Faculty of Resource Science and Technology

Fungi Transformation Methodologies and Cloning of Sago Palm chitinase 2 cDNA

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Fungi Transformation Methodologies and Cloning of Sago Palm chitinase 2 cDNA

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Programme of Resource Biotechnology
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Fungi Transformation Methodologies and Cloning of Sago Palm chitinase 2 cDNA

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ABSTRACT

The sago palm (*Metroxylon sagu*) has become one of the most prevalence source of starch especially in the region of Sarawak, Malaysia. However, fungal infection has become a prevalent problem due to the intensive cultivation in *M.sagu* and crop plants. Fungal infections will eventually lead to wilting of roots which causes crop organs to be damaged and biotic stress in the crop plants will be stimulated. From *M.sagu*, the enzyme chitinase can be extracted where the chitinase are able to breakdown the chitin structure in that made up the wall of fungus. In this research specifically, the chitinase have already been isolated and this research will focus on the cloning of chitinase and genetic transformation in Fungi. The previously cloned samples were revived in both LB Broth and LB Agar with added chloramphenicol, CAM for expression in fungi. Colony PCR were performed to ensure the right colony was revived containing pGSA + chi2. Fungi was chosen for the genetic transformation of chi2 as the plasmid carrying chi2 has a larger size (9.3 kb).

Key words: *Metroxylon sagu*, Fungal infection, Chitinase enzyme, Fungi, Genetic transformation

ABSTRAK

Pokok sagu (*Metroxylon sagu*) merupakan salah satu sumber kanji yang paling lazim terutamanya di wilayah Sarawak, Malaysia. Walaubagaimanpun, jangkitan kulat telah menjadi masalah yang serius industri penanaman pokok sawit sagu dan tanaman-tanaman lain disebabkan oleh penanaman intensif. Jangkitan kulat boleh menyebabkan kelayuan pada akar dan seterusnya memberi impak kerosakan pada organ tanaman, sekaligus merangsangkan tekanan biotik dalam tanaman. Enzim kitinase daripada *M.sagu*, dimana kitinase mempunyai kemampuan untuk memecahkan struktur kitin yang membentuk dinding kulat. Dalam kajian ini secara khususnya, kitinase telahpun diasingkan. Sampel yang diklon sebelum ini telah dihidupkan semula dalam kedua-dua LB Broth dan LB Agar dengan tambahan kloramfenikol, CAM untuk ekspresi dalam kulat. PCR koloni dilakukan untuk memastikan koloni yang betul dihidupkan semula yang mengandungi pGSA chi2. Kulat dipilih untuk transformasi genetik chi2 kerana plasmid yang membawa chi2 mempunyai saiz yang besar (9.3 kb).

Kata kunci: *Metroxylan sagu*, Jangkitan Kulat, Enzim kitinase, Kulat, Transformasi genetik

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LIST OF ABBREVIATIONS

AGE	Agarose Gel Electrophoresis
AMC	Acidic Mammalian Chitinase
AMT	Agrobacterium-mediated Transformation
$CaCl_2$	Calcium chloride
CAM	Chloramphenicol
Cl	Chloride
C	Chitinase
cDNA	Complementary Deoxyribonucleic Acid
CLPs	Chitinase-like-proteins
CTAB	Cetyl trimethylammonium bromide
ddH_2O	Double Distilled Water
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide
LB	Luria Bertani
MsChi2	Chitinase II
NaCl	Sodium Chloride
PCl	Potassium Chloride
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
RNA	Ribonucleic Acid
RT-PCR	Real Time Polymerase Chain Reaction
R.E	Restriction Enzyme

rpm	Rotation per minute
SDS	Sodium Dodecyl Sulphate
SEC	Secretion Translocase
Spp.	Species
vir	Virulence

CHAPTER 1

INTRODUCTION

Commonly known as sago palm, *Metroxylon sagu* are from the group Lepidocaryoid which is the subfamily of Arecaceae (palmae). The *M.sagu* are known to be an important plant in Sarawak as it contributes greatly to the economy of Sarawak. To add, Malaysia and Indonesia are the world's two largest commercial sago producers. Before the 1980s, sago was mostly manufactured in small family-owned enterprises in Malaysia. Along the Mukah and Dalat Rivers, there were about 40 such sago factories. In Malaysia, M.sagu is economically relevant as trunks of *M.sagu* are used to acquire starch that are marketed for human consumptions. Due to its superior high starch yield when compared to commonly found starch sources such as corn, rice, and wheat, the *M.sagu* are also called the palm of many uses here in Malaysia (Lim et al., 2019).

Genetic transformation is known to be essential process for the expansion of gene transfer and large-scale production of protein. In years of research many organisms has been made into model organism to express gene and to improve productivities in industries such as pharmaceuticals, food production and industrial enzyme. The abundance of genetic diversity that are possess by fungi has made it possible to harness them in innovative ways, both as a source of novel genes and as expression hosts. It has been estimated that the global market for industrial enzymes is worth more than $1.6 billion, and that over half of these enzymes are produced through the fermentation of filamentous fungi species. Fungal transformation can be achieved through biological ways and also physical mechanisms. For example, through Agrobacterium-mediated transformation, AMT or protoplast transformation.

Biopesticide are important for industrial applications as problems arises in the crop industries since fungal like diseases such as Rhizoctonia rots may occur. Fungal infections will eventually lead to wilting of roots which causes crop organs to be damaged and biotic stress in the crop plants will be stimulated. Crop plants are also susceptible to fungal infection outbreaks, and this will impact in reduction of crop yield globally and cause quality of crop products to decrease. With the decreasing crop products quality, food safety can be at risk and consuming crops that are contaminated with fungal leads to mycotoxicosis.

In accordance with the observation, Collinge et al. (1993) stated in their article that as a response towards pathogenic microorganisms such as fungus, plants respond by inducing the expression of large number of genes that encodes diversify proteins which are known to be the defence mechanisms. Among those induced protein, chitinase are mainly functional as a defence mechanism for the fungal pathogen infections. This is attributable as the major component of cell wall from many fungi are chitin and chitinase functions by catalysing the hydrolysis of β1,4-linkages of N-acetyl-D-glucosamine, a polymer of chitin (Roslan & Anji, 2011). Hence, destroying the fungi by this action.

In the current study, the objectives is to carry out isolation and retrieve the XL1- Blue and EHA105 host that contains the MsChi2 that was previously cloned and to determine the usage of fungi as a whole as a model organism. AGE, Agarose Gel Electrophoresis will be utilised. That way innovation of chitinase as a biopesticide to fight the fungal infections in plants will be possible which provides an alternative in using the chemical pesticides. Besides, through this paper a review of the usage of fungi in the molecular system as a model organism will be more defined.

CHAPTER 2

LITERATURE REVIEW

2.1 Genetic transformation

Integrating exogenous DNA process into the germline of whole organisms so that it is passed down to subsequent generations is known as genetic transformation (Handler, 2008; Kidwell, 2005; Atkinson et al., 2002). Handler (2008) wrote in his research that this expression can be affected by the presence of extrachromosomal, or episomal, genes in nuclei, which may persist if the introduced DNA has a replication mechanism. Extrachromosomal expression, on the other hand, is frequently transient as DNA is diluted during cell division. Foreign DNA can also be integrated into somatic chromosomes, that can persist throughout the lifespan of the organism but are not inherited. The primary objective of these efforts is for the production of stable manipulation in the target organism's phenotype that is utilised to fill in the knowledge gaps about the physiological, genetic, and other effects of the integrated DNA (Atkinson et al., 2002).

Kidwell (2005) had stated that transferring DNA sequences into eukaryotic species typically requires the use of a bacterium, virus, or transposable element as a vector. As an example, the *Agrobacterium tumefaciens* which is a bacterial pathogen, is used heavily in genetic transformation of plant as a powerful tool for delivering genes of interest into a host plant. Inside the nucleus, the transferred DNA can integrate into the plant genome and be handed down to the next generation (i.e.stable transformation) (Hwang et al., 2017; Cheng et al., 2004). Atkinson et al. (2002) then also stated that in many insect species, genetic transformation is becoming more common. In the 1980s, genetic transformation of the

vinegar fly, Drosophila melanogaster, was increasingly used; the convenience of the methods, coupled with the enormous number of Drosophila mutant stocks and cloned genes, has greatly improved genetic investigation of this insect.

Genetic transformations are also well versed in the fungi kingdom. Molds, which are filamentous fungi that grow well and rapidly on cheap and non complicated medium, are the favoured cell factories due to their extraordinary capacity for recombinant proteins synthesis and secretion with post-translation modification (Li et al., 2017). Some species play critical roles in fields as diverse as medicine, agriculture, and industry.

2.2 Fungi as a model organism in genetic transformation

Saccharomyces cerevisiae cell wall was broken down by an enzymatic treatment, which was referred to as the first trial for fungal genetic transformation (Rivera et al., 2014). Other research groups were unable to duplicate the techniques described in the paper until 1981, when a first report on intact yeast cell transformation utilising auxotrophic markers and Escherichia coli shuttle vectors was published (Rivera et al., 2014). Filamentous fungi on the other hand, are commonly utilised as cell factories in biotechnology to produce chemicals, drugs, and enzymes. Genetic engineering techniques can be effective in helping companies increase their productivity. For industrially relevant filamentous fungi, various transformation techniques as well as DNA- and RNA-based methods for rationally designing metabolic fluxes have been developed (Meyer, 2007).

Fungi play an important role in many degrading processes in nature. Many fungus species are employed in agriculture to control plant pests and illnesses. Fungi are used in medicine to create antibiotics that are used to cure illnesses. Penicillin, for example, is a product of *Penicillium chrysogenum*, whereas cephalosporin is a product of *Cephalosporium*

acremonium (Li et al., 2017). Their exceptionally high capacity to secrete and express proteins becomes the reason why filamentous fungi are indispensable to produce enzymes of fungi origin or non-fungi origin (Punt et al., 2002). Punt et al. (2002) also described that *Aspergillus niger, A. oryzae* and *Trichoderma reesei* are among the fungi that are used for native and recombinant enzymes production currently.

Filamentous fungus are also useful model organisms for studying fundamental cellular and molecular processes. DNA transformation technologies that permitted molecular engineering approaches to further improve these production processes required to be established in order to produce filamentous fungi as hosts for enzyme and metabolite production. Several novel transformation strategies for filamentous fungi, including as CaCl2/PEG-mediated transformation, lithium-acetate therapy, particle bombardment, and electroporation, have been described because of the need over the years (Michielese et al., 2008). Years before, transformation of foreign DNA into filamentous fungi using *A. tumefaciens* have also been described and have been expanding ever since (Hookays et al. 2018). In the paper written by Magana-Ortiz et al. (2013) also discussed about employing shock waves in order to genetically transform fungi.

In 2001, Fusarium *spp.*, *F. circinatum* and *F. oxysporum*, were transformed, giving novel vectors for fungal transformation as well as a plasmid rescue cassette for retrieving DNA sequences emulating the T-DNA insertion in the fungal genome (Idnurm et al., 2017). The paper reviewed by Idnurm et al. (2017) observed an improved transformation efficiencies in the AMT methods compared to the protoplast methods by 300–500 transformants per 106 conidia, paving the groundwork for more advanced Fusarium genetic methods (Mullins et al., 2001). When it comes to large-scale random mutagenesis approaches in *F.oxysporum*, AMT would be the method chosen as well. Mutagenesis studies

that was first recorded with mutants generated in the range of 182-1129 by utilising the REMI and transposon tagging (Idnurm et al., 2017).

Aspergillus niger are recognised as the most significant microbes in biotechnology as they are wide distributed in foods, soil and plant products. Generally regarded as safe (GRAS) status and having capacity of high secretion, *A.niger* have been long utilised in the large number productions of enzyme and organic acids (Li et al., 2013). *A.niger* is also commonly used for industrial scale productions which in time makes it the most prominent transformation host in overexpression of foreign proteins. Although some studies have stated (Park, 2001) that *A.niger* has a lower transformation efficiency due to high concentrations of antibiotic selection, Li et al. (2013), has documented that with an optimised methods the transformation efficiency was accomplished (83 transformants per 10^7 spores). In recent years, *A.niger* has also been utilised to generate cellulase in an effort to efficiently decompose low-cost cellulosic resources into glucose (Li et al., 2017).

Park (2001) discussed in his paper that improvement of AMT with *A.niger* was made by constructing binary vector pBIN19Hg with integration of coding region from bacterial hygromycin B phosphotransferase gene (hgh) in the control of *A. nidulans* trpC promoter as a selectable marker. In this experiment, acetosyringone (AS) was added as a chemical that induces the expression of virulence genes in *A. tumefaciens*. As a result, hygromycin-resistant colonies were produced by co-cultivating A. niger asexual conidia with A. tumefaciens cells expressing pBIN19Hg. There were no transformants found in the absence of AS during co-cultivation. Hence, in Park (2001)'s paper it can be concluded that AS is essential in order for *vir* gene to be transferred. Only when *vir* gene is transferred, foreign gene can be transformed into fungi and transformants can be produced (Groot et al., 1998; Park, 2001).

Sayari et al. (2019) also developed an AMT system for the fungus, *Ceratocystis albifundus*. Their studies was the first successful documented transformation of *Ceratocystis* species using *A. tumefaciens*. An approach established for one genus does not work for other genera in the same family, which is often the case with fungus (Michielse et al., 2008). With the system established by Sayari et al. (2019), multiple *C. albifundus* isolates had single copies of the corresponding antibiotic resistance and GFP expressing genes inserted into their genomes, coupled to the Aspergillus gpdA or trpC promoters. The system utilised in this experiment is reliable and efficient, producing up to 400 transformants per 106 conidia. The morphological results shows different forms as to compared with the wild type. Thus, proving that transformation took place. Changes in morphological features of transformed fungi are a common phenomenon caused by random integration of T-DNA into the genome as according to Lu et al. (2017).

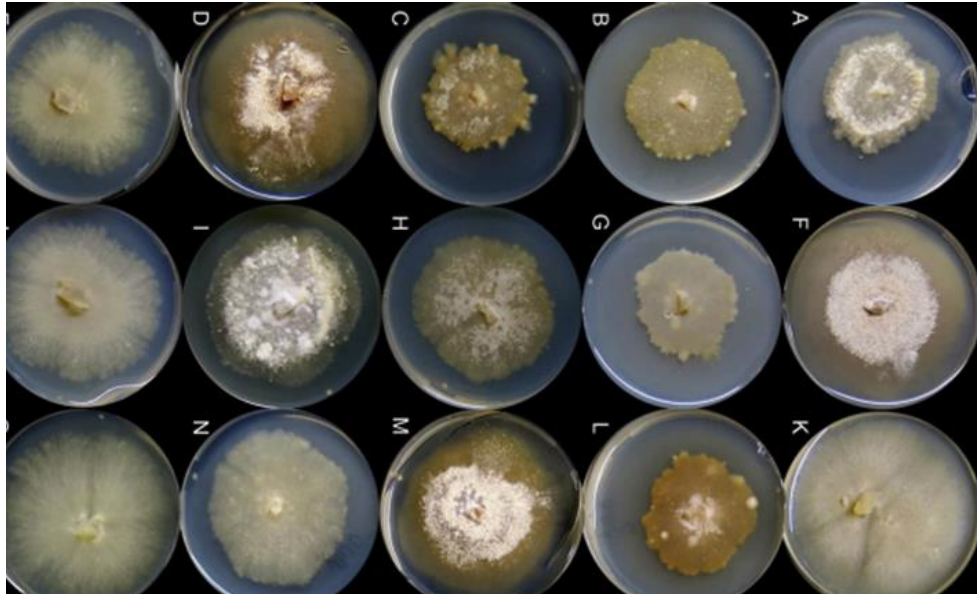

Figure 2.1 Differences between wild types and transformants of Ceratocystis albifundus cultures where the A, F, K (Wild types) and B–E, G–J, L–O (Transformants) (Adapted from Sayari et al., 2019).

2.2.1 Advantages of fungi as model organism

Fungi are known to be important in the agrobiotech industry, clinical pathology and plant pathology. Due to their complex mode of existence and cellular assembly as compared to bacteria, it enables the fungi to be extrapolated to both prokaryotes and higher eukaryotes. Genetic studies are also easier conducted since the genome of fungi is small and tractable (Mohanan & Sahni, 2018). By utilising fungi as model organism, single copy integration were also detected. As stated by Meyer (2007), it is interesting to note that when AMT was employed for the introduction of DNA into filamentous fungi like *A. awamori*, *A. giganteus*, *Calonectria morganii* and *F. oxysporum*. The majority of the integration events that occurred were of the single-copy variety. Remarkably, it has also been demonstrated that AMT is able to increase the frequency of homologous recombination (HR) in *A. awamori* (Michielse et al., 2005).

In order to better understand the functions of specific genes or incorporate new genetic elements into the genome of a strain, scientists can use this technology to precisely target and modify genes. (Martin, 2015). Enzymes that are exuded into the culture medium by the fungi provides a distinct benefit in creating potentially hazardous proteins that accumulates inside the cells. Secretion is particularly advantageous for product purification since it eliminates the need to break down cells and remove all internal proteins, which is a time-consuming and costly operation (Sharma et al., 2009). Hooykaas et al. (2018) also exposed that *Saccharomyces cerevisiae* has a relatively simple genome and has a decline in genetic redundancy which makes it easy to be used for basic molecular processes (eukaryotic cells). This yeast can also elucidate the role of virulence factors in the pathogenesis of pathogens that have effects on plants or animals (Hooykaas et al., 2018).

Yeast cells are transparent and do not contain substantial amounts of endogenous luminous chemicals like chlorophyll, making them ideal for microscopy. These characteristics facilitated the construction of a system based on split GFP to visualise virulence protein translocation into host cells in real time, the results of which could then be employed in plants (Hooykaas et al., 2018). In other words, by developing the transformation of fungi other eukaryotic organisms can also benefit from the expansion of fungal transformation. The availability of the whole genome sequences of a rising number of yeast and fungal species permits the widespread application of contemporary system biology techniques. The idea that fungi are more closely linked to mammals than to plants accentuates the usefulness of these organisms as models for human cells (Van der Klei & Veenhuis, 2006). Utilising fungi can also mean that varying type of tissue such as conidia, germinated conidia and mycelium, may be utilised in certain species of fungi like yeasts (Rivera et al., 2014).

2.2.2 Transformation methodology for fungi

There are two sets of genetic transformation techniques for fungi which are biological and physical. Biological techniques rely on Agrobacterium tumefaciens-mediated transformation, AMT and protoplast transformation with different cell-wall degrading enzymes. Physical genetic transformation technology methods for fungi includes electroporation, biolistic, glass bead agitation, vacuum infiltration, and shock waves. These physical methods have considerably improved capabilities and permitted the construction of genetically altered strains of various fungus (Rivera et al., 2014). AMT has been established as a significant instrument for both random and targeted gene disruption, and it's becoming the system of choice for many fungi (Weld, 2016).

Protoplast transfomation procedure are used for preparation of cells in genetic manipulation of fungi. Presently, protoplast preparation involves the removal of fungal cell wall, that is done primarily using the enzymes (Rodriguez-Iglesias & Schmoll, 2014). Physical procedures such as grinding and supersonic wave shock have also been reported as non-enzyme methods for preparing protoplasts. Besides that, reagents such as PEG are also used in promoting fusion of exogenous nucleic acid and protoplasts. A downside to using this method is that protoplasts can be highly sensitive (Li et al., 2017). Thus, a stable osmotic pressure must be maintained, so that protoplasts will be intact throughout the enzymolysis of cell walls. This can be achieved by using osmotic stabilisers such as sodium, potassium chloride and sorbitol added into all the buffers used in the retrieving the protoplasts. That way the cells will not be ruptured (Li et al., 2017). Nevertheless, Rehman et al. (2016), stated in that protoplasts have been isolated with varying degrees of success from diverse fungal species, depending on the species and environment.

In addition to the digesting enzyme and other parameters, the age of the mycelia influences protoplast isolation efficiency. Mycelia that are fresh and proliferating rapidly are more ideal for protoplast isolation. However, it can vary depending on the species of fungi (Rehman et al., 2016). To ensure good recovery of viable protoplasts, Li et al. (2017) indicated that protoplasts are allowed to recover on a plate with no selection pressure for a defined period of time before being transferred to a selective plate. An osmotic stabiliser should be included in the regeneration culture. The ability of protoplasts to renew cell walls is dependent on maintaining constant osmotic pressure and on the selective medium, which can only generate protoplasts carrying exogenous nucleic acids (Li et al., 2017; Rehman et al., 2016; Rodriguez-Iglesias & Schmoll, 2014). A different way of regenerating transformants can also be done. Transformed protoplasts are frequently transferred straight to media containing an auxotrophic marker or a drug, depending on the selectable marker