Generation of recombinant antibodies specific for *Sclerotinia sclerotiorum*

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Abstract

Sclerotinia sclerotiorum causes disease on numerous economically important crops including oilseeds, vegetables and ornamentals worldwide. Breeding cultivars with durable resistance in oilseed crops is a major challenge in modern agriculture due to the scarcity of innate resistance germ plasm. Antibodies and their fusions have been reported to create fungus-resistant plants as a novel approach developed recently. Fungal antigens prepared from *S. sclerotiorum* were used for immunization of chickens. Polyclonal antibodies were purified from chicken egg yolks, and their reactivity and specificity towards the corresponding fungal antigens were evaluated under optimized conditions. Chicken antibodies were shown a high specificity and reactivity. RNA from immunized chickens were isolated and subjected to cDNA synthesis, which was used for construction of phage display libraries. Several rounds of panning were performed by phage display, resulting in selection of several single-chain antibodies with high affinity. The recombinant antibodies selected were expressed in bacterium upon induction with IPTG and purified by affinity-chromatography. Fungal specific antibodies were characterized by enzyme-linked immunosorbent assays and western blot analyses. To further explore the fungal specific antibodies for plant resistance improvement, the coding sequence of a characterized antibody was used for construction of fusion proteins to anti-fungal peptides. The antibody fusions were introduced into *Arabidopsis thaliana* plants. Transgenic *Arabidopsis* plants expressing the fusions will be evaluated for their resistance response to infection with *S. sclerotiorum*.

Key words: *Sclerotinia sclerotiorum*, single-chain fragment of variable domain, phage display, IgY polyclonal antibodies

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a ubiquitous phytopathogenic fungus that infects an extremely wide range of plants, covering 400 different host plant species (Boland & Hall, 1994). *S. sclerotiorum* induced diseases occur in economically important plants worldwide and cause considerable damage. Innate resistance in plants to this fungus is inadequate and the diseases are difficult to control. Chemical control of the fungus is expensive, is labor intensive, and portends health and environmental risks. Alternatives to classical and chemical crop protection methods are therefore required to circumvent the costs and risks of controlling the pathogen in field. Advances in the development in the molecular biology and plant transformation technologies, and in the understanding of the molecular mechanism underlying host-pathogen interaction have provided alternative strategies for pathogenic fungus control through exploitation of different genetic resources.

Antibodies called immunoglobulins are defense molecules produced by all vertebrates. They recognize and bind to pathogen-specific antigens and thus help to eliminate pathogens from the body. Monoclonal antibodies produced by hybridoma technique (Kohler & Milstein, 1975) display defined specificity and affinity for antigens. Rapid progress in molecular immunology, combined with the polymerase chain reaction, has allowed fragments of antibody genes to be cloned and expressed in bacterial systems. By advanced technologies such as phage display or ribosome display, antibody fragments specific for antigens could be isolated *in vitro* from libraries containing diverse repertoires of antibody V-genes (Hoogenboom *et al*. 1998). These technical advances in recombinant antibody production have been widely applied to research in plant sciences and biotechnology.

Plant-produced antibodies were first demonstrated by Hiatt *et al*. (1989) and Duering *et al*. (1990) that plants can express and assemble functionally active antibodies, opening a new vista in plant biology research. Since then the technology of expressing antibodies in plants has advanced rapidly with a view to utilize it for therapeutic, diagnostic and agricultural purposes (Chen & Chen, 1998; Ma *et al*., 2003; Peschen *et al*., 2004). Various forms of recombinant antibodies have been functionally expressed in a diverse range of plants by using the same pathway for the assembly of light and heavy chains as mammalian cells involving similar signal peptides and successful folding and assembly (Voss *et al*., 1995; Hood *et al*., 2002). Full-size antibodies have been shown to be transported across plasma membrane and cell wall in cell suspension cultures and *in planta* (Fischer *et al*., 1999; Drake *et al*., 2003; Nicholson *et al*., 2005). Expression of plantibodies and their proper assembly and transport in plants have received increasing attention that this technology could be utilized for neutralizing and blocking plant pathogens and thus generate resistant plants (Zimmermann *et al*., 1998; Boonrod *et al*. 2004; Liao *et al*., 2007). In this study, antibodies against *S. sclerotiorum* have been generated that would serve as a basis for resistance improvement in plant to this fungus.

Material and Methods

Fungal strain. The strain of *S. sclerotiorum* was obtained from diseased oilseed plants cultivated in Wuhan, China. Single ascospore isolates were obtained and maintained on potato dextrose agar (PDA). Strain was cultured in potato dextrose broth (PDB) on shake at 22°C for 5 days and the cultures were collected by filtration through mirocloth papers. Mycelium was washed extensively with PBS for the use of antigen preparation.

Antigen preparation and immunization. Mycelium was blended in a PBS-based buffer and used for immunization of chickens. Leghorn chickens were injected intramuscularly with 250μg of the blended mycelium emulsified in complete Freund adjuvant. The chickens were boosted three times at three week intervals with 200μg mycelium in incomplete Freund adjuvant. Eggs were collected from immunized chickens two weeks after the last boost.

Purification of IgY polyclonal antibodies. The egg yolk was separated from the egg white and washed with cold tap water. The yolk suspension was mixed with 4 yolk volumes of PBS and the solution was stirred for 10 min at room temperature. 3.5% of solid PEG6000 was added and the suspension was stirred as described above. The suspension was cooled on ice and centrifuged (12000g/20min/4°C). The supernatant was passed through Whatmann paper and 8.5% PEG6000 was added. After stirring for 20 min at 4°C, the solution was centrifuged as described above and the pellet was dissolved in 2.5 yolk volumes of ice-cold PBS. 12% PEG6000 was added and the suspension was stirred and centrifuged. The pellet was dissolved in 0.25 yolk volumes of ice-cold PBS. An equal volume of precooled 50% ethanol was added to the suspension and centrifuged (15000g/25min/4°C). The IgY pellet was dissolved in 0.25 yolk volumes of ice-cold PBS and the concentration of IgY in PBS was determined spectrophotometrically.

Library construction. Purified fungus-specific IgY antibodies from egg yolks was determined for their titers by enzyme linked immunosorbant assay (ELISA). After a clear boost of immune response was obtained immunized chickens were sacrificed, from which the spleen cells were prepared and subjected to mRNA isolation. The first-strand cDNA was reverse-transcribed and variable (V) regions of heavy or light chains were amplified using the immunoglobulin-specific primers. The PCR amplified V_H and V_L fragments were first separately ligated to a pHEN4III vector after proper digestion with restriction enzymes and then V_H fragments were cloned into V_L or vice versa.

Phage display. Phage library was cultured in 2TY liquid media supplemented with 100μg/ml ampicillin and 1% glucose at 37°C with shaking at 220rpm and M13-K07 helper phages were added. The infected cells were grown with shaking at 30°C overnight and the phages were isolated with PEG6000. After titration of the phages, immunotubes were coated with mycelium in PBS and incubated for 2h at 37°C. The immunotubes were washed three times with PBS and blocked with 2% Marvel for 2h at 37°C. The tubes were washed many times with PBST (0.05% Tween-20) and PBS and the phages were eluted in 100mM triethylamin for 10min at room temperature. The eluted phages were neutralized immediately with 1M Tris-HCl, pH 7.4.

Bacterial expression. A single colony was inoculated into 5ml LB medium supplemented with 100μg/ml ampicillin and 1% glucose and cultured at 37°C with shaking overnight. 2.5ml of the overnight culture were transferred into 500ml 2TY medium containing 100μg/ml ampicillin and 0.1% glucose and incubated at 37°C on a shaker till the OD_{600} value of 0.5. IPTG was added to a final concentration of 1mM for induction of expression at 30°C for 12h. The cells were centrifuged (10min/6000g/4°C) and the pellets were dissolved in 5ml PBS supplemented with 5mM $MgSO_4$, 1mM EDTA, followed by incubation on ice for 15min. After centrifugation (15min/3000g/4°C), the supernatants were pooled and another centrifugation as described above was performed to remove the cell debris. The soluble antibodies were dialysed against PBS for 16h with three changes and purified with affinity chromatography.

Western blot analysis. Bacterially expressed scFv antibodies were separated by 12% SDS-PAGE with two duplicates. Upon completion of electrophoresis one gel was stained with Coomassie Blue dye and dried, while another gel was used for transferring the antibodies to nitrocellulose membrane via semi-dry blotting apparatus. Immunoblots containing scFv antibodies were developed using a 1:2500 diluted mouse monoclonal anti-His tag antibody and a 1:2500 diluted goat anti-mouse antibody conjugated to alkaline phosphatase.

Results

Polyclonal antibodies IgY isolated from egg yolks of immunized chickens were run on 12% SDS-polyacrylamide gel and the results indicated that the PEG precipitation of IgY antibodies from egg yolks produced a high yield and purity. The purified antibodies showed a good integrity on SDS-PAGE (Fig. 1A) and these IgY antibodies could be used for titer determination.

To determine the titers of the purified polyclonal IgY antibodies, coating conditions such as pH and amount of antigens for coating were optimized for *S. sclerotiorum* mycelium for ELISA assays. Neutral conditions were found to be appropriate for the coating of the fungal antigens. The specificity of the polyclonal IgY antibodies was determined and an antibody dilution of 2000 was observed under these conditions (Fig. 1B).

Heavy (V_H) and light (V_L) chains of variable domains amplified from chicken IgY antibodies displayed expected sizes on agarose gel (Fig. 2). V_H and V_L DNA fragments were then digested with SfiI and BstEII, and AscI and NotI, respectively. The digested heavy and light chain fragments were purified and separately ligated into pHEN4III vector. Electroporation competent cells of bacteria were transformed with the ligated products, resulting in the construction of two libraries. After identification of the heavy and light chain libraries, heavy chain fragments in pHEN4III vector were released and ligated to light chains cloned in the same vector to construct a full-size scFv library for selection of scFv antibodies (Fig. 2). The number

of clones of the final full-size scFv library was $2.6x10^5$. Analysis of randomly-selected phage clones digested by restriction enzymes showed that more than 90% clones contained full-sizes of scFvs. These results indicated that the library constructed could be used for further panning and identification of scFvs.

The constructed scFv library was used for panning against *S. sclerotiorum* mycelium. A significant enrichment was obtained after each round of panning. Analysis of the monoclonal soluble scFvs of selected phage clones after three rounds of panning confirmed their reactivity and specificity to the fungal mycelium. DNA finger printing with BstN1 restriction enzyme digestion indicated that all the 10 clones displayed the same pattern, suggesting that they were identical. One of the clones was chosen for expression of soluble scFv in bacteria and the scFv isolated from periplasm showed expected size and integrity (Fig. 3) that could be used for diagnostic analysis of the fungus. The sequence of this clone was determined, which showed typical characters for a chicken antibody in its CDR regions.

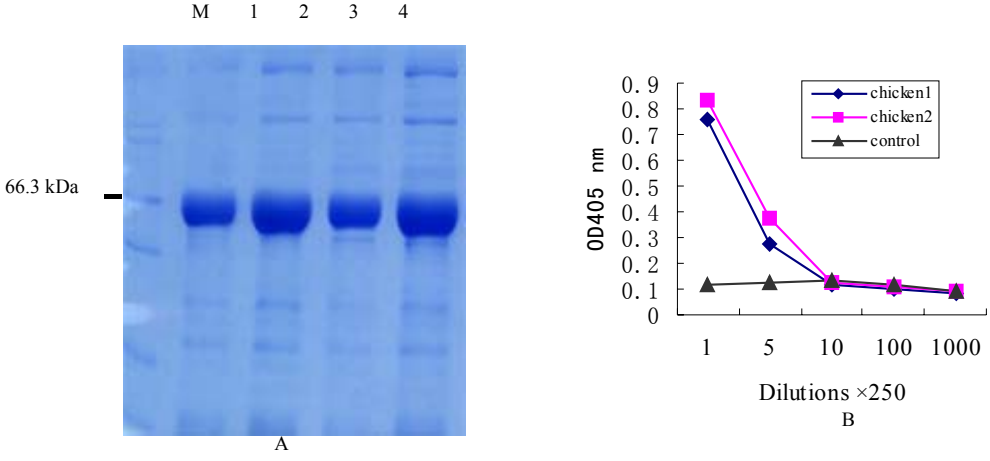

Fig. 1. Analysis of IgY antibodies derived from chickens. A: SDS-PAGE of purified polyclonal antibodies from egg yolks of immunized chickens. Lanes 1 and 2: IgY from chicken 1; Lanes 3 and 4: IgY from chicken 2; Lanes 1 and 3: 10μg; Lanes 2 and 4: 20μg. B: Titer determination of polyclonal antibodies from chicken egg yolks.

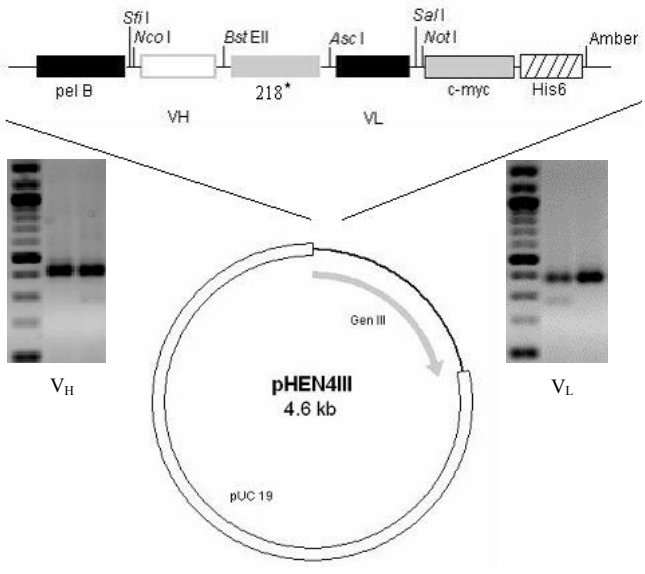

Fig. 2. Partial restriction map of pHEN4III vector used for cloning of scFv antibodies and PCR-amplified V_H and V_L fragments of antibody genes from immunized chickens.

The coding sequence of this scFv antibody was fused to antifungal peptides that were derived from plants and microorganisms and assayed for their inhibitory effects on the growth of this fungus. The fusion protein was expressed in bacterium and plant leaves. Transgenic *Arabidopsis thaliana* plants expressing the antibody fusion gene will be assayed after infection with *S. sclerotiorum* fungus.

Discussion

Different chicken antibodies against various antigens have been generated and used for a number of applications (Andris-Widhopf *et al.* 2000; Peschen *et al.* 2004). The molecular diversification of immunoglobulins in chicken is distinct from that in mammals where the preimmune repertoire is constructed by combinational and junctional diversity during

V-(D)-J recombination. In chicken light chains the construction of V-J recombination is not significant since only one functional V- and J-gene is present for the entire light chain repertoire. The diversity of the chicken light chain repertoire is generated by gene conversion of the rearranged functional V-light gene with pseudo V-light chains clustering upstream of the functional gene. A similar mechanism is for heavy chain. Thus a single pair of primers is required for PCR-amplification of each variable region of V_H and V_L genes. A recombinant scFv antibody specific to *S. sclerotiorum* panned from a phage antibody library suggests that phage display libraries from immunocompetent chickens could be established for selection of antibodies against plant pathogenic fungus. Chicken-derived scFv antibody could be expressed to a high level and were stable in bacterium.

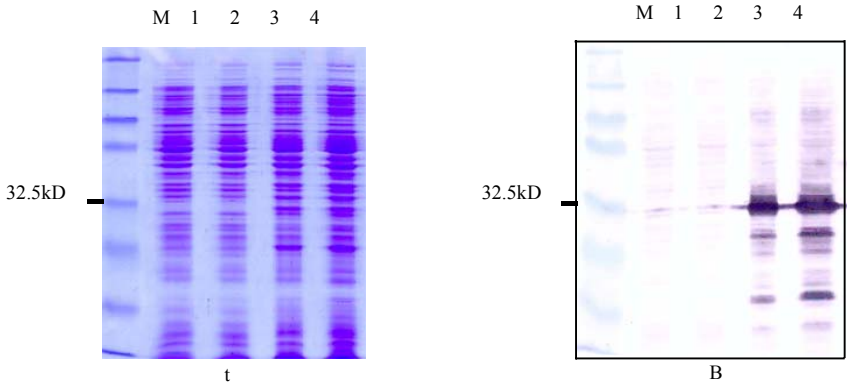

Fig. 3. SDS-PAGE (A) and western blot analysis (B) of a bacterially expressed scFv antibody specific to *S. sclerotiorum*. M: prestained marker; Lanes 1 and 2: non-induced controls; Lanes 3 and 4: IPTG induction for 6 and 8 h respectively.

Conclusion

A recombinant scFv antibody specific for an important phytopathogenic fungus *S. sclerotiorum* has been generated by phage display from a large phage antibody library. Further investigations include characterization of transgenic *Arabidopsis thaliana* plants expressing the scFv antibody -antifungal peptide fusions and bioassay of the plants upon challenge with *S. sclerotiorum* under different conditions. By changing parameters during panning more antibodies would be isolated and used in plant pathology and resistance improvement.

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