CHAPTER 1: INTRODUCTION

Erythropoietin or *EPO* is a glycoprotein hormone composed of four alpha helical bundles and is found in the plasma. It is produced primarily by the kidney in adults and by the liver during fetal life (Lin *et al.,* 1985). It promotes red blood cell formation by promoting erythroid differentiation and initiates haemoglobin synthesis. Any inadequate production of erythropoietin or an impaired ability of the erythron to respond to erythropoietin, results in anaemia (Macdougall and Eckardt, 2006).

Recombinant human *EPO* has been synthesised to help in the correction of renal anaemia and also in other types of anaemia such as anaemia of cancer, chronic diseases or critical illness. Genetic engineering techniques have enabled the production of recombinant human erythropoietin and this was first successfully carried out in the Chinese hamster ovary cells (Lin *et al.,* 1985). However, there are a few patients who have developed neutralizing antibodies against the recombinant *EPO*. Another problem faced in the production of recombinant *EPO* is the difficulty in producing effective generic or biosimilar products. The production of recombinant *EPO* in mammalian cells has also many other disadvantages such as the high cost incurred in the production, concerns about product safety and public acceptance of the recombinant product (Rishi *et al.,* 2001).

Plants are potentially ideal hosts for the expressions of human genes as they are able to produce proteins in large scale at low cost. They are also capable of performing post-translational modifications on the protein synthesised and has been shown to have very high and controlled levels of expression (Rishi *et al.,* 2001). Plants are also excellent hosts for human proteins as they are free of contaminants such as animal viruses and prions (Ban *et al.,* 2004). Plants also provide storage and distribution convenience and

its production can be carried out with the use of renewable resources. Furthermore, there is less concern about product safety and public acceptance (Rishi *et al.,* 2001).

In this study, the human *EPO* gene will be transformed and expressed in banana meristems. Bananas are monocotyledonous herbs growing in humid and sub humid tropical countries. Genetic transformation of bananas has been carried out effectively using *Agrobacterium* mediated transformation (May *et al.,* 1995; Ganapathi *et al.,* 2001), particle bombardment (Sagi *et al.,* 1995; Wong *et al.,* 2005) or electroporation (Sagi *et al.,* 1994). Due to the success of genetic transformation in bananas, gene expression of pharmaceutical proteins can also become a possibility using this crop. Sunil *et al.,* (2005) successfully carried out the expression of hepatitis B surface antigen in transgenic banana plants. This achievement has suggested the real possibility of expressing more pharmaceutical proteins in banana.

1.1 Objective

Plants have showed high potential as biopharming factories. Since the use of erythropoietin is vital in patients with anaemia or anaemia associated diseases, the production of the human *EPO* gene in plants would prove to be a good approach as there are many advantages in the production of recombinant proteins in plants. Therefore, the objective of this research is to express the human *EPO* gene in banana (*Musa acuminata*). The specific objectives are to carry out transformation experiments and to analyse the resulting transformants.

CHAPTER 2: LITERATURE REVIEW

2.1 Micropropagation and genetic transformation

2.1.1 Plant tissue culture

In vitro culture can be defined as the multiplication of plants under sterile (aseptic) conditions, on or in a specific nutrient medium (Preece and Read, 1993). This approach has been used to make *in vitro* propagated plants as model systems in various studies of physiological, biochemical, genetic and structural problems related to plants. Giles and Friesen (1994) also stated the other objectives of micropropagation or *in vitro* culture of plants besides multiplication are the elimination of pathogens, rapid introduction of new or novel genotypes, selection of somaclonal and induced variants and preservation and long term storage of germplasm. The most recent application of tissue culture is its use as explant material for genetic transformation of plants. *In vitro* culture of meristem can be divided into four distinct stages and these include explants establishment, axillary shoot proliferation, pretransplanting and acclimatization to ambient conditions (Preece and Read, 1993).

Plant tissue culture or *in vitro* culture of plants accelerates the process of plant breeding and coupling this with genetic engineering will enable the introduction of useful genes, either for the improvement of the plant itself or for the production of many other useful products for all living things. Hence the availability of a good *in vitro* culture and regeneration system is a prerequisite of most transformation experiments.

2.1.2 Banana and its tissue culture system

Bananas (*Musa* spp.) are monocotyledonous, perennial herbs, cultivated in humid and subhumid tropical regions. They are cheap and are an easy source of energy. They are also rich in certain minerals and in vitamins A, C and B6. Bananas can grow in a range of environments and produce fruit throughout the year. In Malaysia, banana has been traditionally cultivated in smallholdings and also in intercropping systems (Frison *et al.,* 1998; Strosse *et al.,* 2006; Sreeramanan *et al.,* 2006).

Bananas are more than just fruit crop; they are also a source of revenue especially in rural communities. The banana industry in Malaysia has the potential to become one of the most profitable fruit commodities in the future. This is marked by the increase in demand and consumption which is expected to generate an increase in production to over 400 000 tones by the year 2010 (Rohizad, 1998).

Banana belongs to the family Musaceae with 2 major genus; *Musa* and *Ensete*. Musa consists of 4 sections and these include Australiamusa, Callimusa, Eumusa and Rhodochylamys with Eumusa being the biggest and geographically most widely ranged section of the genus (Jalil *et al.,* 2001).

Musa species have different ploidy levels which offer the opportunity to gain insights in crop productivity that often accompany polyploidy. Musa species have a number of autopolyploids (AAA, AAAA, AAAAAA) and different types of allopolyploid (allotriploids AAB, ABB and allotetraploids AABB, AAAB) in addition to the diploid *M. acuminata* (AA) and *M. balbisiana* (BB) and AB hybrids.

The tissue culture system can help overcome many problems faced by banana conventional breeding techniques. Using tissue culture techniques, the rate of

multiplication can also increase rapidly. In the tissue culture of bananas; both organized and unorganized growth are possible (George, 1993).

In the organized *in vitro* growth of banana tissue it is limited to embryo and shoot tip culture. Embryo culture is an important aid for classical breeding of banana and major applications of shoot tip culture are mass clonal propagation and germplasm conservation (Strosse *et al.,* 2001). Shoot cultures of banana start conventionally from any plant part that contains a shoot meristem, i.e. the parental pseudostem, small suckers, peepers and lateral buds (Vuylsteke, 1989). Shoot tips consists of the apical dome covered with several leaf primordial and a thin layer of corm tissue. Rapid multiplication can be obtained using shoot tip cultures. However it has higher susceptibility to blackening and contamination. Meristem culture of bananas on the other hand has the potential of eliminating diseases particularly viral diseases from the plant propagating material. Meristem explants can be obtained by reducing the size of the cube of tissue obtained from suckers to 0.5 – 1mm leaving a meristematic dome with one or two leaf initials (Strosse *et al.,* 2001).

The establisment of embryogenic cell cultures on the other hand is the *in vitro* culture of unorganized tissue in banana. The embryogenic suspensions have high regenerating capacity and can be used for mass clonal propagation and are the only source of regenerable protoplasts in banana. Embryogenic cell suspensions are the most preferred target material for induced mutations and genetic engineering (Strosse *et al.,* 2006; Jalil *et al.,* 2001). Embryogenic cell suspensions of bananas are obtained from different explants sources such as zygotic embryos (Cronauer and Krikorian, 1985; Escalant and Teisson, 1989), rhizome slices and leaf sheaths (Novak *et al.,* 1989), immature female flowers (Côte *et al.,* 1996; Grapin *et al.,* 1996) and multiple meristem cultures (Dhed'a *et al.,* 1991; Schoofs, 1997; Davies, 1995).

2.1.3 Monocot gene delivery system

Monocotyledonous plants are more recalcitrant to genetic transformation. The genetic transformation of monocot plants have been of great interest due to the benefits of crop improvement and human nutrition in these plants. The common method of using *Agrobacterium* mediated transformation has been proven to be unreliable and only some monocots are susceptible to this method of foreign gene delivery (Vain *et al.,* 1995). This is because the lack of binding of *Agrobacterium* to plant cell walls, reduced activity of T-DNA promoters, inhibition of *vir* gene induction and abnormal auxin - cytokinin balance in monocotyledonous cells (Murray, 1993). The first transgenic cereals were obtained from direct transfer of DNA (electroporation, PEG treatment) into regenerating plant protoplasts. This experiment was carried out in maize by Horn *et al.*, (1988) and in rice by Zhang and Wu (1988). However, these days; the particle gun technology is used to obtain nearly all genetically engineered cereals and other monocots as well. This technology was adapted in the transformation of rice (Cao *et al.*, 1990; Christou, 1994), maize (Gordon-Kamm *et al.,* 1990), oat (Somers et al., 1992), sugarcane (Bower and Birch, 1992) and in banana (Sagi *et al.*, 1995).

There are other gene delivery methods for monocotyledonous species. These include macroinjection which is the mechanical injection of naked DNA, the uptake of free DNA into protoplasts by physical (electroporation) or chemical (PEG) permeablility of the plasmic membrane (Lörz *et al.*, 1985; Potrykus *et al.*, 1985; Fromm *et al.,* 1986). *Agrobacterium* mediated transformation of monocotyledonous plants is also an efficient method of gene delivery for certain explants and under specific conditions. This method has successfully transformed asparagus (Bytebier *et al.,* 1987; Delbreil *et al.,* 1993), wheat (Mooney *et al.,* 1991), rice (Raineri *et al.,* 1990) and bananas (May *et al.,* 1995; Wong *et al.,* 2008).

2.1.4 Direct genetic transformation

Direct genetic transformation is the introduction of transgenes with appropriate *cis*-regulatory elements into the host plant cells. The DNA used in such transformations can be agrobacterial plasmids (i.e. Ti plasmids) that are engineered, or any other DNA in the form of plasmids or linearized DNA that include promoters, coding regions, marker genes, terminators, etc. This method of direct transfer of a recombinant DNA is very useful for transient expression. If early detection of transient expression is required then this form of transformation can be exploited. The transgene can be transcribed in the nucleus and translated in the cytosol without the integration of the transgene into the nuclear genome which occurs in *Agrobacterium* mediated transformation. This form of transient expression can be useful for molecular genetic studies and for the production of valuable proteins which can be harvested shortly after direct transformation (Galun and Breiman, 1997). There are many methods in which direct genetic transformation can be carried out and these include with the use of polyethylene glycol, electroporation, particle gun bombardment and macroinjection.

2.1.5 Particle gun bombardment

John Sanford and his team brought about the technology of particle gun bombardment which is the delivery of naked DNA into intact plant cells in the year 1987. (Sanford *et al.,* 1987). This invention paved the opportunity of genetic transformation in plant species which were difficult to be transformed using the common method of *Agrobacterium* mediated transformation and the insertion of foreign genes into the cell's organelles a possibility. The biolistic approach is especially well adopted for transient expression.

Barton (1997) defined biological ballistics or in short biolistic as the process by which biological molecules such as DNA and RNA are accelerated on microcarriers with the help of gun powder, compressed gas or other means. The biological molecules are driven at high velocity into the target which are plant cells or organised plant tissues such as meristems.

Over the years, the biolistic gun went through a series if changes such as from the use of gun powder to a safer helium system, etc. The first application of the biolistic process was made by its inventors (Sanford, Klein, Wolf and Allen) in collaboration with Wu (Klein *et al.,* 1987). This application intended to show that RNA and DNA can be carried into epidermal cells of onion. Then, also using the biolistic method, reports of successful transformation of chloroplasts (in *Chlamydomonas*) and yeast mitochondria became evident (Boynton *et al.,* 1988; Johnston *et al.,* 1988).

High rates of DNA expression in the transformation of plant cells or organised plant tissues can be obtained by optimizing parameters involved in biolistic transformation. These parameters include; microparticle variables such as size, amount and type of microparticle, accelerator parameters such as helium pressure, the distance between the rupture disk and macrocarrier, the distance between the macroprojectile and the stopping screen and the distance between the stopping screen and the biological target (Heiser, 1995).

Stable expression of the foreign gene may be vital but the particle gun bombardment method of transformation allows transient expression in a wide range of tissues and plants of any variety, genus and species making this an ideal choice of transformation for preliminary expression studies as well as the study of the host system for molecular pharming.

Stable transformantion may also be possible with the application of particle bombardment. This is possible with the use of target materials such as somatic embryo cell suspensions which are single cell structures that can be developed into a whole plant. The use of single cell tissue materials can bring about the stable integration of the recombinant DNA into the plant's genome.

2.2 Molecular pharming

2.2.1 Protein expression in plants

'Pharming' refers to the use of plants and animals in the production of recombinant therapeutic proteins (Primrose *et al.,* 2001). These therapeutic proteins are produced in expression systems that are able to perform post-translational modifications which are vital for the functionality of the protein (Dupas *et al.,* 2007). Besides this, the expression system needs to be able to produce mass amounts of the protein which is biosimilar for human consumption. This is the main reason why the mammalian expression systems are currently the preferred host for therapeutic protein production. However large scale culture of animal cells is expensive due to the amount of medium and serum required. It is also necessary for precise and constant growth conditions of the animal cells. Recombinant protein expression in animal cells with animal pathogenic contaminations makes the recombinant protein unsafe for human consumption. Mammalian cell lines have also not been shown to perfectly reproduced the human-type glycosylation. And finally, the purification of recombinant protein in animal cells requires high cost and this makes the large-scale production of recombinant proteins in animal cells an inefficient approach (Primrose *et al.,* 2001; Ko and Koprowski, 2005; Dupas, *et al.,* 2007).

Recombinant protein expression in plants has proven to be a superior approach as there are many advantages and these include; well established protocols for cultivation and downstream processing of plant products, easy scale-up industrial production levels as compared to the use of bioreactors and fermentation systems, low health risks from human pathogen and toxic contaminations and efficient post-translational glycosylation modifications. The other advantages to plant derived therapeutic proteins include convenient storage, potential elimination of hospitals and health professionals for the delivery of the therapeutic proteins and also the use of renewable resources for their production (Daniell *et al.,* 2005; Ko and Koprowski, 2005). Recombinant therapeutic proteins can also be targeted into particular organs and tissues such as seeds and the fruit or in intracellular compartments such as the endoplasmic reticulum and chloroplasts. Additionally, the amount of recombinant product that can be produced in plants approaches industrial-scale levels which make this expression system an ideal choice for the production of therapeutic recombinant proteins (Daniell *et al.*, 2001).

2.2.2 Banana as a host system

Slater *et al.,* (2003) mentioned that bananas would be an ideal plant choice for the production of recombinant therapeutic proteins as it is grown widely in the countries of the developing world and the fruits can be eaten raw. Warzecha and Mason (2003) stated that there are advantages and disadvantages to the production of recombinant proteins in bananas. The advantages of this expression host is that it can be clonally propagated, there is low potential for outcrossing in field and once its breeding is established abundant and inexpensive fruit is available on a 10-12 month cycle. The potential disadvantage of this host system is it has an inefficient transformation system,

little data is available on gene expression especially for fruit specific promoters and high cultivation space is required and this may increase in the cost production of the recombinant protein.

2.2.3 Protein targeting in plant organelles

Protein targeting in organelles are important for the production of active proteins. The protein targeting processes is the basis of maintaining structural and functional integrity in the cell enabling the various subcellular compartments to carry out their unique metabolic roles (Slater *et al.,* 2003; Galun and Breiman, 1997). For intracellular targeting, it is important to engineer the recombinant DNA with a particular targeting sequence that will target the recombinant protein to the respective organelle. Recombinant therapeutic protein targeting eases down-stream processing of the recombinant protein thus reducing the production cost of the protein. Wandelt *et al.,* (1992) stated that the yield of a target protein can be increased by directing the soluble proteins to the specific tissues.

Glycosylation is the covalent linkage of an oligosaccharide side chain to a protein and it is the most widespread post-translational maturation found in both natural and biopharmaceutical proteins. Many human proteins are glycosylated and their functions depend on particular glycoforms (glycans) which affect their plasma half-life, tissue targeting and/or biological activity. Therefore, many biopharmaceutical are to be produced in expression systems with *N*-glycosylation capability. Mammalian cells are the preferred host for therapeutic protein production due to the many advantages of its system however; the cells can never perfectly reproduce the human-type glycosylation (Dupas, *et al.*, 2007).

Plants are able to perform most of the post-translational maturation required for protein activity however, just like the other expression systems, they are unable to perfectly reproduce human-type glycosylation. In plant cells, protein *N*-glycosylation starts in the endoplasmic reticulum (ER). This is the main reason why considerable progress has been made in the glycoengineering of plant-made-pharmaceuticals by targeting the biopharmaceuticals to the ER. However, it is vital to eliminate or minimize plant-specific post-translational modifications to obtain humanized *N*-glycans on plant-made-pharmaceuticals (Dupas, *et al.,* 2007).

2.2.4 Endoplasmic reticulum (ER) targeting of proteins in plants

The plant ER is a complex network of cisternal and tubular structures containing a single internal space (ER lumen). This organelle changes in organization during differential or environmental stress. In seeds of some plant species, the ER is a site of aggregation and accumulation of some classes of storage proteins. The other roles of the ER include anchoring the cytoskeleton, communication between the exterior of the cell and the cytoplasm and communication between contiguous cells of the plant bodies (Galun and Breiman, 1997).

Proteins destined for transport along the secretory pathway are synthesised on ribosomes associated with the ER. The organelles that are in the secretory pathway include the ER, the Golgi complex and the vacuoles. The ER contains proteinaceous structures embedded in their membranes that allow proteins (hydrophilic surface) to pass through without contacting the surrounding hydrophobic lipids in the membrane surface. Co-translational translocation is used for proteins that enter the ER. In this type of translocation, the nascent protein may associate with the translocation apparatus

while it is still being synthesised on the ribosome. It is also observed that in this association, the ribosome is localised to the surface of the ER. The ribosomes are referred to as membrane-bound (Lewin, 2004).

If the proteins are required to reside within the reticuloendothelial system of the ER it needs to be able to recognise the translocation apparatus. This requires the signal for co-translational translocation to be a part of the protein synthesised and once the protein is in the ER, the signal sequence is cleaved. The best defined retention signal for the ER and Golgi proteins is the tetrapeptide His/Lys-Asp-Glu-Leu (HDEL or KDEL) which is present at the C terminus of soluble resident ER proteins such as protein disulfide isomerise (PDI) and BiP (Tillmann *et al.,* 1989; Napier *et al.,* 1992).

2.3 Erythropoietin (*EPO*)

2.3.1 Introduction to erythropoietin

Erythropoietin (*EPO*) a glycoprotein is a principal cytokine involved in the regulation and maintenance of a physiological level of circulating erythrocytes (red blood cells) (Matsumoto *et al.,* 1995). In the human fetus, the liver is the main site of *EPO* gene expression. After the 30th week of gestation renal *EPO* gene expression, the *EPO* production site switches from the fetal liver to the kidneys in adults (Dame *et al.,* 2000). This glycoprotein is maintained in the circulation at a concentration of ~15-30 milliunits/ml of serum or about 0.01 nM under normal physiological conditions (Lin *et al.,* 1985).

According to Ban *et al.,* 2004, the genomic DNA encoding for the human *EPO* gene is 5.4 kb in size and is composed of four introns and five exons which encodes 193 amino

acids. This gene is located on chromosome 7 (7pter-q22). The human *EPO* is heavily glycosylated and carbohydrate comprises of 40% of the total molecular weight of *EPO*. The *in vivo* metabolic fate of this hormone depends highly on the carbohydrate portion of this protein. Mature human *EPO* has three N-linked oligosaccharides at the amino positions 24, 38 and 83 in a total of 166 amino acid residues (Matsumoto *et al.,* 1995). Matsumoto *et al.,* (1995) also mentioned that the N-linked oligosaccharides play an important role in the *in vivo* activity of *EPO* through elongation of its half-life in the circulation but are not necessary for the *in vitro EPO* activity.

Anaemia results from an imbalance in the rates of red-cell loss and formation due to inadequate production of erythropoietin, absence of co-factors needed for red-cell formation or an impaired ability of erythron to respond to erythropoietin. Anaemia of chronic diseases was found in association with malignancy, AIDS and long-standing rheumatoid arthritis (Tabbara, 1993). Although anaemia is a frequent complication of many diseases, its clinical relevance and the necessity and value of its therapeutic correction have long been neglected (Macdougall and Eckardt, 2006). Treatment with recombinant erythropoietin was found to be beneficial in patients with these disorders thus bringing to a need of producing erythropoietin at a large scale.

Many pharmaceutical companies worked have been focused on the production of recombinant *EPO*. This has resulted in epoetin a recombinant human erythropoietin expressed in Chinese hamster ovary cells (Winearls *et al.,* 1986; Eschbach *et al.,* 1987). However several patients who were treated with epoetin developed neutralising antibodies against the recombinant protein and there was also the problem of producing biosimilar epoetin. Injection with epoetin was to be administered two to three times a week which was considered as tedious. This led to the development of another recombinant erythropoietin, Darbepoetin alfa (Egrie and Browne, 2001). This product

had longer-acting erythropoietin analogues that would retain the biological properties of erythropoietin, but require less frequent dosing.

2.3.2 Expression of the *EPO* gene

The *EPO* gene was first expressed in mammalian cells such as COS-1 cells by Jacobs *et al.,* (1985) and in Chinese hamster ovary cells by Lin *et al.,* (1985). Although erythropoietin was found to be biologically active *in vitro* and *in vivo*, but the high cost of production and purification incurred was the downside of this expression system. The *EPO* gene was also introduced in other expression systems such as in insect cells (Quelle *et al.,* 1989), bacteria (Lee Huang, 1984) and in yeast (Elliott *et al.,* 1989).

Expression of recombinant *EPO* in the mammary glands has been carried out but only in small mammals such as mice and rabbits. Toledo *et al.,* (2006) however demonstrated the expression of *EPO* in mammal livestock. This was carried out with the direct transduction of the mammary epithelium of goats with an adenoviral vector carrying the human *EPO* cDNA which observed the expression of the recombinant protein at levels up to 2 g/L. Milk-derived human *EPO* could also be purified with a high efficiency. However, its hematopoietic activity, which was analysed in mice with normocytic anaemia, was very low. This low *in vivo* hematopoietic activity is probably due to the noticeable low sialylation in the glycans on the milk-derived h*EPO*.

In the plant system, the *EPO* gene was first expressed in cultured tobacco BY2 cells via *Agrobacterium tumefaciens* mediated transformation. It was found that the *EPO* produced exhibited biological activities *in vitro* and not *in vivo* (Matsumoto *et al.,* 1995). The production of recombinant *EPO* was also observed in whole plant systems such as in tobacco and *Arabidopsis* plants. The over expression of the *EPO* gene was

then found to be detrimental to the vegetative growth and male sterility in both plants (Ban *et al.*, 2004). Even under these circumstances, the production of recombinant *EPO* is found to be very promising in plants if the appropriate system and condition can be found.

2.4 pCAMBIA 1304

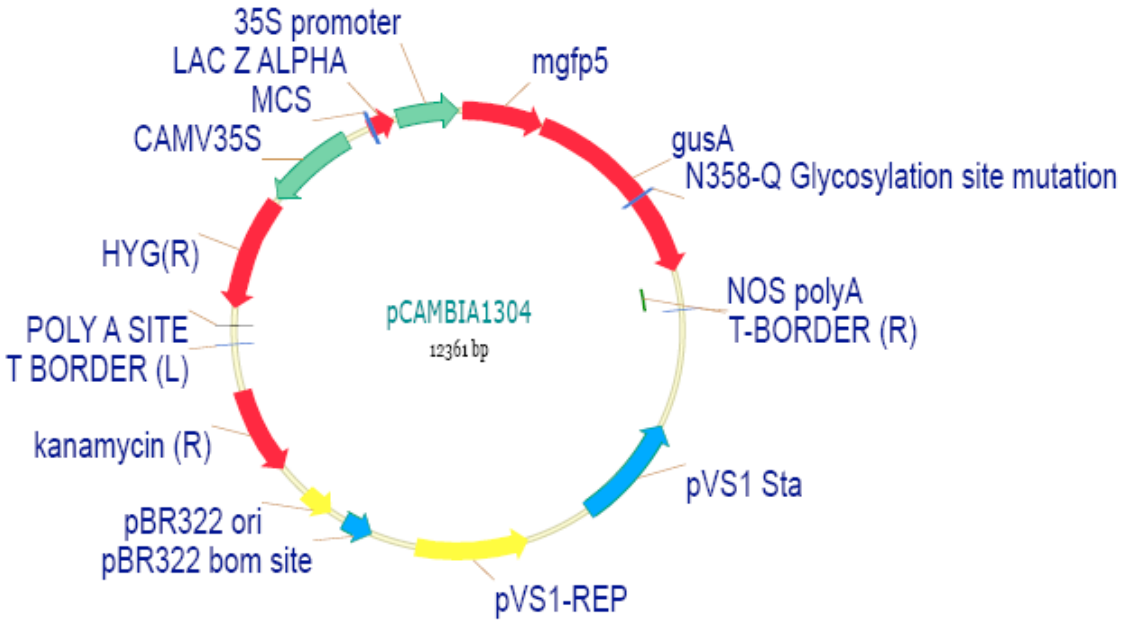

Figure 2.1: pCAMBIA1304 plasmid construct (http://www.cambia.org)

The small, versatile pPZP family of *Agrobacterium* binary vector for plant transformation is used in this study. This plasmid utilises hygromycin resistant for the selection of transformed plants and kanamycin resistant for bacterial selection. The reporter gene that is attached in this plasmid is a fusion of *mgfp5:gusA*. For ER targeting in the explants, the *EPO* gene is attached to the KDEL sequence and is infused between the 35S promoter and the *mgfp5*. In plasmids which are non-targeting, only the

EPO is ligased into the construct. The gene coding for green fluorescent protein (GFP) was incorporated into the plasmid pCAMBIA1304 as a reporter gene.