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Rapid report

Functional analysis of a Wheat Homeodomain protein, TaR1, reveals that host chromatin remodelling influences the dynamics of the switch to necrotrophic growth in the phytopathogenic fungus *Zymoseptoria tritici*

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Summary

- A distinguishing feature of Septoria leaf blotch disease in wheat is the long symptomless growth of the fungus amongst host cells followed by a rapid transition to necrotrophic growth resulting in disease lesions. Global reprogramming of host transcription marks this switch to necrotrophic growth. However no information exists on the components that bring about host transcriptional reprogramming.
- Gene-silencing, confocal-imaging and protein–protein interaction assays where employed to identify a plant homeodomain (PHD) protein, TaR1 in wheat that plays a critical role during the transition from symptomless to necrotrophic growth of Septoria.
- TaR1-silenced wheat show earlier symptom development upon Septoria infection but reduced fungal sporulation indicating that TaR1 is key for prolonging the symptomless phase and facilitating Septoria asexual reproduction. TaR1 is localized to the nucleus and binds to wheat Histone 3. Trimethylation of Histone 3 at lysine 4 (H3K4) and lysine 36 (H3K36) are found on open chromatin with actively transcribed genes, whereas methylation of H3K27 and H3K9 are associated with repressed loci. TaR1 specifically recognizes dimethylated and trimethylated H3K4 peptides suggesting that it regulates transcriptional activation at open chromatin.
- We conclude that TaR1 is an important component for the pathogen life cycle in wheat that promotes successful colonization by Septoria.

Introduction

The hemibiotrophic pathogen *Zymoseptoria tritici* (also known as *Mycosphaerella graminicola* and *Septoria tritici*) the causal agent of Septoria tritici blotch (STB) disease is one of the most devastating foliar pathogens of wheat. STB disease is the most significant threat to yield in Europe, and most other wheat growing regions (Orton *et al.*, 2011). Once on the leaves, Septoria spores begin to produce hyphae, which enter the leaf through the stomata and begin a period of slow intercellular filamentous biotrophic growth, in which it increases its presence within the mesophyll cell layer. While the length of this apparent latent phase can vary, possibly depending on various environmental conditions, it usually lasts for 11–13 d (Dean *et al.*, 2012). An intriguing aspect of Septoria infection that sets it apart from most plant pathogenic fungi is the long symptomless period of fungal growth, which ends in a rapid switch to necrotrophic growth exhibiting characteristics of programmed cell death (PCD) of host tissue (Kema *et al.*, 1996; Keon *et al.*,

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2007; Rudd *et al.*, 2008). The mechanism by which Septoria achieves this extraordinarily lengthy symptomless growth phase in host tissue is unclear although the suppression of the activation of early chitin-triggered immunity through the production of a LysM domain fungal effector protein has been shown to play a role (Marshall *et al.*, 2011; Lee *et al.*, 2014).

The subsequent necrotrophic phase of Septoria infection is marked by global reprogramming of host transcription to generating a cellular environment with high metabolic activity (Yang *et al.*, 2013). The consequent symptom development involves the accumulation of hydrogen peroxide (H_2O_2), degradation of cell-walls, collapse of mesophyll tissue, release of solutes and or nutrients from dying plant cells into the apoplastic spaces (Keon *et al.*, 2007) and rapid increase of fungal biomass leading to the production of spore-filled picnidia in necrotic lesion on leaves. However no information exists on the molecular components in the plant that bring about this rapid transcriptional reprogramming of seemingly thousands of genes during the transition from symptomless growth to necrotrophy.

Chromatin remodelling is a key feature of transcriptional reprogramming in that the compaction of the genome in the context of chromatin physically restricts the accessibility of DNA to transcription factors and RNA polymerase II (reviewed by Petesch & Lis, 2012). DNA accessibility in chromatin can be regulated by a number of mechanisms including modifications of DNA and Histones (reviewed by Bell *et al.*, 2011).

Histones are post-translationally modified via ADP-ribosylation, acetylation, phosphorylation, ubiquitination, sumoylation, or methylation (reviewed by Bannister & Kouzarides, 2011; Zentner & Henikoff, 2013). Whilst Histone modification such as acetylation is more generally correlated with open chromatin and therefore more active transcription (reviewed by Zentner & Henikoff, 2013), by contrast, Histone methylation can result in different transcriptional outcomes, depending on the amino acid modified and the degree of modification (reviewed by Li *et al.*, 2007). Typically trimethylation of Histone 3 at lysine 4 (H3K4) and lysine 36 (H3K36) are found at actively transcribed genes, whereas methylation of H3K27 and H3K9 are associated with repressed loci (reviewed by Zentner & Henikoff, 2013). The functional outcome of these changes in Histone methylations is either alteration of the strength of Histone–DNA interaction or recruitment of non-Histone proteins, called 'readers', to the chromatin (reviewed by Bannister & Kouzarides, 2011; Patel & Wang, 2013; Zentner & Henikoff, 2013). These 'reader' proteins are fundamentally important in regulating the activation of downstream gene expression by allowing the recruitment of transcriptional activator complexes. In depth studies in yeast and mammals have established that plant homeodomain (PHD) proteins act as 'readers' of Histone modifications directly linking chromatin remodelling to gene activation. Here we report the identification of a wheat PHD protein that is important for the control of the phase transition from symptomless to necrotrophic growth during Septoria infection. Wheat plants silenced for *TaR1* show earlier necrotic symptom development but reduced fungal sporulation. We demonstrate that TaR1 is localized to the nucleus in plants cells and binds to wheat Histone 3 *in planta*. TaR1 is able to specifically recognize Histone 3 peptides dimethylated and trimethylated at lysine 4 indicating that TaR1 regulates gene activation at transcriptionally active chromatin.

Materials and Methods

Plant materials and growth conditions

Nicotiana benthamiana plants were grown in environmentally controlled cabinets at 24°C with 16 h : 8 h, light:dark cycles. *Triticum aestivum* cv Avalon plants were grown in an environmentally controlled room at 24°C with 16 h : 8 h, light : dark cycles. All samples for RNA were collected 8 h into the 16 h light cycle.

Quantitative Real-time PCR (qRT-PCR)

All quantitative real-time polymerase chain reactions (qRT-PCRs) were performed in a 15-μl volume containing Rotor-Gene SYBR Green PCR Master Mix (Qiagen) with (1 : 10 v/v) first-strand cDNA as template. For normalization, the wheat ubiquitin gene (TaUb) was used as an endogenous control. Five independent biological repeats and three independent technical replicates were performed for each of the time points.

Pathotests

Zymoseptoria tritici isolate IPO323 was used for all pathology tests as described before (Keon *et al.*, 2007). Spores were grown on yeast extract peptone dextrose plates for 7 d at 18°C. For plant infection, spores were suspended in water containing 0.1% (v/v) Tween20 at a density of 7.5×10^5 spores ml^{-1}. Twenty-five-d-old wheat plants were spore inoculated (14 d after inoculation for silenced plants). Replicates of three leaves each were used to determine the *de novo* spore production within pycnidia after 28 d of infection from leaf washings using a light microscope and haemocytometer counts as previously described (Lee *et al.*, 2014).

Confocal microscopy

The subcelluar localization of yellow fluorescent protein (YFP) was visualized using a confocal laser scanning microscope (Leica SP5 CLSM; Berlin, Germany) with ×63 objective lenses as previously described. 4′6-Diamidino-2-phenylindole (DAPI) staining was carried out by infiltration of leaves with 10 μg ml^{-1} DAPI in 10 mM $MgCl_2$, 20 min before imaging. DAPI was excited at 405 nm and transmission was collected between 420 and 470 nm.

Virus induced gene silencing (VIGS)

Loss-of-function studies in *Triticum aestivum* were carried out through the virus-induced gene silencing (VIGS) system (Baulcombe, 1999) based on the Barley Stripe Mosaic Virus (BSMV) as previously described (Yuan *et al.*, 2011). The specificity and silencing efficiency of the constructs was predicted using siRNA finder si-fi (labtools.ipk-gatersleben.de/index.html).

In vitro histone binding assay

Histone peptide pulldown assays were performed using a method adapted from Lee *et al.* (2009). Since this Lee *et al.* (2009) had previously shown that PHDs nearly identical to TaR1 did not bind to H3k9me3, so we used H3k27me3 peptides instead, as this had not previously been tested.

Agrobacterium mediated transient assays, protein extraction, immunoprecipitation and immunoblotting

Gene constructs were transiently expressed in *Nicotiana benthamiana* plants using *Agrobacterium* mediated transformation (Ewan *et al.*, 2011).

The protein samples extracted from *Nicotiana benthamiana* plants (Ewan *et al.*, 2011) were mixed with 50 µl anti-GFP (Chromotek anti-GFP beads) and incubated on ice for 30 min. The beads were centrifuged down at 10 000 ***g*** for 1 min and washed three times with 1 ml of cold IP buffer. After the last wash 50 µl of pre-heated (95°C) 1× SDS-loading buffer was used to elute the immuno-complex and analysed on 10% SDS-PAGE using immunoblotting methods with Abcam (Cambridge, UK) anti-GFP and anti-HA antibodies.

Chromatin was extracted from *Nicotiana benthamiana* plants using a method adapted from Huang *et al.* (2009). As this protocol used animal cells, nuclei were first extracted using the method adapted for *N. tabacum* from Sikorskaite *et al.* (2013), chromatin was then isolated from these nuclei, using the Huang *et al.* (2009) method.

Sequence analysis

The domains on TaR1 protein were identified based on models from Prosite, Panther, Pfam, Smart, Superfam and Gene3D (Schultz *et al.*, 1998; Letunic *et al.*, 2004) and manually refined. Domain sequences were aligned using CLUSTAL-OMEGA (Sievers *et al.*, 2011).

Accession numbers

Sequence data for the *Triticum aestivum* RING1 cDNA has been deposited in the GenBank database under the accession number KJ472832.

Results

TaR1 is a plant PHD protein

In a screen to identify components of the ubiquitin system involved in Septoria responses in wheat we isolated an mRNA that encoded a potential variant RING domain protein (Sadanandom *et al.*, 2012) designated *Triticum aestivum RING1* (*TaR1*) that had higher expression in Septoria infected leaf tissue. However closer inspection of the RING domain in TaR1 using Clustal Omega alignment with other known canonical PHD and RING domains (Fig. 1a) indicated that TaR1 actually contained a (C4HC3) PHD (Fig. 1a). The PHD in TaR1 contained the main conserved cysteine-histidine zinc-binding backbone present throughout all PHD proteins (in red in Fig. 1a). Further, it also showed that TaR1 contained the critical residues previously indicated to be important for binding to trimethylated lysine 4 on Histone 3 (H3K4me3) by Arabidopsis Alfin-like PHD proteins (Fig. 1a in gold and blue) (Lee *et al.*, 2009). An aspartate and a glutamate residue (Fig. 1a in blue) are also strongly conserved between TaR1 and the Alfin-like group of PHD proteins in plants. These residues were predicted to interact with arginine 2 of Histone 3, and loss of either, by mutation, prevented H3K4me3 binding (Lee *et al.*, 2009).

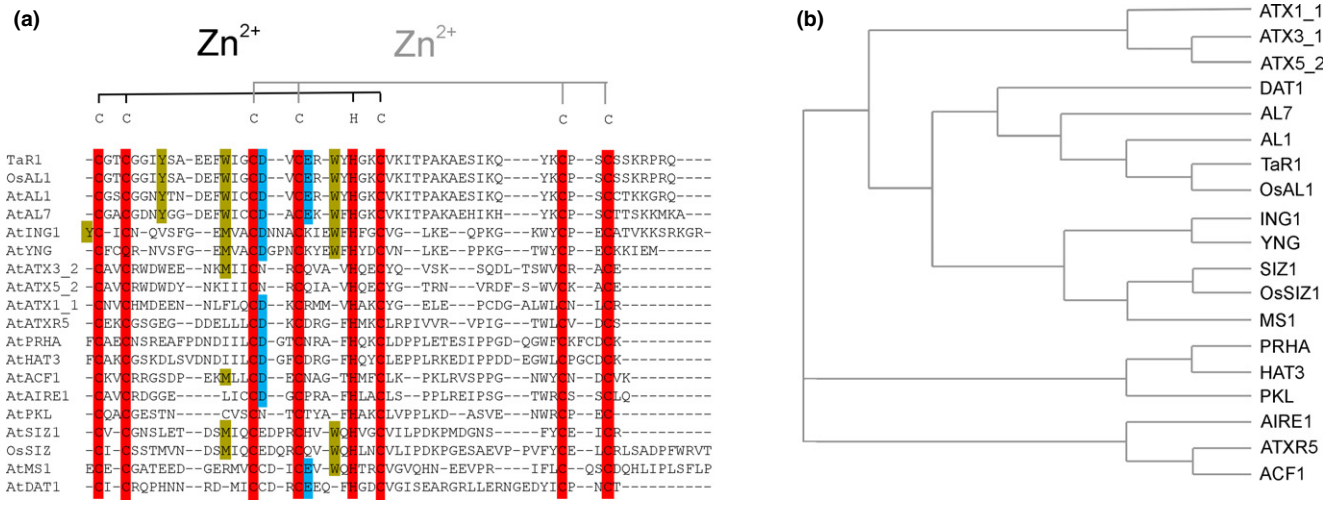

Fig. 1 Sequence analysis reveals that *Triticum aestivum* R1 contains a plant homeodomain (PHD) and an 'alfin-like' domain. (a) Multiple sequence alignment of the PHDs of *Triticum aestivum* (Ta) R1, *Arabidopsis* (At) AL1, *Oryza sativa* (Os) AL1 and AL7, with PHDs from other plant and animal proteins. The alignment was produced using Clustal Omega (1.2.0), then re-colourized. The zinc-binding cysteine-histidine backbone is shown in red. Residues indicated by Lee *et al.* (2009) as important for formation of an aromatic cage (gold) and for Histone 3 Arginine 2 recognition (blue) are also indicated. The alignment was created using protein sequences of the PHDs only. (b) Phylogenetic tree illustrating the grouping of PHDs of TaR1, Arabidopsis Alfin-like 1 and 7 (AtAL1, AtAL7) and rice Alfin-like 1 (OsAL1), amongst other plant and animal PHDs. The phylogenetic tree was created using Clustal O(1.2.0).

Construction of a phylogenetic tree (Fig. 1b) confirmed that, based on the PHD alone, TaR1 was most similar to Alfin-like proteins sharing the greatest homology to the rice protein OsAL1 (98.15% identity) followed by Arabidopsis AL1 (81.48%) and AL7 (64.81) indicating functional conservation. These data strongly suggested that TaR1 represents a Histone binding protein from hexaploid wheat.

Further analysis showed that each of these Alfin-like proteins, as well as the original Alfin1 from alfalfa (MsAlfin1) (Bastola *et al.*, 1998) also share an N-terminal domain of unknown function with TaR1. An alignment of these sequences (Supporting Information Fig. S1a) shows strong conservation of many residues throughout the domain (red). The phylogenetic tree based on this alignment (Supporting Information Fig. S1b) shows that again TaR1 is most

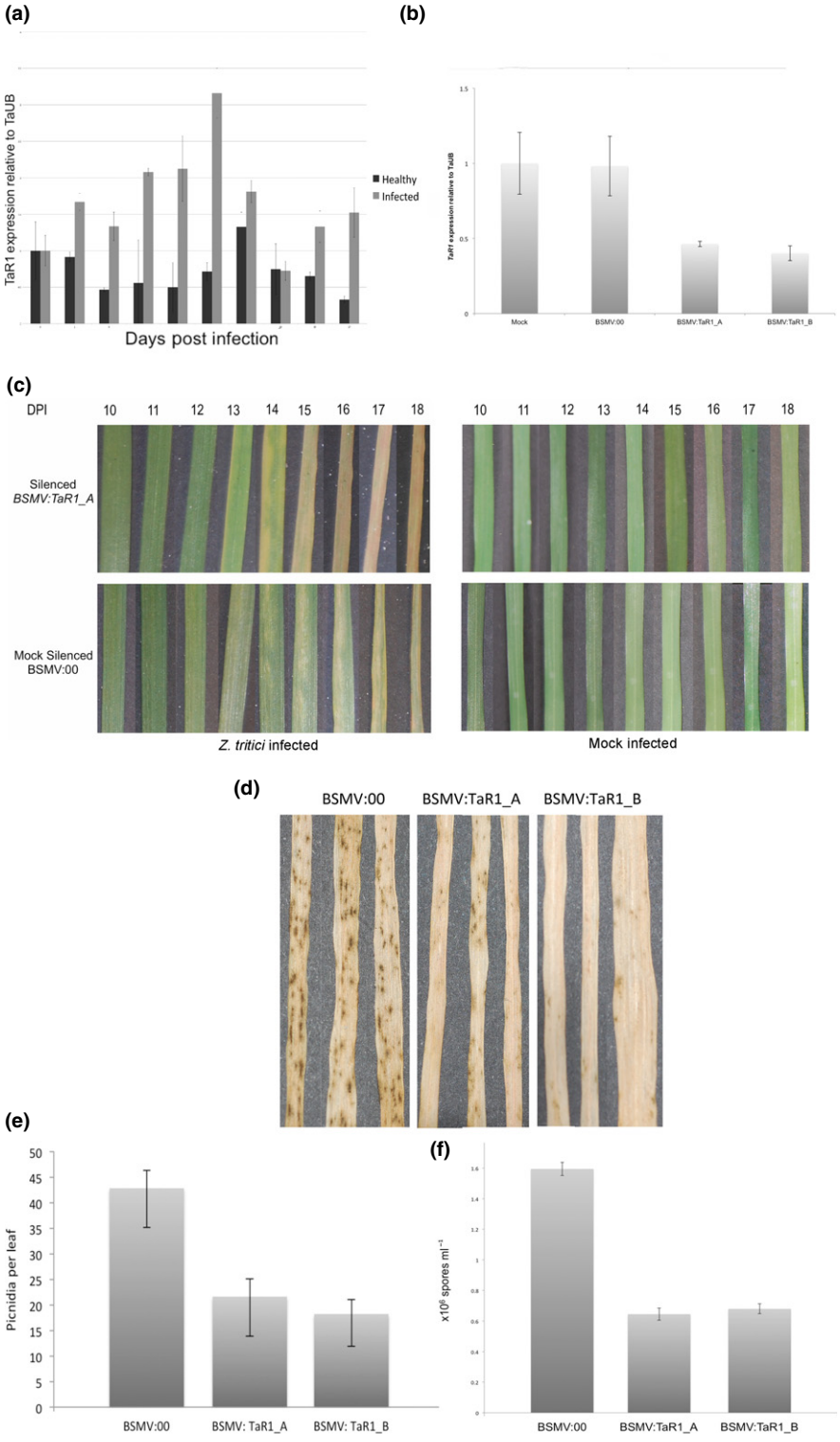

Fig. 2 *Triticum aestivum R1* expression increases on infection. Silencing *TaR1* leads to earlier onset of symptoms and reduced spore production. (a) Real-time polymerase chain reaction (RT-PCR) data shows the expression pattern of *TaR1* in both Septoria infected (■) and healthy (■) plants over 17 d of infection. Error bars, ± standard error (SE) of the mean of raw data. (b) RT-PCR data shows the expression of *TaR1* is reduced in virus-induced gene silencing (VIGS) treated plants silenced by BSMV:TaR1_A and BSMV:TaR1_B, 14 d after Barley Stripe Mosaic Virus (BSMV) treatment, compared to BSMV:00 and wild-type (Mock). Expression in the wild-type sample is set to 1 and all expression levels are given in arbitrary units relative to this. Error bars, ± SE of the mean of raw data. (c) A single leaf of BSMV:TaR1_A silenced and BSMV:00 mock silenced wheat from 10 to 18 d post-infection with *Zymoseptoria tritici* (left), or mock infection (right). Symptoms appear up to 2 d earlier in BSMV:TaR1_A silenced plants (day 13) compared to mock silenced (day 15), while no symptoms appear in either of the mock silenced plants. (d) Representative images showing the level of picnidia formation on mock silenced and TaR1 silenced leaves, 4 wk after infection with *Z. tritici*. (e) The number of picnidia produced on the leaves of TaR1 silenced plants shows about a two-fold reduction compared to mock silenced plants. Student's *t*-tests show a significant difference between the number of picnidia produced on the mock silenced plants and the TaR1_A (P value = 9.9×10^{-3}) and TaR1_B ($P = 5.2 \times 10^{-3}$) silenced lines, but no difference between the two TaR1 silenced lines ($P = 0.23$). Error bars, ± SE of the mean of raw data. (f) Spore washes performed 4 wk after infection show a more than two-fold reduction in spores produced on *TaR1* silenced plants. Student's *t*-tests show a significant difference between the number of spores produced in the mock silenced plants and the TaR1_A (P value = 1.4×10^{-32}) and TaR1_B ($P = 2.4 \times 10^{-34}$) silenced lines, but no difference between the two TaR1 silenced lines ($P = 0.49$). Error bars, ± SE of the mean of raw data.

similar to OsAL1 (92.13% identity), but also has strong homology to AtAL1 (69.53%), AtAL7 (71.09) and MsAlfin1 (70.31) indicating functional conservation.

TaR1 gene expression is induced early during Septoria infection and peaks at the transition period from symptomless to the necrotropic phase

Our preliminary data indicated that *TaR1* transcripts are upregulated in 2-wk-old Septoria infected wheat leaves, however, we wanted to ascertain the duration of the induction of *TaR1* gene expression throughout the Septoria lifecycle in wheat. This might reveal the critical period for TaR1 requirement during Septoria–wheat interaction.

RT-PCR with *TaR1* specific primers on cDNA from samples collected daily throughout a time-course of wheat seedlings (from 2-wk-old) infected with Septoria (Fig. 2a) indicate that TaR1 expression is upregulated early in the infection process. *TaR1* transcript levels are immediately increased by day 1 post-infection, and continue throughout the first week of infection peaking at day 9 where it is up three-fold indicating that *TaR1* expression is stimulated by Septoria even during the symptomless phase. Expression suddenly drops back to match that of uninfected plants on day 13, just when Septoria is postulated to start its life-cycle phase change into necrotrophy, and then increases again at day 17 when symptoms are fully manifested. Our data indicates that the *TaR1* transcript is maximally expressed during the late stages of symptom-free growth, and then suddenly decreases when major transcriptional reprogramming is expected to happen during the symptomless to necrotrophic phase transition of Septoria in wheat. A similar pattern of upregulation during infection in a group of defence related WRKY transcription factor genes was also observed (Supporting Information Fig. S2) confirming the activation of plant immunity pathways.

Silencing TaR1 using VIGS results in early disease symptom formation but reduced *Zymoseptoria tritici* sporulation

VIGS (Matthew, 2004; Watson *et al.*, 2005), was used to study the function of *TaR1*. Gene silencing vectors based on BSMV (Ratcliff *et al.*, 2001; Burch-Smith *et al.*, 2004; Wang & Metzlaff, 2005; Lee *et al.*, 2012), carrying two different and nonoverlapping DNA fragments from the *TaR1* sequence, were used to induce sequence-specific degradation of the endogenous *TaR1* mRNA and therefore a knock down in its gene expression. BLAST analyses confirmed that both cDNA fragments (BSMV:*TaR1_A* and BSMV:*TaR1_B*) were unique to *TaR1*, ruling out potential silencing of closely related 'off target' genes. The siRNA finder software si-fi predicted no off-target silencing and found 21 'effective siRNA hits' for TaR1_A and 78 for TaR1_B. These are in line with predictions for effectively silenced genes in a similar study (Lee *et al.*, 2014). The efficiency of *TaR1* silencing was confirmed by qRT-PCR on mRNA from emerging leaves of plants 14 d after inoculation with the BSMV:*TaR1_A* and BSMV:*TaR1_B* constructs (Fig. 2b). *TaR1*-silenced plants had no obvious morphological phenotype (Supporting Information Fig. S3). Phytoene desaturase (PDS) silencing was used as positive controls for BSMV mediated gene silencing (Lee *et al.*, 2012) (Supporting Information Fig. S4).

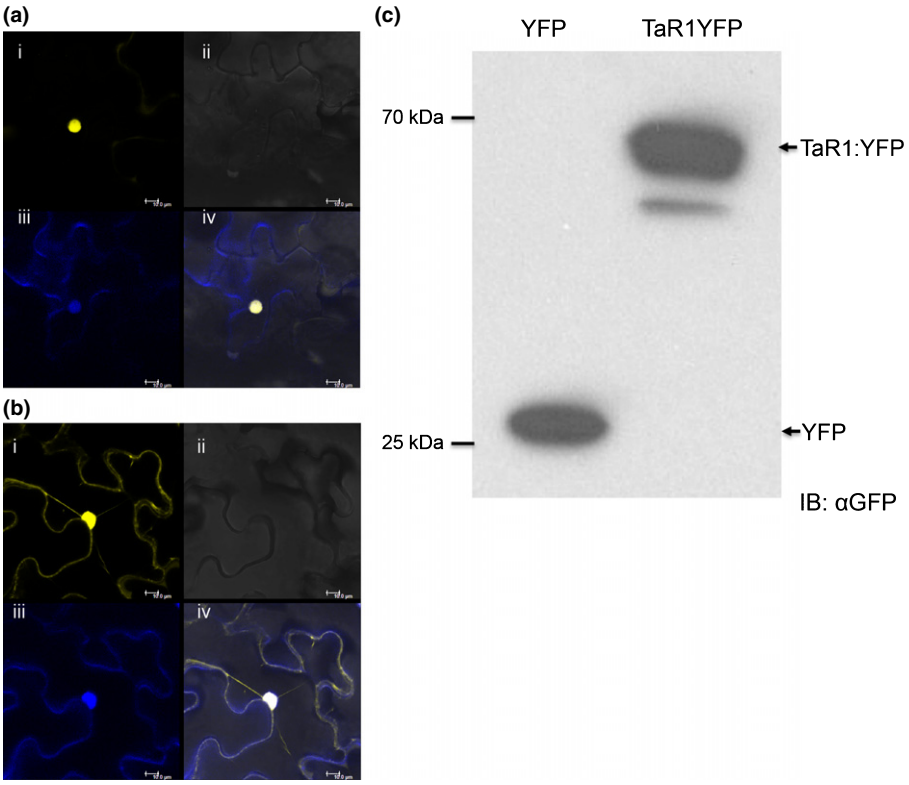

Fig. 3 *Triticum aestivum* R1 localizes to the nucleus. (a) YFP-TaR1 transiently expressed via infiltration of *Nicotiana benthamiana* with Agrobacterium transformed with Pearlygate104-TaR1 contruct. (i) detected at 555–700 nm, (ii) white light, (iii) detected at 420–470 nm, (iv) i–iii overlayed. YFP-TaR1 (yellow) locates specifically to the nucleus, which is marked with DAPI stain (blue). (b) YFP transiently expressed via infiltration in *N. benthamiana* with agrobacterium transformed with empty Pearlygate104 vector. YFP (yellow) locates to the nucleus, which is marked with DAPI stain (blue), but also to the rest of the cell. (c) Western blotting with anti-GFP antibody, which recognizes YFP, shows presence of YFP and YFP-TaR1 in infiltrated *N. benthamiana*.

Pathogen infection studies were carried out with Septoria isolate IPO323, a virulent pathogen of wheat variety Avalon (Arraiano & Brown, 2006). A titre of IPO323 Septoria isolate at 7.5×10^6 spores ml^{-1} was used to infect Avalon that had previously been inoculated with the *TaR1* silencing vectors BSMV: *TaR1_A* and BSMV: *TaR1_B* constructs. No significant difference in disease symptoms development was detected up to 13 d post-infection (DPI) between the control and the *TaR1*-silenced plants. However, at day 14 clear necrotic lesions developed on *TaR1* silenced plants when compared to vector only control plants (Fig. 2c). Further the necrotic lesions were more pronounced in *TaR1* silenced plants compared to controls. Surprisingly *TaR1* silenced plants produced approximately half the number of picnidia and spores (Fig. 2d–f) as mock silenced plants. It has previously been reported that Septoria sporulates within necrotic lesions on infected wheat (Keon *et al.*, 2007). Intriguingly our data demonstrates that accelerated or earlier lesion formation caused by *TaR1* silencing is detrimental to Septoria sporulation. Our data reveals that the host gene *TaR1* influences the ability of Septoria to complete its life cycle in wheat.

TaR1 is a nuclear protein that binds to methylated Histone 3

The observation that TaR1 contains the characteristic features required for H3K4me3/2 binding prompted us first to ascertain whether TaR1 is nuclear localized. The subcellular localization of TaR1 was determined by Agrobacterium mediated transient assays in *Nicotiana benthamiana*. We transiently expressed TaR1 translationally fused to yellow or red fluorescent protein (YFP-TaR1, RFP-TaR1) in tobacco leafs and examined cellular YFP or RFP fluorescence by laser-scanning confocal microscopy. Fluorescence of YFP-TaR1 or RFP-TaR1 was detected only in the nuclei of all cells examined (Fig. 3a, Supporting Information Fig. S5a). Whilst the DNA constructs expressing only YFP or RFP showed fluorescence in the nucleus and cytoplasm (Fig. 3b, Supporting Information Fig. S5b). Immunoblotting experiments with anti-GFP antibodies that cross-react with YFP confirmed the presence of YFP-TaR1 fusion proteins (Fig. 3c).

To ascertain if TaR1 could bind to wheat Histones, Agrobacterium containing the HA epitope tagged wheat Histone 3 (TaH3) and YFP-TaR1 alone driven by 35S CaMV promoter constructs were mixed at a 1 : 1 ratio and infiltrated into *Nicotiana benthamiana* leaves. As a negative control, Agrobacterium containing the HA-tagged Histone 3 construct was co-infiltrated with Agrobacterium containing YFP alone under the same 35S CaMV promoter. The expression of the fusion proteins was confirmed by immunoblotting with anti-HA and anti-GFP antibodies (Fig. 4a). Co-immunoprecipitation assays performed with anti-GFP antibodies showed that HA-TaH3 co-immunoprecipitated with YFP-TaR1 but not with YFP indicating that TaR1 specifically interacts with wheat Histone 3 (Fig. 4a, Supporting Information Fig. S6a). Probing with anti-H3k4me3 antibodies showed that the modified Histone 3 immunoprecipitated with YFP-TaR1 but not with YFP, suggesting it is capable of binding chromatin-associated Histone 3 (Fig. 4a, Supporting Information Fig. S6b).

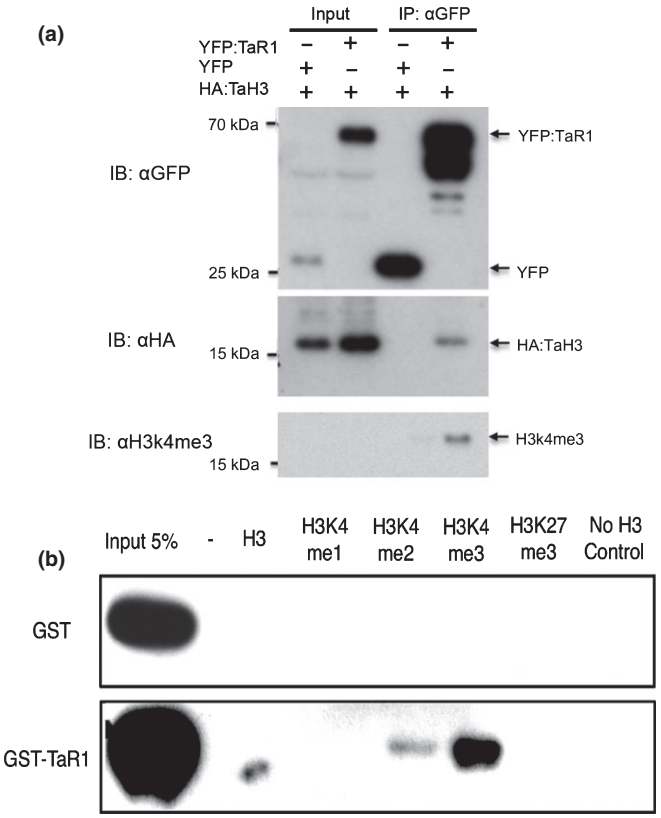

Fig. 4 *Triticum aestivum* R1 interacts with Histone 3 *in vitro* and *in vivo*. (a) GFP pulldown of protein from *Nicotiana benthamiana* tissue co-infiltrated with HA-TaH3 and either YFP-TaR1 or YFP. αGFP Western blot shows presence of YFP-TaR1 and YFP both in the input sample and after αGFP pulldown (αGFP also recognizes YFP). αHA Western blot shows HA-tagged TaH3 is present in the input of both, but is pulled down by YFP-TaR1 and not by YFP. Anit-H3k4me3 Western blot shows that Histone 3 trimethylated on lysine 4 is pulled down by YFP-TaR1 and not by YFP. (b) Histone peptide pulldown assay of GST-TaR1. Purified GST-TaR1 was incubated with biotinylated Histone peptides with no methylation (H3), monomethylated lysine 4 (H3K4me1), dimethylated lysine 4 (H3K4me2), trimethylated lysine 4 (H3K4me3) or trimethylated lysine 27 (H3K27me3), which were pulled down with streptavidin beads. Western blotting with αGST shows TaR1 to bind specifically to Histone 3 peptides, with lysine 4 methylated and particularly to trimethylated lysine 4 (H3K4me3).

Proteins containing PHDs, similar to that which is identified in TaR1, have been shown to bind Histones with specific post-translational modifications (Lee *et al.*, 2009). In particular the main ligand identified for PHDs are dimethylated or trimethylated lysine 4 of Histone 3 (H3K4m3/2). Using biotin pulldown assays with Histone peptides as baits we investigated whether affinity purified recombinant TaR1 could bind H3K4me3/2 (Fig. 4b). TaR1 was found to bind H3K4me3 and to a lesser extent H3K4me2 indicating that a high level of methylation is required for binding. However TaR1 did not recognize H3K27me3 indicating that TaR1 PHD specifically binds to Histones dimethylated and trimethylated at lysine 4.

Discussion

Previous studies have indicated that the long symptomless growth period of Septoria is facilitated by the suppression of plant defences

(Marshall *et al.*, 2011; Lee *et al.*, 2014) and that the sudden switch to necrotrophy and plant cell death is accompanied by transcriptional changes to a large number of genes within the host leaf (Yang *et al.*, 2013), and in the fungus (Keon *et al.*, 2007; Rudd *et al.*, 2010). However, the mechanism behind this seismic transcriptional shift in the plant is still unknown.

Chromatin structure is known to affect transcriptional activity and remodelling to this would be capable of bringing about such a broad variety of changes across the cell. Here we reveal the identity of a Histone interacting protein that could provide a link between chromatin remodelling processes and the development of Septoria leaf blotch disease. Proteins with H3K4me3 binding capability have previously been shown to act as 'readers' of chromatin, which can recruit chromatin remodelling proteins, or protein complexes, onto specific transcriptionally active areas (Wysocka *et al.*, 2006). In doing so, these proteins directly bring about changes to Histone modification and chromatin structure and, through this, affect gene transcription (Shi *et al.*, 2006). We have shown that TaR1 specifically binds to H3K4me3/2. The role of TaR1 in Septoria infection may well rely on a similar function of directly bringing about chromatin remodelling. Further work in wheat to identify the other core components that establish this transcriptional reprogramming would establish a new mechanism that is exploited by Septoria during STB disease.

Our data show that *TaR1* expression is increased almost entirely throughout the period of infection, but at 13 DPI the level returns to that seen in a healthy plant, just as the switch to cell death is about to take place. Silencing of *TaR1* was seen to bring about earlier cell death symptoms in infected leaves, while negatively affecting the reproductive capacity of the fungus. These data suggests that the function of TaR1 is to suppress this response, and that the fungus is using this to its own advantage. By somehow maintaining the function of TaR1, the host's response is delayed until the fungus has reached a critical internal biomass, at which it is able to reproduce at peak efficiency. Such a 'hi-jacking' of a host plant-signalling pathway is not uncommon, and has previously been suggested for this same plant–pathogen interaction (Rudd *et al.*, 2008; Hammond-Kosack & Rudd, 2008; Deller *et al.*, 2011; Dean *et al.*, 2012). However this would be the first example of such a component operating in the crucial transition phase within host nuclei.

Along with *TaR1*, we also saw a similar pattern of upregulation during infection in a group of *WRKY* transcription factors (Supporting Information Fig. S2). In Arabidopsis, WRKYs regulated by HDA19, a Histone deactylase (HDAC), negatively regulates *PR1*, an important pathogen response gene (Kim *et al.*, 2008). Histone methylation by ATX1 has also been shown to regulate a WRKY transcription factor involved in the regulation of salicylic acid (SA) signalling (Alvarez-Venegas *et al.*, 2007). Both ATX1 and HDA19 affect Arabidopsis infection by *Pseudomonas syringae*. Another Arabidopsis HDAC, SRT2, is a suppressor of SA biosynthesis. SRT2 is downregulated upon *P. syringae* infection, allowing SA production and the expression of defence genes (Wang *et al.*, 2010). The TaR1 protein could potentially operate in a similar manner, affecting Histone acetylation, to suppress SA biosynthesis and pathogen responsive genes.

Through the control of the wheat H3K4me3 binding protein TaR1, Septoria may regulate chromatin-remodelling events to delay plant defence responses. This prevents disruption to the pathogens natural life cycle and allows it to reproduce more effectively, and in doing so, spread faster through the field. This work demonstrates a mechanism that may allow Septoria to bypass the natural defences of the plant, and so highlights an area in which future Septoria control measures could be developed.

Acknowledgements

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References

Alvarez-Venegas R, Al Abdallat A, Guo M, Alfano JR, Avramova Z. 2007. Epigenetic control of a transcription factor at the cross section of two antagonistic pathways. *Epigenetics* **2**: 106–113.

Arraiano LS, Brown JKM. 2006. Identification of isolate-specific and partial resistance to septoria tritici blotch in 238 European wheat cultivars and breeding lines. *Plant Pathology* **55**: 726–738.

Bannister AJ, Kouzarides T. 2011. Regulation of chromatin by histone modifications. *Cell Research* **21**: 381–395.

Bastola DR, Pethe VV, Winicov I. 1998. Alfin1, a novel zinc-finger protein in alfalfa roots that binds to promoter elements in the salt-inducible MsPRP2 gene. *Plant Molecular Biology* **38**: 1123–1135.

Baulcombe DC. 1999. Fast forward genetics based on virus-induced gene silencing. *Current Opinion in Plant Biology* **2**: 109–113.

Bell O, Tiwari VK, Thoma NH, Schubeler D. 2011. Determinants and dynamics of genome accessibility. *Nature Reviews Genetics* **12**: 554–564.

Burch-Smith TM, Anderson JC, Martin GB, Dinesh-Kumar SP. 2004. Applications and advantages of virus-induced gene silencing for gene function studies in plants. *Plant Journal* **39**: 734–746.

Dean R, Van Kan J, Pretorious Z, Hammond-Kosack K, Di Pietro A, Spanu P, Rudd J, Dickman M, Kahmann R, Ellis J *et al.* 2012. The top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology* **13**: 414–430.

Deller S, Hammond-Kosack KE, Rudd JJ. 2011. The complex interactions between host immunity and non-biotrophic fungal pathogens of wheat leaves. *Journal of Plant Physiology* **168**: 63–71.

Ewan R, Pangestuti R, Thornber S, Craig A, Carr C, O'Donnell L, Zhang C, Sadanandom A. 2011. Deubiquitinating enzymes AtUBP12 and AtUBP13 and their tobacco homologue NtUBP12 are negative regulators of plant immunity. *New Phytologist* **191**: 92–106.

Hammond-Kosack KE, Rudd JJ. 2008. Plant resistance signalling hijacked by a necrotrophic fungal pathogen. *Plant Signaling & Behavior* **3**: 993–995.

Huang J, Huen MS, Kim H, Leung CC, Glover JN, Yu X, Chen J. 2009. RAD18 transmits DNA damage signalling to elicit homologous recombination repair. *Nature Cell Biology* **11**: 592–603.

Kema GHJ, Yu D, Rijkenberg FHJ, Shaw MW, Baayen RP. 1996. Histology of the pathogenesis of *Mycosphaerella graminicola* in wheat. *Phytopathology* **86**: 777–787.

Keon J, Antoniw J, Carzaniga R, Deller S, Ward JL, Baker JM, Beale MH, Hammond-Kosack K, Rudd JJ. 2007. Transcriptional adaptation of *Mycosphaerella graminicola* to programmed cell death (PCD) of its susceptible wheat host. *Molecular Plant–Microbe Interactions* **20**: 178–193.

Kim KC, Lai ZB, Fan BF, Chen ZX. 2008. Arabidopsis WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defense. *Plant Cell* **20**: 2357–2371.

Lee WY, Lee D, Chung WI, Kwon CS. 2009. Arabidopsis ING and Alfin1-like protein families localize to the nucleus and bind to H3K4me3/2 via plant homeodomain fingers. *Plant Journal* **58**: 511–524.

Lee WS, Hammond-Kosack KE, Kanyuka K. 2012. Barley stripe mosaic virus-mediated tools for investigating gene function in cereal plants and their pathogens: virus-induced gene silencing, host-mediated gene silencing, and virus-mediated overexpression of heterologous protein. *Plant Physiology* **160**: 582–590.

Lee WS, Rudd JJ, Hammond-Kosack KE, Kanyuka K. 2014. *Mycosphaerella graminicola* LysM effector-mediated stealth pathogenesis subverts recognition through both CERK1 and CEBiP homologues in wheat. *Molecular Plant–Microbe Interactions* **27**: 236–243.

Letunic I, Copley RR, Schmidt S, Ciccarelli FD, Doerks T, Schultz J, Ponting CP, Bork P. 2004. SMART 4.0: towards genomic data integration. *Nucleic Acids Research* **32**: D142–D144.

Li B, Carey M, Workman JL. 2007. The role of chromatin during transcription. *Cell* **128**: 707–719.

Marshall R, Kombrink A, Motteram J, Loza-Reyes E, Lucas J, Hammond-Kosack KE, Thomma B, Rudd JJ. 2011. Analysis of two *in planta* expressed LysM effector homologs from the fungus *Mycosphaerella graminicola* reveals novel functional properties and varying contributions to virulence on wheat. *Plant Physiology* **156**: 756–769.

Matthew L. 2004. RNAi for plant functional genomics. *Comparative and Functional Genomics* **5**: 240–244.

Orton E, Deller S, Brown J. 2011. *Mycosphaerella graminicola: from genomics to disease control. Molecular Plant Pathology* **12**: 413–424.

Patel DJ, Wang ZX. 2013. Readout of epigenetic modifications. *Annual Review of Biochemistry* **82**: 81–188.

Petesch S, Lis J. 2012. Overcoming the nucleosome barrier during transcript elongation. *Trends in Genetics* **28**: 285–294.

Ratcliff F, Martin-Hernandez AM, Baulcombe DC. 2001. Tobacco rattle virus as a vector for analysis of gene function by silencing. *Plant Journal* **25**: 237–245.

Rudd JJ, Keon J, Hammond-Kosack KE. 2008. The wheat mitogen-activated protein kinases TaMPK3 and TaMPK6 are differentially regulated at multiple levels during compatible disease interactions with *Mycosphaerella graminicola*. *Plant Physiology* **147**: 802–815.

Rudd JJ, Antoniw J, Marshall R, Motteram J, Fraaije B, Hammond-Kosack R. 2010. Identification and characterisation of *Mycosphaerella graminicola* secreted or surface-associated proteins with variable intragenic coding repeats. *Fungal Genetics and Biology* **47**: 19–32.

Sadanandom A, Bailey M, Ewan R, Lee J, Nelis S. 2012. The ubiquitin-proteasome system: central modifier of plant signalling. *New Phytologist* **196**: 13–28.

Schultz J, Milpetz F, Bork P, Ponting CP. 1998. SMART, a simple modular architecture research tool: identification of signaling domains. *Proceedings of the National Academy of Sciences, USA* **95**: 5857–5864.

Shi XB, Hong T, Walter KL, Ewalt M, Michishita E, Hung T, Carney D, Pena P, Lan F, Kaadige MR *et al.* 2006. ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. *Nature* **442**: 96–99.

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Soeding J *et al.* 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology* **7**: 539.

Sikorskaite S, Rajamaki M-L, Baniulis D, Stanys V, Valkonen JPT. 2013. Optimised methodology for isolation of nuclei from leaves of species in the *Solanaceae* and *Rosaceae* families. *Plant Methods* **9**: 31.

Wang CZ, Gao F, Wu JG, Dai JL, Wei CH, Li Y. 2010. Arabidopsis putative deacetylase AtSRT2 regulates basal defense by suppressing PAD4, EDS5 and SID2 expression. *Plant and Cell Physiology* **51**: 1291–1299.

Wang MB, Metzlaff M. 2005. RNA silencing and antiviral defense in plants. *Current Opinion in Plant Biology* **8**: 216–222.

Watson JM, Fusaro AF, Wang MB, Waterhouse PM. 2005. RNA silencing platforms in plants. *FEBS Letters* **579**: 5982–5987.

Wysocka J, Swigut T, Xiao H, Milne TA, Kwon SY, Landry J, Kauer M, Tackett AJ, Chait BT, Badenhorst P *et al.* 2006. A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. *Nature* **442**: 86–90.

Yang F, Li WS, Jorgensen HJL. 2013. Transcriptional reprogramming of wheat and the hemibiotrophic pathogen *Septoria tritici* during two phases of the compatible interaction. *PLoS ONE* **8**: e81606.

Yuan C, Li C, Yan LJ, Jackson AO, Liu ZY, Han CG, Yu JL, Li DW. 2011. A high throughput barley stripe mosaic virus vector for virus induced gene silencing in monocots and dicots. *PLoS ONE* **6**: e26468.

Zentner GE, Henikoff S. 2013. Regulation of nucleosome dynamics by histone modifications. *Nature Structural & Molecular Biology* **20**: 259–266.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Sequence alignments of alfin-like domains.

Fig. S2 Real-time PCR analysis of TaWRKY transcription factor gene expression in response to Septoria infection.

Fig. S3 Adult wheat plants silencing TaR1 gene expression are not grossly different to unsilenced control plants in their morphology.

Fig. S4 Efficient silencing of phytoene desaturase (PDS) gene expression in wheat.

Fig. S5 Confocal imaging of TaR1-RFP fusion protein.

Fig. S6 Western blot analysis demonstrates *in vivo* interaction of HA-TaR1 and YFP-TaH3.

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