Bulletin of Insectology ***64*** *(Supplement): S75-S76, 2011*
ISSN 1721-8861

Generation of a specific monoclonal recombinant antibody against '*Candidatus* Phytoplasma aurantifolia' using phage display technology

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Abstract

Witches' broom disease of lime (WBDL) is a destructive disease caused by '*Candidatus* Phytoplasma aurantifolia' and is a limiting factor for lime production in Southern Iran. Conventional strategies for disease management have shown little success and new approaches based on genetic engineering need to be considered. Lack of natural resistance against phytoplasma diseases has highlighted the importance of alternative approaches and recombinant antibody mediated resistance is among them. The immunodominant membrane protein (IMP) is a major protein present on the surface of phytoplasma cells and is important for both diagnostics and interactions with plant hosts and insect vectors. Generation of specific recombinant antibodies with high binding ability against IMP proteins is beneficial for both diagnostic purposes and development of recombinant antibody-mediated resistance against disease.

Phage display is a powerful technology for generation of specific recombinant antibodies such as single chain variable fragment antibodies (scFv). This study describes generation of a specific scFv fragment through panning of naïve phage display libraries. For this aim, the gene encoding the IMP protein was isolated and cloned into a bacterial expression vector and recombinant IMP protein was expressed in bacterial cells and purified through affinity chromatography. Purified recombinant IMP protein was used for panning of naïve Tomlinson I and J scFv phage display libraries. Following three rounds of panning for selection and amplification of specific binders, capability of individual clones for production of specific scFvs was evaluated by an ELISA assay. The preliminary results showed generation of specific scFv recombinant antibodies with strong binding ability against the IMP protein. Complementary studies revealed that the scFvs are able to bind native IMP and could detect the presence of phytoplasma in infected plants. Further immunoassay analysis confirmed that generated scFvs are able to detect epitopes along the IMP amino acid residues. As far as we know, this is the first succesful application of phage display libraries for generation of scFv recombinant antibodies against phytoplasma cells.

Key words: phage display, WBDL, IMP, '*Candidatus* phytoplasma aurantifolia', recombinant antibody, scFv.

Introduction

The witches' broom disease of lime (WBDL) caused by '*Candidatus* Phytoplasma aurantifolia', is the most destructive disease in lime tree throughout southern Iran. Phytoplasmas are phloem-limited bacterial pathogens that persistently colonize their plant hosts. They are wall-less prokaryotes belong to Mollicutes. They are known to have specific characteristics such as small genome size from 530 to 1350 Kb, low G+C percent, unculturability in cell free media, and transmission and spread by insect vectors mainly leafhoppers and planthoppers (Lee *et al*., 2000).

Phytoplasmas are surrounded by a single cell membrane. The Immunodominant membrane proteins (IMPs) of phytoplasmas are major proteins located on the external surface of the cell membrane and appear to have important roles in pathogenicity in the host plant and insect cells (Hogenhout *et al*., 2008). Due to the unique behaviour of IMP in pathogenecity of phytoplasma, they are good candidates for suppression of disease through a recombinant antibody-mediated resistance approach. Le Gall *et al.* (1998) generated an scFv fragment binding to the IMP protein of stolbur phytoplasma. The plants expressing this scFv showed the potential for recombinant antibody fragments for suppression of phytoplasma diseases in transgenic plants (Le Gall *et al*., 1998; Malembic-Maher *et al*., 2005).

Phage display is a powerful technology for development of specific binders against almost any antigen. The main advantage of phage display is direct and physical linkage between phenotype and genotype. Several scFv phage display libraries have been developed by amplifying V_L and V_H regions of animal donors and fusing them to the pIII minor coat protein of a filamentous bacteriophage (Hust and Dubel, 2004). This article introduces application of phage display technology for generation of a specific scFv recombinant antibody that could be used for both specific detection of WBDL infected plants and immunomodulation of disease in transgenic plants.

Materials and methods

The gene encoding the IMP protein was isolated and cloned into a bacterial expression vector and the purified recombinant protein was obtained through affinity chromatography as previously described (Shahriyari *et al*., 2010). Phage display processes were carried out by performing three rounds of panning on Tomlinson I and J scFv phage display libraries as previously described (Safarnejad *et al*., 2008).

Panning was performed by immobilizing IMP recombinant protein (~50-100 µg/ml) overnight onto immunotubes (Nunc-Maxisorb), which had been washed with PBS, blocked with skimmed milk and incubated with phage suspension (~ 10^{13} CFU). Phage articles with affinity for the antigen were eluted using triethylamine and used for infection and amplification of exponentially growing *E. coli* (Migula) TG1 cells. The total eluted phage titre was determined after each round of panning. After the third round, individual colonies were randomly selected and analyzed for production of soluble scFv and binding activities against IMP by using blotting and ELISA analysis. Mini-induction of scFv recombinant proteins was performed in *E. coli* strain HB2151 harbouring scFv phagemid and by adding IPTG to a final concentration of 1 mM and further incubation at 30°C overnight. Soluble scFv was obtained by centrifugation and supernatant was applied for further serological analysis. In ELISA, 100 µg/ml antigens in PBS were directly coated on high-binding microtitre plates followed by a blocking step using 2% (W/V) skimmed milk. Around 100 µl of scFv solutions were then applied to the plates and incubated at 37°C for 2 hr. Bound scFs were detected using anti-c-myc monoclonal antibody 9E10 followed by AP conjugated goat-anti-mouse polyclonal antibodies. Colonies with high specificities were selected and subjected to further analysis.

Binding ability of the resultant scFv recombinant antibody against native IMP and intact phytoplasma cells was proved by applying ELISA, western blotting and dot blot immunoassays on WBDL infected plants.

Results and discussion

The IMP protein was produced as a His-tagged fusion protein and purified by affinity chromatography in nickel-agarose columns. The SDS-PAGE results proved integrity and purity of recombinant protein and it was measured at 35 kDa. The total yield of purified IMP in the culture medium was calculated at around 6 mg/l.

The Tomlinson I and J naïve scFv libraries were screened for IMP binders using purified His-tagged IMP recombinant protein. Three rounds of panning were performed with 10^{13} of recombinant phage in each round. The results obtained after each round confirmed enrichment of IMP specific scFv throughout the panning processes. After the third round, individual colonies were randomly selected and their ability for production of soluble scFv was determined by western blot and dot blot assays. Presence of scFv in bacterial supernatant was shown by AP conjugated anti-c-myc monoclonal antibodies. These results revealed that around 50% of clones selected from the Tomlinson I library produced detectable scFv which was measured at 30 kDa. The binding activity of soluble scFv against IMP was measured by indirect ELISA. These results indicated that at least 6 individual clones produce specific scFv with strong binding ability to IMP. There was no detectable binding to negative control samples including GST and BSA proteins. Complementary ELISA and blotting analyses were carried out to determine the binding specificity of scFvs against infected plant samples. These results proved that scFvs could react with phytolasma cells presented in infected plants as well. Furthermore, presence of positive responses in western blot and ELISA analyses indicated that the corresponding epitope is a continuous type and presented on both recombinant and native IMP proteins. Based on the literature, the sole recombinant antibody generated against phytoplasma cells was produced by using pre-established hybridoma clone (Le Gall *et al*., 1998). To our knowledge, this is the first report on obtaining a specific recombinant antibody against phytoplasmas by using scFv phage display libraries. Due to the presence of selective pressure in phage display for obtaining more soluble recombinant antibodies during the panning process, it seems that the resultant scFv fragments could be expressed well in plant cells and are suitable candidates for suppression of WBDL disease *in planta.*

Acknowledgements

We would like to thank the national project of WBDL and Agriculture Biotechnology Research Institute of Iran (ABRII) for providing financial support for this current project.

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