Isolation, Cloning, and Heterologous Maintenance of a Class III Peroxidase Gene from *Solanum tuberosum* and Its Implications for Plant Defense Mechanisms

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ABSTRACT

Plant growth, development, and defense reactions are greatly dependent on the massive multigene family of heme- containing glycoproteins called class III plant peroxidases (EC 1.11.1.7). These enzymes are of prime importance for resistant pathogens, lignification, suberization, and reactive oxygen species (ROS) metabolism. The isolation, amplification, cloning, and bacterial preservation of a peroxidase gene from *Solanum tuberosum* (potato), a globally important crop constantly under attack by biotic and abiotic stress factors, are described in this study. The CTAB method was used for the isolation of genomic DNA from young sprouting buds of potato tubers. Using gene-specific primers, a conserved region of the peroxidase gene was amplified by polymerase chain reaction (PCR). After being ligated into a plasmid vector, the amplified region was heat-shocked to produce *Escherichia coli* DH5α competent cells.

The successful transformation was confirmed by biochemical screening and selection with ampicillin. A useful tool for gene conservation and subsequent applications in molecular breeding and plant genetic engineering is offered by the conservation of the peroxidase gene in a bacterial system. The significance of the peroxidase genes in enhancing plant resistance by genetic engineering and their importance as pathogenesis-related (PR-9) proteins is highlighted in the study. This study enhances our understanding of the role of peroxidase in plants and supports future applications of transgenic technology to improve plant resistance.

Keywords: Class III peroxidase; reactive oxygen species; *Solanum tuberosum*; plant defense; gene cloning; bacterial transformation; pathogenesis-related proteins

INTRODUCTION

Peroxidases (EC 1.11.1.7) are a large family of oxidoreductase enzymes that have an important role in maintaining the redox balance in cells by catalyzing reactions involving hydrogen peroxide and other electron donors. These enzymes are commonly found in fungi, bacteria, plants, and mammals. They have implications in various biological processes such as hormone metabolism, lignin biosynthesis, growth, and reactions under stressed conditions. Peroxidases are one of the most complex enzyme families that are found in plants, which indicates their adaptive significance and multifunctionality (Welinder, 1992; Hiraga et al., 2001). Class III peroxidases are plant-specific enzymes, which are mostly found in the apoplast and vacuoles. Plant peroxidases can be divided into three main classes. These enzymes are encoded by large multigene families. For example, the *Arabidopsis* genome alone contains more than 70 genes for class III peroxidases, which indicate their functional specialization and redundancy (Cosio & Dunand, 2009; Passardi et al., 2005). Despite the fact that these enzymes contain conserved catalytic domains in the prevalent, they possess rather different regulatory regions, which allow them to differently express themselves in reaction to environmental disturbances and developmental signals.

Participation in plant defense mechanisms is one of the most important roles of class III peroxidases. The production and scavenging of reactive oxygen species, lignification of cell walls, cross-linking of phenolic compounds, and the establishment of physical barriers against invading pathogens are some of the processes in which class III peroxidases are involved. Class III peroxidases are known as pathogenesis-related PR-9 proteins due to their inducible aggregation upon

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infection by pathogens, and their expression is highly linked to disease resistance (van Loon et al., 2006; Almagro et al., 2009). A major food crop globally, potatoes (*Solanum tuberosum* L.) are highly susceptible to different biotic factors, including bacterial, viral, and fungal diseases, besides abiotic factors, such as oxidative stress and salt stress. Thus, the use of molecular approaches to enhance the potato plant's natural defense system has become a major research focus. The peroxidase gene is an attractive candidate for genetic engineering approaches aimed at improving crop resistance due to its dual role in stress resistance (Passardi et al., 2004; Yoshida et al., 2003). The heme-containing glycoproteins with molecular weights of about 30 and 45 kDa and the conserved calcium-binding sites essential for the stability of enzymes were found to be plant peroxidases through early biochemical research. Although class III peroxidases were first found to be plant-specific enzymes with only extracellular functions, structural and evolutionary analyses have shown that these enzymes belong to an enormous superfamily that shares characteristics with bacterial and fungal peroxidases (Welinder, 1992; Hiraga et al., 2001).

The important role of peroxidases in cell wall modification, particularly lignification and suberization, has been an important area of investigation. Class III peroxidases accelerate the oxidative coupling of monolignols in lignin biosynthesis, leading to the formation of rigid secondary cell walls that provide strength to plant tissues and protect them from pathogen attack. In vascular tissues and storage organs such as tubers, lignification by peroxidases is particularly important because it greatly increases strength and disease resistance (Ros Barceló, 1997; Joët et al., 2009). Through reactive oxygen species regulation, peroxidases are also crucial in plant defense signaling pathways. The rapid production of hydrogen peroxide in the apoplast is a characteristic of an oxidative burst, which is a reaction launched by plants upon the perception of a pathogen. Class III peroxidases regulate defense signaling and prevent excessive cellular damage through the regulation of ROS production and detoxification (Bolwell et al., 2002; Apel & Hirt, 2004). Through their dual function, peroxidases can function as both defensive antioxidants and signaling molecules.

Several studies have shown that environmental stresses and phytohormones have a significant effect on the expression of the peroxidase gene at the molecular level. There are multiple cis-regulatory elements that are sensitive to oxidative stress, wounding, and pathogen attack that have been found in stress-inducible promoters that have been discovered from crop plants such as sweet potatoes (Kim et al., 2008). Additionally, in transgenic plants, increased susceptibility to salt, drought, and pathogens has been shown to be due to the overexpression of peroxidase genes (Yoshida et al., 2003; Shigeoka et al., 2002). Because of gene redundancy and the inconsistency of substrate specificity, functional studies of individual peroxidase isoenzymes remain challenging despite extensive research efforts. The inability to distinguish and characterize specific peroxidase genes has been made possible through advancements in heterologous expression systems and molecular cloning. The upkeep of cloned genes present in the bacteria makes conduction of functional studies and sequence analysis easy, thereby opening avenues for their applications in molecular breeding and genetic engineering of plants (Passardi et al., 2005; Cosio & Dunand, 2009).

MATERIALS AND METHODS

Plant Material and Induction of Sprouting

The healthy *Solanum tuberosum* with growing eyes were selected, cleaned and cut lengthwise in a way that sample included the meristematic regions of the eyes. The sliced pieces were covered with moist cotton to prevent drying up and placed in sterile petri plates. The Petri plates were kept for incubation for four to five days at 25 ± 2 °C in a biological oxygen demand (BOD) incubator. Emerging young buds that are metabolically active have sufficient number of nucleic acids easily emerged under this controlled incubation. The emerged young buds when sprouted were aseptically cut, covered with aluminium foil and stored at low temperatures.

Genomic DNA Isolation

Genomic DNA was isolated using the cetyltrimethylammonium bromide (CTAB) protocol in which bud material was homogenized with CTAB extraction buffer and then incubated at 65 °C. Polysaccharides were removed by a high salt concentration method and cellular debris was removed by centrifugation. The DNA was precipitated by alcohol precipitation method and then purification was done with 70% ethanol. The DNA pellet obtained after drying was dissolved in sterile distilled water and subjected to agarose gel electrophoresis for verifying its suitability for downstream molecular analysis.

One hundred milligrams of plant culture were taken in a tube and macerated using a pestle, followed by the addition of 500 µl of CTAB buffer. The tube was then inverted several times to allow slow and thorough mixing,

after which the sample was incubated at 65°C for 15 to 20 minutes. Subsequently, 0.2 ml of DNA salt solution was carefully added to the tube and mixed by repeated inversion.

The tube was then centrifuged at 10,000 rpm for 10 minutes, and the supernatant was carefully collected. To this supernatant, 0.8 ml of DNA precipitating solution was added, and the tube was slowly inverted several times while observing for the appearance of white thread-like strands, which indicated successful DNA precipitation. The precipitate was then pelleted by centrifuging at high speed for a minimum of 10 minutes.

Following centrifugation, the supernatant was discarded and the pellet was washed with 0.5 ml of 70% ethanol, followed by another high-speed centrifugation for at least 10 minutes. The ethanol was then carefully discarded, and the tube was left open at room temperature until the pellet was completely dry. Finally, the dried pellet was resuspended in 30 µl of sterile water in preparation for running on an agarose gel.

Gel Electrophoresis

Gel electrophoresis is a method used in clinical chemistry to separate proteins by charge and or size (IEF agarose, essentially size independent) and in biochemistry and molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate proteins by charge. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving. Proteins are separated by charge in agarose because the pores of the gel are too large to sieve proteins. Gel electrophoresis can also be used for separation of nanoparticles.

"Electrophoresis" refers to the electromotive force (EMF) that is used to move the molecules through the gel matrix. By placing the molecules in wells in the gel and applying an electric field, the molecules will move through the matrix at different rates, determined largely by their mass when the charge to mass ratio (Z) of all species is uniform, toward the (negatively charged) cathode if positively charged or toward the (positively charged) anode if negatively charged.

Agarose gel was prepared by dissolving 0.35 g of agarose in 50 ml of 1X TAE buffer, which was made by adding 1 ml of 50X TAE buffer to 49 ml of distilled water. The mixture was heated until the solution became completely transparent, indicating proper dissolution of agarose. It was then allowed to cool to a suitable temperature, after which 2–4 µl of ethidium bromide was added and mixed gently.

The prepared solution was carefully poured into a gel casting tray and left undisturbed for 20–30 minutes to allow proper gel solidification. Meanwhile, the electrophoresis buffer was prepared by mixing 4 ml of 50X TAE buffer with 196 ml of distilled water to obtain 1X TAE buffer. Once the gel was set, it was placed into the electrophoretic unit, and the prepared buffer was poured into the chamber until the gel was completely submerged.

The DNA samples were prepared by adding 10 µl of gel loading dye to each sample, followed by gentle mixing. These samples were then carefully loaded into the wells of the agarose gel. The electrophoresis unit was switched on, allowing the DNA fragments to migrate through the gel matrix according to their molecular weight.

After running the gel for approximately 45–60 minutes, the electrophoresis was stopped, and the gel was carefully removed from the unit. The separated DNA bands were then visualized under a UV transilluminator, where distinct bands corresponding to different DNA fragment sizes were observed.

PCR Amplification of the Peroxidase Gene

PCR was conducted using gene specific primers designed based on the conserved regions of known plant class III peroxidase gene. The PCR mixture contained DNTPs, reaction buffer, $MgCl_2$, Taq DNA polymerase, forward and reverse primers along with genomic DNA as a template. The amplified PCR product was subjected to agarose gel electrophoresis and then analyzed under a UV lamp. Clear amplification was seen by the appearance of a distinct band comparable to the size of the peroxidase gene fragment. After its removal, this region was tied into the plasmid vector. The expected amplicon size was approximately ~900 bp, consistent with conserved regions of class III peroxidase genes. Amplified products were analyzed on 1% agarose gel with DNA ladder.

The two primers designed from the most conserved regions of related sequences were **PX1:** 5’CGTCTCCACTTTCATGACTGC 3' and **PX2:** 5'GAAACCTACCGTGTGTGCACC 3', in sense and antisense orientations, respectively.

A PCR reaction mixture was prepared by taking an appropriate amount of DNA template containing the target region to be amplified in a sterile tube. To this, two primers complementary to the 3′ ends of the sense and

antisense strands of the DNA were added. Subsequently, Taq DNA polymerase, having an optimum activity temperature of around 70°C, was included in the reaction mixture.

Deoxynucleotide triphosphates (dNTPs), which serve as the building blocks for DNA synthesis, were then added, followed by the addition of a suitable buffer solution to maintain optimal chemical conditions for enzyme activity and stability. Divalent cations, primarily magnesium ions (Mg^{2+}), were incorporated into the mixture to facilitate enzyme function; however, manganese ions (Mn^{2+}) may also be used in specific cases such as PCR-mediated mutagenesis, as higher Mn^{2+} concentrations increase the error rate during DNA synthesis.

Finally, monovalent cations, particularly potassium ions, were added to stabilize the reaction conditions. The reaction mixture was gently mixed to ensure uniform distribution of all components and prepared for subsequent amplification under thermal cycling conditions.

Initialization step: This step consists of heating the reaction to temperature of 94-96° C which is held for 1-9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.

Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94-98 ^{0}C for 20-30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single strands of DNA.

Annealing step: The reaction temperature is lowered to 50-65 ^{0}C for 20-40 seconds allowing annealing of the primers of single-stranded DNA template. Typically, the annealing temperature is about 3-5 ^{0}C below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

Extension/elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75- 80 ^{0}C and commonly a temperature of 72^{0}C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5’ to 3’ direction, condensing the 5’-phosphate group of the dNTPs with the 3, -hydroxyl group at the end of the nascent(extending) DNA strand. The extension time is depended upon the both DNA polymerase used and on the length of the DNA fragment to be amplified

Final elongation: This single step is occasionally performed at the temperature of 70 to 74 ^{0}C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Final hold: This step at 4-15 ^{0}C for an indefinite time may be employed for short term storage of the reaction.

PCR METHODOLOGY

All components required for PCR were kept on ice throughout the procedure to maintain enzyme activity and prevent degradation. A PCR tube was labelled appropriately and also kept on ice. To this tube, 10 µl of the isolated genomic DNA sample was added carefully.

Subsequently, the PCR master mix was prepared directly in the same tube by adding 10 µl of 5′ genomic primer, 10 µl of 3′ genomic primer, 20 µl of Taq reaction buffer, 20 µl of dNTPs, 20 µl of Taq polymerase, and 10 µl of PCR-grade water. The contents of the tube were then mixed thoroughly to ensure proper homogenization of all reaction components.

The PCR tube containing the complete reaction mixture was then placed in a thermocycler. The thermocycler was programmed with the following conditions: an initial denaturation step at 96°C for 2 minutes, followed by 33 cycles consisting of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 68°C for 2 minutes. This was followed by a final extension step at 68°C for 7 minutes, and the reaction was then held at 4°C indefinitely.

After completion of the amplification process, the PCR product was collected, and 50 µl of the amplified DNA was used for visualization, typically by agarose gel electrophoresis.

Gram Staining

The cationic dye crystal violet is used to stain the nucleic acid of the microorganisms and background tissues. The crystal violet staining is then lacked with iodine forming a purple complex. Certain microorganism resists differentiation due to the impermeability of their cell walls. However, using a suitable differentiated example

alcohol or acetone, the tissue background and certain species of microorganisms lose their staining, but take up a cationic dye example saffranine of contrasting colour usually red or pink subsequently applied.

The purple staining microorganism are termed “Gram positive”, whereas the microorganism that take up the counter stain red or pink are termed “Gram negative”.

A bacterial smear was first prepared on a clean glass slide, which was then allowed to air dry and subsequently heat-fixed to ensure proper adherence of the cells. The slide was placed on a staining rack and flooded with filtered crystal violet stain for 1 minute, after which it was gently washed with water to remove excess stain. Gram’s iodine was then applied to the smear and allowed to act for 1 minute, followed by a brief washing with water, taking care not to let the smear dry out.

The smear was then decolorized using acetone, allowing the solvent to flow over the slide until the moving dye front passed the lower edge of the smear. Immediately after decolorization, the slide was rinsed with tap water to stop the action of the decolourizer. The smear was subsequently counterstained with safranin for approximately 45 seconds to impart colour to the decolorized cells.
Finally, the slide was washed gently with water to remove excess stain, allowed to air dry completely, and then mounted for microscopic observation.

Plasmid Isolation

Alkaline lysis is the method of choice for isolating circular plasmid DNA, or even RNA from bacterial cells. It is probably one of the most generally useful techniques as it is fast, reliable and relatively clean way to obtain DNA from cells, if necessary, DNA from an alkaline lysis preparation can be further purified.

Alkaline lysis depends on a unique property of plasmid DNA it is able to rapidly anneal following denaturation. This is what allows the plasma DNA to be separated from the bacterial chromosome. Typically, E. coli cells will be grown up that contains the plasmid which has to be isolated, then lyse the cells with alkali and extract the plasmid DNA the cell debris is precipitated using SDS and potassium acetate. This is Spun down, and the pellet is removed. Isopropanol is then used to precipitate the DNA from the supernatant, supernatant is removed and the DNA is resuspended in buffer. A mini preparation usually yields 5-10 ug. This can be scaled upto mini preparation or a maximum preparation, which will yield much larger amounts of DNA and RNA.

The plasmid DNA from the *E. coli* BB4 strain was isolated using the alkaline lysis method which supports supercoiled plasmid DNA but denatures chromosomal DNA, making it possible to easily separate them. The standard ligation method was deployed to ligate the amplified peroxidase gene fragment to the isolated plasmid.

A 2 ml bacterial culture was taken in a centrifuge tube and centrifuged at full speed for 10 minutes. The supernatant was carefully discarded without disturbing the pellet. The bacterial pellet was then resuspended in 200 µl of cell suspension solution by gently flicking the tube to ensure proper mixing. Subsequently, 200 µl of lysis buffer was added, and the contents were mixed gently by inverting the tube several times. The mixture was then incubated at room temperature for about 15 minutes.

After incubation, 200 µl of neutralizing buffer was added and mixed thoroughly by inverting the tube a few times. The sample was then centrifuged at 10,000 rpm for 10 minutes, and the clear supernatant was carefully transferred to a fresh tube. To this supernatant, 0.8 volumes of 100% isopropanol (or alternatively 2.5 volumes of ethanol) were added, followed by gentle inversion to mix the contents. The mixture was then incubated at –20°C for 10 minutes to facilitate DNA precipitation.

The tube was centrifuged again at full speed for 15 minutes, resulting in the formation of a small white pellet. The supernatant was carefully decanted, and the pellet was washed with 300 µl of 70% isopropanol. This was followed by centrifugation for 5 minutes, after which the supernatant was slowly removed using a pipette. The tube was then left open at room temperature to allow complete evaporation of the residual alcohol.

Finally, once the pellet was completely dry, it was resuspended in 50 µl of TE buffer and centrifuged for 2 minutes. The sample was then either used for further downstream applications or stored at –20°C for future use.

Bacterial Transformation

Plasmids were discovered in the late 60 s; it was quickly realized that they could be used to amplify a gene of interest. A plasmid containing resistance to antibiotic is used as a vector. The gene of interest is inserted into the vector plasmid and this newly constructed plasmid is then put into E. coli. The bacteria are then spread over a plate that contains ampicillin. The ampicillin provides a selective pressure because only bacteria that have acquired the plasmid can grow on the plate. Therefore, as long as we grow the bacteria in ampicillin it will need

the plasmid to survive and it will continually replicate it, along with your gene of interest that has been inserted to the plasmid.
There are many different kinds of plasmid commercially available. All of them contain, a selectable marker, an origin of replication, and multiple cloning sites. The multiple cloning sites have many restriction enzyme sites and is used to insert the gene of interest.
The competent E. coli DH5α cells, prepared from the heat shock method, were then transformed with the recombinant plasmid. Selection was done by placing the transformed cells on LB agar aided with ampicillin. The success of the transformation was indicated by the fact that only cells with the recombinant plasmid could grow.

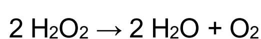
A labelled test tube was first prepared and placed on ice. Two additional fresh tubes along with the provided competent buffer were also kept on ice to maintain low temperature conditions. Subsequently, 3 ml of LB broth was added to a culture tube containing 2 ml of bacterial culture, and the mixture was incubated at 37°C with shaking for one hour to allow bacterial growth. After incubation, the culture was removed and placed on ice for 5 minutes to stabilize the cells.
Following this, 2 ml of the bacterial culture was transferred into two pre-chilled tubes, while the remaining culture was retained as a control. The tubes were then centrifuged at 5000 rpm for 3 minutes to pellet the bacterial cells. The supernatant was carefully discarded without disturbing the pellet. The pellets from both tubes were then combined by adding 0.5 ml of ice-cold competent buffer to one tube and transferring the suspension into the second tube containing the other pellet.
The combined suspension was again centrifuged for 3 minutes to re-pellet the bacteria, and the supernatant was carefully removed. The pellet was then resuspended in 0.2 ml of ice-cold competent buffer by gently pipetting up and down to ensure complete suspension. After this, 10 µl of plasmid DNA was added to the competent cells, and the mixture was incubated on ice for 30 minutes to facilitate DNA uptake.
The tubes were then subjected to heat shock by transferring them to a water bath maintained at 42°C for 90 seconds, followed by immediate transfer back to ice for 2 minutes. Subsequently, 0.5 ml of LB broth was added to the tube, and the cells were incubated at 30°C with shaking for 45 minutes to allow recovery and expression of antibiotic resistance.
Meanwhile, LB agar plates containing ampicillin were prepared and divided into four equal sections, with proper labelling including the sample name and control. Finally, the transformed bacterial culture was plated onto the agar plates, which were then inverted and incubated at 37°C for 24 hours. The plates were observed the following day for the appearance of colonies, indicating successful transformation.

Screening and Biochemical Confirmation

The transformed cells were subjected to biochemical analysis, antimicrobial screening and a hydrogen peroxide catalase/peroxidase activity test was also conducted for expression. The successful presence of the peroxidase gene in the bacterial host was further ascertained by the formation of bubbles, which indicated enzymatic activity.

Catalase Test

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyses the decomposition of hydrogen peroxide to water and oxygen. The reaction of catalase in the decomposition of hydrogen peroxide is:



$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

The presence of catalase in a microbial or tissue sample can be tested by adding a volume of hydrogen peroxide and observing the reaction. The formation of bubbles, oxygen, indicates a positive result. This easy assay, which can be seen with the naked eye, without the aid of instruments, is possible because catalase has a very high specific activity. However, it is important to note that: *E. coli* naturally contains catalase activity.

RESULTS

Young potato tuber slices successfully produced actively growing buds within five days of incubation. These buds served as an efficient source of genomic DNA. CTAB-based DNA extraction yielded intact, high-quality DNA, as evidenced by clear and distinct bands on agarose gels without significant smearing.

PCR amplification using peroxidase-specific primers resulted in a distinct amplification product of the expected size, confirming the presence of the target gene in *S. tuberosum*. The amplified gene fragment was successfully

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ligated into the plasmid vector and transformed into *E. coli* DH5α cells

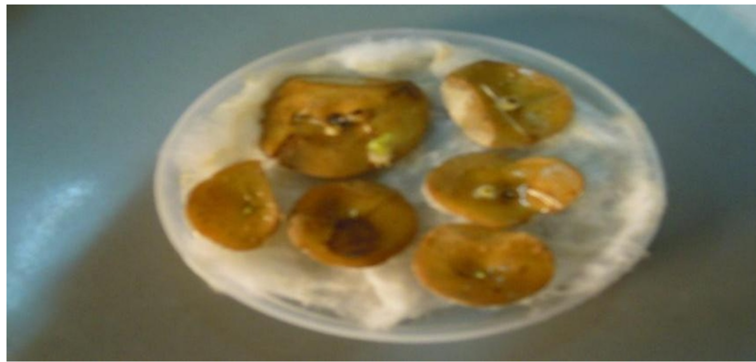

Fig 1.1 Potato slices having young buds
Pictures shows the growth of potato into young buds, these buds are used for peroxidase gene isolation

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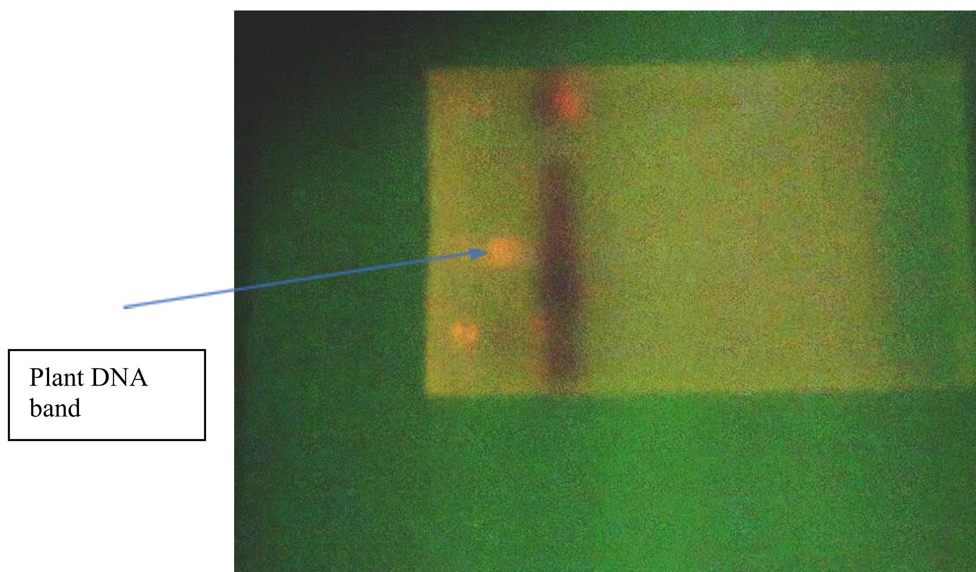

Fig 1.2: DNA of solanum tuberosum
Above picture shows Agarose gel electrophoresis of genomic DNA isolated from Solanum tuberosum showing intact high molecular weight DNA along with DNA ladder for size reference...

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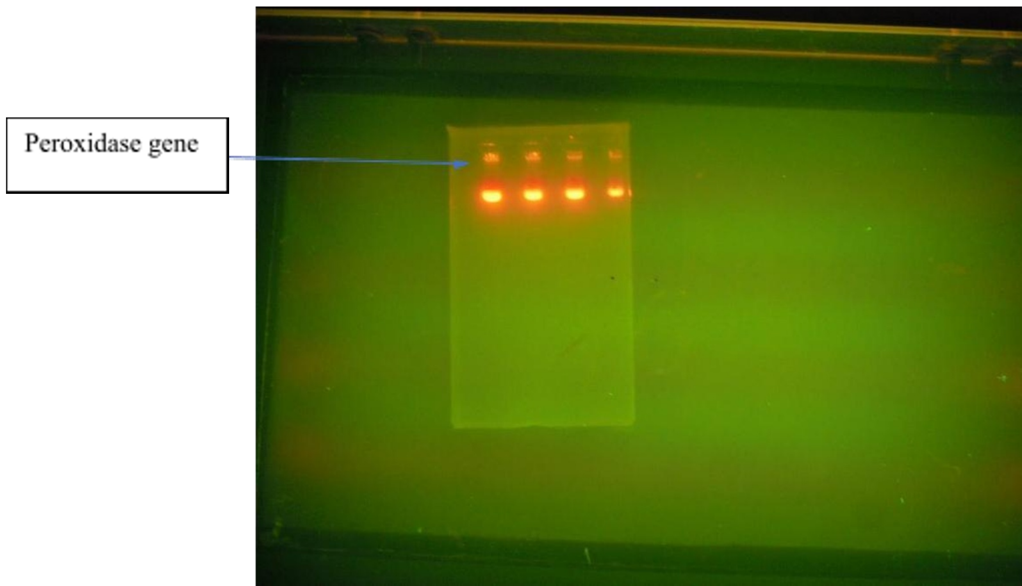

***Fig 1.3**: peroxidase PCR product*
This picture shows PCR amplification of class III peroxidase gene showing a distinct band at ~900 bp compared against DNA ladder.

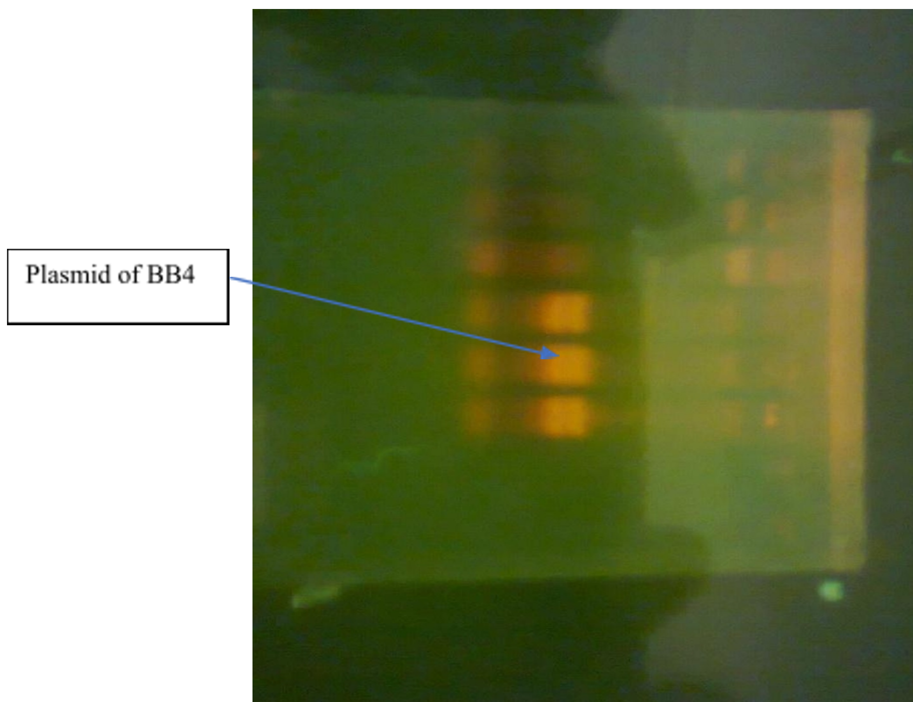

fig 1.4: bands of plasmid
fig 1.4 shows Agarose gel showing isolated plasmid DNA from E. coli BB4 strain.

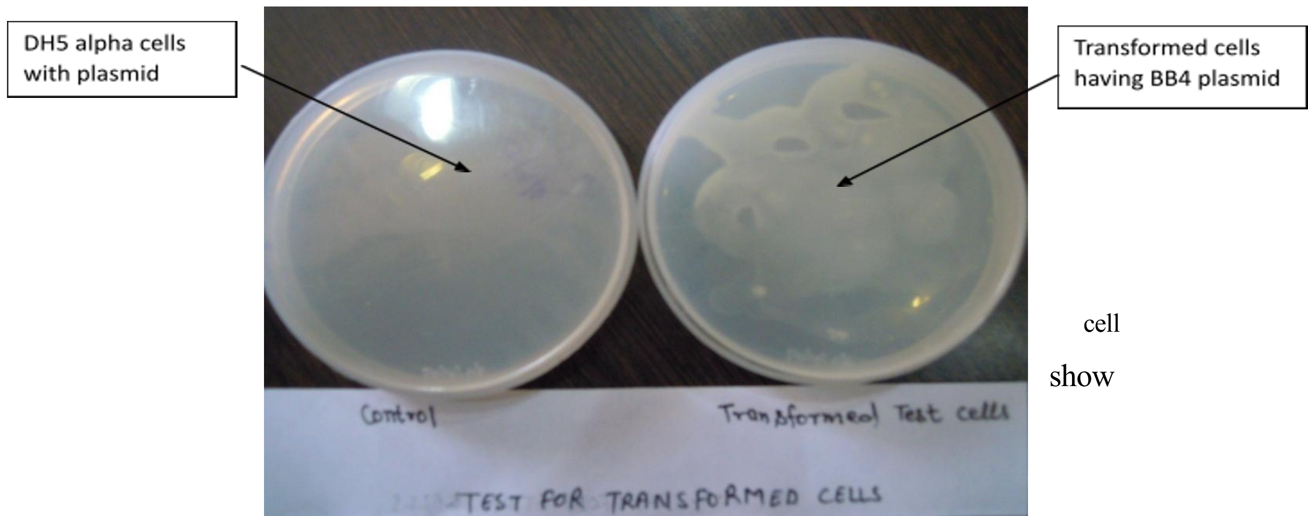

***Fig 1.5**: test for transformed cells*

Growth of transformed E. coli DH5α colonies on ampicillin-containing LB agar indicating plasmid uptake.
This figure shows the difference between normal and transformed DH5 alpha cells. Normal DH5 alpha cells are unable to show resistance against ampicillin. Hence, they cannot grow into the medium containing ampicillin whereas transformed cells have plasmid of BB4 cells, these plasmids have ampicillin resistant gene, so the cells can survive in medium containing ampicillin.

The growth of distinct colonies was observed on ampicillin-containing media clearly indicating stable plasmid uptake. Non-transformed control cells failed to grow, confirming the selection strategy. Biochemical screening further confirmed enzymatic activity, suggesting that the cloned peroxidase gene remained functional in the bacterial system.

Fig 1.6: formation of bubbles over the DH5 alpha cells during catalase test
fig 1.6 Bubble formation during H_2O_2 assay indicating enzymatic activity; however, results may include endogenous catalase activity of E. coli.

Discussion

The successful isolation and cloning of a class III peroxidase gene from *Solanum tuberosum* further support the ubiquity and importance of this enzyme family in plants. The choice of young sprouting buds proved to be particularly useful for genomic DNA isolation, as it is well known that actively dividing tissues are rich in high-quality nucleic acids suitable for molecular studies (Hiraga et al., 2001; Passardi et al., 2004). Class III peroxidases are well known to be involved in lignification, suberization, and oxidative burst responses during pathogen attack. The cloned gene in the current study is likely involved in PR-9-type defense responses, which are rapidly induced under biotic stress conditions. Peroxidase accumulation is associated with reduced disease dissemination and increased resistance, which agrees with similar induction patterns observed in other crop plants (van Loon et al., 2006; Almagro et al., 2009).

The observation of enzymatic activity in transformed *E. coli* suggests potential expression of the cloned gene; however, this conclusion should be interpreted cautiously. The presence of endogenous catalase activity in *E. coli* may contribute to the observed results.
Plant peroxidases have been successfully expressed in microbial systems in previous research, indicating their utility for biochemical characterization and the production of recombinant proteins (Yoshida et al., 2003; Mika & Lüthje, 2003).

Conclusion and Future Perspectives
The present study successfully demonstrates the isolation, amplification, cloning, and maintenance of a putative class III peroxidase gene from *Solanum tuberosum* in a bacterial system. While transformation and preliminary screening indicate successful cloning, further validation through sequencing and expression analysis is necessary to confirm gene identity and functionality. This work provides a foundation for future molecular and functional characterization studies aimed at improving plant stress resistance.

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