Genomic and genetic analysis of the wheat race-specific yellow rust resistance gene Yr5

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Abstract

Yellow rust, caused by the biotrophic fungus Puccinia striiformis f. sp. tritici (Pst), is a significant foliar disease of wheat (Triticum aestivum) and a major target for resistance breeding world-wide. The wheat race-specific yellow rust resistance (R)-gene Yr5 is a valuable source of resistance, still being effective against the pathogen in most wheat growing regions of the world. Through comparative genomic analyses, differential gene transcription and mutation studies, new genetic and genomic insights into this valuable resistance gene are provided. Co-locating DNA markers have been identified that have the potential to provide informative signatures for marker-based Yr5 introgression. Utilising the synteny between wheat and other cereal and grass species for which whole genome sequences are available, primer sets were developed for wheat sequences which co-segregate with Yr5 resistance, located on the long arm of chromosome 2B. Nucleotide and amino acid sequence searches aligned these wheat sequences with both the 5' and 3' ends of rice loci which showed significant homology to NB-LRR type R-genes. These rice loci were located on chromosomes 4 and 7, the rice chromosomes which are syntenic with the wheat group 2 chromosomes. Mutation analysis of the Yr5 locus indicated that loss of the gene had significant effects on the transcription of a number of genes involved in diverse cellular processes, this differential transcription being independent of the presence of the yellow rust pathogen Pst. Mutation analysis also identified loci, unlinked to Yr5, that were required for Yr5-mediated resistance.

Keywords: Wheat, disease resistance, genetics, DNA markers, Triticum aestivum, yellow rust, Yr5 R-gene, mutation, resistance breeding, fungal pathogens, stripe rust, genomics, transcriptomics, Puccinia striiformis, cereal synteny

Introduction

Yellow rust is a significant foliar disease of wheat and a major target for resistance breeding world-wide [1]. The recent discovery of a sexual cycle for Pst [2] and the emergence of new, more aggressive races [1] indicate that the impact of this disease on global wheat production will continue to increase. Yr5, a yellow rust race-specific R-gene effective at both seedling and adult plant growth stages was first identified in a T. aestivum ssp. spelta var. album accession [3]. Monosomic analysis placed the Yr5 gene on the long arm of chromosome 2B [4], being allelic to Yr7, an R-gene introgressed into hexaploid wheat from the tetraploid wheat T. turgidum ssp durum [5]. Yr5 has not been extensively used in wheat breeding programmes and consequently is still potentially effective against Pst on many continents [1].

Previous studies identified two Yr5-linked AFLP markers which showed significant sequence similarity to rice homologues of the bacterial blight NB-LRR R-gene Xa-I [6]. Despite overlap in the nucleotide sequence genetic mapping indicated distinct loci, one AFLP marker co-segregating with Yr5 while the other mapped at a distance of 0.7 cM from the Yr5 gene. This would suggest that Yr5 lies within a region of the wheat genome enriched for NB-LRR-type R-gene analogs. A number of resistance genes effective against fungal pathogens have been isolated from hexaploid wheat, including the leaf rust R-genes Lr1 [7], Lr10 [8] and Lr21 [9], the yellow rust R-gene Yr10 [10], the stem rust R-genes Sr33 and Sr35 [11,12] and the powdery mildew R-gene Pm3b [13]. All belong to the NB-LRR class of R-genes. Non-race-specific resistance genes cloned from wheat include the Lr34/Yr18/Pm38 locus which confers durable resistance against leaf rust, yellow rust and powdery mildew and the yellow rust resistance gene Yr36. Neither encodes a NB-LRR protein, Lr34/Yr18/Pm38 encoding an ATP-binding cassette (ABC)-transporter [14], whereas Yr36 encodes a START-kinase type gene [15].

Numerous genomic and functional genetic tools are now available for cereals. The public release of whole genome sequences of cereals and related grass species has enabled
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exploitation of the syntenic relationships between grass species. The co-linearity between wheat and the small genomes of rice (*Oryzae sativa*) and the model grass *Brachypodium distachyon* has been successfully used to identify and develop markers linked to genes of interest, aiding in the fine mapping of the powdery mildew resistance gene *Pm6* [16] and *Pch1* for resistance to eyespot [17]. High-density oligonucleotide arrays are now available for many crop species allowing the detection of thousands of polymorphisms in a high-throughput and cost effective manner [18]. Most of these array-based technologies use genomic DNA to identify single nucleotide polymorphisms (SNPs), but transcription profiling of RNA levels can be used to detect expression level polymorphisms (ELPs) and single-feature polymorphisms (SFPs) between genotypes. The 55K Affymetrix Wheat1 GeneChip was used to identify 118 SFPs, Yr5 [19]. Microarray technology has also been used for gene discovery, using differences in transcript levels between wild-type and mutant plants to identify candidate transcripts for the gene of interest [20,21].

Here we describe a genomic and genetic analysis of wheat yellow rust resistance conferred by the R-gene Yr5. These studies were undertaken to develop tools and resources to understand the functionality of Yr5 and eventually clone this R-gene. A comparative genomic analysis, which utilised the synteny between wheat and cereal and grass species for which whole genome sequences are available, lead to the identification of NB-LRR-type sequences which co-segregated with Yr5 resistance. These co-segregating DNA markers have the potential to provide informative signatures for marker-based Yr5 introgression. Furthermore the genetic complexity underlying the control of Yr5-mediated yellow rust resistance in wheat was demonstrated by the identification of unlinked gene sequences differentially transcribed, in the absence of the yellow rust pathogen, in lines from which the Yr5 gene had been deleted. Similarly, genetic characterisation of EMS mutants compromised in their Yr5 resistant phenotype identified additional genetic loci required for Yr5-mediated resistance.

### Materials and methods

#### Plant material and mapping populations

A BC1 population ([Lemhi*8*/Yr5 x Lemhi] x Lemhi) [Figure 1] [6] consisting of 248 lines and the doubled haploid (DH) population of the cross Kariega x Avocet S (250 lines) [22] were used to map wheat gene markers to the Yr5 region of chromosome 2B. Wheat lines Lemhi, Avocet S, Kariega, Chinese Spring, *T. spelta* and Lemhi*8*/Yr5 were used as controls.

#### *Puccinia striiformis* f. sp. *tritici* infection tests

The *Pst* isolate WYR81/20 (avir Yr1,5,7,8,9,10,15,17/vir Yr2,3,4,6) was used in all yellow rust infection tests to detect the presence of Yr5. Isolate WYR81/20 is virulent on Lemhi, Avocet S and Chinese Spring (Infection Type (IT) - 4) and avirulent on *T. spelta* and Lemhi*8*/Yr5 (IT0/). All yellow rust infection tests were carried out on seedlings at growth stage 12-13 [23] grown under spore-free conditions [24]. Infection reactions were scored 14-16 days after inoculation using the following IT scale:

- 0 no visible symptoms
- 1 small necrotic flecks
- 2 moderately sized, sporulating uredinia surrounded by chlorotic tissue
- 3 large, sporulating uredinia surrounded by green tissue
- 4 large, sporulating uredinia surrounded by green tissue
- c chlorotic tissue associated with uredinia
- n necrotic tissue associated with uredinia

#### Figure 1

Genetic map of the Yr5 R-gene region on the long arm of wheat chromosome 2B. The marker loci are shown on the right of the map. Distances in centimorgens (cM) are given from the centromeric end of 2BL.

| Distance (cM) | Marker loci |
|--------------|-------------|
| 0.0          | Xgwm120     |
| 7.4          | Xwmc175     |
| 12.0         | Yr5         |
| 12.7         | S23M41-310/STS:S23M41-275 |
| 13.2         | S12M86-210  |
| 14.6         | S12M92-275  |
| 38.4         | Xgwm526     |

Figure 1 | Genetic map of the Yr5 R-gene region on the long arm of wheat chromosome 2B. The marker loci are shown on the right of the map. Distances in centimorgens (cM) are given from the centromeric end of 2BL.
Fast-neutron and EMS-mutation lines compromised for Yr5 resistance

Fast-neutron mutants were generated as previously described [25]. F1 plants monosomic for chromosome 2B:Yr5 were made by crossing Lemhi*8/Yr5 as the male parent to the Chinese Spring 2B monosomic line. The monosomic status of the F1 plants was confirmed using 2B-specific SSR markers GWM388, GWM120 and GWM526, for which Lemhi*8/Yr5 and Chinese Spring carry distinct alleles. F1 plants were selfed to give F2 seed. Five thousand F2 seed were exposed to 3 Gy of fast neutrons. Twenty-five F1 plants displayed a susceptible disease phenotype (IT4) when inoculated with isolate WYR81/20 which was maintained in the F3 generation.

EMS mutants were made by soaking 500 seed of Lemhi*8/Yr5 in ethyl methanesulfonate (0.5% v/v in water) for 2 hours. Excess liquid was removed and the seed left at room temperature for 16 hours, maintaining high humidity. Seed was washed 3 times in ddH2O before sowing in a peat/sand (1:1 v/v) mix. Plants of this M1 generation were allowed to self-pollinate and 20 M2 progeny from each M1 plant tested for resistance. Twelve of the 500 M2 families segregated for a compromised Yr5 resistance.

The selection criteria used to search the rice and *Brachypodium* genome sequences databases were less stringent than that used for the wheat EST analysis to account for cross-species variation. The group 2 wheat sequences from the wheat Whole Chromosome Survey Sequencing database (http://www.wheatgenome.org/Projects/IWGSC-Bread-Wheat-Projects/Sequencing/Whole-Chromosome-Survey-Sequencing) were also interrogated with the wheat gene sequences identified from the comparative genome analysis and the Affymetrix Wheat 1 GeneChip transcriptomics analysis. A stringent cut-off level of $< e^{-50}$ was used to select only highly similar sequences. Nucleotide and amino acid sequences were aligned with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) using the default settings.

Transcriptome analysis of Yr5 mutants Yr5del55 and Yr5del67

Seedlings of Lemhi*8/Yr5, Yr5del55 and Yr5del67 were grown to growth stage 12-13 under spor-e-free conditions [24]. Total RNA was extracted and purified from leaf tissue of six seedlings pooled as one replicate, with three replicates per genotype [26]. Affymetrix Wheat 1 GeneChip processing, including RNA quality control, microarray hybridisation and data acquisition was performed by the research services of the Genome Laboratory, Norwich, U.K. Data was analysed using GeneSpring GX10 (Agilent) and probe sets annotated as previously described [26]. Lemhi*8/Yr5 was compared to Yr5del55 and Yr5del67 to identify probe sets that were differentially expressed in the parental line compared to the mutants, having a fold change $>2$ and a p-value $<0.05$ (Welch T-Test).

Quantitative reverse transcription PCR (qRT-PCR) was used to validate the expression levels of selected probe sets [26]. Total RNA was extracted from leaf samples of Lemhi*8/Yr5, Yr5del55 and Yr5del67 using the RNeasy Plant Mini Kit (QIAGEN) and subsequently treated with TURBO DNA-free (Ambion). cDNA was synthesised using SuperScript III-RTase (Invitrogen-CA, USA), according to the manufacturer’s recommendations and diluted 20-fold with nuclease-free water prior to use. qRT-PCR validation was carried out using gene specific primers (Supplement Table S1). Only primer sets with an amplification efficiency $>80%$ were used for qRT-PCR analysis. The DNA engine Opticon2 Continuous Fluorescence Detector (M.J. Research Inc., Alameda, CA, U.S.A.) was used for PCR amplification with cycling conditions of 95°C for 4 min, followed by 40 cycles of 30 sec at 94°C, 30 sec 60°C and 30 sec at 72°C. Melt curve analysis was used at the end of each reaction to check primer-dimer formation and gene-specific product amplification. Data were analysed using Opticon Monitor™ software v.2.02 (M.J. Research Inc.). cDNA was normalised with geNorm (geNorm program v3.5 http://medgen.ugent.be/~jvdesomp/genorm/) [27] using three reference genes, ubiquitin [28], GAPDH [29] and elongation factor-1α [30] that were all stable...
under our experimental conditions. Relative transcript levels of the genes represented by each probe set were calculated between Lemhi*8/Yr5 and \( Yr5 \) del55 or \( Yr5 \) del67. Where primer sets suitable for qRT-PCR analysis could not be designed end-point RT-PCR was used to assess gene expression. PCR amplification of 50 ng of genomic DNA was used to test for loss of probe set sequences from the mutants \( Yr5 \) del55 and \( Yr5 \) del67. Primers of each probe set were also tested against the Chinese Spring wheat group 2 nulli-tetrasomic lines to identify group 2 homoeologous-specific chromosomal sequences. Mapping of wheat sequences Primer pairs were designed to wheat sequences using the PRIMER3 program (http://www.genome.wi.mit.edu) and screened for polymorphisms between Lemhi, Lemhi*8/Yr5, Kariega and Avocet S, and against the \( Yr5 \) mutants \( Yr5 \) del55 and \( Yr5 \) del67 using Single Strand Confirmation Polymorphism (SSCP) analysis [6]. Markers polymorphic between Lemhi and Lemhi*8/Yr5 were mapped in the BC1 population [(Lemhi*8/Yr5 x Lemhi) x Lemhi] using Joinmap, version 3.0 [31,32]. The map was constructed using a LOD threshold of 3.0 and a maximum recombination frequency of 0.45. The recombination values were converted into genetic distances using the Kosambi mapping function [33]. Markers polymorphic between Kariega x Avocet S were mapped in the DH population [22] using the screening and mapping procedures previously described [34].

**Results**

Identification of gene sequences within the \( Yr5 \) genetic region through comparative genomic analyses

The AFLP markers S19M93-140 and S23M41-275 had previously been mapped to the yellow rust R-gene \( Yr5 \) ([Figure 1] [6]. S19M93-140 co-segregated with \( Yr5 \), while S23M41-275 mapped at a distance of 0.7cM. To specifically identify gene sequences within the region of the wheat genome containing \( Yr5 \) a BlastN search was conducted against the GrainGenes

**Table 1. Genetic, marker and phenotypic assessment of mutations compromised for \( Yr5 \)-mediated yellow rust resistance.**

| Varieties, \( Yr5 \)del & \( Yr5 \)EMS mutants & \( Yr5 \)EMS IT to WYR 81/20 | \( Yr5 \)EMS mutants x AvocetS1 | DNA marker analysis: Alleles present1 |
|----------------|----------------|----------------|----------------|
| \( Yr5 \)EMS 18 | 1* | 4 | 0 | 1 | 19 | 214 | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) |
| \( Yr5 \)EMS 90 | 4 | 4 | 0 | 6 | 15 | 189 | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) |
| \( Yr5 \)EMS 94 | 0* | 0* | 27 | 66 | 39 | 84 | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) |
| \( Yr5 \)EMS 95 | 4 | 4 | 0 | 0 | 234 | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) |
| \( Yr5 \)EMS 98 | 1/2* | 4 | 0 | 34 | 66 | 122 | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) |
| \( Yr5 \)EMS 99 | 4 | 4 | 0 | 0 | 12 | 218 | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) |
| \( Yr5 \)EMS 115 | 4 | 4 | 0 | 0 | 228 | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) |
| \( Yr5 \)EMS 241 | 4 | 4 | 0 | 0 | 212 | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) |
| \( Yr5 \)EMS 287 | 4 | 4 | 0 | 0 | 216 | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) |
| \( Yr5 \)EMS 387 | 4 | 4 | 0 | 0 | 220 | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) |
| \( Yr5 \)EMS 474 | 4 | 4 | 0 | 0 | 218 | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) |
| \( Yr5 \)EMS 500 | 4 | 4 | 0 | 0 | 228 | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) |
| \( Yr5 \)del55 | 4 | - | - | - | - | - | \( DEL \) | \( DEL \) | \( DEL \) | \( DEL \) | \( DEL \) | \( DEL \) |
| \( Yr5 \)del67 | 4 | - | - | - | - | - | \( DEL \) | \( DEL \) | \( DEL \) | \( DEL \) | \( DEL \) | \( DEL \) |

**Controls:**

| Varieties | \( Yr5 \) EMS | \( Yr5 \) EMS | \( Yr5 \) EMS | \( Yr5 \) EMS | \( Yr5 \) EMS | \( Yr5 \) EMS | \( Yr5 \) EMS | \( Yr5 \) EMS | \( Yr5 \) EMS | \( Yr5 \) EMS | \( Yr5 \) EMS | \( Yr5 \) EMS |
|-----------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Lemhi*8/Yr5 | n | 0*1* | 52 | 116 | 2 | 64 | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) |
| Triticum spelta | ; | - | - | - | - | - | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) | \( T.sp \) |
| Lemhi | 4 | - | - | - | - | - | null | L | L | L | L | L |
| Avocet S | 4 | - | - | - | - | - | n/t | n/t | n/t | n/t | n/t | n/t |
| Chinese Spring | 3/4 | - | - | - | - | - | n/t | n/t | n/t | n/t | n/t | n/t |

1. Lemhi*8/Yr5 and the \( Yr5 \)EMS mutants were crossed to the yellow rust susceptible variety AvocetS. The infection type (IT) scores given to the F1 and F2 progeny follow the scheme set out in Materials and Methods.

2. Marker alleles are indicated as T.sp. (as present in *Triticum aestivum* ssp. *spelta* var. *album*), CS (as present in cultivar Chinese Spring), L (as present in cultivar Lemhi), null (no band detected), or DEL (marker absent), n/t (not tested).
wheat sequence database using the nucleotide sequence of S19M93-140 and S23M41-310 as query sequences. Both S19M93-140 and S23M41-310 showed significant matches (e-value) to the same three wheat gene sequences, TaAffx.65234.1.S1_at, BJ255716 and CJ700174 even though the map locations of the two AFLP markers indicated that they represented distinct loci (Supplement Table S2). TaAffx.65234.1.S1_at, BJ255716 and CJ700174 showed 100% identity to each other at the nucleotide level (data not shown) and will be referred to by the probe set name TaAffx.65234.1.S1_at.

The AFLP marker sequences were used to interrogate the rice and *Brachypodium* genome databases, exploiting the co-linearity between wheat and other grass species to identify further candidate gene sequences. S23M41-310 showed significant similarity to the rice loci LOC_Os04g53496 (8.6e-11) and LOC_Os07g04900 (1.0e-16), and to the *Brachypodium* sequence Bradi4g09800.1. S19M93-140 showed significant similarity to the rice sequence LOC_Os11g15670 and to the *Brachypodium* sequences Bradi4g09800.1, Bradi2g36040.1 and Bradi2g36030.1 (Supplement Table S2). As the wheat group 2 chromosomes are syntenous with rice chromosomes 4 and 7 [35] LOC_Os07g04900 and LOC_Os04g53496 were used as query sequences in BlastN searches of the GrainGenes database to identify orthologous wheat sequences. Due to the lack of reported syntenic relationship between rice chromosome 11 and *Brachypodium* chromosomes 2 and 4 [35,36] with wheat chromosome 2B, LOC_Os11g15670, Bradi4g09800.1, Bradi2g36040.1 and Bradi2g36030.1 were not pursued as loci for further wheat sequence analysis.

LOC_Os04g53496 as query sequence identified nine wheat ESTs (significance score < e-35), all of which represented the wheat unigene Ta.28038 (Table S2). LOC_Os07g04900 matched ten wheat ESTs, nine of which (significance score < e-17) were assigned to unigene Ta.28038 (Table S2). The tenth wheat EST sequence showed similarity to Ta.28038 of 2e-12. Ta.28038 was not significant similarity to the rice locus LOC_Os07g04900 and to the *Brachypodium* loci Bradi2g36040.1 and Bradi2g36030.1. Of the 105 up-regulated probe sets, which represented 100 unique gene transcripts, more than half could not be assigned further wheat sequence analysis.

Nucleotide and amino acid sequence alignment of TaAffx.65234.1.S1_at and Ta.28038 indicated that the two wheat sequences aligned to different regions of the rice loci. Ta.28038 aligned to the 5’ end of rice loci LOC_Os04g53496 and LOC_Os07g04900, whilst TaAffx.65234.1.S1_at aligned to the 3’ end of these two rice genes (Supplement figure S1). The two AFLP marker sequences S19M93-140 and S23M41-310, which showed 2e-24 nucleotide similarity to each other, aligned with TaAffx.65234.1.S1_at (Supplement Table S2). A BlastN search of the wheat Whole Chromosome Survey Sequencing database located TaAffx.65234.1.S1_at and Ta.28038 to wheat chromosome 2B (e-value = 0.0), the location of Yr5, although significant matches (e-value < -60) were also found on chromosomes 2A and 2D, implying the presence of homoeologous sequences (Supplement Table S3). While TaAffx.65234.1.S1_at and Ta.28038 both annotated as NB-LRR disease resistance proteins (http://www.plexdb.org/modules/PD_probeset/annotation.php) [37] there was a low degree of similarity between TaAffx.65234.1.S1_at and Ta.28038 at the nucleotide level.

Primers designed to the wheat sequences TaAffx.65234.1.S1_at and Ta.28038 (Supplement Table S1) were used to amplify genomic DNA from *T. spelta*, Lemhi*8/Yr5, Lemhi, Yr5del55 and Yr5del67. No polymorphisms were apparent between *T. spelta*, Lemhi*8/Yr5 and Lemhi using agarose gel separation (data not shown), therefore amplicons were screened using SSCP analysis. SSCPs bands present in *T. spelta* and Lemhi*8/Yr5, but absent in Lemhi, Yr5del55 and Yr5del67 (Supplement figure S2) were used to map TaAffx.65234.1.S1_at and Ta.28038 in the [(Lemhi*8/Yr5 x Lemhi) x Lemhi] BC1 population TaAffx.65234.1.S1_at and Ta.28038 both co-segregated with Yr5 resistance (Figure 1).

Identification of gene sequences within the Yr5 genetic region through transcript profiling of Yr5 fast-neutron mutants

A transcriptome analysis of Lemhi*8/Yr5, Yr5del55 and Yr5del67 was undertaken as an alternative approach to identify wheat gene sequences physically linked to the Yr5 locus. Yr5del55 and Yr5del67 were generated by fast neutron bombardment, displayed a susceptible infection type (IT 4), and had lost the STS markers and wheat gene sequences TaAffx.65234.1.S1_at and Ta.28038 mapped to Yr5 (Table 1). Compared to the parental line Lemhi*8/Yr5, Yr5del55 and Yr5del67 showed significant changes in the transcript levels of 714 and 396 probe sets, respectively (Supplement Table S4 and Supplement figure S3). Only 145 probe sets were in common between the mutants, with 105 probe sets being up-regulated and 40 down-regulated.

Functional classification of the 40 down-regulated probe sets, representing 37 unique gene sequences indicated that the majority were of unknown function (Supplement figure S3). Probe sets with predicted roles in plant defence or stress responses, including superoxide dismutase, cystatin and a non-specific lipid transfer protein, as well as transcripts involved in cellular transport, transcription, energy and metabolic processes were identified (Table 2). However, none of the probe sets were annotated as NB-LRR-type R-genes and the probe sets representing the sequences TaAffx.65234.1.S1_at and Ta.28038 did not show differential transcript levels between Lemhi*8/Yr5 and either Yr5 mutant.

Of the 105 up-regulated probe sets, which represented 100 unique gene transcripts, more than half could not be assigned a potential function (Supplement figure S3). Those that could be assigned a possible function were involved in processes such as plant defence and stress responses, cellular transport, metabolism, energy, cell component biogenesis, transcription,
| Probe Set ID          | Yr5del55 v Lemhi*8/Yr5 | Yr5del67 v Lemhi*8/Yr5 | Annotation from HarvEST/Plexdb                                                                 |
|----------------------|------------------------|------------------------|------------------------------------------------------------------------------------------------|
| Ta.1944.1.S1_at      | p-value: 9.67E-05      | FC: -49.4881           | Superoxide dismutase [Mn] 3.1, mitochondrial precursor                                         |
| Ta.27140.1.S1_at     | p-value: 0.001556      | FC: -6.841019          | Putative MATE efflux protein family protein                                                   |
| Ta.178.2.S1_at       | p-value: 0.018033      | FC: -3.614578          | Cystatin WC-1                                                                                  |
| Ta.178.2.S1_x_at     | p-value: 0.006319      | FC: -3.701423          | Cystatin WC-1                                                                                  |
| Ta.1688.1.S1_at      | p-value: 0.017261      | FC: -2.4441025         | UDP-glucosyltransferase BX8                                                                   |
| Ta.14545.1.S1_at     | p-value: 0.018081      | FC: -6.2296305         | Putative O-methyltransferase                                                                  |
| Ta.1112.1.S1_at      | p-value: 0.0104491    | FC: -13.676553         |                                                                                               |
| TaAffx.14477.1.S1_at | p-value: 2.92E-04      | FC: -3.4393673         |                                                                                               |
| Ta.611.1.A1_at       | p-value: 3.35E-04      | FC: -7.517564          | Hypothetical protein                                                                          |
| Ta.21290.1.A1_s_at   | p-value: 0.0194118    | FC: -3.8935266         |                                                                                               |
| Ta.8757.2.S1_at      | p-value: 0.036228     | FC: -2.5777307         | Phosphoserine phosphatase                                                                     |
| Ta.21005.1.S1_at     | p-value: 0.0143132    | FC: -3.9735072         |                                                                                               |
| Ta.14729.1.S1_at     | p-value: 0.0219907    | FC: -2.1916919         |                                                                                               |
| Ta.30504.1.A1_at     | p-value: 0.0968915    | FC: -3.1662958         | Non-specific lipid transfer protein                                                           |
| TaAffx.73741.1.S1_at | p-value: 0.096054     | FC: -2.3675597         |                                                                                               |
| Ta.17028.1.A1_s_at   | p-value: 0.0485578    | FC: -2.5108774         | Putative HGA1                                                                                  |
| Ta.7149.1.A1_s_at    | p-value: 0.0011034    | FC: -3.3122602         |                                                                                               |
| Ta.611.2.S1_at       | p-value: 0.0392292    | FC: -2.034222          | Hypothetical protein                                                                          |
| TaAffx.66205.2.S1_s_at| p-value: 0.0011056  | FC: -3.7338786         | Intracellular protease, PlpI family protein                                                  |
| TaAffx.100436.1.S1_at| p-value: 0.0086405   | FC: -11.848047         |                                                                                               |
| Ta.16298.1.S1_at     | p-value: 0.0120654    | FC: -2.2416117         |                                                                                               |
| Ta.16582.1.S1_at     | p-value: 0.0380914    | FC: -4.0853386         |                                                                                               |
| Ta.8552.1.A1_at      | p-value: 0.0082964    | FC: -8.785303          |                                                                                               |
| Ta.16476.1.S1_at     | p-value: 0.016985     | FC: -2.2019472         |                                                                                               |
| TaAffx.43914.1.S1_s_at| p-value: 0.0482833  | FC: -2.589973          |                                                                                               |
| Ta.17295.1.S1_at     | p-value: 0.0014027    | FC: -6.2972947         |                                                                                               |
| Ta.9039.2.S1_x_at    | p-value: 3.03E-05     | FC: -4.570216          |                                                                                               |
| TaAffx.53602.1.S1_at | p-value: 0.0419488   | FC: -4.863748          |                                                                                               |
| TaAffx.57738.1.S1_at | p-value: 6.15E-04    | FC: -2.2357112         |                                                                                               |
| TaAffx.51578.1.S1_at | p-value: 0.0383779   | FC: -2.1451643         |                                                                                               |
| TaAffx.7104.1.S1_at  | p-value: 0.0041442   | FC: -3.927811          |                                                                                               |
| Ta.7104.1.S1_x_at    | p-value: 0.0319099   | FC: -2.7847736         |                                                                                               |
| TaAffx.74512.2.S1_s_at| p-value: 0.0128684  | FC: -2.3639898         |                                                                                               |
| Ta.611.2.S1_at       | p-value: 2.18E-04     | FC: -17.154543         |                                                                                               |
| TaAffx.21788.1.S1_at | p-value: 1.99E-04    | FC: -5.1074266         |                                                                                               |
| Ta.19222.1.S1_at     | p-value: 7.20E-04     | FC: -10.351834         |                                                                                               |
| Ta.19222.1.S1_x_at   | p-value: 0.0026437   | FC: -3.3132606         |                                                                                               |
| Ta.20262.2.S1_at     | p-value: 0.0120548   | FC: -6.4605107         |                                                                                               |
| Ta.23203.3.S1_at     | p-value: 9.05E-06     | FC: -9.235376          |                                                                                               |
| TaAffx.22603.1.S1_at | p-value: 0.0068985   | FC: -2.319124          |                                                                                               |

Table 2. Affymetrix Wheat1 GeneChip probe sets showing down-regulated transcript levels in the fast-neutron mutants Yr5del55 and Yr5del67 compared to Lemhi*8/Yr5.
protein synthesis and binding interactions. The transcripts that were found to be down-regulated in both Yr5del55 and Yr5del67 were selected for further analysis.

Probe set transcript levels were validated using qRT-PCR analysis, comparing transcript levels in Lemhi*8/Yr5 to those in Yr5del55 and Yr5del67. Primer sets suitable for qRT-PCR analysis could only be designed for 20 of the 37 unique transcripts (Supplement Table S1). Those primer sets that were not suitable for qRT-PCR had either low amplification efficiency or produced primer dimers, preventing robust assay design. qRT-PCR analysis confirmed down-regulation of 12 of the 20 probe set transcripts (63%) in both Yr5del55 and Yr5del67 (Table 3; Supplement figure S4).

For a further five probe sets qRT-PCR demonstrated transcription repression in only one of the mutants (Table 3). End point RT-PCR was used to detect transcripts for 14 of the probe sets for which qRT-PCR suitable primers could not be designed. For five of these probe sets no transcript was detected in either mutant (Table 3; Supplement figure S5). A transcript for probe set Ta.16582.1.S1_at was absent only from Yr5del67, while the remaining eight probe sets tested by end point RT-PCR produced transcripts in both Yr5del55 and Yr5del67. Two products were observed for probe set TaAffx.21788.1.S1_at in Lemhi*8/Yr5, suggesting that these primers may amplify homoeologous or alternative spliced transcripts produced by this gene.

PCR amplification of genomic DNA was carried out to determine whether probe set sequences had been lost from the genomes of Yr5del55 and Yr5del67. Thirty-three primer sets amplified from the genomes of Lemhi*8/Yr5 and Lemhi, with 28 probe set sequences also being detected in the genomes of the two Yr5 mutants (Table 3). Primers for the probe sets TaAffx.74512.2.S1_s_at; Ta.178.2.S1_at/Ta.178.2.S1_x_at; TaAffx.100436.1.S1_at and Ta.17295.1.S1_at failed to amplify from genomic DNA of either mutant, while probe set Ta.30504.1.A1_at amplified from genomic DNA of Yr5del55, but not from Yr5del67 (Table 3; Supplement figure S6).

Mapping of the down-regulated wheat transcripts to the wheat genome

The syntenic relationship between the genomes of wheat and other cereal and grass species was used to assign the genetic loci represented by the 37 down-regulated probe sets to a chromosomal location through in silico mapping. The sequences of the 37 probe sets were used to investigate the wheat Whole Chromosome Survey Sequencing database, the mapped wheat EST database, and the rice and Brachypodium genome sequences. Nineteen of the transcripts (~51%) had highly significant matches (<e-90) to sequences located on wheat group 2 chromosomes (Supplement Table S3). Of these, 11 (~30%) transcripts had strong matches on all three homoeologous chromosomes, four (~11%) had matches on 2A and 2B, two (~5%) had matches on 2A, one on 2B and one on 2D. Seven probe sets produced significant hits (e-value < e-10) against the mapped wheat EST database, with two mapping to the short arm of wheat chromosome 2B (Ta.27140.1.S1_at and Ta.8757.2.S1_at), and one, Ta.1944.1.S1_at mapping to the long arm of 2B (Supplement Table S3). Twenty-one of the probe set sequences had significant matches on the rice genome, whereas 18 had significant hits to Brachypodium (Supplement Table S3). Ta.1112.1.S1_at showed the best hit to rice chromosome 4, while Ta.27140.1.S1_at, Ta.21290.1.A1_s_at, TaAffx.74512.2.S1_s_at and TaAffx.7104.1.S1_at/TaAffx.7104.1.S1_x_at produced significant hits on rice chromosome 7. Ta.21290.1.A1_s_at and TaAffx.7104.1.S1_at/TaAffx.7104.1.S1_x_at also produced significant hits to sequences located on Brachypodium chromosome 5.

Genomic DNA of the Chinese Spring wheat group 2 null-tetrasomic lines were screened with the 33 primer probe sets by PCR. Only Ta.1944.1.S1_at was assigned solely to wheat chromosome 2B. Ta.30504.1.A1_at, TaAffx.100436.1.S1_at, Ta.17295.1.S1_at, TaAffx.74512.2.S1_s_at and Ta.178.2.S1_at/Ta.178.2.S1_x_at could not be amplified from any of the Chinese Spring null-tetrasomic group 2 lines, while the remaining probe sets amplified from genomic DNA in all the group 2 null-tetrasomic lines (Supplement Table S3).

SSCP analysis failed to identify polymorphisms between Lemhi*8/Yr5 and Lemhi for any of the 33 probe set primers (data not shown). An alternative mapping population, Kariega x Avocet S [22] was therefore used in an attempt to map the probe set transcripts to the wheat genome. While SSCP analysis identified polymorphic bands between Kariega and Avocet S for seven probe sets (Ta.178.2.S1_at, Ta.1112.1.S1_at, Ta.8757.2.S1_at, TaAffx.6162.2.S1_s_at, TaAffx.21788.1.S1_at, Ta.611.2.S1_at and TaAffx.7104.1.S1_at) only Ta.611.2.S1_at could be mapped in the Kariega x Avocet S population. However, Ta.611.2.S1_at did not map to chromosome 2BL, but to the long arm of chromosome 4A, near to an adult plant, slow rusting QTL for yellow rust resistance (Gloudi Agenbag, unpublished data). In silico mapping identified highly significant hits for Ta.611.2.S1_at on both the group 2 and group 4 chromosomes of the Chinese Spring Whole Chromosome Survey Sequencing database, supporting the map location of Ta.611.2.S1_at in the Kariega x Avocet S population.

Genetic and molecular characterisation of EMS-derived mutations compromised for the Yr5 resistance phenotype

As a resource to genetically define the chromosomal region containing the Yr5 locus and subsequently confirm Yr5 candidate genes, EMS mutants were selected that were compromised for Yr5 resistance. Twelve mutants were identified of which nine were fully susceptible to Pst isolate WYR81/20, displaying IT4, while three had partially susceptible phenotypes ranging from IT0 to 1/2 (Table 1; Supplement figure S7).

The Yr5EMS5 mutants were crossed to the yellow rust susceptible variety Avocet S to determine whether the mutation responsible for the altered Yr5 resistance phenotype...
Table 3. PCR analysis of Affymetrix Wheat1 GeneChip probe sets down-regulated in Yr5del55 and Yr5del67 compared to Lemhi*8/Yr5.

| Probe Set ID                      | qRT-PCR a | End point RT-PCR | PCR from genomic DNA |
|-----------------------------------|-----------|------------------|----------------------|
| Ta.1944.1.S1_at                   | 33.5      | -23.7            | + b + + +            |
| Ta.27140.1.S1_at                  | nt c      | nt               | - - + +             |
| Ta.178.2.S1_at/Ta.178.2.S1_x_at   | -2194.5   | -8364.0          | - - - -             |
| Ta.1688.1.S1_at                   | -106.7    | -56.4            | + + + +             |
| Ta.14545.1.S1_at                  | -1294.9   | -1956.8          | - - + +             |
| Ta.11112.1.S1_at                  | -3.1      | -0.9             | + + + +             |
| TaAffx.14477.1.S1_at              | nt        | nt               | + + + +             |
| Ta.611.1.A1_at                    | -42912.3  | -21240           | - - + +             |
| Ta.21290.1.A1_s_at                | -4.3      | -2.7             | + + + +             |
| Ta.8757.2.S1_at                   | -4.4      | -4.6             | + + + +             |
| Ta.21005.1.S1_at                  | -83.8     | -271.3           | - - + +             |
| Ta.14729.1.S1_at                  | 2.8       | -1.0             | + + + +             |
| Ta.30504.1.A1_at                  | -584.8    | -253.0           | - - + -             |
| TaAffx.73741.1.S1_at              | nt        | nt               | - - + +             |
| Ta.17028.1.A1_s_at                | nt        | nt               | + + + +             |
| Ta.7149.1.A1_s_at                 | nt        | nt               | + + + +             |
| TaAffx.66205.2.S1_s_at            | 0.0       | -2.2             | + + + +             |
| TaAffx.100436.1.S1_at             | nt        | nt               | + + + +             |
| Ta.16298.1.S1_at                  | nt        | nt               | + + + +             |
| Ta.16582.1.S1_at                  | nt        | nt               | + - + +             |
| Ta.8552.1.A1_at                   | -231.5    | -154.1           | - - + +             |
| Ta.16476.1.S1_at                  | -3.3      | -0.7             | + + + +             |
| TaAffx.43914.1.S1_s_at            | nt        | nt               | - - + +             |
| Ta.17295.1.S1_at                  | nt        | nt               | - - - -             |
| Ta.9039.2.S1_x_at                 | -13.0     | -11.9            | + + + +             |
| TaAffx.53602.1.S1_at              | nt        | nt               | nt nt nt             |
| TaAffx.57738.1.S1_at              | nt        | nt               | + + + +             |
| TaAffx.51578.1.S1_at              | nt        | nt               | nt nt nt             |
| TaAffx.7104.1.S1_at/TaAffx.7104.1.S1_x_at | nt        | nt               | nt nt nt             |
| TaAffx.74512.2.S1_x_at            | -530.9    | -300.0           | - - - -             |
| Ta.611.2.S1_at                    | -28521.9  | -10866.1         | - - + +             |
| TaAffx.21788.1.S1_at              | nt        | nt               | - - + +             |
| Ta.19222.1.S1_at/Ta.19222.1.S1_x_at | -1.1   | -10.8            | + + + +             |
| Ta.20262.2.S1_at                  | nt        | nt               | + + + +             |
| Ta.23203.3.S1_at                  | nt        | nt               | nt nt nt             |
| TaAffx.22603.1.S1_at              | 0.0       | -1.3             | + + + +             |

aTranscript levels expressed as fold change differences in deletion mutant relative to Lemhi*8/Yr5. Underlined expression values highlight those probe sets where qRT-PCR analysis was used to validate the microarray data.

b+ = PCR amplicon detected in mutant
c- = PCR amplicon absent from mutant; dnt = not tested
was within the Yr5 gene itself, or at a second, independent locus required for the expression of Yr5 resistance. Seven mutants produced only yellow rust susceptible F_2 progeny, indicating that the mutation event directly affected the Yr5 locus (Table 1). All seven mutants had a fully susceptible infection type (IT4) reaction to isolate WYR81/20. These seven Yr5EMS mutants retained not only the linked STS and SSR markers mapped to Yr5, but also TaAffx.65234.1.S1_at and Ta.28038 (Table 1). Primers for TaAffx.65234.1.S1_at and Ta.28038 produced amplicons with identical SSCP band patterns in Lemhi*8/Yr5 and all 12 Yr5EMS mutants (Table 1).

The remaining five Yr5EMS mutants segregated F_2 individuals expressing a more resistant phenotype than that shown by the parental mutation (Table 1). This would indicate a mutation within a second locus, required for Yr5-mediated resistance. In the control cross, Lemhi*8/Yr5 (ITn) x Avocet S (IT4), Yr5 expressed as a semi-dominant gene, the heterozygous F_2 progeny (IT 00/) appearing less resistant to isolate WYR81/20 than the Yr5 donor parent Lemhi*8/Yr5 (Table 1). This made it difficult to confidently predict the number of additional mutant loci affecting the Yr5 resistance phenotype based on an analysis of segregation ratios. Similar segregation ratios were obtained in both populations made from each Yr5EMS mutant M3 family (data not shown).

**Discussion**

To date, virulence for the wheat race-specific yellow rust R-gene Yr5 has only been confirmed in Australia [38]. Yr5 is therefore a valuable resource, which used in combination with other R-genes, as well as quantitative sources of yellow rust resistance, would support breeding for durable yellow rust control in wheat. Deploying multiple R-genes in combination with more durable, but partial resistance genes such as the Lr34/ Yr18/Pm38 locus would provide a more sustainable approach to disease protection in regions of the world where yellow rust disease pressure is high [39,40]. Here we successfully use comparative genomics analyses between wheat and related cereal and grass genomes, transcriptomics analysis and in silico mapping to locate candidate wheat gene linked to the Yr5 locus. Two previously described AFLP markers that map to Yr5 [6] were initially used in cross-species comparative sequence analyses. This led to the identification of two rice NB-LRR-type genes, one on rice chromosome 4 and the other on chromosome 7, which subsequently supported the identification of wheat gene sequences that were shown to co-segregate with Yr5 resistance.

The co-linearity between wheat and other cereal and grass species for which a full genomic sequence is available provides a valuable resource by which to interrogate the genomic region around a gene of interest. Co-linearity provides a way to identify additional wheat sequences, flanking the gene of interest, which can be used for additional marker development [41], with the use of more than one genomic sequence providing greater confidence in the inferred syntenic relationships [16,17].

Previously, two genetically distinct AFLP markers linked to Yr5 resistance where shown to have significant sequence similarity to rice homologues of the rice bacterial blight NB-LRR R-gene Xa-I [6]. In this study, comparative genomic analysis with rice further places the Yr5 gene within a region of the wheat genome enriched with NB-LRR-type R-gene sequences. While two wheat gene sequences annotated as NB-LRR-type R-genes, TaAffx.65234.1.S1_at and Ta.28038 were shown to co-locate with Yr5. Yr5 may therefore represent another NB-LRR-type R-gene similar to many of the R-genes already cloned from wheat [8-13]. It is not uncommon for NB-LRR-type R-genes to occur in clusters within plant genomes [42] and the development of resistance gene-analog polymorphism (RGAP) markers which co-segregated with Yr5 also support these observations [43].

Microarray-based transcription profiling has shown potential as a tool to assist in the cloning of genes of agronomically important traits, successfully identifying genes involved in symbiotic interactions in Medicago truncatula [20] and defence responses against stem rust in barley [21]. The Affymetrix Wheat1 GeneChip was therefore used to identify gene transcripts down-regulated in two Yr5 fast-neutron mutants, Yr5del55 and Yr5del67 compared to the parental line Lemhi*8/Yr5. However, while microarray-based transcription profiling identified a number of wheat gene transcripts which located to chromosome 2B, the polyploidy nature of hexaploid wheat limited the potential of this approach as a tool to identify wheat gene transcripts that lay within the genomic region of the Yr5 locus. Of the 37 unique gene transcripts down-regulated 28 amplified from genomic DNA in both Yr5del55 and Yr5del67. In silico mapping to the wheat group 2 chromosomes indicated that 12 of these gene transcripts had homoeologous sequences on more than one of the group 2 chromosomes, while Ta.611.2.S1_at was also shown to have homoeologous sequences on the group 4 chromosomes. The probe sets representing the wheat sequences TaAffx.65234.1.S1_at and Ta.28038, which had been shown to co-localize with Yr5, did not show differential transcript levels between Lemhi*8/Yr5 and either Yr5del55 and Yr5del67. Again this observation was supported by the presence of homoeologues on chromosomes 2A and 2D, transcripts from which may have masked the loss of the loci on chromosome 2B in Yr5del55 and Yr5del67.

While 28 probe sets amplified from genomic DNA in both Yr5del55 and Yr5del67, down-regulation was confirmed for 12 transcripts by qRT-PCR in both mutants, and for a further five probe sets in one of the mutants. The function of many of the genes represented by these probe sets is unknown, but defence related proteins, including superoxide dismutase, cystatin and a non-specific lipid transfer protein were confirmed as down-regulated.

Interestingly, primers for five probe sets failed to amplify from genomic DNA in one or both Yr5 fast-neutron mutants, while successfully amplifying DNA from Lemhi and Lemhi*8/
Yr5. These five probe sets also failed to amplify from genomic DNA of the Chinese Spring nulli-tetrasomic group 2 lines and did not show hits against the wheat Whole Chromosome Survey Sequencing database. This would suggest that these genes, while present in Lemhi and Lemhi*8/Yr5 are not present in Chinese Spring, from which the wheat Whole Chromosome Survey Sequence was generated.

EMS mutagenesis of Lemhi*8/Yr5 identified twelve mutants, seven of which were within the Yr5 locus and five in a second gene required for Yr5 resistance. A number of genes have been identified which are required for the full expression of NB-LRR-type R-gene-mediated resistance. RAR1, SGT1 and the cytosolic molecular chaperone HSP90 have all been shown to be required for Lr21 wheat leaf rust resistance [44], while Lr10-mediated leaf rust resistance requires the presence of a second Resistance Gene Analogue [45]. Mutations to genes such as RAR1, SGT1, HSP90 or other currently unknown regulators of R-gene function may be responsible for the loss of Yr5 resistance in these five EMS-derived Yr5 mutants.

Conclusions

Yr5 still has potential as an effective source of yellow rust resistance if deployed in combination with other R-genes and sources of partial, but potentially more durable yellow rust resistance. Two approaches were undertaken to identify gene sequences within the genetic region defining the Yr5 gene, demonstrating the power of comparative genomic analyses and in silico mapping to identify and locate genes. While DNA markers have been published for Yr5 there are no guarantees that a given marker will be polymorphic between the wheat genotypes within a breeding program, so the DNA sequences shown here to co-locate with Yr5 provide a potential new resource of informative DNA sequences for Yr5 marker introgression. The ideal marker is always within the gene itself, and results for this study and others [43] would indicate that Yr5 lies within a gene region of the wheat genome rich in NB-LRR-type sequences, potentially being a NB-LRR-type R-gene. The genetic and transcriptomic analyses of the mutants compromised for Yr5-mediated resistance indicates the complexity of this yellow rust R-gene, and the dependence of Yr5 resistance on additional genes for normal function. The mutants described also provide a valuable resource for further work on the functional genomics of Yr5 resistance.

Supplement Table S1
Supplement Table S2
Supplement Table S3
Supplement Table S4
Supplement figure S1
Supplement figure S2
Supplement figure S3
Supplement figure S4
Supplement figure S5
Supplement figure S6
Supplement figure S7

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions

| Authors' contributions | GBDM | PHS | CB | GRM | TNC | RM | EW | GA | LAB |
|------------------------|------|-----|----|-----|-----|----|-----|-----|-----|
| Research concept and design | ✓ | | | | | | | | ✓ |
| Collection and/or assembly of data | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | | | ✓ |
| Data analysis and interpretation | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | | | ✓ |
| Writing the article | ✓ | | | | | | | | ✓ |
| Critical revision of the article | ✓ | | | | | | | | ✓ |
| Final approval of article | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | | | ✓ |
| Statistical analysis | ✓ | | | | | | | | ✓ |

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