1.0 Introduction

1.1 Literature review

Orchidaceae, or orchids as they are commonly known, belong to the largest family of angiosperms. Of the over 25 000 species found worldwide, 854 species are said to be indigenous to Peninsular Malaysia (Seidenfaden and Wood, 1992) and over 1400 species to Borneo (Chan *et al.*, 1994).

Orchid flowers come in many different arrays of colors, patterns and sizes and are regarded as the most highly diverse and evolved plant family in the world. The structure of orchid flowers give them their character, a typical orchid flower has an outer whorl of three sepals and an inner whorl of three petals that look like any ordinary flower however one thing that sets orchids apart is that all orchids have their middle petal transformed into a unique structure called the labellum/lip and this petal is always quite different from the other two petals (Dressler, 1981). These herbaceous plants are highly adaptable and inhabit most surfaces and environments in the wild and this diversity gives orchids an edge over other plants making them more attractive for cut flowers, ornamental purposes as well as other commercial uses in medicine, arts, crafts and cosmetics (Arditti, 1992).

The orchid industry is among the most important sectors in Malaysia, contributing a total floriculture export worth of RM 22 million in 1997 and increased to RM104 million in the year 2010 with still an increasing opportunity to penetrate new markets and expand market shares (Kementerian Pertanian & Industri Asas Tani Malaysia, 2010). These figures demonstrate the importance of the floral industry to our

economy hence it is important for us to protect our local orchid industry by producing high quality orchids that are free from disease.

One of the major viruses of concern to the orchid industry is Cymbidium mosaic virus (CymMV) which is among the most prevalent and economically important viruses infecting orchids globally (Wong *et al.*, 1994; Ryu and Park, 1995; Khenry *et al*., 2006). The virus is extremely stable and is generally restricted to the orchid species where it is found in high concentrations. (Jensen, 1950). CymMV infected orchids often show smaller flower size, are of poor quality, show necrosis and flower deformity as well as foliage symptoms and reducing plant vigor thus resulting in huge economic losses (Seoh *et al.,* 1998; Inouye, 2008). A number of orchid species is believed to have fallen to a crtical level and thus conserving these orchids has becoming an important issue in recent decades (Kull *et al*., 2006).

1.2 Cymbidium Mosaic Virus (CymMV)

CymMV is an RNA virus belonging to the group of flexuous rod-shaped potexviruses. CymMV was first described by Jensen in 1951. The genomic nucleic acid of CymMV which was first isolated by Frowd and Tremaine in 1997 had the base composition of 21.1% G; 28.9% A; 24.4% C and 25.6% U. It is a single stranded RNA-containing virus with flexuous non-enveloped filamentous virions, which are approximately 475–490 nm in length (Steinhart and Oshiro 1990) and 13 nm wide. The axial canal is obscure with an obvious basic helix. The pitch of the basic helix is 2.8 nm. CymMV comprises of about 6–7 kb of positive-sense genomic RNA that is capped and polyadenylated (Francki *et al.,* 1985; Srifah *et al.,* 1996). The protein subunits of 257 amino acid residues have a molecular weight of 27,600 daltons. There is only one

sedimenting component in purified CymMV preparations with the sedimentation coefficient at 121 S. The virions contain 5.6% nucleic acid; 94% protein and no lipid. Figure 1.1 shows an electron mircograph of partially purified CymMV at 81 000 times magnification (Sherpa *et al.*, 2003).

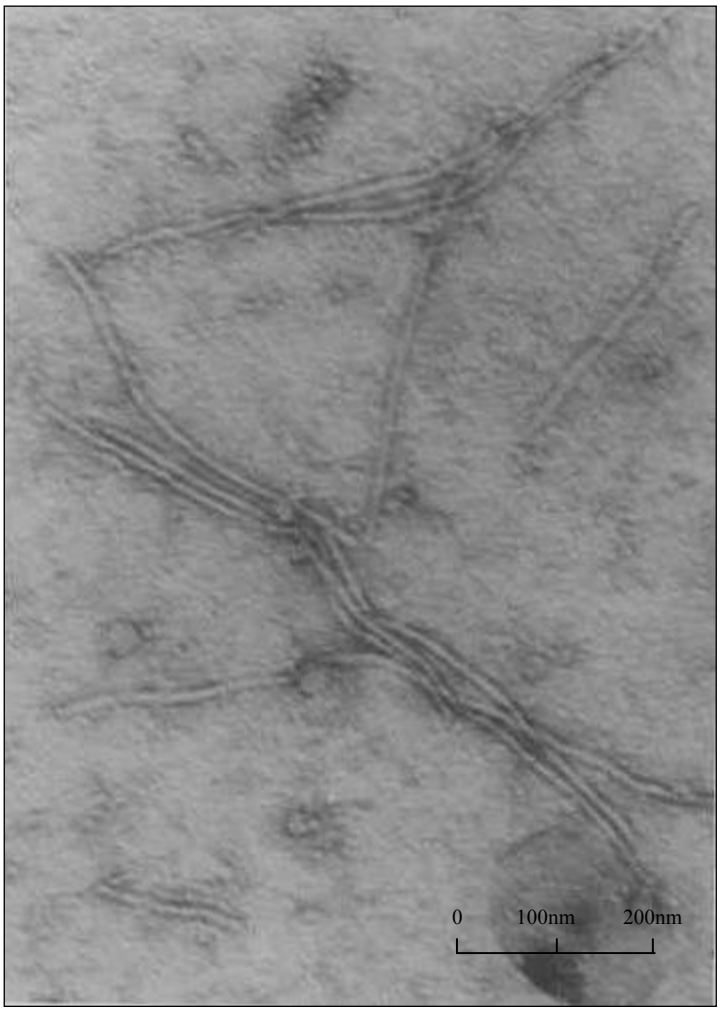


Figure 1. 1: Electron micrograph of partially purified CymMV at 81 000 times magnification (Sherpa *et al.*, 2003)

The complete nucleotide sequence of the genomic RNA of CymMV was determined to be 6227 nucleotides in length excluding the poly (A) tail at the 3' terminus as shown in Figure 1.2 (Wong *et al*., 1997). Similar to other potexviruses, its genome organization is comprised of five major open reading frames (ORFs 1 to 5); with the first ORF encoding a *M*r 160KDa putative RNA-dependent RNA polymerase (RdRp), the second to fourth ORFs are a *M*r 26 KDa, 13 KDa and 10KDa respectively called the triple-gene-block (TGB). Whereas, the fifth ORF is a *M*r 24KDa, a coat protein which is located at the 3′ end (Chia *et al.,* 1992). All ORFs of potexviruses encode proteins containing consensus motifs except ORF4 (Wong *et al.*, 1997).

Figure 1.2 Reading frames of the CymMV genomic RNA. (National Center for Biotechnology Information)

CymMV is a stable and highly infectious virus. The virus is transmitted either by mechanical inoculation or by contact between plants but not by a vector. It was first reported in Cymbidium species from California, USA by Jensen in 1950. The symptoms caused by this virus vary depending on the orchid species, the strain of the virus as well as environmental factors as CymMV systematically infects all parts of the orchid plant including leaves, flowers, pollinia, roots and epidermal cells (Kado and Jensen, 1964; Lawson and Hearon, 1974). There are however some symptoms that are frequently associated with CymMV where when expressed, foliar symptoms include chlorotic mosaic and necrosis and floral symptoms such as necrotic flecks, color break and flower distortion (Lawson, 1995; Okemura *et al*., Kamemoto and Ishii, 1984; Yuen *et al*,. 1979).

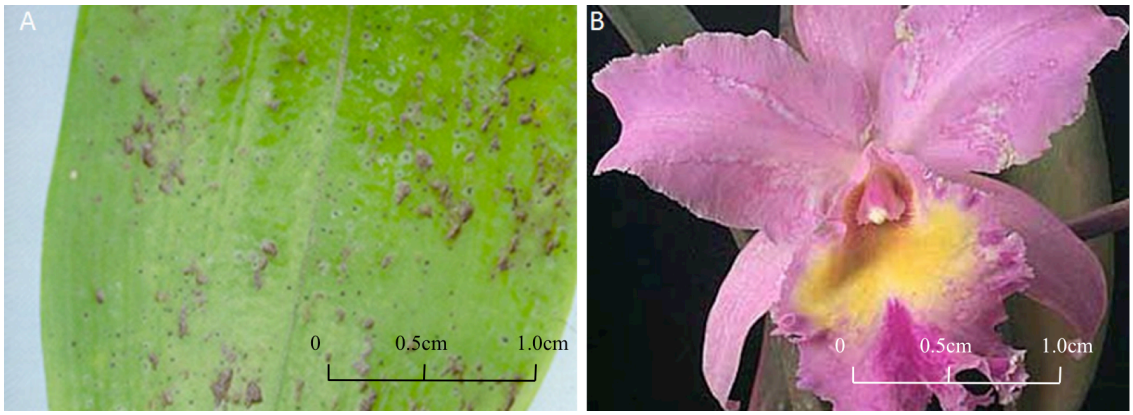


Figure 1.3 Orchid showing CymMV symptoms; (A) necrotic leaf and (B) flower color breaking (Pictures courtesy of Nan-Jing Ko reproduced from http://www.freshfromflorida.com/pi/enpp/pathology/florida_viruses/Orchidacae/Orchidvirus.html and American Orchid Society).

1.3 Detection methods for plant viruses

Viruses are among the smallest organisms that can cause plant diseases and are constantly infecting plants. Viral diseases in plants especially orchids caused a range of symptoms affecting orchid quality and thus place a significant economic drain on the local orchid industry (Wong *et al*., 1994). Unlike other pests and diseases, which can be controlled with agrochemicals, once plants are infected with viruses, there is no cure except by tissue culture. In tissue culture, the material of the plant can still be rescued however it can be quite costly in terms of time and money (Kaper and Waterworth, 1981; Arditti, 1992; Sutic *et al*., 1999). Also, since plants may be asymptomatic and viruses are easily transmitted it is very important that the infected plants to be detected as early as possible in order to prevent the infection from further spreading. Hence a good detection method for important orchid pathogens such as cymbidium mosaic potexvirus (CymMV) is of high importance to the orchid industry.

Several techniques are available for plant virus detection, each with its own pros and cons namely; serological techniques, electron microscopy as well as nucleic acid procedures. Serological techniques are based on the utilization of the nature of antibodies, which bind specifically to their antigens (Gibbs and Harisson, 1976). These include enzyme-linked immunosorbent assay (ELISA), tissue blot immunoassay (TIBA) and quartz crystal microbalance (QCM) immunosensors (Webster *et al*., 2004). ELISA is based on antigen-antibody interaction where it exploits the ability of antibodies to identify proteins such as coat protein of the virus of interest. One of the advantages of ELISA is that either multiple plants or a single plant can be tested concurrently on a single plate. This can be done by coating each well with different type of antibodies thus allowing simultaneous detection of different antibodies and or

antigens with different antibodies and or antigens (Webster *et al*., 2004). As for TIBA; labeled probe is used to detect viruses from plat tissue sap that was expressed onto blotting paper, nitrocellulose or nylon membranes (Webster *et al.,* 2004).). QCM on the other hand provides a novel technique for plant virus detection. This was done by using quartz crystal disk coated with virus specific antibodies that yielded results in both quantitative and qualitative mode. Voltage applied across the disk causes the adsorption of virus particles to the crystal surface (Webster *et al*., 2004).

Electron microscopy (Matthews, 1991) is based on the electron beam passing from an electrode through the specimen to the anode where an image of the specimen will be produced when the electrons are blocked by the specimen. Viruses can be identified by their distinct morphological characteristics and sizes.

Nucleic acid procedures include reverse transcription–polymerase chain reaction (RT–PCR) and PCR, restriction fragment length polymorphism (RFLP), nucleic acid hybridization labeled probes, arrays and probes for microarrays (Webster *et al*., 2004). RT–PCR and PCR basically involves a repeated series of cycles of DNA amplification that comprises of template denaturation, primer annealing and the extension of the bound primers by Taq DNA polymerase using sequence specific primers (Ryu and Park, 1995; Seoh *et al*., 1998). Both of these methods are extremely sensitive procedures but both methods require a thermocycler, which can be expensive, and prior knowledge on the sequence for design of primers (Wylie *et al*., 1993). RFLP are often referred to as a single nucleotide polymorphisms is a procedure used in combination with PCR to identify differences between viruses based on the presence or absence of restriction enzyme-recognition sites (Hu and Wong, 1998) it allows differentiation of

varying isolates of viruses without the expense of cloning and sequencing (Webster *et al*., 2004). Nucleic acid hybridization (Hu and Wong, 1998; Tijssen, 1993) is based on the annealing of a sequence specific complementary single-stranded DNA to a double stranded DNA or RNA followed by appropriate detection methods by labeled probe (Webster *et al*., 2004). More recently, both macro and microarrays are coming more popular (Webster *et al*., 2004). Arrays printed with probes corresponding to a large number of virus species can be utilized to simultaneously detect all those viruses within the tissue of an infected host. The position of a visible spot corresponds to the presence of a particular virus in the plant sample (Webster *et al*., 2004).

1.4 Pathogen Derived Resistance

Genetic engineering is proving to be highly effective for controlling virus diseases in a wide range of crops grown worldwide as it offers the most advanced applications of biotechnology for the management of plant pathogens (Wilson, 1993; Gonsalves and Slightom, 1992, James, 2009). Tremendous advances have taken place in understanding of plant-virus interaction in the process of pathogenesis and resistance. This, along with associated advances in the genetic transformation of a number of crop plants, have opened up the possibility of an entirely new approach of genetic engineering towards controlling plant virus diseases (Dasgupta *et al*., 2003).

Historically the idea of creating virus-resistant crops evolved from observation of infecting mild, symptomless or attenuated strains of viruses has protected field crops against virulent strains (Hamilton, 1980; Sanford and Johnston, 1985; and Powell Abel et al., 1986). Later in 1985, Sanford and Johnston proposed that the expression of part of a pathogen's genome in plant would render it resistant to that particular pathogen.

Based on this pathogen derived resistance (PDR) model by Sanford and Johnston, the first concept that referred to the expression of nucleotide sequence from plant viruses in transgenic plants being able to induce protection against disease was introduced (Lomonossoff, 1995). This strategy has been proven to be sucessful against plant viruses as shown by Powell-Abel et al., in 1986 that successfully produced tobacco mosaic virus (TMV) resistant tobacco plants through expression of the TMV coat protein gene as well as papaya ringspot virus (PRSV) in papaya (Gonsalves, 1998) and potato virus X (Baulcombe, 1999). Subsequently, there have been numerous attempts to generate virus-derived genes or genomes fragments (Beachy, 1993; Wilson, 1993; Baulcombe, 1994b; Lomonossoff, 1995). Status of research and applications of crop biotechnologies is shown in Table 1.1.

Table 1.1: Number of initiatives to develop GMOs with resistance to pathogens (reproduced from FAO, Corporate Document Repository)

	Africa					Asia					Eastern Europe					Latin America					Near East					Total				
	C	F	E	U	N	C	F	E	U	N	C	F	E	U	N	C	F	E	U	N	C	F	E	U	N	C	F	E	U	N
Virus	-	2	3	-	2	2	17	14	9	9	-	-	-	-	-	-	16	26	-	9	-	9	2	-	3	2	44	45	9	23
Bacteria	-	-	-	-	-	-	1	3	5	5	-	-	-	-	-	-	-	1	-	1	-	-	-	-	-	-	1	4	5	6
Fungi	-	1	1	-	1	1	1	6	-	5	-	-	3	-	1	-	6	16	-	8	-	-	-	-	-	-	8	26	-	15
Other	-	-	-	-	-	-	-	7	-	4	-	-	1	-	1	-	3	8	-	4	-	-	-	-	-	-	3	15	-	8
Total	-	3	4	-	3	3	19	35	14	9	-	-	4	-	1	-	25	51	-	9	-	9	2	-	3	2	56	96	14	25

C: number of GM variesties released as commercial varieties; F: number of GM varieties in field tirals; E: number of activities at experimental level (including laboratory of glasshouse activities);

U: number of GM varieties at unknown status; N: number of countries involved (for the total column of N, if more than one activity is being carried out by a given country, the country is only counted once)

In a number of crops, transgenic resistance to infective virus has been developed by introducing a sequence of the viral genome in the target crop by genetic transformation. Virus-resistant transgenics have been achieved by introducing either viral coat protein (CP) or replicase gene encoding sequence (Dasgupta *et al*., 2003). Resistance obtained by using CP is conventionally called coat-protein mediated protection (CPMP) whereas replicase-mediated resistance that have been pursued in several laboratories; the resistance that was shown to be due to an inherent plant response known as post-transcriptional gene silencing (PTGS). Other PDR approaches have also been developed including through movement protein, satellite RNA and defective-interfering viral nucleic acids.

The use of viral CP as a transgene for producing virus-resistant plants is one of the most spectacular successes achieved in plant biotechnology. The CP gene of tobacco mosaic virus (TMV) was used in the first demonstration of virus-derived resistance in transgenic plants (Powell-Abel *et al*., 1986). The resistance was manifested as delayed appearance of symptoms as well as a reduced titre of virus in the infected transgenic plants, as compare to controls. The resistance against TMV using TMV CP in tobacco was also reported to be effective against other tobamoviruses whose CP was closely related to that of TMV but not effective against viruses which were distantly related to TMV (Masuta *et al*., 1995). Transgenic potato, expressing the CP of patato virus X (PVX) also showed resistance against PVX (Hemenway *et al*., 1988). However, in marked contrast to TMV, this resistance was not broken down when PVX RNA was used as the inoculum, thus indicating several possible mechanisms of CPMP.

The stage of the viral life cycle at which the CPMP is effective has been shown to vary. In TMV, it is at the virus disassembly and in the long-distance transport stage (Wisniewski *et al*., 1990). In the case of alfalfa mosaic virus (AMV), it is only at the disassembly stage whereas in PVX, it is at multiple stages, including replication, cell-to-cell and systemic movement stages. In tospoviruses, the stage affected most by CPMP is believed to be replication. Although some of the mechanisms of CPMP are unclear, there is now enough evidence to suggest that CPMP results from the propensity of the transgenically expressed CP to be mutated such that there was an increase in inter-subunit interactions, the transgenic plant expressed higher levels of resistance (Bendahmane *et al.*, 1997; Lu *et al*., 1998). In the case of resistance to TMV, the transgenically expressed CP sub-units are believed to re-coat the nascent disassembled viral RNA which leads to a decreased pool of the available viral RNA for translation (Lu *et al.,* 1998; Beachy *et al.,* 1999), resulting in resistance.

Apart from the interactions involving viral proteins, some of the most interesting developments in the area of PDR are related to RNA effects and the most important phenomenon associated with RNA-mediated PDR is RNA silencing.

1.5 RNA Interference

RNA interference or RNAi is a highly evolutionary conserved mechanism of gene silencing in a sequence specific manner at the post –transcriptional level. Prior to the discovery of RNAi *Caenorhabditis elegans* by Andrew Fire and Craig Mello in 1998, an earlier observation by David Baulcombe's group on the similarity of viral defense and gene silencing in plants (Ratcliff *et al*., 1997) led to studies showing that small RNA were generated during post-transcriptional gene silencing (PTGS); as referred as RNAi in plants (Hamilton and Baulcombe, 1999). It was then recognized,

that the previously discovered microRNA in *C. elegans* (Fire and Mello, 1998) was of the same size. This small silencing RNA are likely common in plants and animals are indeed the mediators of RNAi. Baulcombe's research group subsequently demonstrated that while some viruses can induce gene silencing, other viruses are able to encode proteins that suppress gene silencing (Hamilton and Baulcombe, 1999). After these initial observations, many laboratories around the world searched for the occurrence of this phenomenon in other organisms.

Two distinct gene silencing phenomena are observed in plants: transcriptional gene silencing (TGS), which involves decreased RNA synthesis because promoter methylation (Cogoni and Macino, 2000; Wassengegger *et al*., 1994), and post-transcriptional gene silencing (PTGS), that involves sequence specific RNA degradation (Fire, 1999). As explained by Khraiwesh *et al*., 2011 in Figure 1.4, the double stranded RNA (dsRNA) induces gene silencing by targeting complementary mRNA for degradation. A RNase – III (Ribonuclease-III) family member; DCL cleaves the long dsRNA into 21-23 nucleotide of short pieces of small interfering RNA (siRNA). siRNAs processed by one of four DCLs proteins as well as dsRNA-binding proteins and HEN1-mediated siRNA stabilization guide AGO-containing RNA-Inducing Silencing Complex (RISC). The siRNA are then transported to the RISC (Yu *et al*., 2002); where the AGO proteins bind these small RNAs. Once unwound, the single-stranded antisense strand guides the RISC to mRNA that has a complementary sequence and subsequently effect endonucleolytic cleavage of target mRNA (gene silencing), translational repression of mRNAs, or DNA methylation (Dykxhoorn *et al*., 2003 and Voinnet, 2009). DCL proteins targeted the highly tructured or dsRNA of viruses to generate virus-derived small interference RNAs (vsiRNAs). These vsiRNAs can be incorporated into virus-induced RISC complexes that target viral RNAs, thus making RNA silencing

a doubly effective antiviral mechanism (Ding and Voinnet, 2007; Omarov *et al*., 2007). One important aspect of RNA silencing in plants is that it can be triggered locally and then spread via a mobile silencing signal (Vance and Vaucheret, 2001).

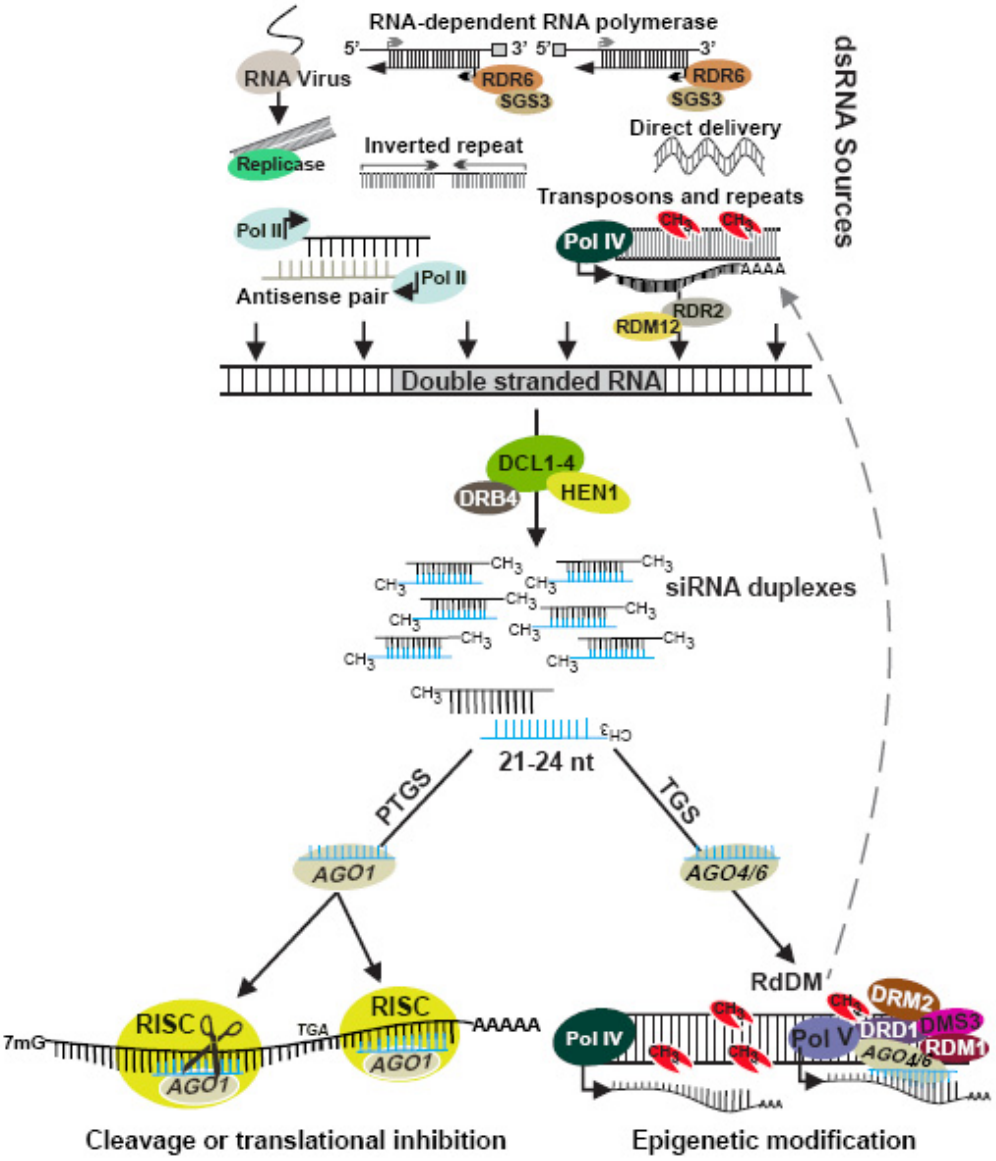


Figure 1.4 siRNA biogenesis (reproduced from Khraiwesh *et al.*, 2011).

RNA silencing machinery limits the transcript level either by suppressing transcription (transcriptional gene silencing, TGS) or by activating a sequence-specific RNA degradation process (Agrawal *et al*., 2003). RNA silencing in the animal kingdom is called RNA interference (RNAi), whereas in plants it was first known as either co-supression or post-transcriptional gene silencing (PTGS) (Agrawal *et al*., 2003; Vanitharani *et al*., 2003). Due to sequence-dependence, it gives a narrow range of resistance (Baulcombe, 1996b). Transgenic plants carrying a viral sequence show suppression of transgene mRNA accumulation. The transgenes can result in protection to the transgenic plants against any viral RNA having a sequence similarity to the transgene (English *et al.,* 1996).

To specifically silence or knock down the expression of targeted gene in plants several approaches of PTGS have been developed, one of which is virus induced gene silencing (VIGS). VIGS was being described as the resistance event against viral infection by Van Kammen in 1997 is one of the tools to suppress expression level of the gene of interest in plants (Van Kammen 1997, Dinesh-Kumar *et al*., 2003). Plants infected by many viruses induce RNA-mediated defense which targets viral RNAs and any transgene RNA products inserted into it (Voinnet, 2001). As this phenomenon is a homology dependent process, not only viral RNA but also the mRNA corresponding to the inserted plant sequence will be degraded. The virus was shown to spread and is silenced systemically due to the movement of a systemic silencing signal in the recovery phase (Mlotshwa *et al*., 2008, Padmanabhan and Dinesh-Kumar, 2008).

Short hairpin RNA (shRNA) is another useful tool for RNAi studies. DNA molecules are cloned into expression vectors to express siRNA. These shRNA has

special features such as they are short nucleotide sequence ranging from 19-29 nucleotides derived from the target gene, a short of 4-5 nucleotides to form a loop and another 19-29 nucleotide sequence that is the reverse complement of the initial target sequence. This loop or hairpin is used to silence gene expression via RNA interference. This happens as the hairpin is cleaved by the cellular machinery into siRNA, which will the bound to the RNA-induced silencing complex (RISC). This complex will later binds to and cleaves mRNA which match the siRNA that is bound to it (McIntyre and Fanning, 2006).

Similar work has been done by Chang *et al.* in year 2005 where they managed to generate CymMV virus-resistant Dendrobium varieties, with the transgenic *Dendrobium* contains the CymMV CP gene showed to express a very low level of virus accumulation four months post-inoculation with CymMV.

1.6 Plantibody

Antibodies or antibody fragment produced in transgenic plants are referred to as 'plantibodies'. Using molecular technique, it allows the interference with cellular metabolism or pathogen infectivity by the ectopic expression of genes encoding antibodies. The expression of complete or engineered single-chain Fv antibody is particularly suitable for expression in plants as demonstrated by the plantibody-mediated resistance using *Potato leafroll virus* (PLRV) (Nickle *et al.*, 2008) because of its small size, ease of manipulation and the lack of assembly requirements. This approach, which has also shown convincing results in the development of plant viral resistance involves expressing an antibody against the viral protein (coat protein) within the host plant (Whitelam and Cockburn, 1997). The utilization single-chain variable fragments (ScFv) antibodies in plants is in contrast to previous approaches that used parts of the viral genome to confer viral resistance in transgenic plants (Tavladoraki *et al*., 1993; Zimmermann *et al*., 1998). Two studies utilizing this approach showed expression of ScFv antibodies against artichoke mottles crinkle virus (AMCV) and tobacco mosaic virus (TMV) respectively, which resulted in tolerance or decreased sensitivity to the viral infection (Tavladoraki *et al*., 1993; Zimmermann *et al*., 1998).

Although several reports have proven the value of this tool in plant research such as for modulation of phytohormome activity and for blocking plant-pathogen infection (De Jaeger *et al.*, 2000), the exploitation of this strategy is limited due to lack of generality and perceived viral genes expression risk issues.

1.6.1 Phage Display

Over the past decade, various types of combinatorial methodologies for protein screening and selection have been developed. Among these, phage display represents the most preferred method of choice for screening either protein interaction or selecting antibodies for diagnostic and therapeutic purposes (Mersich *et al.,* 2008). Phage display is a molecular technique by which foreign proteins are expressed at the surface of the phage particles (Smith, 1985; Winter *et al.,* 1994; Kay and Hoess, 1996). Such phage thereby become vehicle for expression that not only carry within them the nucleotide sequence encoding expressed proteins, but also have the capacity to replicate and is becoming an extremely powerful tool for selecting peptides or proteins with specific binding properties from vast number of variants (Willats, 2002).

The expression of peptides, proteins or antibody on the surface of phage particles is accomplished by incorporation of the nucleotide sequence encoding the protein to be displayed into a phage or phagemid genome as fusion to a gene encoding a phage coat protein. As the fusion is to the coat protein, this ensures that the protein is present on the surface of the mature phage (Willats, 2002).

Generally there are two common types of libraries that can be constructed usingthe filamentous bacteriophage system. The libraries are peptide libraries where the surface of the phage particles are decorated with random-sequences of oligopeptide (Smith and Scott, 1995; Burritt *et al*., 1996) and antibodies libraries where the antibody is expressed at the surface of each individual phage (McCafferty *et al*., 1990; Clackson *et al*., 1991). Both types of libraries permit fast selection and identification of the interaction between the target and the expressed protein on the phage surface thus are

useful for the confirmation of structure and function of the molecular system. The peptide phage-display library is mainly used to obtain information about the peptide sequences that interact with a particular molecule whereas the antibody phage-display are mainly used for isolating antibodies against a particular target or antigen as well as being useful for development inhibitor, vaccine or diagnostic antibodies (McCafferty *et al*., 1990; Clackson *et al*., 1991).

To date, most popular application of this approach involves identification of novel allergens, potential biomarkers in autoimmune disorders (Waterman *et al*., 2010 and Somers *et al.,* 2011), cancer (Ran *et al*., 2008; Alsoe *et al*., 2008) and vaccine (Pereboeva *et al*., 2000) development. This is because not only phage-display is able to generate cDNA and ORF expression products on phage but it is also able to increase library quality, produced indepth analysis of the enrichment process as well as providing a faster, more convenient and reasonably priced system.

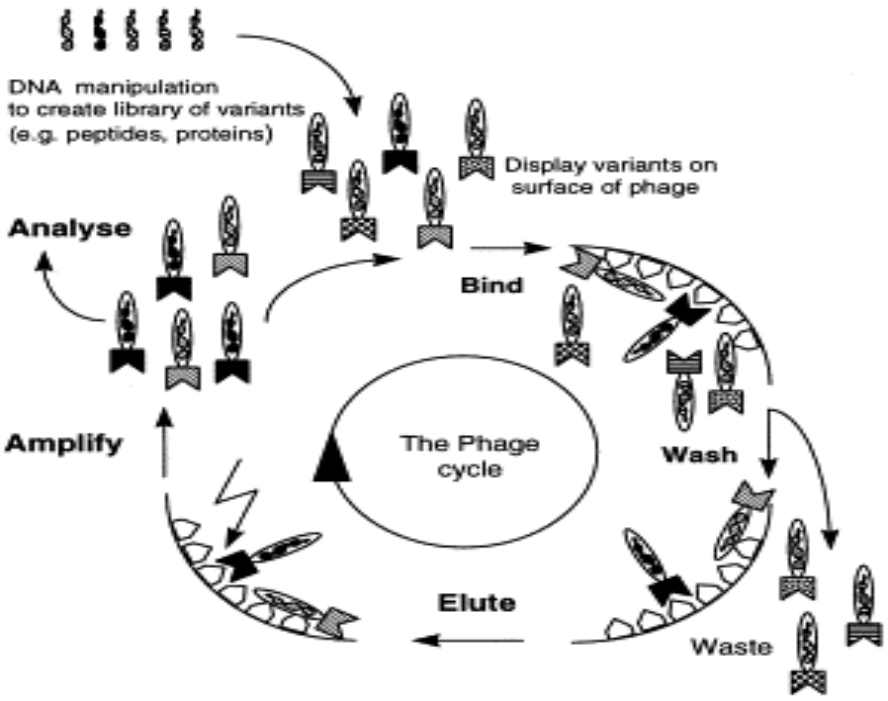


Figure 1.5: The phage display cycle (Reproduced from Hoogenboom, 1998)

1.6.2 Single chain variable fragment (ScFv) antibody library

The development of hybridoma technology allowed the generation and production of monoclonal antibody with predefined specificities (Kohler and Milstein, 1975). A phage display library is a collection of recombinant phage, each displaying a different antigen-binding domain on its surface. Antibody phage display is a very powerful technique for selecting recombinant antibodies from a large library (Marks *et al*., 1991; Nissim *et al*., 1994; Winter *et al*., 1994). It offers a good platform in studying protein function, protein-protein interactions, protein localization and also useful for diagnostic and therapeutic purposes (Agaton *et al*., 2004; Uhlen *et al*., 2005; Liu *et al*., 2002; Haab, 2001; Haab, 2006; Kalyn, 2007; Glennie and Johnson, 2000 and Babel *et al*., 2011).

Several advantages of recombinant antibodies include antibodies can be produced in bacteria, yeast or plant (Miller *et al*., 2005; Cabezas *et al*., 2008 and Almquist *et al*., 2006), immunization is not required and intrincis properties sich as immunogenecity, affinity, specificity and stability of antibodies can be improved via various mutagenesis technologies (Gram *et al*., 1992 and Valjakka *et al*., 2002).

An antibody phage library consists of the variable regions of heavy (V_H) and light (V_L) chains; which are randomly combined and linked together by polypeptide liner in order to form a single chain fragment (ScFv). These recombinant antibodies were expressed on the surface of phage. Subsequently, phage bearing antibody with high affinity against the target antigen is enriched after selection and infect to bacterial cells for soluble antibody expression. Variable fragment (Fv) plays a role in antigen-binding activities of an immunoglobulin molecule. As it is the smallest unit of immunoglobulin, Fv is easier to manipulate than a whole antibody molecule. (Batra *et*

al., 1992; Freidman *et al*., 1993; Kreitman *et al*., 1992), have high tissue penetrability, while maintaining their affinity and specificity (Dubel, 2007; Holt *et al*., 2003 and Schrama *et al*., 2006), and are also easier and faster to produce in recombinant form. These libraries can be used to select antibodies against any given antigen, including foreign antigens, self antigens, non-immunogenic antigens and toxic antigens (Winter *et al*., 1994). Phage antibodies are selected by panning the library for several rounds on an immobilized antigen.

However it has been known that producing enough antibodies in order to establish high throughput yield has always been a challenge. This high throughput antibody production is required to test multiple antibodies against different proteins. Therefore a high-throughput antibody development with lower antigen consumption that is also time saving would be desirable. A new methodology developed by Babel *et al*, 2011 was able to develop fast way to produce recombinant antibodies to any potential protein target with only minimal protein required. This technique not only saves time but it is also useful for production of difficult-to-express or toxic protein.

Currently, studies have been done to help improving the phage display and periplasmic folding of ScFv fragments. This have led to the discovery of accessory proteins for decrease aggregation and also facilitated the folding of the ScFv antibody fragments (Bothmann & Plückthun, 1998; Bothmann & Plückthun, 2000; Ramm & Plückthun, 2000; Aavula, *et al*., 2011).

Another significant application of the ScFv is as diagnostic reagents where, specific recombinant ScFv antibodies produced in bacteria have become potential alternatives to these “conventional” immunodiagnostic reagents (Chowdhury, *et al*. 2005). Monoclonal antibody especially ScFv antibody is able to bind to a variety of

antigens such as haptens, proteins, even the whole pathogens, and they can also be used in the enzyme-linked immunosorbent assay (ELISA) (Luka, *et al*., 2011).

1.7 Objective of This Study

The overall aim for this research was to use molecular tools for control of Cymbidium mosaic virus (CymMV) with the following specific objectives:

1. To construct plant transformation vector(s) with part of the CymMV coat protein in sense and antisense orientation and to test the expression of this in orchid species
2. Selection of a specific antibody to CymMV through biopanning experiments against Toxoplasmaa gondii-immunized single-chain variable fragment (ScFv) antibody phage-display library
3. Sequence analysis and homology modeling of the candidate ScFv antibody(s) isolated.

2.0 Materials and Methods

2.1 Materials

2.1.1 Plasmids

CymMv.pBlueScript plasmid containing the viral coat protein complete cDNA clone, was obtained from the National University of Singapore and kindly provided by Professor Wong Sek Man.

pGEM-T Easy Vector System was by Promega (USA) whereas pCAMBIA 1304 obtained from CAMBIA, Australia.

2.1.2 Orchid explants

Dendrobium sp explants used for this research were kindly provided from Plant Biotechnology Incubator Unit, University of Malaya, Kuala Lumpur, Malaysia. Explant samples were prepared from in vitro Dendrobium sp after 20 weeks of culture. Young leaves and roots of up to 5.0 cm in length were cut into 0.5-1.0 cm long segments and used as explants.

2.1.3 Cymbidium mosaic virus

The cymbidium mosaic virus was obtained from a research associate from Malaysia University of Science & Technology. The virus was originally sourced from the American Type Culture (accession number PV-82) and was provided in the form of lyophilized leaves following propagation in *Cucumis sativus*.

2.1.4 Cucumber plants

Cucumis sativus seeds were obtained from the Kelana Jaya Nursery, Selangor, Malaysia.

2.1.5 ScFv antibody phage display library

The *Toxoplasma gondii*-immunized single-chain variable fragment (ScFv) antibody phage display library was prepared and kindly provided by a research associate from University of Malaya, Kuala Lumpur, Malaysia.

2.2 Polymerase chain reaction

Polymerase chain reaction (PCR) was carried out to amplify the CymMV coat protein cDNA from the construct in pBluescript (Wong, 2004) using primers designed with the software Primer 3, Whitehead Institute for Biomedical Research. The forward and reverse primer sequences were designed to have *Nco*I restriction site on both strands to ease future ligation. The reaction mixture contained a final concentration of 1X reaction buffer, 2mM $MgCl_2$, 0.2mM dNTP mix, 5 units of *Taq* polymerase (Invitrogen, USA), 1µM of forward (5' ATT ACC ATG GAC CCC CGA AGA AAT CAA GAC 3') and reverse (3' GAT TCC ATG GTT GGT GAT GAG GTT GCG CAG 5') primers each, 20ng of template CymMV cDNA and nuclease-free water to a final volume of 50µl. The reaction was run in a thermocycler using the following profile:

1. Initial denaturation	94ºC	2 min
2. Denaturation	94ºC	1 min
3. Annealing	60ºC	1 min
4. Extension	72˚C	2 min
5. Repeat step 2-4 for 30 cycles		
6. Final extension	72ºC	10 min
7. Hold	4ºC	forever

2.3 Gel electrophoresis

The PCR products to be analyzed were mixed with one fifth volume of 6X loading dye (Fermentas, Lithuania) and separated by electrophoresis on an 0.8% agarose gel containing 1X TBE buffer [Tris Base 121.1 g/L, boric acid 55.0 g/L, 0.5mM EDTA (pH 8.0)] as well as 1 μg/ml of ethidium bromide. The gels were run at a constant voltage of 120V for 28 minutes or until loading dye was about 70% along the length of the gels in the same 1X TBE which was also used as the reservoir buffer. A 1 kb DNA ladder (Fermentas, Lithuania) was used as DNA size marker and the DNA bands were visualized under UV light in a ChemiImager^{TM} gel documentation system (Alpha Innotech, USA).

2.4 Isolation and purification of DNA bands from agarose gel

Isolation and purification of DNA bands from agarose gel were done by using QIAquick Gel Extraction Kit (Qiagen, Netherlands). The bands with sizes of interest were excised from the gel with a scalpel and purified according to the protocol and solutions provided in the kit. The weight of the gel slices were determined and three volumes of buffer QG were added to one volume of gel; where 1.0g of gel equals to 1.0ml and later incubated at 50ºC for 10 min or until the gel was dissolved completely. This was followed by adding one gel volume of isopropanol to the samples and mixing well. The samples were then applied into a QIAquick spin column which was placed in a 2.0ml collection tube and centrifuged for 1 min at 10 000 x g. The flow-through were discarded and the column replaced back into the same collection tube, after that 0.75mL of buffer PE was added to the column as a washing step and centrifuged again at 10 000 x g for another minute. In order to elute the DNA, the column was then placed on a clean 1.5mL microcentrifuge tube and 30µL of buffer EB was added and the column was centrifuged again before being kept in -20ºC for further use.

2.5 Preparation of *E.coli* competent cells

A single colony of *E.coli* DH5α was inoculated in 10mL LB broth and grown overnight at 37ºC. The following day, 400µL of the overnight inoculum was diluted in 40mL LB broth and incubated at 37ºC with shaking at 250rpm until the exponential phase of OD_{600} approximately 0.4 was reached. The culture was then harvested by centrifugation at 3000 x g at 4ºC for 10 minutes. The pellet was then resuspended in 20mL of ice-cold 0.1M $CaCl_2$ and incubated on ice for 30 min followed by centrifugation at 3000 x g for another 5 min. The collected pellet was then resuspended

in 4mL of ice-cold 25mM $CaCl_2$, aliquoted 100µL per tube in 1.5mL microcentrifuge tubes and stored at -80ºC in 15% glycerol or used immediately.

2.6 Transformation of *E.coli* and Selection of Tranformants

Approximately 1-2 µL of ligation product (see Section 2.8) was transformed into prepared competent *E.coli* DH5α and incubated for 30 min. The mixture was then heat-shocked at 42ºC for 90 seconds. Followed immediately by incubation on ice for 2 min. 900µL of pre-warmed LB broth was added to the mixture and incubated for 1 hour at 37ºC with shaking motion to allow the recovery of the bacteria. The bacteria suspension (50-100µL) was plated onto LB agar plates supplemented with appropriate antibiotic and X-gal (40µg/mL); and incubated overnight at 37ºC. Successful transformants were selected by blue-white screening and confirmed by restriction enzyme digestion with *Nco*I to release the insert.

2.7 Restriction enzyme digestion

Restriction enzyme digestion was performed using up to 1µg DNA, 2µl 10 x restriction buffer 1µl of 10U/µl of restriction enzyme (New England Biolabs, USA) and up to 20µl dH_20 for 1 hour at the appropriate temperature.

2.8 Ligation

Ligation reactions were performed using 4 µl of 5 x ligase reaction buffer, 1U of T4 DNA ligase (Roche, Switzerland), 10ng vector DNA and 30-50ng insert DNA with vector to insert ratio range 1:3 to 1:5 used. Reactions were incubated overnight at 4ºC for optimum ligation.

2.9 Calf Intestinal Phosphatase Treatment

Calf intestinal treatment removes 5'phosphate groups from DNA. Since CIP-treated fragments lack 5'phosphoryl termini required by ligases, it would avoid self-ligation. This is used to decrease the vector background in cloning strategies. Reaction mixture of 4 µl of 1 x NEB buffer 3, 1U of Calf Intestine Phosphatase (CIP) (NEB, New England), 10 ng vector DNA and 30-50 ng insert. Reactions were incubated overnight at 4ºC.

2.10 Small scale preparation of plasmid DNA

The Purelink ^{TM} Quick Miniprep Kit (Invitrogen, USA) was used for small scale preparation of plasmid DNA according to the protocol provided by the manufacturer. Ten ml of LB broth containing appropriate antibiotic was inoculated with a single bacterial colony and incubated overnight at 37˚C with shaking at 220 rpm. The overnight culture was pelleted by centrifugation for 10 min at maximum speed. The pellet was thoroughly resuspended in 250µl Resuspension solution (R3) and 250µl of Lysis buffer (L7) was then added to the mixture and incubated at room temperature for 5 min. After that, 0.35ml of Precipitation solution (N4) was slowly added and mixed gently by inverting to allow precipitation of DNA and protein to form by incubating on

ice for 5 min. Following this, the mixture was centrifuged at top speed for 10 min, the supernatant was then loaded into a spin column which was placed onto a wash tube and subjected to centrifugation at 12 000 x g for 1 min. The flow-through was discarded and 500µl of Wash buffer (W10) was added to the column, incubated at room temperature for 1 min and centrifuged at 12 000 x g for 1 min. the flow-through was again discarded and the column was later washed with 700 µl of Wash buffer followed by centrifugation at 12 000 x g for another 1 min. Additional centrifugation was done for another 1 min in order to remove any remaining residue. After that, the column was placed in a clean 1.5ml microcentrifuge tube and eluted with 50µl of TE buffer by centrifugation at 12 000 x g for 1 min.

2.11 DNA Sequencing and analysis

Plasmids prepared from selected transformants were sent to First Base Laboratories Sdn. Bhd. for sequencing in order to confirm the correct insert of the constructs prior to plant transformation by particle bombardment. Sequencing results were analysed using BioEdit Sequence Alignment editor (version 7.0.8.0) software (http://www.mbio.ncsu.edu/bioedit/bioedit.html) via pairwise alignment with CymMV coat protein sequence from the NCBI database (accession no: X62665) as template of comparison.

2.12 Plant transformation by microprojectile bombardment

PDS-1000/He System (Bio-Rad, USA) was used during the biolistic bombardment to transform the constructed plant transformation vector into the plant materials. The experiment was done according to the protocol provided in the Biolistic® PDS-1000/He Particle Delivery System by Bio-Rad.

2.12.1 Coating the washed gold microcarriers with DNA

The following procedure is sufficient for six bombardments, the quantities were adjusted to the amount of bombardment needed. Microcarriers in 50% glycerol (30 mg/ml) were vortexed for 5 min to resuspend and disrupt agglomerated particles. It is important that the tube containing the microcarriers to be continuously vortexed in order to maximize uniform sampling.

Five µl of DNA (1mg/ml) was mixed with 50µl of gold suspension in miccrocentrifuge tube. Continuous agitation of the microcarriers was needed for uniform DNA precipitation onto microcarriers. Next, 50µl of 2.5M $CaCl_2$ and 50µl of 100mM Spermidine were added into the tube and vortexed for 3 minutes at maximum speed. The mixture was allowed to settle for 1 min. After that, the tube was centrifuged at 8000 x g for 10 seconds. The supernatant was discarded as much as possible. Then, the pellet was resuspended in 250µl 100% ethanol and vortexed at maximum speed for 1 minute. Later, the tube was centrifuged at 8000 x g for 10 seconds. The supernatant was discarded and resuspend the pellet in 60µl 100% ethanol.

Gold-DNA suspension was prepared last. Then, macrocarriers were sterilized by dipping into 95% ethanol and air dried. Later, 10µl of the gold–DNA suspension was pipetted on the center of macrocarrier and air dried. The rupture disc was wiped with 70% ethanol before use. The rupture disc was then placed in the rupture disc holder and screwed tightly to the gas acceleration tube. In the macrocarrier launch assembly plate, a piece of stopping screen and the macrocarrier were placed, face down to the screen. Next, the macrocarrier cover lid was closed tightly. After that, the macrocarrier launch assembly was placed in the chamber. Lastly, the orchid root and shoot explants were placed on the stage and the door was closed and bombardment was started.

2.13 Performing a bombardment

Particle bombardment was performed according to the manufacturer's Biolositic® PDS-1000/He Particle Delivery System protocol.

22.13.1 Before the bombardment

Bombardment parameters for gap distance between rupture disk retaining cap and microcarrier launch assembly were adjusted with the stopping screen support placed in proper position inside a fixed nest of microcarrier launch assembly. Helium supply was checked where there should be 200 psi in excess of desired rupture pressure needed. Equipment and consumables such as rupture disk retaining cap, microcarrier launch assembly, macrocarriers and macrocarrier holders were clean and sterilized. Microcarriers were washed and resuspend in 50% glycerol and later the microcarriers were coated with DNA and loaded onto sterile macrocarrier/macrocarrier holder on the day of the experiment.

2.13.2 Firing the device

Chamber walls were sterilized with 70% ethanol. A sterile rupture disk was later loaded into a sterile retaining cap. The retaining cap was secured to the end of gas acceleration tube (inside, top of bombardment chamber) and tightened with a torque wrench. Macrocarrier and stopping screen were loaded into microcarrier launch assembly. The microcarrier, launch assembly and target cells were placed in the chamber and the door closed. The chamber was then evacuated and a vacuum was held at the desired level (minimum 5 inches of mercury) and the sample bombard by pressing the Fire button continuously until the rupture disk burst. The Fire button was released when the helium measure gauge dropped to zero.

2.13.3 After the bombardment

The vacuum was released and target cells were removed from the chamber. Microcarrier and stopping screen were unloaded from microcarrier launch assembly. Spent rupture disk was also removed as well as the helium pressure from the system (after all experiments were completed for the day).

2.14 Histochemical analysis of GUS expression

This technique was performed to analyze the expression of the reporter gene (in this case GUS) through visualization of its activity in different tissues. It is a method based on beta-glucuronidase, an enzyme from *E. coli*, which catalyses conversion of specific substrates into colored or fluorescent products (Jefferson, 1986).

After microprojectile bombardment, the orchid roots and leaf explants were incubated for at least two days in order to differentiate good quality samples from damaged tissues (Jefferson, 1987). The samples were later transferred into a histochemical reagent containing 0.1M phosphate buffer, 0.5 mM ferrocyanide, 0.1% triton X-100, 10.0 mM EDTA, 20 methanol and 1.0mM 5-bromo-3-indolyl-glucuronide (X-gluc) (Clonetech, USA) and incubated for 18 to 24 hours at 37˚C until the tissues were evaluated for their level of GUS expression.

2.15 Observation of Green fluorescent protein (GFP) expression

The transformed orchid root and leaf explants tissues were left at room temperature for two week to allow GFP expression in the samples and then visualized under a fluorescence stereo microscope Olympus IX71, IX2 series with a GFP Plus filter system (excitation filter: 480/40 nm, emission filter: 510 nm) and a GFP Plant filter system (excitation filter: 470/40 nm, emission filter:525/50 nm). The GFP Plant filter system is able to cut out the red color of the auto-fluorescence of chlorophyll. Photographs of GFP expression were taken using the Olympus IX71 IX2 series digital camera system.

2.16 RNA extraction

Approximately 30-50 mg of liquid nitrogen (N_2) frozen material or lyophilized tissue was used for small scale RNA extraction in 500µL buffer. It is important to make sure that the temperature of the extraction buffer to be at 65 ˚C. The sample was ground using mortar and pestle containing liquid nitrogen. The ground tissue in powdered form was transferred into 2mL microcentrifuge tubes and 500µL of pre-warmed extraction

buffer was added. 250µL of chlorofoam/isoamylalcohol (24:1 v/v) was added and vortexed at room temperature for 10 min. The mixture was later centrifuged for 5 min at 4˚C at 13 000 x g. To increase the purity of the RNA sample, it is advised to repeat the step by transferring the supernatant to a 2mL microcentrifuge tube and adding 125µL chlorofoam/isoamylalcohol before vortexing at room temperature and centrifuged again for 2 min at 4 ˚C at 13 000 x g. After that, the supernatant was transferred to a new 2mL microcentrifuge tube and 2 volume of cold isopropanol was added and incubated for 5 min on ice and later centrifuged for 5 min at 4˚C at 13 000 x g. The pellet was later dissolved in 13µL H_2O and 2µL of DNase buffer and 5µL of 5U DNase I were later added and incubated for 15 min at 37 ˚C. Two volume of cold isopropanol was added, mixed and centrifuged for 2 min at 4 ˚C at 13 000 x g. The pellet was washed with 1mL of cold 70% ethanol (v/v) and centrifuged for 5 min at 4 ˚C at 13 000 x g. Supernatant were aspirated and the pellet was air dried for 20 min before dissolving in 50µL RNase free H_2O.

2.17 Reverse transcriptase PCR (RT-PCR)

RNA samples were subjected to RT-PCR analysis for detection of the CymMV post particle bombardment. The reaction mixture consisted of 5X reaction buffer, 25mM $MgCl_2$, 10mM dNTP mix, 1U of reverse transcriptase SuperScript III (Invitrogen, USA), 0.5µg/µl random primers and 0.5µg/µl RNA template and was topped up with nuclease free water to 50µl. The reaction mixture was incubated at 25°C for 15 min for annealing reactions to take place followed by incubation at 42°C for 1 hr for extension. The reverse transcriptase was then thermally inactivated by heating the mixture to 70°C for 15 min. The cDNA synthesized was then subjected to PCR using the reaction mixture and temperature profile as described in section 2.2.

2.18 Preparation of cymbidium mosaic virus antigen

Cucumber plants (*Cucumis sativus*) were planted to propagate the Cymbidium mosaic virus because it is susceptible to CymMV, fast growing and has large leaves which make it a suitable host for propagation of the virus.

Seeds were germinated by placing them on a Petri dish laid with tissue paper wet with distilled water. The seeds watered daily with distilled water. The seeds were germinated for approximately 2-3 days and were later transferred to soil once the roots and shoots were visible. The plants were later transferred to a growth chamber. Glass rods were placed to support the plants and plants were maintained at 16 hour photoperiod at room temperature and watered every two days.

2.18.1 Mechanical inoculation

Infected cucumber leaves obtained from research associate (Ooi *et al*, 2006) were ground in a mortar with a small spatula of cellite as an abrasive to injure the leaves and 1-2 mL of 0.1M phosphate buffer (K_2HPO_4, pH 7.4) to make a thin paste. Mechanical inoculation was carried out on plants with four or more leaves and was done by gently rubbing the mixture onto the leaves using a finger covered with a disposable glove. The plants were incubated for 5 min before rinsing off any debris under slow running tap water (AbouHaidar *et al*. 1998). The plants were covered overnight with black plastic bags to exclude light. The next day, the cover was removed and plants were allowed to grow as usual under greenhouse conditions with 16 hour photoperiod at room temperature (AbouHaidar *et al*. 1998).

Infected plants and controls were observed for symptoms. The infected leaf tissues were collected 10-14 days post-inoculation. The infection was later confirmed by ELISA assay.

2.18.2 Purification Purification of CymMV from infected plants

Purified CymMV was prepared from infected plant materials using method of Wong *et al*. 1994 as described below.

Infected leaves were collected and homogenized in extraction buffer (0.01M EDTA, 0.1M sodium phosphate and 0.1% β-mercaptoethanol) for 5 min using approximately 1-2 mL of buffer per gram of leaves. Subsequent steps were performed at 4ºC. The homogenized tissues were blended and the extracts were clarified using 100mL of chloroform. The mixture was centrifuged at 8000 x g for 10 min. The supernatant was filtered with cheesecloth. PEG and NaCl were added to a final concentration of 0.25% and 4% respectively. The mixture was incubated for 30-60 min at 4ºC before centrifugation again at 8000 x g for 20 min. Following centrifugation, the pellet was resuspended in 5 mL of resuspension buffer (1% Triton X-100 and 0.1M sodium borate buffer pH 8.0) and stirred slowly for 12 hours at 4ºC. The overnight virus solution was then pelleted at 90 000 x g at 4ºC for 2.5 hours. The pellet was re-dissolved in 0.01M sodium borate buffer pH 7.5 and kept at -20ºC.

2.19 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is an enzyme-linked immunosorbent assay which is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. ELISA reagent set from Agdia (Agdia CymMV ELISA Reagent Set, Cat no. SRA 89500/0500, USA) was used to detect and verify the presence of

CymMV from infected leaves as well as the purified virus samples. Both positive and negative controls were provided with the ELISA reagent set. The number of single wells needed for analysis was determined, the sample blanks and controls were analyzed in triplicate.

The following protocol was obtained from the instruction manual provided in the ELISA set. Firstly, the microtiter plate was coated with CymMV antibody. It is important to coat the plate immediately after preparing the coating antibody as some coating antibody may be lost if too much time elapses between diluting the coating antibody and coating the plate. The 1X coating buffer (0.05M sodium carbonate, pH 9.6) was used to dilute the concentrated coating antibody into 1:200 dilutions. The volume of coating buffer required depends on the number of single wells used for analysis which was predetermined earlier, 100 µL of diluted coating antibody was pipetted into each well and the coated plate was left either at room temperature for 4 hours or 4 ºC overnight.

After incubation, the coating solutions were emptied from the wells. The plate was washed with washing solution (50 mM Tris, 0.14M NaCl 0.05% Tween 20, pH 8.0) by overflowing the wells and emptying completely after each wash. The washing steps were repeated 4-8 times and later the plate was dried by tapping it firmly onto paper towels.

Samples of infected leaves were prepared by grinding the leaves showing symptoms with mortar and pestle in phosphate buffer (0.03M KH_2PO_4, pH 7.0). Sample extracts (100µL) were dispensed into each test well along with 100µL of positive and negative controls were dispensed into positive and negative control wells respectively. All samples were prepared in triplicate. The plate was incubated in a humid box for 2 hours at room temperature or alternatively overnight at 4^oC.

The enzyme conjugate was prepared just a few minutes (less than 10 minutes) before the incubation was complete. The enzyme conjugate was prepared by dispensing the enzyme conjugate diluent into a test tube with cap. Equal amounts of peroxidase enzyme conjugate from both bottles A and B were added according to the 1:200 dilutions. The mixture was mixed thoroughly by inversion and 100 µL of enzyme conjugate diluent was dispensed into each test well.

When the first incubation was completed, the plate was washed and dried as described above. Care was taken so as not to mix the contents of different wells. The prepared enzyme conjugate (100µL) was dispensed into each test well. The plate was then incubated in a humid box for 2 hours at room temperature.

Just before (not more than two minutes) the incubation is completed, the OPD solution was prepared. Each OPD stick will make 10mL of OPD solution, which is enough for twelve 8-well strips. Ten mL of 1X OPD buffer (4.1 g 30 % hydrogen peroxide, 5.1 g anhydrous citric acid, 7.33 g anhydrous dibasic sodium phosphate) was prepared at room temperature. The paper end of the OPD stick (s) was immersed in the

buffer for 2 minutes. The buffer was stirred lightly with the sticks. Care must be taken as OPD is a carcinogen.

The plate was incubated for another 15 to 30 minutes. The reaction was later stopped by adding 50μL of 3M sulfuric acid. The processed plates were measured using an ELISA plate reader TECAN GENious at 490 nm.

2.20 Colorimetric assay

The Bradford protein assay is one of several simple methods commonly used to determine the total protein concentration in solutions and was first described by Dr Marion Bradford in 1976. The assay has become the preferred method for quantifying protein as it is less susceptible to interference by common reagents and non-protein components of biological samples. In addition this technique is also simpler, faster and sensitive as compared to other assay methods.

The Bradford assay relies on the equilibrium between three different forms of binding capabilities of the dye Coomassie Blue G-250 to protein; under strongly acid conditions, the dye is most stable as a doubly-protonated red form. Upon binding to protein, though, it is most stable as an unprotonated, blue form. Furthermore, the assay is colorimetric; as the protein concentration increases, the color of the test sample becomes darker. Coomassie absorbs at 595 nm. The protein concentration of a test sample is determined by comparison to that of a series of protein standards known to reproducibly exhibit a linear absorbance profile in this assay.

Although different protein standards can be used, Bovine Serum Albumin (BSA) was chosen as a standard as it being the most widely used and easy to obtain. The Bio-Rad Protein Assay was used with a microplate reader. Firstly, dye reagent was prepared by diluting one part of concentrated reagent with four parts of distilled water. The diluted reagent may be used for about two weeks when kept at room temperature. Three to five dilutions of protein standard (BSA) were prepared. These are representative of the protein solution to be tested. The linear range of this microtiter plate assay is 0.05mg/ml to approximately 0.5mg/ml. The protein solutions are assayed in duplicate.

Next, 10µl of each standard and sample solution were pipetted into separate microtiter plate wells. Then 200µl of diluted dye reagent were added to each well and were mixed with multi-channel pipette. The microtiter plate was later incubated at room temperature for at least five minutes; as absorbance will increase over time, samples should not be left at room temperature for more than one hour. The absorbance was measured at 595 nm using TECAN GENios.

2.21 Biopanning for selection of candidate antibody

2.21.1 Rescue of *Toxoplasma gondii*-immunized single-chain variable fragment (ScFV) antibody phage-display library

Toxoplasma gondii-immunized single-chain variable fragment (ScFV) antibody phage-display library, with complexity of 1.62 X 10^4 independent transformants was prepared from Recombinant Phage Antibody System (RPAS) by Amersham Biosciences by a research associate (Lim *et al*, 2011, personal comm) was used as the phage display library in this research. The helper phage M13KO7 was used to rescue the phagemid with its antibody SfcFv gene insert from *E. coli* TG1 cells. M13KO7 provides the necessary genetic information to package the recombinant pCANTAB 5E phagemid which encodes the recombinant antibody expressed on the phage tip. The transformation of *E. coli* TG1 with pCANTAB 5E containing the ScFV insert was done prior to phage rescue.

The rescue was performed using 900µl of the undiluted transformed cells with the remaining 100µl diluted and used to determine transformation efficiency and to prepare a glycerol stock. First, 9.1ml of 2x YT-G medium (2x YT medium containing 100µg/ml ampicillin and 2% glucose) was added to the 900µl of transformed cells. Polypropylene tubes are recommended for this procedure as phage may adsorb non-specifically to other plastics. The culture was incubated for 1 hour at 37°C with shaking at 250 rpm. It is important to make sure that the culture does not overgrow since the seed culture will be rather dense. Next, 50µl of 20 mg/ml ampicillin was later added; together with 4 x 10^{10} pfu of M13KO7 to the cell suspension (volume of stock to add = 4 x 1010 pfu ÷ M13KO7 pfu/ml). Next, the culture was incubated for 1 hour at 37°C with shaking at 250 rpm and followed by spinning at 1 000 x g in a clinical centrifuge for 10 minutes to sediment the cells. Supernatant was carefully removed and discarded.

The entire sample was later resuspended in a sterile 50ml disposable polypropylene centrifuge tube containing 10 ml of 2x YT-AK medium (2x YT medium containing 100µg/ml ampicillin and 50 µg/ml kanamycin). The culture was incubated overnight at 37°C with shaking at 250 rpm. Next, the cells were sedimented by centrifuging at 1 000 x g for 20 minutes in a clinical centrifuge. The supernatant which contains the recombinant phage was transferred to a sterile 50ml disposable polypropylene centrifuge tube and stored at 4°C or directly panned. It is recommended that the supernatant be filtered through a 0.45µm filter if it will be stored.

2.21.2 Phage titering

5-10 mL of 2xYT medium were inoculated with a single colony of TG1 cell and incubated with shaking until mid-log phase (OD600 ~ 0.5). While the cells were growing, serial dilution of the M13K07 phage culture was prepared by labeling five tubes containing 2x YT medium with $T10^{-2}$, $T10^{-3}$, to $T10^{-6}$ and six SOBAG plates as $P10^{-3}$, $P10^{-4}$ to $P10^{-7}$ and one control plate PC. 1mL of the phage culture was then transferred to the tube labeled $T10^{-2}$ and the dilutions were mixed by drawing the liquid up and down several times. It is very important that the dilutions are well mixed. A new sterile pipet was used to transfer 1 mL culture from tube $T10^{-2}$ to tube $T10^{-3}$ and again the dilutions were thoroughly mixed. This diluting procedure was continued until tube $T10^{-6}$. Each dilution was kept at 42ºC until ready for use.

0.1 mL of phage dilution was removed from tube $T10^{-2}$ and added to tube containing pre-warmed agar mix. 0.25 mL of log phase of TG1 were then dispensed into the same tube. After that, the content was poured onto SOBAG plate $P10^{-3}$. The plate was swirled to make sure the content was evenly distributed. This process was repeated for each dilution. The control plate was prepared by pipeting 0.1 mL of plain 2X YT medium, pre-warmed agar mix and 0.25 mL of *E.coli* and poured onto control SOBAG plate PC. The plates were incubated overnight at 30ºC. The number of phage plaques for each plates were counted to determine the number of phage particles per mL which is also known as phage titer.

2.21.3 Panning to select for antigen-positive recombinant phage antibodies

Panning or selection is done to selectively capture antigen-positive recombinant phage antibodies with an antigen bound to a solid support or with antigen in solution. It is highly recommended as the incorporation of one or more panning steps will greatly reduce the number of clones that must be screened by ELISA. Recombinant phage are precipitated from solution with PEG to separate them from soluble antibodies prior to panning it against the antigen. The recombinant phage that bind to the antigen are retained while those that fail to react with the antigen are removed during the subsequent wash steps. Once the log phase *E. coli* TG1 cells are added to the panning vessel, the antigen-reactive phage will start to infect the cells which can later be rescued and selected again or plated onto SOBAG (20g Bacto-tryptone, 5g Bacto-yeast extract, 0.5g NaCl, 10ml sterile 1M $MgCl_2$, 55.6ml sterile 2M glucose and 5ml filter-sterilized 20 mg/ml ampicillin and 15g Bacto-agar for plates to a final volume of 1L) plates for screening of individual colonies.

2.21.4 Advanced preparation

Prior to selection of antigen-positive phage and screening of individual clones, an immunoassay should be performed to determine optimal binding conditions for the antigen of interest. A TG1 log phase were prepared by transferring a colony from minimal medium plate to 5ml of 2x YT medium (17g Bacto-tryptone, 10g Bacto-yeast extract and 5g NaCl) and incubating at 37^{o}C with shaking at 250 rpm. Next, 10ml of fresh 2x YT medium were later inoculated with 100µl of the overnight culture and later being incubated at 37˚C with shaking at 250 rpm until the culture reached an A_{600} of 0.3.

2.21.5 PEG Precipitation of recombinant phage

PEG precipitating of the phage-displayed recombinant antibodies will purify the recombinant phage from soluble antibodies which will compete for antigen during selection. 2ml of PEG/NaCl (200g Polyethylene glycol 8000 and 146.1g NaCl, added to distilled water up to 1L) was added to the 10ml of recombinant phage antibody supernatant and mixed well before placing it on ice for 30-60 min. the mixture was later spun at 10 000 x g in a Beckman JA-20 rotor for 20 min at 4˚C. The supernantant was discarded and the tube was inverted over a clean paper towel to remove all of the buffer. The pellet which may be easily visible was resuspended in 16ml of 2x YT medium and filtered through a 0.45µm filter before being stored at 4˚C.

2.21.6 Panning of CymMV against recombinant phage antibody library

A 25cm^2 tissue culture flask was coated with 5 ml of antigen of interest diluted to 10 µg/ml in PBS (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4, pH 7.4). The coating of antigen could be performed for 1-2 hours at room temperature or overnight at 4˚C. The flask was washed three times with PBS, emptying it completely after each

wash. The flask was later filled in completely with blocking buffer (1XPBS containing 10% non-fat dry milk) to block any remaining sites on the flask surface and later incubated at room temperature for 1 hour. After that the flask was washed three times with PBS, emptying it completely after each wash. The 16 ml of PEG-precipitated recombinant phage prepared earlier was diluted with 14 ml of blocking buffer which contains 0.01% sodium azide as preservative and incubated at room temperature for 10-15 min. Non-specific, hydrophobic protein-protein interactions that may occur between native M13 phage proteins and some antigens during the panning step can be reduced by adding Triton X-100 to the diluted phage supernantant to a final concentration of 0.1%. 20 ml of the diluted recombinant phage was added to the flask and incubated for 2 hours at 37˚C. The flask was later emptied and washed 20 times with 30-50ml PBS and 20 times with PBST (1XPBS, 2ml Tween-20, pH7.2 for 1L). The flask was emptied completely each time. Isolation of colonies for small scale rescue was done by reinfecting *E. coli* TG1 cells with bound phage directly in the panning vessel and plating the infected cells.

Isolation of colonies for small scale rescue was done by reinfecting *E. coli* TG1 cells with bound phage directly in the panning vessel and plating the infected cells. As subsequent rounds of panning are to be performed, rescue of phagemid library was done as follows. The entire 10ml of log-phase TG1 cells were added to the flask or panning vessel and incubated at 37 ˚C for 1 hour with shaking at 250 rpm. The cell was later transferred into a sterile 50ml disposable polypropylene centrifuge tube with ampicillin and glucose added to a final concentration of 100 µg/ml and 2% respectively. A 4 x 10^{10} pfu/ml of M13K07 was also added to the cell suspension and incubated for 1 hour at 37 ºC with shaking at 250 rpm. The cells was later sedimented my centrifugation at 1000 x g for 10 minutes and the supernatant was carefully removed and discarded. Next, the entire sample was resuspended gently in 10ml of 2x YT-AK medium in a sterile 50 ml

disposable polypropylene centrifuge tube and incubated overnight at 37 ˚C with shaking at 250 rpm. Cell suspension was spun at 1 000 x g for 20 minutes the next day and the supernatant which contains the recombinant phage was transferred to a 50ml polypropylene centrifuge tube and to be stored at 4 ˚C or proceed straight to panning. However, it is recommended that the supernatant to be filtered through a 0.45µ, filter if it will be stored.

2.21.7 Reinfection of *E.coli* with enriched phage clones

The entire 10ml of TG1 cells was added to the flask and incubated at 37˚C for 1 hour. After 1 hour, 100µl of the 10ml cell suspension was removed to prepare tenfold dilutions in 2x YT medium (1:10, 1:100, 1:1000). An aliquot of 100µl of undiluted cells of each dilution was plated onto separate SOBAG plates using glass spreader and later incubated overnight at 30˚C. The plates were left for additional 4-8 hours if the colonies were too small to be picked. Frozen stock culture were prepared by adding 800µl of reinfected cells from the flask to 200µl of sterile 80% glycerol and stored at -70˚C. The remainder of the infected cells can be stored for several days at 4˚C in a polypropylene centrifuge tube for later use if the plating was unsuccessful.

2.21.8 Preparation for screening

The ELISA (see Section 2.17) to be used for screening was optimized before preparing phage-displayed and soluble recombinant antibodies where the amount of antigen to be used for screening was determined empirically and depends on the nature of both the antigen and the specific antibody used. Generally concentrations between 1 and 100 µg/ml in a volume of 50 to 200µl was needed for coating the wells. Next, 400µl of 2x YT-AG medium was added to each cluster tube (96 tubes in a microtiter format, Costar #4411). Individual well-isolated colonies were transferred to separate tubes using sterile toothpick or pipet tips. The plate is labeled as Master plate where it will serve as the source from which phage recombinant antibodies and soluble antibodies were prepared for screening. Plate was incubated overnight 30˚C with shaking at 250 rpm.

2.22 Analysis of cloned sequences

DNA sequence of the candidate antibodies was obtained via automated sequencing. The purified clones were sequenced using the pCANTAB 5 sequencing primers provided in the Expression Module/Recombinant Phage Antibody System kit by GE Healthcare (USA).

The sequences of the primers are as follows:

pCANTAB 5-S1 (Forward): 5' CAACGTGAAAAAATTATTATTCGC 3'

pCANTAB 5-S6 (Reverse): 5' GTAAATGAATTTTCTGTATGAGG 3'

The deduced amino acid sequences were analyzed first by aligning the sequences using online software Clustal W and then by using nucleotide using BLAST and BLASTX program and compared with the published sequences in the Gene Bank database.

The significant amino acid sequence with high similarity to the published sequence was picked and used to build a structure modeling by using the internet-based program RASMOL (httlp://rasmol.org).

3.0 Results

3.1 PCR amplification of CymMV for plant transformation vector construction

The sense and antisense coat protein (CP) CymMV construct were designed in such a way that they would yield DNA constructs with CymMV sequences in both forward and reverse orientation with respect to the T7 promoter. This was done by designing specific PCR primers that were able to amplify part of the CymMV CP from the CymMV CP cDNA template supplied in CymMV.pBlueScript (Wong, 2004) by using the PCR reaction mixture and profile as described in Section 2.2. The forward and reverse primer sequences were designed to have *Nco*I restriction site on both strands to ease the future ligation onto the pCAMBIA 1304 vector as described in Section 2.2 earlier.

Figure 3.1 CymMV CP cDNA sequence. Grey highlights the primer regions and black, the amplified region.

1 ATGGGAGAGC CCACTCCAAC TCCAGCTGCC ACTTACTCCG CTGCCGACCC
51 CACCTCTGCA CCCAAGTTGG CCGACCTGGC TGCCATTAAG TACTCGCCTG
101 TCACCTCCTC CATCGCCACC CCCGAAGAAA TCAAGGCCAT AACCCAATTG
151 TGGGTTAACA ACCTTGGCCT CCCCGCTGAT ACCGTAGGTA CCGCGGCCAT
201 TGACCTGGCC CGCGCCTACG CTGACGTTGG GGCGTCCAAG AGTGCTACCC
251 TGCTCGGTTT CTGCCCTACG AAACCTGATG TCCGTCGCGC CGCTCTTGCC
301 GCGCAGATCT TCGTGGCCAA CGTCACCCCC CGCCAGTTTT GCGCTTACTA
351 CGCAAAAGTG GTGTGGAATC TGATGCTGGC CACTAACGAT CCGCCTGCCA
401 ACTGGGCCAA GGCTGGTTTC CAGGAGGATA CCCGGTTTGC CGCCTTTGAC
451 TTCTTCGATG CTGTCGATTC CACTGCCGCG CTGGAGCCTG CTGAATGCAA
501 CGGCCGCCCT ACTGACCGCG AACGTGCTGC GCACTCTATC GGGAAGTACG
551 GCGCCCTTGC CCGTCAGCGT ATCCAAAACG GCAACCTCAT CACCAACATC
601 GCCGAGGTCA CCAAGGGCCA TCTTGGCTCC ACCAACACTC TCTATGCTCT
651 GCCTGCACCC CCTACTGAAT AACGCCAAAC TTAATAAGGC GTGTGGTTTT
701 CTAAAGTTTG TTTCCACTAC TGGCATAATA TACTTAGCCA GCTTAAATA

The gel electrophoresis of the PCR product yielded a fragment of the expected size of 480 bp as shown in Figure 3.2. DNA sequence of the PCR product aligned against the DNA sequence of CymMV CP in the NCBI database (accession number X62665) using BioEdit Sequence Alignment editor (version 7.0.8.0) software showed them to be the same (See Appendix 1).

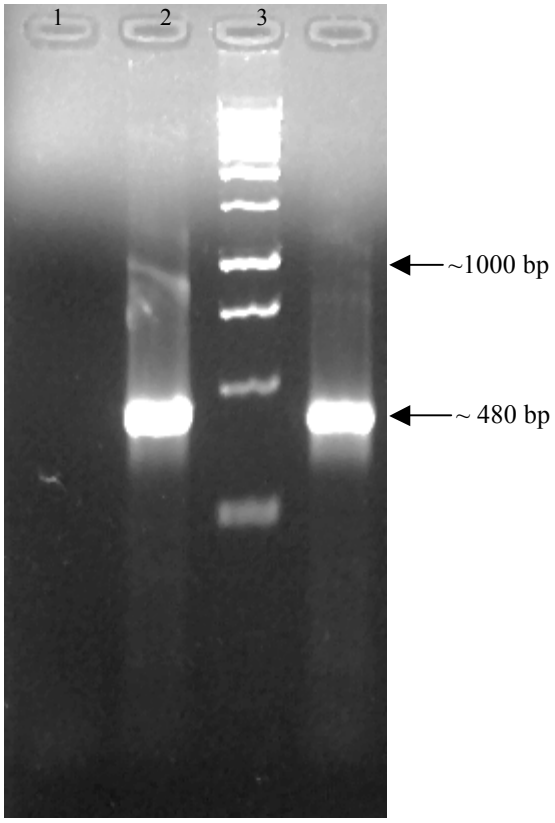


Figure 3.2 CymMV CP PCR product

Lane 1: Negative control (PCR mix without DNA sample)

Lane 2: PCR product (approximately 480 bp)

Lane 3: 1 kb DNA marker

Lane 4: PCR product [approximately 480 bp (duplicate sample)]

3.1.1 Cloning into pGEM-T Easy Vector and Sequence Confirmation

The PCR product obtained was later cloned into pGEM-T Easy Vector (Promega, USA) prior to cloning it into pCAMBIA 1304 plant transformation vector (CAMBIA, Australia). The pGEM-T Easy vector comes in a linearized form with 3'-T overhangs at the insertion site which allows insertion and ligation of the PCR product containing 5'-A overhangs synthesized by Taq polymerase. Transformants were screened by blue/white selection on X-gal plates where the lacZ gene in the pGEM-T multiple cloning site (MCS) encodes for β-galactosidase enzyme. This enzyme will metabolize X-gal into a blue colored substrate. With a successful insertion/cloning, the inserted product will disrupt lacZ gene and thus producing white colonies of transformants.

Based on this selection method, white colony plasmids were extracted and digested using *Nco*I restriction enzyme to check for the correct insert size followed by gel electrophoresis. The inserted fragment was later sent for DNA sequencing analysis which confirmed it to be the desired clone (See Appendix 2).

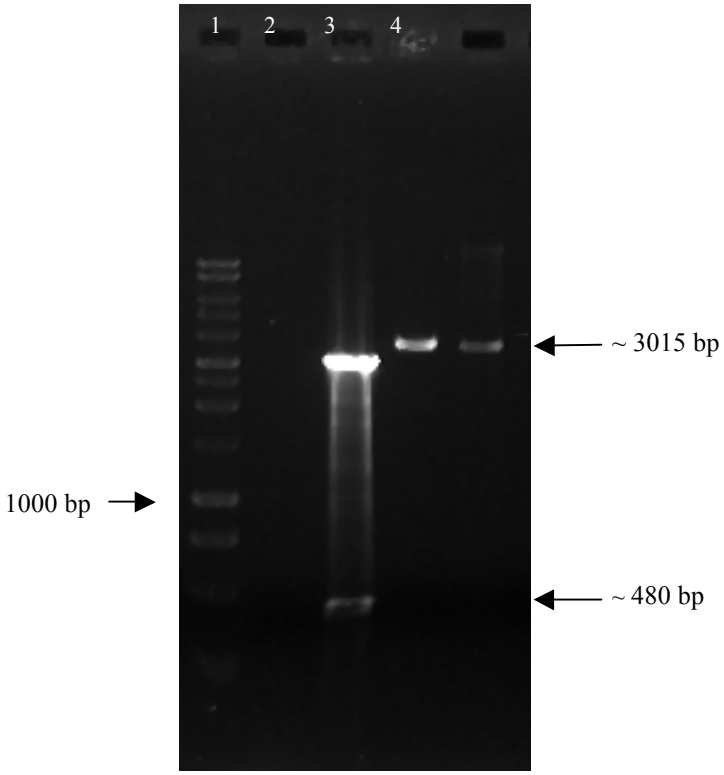


Figure 3.3 Screening of pGEM-T Easy Vector with inserted CymMV CP fragment

Lane 1: 1 kb DNA marker

Lane 2: Empty lane

Lane 3: pGEM-T with CymMV insert

Lane 4: pGEM-T without CymMV insert

Lane 5: pGEM-T without CymMV insert

3.1.2 Construction and sequence confirmation of CymMV.pCAMBIA 1304

Construction of CymMV.pCAMBIA1304 plant transformation vector was done by excising the construct initially cloned into pGEM-T Easy vector using *Nco*I restriction enzyme sites (incorporated into the construct via the PCR primers) after the inserted sequence was confirmed by DNA sequencing analysis (as previously described in section 2.11). The digested fragment was then ligated into pCAMBIA 1304 transformation vector and selection for the positive transformants was done by screening the plasmid colonies using *Nco*I restriction enzyme digestion to select for fragments with correct insert size (480 bp) as shown in Figure 3.5.

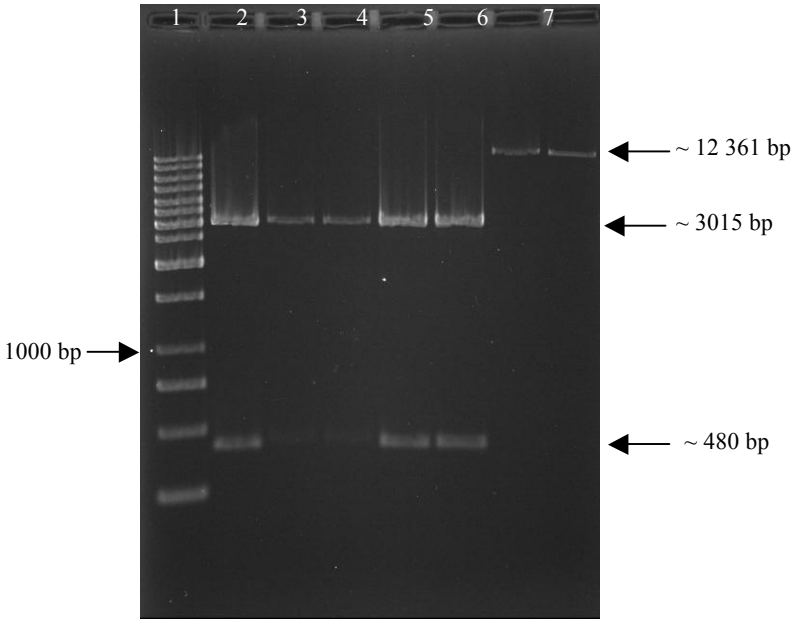


Figure 3.4 Products for cloning

Lane1: 1 kb DNA marker

Lane 2-6: Post digestion pGEM-T and CymMV CP fragment

Lane 7-8: Post digestion pCAMBIA 1304 vector ready for ligation

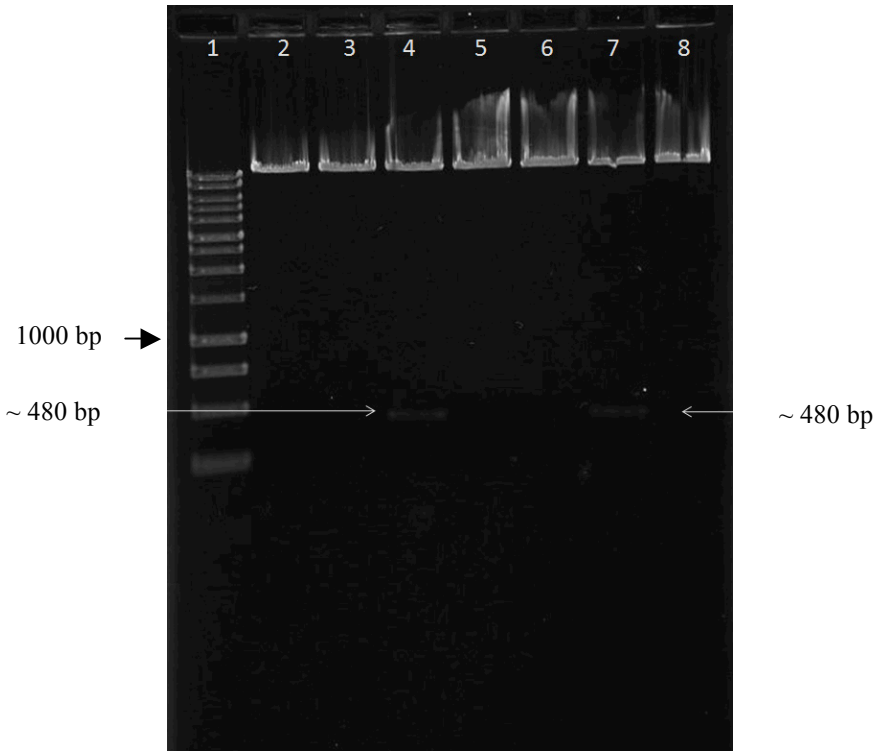


Figure 3.5 Screening of pCAMBIA 1304 vector with inserted CymMV fragment

Lane 1: 1 kb DNA marker

Lane 2: pCAMBIA 1304 vector without insert

Lane 3: pCAMBIA 1304 vector without insert

Lane 4: pCAMBIA 1304 with insert

Lane 5: pCAMBIA 1304 vector without insert

Lane 6: pCAMBIA 1304 vector without insert

Lane 7: pCAMBIA 1304 with insert

The cloning results were confirmed by DNA sequencing to verify that the positive transformant was indeed carrying the desired pCAMBIA 1304 plasmid with the correct insert of CymMV CP. The sequencing result is as in Figure 3.6. Analysis was done by comparing the sequence from the sequencing results with the expected CymMV CP obtained from the PCR product sequence that was corresponding to the CymMV CP sequence from NCBI database as done previously. The comparison was done using ClustalW multiple Alignment function of BioEdit Software.

Figure 3.6: Alignment of pCAMBIA 1304 vector with CymMV insert against CymMV CP

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
CAMBIA.CymMV    AATTGGTGGG TTAACAACCC TTGGCCTCCC CGCTGACACC GTAGGTACCG
CymMV CP        aattg-tggg ttaacaaccc ttggcctccc cgctgatacc gtaggtaccg

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
CAMBIA.CymMV    CGGCCATTGA CCTGGCCCGT GCCTACGCTG ACGTCGGGGC GTCCAAGAGT
CymMV CP        cggccattga cctggcccgc gcctacgctg acgttggggc gtccaagagt

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
CAMBIA.CymMV    GCTACCCTGC TCGGTTTCTG CCCCACGAAA CCTGATGTCC GTCGCGCCGC
CymMV CP        gctaccctgc tcggtttctg ccctacgaaa cctgatgtcc gtcgcgccgc

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
CAMBIA.CymMV    TCTTGCCGCG CAGATCTTCG TAGCCAACGT CACCCCCCGC CAGTTTTGCG
CymMV CP        tcttgccgcg cagatcttcg tggccaacgt caccccccgc cagttttgcg

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
CAMBIA.CymMV    CTTACTACGC AAAAGTGGTG TGGAATCTGA TGCTGGCCAC TAACGATCCG
CymMV CP        cttactacgc aaaagtggtg tggaatctga tgctggccac taacgatccg

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
CAMBIA.CymMV    CCCGCCAACT GGGCTAAGGC TGGTTTCCAG GAGGATACCC GGTTTGCCGC
CymMV CP        cctgccaact gggccaaggc tggtttccag gaggataccc ggtttgccgc

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
CAMBIA.CymMV    CTTTGACTTC TTCGATGCCG TCGATTCCAC TGCCGCGCTG GAGCCTGCTG
CymMV CP        ctttgacttc ttcgatgctg tcgattccac tgccgcgctg gagcctgctg
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        360        370        380        390        400
CAMBIA.CymMV    AATGGCAGCG CCGCCCTACT GACCGTGAAC GTGGGTCCAT GGTAGATCTG
CymMV CP        aatgcaacgg ccgccctact gaccgcgaac gtg------- ----------
```

However for some unknown reason, the antisense plasmid was unable to be obtained even after many attempts of cloning and screening. The diagram in Figure 3.7 showed the designed construct with CymMV CP insert in both forward and reverse orientation.

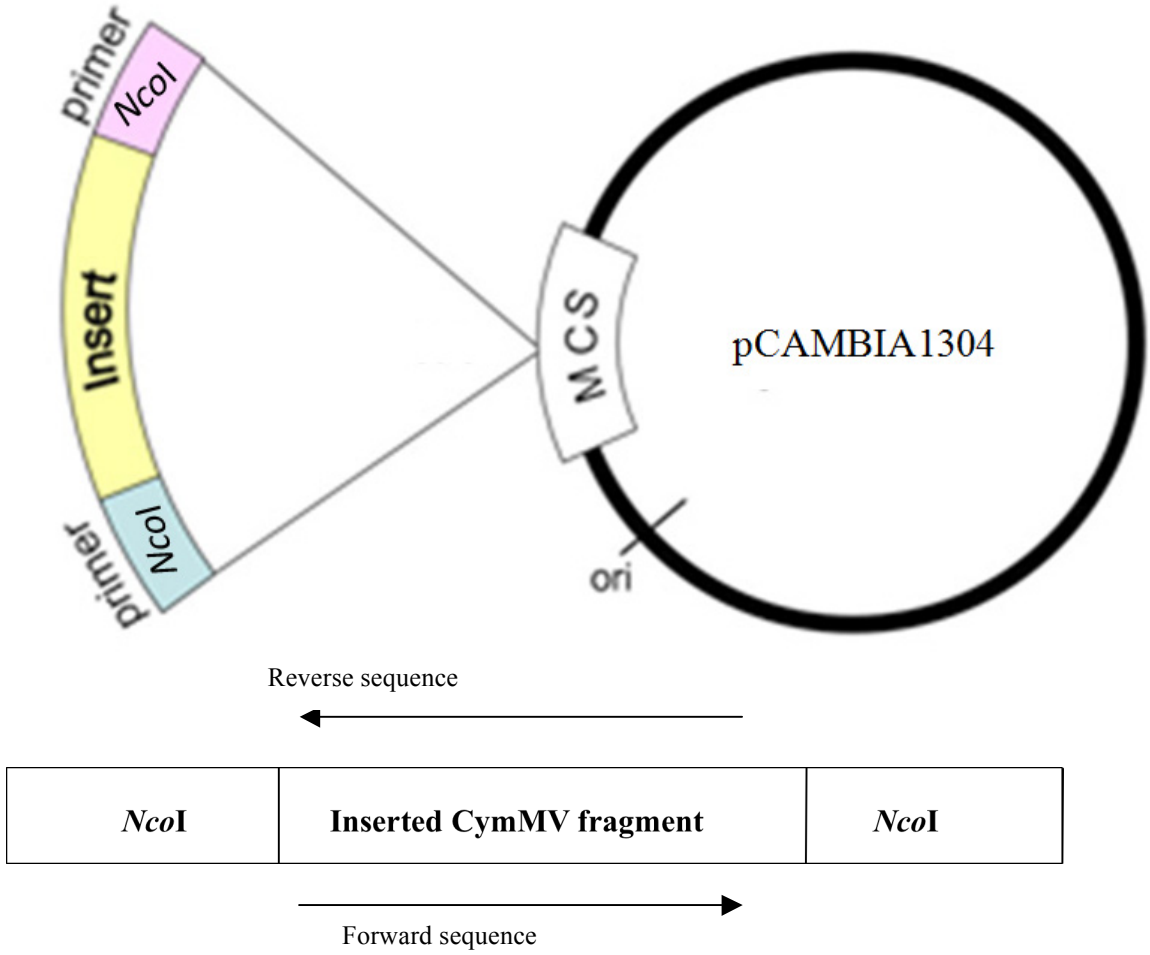


Figure 3.7 Diagram showing the construction of the pCAMBIA 1304-CymMV plant transformation vector construct with CymMV CP fragment inserted in forward and reverse orientation.

3.2 Testing the expression of the pCAMBIA 1304.CymMV-fwd construct on Dendrobium sp orchid sample

3.2.1 Plant transformation and histochemical analysis of GUS expression

Microprojectile bombardment of the orchid leaf and root explants of dendrobium sp were later analyzed for GUS expression and by reverse transcriptase PCR (RT PCR) as described earlier. Transient expression of the GUS gene at different target tissue distances are shown in Figure 3.8 and 3.9.

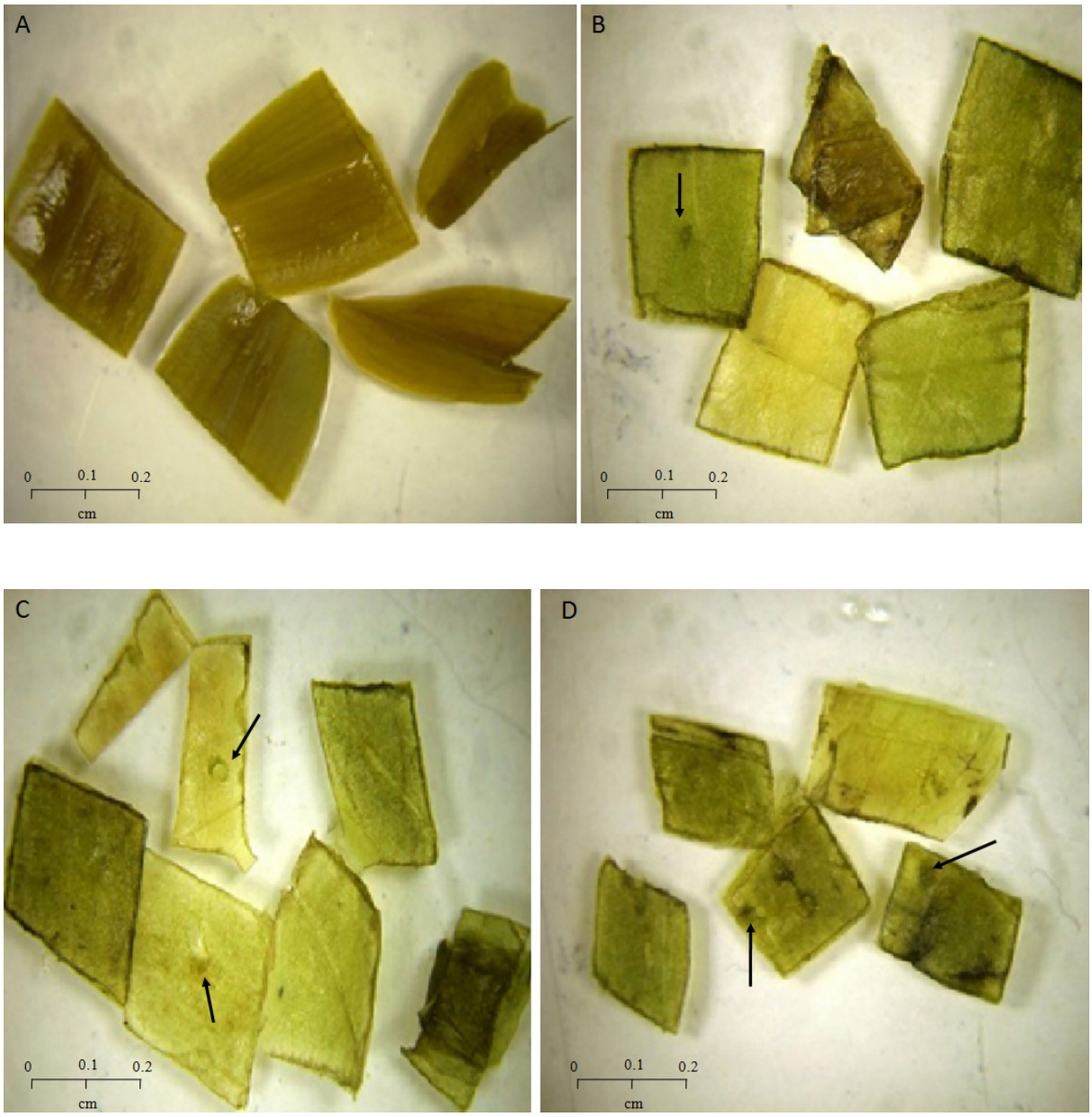


Figure 3.8 Orchid leaf explants at different biolistic target distances with arrow indicating dark blue color. (A) control (untransformed explants), (B) 6 cm, (C) 9 cm and (D) 12 cm.

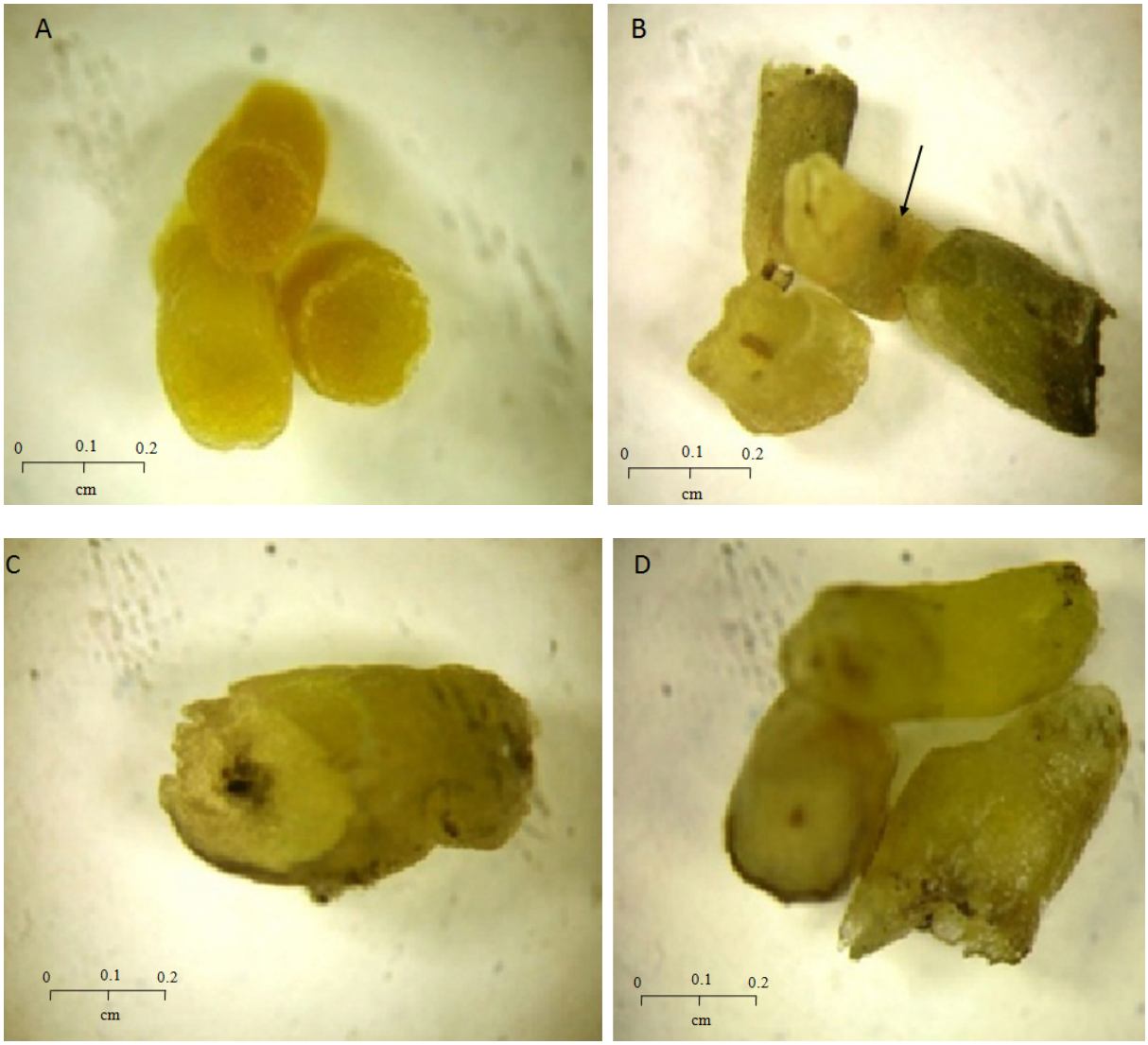


Figure 3.9 Orchid root explants at different biolistic target distances with arrow indicating dark blue color. (A) control (untransformed explants), (B) 6 cm, (C) 9 cm and (D) 12 cm.

3.2.2 Observation of GFP expression

The root and leaf explants were examined under a florescent microscope(Olympus Advanced Research MicroscopeIX71) at different target tissue distances.

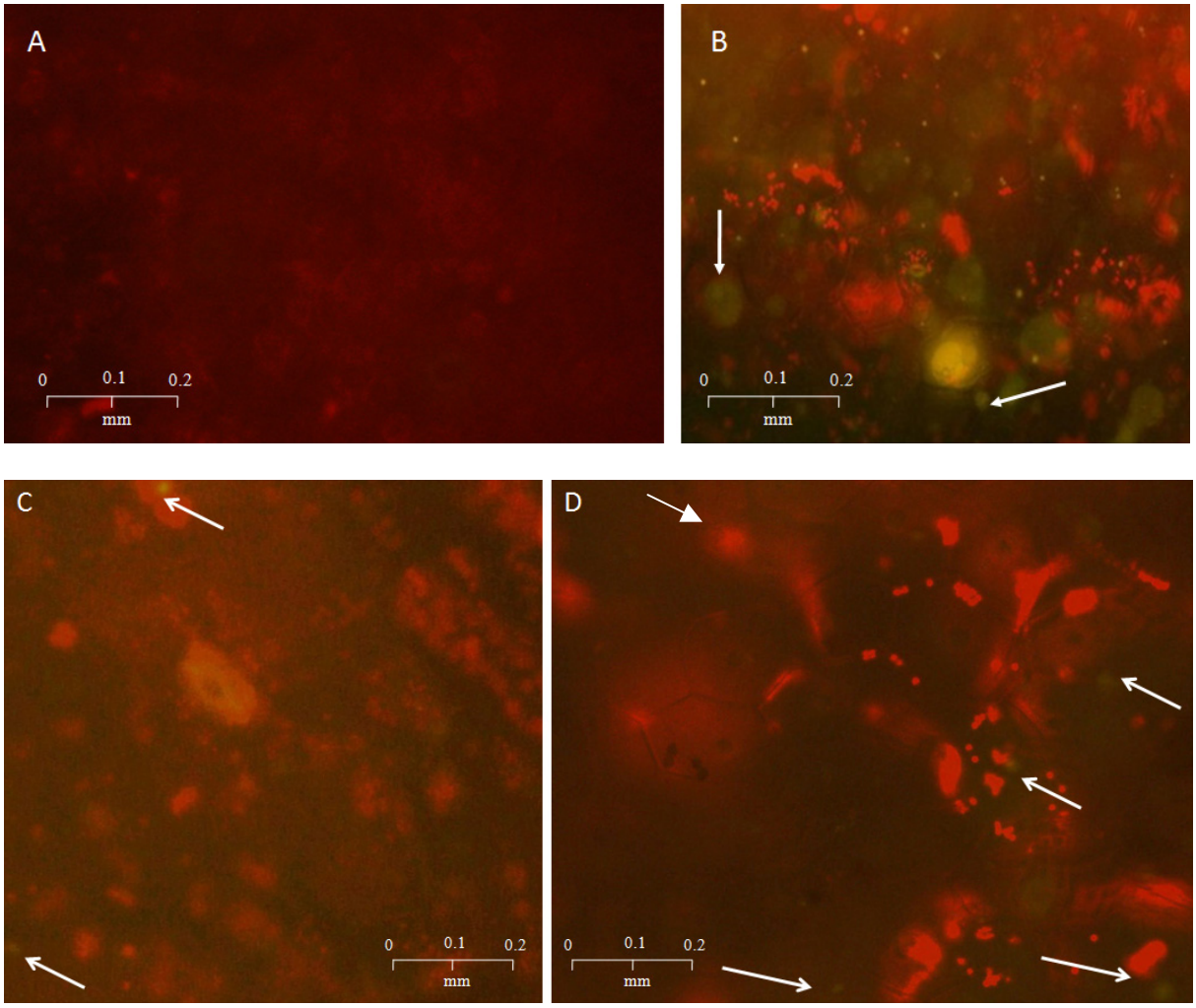


Figure 3.10 Orchid leaf explants at different biolistic target distances with arrow indicating florescent signal. (A) control, (B) 6 cm, (C) 9 cm and (D) 12 cm.

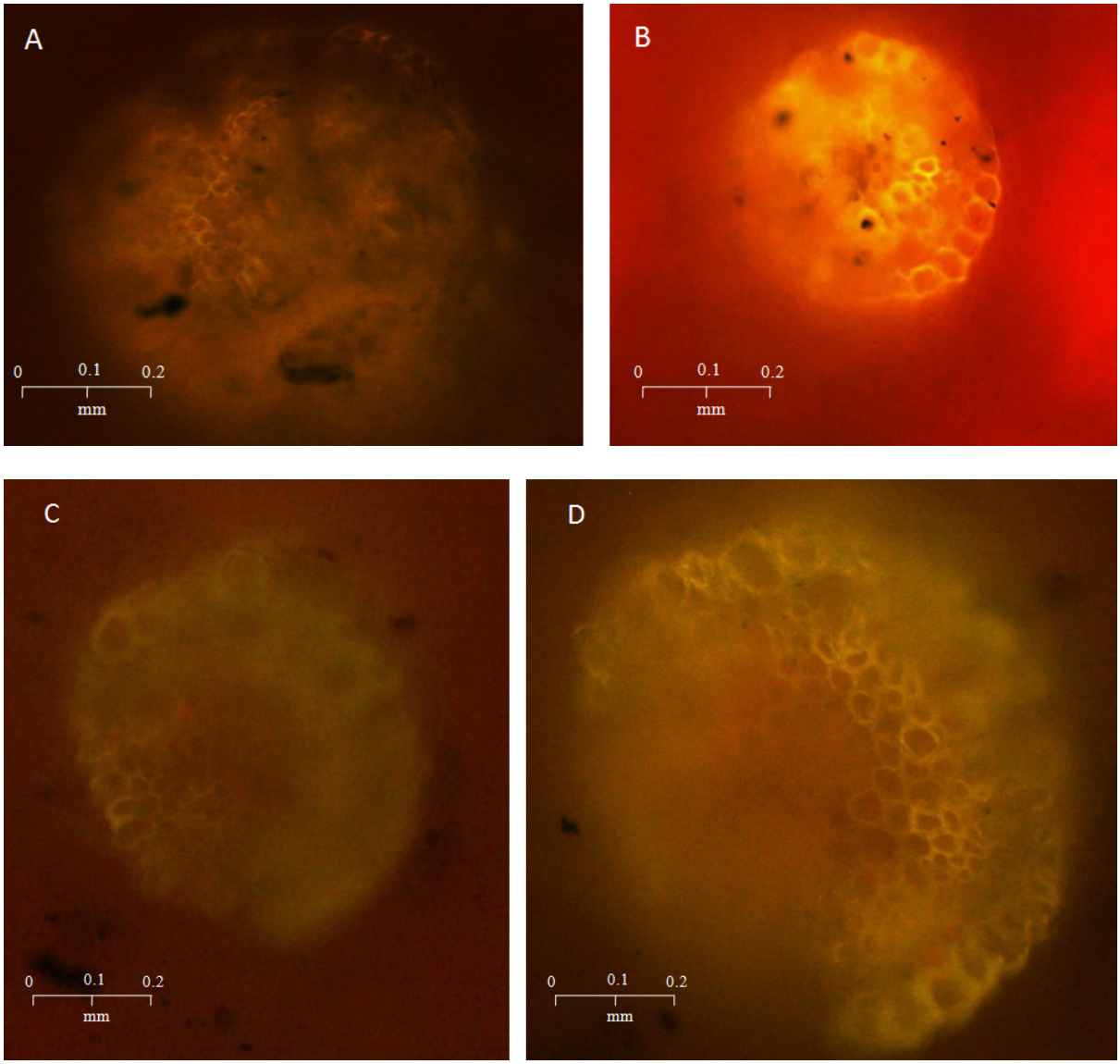


Figure 3.11 Orchid root explants at different biolistic target distances. (A) control, (B) 6 cm, (C) 9 cm and (D) 12 cm.

3.2.3 Reverse transcriptase PCR

Figure 3.12 shows the RNA extraction and Figure 3.13 shows the RT-PCR results for the explant samples of orchid explants post transformation with pCAMBIA 1304-CymMV-fwd construct.

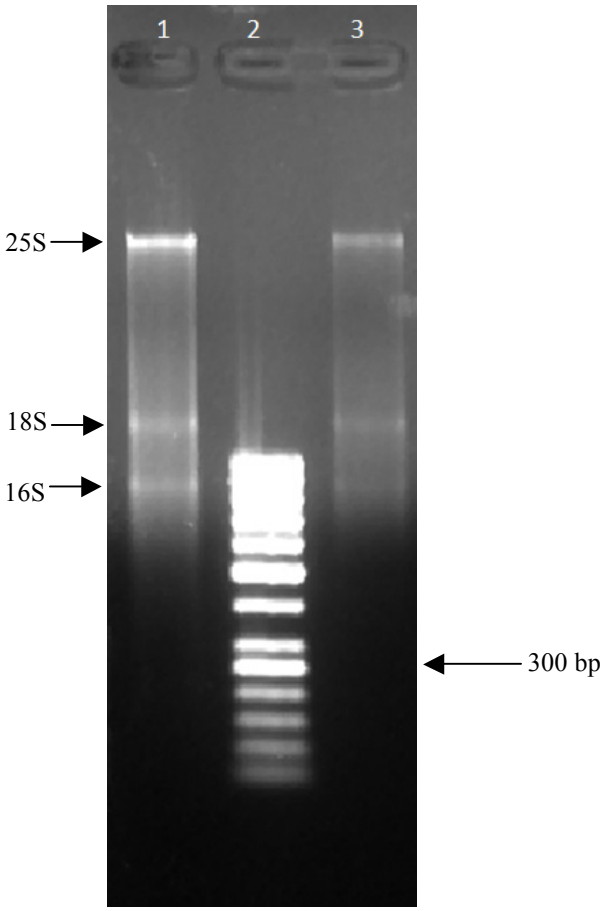


Figure 3.12 RNA extraction products of orchid explants post transformation with pCAMBIA 1304.CymMV-fwd construct

Lane 1: RNA extracted from orchid leaf explants

Lane 2: 100 bp DNA marker

Lane 3: RNA extracted from orchid leaf explants (duplicate)

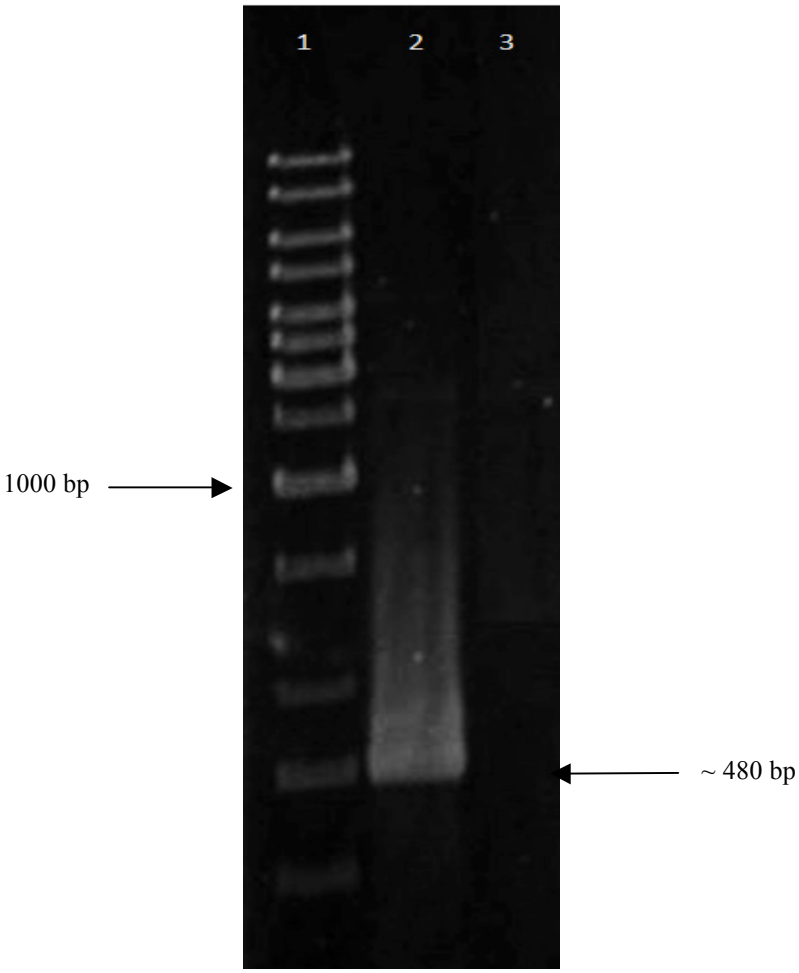


Figure 3.13 Products of RT-PCR orchid explants post transformation with pCAMBIA 1304.CymMV-fwd construct

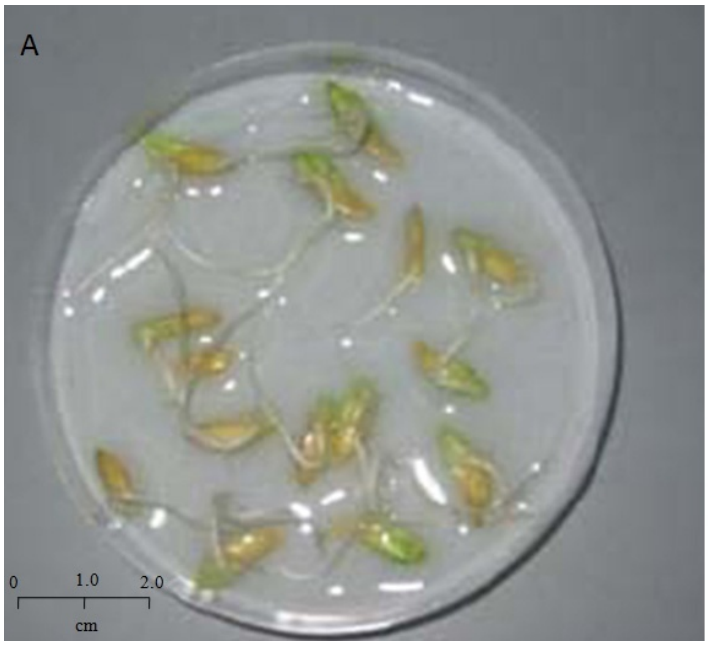
Lane 1: 1 kb DNA marker

Lane 2: RT-PCR of orchid leaf explants

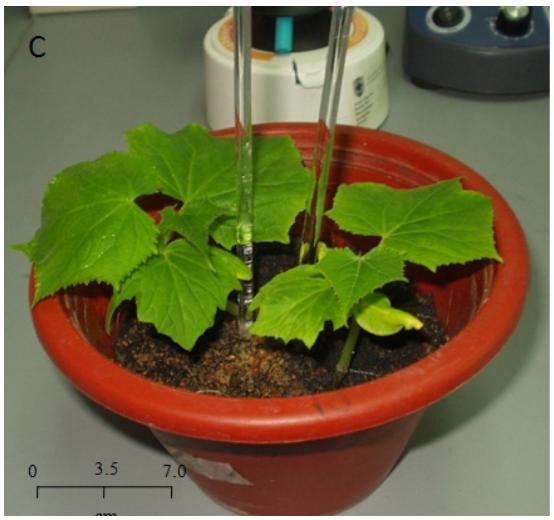
Lane 3: Negative control (PCR mix without DNA)

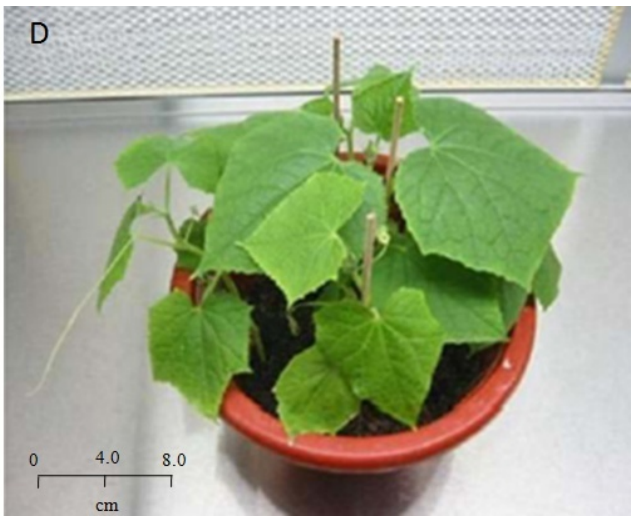
3.3 Selection of the specific antibody through biopanning experiments against the ScFV antibody library

Cucumber plants (*Cucumis sativus*) were used to propagate the CymMV for use as antigen in biopanning experiments with the ScFV library, as they are susceptible to the virus and fast growing compared to the orchid plants. It takes approximately up to two months to reach the four leaves stage for the plant to be ready to be inoculated with virus. The infected plants produced necrotic, yellowing and wrinkling symptoms 14 days post inoculation (dpi) as shown in Figure 3.14.


A
0
1.0
2.0
cm


B
0
0.5
1.0
cm


C
0
3.5
7.0


D
0
4.0
8.0
cm

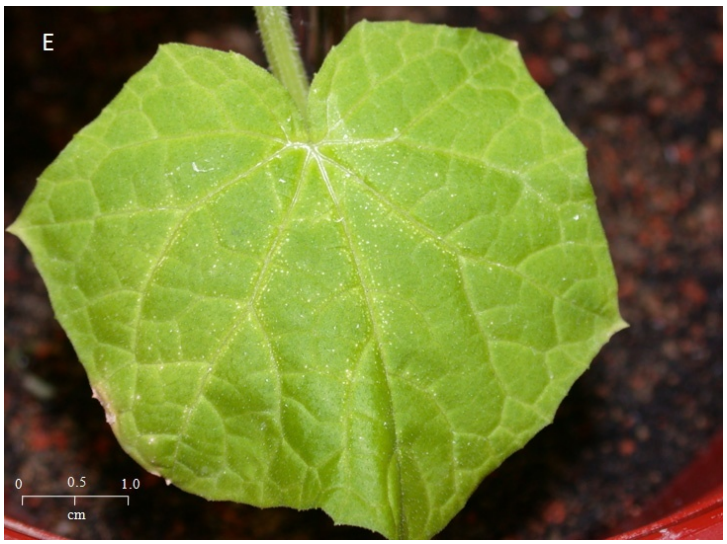

E
0
0.5
1.0
cm

Figure 3.14 Propogation of CymMV in *Cucumis sativus*

A: *Cucumis sativus* seeds, two days after germination

B: *Cucumis sativus* in MS media, 5 days old

C: *Cucumis sativus* transferred in soil, 14 days old (1 day post infection)

D: *Cucumis sativus* post infection, 3 weeks old

E: *Cucumis sativus* showing symptoms, 2 weeks post infection

3.3.1 Purification of CymMV from infected plants

Based on the results, the absorbance at 595 nm wavelength of the purified virus was 0.2915 which corresponded to a protein concentration of 0.1606 mg/ml.

Table 3.1: Verification of CymMV presence via ELISA

Samples	Absorbance at 490 nm		
	1st reading	2nd reading	Average
Positive control	0.817	0.956	0.8865
Negative control	0.036	0.033	0.0345
Uninfected plant	0.044	0.029	0.0365
Infected sample	1.578	1.665	1.6215
Purified sample	0.593	0.556	0.5745

*Positive and negative controlswere provided in ELISA reagent set Cat no. SRA 89500/0500!

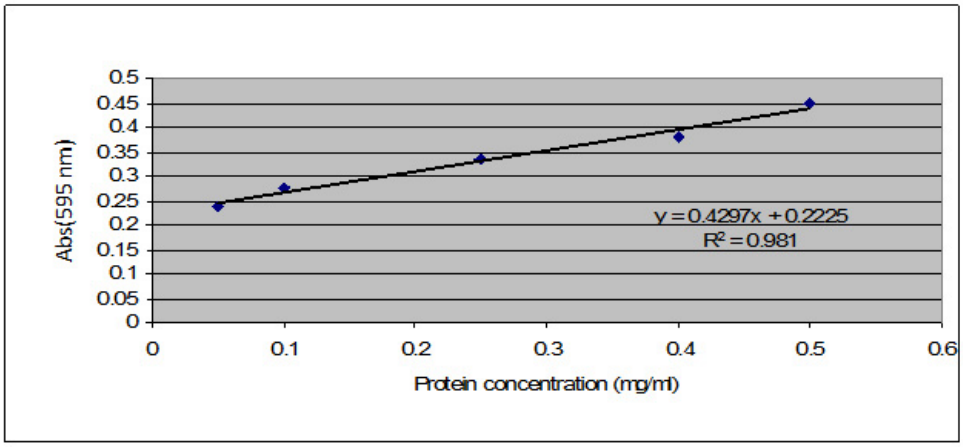


Figure 3.15: Quantification of CymMV concentration using Bradford Protein Assay

3.4 Antigen-positive recombinant phage antibodies selection

The *Toxoplasma gondii*-immunized single-chain variable fragment (ScFV) antibody phage display library was estimated to be 2 x 10^8 cfu ml^{-1} in size after the bacterial library was infected by TG1 with final titer of 3 x 10^9 pfu ml^{-1} of MK13K07 phage as analyzed by direct colony count (Lim, 2012, personal communication).

The phage-display library was used for three rounds of panning against purified CymMV with 25 colonies selected from the first round, 63 colonies in the second round and 18 selected after the third round of biopanning. ELISA was used to screen all 17 colonies from the final round for the affinity between obtained clones and CymMV antibody (see Section 2.21.8) and four clones (clone A, B, C and D) with average binding ratio more than 3.00 as shown in Figure 3.16 were selected to be sent for DNA sequencing of the encoding phagemids and alignment by Clustal W software (see results Figure 3.17) using the primers S1 and S6 provided as described in Section 2.22.

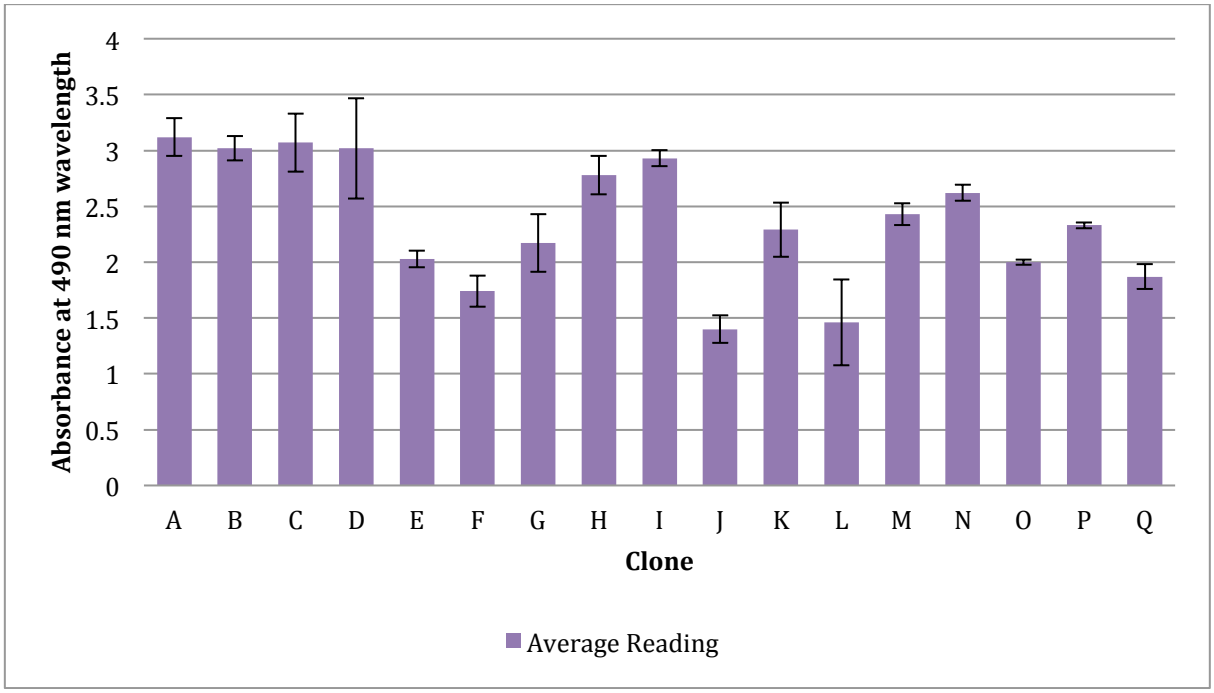


Figure 3.16: Average binding ratio of seventeenclones by ELISA screening under absorbance at 490 nm wavelength

3.5 Results from alignment of the four selected sequences

Four clones were selected based on their affinity binding capability to CymMV(from in section 3.4) and aligned using Multiple Sequence Alignment, CLUSTAL W (as described in section 2.22). As the results in Figure 3.16 showed, we were unable to get good alignment between the four sequences except for sequence c and sequence d where they showed significant similarities with the score of 78. We decided to proceed to BLAST and BLAST-X sequence c (from Clone C) as sequence c gave us longer sequencing results of 500 bp as compare to sequence d of 238 bp.

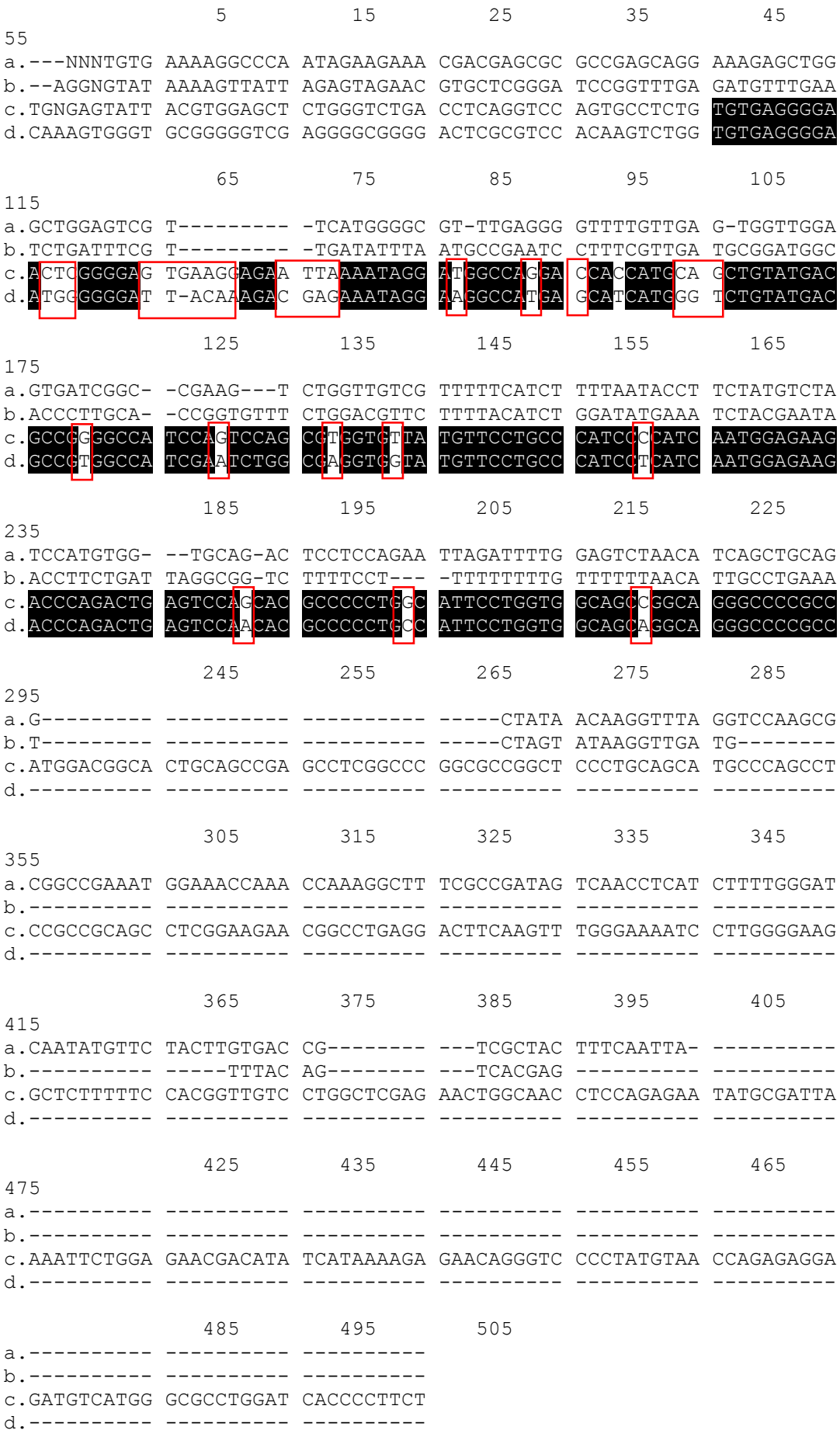


Figure 3.17: Multiple sequence alignment of candidate antibody clones

Figure 3.18: Nucleotide BLAST alignment of clone C

Gene ID: 5170 PDPK1 3-phosphoinositide dependent protein kinase-1 [Homo sapiens]

Score = 702 bits (380), expect = 0.0

Identities = 411/425 (97%), Gaps = 6/425

(1%), strand = Plus/Plus

```
 Score =  702 bits (380),  Expect = 0.0
 Identities = 411/425 (97%), Gaps = 6/425 (1%)
 Strand=Plus/Plus

Query  91   ATGGCCAGGACCACCATG-CAGCTGTATGACGCCGGGGCCATCCAGTCCAGCGTGGTGTT  149
            |||||||||||||||| | |||||||||||||||| | ||||||||||||||||||||||
Sbjct  145  ATGGCCAGGACCACCA-GCCAGCTGTATGACGCCGTGCCCATCCAGTCCAGCGTGGTGTT  203

Query  150  ATGTTCCTGCCCATCCCCATCAATGGAGAAGACCCAGACTGAGTCCAGCACGCCCCCTGG  209
            |||||||||||||||||||||||| || ||||||||||||||||||||||||||||||||
Sbjct  204  ATGTTCCTGCCCATCCCCATCAATGGTGAGGACCCAGACTGAGTCCAGCACGCCCCCTGG  263

Query  210  CATTCCTGGTGGCAGCCGGCAGGGCCCCGCCATGGACGGCACTGCAGCCGAGCCTCGGCC  269
            ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  264  CATTCCTGGTGGCAGCAGGCAGGGCCCCGCCATGGACGGCACTGCAGCCGAGCCTCGGCC  323

Query  270  CGGCGCCGGCTCCCTGCAGCATGCCCAGCCTCCGCCGCAGCCTCGGAAGAA-CGGCCTGA  328
            |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  324  CGGCGCCGGCTCCCTGCAGCATGCCCAGCCTCCGCCGCAGCCTCGGAAGAAGCGGCCTGA  383

Query  329  GGACTTCAAGTTTGGGAAAATCCTTGGGGAAGGCTCTTTTTCCACGGTTGTCCTGGCTCG  388
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  384  GGACTTCAAGTTTGGGAAAATCCTTGGGGAAGGCTCTTTTTCCACGGTTGTCCTGGCTCG  443

Query  389  AGAACTGGCAACCTCCAGAGAATATGCGATTAAAATTCTGGAGAA-CGACATATCATAAA  447
            |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  444  AGAACTGGCAACCTCCAGAGAATATGCGATTAAAATTCTGGAGAAGCGACATATCATAAA  503

Query  448  AGAGAACAGGGTCCCCTATGTAACCAGAGAG-GAGATGTCATGG-GCGCCTGGATCACCC  505
            |||||||| |||||||||||||||||||| | |||||||||  ||||||||||||||||
Sbjct  504  AGAGAACAAGGTCCCCTATGTAACCAGAGAGCGGGATGTCATGTCGCGCCTGGATCACCC  563

Query  506  CTTCT  510
            |||||
Sbjct  564  CTTCT  568
```

Figure 3.19: BLASTX alignment for clone C

Gene ID: 5170 PDPK1 3-phosphoinositide Independent protein kinase-1 [Homo sapiens] Score = 702 bits (380), Expect = 0.0 Identities = 411/425

```
 Score =  122 bits (306),  Expect = 2e-32
 Identities = 75/93 (81%), Positives = 77/93 (83%), Gaps = 0/93 (0%)
 Frame = +1

Query  91   MARTTMQLYDAGAIQssvvlcscpspsMEKTQTESSTPPGIPGGSRQGPAMDGTAAEPRP  270
            MARTT QLYDA  IQSSVVLCSCPSPSM +TQTESSTPPGIPGGSRQGPAMDGTAA PRP
Sbjct  1    MARTTSQLYDAVPIQSSVVLCSCPSPSMVRTQTESSTPPGIPGGSRQGPAMDGTAAGPRP  60

Query  271  GAGSLQHAQPPPQPRKNGLRTSSLGKSLGKALF  369
            GAGSLQHAQPPPQPRK        GK LG+  F
Sbjct  61   GAGSLQHAQPPPQPRKKRPEDFKFGKILGEGSF  93


 Score = 59.3 bits (142),  Expect = 7e-09
 Identities = 29/29 (100%), Positives = 29/29 (100%), Gaps = 0/29 (0%)
 Frame = +3

Query  330  DFKFGKILGEGSFSTVVLARELATSREYA  416
            DFKFGKILGEGSFSTVVLARELATSREYA
Sbjct  81   DFKFGKILGEGSFSTVVLARELATSREYA  109
```

3.6 BLAST Analysis of antigen-positive recombinant phage antibodies clone

Based on the sequencing data obtained, out of the four clones of the candidate antibody for CymMV that were sent for sequencing only clone C was able to provide a significant alignment with a published sequence (see Figure 3.17 and 3.18) as the others did not show any significant similarities in the database. Both nucleotide BLAST and BLASTX hit the same target of PDPK1 3-phosphoinositide dependent protein kinase-1 [*Homo sapiens*].

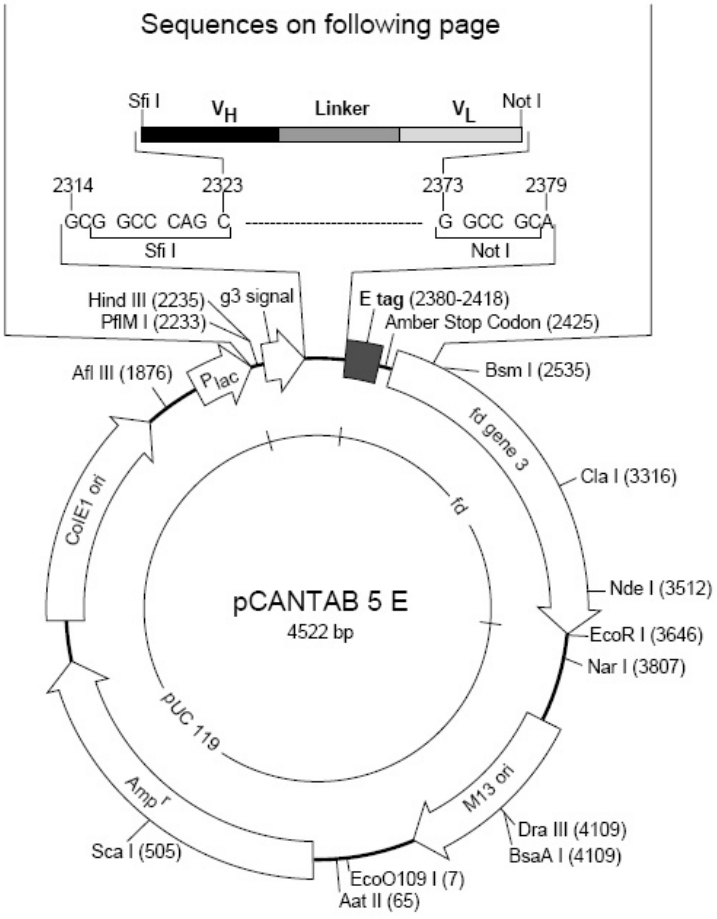


Figure 3.20: Vector map of pCANTAB 5E that was used in producing ScFV

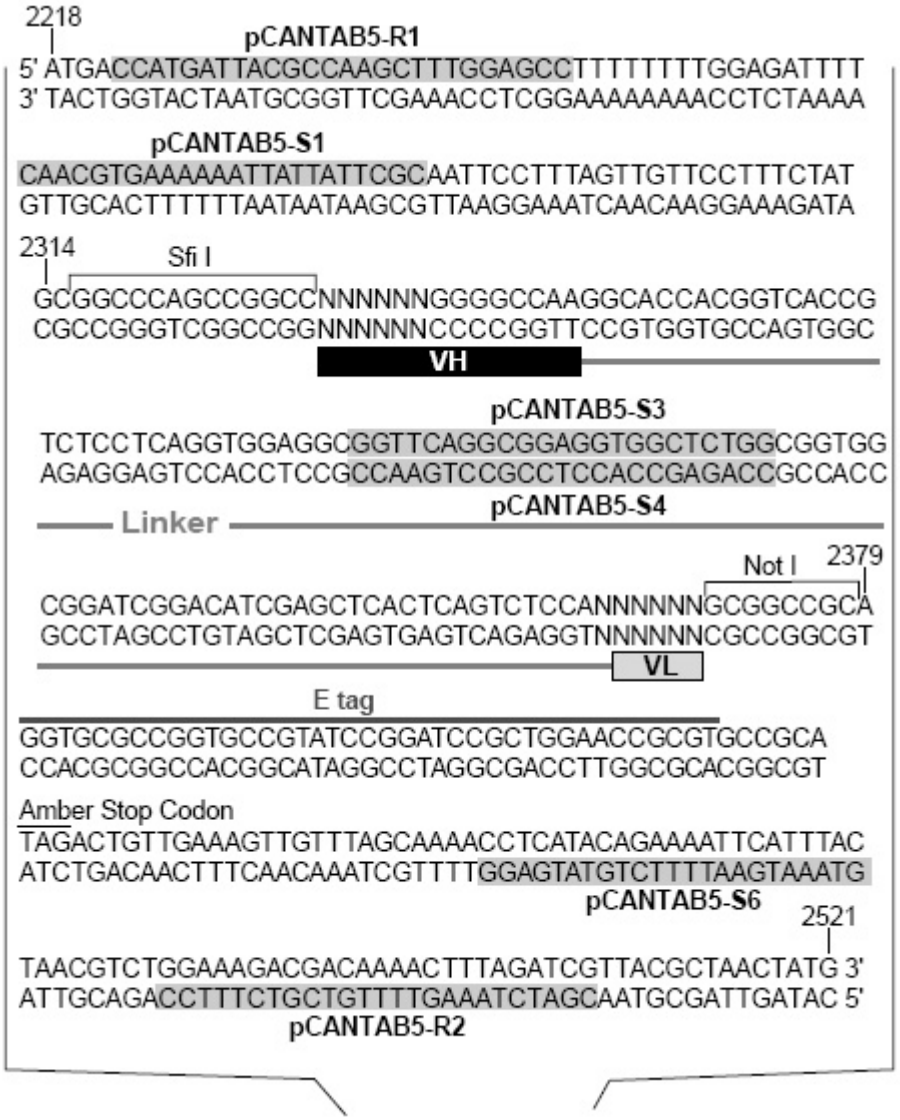


Figure 3.21 Sequence of heavy chain and light chain as part of the pCANTAB 5E vector

Figure 3.20 and 3.21 show the vector map (VH-Linker-VL) depicts the orientation of a hypothetical ScFV fragment cloned into pCANTAB 5 E. Based on Figure 3.21, the clone obtained from the phage antibody screening should be in between pCANTAB5-S1 and pCANTAB5-S6 which should include the heavy and light chain and attached with a linker in between.

3.6 Model of the CymMV candidate antibody obtained from the *Toxoplasma gondii*-immunized single-chain variable fragment (ScFV) antibody phage selection

We decided to build a structure model of the PDPK1 3-phosphoinositide dependent protein kinase-1 [*Homo sapiens*] obtained from the library screening using RASMOL program as shown in Figure 3.22. RasMol as it is a widely used molecular graphics program for visualizing three-dimensional structures of proteins, nucleic acids and small molecules. From Figure 3.22, we are able to predict the molecular structure and potential useful binding sites that might come in handy for further research.

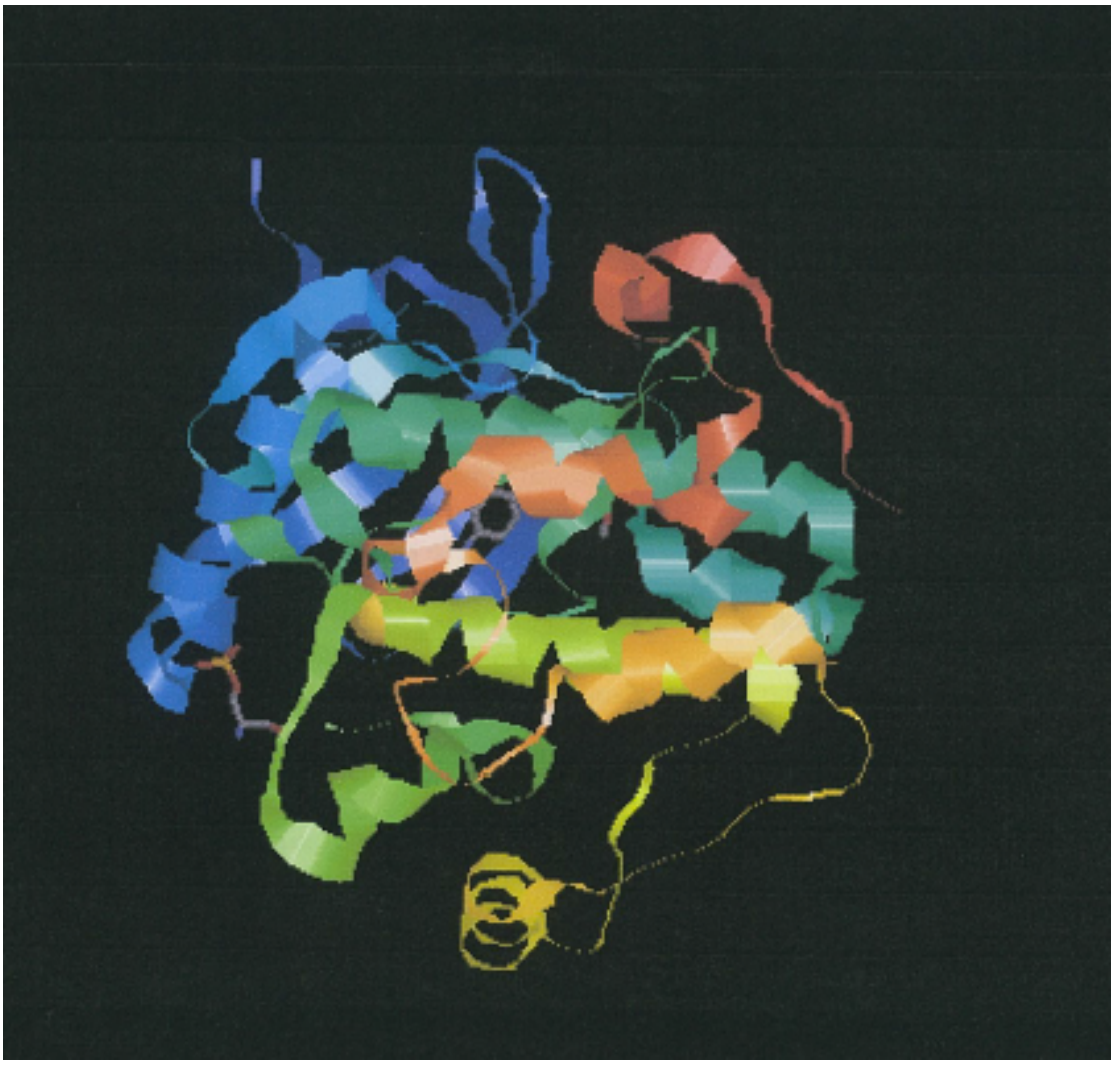


Figure 3.22: Model structure of the Homo sapiens PDK1 gene for 3-phosphoinositide dependent protein kinase 1 using RASMOL model. This is a graphical view (Ribbon Model) representing all the helixes and coils of the model. A-helix represented as a coiled structure and β-strand as pleated structure. Each color represents different chains contain in this structure.

4.0 Discussion

Orchids, amongst the most popular flowers in the world, play a major role in the economy of many Asian countries. However detrimental effects by various viruses can reduce the economic value of this horticultural crop. There have been a number of viruses known to occur in orchids globally with the most common being Cymbidium mosaic virus (CymMV) and Odontoglossum ringspot virus (ORSV). Whilst good plant hygiene and careful management practices by orchid growers can help lessen the spread of these viruses, the pathogens are already prevalent in cultivated orchids worldwide, easily transmitted by mechanical means and are usually asymptomatic during early stages of infection (Jensen, 1950; Lawson and Brannigan, 1986). Current molecular based tests are expensive and often require access to laboratory. Among the possible existing molecular tests, antibody and other ligand based tests are often preferred as they are less expensive and can be developed into user-friendly dipstick type kits. Recently several companies have developed test kits for both ORSV and CymMV such as Mukoyama Orchids in Japan, Forsite Diagnostics (Great Britain) and Agdia Inc (USA). These kits are very efficient as they come in small sizes hence they are mobile and convenient, only require small amount of samples and the results can be obtained within 10 minutes. Similar to the ELISA detection method used in the laboratory, these kits also uses antigen-antibody reaction to detect the presence of viruses where the virus-infected samples will be captured and detected by the specific antibodies that have been tagged with coloured particles (Batchman, 2008).

Liao *et al*., 2004 have established a gene transformation system for Phalaenopsis orchid that is resistance to CymMV. The author had used the post-transcriptional gene silencing (PTGS) at the small interfering RNA (siRNA)-mediated RNA level approach where the construct with CymMV CP cDNA fragment and nos terminator was placed

downstream of a maize ubiquitin promoter and was transformed into Phalaenopsis via particle bombardment. Results showed that although low level of CP mRNA transcript signal was observed in the transgenic orchid plants, CP was not detected and the plants were shown to have enhanced protection against CymMV infection because of RNA-mediated resistance through a PTGS mechanism (Liao, 2004). Later in 2005, the first Phalaenopsis orchid with dual resistance to phytopathogens was reported. This study had managed to confer both CymMV and *Erwinia carotova* resistant traits in Phalaenopsis orchid by double transformation using gene stacking approach. This was done one transformation after another where they initially transformed the orchid with CymMV CP cDNA and then re-transformed for the second round with sweet pepper ferredoxin-like protein cDNA (Pflp) by *Agrobacterium tumefaciens* (Chan, 2005).

These two studies had showed techniques on improving the resistance of CymMV in orchid plants. Since there is an interest in the development of orchid varieties with better resistance to viruses and with improved understanding of the mechanisms involved in RNA mediated gene silencing, we decided to focus our research to develop a multiple strategy approach towards the control of CymMV in orchids by using modified concept of pathogen derived resistance and antibody mediated resistance. There has been concern expressed that any resistance resulting from a single introduced gene might be rather narrow which is specific to only certain virus sub-types and could easily be broken down due to virus evolution. A recent study by Harvey, 2011 proposed that there are multiple layers to RNA mediated defense and counter defense in the interactions between plants and their viruses. They proposed that AGO1 which functions to target cleavage or translational suppression of complementary RNA is RNA silencing as the first layer and in plant RNAi defense and

for some viruses such as cucumber mosaic virus (CMV) and turnip crinkle virus (TCV), this first layer is usually overcome by virally encoded suppressors of silencing that target AGO1. A second layer that involves AGO2 is then activated in the plant as a direct consequence of the loss of first layer of defense thus giving another level of defense and limiting the virus accumulation. This also suggest that different AGO protein may vary in the effectiveness in targeting different viral RNA genomes (Jaubert *et al*., 2011).

Although numerous methods have been applied to control the spread of CymMV, it is believe that none have combined these two different concept of PDR abd antibody mediated resistance into one vector model approach as we proposed, thus making ours a potentially novel system. Through recent study, it was found that the outcome of evolution in plant-virus interaction meant that there is a balance between the ability of the virus to exploit host cellular mechanism and the multiple defense mechanism employed against it by the host. Plant viruses have over the years evolved to express viral suppressors of RNA silencing in response to the pressure applied by RNA silencing of host plant (Diaz-Pendon and Ding, 2008; Alvarado and Scholthof, 2009). A combination of infection by two viruses was shown to be able to broaden the host range, increase virus accumulation and aggravate viral symptoms (Latham and Wilson, 2008). Potato virus X (PVX) was shown to be able to infect Arabidopsis thaliana, which normally a non-host for PVX if being co-infected with a second virus, Pepper ringspot virus. P25 is a critical property of Potato virus X (PVX) VSR as it has the ability to express silencing signal (Voinnet *et al*., 2000; Himber *et al*., 2003; Bayne *et al*., 2005). Previous report showed that P25 is able to suppress silencing induced by RNA hairpin both *N. benthamiana* and Arabidopsis. The ability of PVX to infect Arabidopsis suggests that the P25 protein may not fully function as effective as VSR in the host. It is

suggested that the ability of PVX to infect Arabidopsis might be due to combination of factors together with the inhibition or ablation of silencing that allows it to overcome a threshold required for systemic infection. As it is generally known that dicer protein alone is not sufficient for viral degradation as their vsiRNA products must be included into RISC complex to target RNAs. Through the study by Jaubert *et al*., 2011, they suggested that AGO protein is important as it can limit virus infection rather than playing a secondary role. Based on this, they suggest that AGO2 is the other component that is required for systemic infection as previously noted. This discovery showed the evolution in the virus system whereby the pepper ringspot virus functions as a suppressor of silencing that appears to enable PVX to infect Arabidopsis (Jaubert *et al*., 2011).

In this study, our strategy to increase the durability of resistance was to incorporate multiple resistance genes into a single vector where we aimed to develop molecular tools in controlling Cymbidium mosaic virus in the form of plant transformation constructs. The plant transformation vector pCAMBIA 1304 was used in this study because it contains both GFP and GUS reporter genes. The construct was designed by combining two different research ideas towards developing what we called a pyramid vector. Gene pyramiding involves stacking multiple genes leading to the simultaneous expression of more than one gene in variety to enhance the durability of disease resistance and to broaden the spectrum of resistance capabilities (Joshi and Nayak, 2008 and Ye and Smith, 2008).

Initially the aim was to develop a pCAMBIA 1304-CymMV construct with coat protein (CP) fragment inboth forward and reverse orientation. We used the CymMV CP to construct sequences of RNA that would render a haipin structure of transcribed RNA with the intention of silencing the gene expression of the viral CP in transformed plant via RNAi. This short hairpin RNA (shRNA) would contain a sense and antisense strand as well as a short loop sequence in between to allow folding and complementary base pairing of the sense and antisense fragments. The shRNA was to be cloned into the pCAMBIA 1304 plant transformation vector to allow expression by Pol II type promoter. Expressed shRNA should be cleaved by cellular machinery into siRNA which is then incorporated into the RISC.

Together with the shRNA approach, we also aimed to include an antibody specific to CymMV in the plant transformation vector construct as antibody mediated resistance approach. This approach utilizes the expression of an antibody against the viral protein (Whitelam and Cockburn, 1997) and this concept of exploiting single-chain variable fragments (ScFV) antibodies in plants is in contrast to previous approaches that used parts of the viral genome to confer viral resistance in transgenic plants (Tavladoraki *et al*., 1993). The combination of the two gene constructs (i.e shRNA and ScFv targeting the coat protein of CymMV) thus developing a more efficient CymMV silencing vector. In characterization of the CymMV CP gene, the cloned DNA fragment of about 480 bp was successfully cloned into pCAMBIA 1304 vector and the DNA sequencing results confirmed that the inserted fragment was equivalent to the published CymMV coat protein sequence in NCBI database (accession number X62665). However we observed the pCAMBIA 1304-CymmV cloned obtained were all in the forward (sense) orientation. Even after many attempts, for some unknown reason, we were unable to obtain any clone with the fragment in a reverse or

antisense orientation. Different cloning strategies with several different insert tto vector ratios were employed but we were unable to get the clone with CymMV fragment in a reverse orientation. As it is known that transformation into *E. coli* is an inefficient procedure, however carefully the cell have been prepared with less than 0.01% obtained among 1,000-10,000 transformed cells (Brown, 1998) hence we also tried using commercially available JM 109 competent cells (Promega, USA) with competency of 10^8 cfu/μg, but were still unable to obtain the desired clone. Although we don't believe self ligation was the issue as we still managed to obtain vectors with CymMV fragment in forward orientation, the pCAMBIA 1304 was also treated with alkaline phosphatase (CIP) following digestion to ensure the colonies picked for screening would contain CymMV insert. We also tried to eliminate all the other cloning inefficiency by making sure all of our enzymes and buffers were in good condition, active and usable, as some restriction enzyme digestion procedures can cause digested vectors ends to be incompatible with the inserted fragment, this is due to the fact restriction enzyme sites that are adjacent to the multiple cloning regions or near to the ends can prove difficult to digest completely. Even after all these trouble shooting measures and screening more than 500 colonies we were still unable to obtain the desired clone. A possible reason could be that the reverse fragment might produce toxicity during transformation in *E. coli* thus making it unfavorable to the growth of the bacterial cells. Consequently, causing bacterial with pCAMBIA 1304 that contains reverse CymMV CP fragment to be non-viable.

Due to the difficulties described above, we decided to proceed to transform plant using pCAMBIA 1304-CymMV containing the CP sequences in forward (sense) orientation. This was carried out in orchid leaf and root explants using biolistic bombardment. Analysis of the transient transformation of orchid root and leaf explants with pCAMBIA 1304-CymMV fwd showed that the two maker genes Green Fluorescent Protein (GFP) and β–glucuronidase were expressed in both explants (See Figure 3.8 to 3.11). the two reporter genes β–glucuronidase and mGFP5 gene are both located on the pCAMBIA 1304 vector. GUS assay is extremely sensitive, simple and is a non-radioactive method. The mGFP5 gene is a mutant version of the green fluorescent protein gene from the jellyfish *Aequorea victoria* that provides fluorescent signals that are up to 100-fold brighter than wild-type GFP (Taniguchi *et al*., 2005). In this study, expression of marker genes GUS and GFP indicate that transient transformation was successful except for the GFP expression in the orchid root explants. This could be due to technical problem of the vector as explained by CAMBIA website, where the mGFP5 proteins produced by their initial vectors were quite faint as compared to their improved vectors that use the egfp gene. Another possibility is the short hairpin RNA (shRNA) was cloned upstream of the transcriptional start site of the pol II promoter resulting the siRNA sequence to be present in the mRNA expressing the marker gene thus producing somewhat lower expression of the marker genes. This can be overcome by alternatively positioning the silencing cassette to be upstream of the marker expression cassette to avoid down-regulation of the marker (Tiscornia *et al*., 2008). Reverse transcriptase PCR (RT-PCR) analysis showed that the expression of the pathogen derived resistance sequences was successful (see Figure 3.13). However we would suggest that Northern hybridization to be done in order to provide a more reliable result in confirming that the CymMV CP gene sequence are expressed in these plants. Since transient transformation does not usually lead to a stable event in plants therefore, future studies should include

creating stable transformants. These transformants will then be challenge with CymMV to verify that the construct is able to interfere with viral assembly. This is to be carried out as confirmation that the pathogen derived resistance sequences are expressed.

For second part of this study, we used a *Toxoplasma gondii*- immunized single chain variable fragment (ScFv) antibody phage-display library to select candidate antibody specific to CymMV to be used for an antibody mediated resistance approach. The antibody phage display was chosen as it is the most successful *in vitro* antibody selection technology (Schirrmann *et al*., 2011). It allows identification of immunogenic proteins such as from pathogens most notably of *Mycoplasmaspp hyopneumoniae*, a pathogen that caused respiratory diseases (Kügler *et al*., 2008; Naseem *et al*., 2010; Miltiadou *et al*., 2009). So far many examples of antibodies derived by phage display application are available (Meyer *et al*., 2011; Pitaksajjakul *et al*., 2010 and Schütte *et al*., 2009) as one advantage of antibody phage display is the direct access to the genetic information of the binder, allowing a fast adaption of the antibody system such as IgG, ScFv-Fc, biotinylated antibody or even ScFv-phoA fusion (Hust *et al.,* 2011).

The cymbidium mosaic virus was propagated in *Cucumis sativus* (cucumber plants) as they are susceptible to the virus and fast growing compared to the orchid plants. ELISA test confirmed the presence of CymMV in the infected plants (see Table 3.1). Purified CymMV has become antigen of choice to screen the antibody phage-display library via biopanning experiment due to its antigencity (Chua, 2002). Biopanning is referred to as affinity selection process where repertoires of the antibody fragment obtained from an antibody combinatorial library are displayed on the surface of the filamentous phage. This technique can also be used in peptide library for peptide selection based on affinity as well. This approach was selected rather than using just the

viral coat protein as an antigen as its shown to be time-saving, provides stronger immunogenicity and problems with PCR introduced mutations during coat protein gene cloning can be eliminated (Michael *et al*., 1998; Ooi, 2006; Buckler *et al*., 2008; Plummer, 2011).

Previous study by Ooi, 2006 described the identification of peptide with specificity for CymMV. The author also used a biopanning technique against the whole CymMV viral particle as an antigen, but this time in selecting peptide from a commercial 12-mer random peptide display library. The authors revised the solution phase panning method by using streptavidin coated superparamagnetic beads to enable a more efficient biopanning technique as compared to immobilized target panning. Solution phase panning has several advantages as a significantly higher number of plaque forming units were recovered and it permitted a higher concentration of target ligand to be used a s compared to the conventional method. They managed to isolate several peptides with positive binding to both CymmV and ORSV and one out of the seven peptides obtained was found to be specific to CymMV.

In the current study where an antibody phage-display library was used, a total of 18 colonies representing candidate anti-CymMV antibodies were selected after three rounds of biopanning. However out of these 18 clones, only four ScFv showed an average binding ratio of more than 3.00 after being tested by ELISA with CymMV as the antigen. We decided to select these four clones (A, B. C and D) for further analysis as those with average binding ration less than 3.00 do not show sufficient binding specificity to the antigen as suggested based on model experiment in measuring the performance of the phage-display system where the set-up mimic the situationoccurring in the various stages of panning genuine library (McCafferty, 1990; Kay *et al*., 1996).

Interestingly, the nucleotide BLAST and BLASTX results of the candidate antibody showed high similarity to Homo sapiens PDPK1 gene for 3-phosphoinositide dependent protein kinase 1. PDPK1 as is commonly known is from the family of protein kinases is thought to play a general role in signaling processes and in development of human. However this construct might be different from the wild-type gene and any similarity between the ScFv sequence in this study might be coincidental and without functional relevant. A structure model was build based on the deduced protein sequence using RASMOL. We would suggest that a further investigation to be done by predicting the structure and to do homology modeling against antibody database such as Rosetta Antibody Modeling that is available online. Protein docking would be another step that we are looking at as well to study the protein-protein interaction and the 3-D structure and to predict the orientation and position of the antibody. Since both nucleotide BLAST and BLASTX produced the same results it is interesting to see the relation between CymMV and the anti-CymMV isolated from the antibody library. Further investigation such as assays would be required for verification before proceeding further.

Apart from that we could also proceed with our initial aim to include the anti-CymMV obtained from the biopanning to be pyramided into our constructed plant transformation vector pCAMBIA 1304-CymMV-fwd in order to build the pyramid vector. We could also be doing functional studies to test the expression of the pCAMBIA 1304-fwd-anti CymMV in a plant system to confirm that the construct able to interfere and thus producing resistance towards CymMV.

5.0 Conclusion

Viruses are constantly infecting plants and once infected they can never be cured. These plant viruses have caused devastating diseases to food crops and of valuable horticulture plants like orchids. There have been a number of viruses known to occur in orchids globally with the most common being Cymbidium mosaic virus (CymMV) and Odontoglossum ringspot virus (ORSV) thus it is important to develop orchid varieties with improved resistance to viruses. Various strategies have been applied to the development of virus resistance plants and coat protein (CP) mediated protection is proving to be one of the most effective tools to provide resistance towards a particular pathogen. Nevertheless, a single construct mediated viral resistance has high chance to be broken down as viruses mutate and escape resistance. Thus in this study it was aimed to include and antibody mediated resistance approach to be used alongside the pathogen derived resistance strategy.

This thesis aimed to develop molecular control of CymMV using the said concepts and will eventually combined in order to develop pyramid vector with resistance to CymMV. We managed to achieve good preliminary data that has a lot of potential with the CP construct successfully introduced into plants and the isolation of an anti-CymMV ScFv with affinity to the virus. However a more comprehensive testing is required before this model can be of general use.

6.0 References

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