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Fusion proteins comprising a *Fusarium*-specific antibody linked to antifungal peptides protect plants against a fungal pathogen

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In planta **expression of recombinant antibodies recognizing pathogen-specific antigens has been proposed as a strategy for crop protection. We report the expression of fusion proteins comprising a *Fusarium*-specific recombinant antibody linked to one of three antifungal peptides (AFPs) as a method for protecting plants against fungal diseases. A chicken-derived single-chain antibody specific to antigens displayed on the *Fusarium* cell surface was isolated from a pooled immunocompetent phage display library. This recombinant antibody inhibited fungal growth *in vitro* when fused to any of the three AFPs. Expression of the fusion proteins in transgenic *Arabidopsis thaliana* plants conferred high levels of protection against *Fusarium oxysporum* f.sp. *matthiolae*, whereas plants expressing either the fungus-specific antibody or AFPs alone exhibited only moderate resistance. Our results demonstrate that antibody fusion proteins may be used as effective and versatile tools for the protection of crop plants against fungal infection.**

Fusarium is an important genus of fungal pathogens, responsible for devastating diseases such as cereal scab, which has reached epidemic levels[1,2]. There are 17 known species of *Fusarium* infecting all members of the Gramineae and most genera of other cultivated plants[3]. These fungi cause huge crop losses and produce mycotoxins that are detrimental to human and animal health[2–5]. The best control strategy is the prevention of infection in the field and during storage. However, natural resistance against *Fusarium* is inadequate[2,3]. Current protective measures rely on chemical control, producing undesirable environmental consequences. Alternatives must be found to protect plants from fungal pathogens and to reduce or eliminate mycotoxin production.

AFPs have been expressed in plants to confer disease resistance[6–9]. In most cases, the expression of individual AFPs only delays the appearance of disease symptoms and does not provide effective control of the disease. Monoclonal and recombinant antibodies have been produced in various expression systems including plants[10–13]. In many cases, plant-derived antibodies have been developed for therapeutic applications, but they have also been used for immunomodulation[13] and the protection of plants against pathogens[14–17]. For example, cytoplasmic expression of a single-chain (scFv) antibody against artichoke mottled crinkle virus in transgenic tobacco was shown to reduce viral infection and delay the progression of disease symptoms[15]. However, antibody-mediated resistance against fungal pathogens in transgenic plants has not been demonstrated.

To evaluate the potential of antibody-mediated fungal resistance in plants, we generated specific antibodies against *Fusarium graminearum*. No germ plasm exists that provides effective innate resistance to this pathogen under high disease pressure[2,3,18]. Our strategy was based on the use of chicken antibodies that target AFPs to the site of infection by binding to surface components of the invading fungus, thus interfering with fungal growth and development. Recombinant proteins were produced by fusing a *Fusarium*-specific scFv antibody to AFPs, which specifically inactivate fungal pathogens. The fusion proteins showed strong inhibitory effects on the growth of *Fusarium* spp. *in vitro*, whereas transgenic *A. thaliana* plants expressing the fusions showed substantially enhanced resistance to the pathogen. Our results demonstrate that engineered antibody-fusion proteins expressed in transgenic plants can control *Fusarium* infection, providing a new strategy for the development of environment-friendly pathogen resistance management practices.

RESULTS

Production and characterization of *Fusarium* specific scFvs

Three phage display libraries were created with mRNA from chickens immunized with three different *F. graminearum* antigens: cell wall–bound proteins (CWPs), mycelium surface proteins and germinated spores. The libraries were pooled to increase the likelihood that the best binders would be selected, and were screened against CWPs. After three rounds of panning, 100 monoclonal phages reactive to CWPs were randomly selected and analyzed using an enzyme-linked immunosorbent assay (ELISA). Fingerprinting of the ten most reactive clones identified one scFv (which we named CWP2) that was

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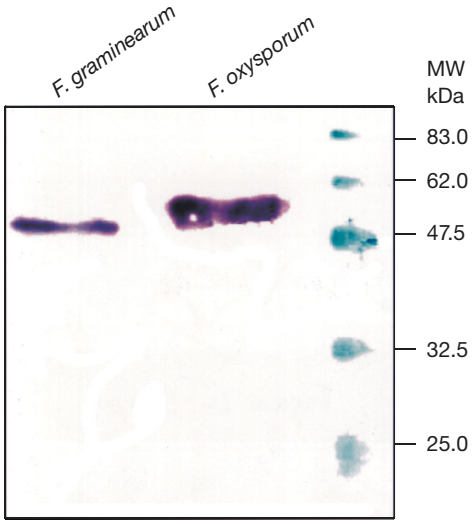

Figure 1 Immunoblot of CWP from *F. graminearum* and *F. oxysporum* f.sp. *matthiolae* detected with the chicken-derived scFv antibody CWP2. Twenty microliters of CWPs (about 50 to 100 ng of total protein) was loaded on a 12% SDS-PAGE gel, separated and blotted onto a nitrocellulose membrane. After blocking with 1% (wt/vol) BSA, the membrane was incubated with 50 ng/ml bacterially expressed CWP2 followed by the addition of a mouse anti-c-myc antibody and a goat anti-mouse IgG (H+L) conjugated to alkaline phosphatase. The colorimetric reaction was carried out by adding NBT/BCIP substrate.

highly reactive towards CWPs. Sequence analysis indicated that, compared to other chicken antibody sequences in the database, this scFv antibody contained conserved sequences in the framework regions but variable sequences in the complementarity determining regions (data not shown).

CWP2 was expressed in bacteria and purified by affinity chromatography. Western blot analysis showed that the antibody bound to one protein with a molecular mass of approximately 50 kDa from cell wall preparations of both *F. graminearum* and *F. oxysporum* f.sp. *matthiolae* (**Fig. 1**). These results suggest that CWP2 recognizes the same epitopes in the cell wall protein fractions of both fungi.

To evaluate the specificity of CWP2, a number of different *Fusarium* spp. and fungi from other genera were analyzed by ELISA. CWP2 recognized all the *Fusarium* spp. with similar reactivity but did not cross-react with *Sclerotinia sclerotiorum*, *Verticillium dahliae* or *Phoma lingam* (**Table 1**). These results showed that CWP2 was *Fusarium* genus–specific.

In vitro assay of AFP-scFv fusions

To construct antibody fusion proteins, the CWP2 coding region was genetically fused to the coding regions of three AFPs (**Fig. 2**): a wheat class I chitinase (Chi)[19], an AFP from *Raphanus sativus* (RS)[20] and an AFP from *Aspergillus giganteus* (AG)[21]. The fusion proteins were assayed *in vitro* for their ability to inhibit the mycelial growth of *F. graminearum* and *F. oxysporium* f.sp. *matthiolae*.

To ascertain whether CWP2 mediated a generic, nonspecific antifungal effect or more specific inhibitory activity, the human chorionic gonadotropin (hCG)-specific scFv antibody, PIPP[22], was fused to the AFPs and the resulting AFP-PIPP constructs were used as controls. **Figure 3** shows the results obtained using the AFP-CWP2 fusions, the AFP-PIPP controls, individual AFPs, CWP2 alone and combinations of CWP with individual AFPs supplied as separate entities. From the

Table 1 Specificity and cross-reactivity of CWP2 to different *Fusarium* and non-*Fusarium* fungi determined by ELISA

Species	Reactivity of scFv CWP2
F. graminearum	+++
F. culmorum	+++
F. N5331	+++
F. T3	+++
F. avenaceum	+++
F. solani	+++
F. oxysporum f.sp. *cyclaminis*	+++
F. oxysporum f.sp. *lycopersici*	+++
F. oxysporum f.sp. *matthiolae*	+++
S. sclerotiorum	–
V. dahliae	–
P. lingam	–
noninfected plant material	–

High-binding ELISA plates were coated with germinated conidiospores from each *Fusarium* species, as described above. Colorimetric reactions were done upon adding *p*-nitrophenyl phosphate substrate and measured at 405 nm. The scale presents an arbitrary set of OD_{405nm} readings (<0.1 OD, –; 0.1–0.8 OD, +; 0.81–2.5 OD, ++; 2.51–4.0 OD, +++) after 1 h substrate reaction.

13 recombinant proteins and combinations assayed, only the three AFP-CWP2 fusions (AG-CWP2, RS-CWP2 and Chi-CWP2) strongly inhibited the growth of *F. graminearum* and *F. oxysporum* f.sp. *matthiolae in vitro*. At a concentration of 100 ng/ml, these fusion proteins exhibited very significant ($P < 0.01\%$) inhibitory activity comparable to that of the antimicrobial agent sodium azide. Moreover, a significant ($P < 5\%$) inhibitory effect on *F. graminearum* was seen for Chi-CWP2 at concentrations as low as 20 ng/ml. In contrast, and under the same conditions, none of the AFP-PIPP fusion-proteins, or CWP2 alone, or any of the individual AFPs, or any AFP + CWP2 combination showed visible inhibitory effects on fungal growth. These results indicated that there is no synergistic effect between the AFPs and CWP2. The strong inhibition observed in the case of the fusion proteins means that physical association between the scFv and AFP is a key determinant of antifungal activity.

Analysis of antifungal activity by immunofluorescence labeling

Confocal immunofluorescence labeling was used to identify the site of AFP-CWP2 binding and to determine whether binding was associated with any morphological changes in the fungus (**Fig. 4**). **Figure 4a** shows the normal morphology of germinating *F. graminearum* conidiospores. **Figure 4b** shows that the binding of CWP2 (control construct, without AFP fusion) localized with the brightest, most continuous fluorescence intensity to the cell walls of germinated tubes, confirming the specificity of this antibody for a cell surface target. For the AG-CWP2 fusion, fluorescence staining on the mycelium (particularly around the cell wall) was much less intense as a result of antifungal activity (**Fig. 4c,d**). In the case of RS-CWP2 (**Fig. 4e,f**), more severe disruption of the mycelial cell walls was seen and the fluorescence staining was more dispersed. The Chi-CWP2 fusion was the most active inhibitor of the two *Fusarium* spp. completely destroying the germinated tube structure so that only mycelium debris was detectable (**Fig. 4g,h**). No fluorescent labeling was visible after incubation with the nonspecific scFv antibody PIPP (**Fig. 4i,j**). From these data we concluded that Chi-CWP2 displayed the strongest *in vitro* antifungal activity of the three AFP-CWP2 fusions, followed by RS-CWP2 and AG-CWP2. These results, in conjunction with the assay for antifungal activity, confirm that CWP2 plays an important role in mediating the

antifungal effects of the fusion proteins because the AFPs alone had no discernible effect on fungal growth *in vitro* (**Fig. 3**).

Expression of AFP-scFv fusions in transgenic plants

To determine whether AFP-CWP2 fusions were effective against fungal infection *in planta*, the constructs described above were introduced into *A. thaliana* using *Agrobacterium tumefaciens*–mediated gene transfer by floral dipping[23]. The plants were grown and T1 seeds harvested. Putative transformants were selected on kanamycin-supplemented medium and transplanted to soil. ELISA, PCR and Southern blot hybridization experiments were done to confirm the presence of the transgenes and to establish their expression and segregation ratios. Transgenic plants with a single integration locus were selected for further analysis. Forty T1 seedlings were grown for each transformant. In these plants, expression of the AFP-scFv fusions was verified by ELISA, and the size and integrity of the immunoreactive polypeptides were confirmed by western blot analysis of plant leaf extracts (data not shown). Three lines of T2 transgenic plants per construct, chosen for their comparable, high levels of transgene expression, were used for *Fusarium* inoculation. Ten plants were used from each line, making a total of 30 plants for each construct. The level of recombinant protein in each plant was quantified by ELISA, and ranged from 198.8 to 689.6 ng/g leaf tissue (**Fig. 5**). The morphology of the transgenic plants was similar to that of the nontransgenic controls.

Response of transgenic plants to infection with fungi

The resistance of T2 transgenic plants against infection with *F. oxysporum* f.sp. *matthiolae*[24] was evaluated at 7, 14 and 21 d post inoculation (d.p.i.). Responses were classified as follows: (i) no symptoms; (ii) disease symptoms present; and (iii) death of plants. Disease severity was scored as the mean disease index, which indicated that transgenic plants containing CWP2 alone, AFPs alone or CWP2-AFP fusions displayed an increased resistance to *F. oxysporum* compared to controls. To confirm that the increased resistance was due to the presence of CWP2, disease index values were compared in two groups. In the first group, GFP-transgenic plants[25] were used as controls for comparison with the transgenic plants expressing either CWP2 or individual AFPs, whereas in another group, AFP-CWP2 fusions were compared to corresponding AFP-PIPP fusions (**Table 2**).

Table 2 Resistance of transgenic T2 *A. thaliana* plants after inoculation with *F. oxysporum* and *S. sclerotiorum*

Constructs	*F. oxysporum* disease index (%)[a]			*S. sclerotiorum*
	7 d.p.i	14 d.p.i.	21 d.p.i.	Lesion diameter (mm)[b]
WT (nontransgenic)	60	100	100	15.4 ± 1.31
GFP	60	95	97	n.d.
CWP2	20[c,e]	55[c,(c),e]	82	15.2 ± 1.40
AG	38[c,e]	50[c,(c),e]	92	15.3 ± 1.08
RS	20[c,e]	60[c,(c),e]	75	13.7 ± 1.81
Chi	20[c,e]	52[c,(c),e]	75	14.5 ± 1.73
AG-PIPP	40	85	95	14.2 ± 1.17
RS-PIPP	30	80	80	14.2 ± 1.83
Chi-PIPP	35	75	88	14.5 ± 1.52
AG-CWP2	5[d,e]	10[d,e]	45[d,(d),e]	14.8 ± 1.87
RS-CWP2	0[d,e]	0[d,e]	18[d,(c),e]	13.9 ± 1.29
Chi-CWP2	5[d,e]	5[d,e]	40[d,(d),e]	14.2 ± 1.67

Ten T2 *A. thaliana* plants from each line were inoculated with 1×10^5 spores of *F. oxysporum*.

[a]Resistance was evaluated by measuring the percentage of diseased plants and scoring the severity of inoculated plants at 7-d intervals until 21 d.p.i. Means of the disease index were calculated for three independent experiments involving ten plants per construct derived from three different lines using the formula:

$$\text{Disease index (\%)} = \frac{\Sigma(n\times 0 + n\times 1 + n\times 2)}{N\times 2} \times 100\%$$

where Σ is the sum total, n the numbers of plants in the class, N the total number of plants, and 1, 2 and 3 the class of symptom severity as determined: 1, healthy plants with no symptoms; 2, plants with disease symptoms; 3, dead plants. [b]Lesion diameters of *S. sclerotiorum* are the mean values from ten plants per line (one leaf per plant) measured 2 d.p.i. N.d., not determined. [c]Significant differences between transgenic plants expressing either CWP2 or AFP alone and GFP-transgenic plants based on analysis of variance using the GLM procedure with SAS system. GLM, General Linear Model; SAS, Statistics Analysis System. [d]Significant differences between transgenic plants expressing AFP-CWP2 and AFP-PIPP constructs, determined as described above. Letters in parentheses are the results from statistical analyses within each group consisting of an individual scFv antibody, one of the AFPs, or one of the AFP-scFv CWP2 fusions respectively. Different letters in the parentheses represent significant differences at <1% whereas identical letters indicate no significant differences. [e]Significant at the 1% level.

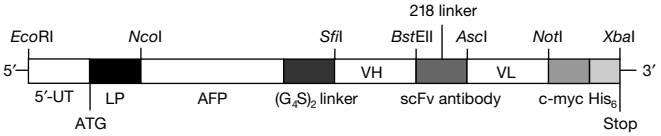

Figure 2 Structure of AFP-scFv fusion constructs. AFP and single-chain Fv (scFv) antibody coding regions were connected by a sequence encoding a ten-amino-acid glycine-serine linker. The variable heavy-chain (V_H) and light-chain (V_L) coding regions were connected by a sequence encoding the 18-amino-acid 218 linker. AFP-scFv fusion constructs were inserted into the bacterial expression vector pET22b using the *Nco*I and *Xba*I sites, and into the plant expression vector pTRAkc[39] using the *Eco*RI and *Xba*I sites. 5′-UT, 5′-untranslated region of the petunia chalcone synthase gene[16]; LP, original mouse leader peptide sequence[16]; c-myc, c-myc epitope tag; His_6, histidine 6 tag.

A statistically significant ($P < 1\%$) difference was observed between transgenic plants expressing CWP2 alone and the GFP-transgenic control plants. The disease index of transgenic plants expressing CWP2 was 20% at 7 d.p.i. (a reduction of 67% compared to GFP-controls) and 55% at 14 d.p.i. (a reduction of 42% compared to GFP-controls). Plants expressing AFPs alone also showed a significant ($P < 1\%$) reduction in disease index compared to GFP plants, with values similar to the CWP2 transgenics.

More importantly, plants expressing the AFP-CWP2 fusions displayed a particularly high level of resistance compared to those expressing the control AFP-PIPP fusions. The disease index ranged from 0% to 10%, a reduction of 88% to 100% between 7 and 14 d.p.i. compared to AFP-PIPP controls. After 21 d.p.i. the disease indices of plants expressing AFP-CWP2 fusions were 53%–78% lower than those of corresponding plants expressing AFP-PIPP fusions. At the end of the assay period, about 10% of the AG-CWP2 plants, 65% of the RS-CWP2 plants and 50% of the Chi-CWP2 plants set viable seeds. To further verify that the resistance mediated by CWP2 was specific for *Fusarium*, another ten plants per each transgenic line were inoculated with a non-*Fusarium* fungus, *S. sclerotiorum*. The results showed no significant difference in lesion sizes among the transgenic plants expressing the different fusion and control proteins at 2 d.p.i. (**Table 2**). Examples of transgenic plants inoculated with *F. oxysporum* at 21 d.p.i. and *S. sclerotiorum* at 2 d.p.i. are shown in **Figure 6**. These results show that the AFP-CWP2 fusions provided the highest level of resistance against *Fusarium* whereas plants expressing either CWP2 or the AFPs alone displayed a moderate level of resistance. None of the constructs provided resistance against *S. sclerotiorum*.

DISCUSSION

The enhancement of fungal resistance in crop plants has been a major challenge because of the limited amount of available germ plasm with natural resistance to fungal diseases. Further hurdles include difficulties associated with the synthesis of recombinant proteins that have

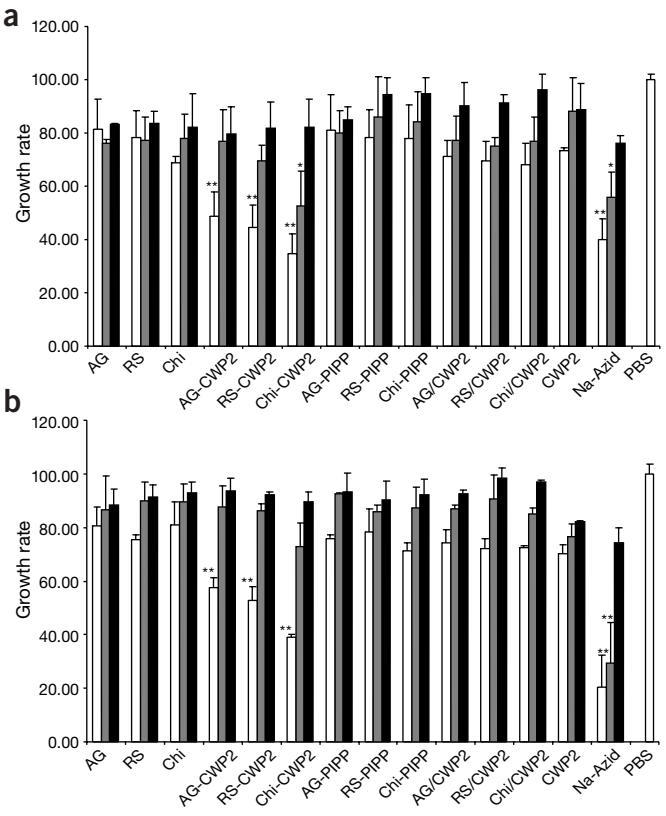

Figure 3 Inhibitory effects of AFPs, scFv and AFP-scFv fusion proteins on the growth of *Fusarium in vitro*. (**a**,**b**) One hundred microliters of spores (1×10^5/ml) from *F. graminearum* (**a**) or *F. oxysporum* (**b**) was placed into sterile 96-well plates and germinated for 16 h at 28 °C in PDB medium. 100 ng/ml (blank boxes), 20 ng/ml (gray boxes) or 4 ng/ml (black boxes) of proteins or fusions were used for the assays. Results of each treatment were expressed as fungal growth rate and determined from the ELISA plates, which were scanned using a flat bed scanner and stored as a grayscale image. The intensity range was inverted. The integrated intensity for each well was measured by defining local regions of interest using Adobe Photoshop and calculated using the wells containing PBS as controls (100%). Two replicates were used for ANOVA analysis with Analyse-it software (www.analyse-it.com). *, significant at the 5% level; **, significant at the 0.01% level.

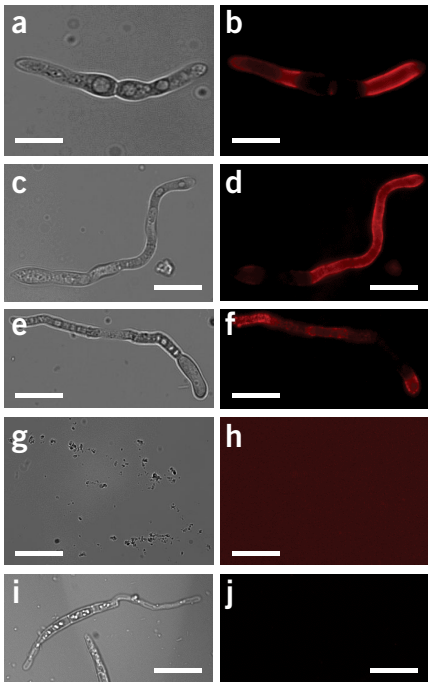

Figure 4 Immunofluorescence labeling of germinated tubes of *F. graminearum* with scFv antibodies and AFP-scFv fusions. (**a**–**j**) Conidiospores of *F. graminearum* were germinated for 16 h at 28 °C and exposed to the bacterially expressed CWP2 (**a**,**b**), AG-CWP2 fusion protein (**c**, **d**), RS-CWP2 fusion protein (**e**, **f**), Chi-CWP2 fusion protein (**g**, **h**) and a nonspecific scFv antibody PIPP as a negative control (**i**, **j**). This was followed by the addition of mouse anti-c-myc antibody and goat anti-mouse IgG (H+L) labeled with Alexa Fluor 568 (578/603). Transmission photographs (**a**, **c**, **e**, **g** and **i**) are on the left and fluorescence photographs (**b**, **d**, **f**, **h** and **j**) are on the right. Bar, 20 μm.

antifungal activity *in vivo*. The goal of this study was to determine whether fusion proteins comprising a pathogen-specific antibody and an AFP could protect plants from fungal infection more efficiently. Our approach was to generate and isolate recombinant chicken antibodies that recognized surface-exposed fungal antigens using phage display and then to exploit these antibodies to deliver AFPs to the invading fungal pathogen at the site of infection in transgenic plants.

Fusarium CWPs were selected as the target for the protective antibodies, because these proteins are displayed on the pathogen surface during infection. Using phage display, we identified CWP2, a high-affinity antibody that reacted strongly with CWPs. The selected scFv antibody was fused to three AFPs, and the resulting constructs (Chi-CWP2, AG-CWP2 and RS-CWP2) were expressed in bacteria to evaluate their antifungal activities.

F. graminearum and *F. oxysporum* f.sp. *matthiolae* mycelial growth was severely inhibited by each of the three fusion proteins *in vitro* (**Fig. 3**). In contrast, no inhibitory effect was detected with the corresponding AFP-PIPP fusions, which carry the same AFPs but are fused to a single-chain antibody (PIPP) specific for a nonfungal antigen[22]. This suggested that the presence of CWP2 in the fusions played an essential role in the inhibition of fungal growth *in vitro*. Immunofluorescence labeling further verified that CWP2 bound to the surface components of *F. graminearum* (**Fig. 4b**). Disruption of fungal wall structures by the AFP-CWP2 fusion proteins was clearly demonstrated by mycelium fluorescence labeling (**Fig. 4d,f,h**) providing visual evidence of the inhibitory activity. The failure of either CWP2 alone, or any of the AFPs alone, or any combination of CWP2 and AFPs supplied as separate entities, to elicit an antifungal response of the magnitude seen with the fusion proteins, indicated that physical association between the antibody and the AFP was essential for maximal protection.

The effectiveness of CWP2 and AFP-CWP2 fusion proteins against fungal infection *in planta* was assessed in transgenic *A. thaliana* plants. As it is difficult to compare the degree of resistance in plants infected with *F. oxysporum*, we used a highly stringent bioassay for disease resistance with 100% of the wild-type plants dying by 14 d.p.i. (**Table 2**). Under such conditions, transgenic plants expressing CWP2 or individual AFPs exhibited a moderate resistance to *F. oxysporum* f.sp. *matthiolae*, compared to control plants expressing GFP. This suggests that CWP2 bound to mycelium surface antigens and actively interfered with fungal growth. More importantly, plants expressing the AFP-CWP2 fusion proteins showed significantly enhanced resistance to the fungus compared to plants containing AFP-PIPP fusions[22]. This comparison excluded the possibility that the scFv antibody

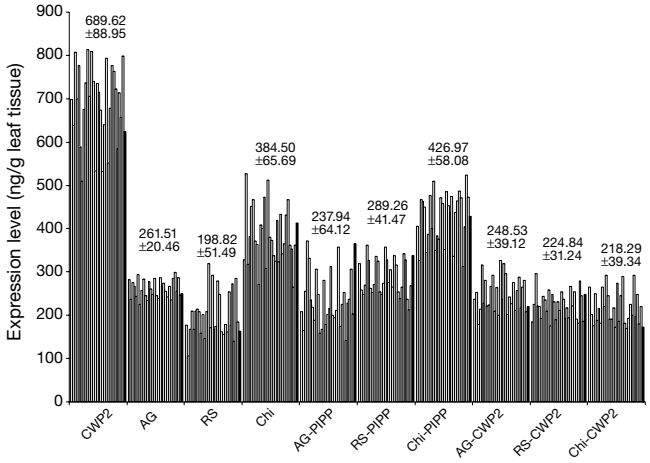

Figure 5 Expression levels of CWP2, AFPs and AFP-scFv fusions in transgenic *A. thaliana* plants. Total soluble protein was isolated from the leaves of 30 T2 plants per construct. Protein levels were determined by ELISA and mean values of two measurements for each plant were presented in ng/g leaf tissue. Average levels of each construct are indicated with standard deviations.

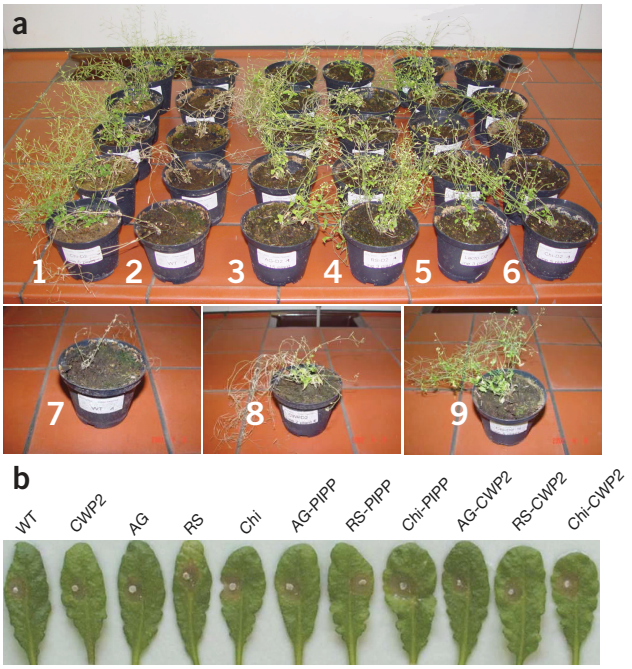

Figure 6 Phenotype of transgenic *A. thaliana* plants infected with *F. oxysporum* (21 d.p.i) and with *S. sclerotiorum* (2 d.p.i.). (**a**) Representative plants from different lines used in this investigation. Top panel shows five representative plants from each of the following six lines: (1) wild type (noninfected); (2) wild type (infected); (3) AG-CWP2 transgenic plants (infected); (4) RS-CWP2 transgenic plants (infected); (5) Lacto-CWP2 transgenic plants (infected). Lacto is a 55-amino-acid peptide derived from the N terminus of human lactoferrin[41]. This short peptide has shown antimicrobial activity and was used here as a further control to check if it confers fungal resistance *in planta* when fused to CWP2; (6) Chi-CWP2 transgenic plants (infected). Bottom panels show individual plants from the following lines to represent the spectrum of resistance we demonstrated: (7) wild type (infected); (8) CWP2 transgenic (infected); (9) Chi-CWP2 transgenic (infected). The plants in panels 2–9 were inoculated with *F. oxysporum* and the photos were taken at 21 d.p.i. (**b**) *A. thaliana* leaves inoculated with PDA-plugs colonized with *S. sclerotiorum*. The photos were taken at 2 d.p.i. and one representative leaf from each line is shown.

component of the fusion played a generic role, such as stabilizing the fusion proteins. Furthermore, the infection assay with *S. sclerotiorum* confirmed that the enhanced resistance conferred by the AFP-CWP2 fusions was *Fusarium* specific. Plants expressing CWP2 or any of the individual AFPs were as susceptible as nontransgenic plants to *S. sclerotiorum* infection (**Fig. 6** and **Table 2**). Furthermore, plants expressing the AFP-CWP2 fusion proteins showed *S. sclerotiorum* disease lesions of similar size to control plants (**Fig. 6** and **Table 2**). These results clearly demonstrate that the CWP2 antibody was essential for enhanced *Fusarium*-specific resistance *in planta*.

There appeared to be no direct correlation between the level of protein expression and the degree of disease resistance observed in transgenic plants. Among the four nonfusion proteins, CWP2 accumulated to the highest level in transgenic plants but conferred moderate *Fusarium* resistance similar to that of the individual AFPs, which were expressed at lower levels (**Fig. 5** and **Table 2**). The three AFP-CWP2 fusion proteins accumulated to comparable levels but conferred differing degrees of resistance, with RS-CWP2 providing the highest level of protection at 21 d.p.i., followed by Chi-CWP2 and AG-CWP2. These results suggest that the resistance level *in planta* is mainly dependent on the particular transgene rather than its expression level. The variable resistance levels provided by the different constructs are likely to reflect the action modes and effects of the corresponding proteins on the target pathogen. A comparison of the disease indices in **Table 2** with the antifungal activity of each AFP-CWP2 as visualized by immunofluorescence labeling (**Fig. 4**) shows a good correlation. More severe disruption of the mycelia was seen for RS-CWP2 and Chi-CWP2 fusions than for AG-CWP2, and the first two fusions also conferred stronger resistance in transgenic plants.

Individual AFPs and the CWP2 scFv antibody alone showed no detectable antifungal effect *in vitro* whereas they conferred moderate resistance *in planta*. This discrepancy may reflect the different conditions for fungal growth in the two environments. The fungi grow very fast in potato dextrose broth (PDB) medium at 28 °C and achieve a large biomass within a few days. The amounts of AFPs and scFv antibody molecules applied to the cultures might not be sufficient to block fungal growth under these conditions. On the other hand, the constitutive expression of these proteins and their continual secretion to the apoplast in transgenic plants (achieved using a plant or animal leader peptide sequence contained in the expression constructs[16]) could interfere with fungal growth and development more efficiently, where the pathogen is present in lower amounts, thus preventing the fungus getting a 'foothold' in the transgenic plants.

Chitinase has been shown to degrade the fungal cell wall, which is composed of chitin polymers[26]. AFP RS has been shown to interfere with membrane potential by interacting with the plasma membrane proton pump[27]. The positively charged AG peptide from *A. giganteus* is thought to interact with negatively charged phospholipids of fungal membranes[28]. It is likely that the striking antifungal activity we observed was due to the CWP2 antibody binding to surface antigens and delivering the AFPs efficiently to their normal site of action. Therefore, the AFP-CWP2 fusion proteins were more effective antifungal agents than the AFPs alone (**Table 2**). Although monoclonal antibodies specific for *Neotyphodium coenophialum* have been shown to reduce fungal growth *in vitro*[29], here we demonstrate the ability of a single-chain antibody molecule to confer resistance *in planta* and therefore demonstrate the utilization of antibody fusion proteins as a

tool for enhancing fungal resistance. Our findings show that antibody fusion proteins provide an attractive method for the production of transgenic crop plants that are intrinsically resistant to fungal pathogens. Such plants could contribute to sustainable agriculture by reducing the pathogen load and mycotoxin levels.

METHODS

Antigen preparation and immunization. *F. graminearum* isolate Wuchang 1, a predominantly infectious strain of wheat head blight from Wuhan, China, was used for antigen preparations. Mycelium from the fungus was cultured in PDB medium (DIFCO) in a fermenter at 28 °C for 5 d. CWPs were prepared from the mycelium as described[30]. The mycelium surface proteins were prepared from Petri dish cultures upon fungal cultivation for 5 d at 24 °C[31]. Conidiospores produced in complex medium[32] were germinated in PDB for 16 h at 28 °C, producing germinated spores. 'White leghorn' chickens (*Gallus domesticus*)[33] were given intramuscular injections comprising 100 μg antigen or 500 μl germinated spores (2×10^5 spores/ml) in Gerbu 100 adjuvant. Two additional injections were given at 1-week intervals and a final boost performed 6 d before spleen cell isolation.

Construction of phage display libraries and isolation of scFv antibodies. RNA was isolated from the spleen cells of immunized chickens using the RNeasy isolation kit (Qiagen) and the mRNA was purified using the Oligotex mRNA isolation kit (Qiagen). The first strand cDNAs were synthesized with two primers, V_H-cDNA (5′-CGGTGGGGGACATCTGAGTGGG-3′) and V_L-cDNA (5′-AGGGGTGGAGGACCTGCACCTC-3′) respectively, using the Superscript kit (Gibco BRL). The cDNAs were subsequently used for amplification of avian variable heavy and light chains. One pair of primers was used for the heavy chain, (VHF: 5′-ATCTAGGCATCCCTTGGCCCAGCCGGCCATGGCTGC CGTGACGTTGGACGAGTCC-3′; VHB: 5′-CTAGTGCATGCTGGAGGTGAC CTCGGTCCCGTGGCCCCATGCGTC-3′) and one for the light chain (VLF: 5′-TCCACACATGCTCCAGGCGCGCCTGCGCTGACTCAGCCGTCCTCG GTG-3′; VLB: 5′-TGACCTTCGAGGATGCGCGGCCGCGTCGACGGGCTG GCCTAGGACGGTCAG-3′). The amplified V_H and V_L domains were digested with *Sfi*I-*Bst*EII and *Asc*I-*Not*I, and cloned in the pHENHi phagemid containing the backbone sequence of pHEN1 (ref. 34) and a C-terminal His_6 tag to facilitate affinity purification. The $(Gly_4Ser)_3$ linker contained in pHEN1 was replaced by the 218 linker[35]. The resulting recombinant phagemids were introduced into Escherichia coli XL1-Blue MRF′ (Stratagene) by electroporation (Bio-Rad Laboratories). Two libraries for each antigen, designated pHENHi+LH and pHENHi+HL, were constructed by recovering V_H fragments from the V_H library and cloning in the V_L library, and vice versa. The two libraries were combined as one scFv library for subsequent use, which had a complexity of ~1.0×10^7 independent clones with a high level of diversity.

Solid phase panning[36] was carried out using antigens at a concentration of 250 μg/ml for CWPs. ELISA-positive phage clones with high OD values were selected for fingerprinting by *Bst*NI digestion. They were sequenced using the Thermosequenase kit (Amersham Biosciences) and a LI-COR 4200 IR2 automated DNA sequencer (MWG).

ELISA and immunoblot analysis. For selection and characterization of scFv antibodies, antigens were coated onto 96-well plates for 1 h at 37 °C. The appropriate scFv was added and detected in a two-step procedure using an anti-His_6 antibody and a goat anti-mouse IgG (H+L) conjugated to alkaline phosphatase (Jackson ImmunoResearch) with colorimetric detection[33]. For analysis of scFv specificity and cross-reactivity with different fungi, wells were coated with *Fusarium* conidiospores (1×10^5/ml) or mycelia from *S. sclerotiorum*, *V. dahliae* or *P. lingam* (200 μg/ml), and then assayed as described for antibody selection. *Fusarium culmorum*, *F. oxysporum* f.sp. *lycopersici*, *Fusarium avenaceum*, *F. oxysporum* f.sp. *cyclaminis* and *Fusarium solani* f.sp. *eumartii* were obtained from H. Nirenberg (Biologische Bundesanstalt, Berlin, Germany). *F. oxysporum* f.sp. *matthiolae* was provided by B.P.H.J. Thomma (Katholieke Univ. Leuven, Belgium)[24]. The banana pathogens *F. N5331* and *F. T3* were obtained from S. Schillberg (Fraunhofer IME, Aachen, Germany). *Verticillium dahliae*, *S. sclerotiorum* and *P. lingam* were provided by P. Bekommen (Paderborn Univ., Germany).

For immunoblot assays, cell wall proteins from *F. graminearum* and *F. oxysporum* or leaf extracts from transgenic plants were separated by 12% (wt/vol) SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots containing CWPs were developed using the isolated scFv antibody, a 1:5,000 diluted mouse monoclonal anti-c-myc antibody and a 1:5,000 diluted goat anti-mouse antibody conjugated to alkaline phosphatase. The membranes with plant extracts were developed as described above omitting the scFv antibody step. Quantification of expression levels in transgenic plants was performed by ELISA and western blot analysis. Leaf extracts were coated on plates and detected as described above for antibody selection. Various known amounts of bacterially expressed proteins were extracted together with nontransgenic leaves and used in ELISA and western blots as standards. Western blots were scanned with Fuji FLA-3000 scanner.

Construction of AFP-scFv fusions. The coding sequences of three AFPs—Chi, RS and AG[19–21]—were joined to the 5′ end of the CWP2 coding sequence to make AFP-scFv fusion constructs (Fig. 2). As controls, all three AFPs were fused to the PIPP scFv antibody which recognizes the human antigen hCG[22]. The resulting AFP-scFv fusions were cloned in bacterial expression vector pET22b following standard methods[37]. The coding sequences for the AFP-scFv fusions, AFPs, CWP2 and green fluorescent protein (GFP)[38] were introduced into the plant expression vector pTRAkc for stable transformation of plants[39].

Expression of CWP2 and AFP-scFv fusions. A single colony of recombinant *E. coli* was transferred to 2× TY medium plus ampicillin and 1% (wt/vol) glucose and incubated overnight at 37 °C with shaking (200 r.p.m.). Two milliliters of overnight culture was transferred to 100 ml 2× TY (10 g Tryptone, 1 g yeast) medium containing ampicillin and 0.1% (wt/vol) glucose and grown to an OD_{600} value of 0.7–0.9. After adding β-D-isopropyl-thiogalactopyranoside to a final concentration of 1 mM, the culture was incubated at 28 °C overnight. Soluble scFv antibody fragments or fusions were affinity-purified by immobilized metal affinity chromatography (IMAC; Qiagen).

***In vitro* antifungal assay.** One hundred microliter aliquots of *Fusarium* conidiospores (1×10^5/ml) in PDB were germinated in the wells of ELISA plates for 16 h at 28 °C. One hundred microliters of affinity-purified CWP2, AFPs, combinations thereof (AFP/CWP2) or AFP-scFv fusion proteins (at different concentrations) was then added to the wells. Sodium azide (0.04%, 0.008% and 0.0016% respectively, wt/vol) was used as a positive control. The plates were incubated at 28 °C in the dark for 2 weeks and the antifungal effect on mycelium growth was evaluated.

Immunofluorescence labeling. Circular coverslips (18 mm diameter) were rinsed with 2 N NaOH and coated with poly-L-lysine (500 μg/ml). The coverslips were placed into 12-well plates (Fisher Scientific) and blocked with 1% (wt/vol) bovine serum albumin (BSA). Germinated spores (1×10^5/ml) or mycelia (50 mg/ml) were added to the wells, and CWP2 or the AFP-scFv fusions (50 μg/ml) were added after centrifugation (15 min, 2,000*g*, 23 °C). The reaction mixtures were incubated at 23 °C overnight. The plates were washed twice for 5 min with 1% (wt/vol) BSA, followed by the addition of a 1:5,000 diluted c-myc-specific mouse antibody and a 1:5,000 diluted goat anti-mouse α IgG (H+L) antibody labeled with Alexa Flour 568 (578/603) (MoBiTec). The coverslips were analyzed using a Leica DM R fluorescence microscope or a Leica TCSSP confocal microscope (Leica).

Plant transformation. All constructs were introduced into the *A. tumefaciens* strain GV3101 (ref. 39). *Arabidopsis thaliana* cv. *Columbia* was transformed by floral dipping[23] and transgenic plants were selected on half strength Murashige and Skoog (MS) medium (DUCHEFA) containing 50 mg/l kanamycin.

Fungal bioassay. After germination on MS medium in Petri dishes for 2 weeks, transgenic *A. thaliana* T2 plants were transplanted to soil and grown for 4–5 weeks in a growth chamber with a 12-h photoperiod at 23 °C, 10000 Lux. The plants were then sprayed once with a *F. oxysporum* f.sp. *matthiolae* conidiospore suspension (which is infectious to *A. thaliana*[24]) containing 1×10^5 spores/ml. The plants were incubated under the same conditions at 85% relative humidity. For the *S. sclerotiorum* bioassay, individual leaves were inoculated with a single mycelia-colonized agar plug (2-mm diameter) taken from the growing edge of a 3-d-old culture[40]. Inoculated leaves were maintained at 100% relative humidity in the same growth chamber as described above.

Accession numbers. CWP2 (European Molecular Biology Laboratory), AJ517190; *F. oxysporum* f.sp. *matthiolae* (Biologische Bundesanstalt für Land- und Forstwirtschaft), BBA 62308; *F. graminearum* (BBA), BBA 72297.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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