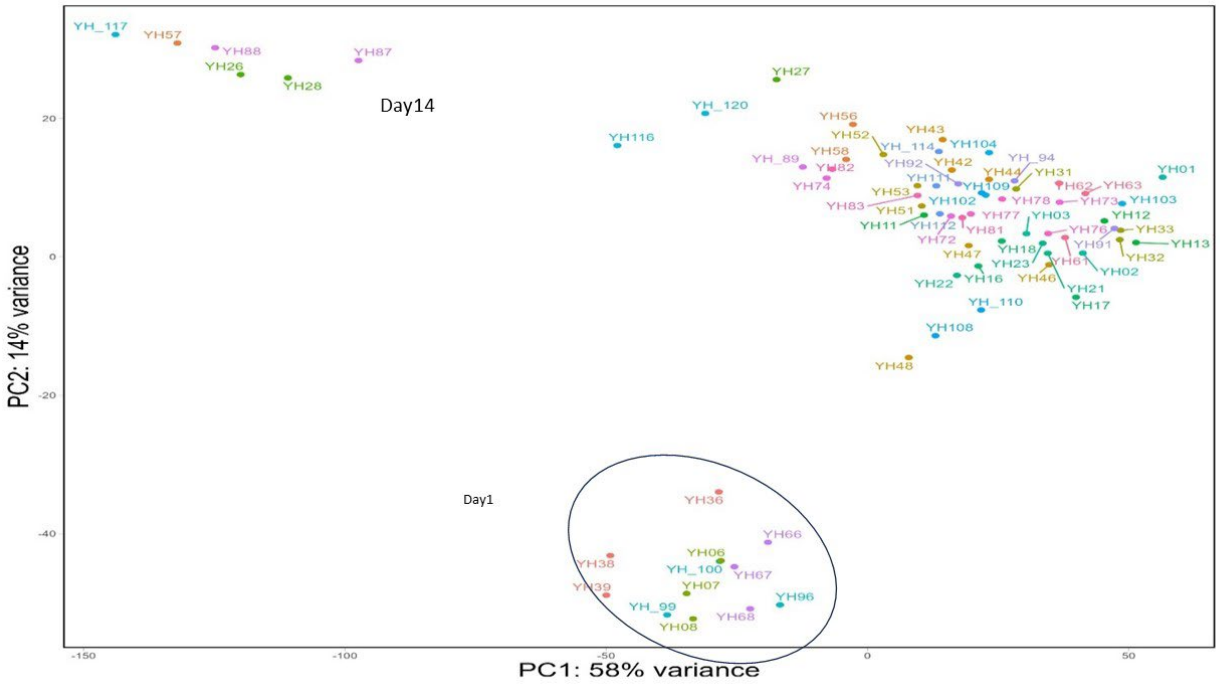
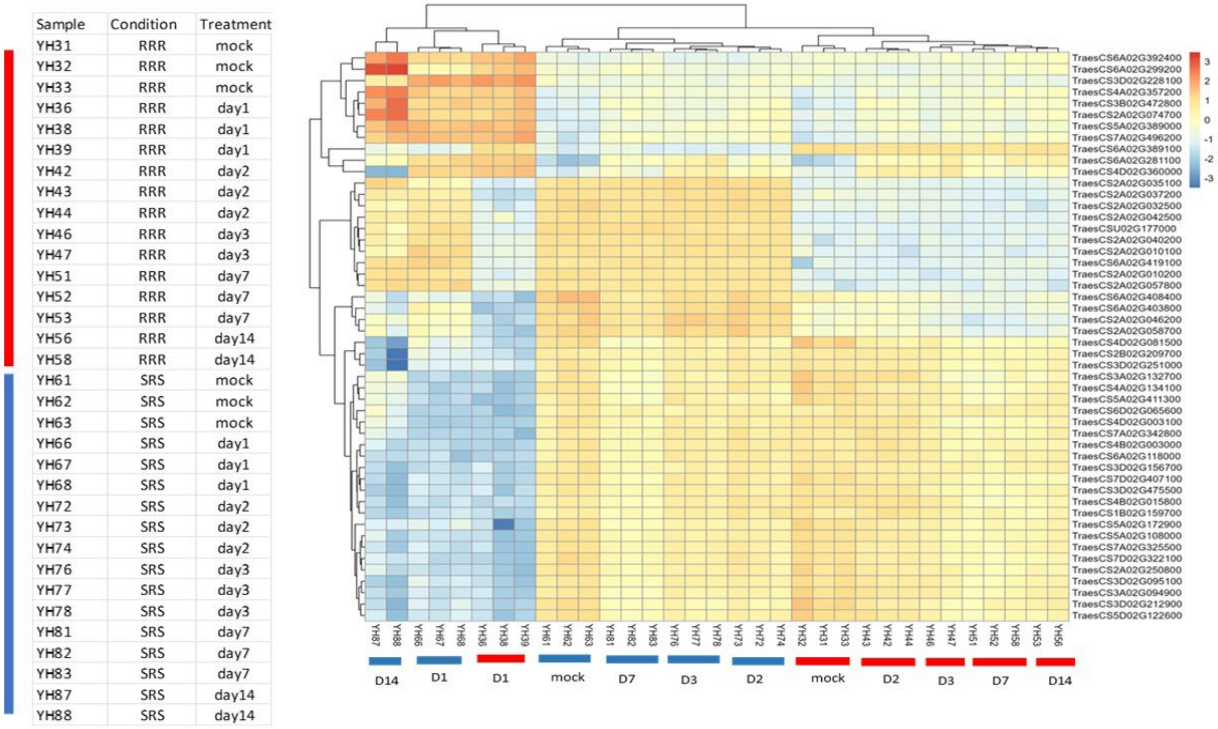


Figure 8. Principal component analysis comparing the three biological replicates of the uninoculated control and *Pst* inoculated flag leaf samples of the Limagrain NIL RRR, RRS, SRR and SRS at 1, 2, 3, 7 and 14dai.



Sample	Condition	Treatment
YH31	RRR	mock
YH32	RRR	mock
YH33	RRR	mock
YH36	RRR	day1
YH38	RRR	day1
YH39	RRR	day1
YH42	RRR	day2
YH43	RRR	day2
YH44	RRR	day2
YH46	RRR	day3
YH47	RRR	day3
YH51	RRR	day7
YH52	RRR	day7
YH53	RRR	day7
YH56	RRR	day14
YH58	RRR	day14
YH61	SRS	mock
YH62	SRS	mock
YH63	SRS	mock
YH66	SRS	day1
YH67	SRS	day1
YH68	SRS	day1
YH72	SRS	day2
YH73	SRS	day2
YH74	SRS	day2
YH76	SRS	day3
YH77	SRS	day3
YH78	SRS	day3
YH81	SRS	day7
YH82	SRS	day7
YH83	SRS	day7
YH87	SRS	day14
YH88	SRS	day14

Figure 9. Top 50 genes different expressed between NIL RRR and SRS.

Table 9. Top different expressed genes identified between comparison of Limagrain NILs RRR and SRS, as found on chromosomes 2A and 6A within the genomic regions covering HIT1 (2A019) and HIT2.1 (6A612).

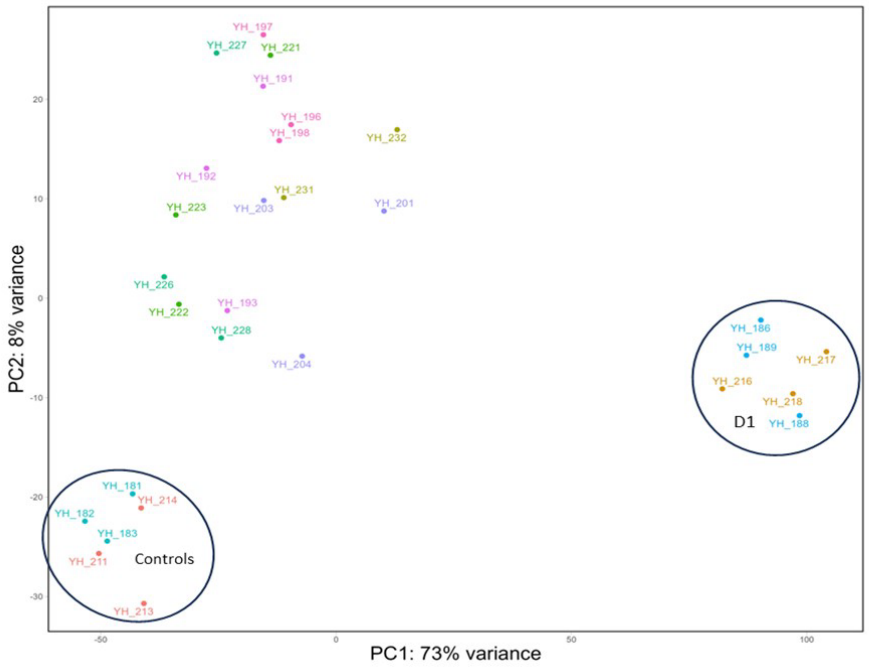
Position	Gene	Chrm	Start_RefS	End_RefSe	Annotation
27	TraesCS2A02G010100	chr2A	3959343	3962913	Pyruvate dehydrogenase E1 component subunit alpha
2	TraesCS2A02G010200	chr2A	3964835	3968074	#N/A
5	TraesCS2A02G032500	chr2A	14808977	14813420	ATP sulfurylase (Sulfate adenylyltransferase)
1	TraesCS2A02G035100	chr2A	15269852	15270683	Glyceraldehyde-3-phosphate dehydrogenase
4	TraesCS2A02G037200	chr2A	15874833	15876687	dimethylallyl, adenosine tRNA methylthiotransferase
7	TraesCS2A02G040200	chr2A	16554144	16557226	Eukaryotic translation initiation factor 3 subunit M
47	TraesCS2A02G042500	chr2A	17025906	17033628	NBS-LRR disease resistance protein
26	TraesCS2A02G046200	chr2A	18058353	18059469	Nuclear transport factor 2 (NTF2)-like protein
19	TraesCS2A02G057800	chr2A	24278547	24285255	#N/A
29	TraesCS2A02G058700	chr2A	24530232	24532708	Chaperone protein DnaJ
46	TraesCS2A02G074700	chr2A	33041451	33044917	Acetyltransferase component of pyruvate dehydrogenase c
43	TraesCS6A02G389100	chr6A	6.05E+08	6.05E+08	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex sub
22	TraesCS6A02G392400	chr6A	6.07E+08	6.07E+08	3-ketoacyl-CoA thiolase-like protein
3	TraesCS6A02G403800	chr6A	6.11E+08	6.11E+08	Divinyl reductase
48	TraesCS6A02G408400	chr6A	6.13E+08	6.13E+08	Photosystem II 22 kDa, chloroplastic
36	TraesCS6A02G419100	chr6A	6.17E+08	6.17E+08	Alpha-1,3-mannosyl-glycoprotein 2-beta-N-acetylglucosam

4.5.4.3. RAGT lines 19PD1020.016.028 (HIT2.2+) and 19PD1020.016.050 (HIT2.2)

The NILs 19PD1020.016.028 and 19PD1020.016.050 were developed by RAGT and were thought to differ for WAGTAIL HIT2, corresponding to the Yellowhammer MTA 6A612. These lines were genotyped using KASP markers (see results section 4.5) to check for the presence of the 6A612 MTA. However, these markers indicated that the yellow rust resistance in the RAGT NIL 19PD1020.016.028 is not the same yellow rust resistance locus as in the Limagrain NIL. We therefore renamed the yellow rust resistance in the NIL 19PD1020.016.028 as HIT2.2.

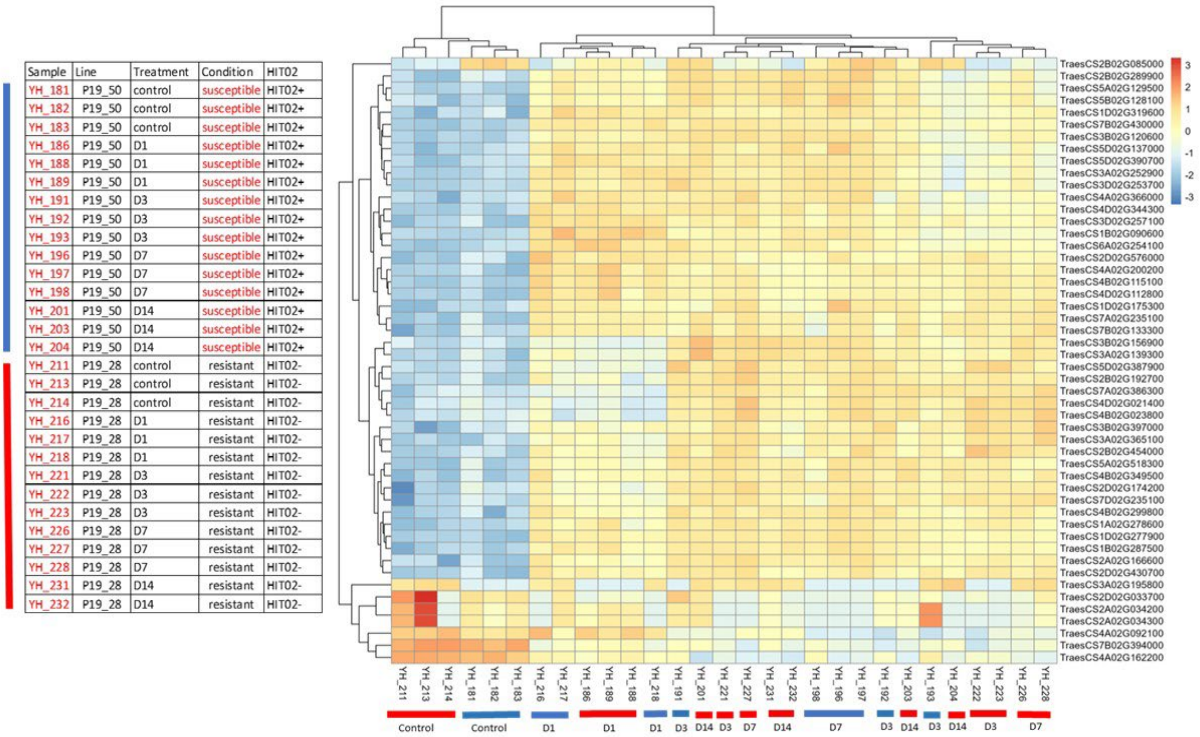
Pst inoculated flag leaves of 19PD1020.016.028 and 19PD1020.016.050 were sampled at 1, 3, 7 and 14 dai from three biological samples of each NIL. Uninoculated flag leaves were sampled as a control. Paired-end reads were mapped against the Chinese Spring IWGSC cDNA RefSeq v2 reference. The libraries were normalized and a PCA analyse was performed. PC1 explained 73% and PC2 explained 8% of the variance (Figure 10). Library YH_233 (P19_28 - 14 dai) was removed from the analysis due to low similarities among the biological replicates.

Analysis of 19PD1020.016.028 (HIT2.2) to 19PD1020.016.050 did not show a concentration of top DEG on chromosome 6A, (Figure 11) as was found for HIT2.1. Only two candidate NBS-LRR genes were found in the top 50 DEG, and these were located on chromosomes 2B and 2D. In total 83 genes on chromosome 6A were found to be differentially expressed between 19PD1020.016.028 (HIT2.2) and 19PD1020.016.050, of which 53 were in common with DEG found between the comparison RRR (HIT1, HIT4/5, HIT2.1) and SRS (hit1, HIT4/5, hit2.1).



Sample	Line	Treatment	Condition	HIT02
YH_181	P19_50	control	susceptible	HIT2.2-
YH_182	P19_50	control	susceptible	HIT2.2-
YH_183	P19_50	control	susceptible	HIT2.2-
YH_186	P19_50	D1	susceptible	HIT2.2-
YH_188	P19_50	D1	susceptible	HIT2.2-
YH_189	P19_50	D1	susceptible	HIT2.2-
YH_191	P19_50	D3	susceptible	HIT2.2-
YH_192	P19_50	D3	susceptible	HIT2.2-
YH_193	P19_50	D3	susceptible	HIT2.2-
YH_196	P19_50	D7	susceptible	HIT2.2-
YH_197	P19_50	D7	susceptible	HIT2.2-
YH_198	P19_50	D7	susceptible	HIT2.2-
YH_201	P19_50	D14	susceptible	HIT2.2-
YH_203	P19_50	D14	susceptible	HIT2.2-
YH_204	P19_50	D14	susceptible	HIT2.2-
YH_211	P19_28	control	resistant	HIT2.2+
YH_213	P19_28	control	resistant	HIT2.2+
YH_214	P19_28	control	resistant	HIT2.2+
YH_216	P19_28	D1	resistant	HIT2.2+
YH_217	P19_28	D1	resistant	HIT2.2+
YH_218	P19_28	D1	resistant	HIT2.2+
YH_221	P19_28	D3	resistant	HIT2.2+
YH_222	P19_28	D3	resistant	HIT2.2+
YH_223	P19_28	D3	resistant	HIT2.2+
YH_226	P19_28	D7	resistant	HIT2.2+
YH_227	P19_28	D7	resistant	HIT2.2+
YH_228	P19_28	D7	resistant	HIT2.2+
YH_231	P19_28	D14	resistant	HIT2.2+
YH_232	P19_28	D14	resistant	HIT2.2+

Figure 10. Principal component analysis comparing the three biological replicates of the uninoculated control and *Pst* inoculated flag leaf samples of the RAGT NILs 19PD1020.016.028 (HIT2.2+ - P19_28) and 19PD1020.016.050 (P19_50) at 1, 3, 7 and 14dai.



Sample	Line	Treatment	Condition	HIT02
YH_181	P19_50	control	susceptible	HIT02+
YH_182	P19_50	control	susceptible	HIT02+
YH_183	P19_50	control	susceptible	HIT02+
YH_186	P19_50	D1	susceptible	HIT02+
YH_188	P19_50	D1	susceptible	HIT02+
YH_189	P19_50	D1	susceptible	HIT02+
YH_191	P19_50	D3	susceptible	HIT02+
YH_192	P19_50	D3	susceptible	HIT02+
YH_193	P19_50	D3	susceptible	HIT02+
YH_196	P19_50	D7	susceptible	HIT02+
YH_197	P19_50	D7	susceptible	HIT02+
YH_198	P19_50	D7	susceptible	HIT02+
YH_201	P19_50	D14	susceptible	HIT02+
YH_203	P19_50	D14	susceptible	HIT02+
YH_204	P19_50	D14	susceptible	HIT02+
YH_211	P19_28	control	resistant	HIT02-
YH_213	P19_28	control	resistant	HIT02-
YH_214	P19_28	control	resistant	HIT02-
YH_216	P19_28	D1	resistant	HIT02-
YH_217	P19_28	D1	resistant	HIT02-
YH_218	P19_28	D1	resistant	HIT02-
YH_221	P19_28	D3	resistant	HIT02-
YH_222	P19_28	D3	resistant	HIT02-
YH_223	P19_28	D3	resistant	HIT02-
YH_226	P19_28	D7	resistant	HIT02-
YH_227	P19_28	D7	resistant	HIT02-
YH_228	P19_28	D7	resistant	HIT02-
YH_231	P19_28	D14	resistant	HIT02-
YH_232	P19_28	D14	resistant	HIT02-

Figure 11. Top 50 genes different expressed between NILs 19PD1020.016.028 (HIT2.2+: P19_28) and 19PD1020.016.050 (P19_50)

5. Discussion

A comprehensive assessment of yellow rust field resistance was undertaken in 427 UK and northern European winter wheat varieties between 2018 and 2022 across multiple field trial sites in the UK, France, Germany, Denmark and Sweden. Variation was seen in the levels of yellow rust infection between the wheat varieties across field trial locations and years. This may reflect the different environments, some locations being more conducive to yellow rust infection, but may principally be due to differences in the race composition of the populations of *Pst* present at each location.

While numerous associations between SNPs and yellow rust resistance were detected by the GWAS, the following cut-off threshold for calling an MTA as significant, and for inclusion in this report, was considered as: An MTA was considered a potentially genuine yellow rust resistance locus when (1) it had a significance score above threshold of > 3.0, or (2) had a significance score above threshold of > 1.5 in more than one season or location. The MTA 3A010 was also included in the final list, as despite not meeting the criteria for inclusion, it was subsequently detected in the bi-parental mapping population Reflection x Dickens. The MTA 3A241 was included as the significance score above threshold (2.92) was close to the threshold value of 3.0. Of these 45 MTAs, 29 were present in over 90% of the GWAS panel lines, suggesting that these yellow rust resistance loci have become fixed in UK and northern European winter wheat germplasm. MTAs that occurred at a low frequency (less than 25%) were 1A011 (18.5%), 2B153 (17.3%), 3B004 (21.7%) and UN004 (6.25%). Other MTAs present in the Yellowhammer panel at lower frequencies, and therefore of potential value to winter wheat breeding, are 2A647 (43.7%), 2D999 (30.7%), 3A010 (57.4%), 3A241(61.1%), 4B605 (38.4%) and 6A612 (43.1%). The MTAs with the highest significance scores were, in descending order, 2A019 (22.78), 2B298 (12.67), 6A612 (11.29), 2B236 (10.35), 5A607 (10.31), UN004 (8.64) and 4B672 (8.41).

The **MTA 2A019** is believed to be present on the *Ae. ventricosa* translocation 2NS/2AS. The 2NS/2AS translocation introduced the yellow rust resistance gene *Yr17* into the winter wheat gene pool, which became ineffective in 1996. KASP markers diagnostic for *Yr17* confirmed the presence of the 2NS/2AS translocation in all the Yellowhammer panel, with all varieties positive for *Yr17* also contained MTA 2A019, supporting the likelihood that 2A019 was also present on 2NS/2AS. However, while 2A019 was effective in 2018 and 2019 it became less effective in subsequent years in the GWAS analyses. However, a residual yellow rust resistance conferred by 2A019 was detected in the bi-parental mapping populations Reflection x Dickens in 2022, Revelation x Solstice in 2020 and 2021, and in Gladiator x Vuka in 2021 and 2022. 2A019 located to the same position as YR_HIT1 identified in the WAGTAIL project. Microphenotyping indicated that HIT1 appeared to primarily stop *Pst* development at development stage Pst4, i.e., formation of runner hyphae. In the NIL without HIT1 more spores were found to have developed further, forming longer runner hyphae and infecting additional host cells, as well as developing pustules, than in the NIL with HIT 1. RNAseq analysis of differential wheat gene expression identified 30 DEG annotated as NBS-LRR genes and 16 as

kinases within the genomic region defining HIT1 (2A019). These genes represent good candidates for the gene/s responsible for the HIT1 (2A019) yellow rust resistance, but also show that this 2NS/2AS translocation from *Ae. ventricosa* is a rich source of R-type disease resistance genes.

MTA 2B298 exhibited a significant value above threshold, presenting as a major yellow rust resistance gene, and potentially a R-gene type of resistance. This MTA was principally found in France and the UK, where it was effective throughout the study period (2019 to 2022), although being less effective in 2022, suggesting that virulence towards 2B298 may be increasing in the *Pst* populations in France and the UK. MTA 2B298 was detected as significant in the GWAS in Denmark in 2019 and Sweden in 2021, which may reflect differences in the virulence profiles of the *Pst* populations in each country. The value of 2B298 for future wheat breeding is less clear, as this MTA was found to be present in 96.7% of the Yellowhammer GWAS panel, and therefore is relatively fixed within the winter wheat gene pool used in the UK and northern Europe.

The **MTA 6A612** was detected in all countries, except Sweden. It was detected in every year of the GWAS study, and was most effective in France and the UK, followed by Germany and Denmark, again reflecting similarities in the virulence profiles of the *Pst* populations in France and the UK. The SNP markers defining 6A612 covered a considerable length of the chromosome 6A, indicating that like MTA 2A019, MTA 6A612 may be located on a translocation. However, high synteny between Chinese Spring and the genome reference sequence of the European winter wheat cv. Attraktion, in the vicinity of MTA 6A612, ruled out the presence of an alien introgression (Kale et al., 2022). 6A612 contributed a large yellow rust resistance effect, thus indicating a probably major, R-gene resistance. MTA 6A612 was found in 43.1% of the wheat lines within the Yellowhammer GWAS panel, and its presence was confirmed through bi-parental QTL mapping in the wheat varieties Dickens and Graham. MTA 6A612 was believed to correspond to HIT2.1 identified in the WAGTAIL GWAS. NIL with and without HIT2.1 indicated that this source of yellow rust resistance, while allowing *Pst* to infect and develop to a fairly advanced state, i.e., producing runner hyphae and infecting multiple host plant cells, restricted the development of pustules. RNAseq analysis of wheat differential gene expression identified nine DEG annotated as NBS-LRR genes and 18 as kinases within the HIT2.1 genomic region, each providing good candidates for the gene/s responsible for the HIT2.1 (6A612) yellow rust resistance. An MTA was detected in the same region on chromosome 6A in a GWAS of Nordic spring wheats, QYr.nmbu.6A (Lin et al., 2023) and In a GWAS of 908 genotypes representing northern European winter wheats (Kale et al., 2022). While the Nordic population was not tested in Sweden (6A612 not effective in Sweden in this Yellowhammer study), QYr.nmbu.6A was effective in Norway in field trials carried out from 2015 to 2019. Kale et al. (2022) interrogated the 6A genomic region in the genome assembly constructed for the cv. Attraktion, identifying seven candidate NLR-type R-genes.

The **MTA 2B236** was found in France in 2020 and 2021, being more effective in 2020 than 2021. It was also detected in the UK in 2019, 2020 and 2022, being most effective in 2019. MTA

2B236 was present in 96.5% of the wheat varieties of the Yellowhammer panel, so is basically fixed within the winter wheat gene pool within the UK and northern Europe.

The **MTA 5A607** was detected as a major effect in Sweden in 2022, but at a lower level of significance in Sweden in 2021, Germany in 2020 and the UK in 2020 and 2022. The **MTA 4B672** was only detected in Sweden in 2019. The differences in the effectiveness of yellow rust resistance MTAs between Sweden and the other European countries where field trials were undertaken could be due to the servere winters experienced in Sweden. Larger shifts in the virulence profile of the *Pst* population in Sweden being seen due to eradication of all existing isolates at the end of one season, yellow rust infection in the following year being due to migration of new *Pst* isolates into Sweden. The MTA 4B672 was present in 95.8% of the Yellowhammer panel.

The **MTA UN004** was detected in the UK, Germany and Denmark between the year of 2019 to 2022. Although UN004 was only detected in 6.25% of the Yellowhammer panel, we were able to confirm the presence of this MTA in wheat varieties Crusoe and Mallet, contributing a major yellow rust resistance in 2020, 2021 and 2022. As MTA UN004 has been relatively underutilised in UK and northern European winter wheat varieties it presents as a very valuable yellow rust resistance locus of future breeding efforts.

A number of MTAs were detected that had significance values above threshold in the range of 4.0 to 7.0. This included five MTA on chromosome 2B, all of which were present at over 90% within the Yellowhammer panel: **MTAs 2B051, 2B559, 2B592, 2B657 and 2B752**. As chromosome 2B is well known for being rich in R-gene type disease resistance genes, these MTAs may well represent R-genes to which virulence exists within the *Pst* population, but that still express a residual yellow rust resistance phenotype. Other MTAs, of similar effect and present at similar frequencies within the Yellowhammer panel, included the **MTAs 3B050, 4A713, 5A475, 5B478, 7A730, 7D548 and UN025.**

MTAs that were present at a low frequency within the Yellowhammer panel, and which may be of value to future winter wheat breeding in the UK and northern Europe, are: **MTA UN004** (already discussed above), which was only present in 6.25% of the Yellowhammer panel. The **MTA 1A011** was primarily effective in the UK, although was significant in Germany and Denmark in 2019. In the WAGTAIL project a yellow rust resistance was identified in the same location – YR_RARESUS03. This MTA was only found in 18.5% of the Yellowhammer panel. The **MTA 2B153** was only detected 17.3% of the Yellowhammer panel. **MTA 2B153** was not detected in the original analysis of the WAGTAIL yellow rust data, but was picked up in the reanalysis. The **MTA 3B004** conferred a smaller, but constant effect on yellow rust resistance in the UK and Denmark, being present in 21.7% of the Yellowhammer panel. The **MTA 2D999** was only detected in 30.7% of the Yellowhammer panel, being effective in Denmark in 2020 and in the UK in 2019. The **MTA 4B605** was found in 38.4% of the Yellowhammer lines, and exhibited low levels of significance above the threshold, appearing in the UK, Denmark and Germany in 2019, and again in the UK, Denmark and France in 2022.

Comparison of MTAs identified in the Yellowhammer panel with HITs identified in the original GWAS analysis of the WAGTAIL project: The WAGTAIL project (unpublished data) assessed 480 wheat varieties and breeding lines assembled by the partners, representing UK and northern European wheat gene pool. This panel was assessed for a number of fungal diseases, including yellow rust, in 2012 and 2013. The original GWAS analyses were undertaken before the availability of the Chinese Spring Refseq chromosomal assembly, so exact locations of the SNP markers to wheat chromosomal regions was not possible. At NIAB we have now reanalysed the WAGTAIL data (publication in preparation), locating the HITs identified in WAGTAIL to the wheat reference genome. This has also enabled us to make a direct comparison between the yellow rust resistance MTAs identified in Yellowhammer with those identified in WAGTAIL.

The MTAs found in the Yellowhammer panel and in the original WAGTAIL analysis were MTAs 1A011 (RareSUS03), 2A019 (HIT1), 2A647 (RareSUS23), 2B051 (HIT8), 2D999 (HIT4/5), 3B004 (Marginal HIT22), 4B672 (MarginalRareSUS79), 5A020 (RareSUS17), 5A702 (WeakRareSUS36), 5B697 (MarginalRareSUS74), 6A612 (HIT2), 7A076 (MarginalRareSUS52) and 7D548 (RareSUS06).

The following MTAs were found in the Yellowhammer panel but not in the original or reanalysis of the WAGTAIL data: MTAs 1A524, 1B545, 1D002, 2A087, 2B236, 2B298, 2B559, 2B592, 2B657, 2B752, 2D619, 3A045, 3A241, 3B050, 3B561, 4B605, 5A048, 5A475, 5B478, 6B450, 6B718, UN004 and UN025. However, the MTAs 2B153, 3A010, 4A713, 5A607, 7A730 and 7B033, while not found in the original WAGTAIL analysis, were detected in the reanalysis using the Chinese Spring Refseq genome reference to locate the significant SNPs.

Comparison of MTAs identified in the Yellowhammer panel with yellow rust resistance QTL identified in the NIAB eight founder multi-parent population – MAGIC population: QTL mapping of yellow rust resistance in the NIAB 8 founder multi-parent MAGIC population identified 14 yellow rust resistance QTL (Bouvet et al., 2022). The MAGIC population was assessed for yellow rust resistance at three locations in the UK in 2015 and 2016. Six of the eight founder wheat varieties that were used to generate the NIAB MAGIC mapping population were included in the Yellowhammer GWAS panel, namely Alchemy, Claire, Hereward, Robigus, Soissons and Xi_19. Comparing the yellow rust resistance QTL identified in the MAGIC mapping population with the location of the Yellowhammer MTAs, based on the location of identifying markers on the CS Refseq genome assembly, identified a link between the MAGIC yellow rust resistance QTL and the Yellowhammer MTAs shown in Table 10. While nine of the QTL collocated with MTAs in the Yellowhammer GWAS, five of the MTAs were not called as significant based on the threshold levels applied in the Yellowhammer GWAS analysis (i.e., 1A581; 2A767; 2B673;4D491; 6A048). This may be that in 2015 and 2016 these yellow rust resistance QTL were more effective against the then current *Pst* population in the UK than between 2018 to 2022 when the Yellowhammer population was assessed for yellow rust resistance. No matching Yellowhammer MTA could be found for the

five QTL *QYr.niab-2A.1, QYr.niab-3D.1, QYr.niab-4B.1, QYr.niab-6A.1*, and *QYr.niab-6B.1*. However, a match could be found for the QTL *QYr.niab-2D.1* with the MTA 2D619 and the QTL *QYr.niab-5A.1* with the MTA 5A702, the resistant allele being present in all 6 founder varieties in common between the MAGIC population and the Yellowhammer panel. A match was also found between *QYr.niab-3A.1* and 3A010, the resistant allele being found in Hereward and Soissons, and between *QYr.niab-6A.3* and 6A612, the resistant allele being present only in Soissons.

QTL identified in MAGIC pop.	Chrom	Marker defining MAGIC Yr QTL	Genetic position	Physical position	QTL interval (cM)	YH_MTA found in same genomic region
QYr.niab-1A.1	1A	RAC875_rep_c71093_1070	185.8	568.013	17.17	1A581
QYr.niab-2A.1	2A	BS00022903_51	140.26	607.827	19.29	None
QYr.niab-2A.2	2A	BS00011599_51	259.39	762.29	2.56	2A767
QYr.niab-2B.1	2B	Kukri_c9118_1774	271.92	683.048	18.62	2B673
QYr.niab-2D.1	2D	Ra_c21099_1781	197.36	638.376	10.85	Possibly 2D619
QYr.niab-3A.1	3A	Kukri_c28650_111	3.02	7.921	16.64	3A010
QYr.niab-3D.1	3D	BS00004334_51	162.2	574.773	0	None
QYr.niab-4B.1	4B	Ra_c26080_461	50.66	36.643	17.73	None
QYr.niab-4D.1	4D	D_GDRF1KQ02H66WD_341	125.78	499.107	26.56	Possibly 4D491
QYr.niab-5A.1	5A	IAAV3916	301.25	683.343	13.32	Possibly 5A702
QYr.niab-6A.1	6A	BS00011010_51	55.51	18.713	18.24	None
QYr.niab-6A.2	6A	Kukri_c21743_269	75.69	27.108	2.05	Possibly 6A048
QYr.niab-6A.3	6A	wsnp_Ex_rep_c101766_87073440	220.32	596.521	17.96	6A612
QYr.niab-6B.1	6B	BS00068615_51	60.56	54.662	13.37	None

Table 10. Linkage between the yellow rust resistance QTL identified in the MAGIC population and the yellow rust resistance MTA identified in the GWAS analysis of the Yellowhammer panel

The value and robustness of the GWAS approach to identifying source of disease resistance within large collections of wheat genotypes has now been well demonstrated (Lin et al., 2023, Kale et al., 2022). In the Yellowhammer project we have identified 45 genomic regions conferring yellow rust field resistance. Bi-parental QTL mapping, in a small selection of wheat crosses, enabled us to confirm the presence of 13 of these MTAs. Screening of the Yellowhammer panel with published KASP markers for cloned yellow rust resistance genes served to highlight the need to develop such markers using the UK and northern European winter wheat gene pool. Many of the KASP primers failed to distinguish between the positive and negative control DNAs, while in the case of KASP markers for *Yr36* the allelic variants within the Yellowhammer panel were substantially different from

the positive and negative control DNAs. Finally, RNAseq analysis of wheat genes differentially expressed in response to *Pst* inoculation in NIL with and without a target *Yr* gene showed the value of this approach to identifying the gene/s responsible for target yellow rust resistance loci.

The Yellowhammer project has provided wheat breeders with a holistic view of the sources of yellow rust resistance they have available to them within their winter wheat breeding programmes. This includes knowledge of resistances that are principally fixed in their wheat materials, as well as resistance loci that are underutilised, with some sources of resistance only being present in 2.3% and 6.25% of the wheat varieties, i.e., *Yr15* and UN004, respectively. The partners have been able to take the SNP markers identifying these sources of yellow rust resistance to develop DNA markers that are diagnostic for each locus and use these in their breeding programmes to deliver new wheat varieties with effective yellow rust resistance. The microscopic development of the yellow rust pathogen has shown that different sources of yellow rust resistance interact with the pathogen in different ways, indicating potentially different resistance mechanisms. By combining resistances that function through different mechanisms it is believed that more durable resistance can be achieve.

6. References

Allen, A.M.; Winfield, M.O.; Burridge, A.J.; Downie, R.C.; Benbow, H.R.; Barker, G.L.; Wilkinson, P.A.; Coghill, J.; Waterfall, C.; Davassi, A.; et al. Characterization of a Wheat Breeders' Array suitable for high-throughput SNP genotyping of global accessions of hexaploid bread wheat (*Triticum aestivum*). *Plant Biotechnol. J.* **2017**, *15*, 390–401.

Aranda, P. S., LaJoie, D. M., & Jorcyk, C. L. (2012). Bleach gel: a simple agarose gel for analyzing RNA quality. Electrophoresis, 33(2), 366-369.

Bariana HS, McIntosh RA (1993) Cytogenetic studies in wheat. XV. Location of rust resistance genes in VPMl and their genetic linkage with other disease resistance genes in chromosome 2A. Genome 36:476–482.

Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society: Series B (Methodological) 57: 289-300.

Bouvet, L., Percival-Alwyn, L., Berry, S., Fenwick, P., Mantello, C. C., Sharma, R., ... & Cockram, J. (2022). Wheat genetic loci conferring resistance to stripe rust in the face of genetically diverse races of the fungus Puccinia striiformis f. sp. tritici. *Theoretical and Applied Genetics*, 1-19.

Bray NL, Pimentel H, Melsted P, Pachter L (2016) Near-optimal probabilistic RNA-seq quantification, Nature Biotechnology 34, 525–527.

Breiman, L. (2001). Random Forests. Machine Learning 45: 5–32.

Broman KW, Wu H, Sen S, Churchill GA (2003) R/qtl: QTL mapping in experimental crosses. Bioinformatics 19(7): 889-890.

Fang, T., Lei, L., Li, G., Powers, C., Hunger, R. M., Carver, B. F., & Yan, L. (2020). Development and deployment of KASP markers for multiple alleles of Lr34 in wheat. *Theoretical and Applied Genetics*, *133*, 2183-2195.

Forrest, K., Pujol, V., Bulli, P., Pumphrey, M., Wellings, C., Herrera-Foessel, S., ... & Spielmeyer, W. (2014). Development of a SNP marker assay for the Lr67 gene of wheat using a genotyping by sequencing approach. *Molecular Breeding*, *34*, 2109-2118.

Fu, D., Uauy, C., Distelfeld, A., Blechl, A., Epstein, L., Chen, X., ... & Dubcovsky, J. (2009). A kinase-START gene confers temperature-dependent resistance to wheat stripe rust. *science*, *323*(5919), 1357-1360.

Fulton TM, Chunwongse J, Tanksley SD (1995). Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Molecular Biology Reporter, 13: 207–209.

Helguera, M., Khan, I. A., Kolmer, J., Lijavetzky, D., Zhong-Qi, L., & Dubcovsky, J. (2003). PCR assays for the Lr37-Yr17-Sr38 cluster of rust resistance genes and their use to develop isogenic hard red spring wheat lines. *Crop science*, *43*(5), 1839-1847.

M. S. Hovmøller, S. Walter, R. A. Bayles, A. Hubbard, K. Flath, N. Sommerfeldt, M. Leconte, P. Czembor, J. Rodriguez-Algaba, T. Thach, J. G. Hansen, P. Lassen, A. F. Justesen, S. Ali, C. de Vallavieille-Pope (2016) Replacement of the European wheat yellow rust population by new races from the centre of diversity in the near-Himalayan region. Plant Pathology 65: 402-411. https://doi.org/10.1111/ppa.12433

Kale SM, Schulthess AW, Padmarasu S, Boeven PHG, Schacht J, Himmelbach A et al (2022) A catalogue of resistance gene homologs and a chromosome-scale reference sequence support resistance gene mapping in winter wheat. Plant Biotechnology Journal (2022) 20: 1730–1742. doi: 10.1111/pbi.13843

Klymiuk V, Yaniv E, Huang L, Raats D, Fatiukha A, Chen S et al (2018) Cloning of the wheat *Yr15* resistance gene sheds light on the plant tandem kinase-pseudokinase family. Nat. Commun. 9(1):3735.

Kosambi, DD (1943) The estimation of map distances from recombination values. Ann Eugen. 12: 172-175.

Lin M, ·Dieseth JA, Alsheikh M, Yang E, Holzapfel J, · Schürmann F, Morales L, Michel S, Buerstmayr H, Bhavani S, Lillemo M. (2023). A major yellow rust resistance QTL on chromosome 6A shows increased frequency in recent Norwegian spring wheat cultivars and breeding lines. Theoretical and Applied Genetics, 136:164 https://doi.org/10.1007/s00122-023-04397-9

Love MI, Huber W, Anders S (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology, 15, 550.

Marchal, C., Zhang, J., Zhang, P., Fenwick, P., Steuernagel, B., Adamski, N. M., ... & Uauy, C. (2018). BED-domain-containing immune receptors confer diverse resistance spectra to yellow rust. Nat Plants 4: 662–668.

Moore, J. W., Herrera-Foessel, S., Lan, C., Schnippenkoetter, W., Ayliffe, M., Huerta-Espino, J., ... & Lagudah, E. (2015). A recently evolved hexose transporter variant confers resistance to multiple pathogens in wheat. *Nature genetics*, *47*(12), 1494-1498.

Ramirez-Gonzalez, R. H., Segovia, V., Bird, N., Fenwick, P., Holdgate, S., Berry, S., ... & Uauy, C. (2015). RNA-S eq bulked segregant analysis enables the identification of high-resolution genetic markers for breeding in hexaploid wheat. *Plant biotechnology journal*, *13*(5), 613-624.

Rosyara UR, De Jong WS, Douches DS, Endelman JB (2016) Software for Genome Wide Association Studies in autopolyploids and its application to potato. The Plant Genome 9: 1-10.

Stekhoven D, Buehlmann P (2012). MissForest - non-parametric missing value imputation for mixed-type data. Bioinformatics, 28: 112–118.

Wickham H (2016). ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York. ISBN 978-3-319-24277-4, https://ggplot2.tidyverse.org.