Development of a Competitive Dot-blot Assay for Tungro Disease Detection using Recombinant Polyclonal Antibodies

Description

This dot-blot is designed to detect the presence of tungro in rice plants by exerting the antigen present in plant sample as the inhibitor. In this case, the inhibitor competes with the recombinant CPs of the tungro viruses for binding with the corresponding polyclonal antibodies. The polyclonal antibodies of RTBV-rCP and RTSV-rCP3 were first incubated with the sample to allow primary binding of the antibodies to form antigen-antibody complexes. Once added to the competitor (RTBV-rCP and RTSV-rCP3) present on the blots, only the unbound polyclonal antibodies from the antigen-antibody mixture can bind to the recombinant CPs on the blots. Hence, the more antigen is present in the sample, the less antibody is available to bind to the recombinant antigen on the blots. Therefore, if the mixture is prepared with plants infected with tungro, the blots will deposit no color. In contrast, color will be detected on the immunoblots with the healthy plants.

Brief Methodology

A) Production of recombinant coat proteins of the tungro viruses, RTBV-rCP and RTSV-rCP3

Rice tungro disease is caused by a mix infection from two types of virus; rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV). RTBV has a circular double-stranded deoxyribonucleic acid of about 8 kb. The genome has four open reading frames (ORFs) namely ORF1, ORF2, ORF3 and ORF4. Only ORF2 and ORF3 have known functions. ORF2 encodes a protein that is involved in capsid protein assembly whereas ORF3 encodes movement protein (MP), coat protein (CP), aspartate transferase (AT) and reverse transcriptase/ ribonuclease H (RT/RNase H) which are involved in virus replication and assembling. On the other hand, RTSV has a positive-sense single-stranded, polyadenylated RNA genome of approximately 12 kb contained within a capsid composed of three coat proteins (CP). The genome encodes a single

large polyprotein of 3473 amino acids which included the three CPs namely CP1, CP2 and CP3.

The coat protein (CP) gene of RTBV and CP3 gene of RTSV were cloned and expressed using the SUMO fusion expression system. The CP genes of the tungro viruses were first amplified and ligated into pET SUMO vector. The recombinant plasmids obtained were transformed into competent One Shot Mach1-T1 E.coli. Next the colonies formed were screened, and the nucleotide sequences of selected positive clones were determined. The confirmed selective positive clones were transformed into expression host, competent One Shot BL21 (DE3) E.coli. A single colony transformant was selected and induced to express the recombinant protein of interest. The expression of the recombinant coat proteins were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot. Finally, the expressed recombinant coat proteins were purified using affinity column chromatography.

B) Recombinant polyclonal antibody production

Polyclonal antibodies were produced in rabbits by immunization with the purified rCPs of RTBV CP and RTSV CP3. Immunoglobulin (IgG) fractions were purified from crude antisera by ammonium sulfate precipitation and column chromatography.

C) Plant sample preparation

Leaves samples collected are kept dried and stored in -80 ^{o}C. Leaf sap is extracted by grinding 0.1 g of cut leaves using an ice-cold mortar and pestle. The leaves is ground again in 600 µl of 1X phosphate-buffered saline (PBS). The mixture is placed into a 1.5 ml microcentrifuge tube and incubated in a 40 ^{o}C water bath for 10 minutes. The mixture is re-ground using the end of a blunted pipette tip and vortexed to mix. The mixture is clarified by spinning at 13,400 rpm for 5 minutes. The supernatant was aliquoted into a new 1.5 ml microcentrifuge tube and stored at -80 ^{o}C.

D) Preparation of antibody

Cross-reactivity of the polyclonal antibodies of RTBV-rCP and RTSV-rCP3 to healthy plant proteins is prevented by direct cross-adsorption with sap extracts from healthy rice plants. One volume of antiserum is incubated with two volumes of healthy plant extract for a three hours incubation period at 37 ^{o}C. The adsorbed antibodies is then clarified by short spinning at 13,400 rpm and supernatant is collected into new microcentrifuge tubes.

E) Primary incubation of sample and polyclonal antibodies

The antibody is first incubated with the leaf sap for 1 hour incubation period at 37 ^{o}C. This adequately allow the primary binding of antibody to the targeted analyte present in the sample forming antigen-antibody complexes. One volume of the leaf sap extracts, either from healthy or infected plants is mix equally with the polyclonal antibody. Prior to this, both parts is appropriately diluted in 5% non-flat skimmed milk in 1XPBS (1XPBS-SKM). The optimal dilution factor for both the sap extracts and the polyclonal antibody has been established. At a 1:4 dilution factor for plant sap preparation, the polyclonal antibody is able to differentiate between infected and healthy plant. Supplementary, the working dilution for the polyclonal antibody is at 1:5000.

F) Competitive dot-blot immunoassay (CDBIA)

A nitrocellulose membrane (NCM) strip (L X W; 1.5 cm x 2.0 cm) is dotted with 2 µl of antigen (RTBV-rCP and RTSV-rCP3) and SUMO control protein. The strip is oven-dried for 5 minutes at 60 ^{o}C and block with 5% non-flat skimmed milk in 1XPBS (1XPBS-SKM) at room temperature for 30 minutes. By using a 10-well plate, one strip of dotted membrane is immersed in 100 µl of the prepared antigen-antibody mixture. The membrane is left incubated in the solution for 1 hour at 37 ^{o}C and washed three times with 1XPBS-Tween20 with an interval time of 10 minutes. HRP-conjugated swine anti-rabbit immunoglobulins (Dako, Glostrup, Denmark) diluted 1:1000 in 1XPBS-SKM is then added into the well and incubated for another 2 hours at room temperature. The strip membrane is again washed thrice in wash buffer. Color is developed with

chromogenic substrate containing 1-Step^{TM}Chloronaphtol (Thermo Fisher Scientific, Inc., MA, USA) in hydrogen peroxide substrate.