In planta expression screens of candidate effector proteins from the wheat yellow rust fungus reveal processing bodies as a pathogen-targeted plant cell compartment

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ABSTRACT
Rust fungal pathogens of wheat (Triticum spp.) affect crop yields worldwide. The molecular mechanisms underlying the virulence of these pathogens remain elusive, due to the limited availability of suitable molecular genetic research tools. Notably, the inability to perform high-throughput analyses of candidate virulence proteins (also known as effectors) impairs progress. We previously established a pipeline for the fast-forward screens of rust fungal effectors in the model plant Nicotiana benthamiana. This pipeline involves selecting candidate effectors in silico and performing cell biology and protein-protein interaction assays in planta to gain insight into the putative functions of candidate effectors. In this study, we used this pipeline to identify and characterize sixteen candidate effectors from the wheat yellow rust fungal pathogen Puccinia striiformis f.sp tritici. Nine candidate effectors targeted a specific plant subcellular compartment or protein complex, providing valuable information on their putative functions in plant cells. One candidate effector, PST02549, accumulated in processing bodies (P-bodies), protein complexes involved in mRNA decapping, degradation, and storage. PST02549 also associates with the P-body-resident ENHANCER OF mRNA DECAPPING PROTEIN 4 (EDC4) from N. benthamiana and wheat. Our work identifies P-bodies as a novel plant cell compartment targeted by pathogen effectors.

KEY WORDS
Agrobacterium tumefaciens, coimmunoprecipitation, confocal microscopy, live-cell imaging, mass spectrometry, Pucciniales, Triticum aestivum, varicose, wheat stripe rust

INTRODUCTION
Plant pathogens colonize hosts by deploying virulence proteins known as effectors that manipulate plant cell structures and functions (Dodds and Rathjen, 2010; Win et al., 2012). Once delivered into host tissues, effectors reside in the extracellular space (apoplastic effectors) or translocate into the plant cells (cytoplasmic effectors). Unravelling how effectors function in the host is key to understanding parasitism and to developing resistant plants (Dangl et al., 2013). Pathogen effectors are operationally plant proteins; they function in plant tissues, they associate with plant molecules, and their phenotypic expression in plants drives their evolution (Hogenhout et al., 2009). The field of effector biology has rapidly advanced in recent years due in large part to the availability of host plants that are amenable to molecular genetics and in which effectors can be heterologously expressed and studied (for comprehensive reviews, see Martin and Kamoun, 2012). However, due to the limited availability of functional genetic resources for crop species, characterising the effectors of crop pathogens remains challenging (Petre et al., 2014; Upadhyaya et al., 2014). To study crop pathogen effectors, an alternative approach is the use a surrogate experimental plant system, such as Nicotiana benthamiana (Petre et al., 2015a).

Nicotiana benthamiana (Solanaceae) is a well-established experimental system to study proteins in planta (Goodin et al., 2008; Bombarely et al., 2012). The agroinfiltration
method allows transient expression of proteins in leaf cells, and a wide range of assays is available for functional investigations. Thus, *N. benthamiana* is extensively used in effector biology (Pais et al., 2014). We recently used this plant to set up an effectoromics pipeline aimed at determining the plant cell compartments and protein complexes targeted by candidate effectors of rust fungi (Petre et al., 2015a; Petre et al., 2015b). Such pipeline is a valuable tool for the rapid screening of candidate effectors.

Rust fungi (Pucciniales) are notorious for being destructive crop pathogens (Dean et al., 2012). The species that infect wheat pose a constant threat to global food security (Beddow et al., 2015). These fungal pathogens include the yellow rust fungus *Puccinia striiformis* f sp *tritici* (Chen et al., 2014; Hubbard et al., 2015). To date, effectors have not been functionally characterized for this species. However, genome and transcriptome analyses have predicted hundreds of candidate effectors, most of which are secreted proteins of unknown function (Cantu et al., 2011; Garnica et al., 2013; Zheng et al., 2013). Cantu and colleagues recently combined genome and *in silico* analyses to prioritize candidate effectors for further functional analyses (Cantu et al., 2013).

Processing bodies (P-bodies) are protein/RNA complexes that reside in the cytosol of eukaryotic cells. They control the decapping, degradation, and storage of mRNA molecules (Chan and Fritzlers, 2012). In plants, P-bodies and P-body-resident proteins have important roles in post-embryonic development (Xu et al., 2006; Xu and Chua, 2009), salt stress tolerance (Steffens et al., 2015), and immune responses (Maldonado-Bonilla et al., 2014). In animals and yeast, limited evidence suggests that pathogenic bacteria and viruses target P-bodies (Eulalio et al., 2011; Reineke and Lloyd, 2013). To date, no connection has been made between P-bodies and filamentous pathogens.

In this study, we investigated sixteen candidate effectors of the wheat yellow rust fungus *P. striiformis* f sp *tritici* using the *N. benthamiana* effectoromics pipeline we previously developed (Petre et al., 2015a). We discovered that nine candidate effectors accumulate in distinct plant cell compartments and associate with specific protein complexes. Notably, the candidate effector PST02549 accumulates in P-bodies and associates with the wheat enhancer of mRNA decapping protein 4. Our findings suggest that P-bodies are a plant compartment targeted by pathogen effectors. We also conclude that *N. benthamiana* can be used as an experimental system to screen candidate effectors of pathogens of monocot plants, including obligate biotrophic pathogens of wheat.

**RESULTS**

**Selection of 16 candidate effectors from *Puccinia striiformis* f sp *tritici***

The predicted effector complement of *P. striiformis* f sp *tritici* consists of hundreds of secreted proteins (Cantu et al., 2013). To select candidate effectors for functional investigations, we leveraged our recently developed pipeline (Petre et al., 2015a) to select eleven proteins, using transcript enrichment in purified haustoria as the principal criterion for selection. We also included five proteins previously flagged as promising candidates (Cantu et al., 2015) to obtain a final list of sixteen candidate effectors (Table 1, Table S1). These sixteen candidates are Pucciniales-specific, and only seven show some sequence similarity to proteins of the wheat stem rust fungus *Puccinia graminis* f sp *tritici* (Table 1). This finding suggests that most of these candidate effectors recently emerged in the Pucciniaceae family.

**Candidate effector–fluorescent protein fusions accumulate in *N. benthamiana* leaf cells**

To test whether the mature form (i.e., without the signal peptide) of the candidate effectors could be expressed in dicot cells, we generated candidate effector–green fluorescent protein (GFP) fusions and expressed them in *N. benthamiana* by agroinfiltration. Live-cell imaging and immunoblotting assays revealed that the sixteen fusion proteins accumulate in leaf cells at detectable levels, with no obvious sign of aggregation or degradation (Figure 1; Figure S1). Some fusions showed a band signal at a lower molecular weight, in addition to the band signal at the theoretical size (PST18220, PST03196, and PST12160), or a band signal at a higher molecular weight than expected (PST02549 and PST05023), suggesting post-translational modifications (Figure S1). As the proteins effectively accumulated in leaf cells, we inferred that transient assays in *N. benthamiana* are suitable for further *in planta* analyses.

**Seven candidate effectors accumulate in specific plant cell compartments**

To identify the plant cell compartments in which the candidate effectors accumulate, we performed live-cell imaging of cells expressing effector-GFP fusions. Seven out of the
sixteen fusion proteins displayed an informative distribution in leaf cells (Figure 1). The fluorescence signal from PST02549-GFP and PST03196-GFP accumulated in small cytosolic bodies and chloroplasts, respectively, as well as in the nucleus and the cytosol (Figure 1A-B). The fluorescence signal from PST18220-GFP labelled both chloroplasts and nuclei, suggesting a dual targeting to the two organelles (Figure 1C). The fluorescence signal from PST18447-GFP, PST11721-GFP, and PST15391-GFP specifically accumulated in nuclei, and PST11721-GFP also labelled nuclear foci in some rare cases (Figure 1D-F, Figure S2). Finally, the fluorescence signal from PST05023-GFP labelled endomembrane compartments (Figure 1G). The fluorescence signal from the remaining nine fusion proteins had a non-informative distribution in the nucleus and the cytosol, similar to the distribution of a free GFP control (Figure 1H-P).

To explain the specific accumulation patterns observed, we examined the candidate effectors for subcellular targeting sequences. We focused on PST15391 and PST18447, because they showed specific, robust accumulation in nuclei (Figure 1D and 1F; Figure 2). Previous analysis failed to identify a nuclear-localisation signal (NLS) for PST15391 and PST18447 (Cantu et al., 2013). However, we noted that both carry NLS-like stretches of amino acids enriched in positively charged residues at the C-terminus and N-terminus of their mature forms, respectively (Figure 2A). Truncations lacking the NLS-like sequences accumulated mainly in the cytosol and only showed background accumulation in the nucleus, demonstrating that these regions are necessary for specific nuclear accumulation (Figure 2B).

This set of experiments suggests that P. striiformis f. sp. tritici effectors use targeting sequences to traffic within plant cells.

Six candidate effectors specifically and reliably associate with plant proteins

To gain further insight into the putative functions of the candidate effectors, we next aimed to identify the plant proteins they interact with in planta using anti-GFP coimmunoprecipitation/liquid chromatography-tandem mass spectrometry (coIP/MS) (Petre et al., 2015a). Using this approach, we identified 439 N. benthamiana proteins as potential interactors of the 16 candidate effectors (Table S2, Figure S3). A candidate effector associated with an average of 98 proteins, ranging from 20 to 236 (Figure 3A). Conversely, a plant protein associated with an average of 3.5 candidate effectors, ranging from 1 to 16 (Figure 3B). Given the high complexity of the dataset, we used a scoring method we previously developed to discriminate reliable and specific interactors (high score) from redundant and non-specific ones (low score) (Petre et al., 2015a). Scores ranged from 0.003 to 108, with an average value of 0.91 (Figure 3C). Eighteen proteins had a score of ≥3, and all specifically coimmunoprecipitated with a single candidate effector (Figure 3C, Figure 4). For instance, the protein with the highest score (108) was an enhancer of mRNA decapping protein 4 (NbEDC4) that specifically and robustly immunoprecipitated with PST02549 (Table S2).

PST02549 associates with the wheat enhancer of mRNA decapping protein 4 (TaEDC4) in P-bodies

Our coIP/MS assays showed that PST02549 specifically associated with NbEDC4. To evaluate the biological significance of this association, we first identified and cloned the protein with the highest amino acid sequence similarity to NbEDC4 in bread wheat (Triticum aestivum), and named it TaEDC4. NbEDC4, TaEDC4, and Arabidopsis thaliana EDC4 (AtEDC4, also known as VARICOSE or VCS) are of a similar length (1203 to 1349 amino acids) and exhibit a pairwise amino acid sequence identity of between 42 and 46% (Figure 5A). The amino acid sequence identity between these proteins reaches 75% in the N-terminal WD40 domain (Figure 5B). Next, we expressed a TaEDC4-mCherry fusion in N. benthamiana leaf cells. Confocal microscopy revealed that TaEDC4-mCherry accumulated in cytosolic foci in addition to the cytosol. Since EDC4 is a component of P-bodies, we hypothesized that the foci we observed were P-bodies. To test this hypothesis, we co-expressed TaEDC4-mCherry with YFP-VCS, a marker of P-bodies (Xu et al., 2006). Confocal microscopy revealed perfectly overlapping signals in cytosolic foci, confirming that TaEDC4 accumulates in P-bodies in N. benthamiana leaf cells (Figure 5C).

To determine whether PST02549 and TaEDC4 associate in planta, we co-expressed PST02549-GFP and TaEDC4-mCherry fusion proteins in N. benthamiana leaf cells and performed anti-GFP coimmunoprecipitation followed by immunoblotting or sodium dodecyl...
sulphate polyacrylamide gel electrophoresis/Coomassie Brilliant Blue (SDS-PAGE/CBB) staining. Both the anti-mCherry immunoblot and the SDS-PAGE/CBB assays revealed a specific band signal matching the predicted size of TaEDC4-mCherry in protein complexes immunoprecipitated with PST02549-GFP, indicating a strong and robust association between the two proteins (Figure 6). As negative controls, we used three GFP and three mCherry fusion proteins available in the lab (see Materials and Methods for details); none of these control proteins associated with either PST02549 or TaEDC4. Confocal microscopy revealed that the fluorescence signals from PST02549-GFP and TaEDC4-mCherry perfectly overlapped in cytosolic foci, indicating co-accumulation in P-bodies (Figure 7A). From this set of experiments, we conclude that PST02539 and TaEDC4 specifically and robustly associate in P-bodies in N. benthamiana leaf cells.

Co-expression of PST02549 and TaEDC4 increases the size of P-bodies

During confocal microscopy assays of N. benthamiana leaf cells co-expressing PST02549-GFP and TaEDC4-mCherry, we noted that the P-bodies appeared larger than usual (Figure 7A). To quantify this phenomenon, we measured the diameter of P-bodies from confocal microscopy images. When PST02549-GFP was co-expressed with TaEDC4-mCherry or an untagged version of TaEDC4, the average diameters of the P-bodies were 4.5 ± 3.5 µm and 4.9 ± 2.2 µm, respectively (Figure 7B, Table S4). By contrast, when PST02549-GFP and TaEDC4-mCherry were expressed independently and/or with other control proteins, the average diameter of a P-body was 1.3 ± 0.6 µm. CoIP/MS assays confirmed the presence of the untagged TaEDC4, as well as the presence of the endogenous NbEDC4, in complex with PST02549 (Table S5). None of the negative controls we tested co-localised with PST02549-GFP or TaEDC4-mCherry or triggered the formation of large P-bodies (Figure 7B and C, Table S4). We conclude that co-expression of PST02549 and TaEDC4 specifically increases the size of P-bodies.

DISCUSSION

In this study, we found that PST02549 accumulates in plant cell P-bodies and associates with a P-body-derived protein. This observation suggests that an effector that targets plant P-bodies has evolved in P. striiformis f sp tritici. To our knowledge, a connection between filamentous plant pathogens and P-bodies has not previously been established.

How would a pathogen benefit from manipulating host P-bodies? Some plant pathogen effectors target components of the host RNA silencing machinery (Weiberg et al., 2011; Perez-Vilaro et al., 2012). Therefore, diverse parasites of eukaryotes have evolved to target host P-bodies. Further mechanistic investigations of the pathogen effector/P-body interplay should reveal the biological significance of this phenomenon.

We observed an increase in P-body size upon co-expression of PST02549 and TaEDC4. The depletion or overexpression of P-body components is known to modify P-body integrity, which can lead to an increase in size (Eulalio et al., 2007). It is therefore possible that the increase in P-body size observed in our study is due to over-accumulation of P-body-resident proteins such as PST02549 or TaEDC4. However, we observed this phenomenon only when the two proteins co-accumulated, indicating that both are required to increase P-body size. The biological significance of the association between PST02549 and TaEDC4 as well as the increase in P-body size remain to be further investigated in wheat.

The pipeline we used in this study allowed us to retrieve informative data for more than 50% of the candidate effectors we tested. We recently obtained informative data for 40% of a set of candidate effectors from another rust species (Petre et al., 2015a). Thus, N. benthamiana is a valuable heterologous system for fast-forward effectoromic analysis of plant pathogens, regardless of their host plant.

We identified plant interactors of candidate effectors, some of which may represent "bone fide" effector targets. Growing evidence suggests that during evolution domains from effector targets have been incorporated into immune receptors such as nucleotide binding-leucine rich repeat (NB-LRR, also referred to as NLR) proteins to become ‘sensor domains’
that mediate recognition of specific effectors (Cesari et al., 2014; Wu et al., 2015; Sarris et al., 2015). A recent genome-wide analysis predicted many NLR gene models in which protein domains that differ from typical NLR domains have been incorporated (Sarris et al., in review). Interestingly, six of the eighteen top scoring effector interactors identified in our study carry a protein domain that is predicted to be integrated into a plant NLR, including the WD40 protein domain of EDC4 (Table S2). Therefore, our predicted host targets can be a valuable source of new ‘baits’ for engineering NLR genes with sensor domains.

**MATERIALS AND METHODS**

**In silico analyses**

Predicted protein sequences were retrieved from the following sources: *P. striiformis f. sp. tritici* (http://yellowrust.com/; Cantu et al., 2013), *N. benthamiana* (http://solgenomics.net/; Bombarely et al., 2012), and *T. aestivum* (http://www.plantgdb.org/TaGDB/). Protein sequence analysis was performed with ClustalX and Jalview programmes. Homology searches were performed with the BLAST+ programme. The most stringent criterion for selection of the candidate effectors was transcript enrichment in purified haustoria compared to infected tissues (Cantu et al., 2013). The set of candidate effectors selected via the pipeline was manually analysed to remove redundant family members. PST05258 and PST15391 from Tribe 54, as well as PST18220 and PST18221 from Tribe 238, were both retained due to high levels of polymorphism (Cantu et al., 2013).

**Cloning procedures and plasmids**

The open reading frame (ORF) encoding the mature form (i.e. without the signal peptide) of *P. striiformis f. sp. tritici* small-secreted proteins or the full-length of *T. aestivum* EDC4 (Traes_6DL_3FBA5B70E.1) were amplified by polymerase chain reaction (PCR) using cDNA isolated from wheat leaves 14 days after inoculation with a virulent isolate of *P. striiformis f. sp. tritici* (isolate PST-08/21; Cantu et al., 2013), or were obtained through gene synthesis (Genewiz, London, UK), with codon optimization for plant expression and removal of internal BbsI and BsaI restriction sites. Primers and synthetic genes were designed to be compatible with the suite of Golden Gate vectors, as previously described (Petre et al., 2015a; Table S1). Truncated versions of PST15391 and PST18447 were obtained by PCR cloning. All PCR-generated DNA fragments were verified by sequencing after cloning into level 0 Golden Gate vectors. Plasmids were multiplied and conserved in *Escherichia coli* (Subcloning Efficiency DH5α Competent Cells; Invitrogen, Carlsbad, California, USA) as previously described (Petre et al., 2015a). The fusion proteins built with candidate effectors from the poplar rust fungus *Melampsora larici-populina* (MLP124111, MLP123218, MLP123438 and MLP124202CT) were obtained in previous studies (Petre et al., 2015a, Petre et al., 2015b, Petre et al., unpublished) and were used as negative controls in coIP and confocal microscopy assays.

**Transient protein expression in *N. benthamiana* leaf cells**

*Agrobacterium tumefaciens* (electrocompetent strain GV3101) was used to deliver T-DNA constructs in leaf cells of three- to four-week-old *N. benthamiana* plants, following the agroinfiltration method previously described (Petre et al., 2015a). The leaves were collected two days after infiltration for further protein isolation or microscopy.

**Live-cell imaging by laser-scanning confocal microscopy**

Confocal microscopy was performed as previously reported (Petre et al., 2015a) with a Leica DM6000B/TCS SP5 laser-scanning confocal microscope (Leica Microsystems, Bucks, UK), using 10x (air) and 63x (water immersion) objectives. Each construct gave a similar localisation pattern in at least three independent observations. Image analysis was performed using the Fiji plugin of Image J 2.0.0 (http://fiji.sc/Fiji). To quantify the diameter of P-bodies, the ‘measure’ tool of Fiji was used to measure manually-drawn lines matching the apparent diameter of P-bodies in all the single optical section confocal images acquired in the course of this project. Categorical scatterplots were generated with R, using the ggplot2 package and an in-house developed script (Text S1).
Protein isolation and immunoblot analyses

Frozen leaves were ground to a powder using a mortar and pestle. Total proteins were extracted as previously described (Petre et al., 2015a). Ten microliters of isolated protein was separated on a 15% SDS-PAGE gel, and the protein content was estimated by Coomassie Brilliant Blue (CBB) staining. Immunoblot analysis was performed as previously described (Petre et al., 2015a), using GFP (B2):sc-9996 HRP-conjugated antibody (Santa-Cruz Biotechnology), rat anti-RFP 5F8 antibody (Chromotek, Munich, Germany) and a HRP-conjugated anti-rat antibody.

Coimmunoprecipitation and LC-MS/MS analyses

Coimmunoprecipitation procedures were performed as reported by Win and colleagues (2011), with the adaptation described in Petre et al., 2015a, using GFP_Trap_A beads (Chromotek, Munich, Germany). GFP and mCherry fusion proteins (Petre et al., 2015a, Petre et al., 2015b, Petre et al., unpublished) selected based on their ability to generate cytosolic aggregates, their similarity to tested proteins, or their ability to associate with a high number of proteins (i.e. their ‘stickyness’) in coIP assays were used as negative controls. Sample preparation, liquid chromatography / tandem mass spectrometry (LC-MS/MS) and data analyses were performed as described in Petre et al., 2015a, using a hybrid mass spectrometer LTQ-Orbitrap XL (ThermoFisher Scientific, Carlsbad, California, USA) and a nanoflow-UHPLC system (NanoAcquity Waters Corp., Burnsville, Minnesota, USA). LC-MS/MS data were processed and scored as previously described (Petre et al., 2015a; Table S2).

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**LEGENDS**

**TABLE 1. Puccinia striiformis f. sp. tritici candidate effectors analysed in this study**

| Protein IDs | Description |
|-------------|-------------|
| a | Protein IDs were adapted from Cantu et al., 2013 by removing the isolate ID for simplicity. |
| b | Data mined from Cantu et al., 2013. |
| c | Number of amino acids in the mature form of the protein (i.e. without the signal peptide) |
| d | Number of cysteine residues in the mature form of the protein (i.e. without the signal peptide) |

**FIGURE 1. Seven candidate effectors show specific accumulation patterns in leaf cells**

Live-cell imaging of the 16 candidate effector-GFP fusion proteins accumulating in distinct subcellular compartments of N. benthamiana leaf cells. Proteins were transiently expressed in N. benthamiana leaf cells by agroinfiltration. Live-cell imaging was performed with a laser-scanning confocal microscope two days after infiltration. GFP and chlorophyll were excited at 488 nm. GFP (green) and chlorophyll (blue) fluorescence were collected at 505-525 nm and 680-700 nm, respectively. Images are single optical sections of 0.8 µm or a maximal projection of up to 47 optical sections (max. z-stack of 37.6 µm). Images displayed are overlays of the GFP signal, the chlorophyll signal, and bright field. For A-G, specific cellular compartments in which the GFP signal accumulates are indicated. White arrowheads indicate GFP-labelled cytosolic bodies (A), chloroplasts (B-C), nuclei (D-F), nuclear surrounding (G), or cytosolic fractions (H-P). Black arrowheads indicate GFP-labelled small cytosolic bodies (A), a stromule (B), a nucleus (C), the plasma membrane (G), or nuclei (H-P). In (P), the low level of accumulation of the fusion protein imposed higher laser power and gain, which resulted in non-specific signal for the GFP channel being visible in chloroplasts and ostiole edges.

**FIGURE 2. PST15391 and PST18447 carry functional nuclear-localisation signals**

(A) Schematic representation of the protein primary structure of PST15391 and PST18447. Yellow: predicted signal peptide for secretion; red: amino acid sequence necessary for nuclear accumulation; blue: positively charged residues (net charge is indicated in parentheses). Numbers indicate amino acid positions.

(B) Live-cell imaging of GFP-PST15391, GFP-PST15391Δ9CT, GFP-PST18447, and GFP-PST18447Δ8NT in N. benthamiana leaf cells. The cellular compartments in which the GFP
signal accumulates are indicated. Proteins were transiently expressed in *N. benthamiana* leaf
cells by agroinfiltration. Live-cell imaging was performed with a laser-scanning confocal
microscope two days after infiltration. GFP and chlorophyll were excited at 488 nm. GFP
(green) and chlorophyll (blue) fluorescence were collected at 505-525 nm and 680-700 nm,
respectively. Images are single optical sections of 0.8 µm or maximal projections of up to 3
optical sections (max. z-stack of 2.4 µm). White arrowheads: nuclei; black arrowheads: cytosol.

**FIGURE 3. Candidate effectors associate with distinct plant protein complexes**

(A) Number of *N. benthamiana* proteins associating with each candidate effector. Candidate
effectors are arranged from left to right in descending order according to the number of
interactors.

(B) Number of candidate effectors associating with each *N. benthamiana* protein. The 439
interactors are arranged from left to right in descending order according to the number of
associated candidate effectors. The X-axis legend indicates (from right to left) the number of
*N. benthamiana* proteins that associated with at least one (439), two (328), three (204), five
(99), and ten (31) candidate effectors.

(C) For each *N. benthamiana* protein identified, we calculated a score following the formula

\[ \text{protein score} = \frac{\text{maximal peptide count}}{(\text{redundancy})^2} \]

The redundancy value was calculated by integrating the coIP/MS data from *Petre* et al., 2015a. Proteins are arranged from left to
right in descending order based on their score. Selected proteins are indicated on the graph.

Proteins were transiently expressed in *N. benthamiana* leaf cells by agroinfiltration. Total
proteins were isolated two days after infiltration. Plant protein complexes associated with the
candidate effector-GFP fusions were purified by anti-GFP coimmunoprecipitation, separated
with SDS-PAGE, and digested with trypsin. Trypsin-digested peptides were processed by LC-
MS/MS and collected peaks were used to search a database containing the predicted
proteome of *N. benthamiana*. After filtering out contaminants and proteins supported by a
single peptide, and clustering similar proteins, a total of 439 non-redundant protein interactors
were retained. The full dataset used to draw these figures is shown in Table S2.

**FIGURE 4. Nine candidate effectors have a specific subcellular localisation and/or a
high-scoring plant protein interactor**
The 16 candidate effectors used in this study are shown in the middle column. Colours
indicate specific subcellular localisation. The 16 plant proteins with the lowest scores (≤ 0.01;
termed ‘usual suspects’) and the 18 plant proteins with the highest scores (≥ 3; termed
‘specific interactors’) are shown on the left- and right-hand sides, respectively. Black lines
indicate the association between a candidate effector and a plant protein as detected by
colIP/MS. For each *N. benthamiana* protein, the most similar wheat protein was identified by
protein sequence similarity searches against the predicted proteome of *Triticum aestivum* L. using the BLASTp algorithm.

**FIGURE 5. TaEDC4 accumulates in P-bodies**

(A) Amino acid alignment of EDC4 of *Arabidopsis thaliana* (AtEDC4, AT3G13300.2),
*Nicotiana benthamiana* (NbEDC4, NbS00023257g0003.1), and *Triticum aestivum* (TaEDC4,
Traes_6DL_3FBA5B70E.1). Alignment was performed with ClustalX. Amino acid residues are
colored according to the ClustalX scheme.

(B) Schematic representation of the protein primary structure of AtEDC4, NbEDC4, and
TaEDC4. Numbers indicate amino acid positions. The percentage of pairwise amino acid
sequence identity is indicated to the right of the diagram.

(C) Live-cell imaging of TaEDC4-mCherry and YFP-VCSc in *N. benthamiana* leaf cells.
Images show a single optical section of 0.8 µm. Proteins were transiently expressed in *N.
benthamiana* leaf cells by agroinfiltration. Live-cell imaging was performed with a laser-
scanning confocal microscope with a sequential scanning mode two days after infiltration. The
YFP was excited at 514 nm; mCherry and chlorophyll were excited at 561 nm. YFP (yellow),
mCherry (red), and chlorophyll (blue) fluorescence were collected at 525-550 nm, 580-620
nm, and 680-700 nm, respectively. White arrowhead: nuclei; black arrowhead: P-bodies. The
intensity plot in the top right corner shows YFP and mCherry (RFP) relative fluorescence
signal intensity along the white line connecting points a and b in the overlay image.

**FIGURE 6. PST02549 associates with TaEDC4 in planta**
FIGURE S2. PST11721-GFP labels nuclei foci indicated in kDa in parentheses. White asterisks indicate specific protein bands. Immunodetection was performed with anti-GFP or anti-red fluorescent protein (RFP) antibodies, and immunoblots were revealed with a chemiluminescent imager. Theoretical protein size is indicated in parentheses (in kilodalton, kDa) for each fusion protein. Numbers to the left of the gel images indicate protein size in kDa. In the immunoblot images, red asterisks indicate specific protein bands. In the gel image, asterisks indicate specific protein bands (red: TaEDC4-mCherry; black: GFP fusions); the PageRuler ladder is shown to the left of the image. IP: immunoprecipitation. In the IP-GFP/α-RFP blot, note that the weak band signals observed on the right side between 25 and 40 kDa are due to nonspecific background detection of abundant GFP fusions by the anti-RFP antibodies.

FIGURE S1. Immunoblots confirm the accumulation of the fusion proteins in N. benthamiana leaf cells. Probes were transiently expressed in N. benthamiana leaf cells by agroinfiltration. Total proteins were isolated two days after infiltration, and immediately used for anti-GFP immunoprecipitation. Immunoprecipitated protein mixtures were separated with SDS-PAGE. For direct protein visualization, the acrylamide gel was stained with CBB. For immunoblotting, proteins were electrotransferred onto polyvinylidene fluoride (PVDF) membranes. Immunodetection was performed with anti-GFP or anti-red fluorescent protein (RFP) antibodies, and immunoblots were revealed with a chemiluminescent imager. Protein size is indicated in parentheses (in kilodalton, kDa) for each fusion protein. Numbers to the left of the blot and gel images indicate protein size in kDa. In the immunoblot images, red asterisks indicate specific protein bands. In the gel image, asterisks indicate specific protein bands (red: TaEDC4-mCherry; black: GFP fusions); the PageRuler ladder is shown to the left of the image. IP: immunoprecipitation. In the IP-GFP/α-RFP blot, note that the weak band signals observed on the right side between 25 and 40 kDa are due to nonspecific background detection of abundant GFP fusions by the anti-RFP antibodies.

FIGURE 7. PST02549 and TaEDC4 co-accumulate in large P-bodies. (A) Live-cell imaging of PST02549-GFP and TaEDC4-mCherry in N. benthamiana leaf cells. Images show a single optical section of 0.8 µm. The white asterisk indicates a pavement cell expressing only the TaEDC4-mCherry fusion, in which no large P-body was detected. (B) Categorical scatterplots showing the diameter of P-bodies labelled by PST02549-GFP and/or TaEDC4-mCherry in leaf cells. Boxes depict the interquartile range and the median, vertical bars indicate the first and fourth quartile range, and outlier data points are depicted in black. P-body diameters were measured from laser scanning confocal microscope images acquired through two to eight independent agroinfiltration assays. The different colours correspond to independent observations (repeats). The following numbers of P-bodies were scored: PST02549-GFP (n=150); TaEDC4-mCherry (n=20), PST02549-GFP/TaEDC4-mCherry (n=303), PST02549-GFP/TaEDC4 (n=96). For treatments ‘PST02549-GFP’ and ‘TaEDC4-mCherry’, the fusion proteins were expressed alone or with additional control fusion proteins (see Table S4 for raw data). (C) Live-cell imaging of various GFP and mCherry fusion proteins in N. benthamiana leaf cells. Images present a single optical section of 0.8 µm of a maximal projection of up to 6 optical sections (max. z-stack of 4.8 µm). Overlay images merge GFP, mCherry, chlorophyll, and bright field signals. Note that for the PST02549-GFP/TaEDC4, TaEDC4 was untagged and the mCherry fluorescence signal was not recorded. For (A) and (C), proteins were transiently expressed in N. benthamiana leaf cells by agroinfiltration. Live-cell imaging was performed with a laser-scanning confocal microscope with a sequential scanning mode two days after infiltration. GFP and the chlorophyll were excited at 488 nm; the mCherry was excited at 561 nm. GFP (green), mCherry (red), and chlorophyll (blue) fluorescence were collected at 505-525 nm, 580-620 nm and 680-700 nm, respectively. Black arrowheads indicate P-bodies. White arrowheads: nuclei. Note that the large protein aggregates formed by MLP124111-GFP do not show any TaEDC4-mCherry signal.

FIGURE S2. PST11721-GFP labels nuclei foci
Live-cell imaging of PST11721-GFP in *N. benthamiana* leaf cells. Proteins were transiently expressed in *N. benthamiana* leaf cells by agroinfiltration. Live-cell imaging was performed with a laser-scanning confocal microscope two days after infiltration. The GFP was excited at 488 nm. GFP (green) fluorescence was collected at 505-550 nm. The image is a single optical section of 0.8 µm, showing an overlay of the GFP and bright field channels. The black arrowheads indicate GFP-labelled nuclear foci.

**FIGURE S3. In planta coimmunoprecipitation efficiently purifies fusion proteins**

Protein mixtures isolated by anti-GFP immunoprecipitation were reduced and denatured in a Laemmli buffer. Proteins were separated with SDS-PAGE and stained with Coomassie Brilliant Blue. Trypsin-digested peptides were processed by LC-MS/MS and collected peaks were used to search a database containing the GFP sequence. The theoretical size of each fusion protein is indicated in parentheses in kilodalton (kDa). The number of peptides identified by LC-MS/MS and matching the GFP is indicated for each fusion protein between brackets. The size of the PageRuler ladder bands is indicated in kDa. Images originating from the same gel are grouped together. Black asterisks indicate detectable and specific protein bands.

**TEXT S1. R script to generate categorical scatterplots**

**TABLE S1. Cloning and protein details**

**TABLE S2. The *P. striiformis f sp tritici* candidate effector interactome**

**TABLE S3. Overlap between CoIP/MS replicates**

**TABLE S4. P-body diameter values**

**TABLE S5. Detection of TaEDC4 in *N. benthamiana* leaves by coIP/MS**
A

PST15391  

1 19

SP

247 256

NAPKRRKL (+4)

PST18447  

1 24 32

SP

SKKRKCRG (+5)

B

|       | overlay | GFP     | chlorophyll | bright field |
|-------|---------|---------|-------------|--------------|
| GFP-PST15391 nucleus | ![Image](overlay) | ![Image](GFP) | ![Image](chlorophyll) | ![Image](bright field) |
| GFP-PST15391Δ9CT nucleus, cytosol | ![Image](overlay) | ![Image](GFP) | ![Image](chlorophyll) | ![Image](bright field) |
| GFP-PST18447 nucleus | ![Image](overlay) | ![Image](GFP) | ![Image](chlorophyll) | ![Image](bright field) |
| GFP-PST18447Δ8NT nucleus, cytosol | ![Image](overlay) | ![Image](GFP) | ![Image](chlorophyll) | ![Image](bright field) |
