Transcriptome sequencing identifies novel persistent viruses in herbicide resistant wild-grasses

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Herbicide resistance in wild grasses is widespread in the UK, with non-target site resistance (NTSR) to multiple chemistries being particularly problematic in weed control. As a complex trait, NTSR is driven by complex evolutionary pressures and the growing awareness of the role of the phytobiome in plant abiotic stress tolerance, led us to sequence the transcriptomes of herbicide resistant and susceptible populations of black-grass and annual rye-grass for the presence of endophytes. Black-grass (Alopecurus myosuroides; Am) populations, displaying no overt disease symptoms, contained three previously undescribed viruses belonging to the Partititiviridae (AMPV1 and AMPV2) and Rhabdoviridae (AMVV1) families. These infections were widespread in UK black-grass populations and evidence was obtained for similar viruses being present in annual rye grass (Lolium rigidum), perennial rye-grass (Lolium perenne) and meadow fescue (Festuca pratensis). In black-grass, while no direct causative link was established linking viral infection to herbicide resistance, transcriptome sequencing showed a high incidence of infection in the NTSR Peldon population. The widespread infection of these weeds by little characterised and persistent viruses and their potential evolutionary role in enhancing plant stress tolerance mechanisms including NTSR warrants further investigation.

As our knowledge of the complexity of the microbial community associated with plants (the phytobiome) grows, there is an increasing awareness of the unexpected benefits to the host of these interactions1. In addition to the well-studied classic symbioses (eg: nodulation), members of the phytobiome can contribute by largely undefined mechanisms, to plant vigour, stress tolerance and disease resistance1,2. Within the scope of enhancing abiotic stress tolerance, we have recently reviewed the evidence that endophytic microbes influence herbicide tolerance, either through directly metabolizing toxic xenobiotics, or by inducing more generic plant defence mechanisms3. Following on from this line of enquiry, we have become interested in any potential role for the phytobiome in herbicide resistance in weed populations.

The chemical control of black-grass (Alopecurus myosuroides) and annual rye-grass (Lolium rigidum) in cereal crops is an increasing challenge to sustainable arable production, due the steady rise of herbicide resistance4–6. In the UK, herbicide resistance in black-grass has been reported on over 16000 farms and can develop through two major mechanisms7. Target site-based resistance (TSR), arises from point mutations in plant genes encoding proteins targeted by herbicides, rendering them insensitive to inhibition. In the case of black-grass and annual rye-grass the most important current TSR mutations arise in acetolactate synthase (ALS) and acetyl CoA carboxylase (ACCase); these lead to a reduced sensitivity to inhibition to herbicides from the sulphonylurea and aryloxyphenoxypropionate/cyclohexanedione classes respectively7. TSR can be counteracted by alternating the use of herbicides with different modes of action. In contrast, non-target site resistance (NTSR) results from the co-ordinated enhanced expression of multiple genes involved in xenobiotic detoxification, resulting in resistance to herbicides, irrespective of their mode of action8,9. In wild–grasses, both TSR and NTSR are selected for following repeated exposure to graminicides9. Whereas TSR behaves as a Mendelian trait, linked to mutations in a single gene, NTSR is inherited in a complex manner, behaving as a multigenic quantitative trait. As such, the inheritance of NTSR in wild grasses is influenced by environmental factors in addition to herbicide selection10. With an interest in the potential role of microorganisms as an environmental driver of NTSR, we have used a

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non-targeted next generation sequencing approach to examine the phytobiume of black-grass and rye-grass. We have then examined the incidence of the identified endophytes in weed populations independently isolated from different sites in the UK that vary in their resistance to sulphonylurea and aryloxyphonoxypropionate/cyclohexanedione graminicides.

Results
Identification of persistent viruses in black-grass. RNA extracted from leaves of apparently healthy (symptomless) black-grass and annual rye-grass was sequenced using a GS-FLX (Roche). For black-grass, the wild-type sensitive (WTS) Rothamsted and NTSR Peldon populations were used, while for annual rye-grass commercially available WTS and SLR31 resistant plants were tested. The assembled contigs were analysed for the presence of non-plant sequences by comparing the sequences to the NCBI nr database using blastx v2.2.2810-12, followed by taxonomic determination using MEGAN13. Based on this analysis, while no evidence for the presence of bacterial or fungal RNA were found, a large number of sequences of viral origin were readily identified with amino-acid sequence homology to viruses belonging to the Alphapartitivirus and Varicosavirus genera.

In black-grass, low coverage, long read data generated using the GS-FLX gave only partial sequence coverage for the new viruses and it was not obvious whether the plants were infected with a single virus from each genera, or several closely related viruses. To extend the coverage of the viral genomes, high coverage, short read datasets were produced using RNAseq (Illumina). Three replicates were taken from each of the WTS (Roth 09), NTSR (Peldon 05) and TSR (Notts 05) populations characterized previously7. The improved coverage, allowed the reconstruction of the complete RNA genomes of the three viruses (Fig. 1A). Each genome was bipartite, comprising an RNA1, coding for an RNA-dependent RNA polymerase (RdRP) and an RNA2, coding for (at least) one coat protein. The respective RNA1 and RNA2 sequences were each paired to a single virus based on DNA sequence identity in the un-translated linker regions (UTRs).

The sequence of the assembled virus genomes were subjected to BlastX annotation and phylogenetic analysis based on the RNA1 sequences (Fig. 2). Of the three viruses, two were homologous to the dsRNA family of Partitiviridae and were called Alpecurus myosuroides partitivirus 1 (AMPV1) and Alpecurus myosuroides partitivirus 2 (AMPV2) respectively. The Partitiviridae sequences identified in black-grass were most closely related to members of the plant-infecting Alphapartitivirus genera, but were sufficiently diverse to fall into two different clades (Fig. 2).

The third viral genome found in black-grass had some protein sequence similarity to members of the Rhabdoviridae family, but was most similar to the Varicosavirus Lettuce big-vein associated virus (LBVaV) (Fig. 2), with which it shares a bi-partite genome structure14,15. The virus was called Alpecurus myosuroides varicosivirus 1 (AMVV1). Within the bipartite AMVV1 genome, RNA1 encoded a poly-protein made up of an RNA-dependent RNA polymerase, a capping region and a capping enzyme, while RNA2 coded for three putative coat proteins (Fig. 1A). BlastX analysis of the RNA2 of AMVV1 found low-homology protein matches to the coat protein 1 of Lettuce big-vein associated virus (LBVaV) and Tobacco stunt virus (TSV), both members of the genus Varicosavirus. No homology or function could be associated with the other putative coding regions in AMVV1, with similar ambiguous links to function determined with the orthologous proteins of LBVaV16.

Identification of persistent viruses in other wild-grass species. Transcriptome sequencing was also used to identify any viral sequences present in annual rye-grass (Lolium rigidum) NTSR (SLR31) and WTS populations. De novo assembly of the transcriptome derived from long read, low coverage sequencing (GS-FLX) produced the complete RNA2 sequence of three putative members of the Partitiviridae (Fig. 1B). Protein alignments showed that two of these sequences (accessions: HG005148 and HG005149) shared homology with the RNA2 of AMPV2 and would likely be classified as members of the genus Alphapartitivirus. These viruses were tentatively named Lolium rigidum partitiviruses 1–3 (LRPV1, LRPV2 and LRPV3), although the absence of RNA1 for each meant the full identity and classification of these viruses was not possible (Fig. 1B).

The presence of related viral sequences in black-grass and annual rye-grass suggested that further wild grasses could harbour uncharacterised persistent viruses. The assembled genomes of the viruses found in black-grass and annual ryegrass were used to search the transcriptome and genome databases of wild grasses available on NCBI. BlastN searches against the Transcriptome Shotgun Assembly (TSA) and Expressed Sequence Tag (EST) databases using the RNA1 and RNA2 sequence of AMVV1 identified several un-annotated orthologous sequences in perennial ryegrass (Lolium perenne) and meadow fescue (Festuca pratensis = Alopecurus pratensis). While the sequences from F. pratensis were too fragmented for reassembly, the AMVV1 scaffold did allow for the reconstruction of the majority of the putative RNA1 of the related virus from L. perenne. Further searches revealed the full length RNA2 was already available un-annotated in public TSA databases in two versions (GAYX01053802.1; GAYX01053803.1). One of these sequences coded for a protein 2 with the same length as that predicted for AMVV1, whilst the other version had an additional 41 amino acids (data not shown).

Occurrence and abundance of persistent viruses in wild-grass populations. After normalising the EST counts within the RNAseq libraries, it was apparent that the abundance of AMVV1 sequences in black-grass was higher in NTSR compared to WTS plants (Table 1). To allow for a more extensive screen for the presence of these viral sequences in black-grass populations, oligonucleotide primers were designed for RT-PCR to amplify the viral sequences corresponding to the coat proteins of RNA2 for AMPV1, AMPV2 and AMVV1 respectively (Table 2). RNA was extracted from leaves of black-grass plants derived from 10 populations sampled from different locations in the UK (Table 3). These populations had previously been characterised as either being WTS, TSR or NTSR with respect to their resistance to herbicides17. To confirm their herbicide resistance phenotype, the 10 samples were tested for their susceptibility to two commercially available graminicides, Atlantis WG® and
Cheetah® Gold. acting on ALS and ACCase targets respectively (Table 3). In addition, each population had been assessed previously for their relative enhancement in herbicide detoxification associated with NTSR (Table 3)7,17.

As a wild out-crossing weed, black-grass plants isolated from field sites are by their nature highly heterogeneous, with individuals within herbicide-resistant populations varying in their tolerance to graminicides. Thus, within the population tested it was already known that individuals varied in their susceptibility to herbicides due to variations in the incidence of TSR mutations7. To test whether these populations varied in their incidence of viral infection, individual plants from six different populations were tested using RT-PCR for the presence of AMVV1, AMPV1 and AMPV2 (Table 4). These results were of interest, as they presented an opportunity to examine the frequency of viral infection in individual plants present in identifiable populations. Following virus testing, individual infected plants were found in all populations, demonstrating that these viruses are widespread in the UK (Table 4). While the presence and abundance of each of the viruses varied within each set sampled, the well described NTSR Peldon population showed a high incidence of infection with all three viruses (Table 4).

Figure 1. Schematic representation of the fully assembled genome sequences of the viruses found in (A) black-grass (AMPV1/2 & AMVV1) and (B) annual rye-grass (LRPV). Total nucleotide and protein length of the coded ORFs are indicated, together with the putative function and the accession number.
Discussion
The results presented identify several previously undescribed persistent viruses which are widespread in black-grass populations in the UK. Related sequences were also present in other wild grasses, including annual rye-grass, which like black-grass can be a major problem weed in cereal production. The most abundant viral sequences belonged to AMPV1 and AMPV2, members of the Alphapartitivirus genus within the Partitiviridae family. AMPV1 and AMPV2 belong to the Alphapartitivirus genus within the Partitiviridae family. These viruses are vertically transmitted via transfer from pollen into embryos in many crops and wild species and are highly stable within their hosts, surviving both thermotherapy and meristem culturing. Members of the Partitiviridae family are widespread in plants, fungi and protists and have previously been identified in a number of metagenomic studies. In addition, the previously undescribed virus AMVV1 was identified in black-grass. AMVV1 was tentatively assigned to the genus Varicosavirus, based on similarity to the type member Lettuce big-vein associated virus (LBVaV). AMVV1 provoked no overt disease symptom in black-grass. Although the source of AMVV1 was not investigated, LBVaV is transmitted to plants through fungal infection by members of the Chytridiomycetes, notably Olpidium virulentus. In addition to being widespread, O. virulentus can persist in the soil for more than 20 years and is known to infect monocots. It is therefore a plausible vector for AMVV1 infection in grass weeds.
With all the viruses identified, while their respective sequences were readily amplified from cDNA prepared from total RNA, no amplification products were obtained from genomic DNA. This suggested that neither AMPV or AMVV viruses had integrated into the host plant chromatin.

While widespread in the grass weeds tested, it was not clear whether or not the hosts derived any benefit from these viral infections. There are examples of plant-infecting partitiviruses having a mutualistic relationship with the plant host. One study on salt stress in *Lolium perenne* identified evidence of a greater prevalence of a deltapartitivirus within tolerant populations. In the current study, we have been interested in the relationship between the abundance of persistent viruses in black-grass and changes in abiotic stress tolerance linked to NTSR-based herbicide resistance. While the incidence of infection to all three viruses was particularly marked in the well-studied Peldon NTSR population (Table 4), no further associative link between infection and NTSR could be established in black-grass. By manipulating innate defence mechanisms in the host, plant viruses are known to invoke broad ranging stress tolerance pathways in plants. This protection can extend to induced herbicide resistance. Thus, the baculovirus p35 gene can suppress apoptosis in passion fruit and as a consequence, enhance tolerance to the herbicide glufosinate, by suppressing cell death caused by the secondary effects of chemical injury. The basis for this virally-induced activation of host defences is poorly understood, but has been linked to the induction of

### Table 1. Relative abundance of virus reads as a percentage of total reads following transcriptome analysis (using Illumina) for three populations of black-grass (Peldon 05, Notts 05 and Roth 09).

| Sample/replicate | Total number of reads | Relative abundance of virus reads (%) |
|------------------|----------------------|--------------------------------------|
|                  |                      | AMPV1 | AMPV2 | AMVV1 |
| Peldon 05/1      | 13556109             | 0.084 | 0.154 | 0.035 |
| Peldon 05/2      | 14405457             | 0.190 | 0.053 | 0.055 |
| Peldon 05/3      | 14674                | 0.190 | 0.204 | 0.075 |
| Notts 05/1       | 7625139              | 0.421 | 0.116 | 0.035 |
| Notts 05/2       | 6362601              | 0.091 | 0.039 | 3.143 \times 10^{-5} |
| Notts 05/3       | 10446490             | 0.084 | 0.111 | 4.786 \times 10^{-5} |
| Roth 09/1        | 4609793              | 0.146 | 0.065 | 4.338 \times 10^{-5} |
| Roth 09/2        | 3876546              | 0.231 | 0.089 | 5.159 \times 10^{-5} |
| Roth 09/3        | 8917820              | 0.048 | 0.005 | 2.242 \times 10^{-5} |

### Table 2. Primer sequences designed to RNA 2 of AMVV1, AMPV1 and AMPV2.

| Virus (accession number) | Primer | Sequence (5'→3') |
|--------------------------|--------|------------------|
| AMVV1 (LN713934)         | AMVV1 F | TACCTGACTCTGACAACTCAAAGGAGCCAGG |
| AMVV1 (LN713934)         | AMVV1 R | TAGTCTCTGTGCCAGAGTCCACTGCTAGT |
| AMPV1 (LN713935)         | AMPV1 F | AGGCCACTGAAAACATCTATTGGAGC |
| AMPV1 (LN713935)         | AMPV1 R | TTGAGCCGACGAAAGCGAAGCTGAGC |
| AMPV2 (LN713937)         | AMPV2 F | TCACCCGCTTTGGATATTGTGTGGTTG |
| AMPV2 (LN713937)         | AMPV2 R | ATCAAGCCCTATGTGGGCGTCCTTGTGACTCTAG |

### Table 3. Results following testing of black-grass population for susceptibility to herbicides Atlantis and Cheetah. Further details of these populations and the identification of the mutations in their target site proteins are as referenced. NTSR (enhanced metabolism refers to the relative rates of herbicide detoxification reported as H = high, M = medium, L = Low, — = not reported).

| Population | County | Location | ALS Mutations (%) | ACCase Mutation (%) | NTSR (Enhanced Metabolism) | Lethal Dose ATLANTIS (400 g/Ha) % damage 21 DAT | Lethal Dose Cheetah (1.25 l/Ha) % damage 21 DAT |
|------------|--------|----------|-------------------|--------------------|---------------------------|-----------------------------------------------|-----------------------------------------------|
| Roth 09    | Hertfordshire | Broadbalk | 0 | 0 | L | 85 | 100 |
| LongC 08   | Oxfordshire | Chalgrove | 42 | 8.5 | M | 28 | 15 |
| Notts 05   | Nottinghamshire | Notts | 0 | 22.5 | L | 81 | 6 |
| Kent 02    | Kent | Kent-Survey | 0 | 0 | H | 75 | 13 |
| Hor 08     | Oxfordshire | Oxford | 50 | 25 | — | 16 | 28 |
| Suffolk 09 | Suffolk | Suffolk survey | 0 | 27.5 | H | 33 | 17 |
| Peldon 05  | Essex | Peldon2 Suffolk survey | 50 | 0 | H | 25 | 17 |
| Vele 08    | Lincolnshire | Velcourt | 32.5 | 31.5 | — | 25 | 10 |
| Warren 09  | Bedfordshire | Conts All G/ Ha60 | 31.5 | 18.75 | M | 49 | 21 |
| R30 08     | Cambridgeshire | Huntingdon | 45 | 27.5 | — | 25 | 15 |
contigs was carried out using MetaGeneMark with default settings 26. Data from each of the four samples was assembled, with isoforms raising the total number of components (genes) to 180206. A total of 180206 components were assembled, with isoforms raising the total number of components to 373555. Non-host derived contigs were identified using Blastx to search the viral genome fragments. Results of RT-PCR testing of individual plants for AMVV1, AMPV1 and AMPV2 from RNA extracted from 6 black-grass populations. Extracts were tested for the Cp region of RNA2 for each virus.

| Population         | Numbers of infected plants/plants tested |
|-------------------|------------------------------------------|
|                   | AMVV1 | AMPV1 | AMPV2 |
| Feldon 05         | 21/36 | 23/36 | 16/36 |
| Cambridge 08      | 0/5   | 4/5   | 3/5   |
| Feldon 07         | 5/5   | 4/5   | 3/5   |
| Notts 05          | 0/5   | 2/5   | 2/5   |
| Roth 09           | 0/5   | 0/5   | 2/5   |
| Kent 02           | 0/5   | 2/5   | 1/5   |

Table 4. Results of RT-PCR testing of individual plants for AMVV1, AMPV1 and AMPV1 from RNA extracted from 6 black-grass populations. Extracts were tested for the Cp region of RNA2 for each virus.

Materials and Methods

Plant analysis. Seeds from herbicide-susceptible and -resistant black-grass and annual rye-grass derived from previously described populations (Table 3) were heat treated at 30°C for 14 d prior to use. For transcriptome studies, seeds were sown into potting medium (John Innes No. 2) and germinated in a growth room (20°C, under a 16:8 h light:dark photoperiod). Plants were then harvested after 14 days. For all other studies, plants were sown (20 seeds per 12 cm i.d. pot) in peat-based compost and grown under glass. To determine their tolerance to herbicides, black-grass populations were sprayed with Atlantis WG® and Cheetah® Gold (Bayer CropScience, UK). Spray trials were performed in triplicate, with each population of Alopecurus myosuroides treated with five different concentrations of the two herbicides. Atlantis WG® was applied at rates equivalent to 0, 100, 400, 800 and 1600 g/ha respectively (normal field rate = 400 g/ha). Cheetah® Gold was applied at; 0, 0.625, 2.5, 10 and 20 l/ha (normal field rate = 1.25 l/ha). Treatments were assembled in a randomised order and phytotoxicity assessments scored visually in comparison to formulation treatments at 7, 14 and 21 days after treatment (DAT). RNA samples for PCR and next generation sequencing were prepared from individual shoots (150 mg) in triplicate after freezing in liquid Nitrogen, homogenizing with a pestle and mortar then extracting with the RNeasy kit with protective antioxidant responses in some species 25. As viral infection is known to enhance tolerance to abiotic stress and enhance fitness in a wide variety of plants 2, we speculate that the presence of these viruses could provide an improved biochemical and genetic background for NTSR to evolve in wild grasses in the field. Given the widespread occurrence of NTSR in grass weeds and our lack of understanding of its functional links to stress tolerance, these findings suggest that further study of the links between herbicide resistance and infection by persistent viruses is warranted.

Transcriptome sequencing (low coverage – long read). RNA samples were used to generate double-stranded cDNA using the SMARTer™ PCR cDNA Synthesis Kit and Advantage® 2 PCR Kit (Clontech, Mountain View, CA). Double stranded cDNA was fractionated into smaller fragments (500–800 bp) by nebulisation. Emulsion PCR was carried out using the Lib-L-LV emPCR Kit with samples run on a GS-FLX sequencer (Roche). For sequence analysis, after quality control (QC) and removal of SMART cDNA amplification primers, each of the four datasets was subjected to de novo genome assembly with Newbler v2.6 (Roche/454 Life Sciences). The only deviation from default settings was the application of the [–urt] option to improve the production of contigs in low depth regions of the assembly. BlastN and BlastX searches were carried out using the Lib-L-LV emPCR Kit with samples run on a GS-FLX sequencer (Roche). For sequence analysis, after quality control (QC) and removal of SMART cDNA amplification primers, each of the four datasets was subjected to de novo genome assembly with Newbler v2.6 (Roche/454 Life Sciences). The only deviation from default settings was the application of the [–urt] option to improve the production of contigs in low depth regions of the assembly. BlastN and BlastX searches were carried out using a local copy of Genbank (downloaded 30/11/2011). Blast results were visualised with MEGAN v4.6313 with the following LCA parameters: Minimum support = 1, minimum score = 50, top percent = 10, win score = 0 and minimum complexity = 0. Confirmation of protein coding regions in selected contigs was carried out using MetaGeneMark with default settings 25. Data from each of the four samples was passed through a basic quality check with all sequences with an average quality (phred) score of <20 and length <40 bp discarded. Multiplex identifiers (MIDs) and SMART cDNA amplification primers were then identified and removed. Genomic datasets were assembled in preference to cDNA to improve the quantity and quality of non-host contigs.

Transcriptome sequencing (high coverage – short read). Total RNA (4 μg) was used to produce a poly-A enriched, indexed, TruSeq V2 Illumina sequencing library following the manufacturer’s instructions. Libraries were quantified using Qubit hs-double stranded DNA kits (Invitrogen, UK). They were then pooled in equimolar quantities and purified using Ampure XP beads (Truseq manual), prior to checking for size and quality using a Tapestation (Agilent). The two resulting libraries were sequenced on two lanes of a HiSeq sequencer (Illumina). After an initial QC step to trim poor quality regions with a Phred quality score of <25 and remove reads <50 bp, the remaining 302254761 sequences were assembled with the transcriptome assembler Trinity using default settings 27. A total of 180206 components (genes) were assembled, with isoforms raising the total number of contigs to 373555. Non-host derived contigs were identified using Blastx to search the viral genome fragments identified from the GS-FLX data and further verified using a Blastx search against the NCBI nr database. Alignment of the amino acid sequences of the viral RNA-dependent RNA polymerases was performed with pairwise global alignments using BLASTP 28,29.
ClustalW within the MEGA6 package\textsuperscript{28}, prior to the construction of neighbour-joining trees with 1000 bootstrap replcicates.

Reverse transcriptase-PCR (RT-PCR). All RNA extracts were tested using primers targeting the coat protein region of the RNA2 of AMVV1, AMPV1 and AMPV2 (Table 2). Total RNA (1 \(\mu\)l) was added to a 24 \(\mu\)l reaction containing \textregistered3\textsuperscript{TM} 1-Step RT-PCR ReddyMix™ Kit (Thermo Scientific) and 400 mM of each primer. The assays were performed in a Bio-Rad C1000\textsuperscript{TM} thermal cycler (Bio-Rad laboratories) using the cycling conditions: 48 °C for 30 mins for the reverse transcription step, 98 °C for 2 mins, followed by 35 cycles of 98 °C for 10 s, 63 °C for 30 s and 72 °C for 1 min and a final step of 72 °C for 5 min. Amplification products were separated on a 1.2% agarose gel stained with ethidium bromide and visualised on a UV transilluminator.

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Author Contributions

F.S., R.G., R.S., Z.R.-A. and I.A. performed the experimental work described and contributed to the preparation of the manuscript and figures, N.B., R.M. and R.E. wrote the main text of the MS. All authors reviewed the manuscript.
**Additional Information**

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