DETECTION OF PROTEIN- PROTEIN INTERACTIONS *IN PLANTA* BY BiFC AND SPLIT LUCIFERASE ASSAYS

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Tiivistelmä — Referat — Abstract

Molecular biology has created a new pathway for plant breeding in cut flower industry. It focuses on studying flower gene functions and provides a more direct and effective way of breeding new flower cultivars using genetic transformation. Besides flower color, disease resistance, quality and vase life, modification of flower architecture is an important target for flower breeding. Previous studies have showed that various transcription factors encoded by the corresponding genes are involved regulating flower development and flower architecture. The most studied are MADS domain and TCP domain transcription factors. For targeted breeding, it is crucial to study the functions of the corresponding genes in detail. For both MADS and TCP domain proteins, previous studies have indicated that protein-protein interactions are important for their function.

GhCYC1, GhCYC2, GhCYC3 and *GhCYC4,* isolated from gerbera (*Gerbera hybrida*), are *CYCLOIDEA* –like genes affecting inflorescence development. The protein-protein interactions among these four genes have previously been studied by yeast two-hybrid system. The aim of this thesis was to verify the interactions in living plant cells, using both BiFC and split luciferase assays. Protoplast electroporation and agroinfiltration were used to introduce the genes *in planta.* The results from the two assays were compared in order to find an effective *in planta* method for detecting protein-protein interactions. The experiment also provided information about DNA transformation efficiency using protoplast electroporation and agroinfiltration.

The results of the split luciferase assay showed that GhCYC1+GhCYC4, GhCYC3+GhCYC4 as well as GhCYC4+GhCYC4 interacted quite strongly in plant cells while GhCYC1+GhCYC1, GhCYC2+GhCYC2 as well as GhCYC4+GhCYC2 had almost no interactions. The interactions between GhCYC3+GhCYC4, and GhCYC4+ GhCYC4 were also shown in yeast two-hybrid, but the other results were different. According to the BiFC assay, no signals of interactions were detected from GhCYC2+GhCYC2, while strong signals were observed from GhCYC2+GhCYC3, and weak signals were seen from GhCYC2+GhCYC4. The interactions between GhCYC2+GhCYC3, GhCYC2+GhCYC4 were also observed in yeast two-hybrid, but the other results were unconfirmed. Large standard deviations were observed in the split luciferase assay and thereby reliable conclusions cannot be drawn from it. However, BiFC turned out to be a better method to detect the protein-protein interactions *in planta* and clear signals from interactions could be observed. Comparison of the transformation methods indicated that agroinfiltration is a better way of introducing DNA into plant cells than protoplast electroporation. For further study, BiFC assay still needs to be repeated to confirm the efficiency of this assay, and factors affecting the transformation efficiency in protoplast electroporation need to be optimized in the future studies.

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1 INTRODUCTION

1.1 Molecular breeding of cut flowers

1.1.1 Global situation of molecular breeding of cut flowers

The global flower industry has developed rapidly in recent years and cut flowers account for one third of the global ornamental horticulture market. Altogether, 50% of the cut flower market is made up of roses, chrysanthemums and carnations (Tanaka et al., 2005). Producing new and popular varieties is the first and foremost target of flower industry. Domestication of wild species and breeding by crossing and selection are two primary methods to acquire new varieties. However, the disadvantages of these two methods are gradually becoming obvious as compared with genetic engineering. The domestication of wild species and classical breeding are slow processes. For example, it would take at least a thousand years for cereals to have some obvious morphological changes (Gepts, 2002). However, one of the main disadvantages of domestication and classical breeding is that the gene pool of one species is too limited for all genes to be found in it, which is also the reason why there are no natural orange petunias and blue roses (Mol et al., 1995).

Biotechnology, including genetic modification and tissue culture, has been widely applied in flower production industry. Tissue culture techniques have greatly contributed to mass production of ornamentals while genetic modification technology offers great advantages for modern plant breeding. The best example is the 'moon' series carnation, which is a new violet carnation cultivar produced by genetic transformation. It has great markets in North America, Australia and Japan (Tanaka et al., 2005). In European Union, however, area of commercial production of genetically modified plants is much smaller, compared with the U.S. Until 2001, the total area in the EU for genetically modified plant growth was just a few thousand hectares, which was merely 0.03% of the global GM plant production (Brandt, 2003). However, the 'moon' series carnation can also be found in many European countries since first introduced in Australia in 1996 (Mol et al., 1999).

1.1.2 Applications of molecular breeding in ornamentals

Molecular breeding has been applied not only to modify flower colour, but also many other important traits in flowers. A longer vase life is one of the most important features of a high-quality cut flower. Together with flower colour, vase life is also highly demanded by the consumers (Mol et al., 1999). The synthesis of ethylene is a crucial factor in senescence of some cut flower species. In the pathway of ethylene synthesis, ACC (1-aminocyclopropane-1-carboxylic acid) is very important and will be converted to ethylene after oxidization with the help of ACC oxidase. The vase life could be extended through a successful suppression of the activity of ACC oxidase and ethylene could not be synthesised anymore. In the experiment of Bovy and the co-workers, carnations without ACC oxidase activity were successfully produced and the vase life was twice the length of that of the wild type (Bovy et al., 2004).

Disease has always been a significant problem in production of any plant species. Today, disease resistant plants including various cut flowers can be produced with the help of modern gene technology. For example in rose, blackspot is one of the most troublesome diseases, and is caused by the fungus *Diplocarpon rosae*. The major component of the fungal cell wall is chitin that can be hydrolysed with the help of chitinase. Usually, a plant can synthesise some protein to defend pathogen invasion. However, none of the modern commercial rose cultivars are completely resistant to the pathogen causing the blackspot. One gene from rice encoding chitinase was introduced into a rose cultivar by Marchant's et al. (1998), and the severity of blackspot was reduced by 13-43% (Marchant et al., 1998).

Although long vase life and disease resistance are essential for high quality cut flower, the ornamental and visual appearances, especially flower colour, are also vital features of cut flowers. Flavonoids, carotenoids and betalains are the three major substances contributing to various flower colours. The pathway of flavonoid metabolism has been widely studied in many flower species, such as snapdragon, petunia, carnations and gerbera (Tanaka et al., 1998). New flower colours are manipulated by modifying the flavonoid metabolic pathway. Scientists manipulate flower colours by modifying the related biosynthetic genes involved in metabolic pathways and secondary metabolism, but also transcription factors are crucial targets for modification. These transcription factors activate or repress the expression of enzyme

encoding genes in specific metabolic pathways, which makes it easier to manipulate the whole metabolic pathways by modifying the related transcription factors (Iwase et al., 2009).

Besides the aspects of cut flower quality mentioned above, plant morphology and floral architecture can be modified by genetic engineering. Recent studies have focused on transcription factors involved in developmental regulation. Multiple transcription factors are involved in regulating plant development or function through regulation of plant hormone synthesis to regulate floral architecture and plant morphology (Tanaka et al., 2005). The *CENTRORADIALIS (CEN)* gene is isolated from *Antirrhinum*, and it encodes the protein involved in the indeterminate growth of *Antirrhinum* inflorescence. When transformed and overexpressed in tobacco, which is a determinate species, the CEN protein prolong the vegetative growth and change the height and the number of leaves of the plant (Amaya et al., 1999). Another example is the Lateral-shoot Inducing Factor (*LIF*) that is a zinc-finger type transcription factor from petunia. Controlled by the promoter CaMV35S, overexpression of *LIF* can change the cytokinin levels in the leaves of petunia and makes the plant to produce more lateral shoots than the wild type suggesting that the LIF perform its function by alter the phytohormone biosynthesis (Nakagawa et al., 2005).

1.2 MADS-box genes and TCP transcription factors in plants

Transcription factors are proteins that bind to certain DNA sequences and control the transcription of genetic information from DNA to RNA (Zhang, 2003). Transcription factors can perform their functions alone or together with other proteins in a complex. They can promote or block the recruitment of RNA polymerase to specific genes. As in case of other proteins, transcription factors need to be transcribed from a certain gene on a chromosome into RNA, which will then be translated into protein. In this process, there is a high possibility that the transcription factors could be influenced by any regulators. Based on this theory, the transcription factors can even regulate themselves and the transcriptional level will affect the expression of transcription factors (Liu et al., 1999).

Regulation of metabolic pathways is not the only work for transcription factors. Some transcription factors regulate plant branching, while some others regulate the leaf and flower development (Crawford et al., 2004; Aguilar-Martínez, et al., 2007).

Transcription factors encoded by MADS-box gene family control various plant developmental processes ranging from flower development to root formation (Becker and Theiβen, 2003). The TCP-domain transcription factors belong to another important gene family controlling floral symmetry, flower development and growth of axillary branches (Carpenter and Coen, 1990; Doebley et al., 1997).

1.2.1 MADS – box genes and ABCDE model of flower development

MADS-box genes are a group of genes that are named according to *MINICHOROMOSOME MAINTENANCE1 (MCM1)* genes in yeast, *AGAMOUS (AG)* in Arabidopsis, *DEFICIENS (DEF)* in *Antirrhinum* and serum response factor (*SRF*) in humans (Ng and Yanofsky, 2001). MADS-box genes widely exist in many kinds of life forms, including plants, animals and fungi. These genes can be categorized into two MADS-box lineages (type I and type II) (Alvarez-Buylla et al., 2000). Most plant MADS-box genes belong to type II lineage. The common feature of type II lineage is that all the proteins have a carboxy-terminal domain, which extends beyond the two hundredth amino acid protein, and functions as a transactivation domain and to stabilize the protein-protein interactions (Riechmann and Meyerowitz, 1997).

The MADS-box genes in flowering plants have various functions in different stages of plant growth and development, such as, regulating flowering time, vegetative development and flower architecture, and many MADS-box genes are involved in differentiation of meristems and floral primordia. Studies of organ identity mutations in *Arabidopsis* and *Antirrhinum* suggested that there are three classes of genes responsible for regulating organ identity. These three classes were originally described in the ABC model of flower development (Coen and Meyerowitz, 1991).

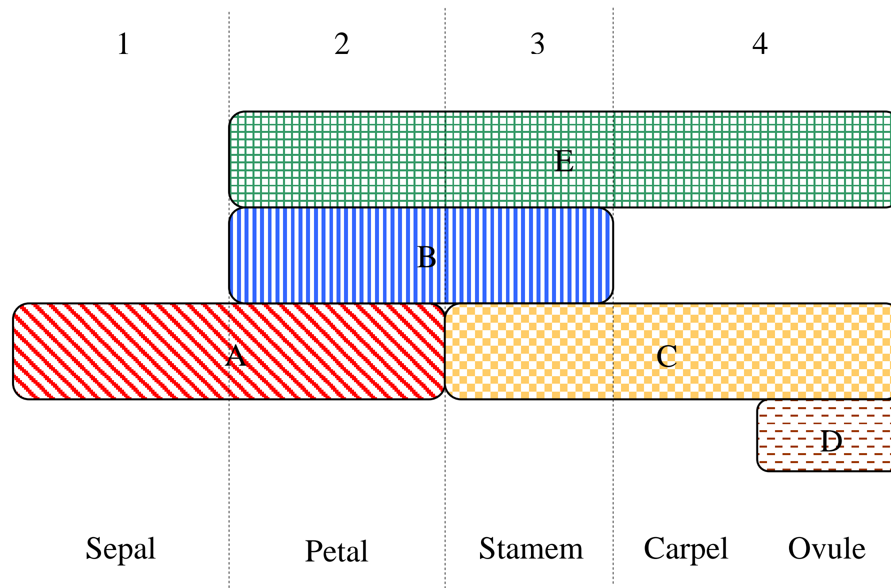


Figure 1. ABCDE model of flower development and the functions of A, B, C, D and E genes. If A gene express alone, the primordia will develop into sepal, but together with B and E function genes, the primordia will develop to petal. If B, C and E genes all expressed, the primordia will become a stamen, if B genes are absent, C and E genes will make primordia become a carpel. C, D and E genes are all required for ovule development.

The original ABC model of flower development was based on the studies of *Arabidopsis* and *Antirrhinum*, and this model applies to the flower development of many angiosperm plant species (Causier et al., 2010). The angiosperm flowers usually have four whorls of organs: sepals, petals, stamen and carpel (ovules). A, B and C function genes will induce the primordia to develop into the four different organ whorls by expressing alone or together. However, additional D and E function genes were found and have been proven to be involved in flower organ development and the ABCED model is the extension of the old ABC model (Fig. 1) (Glover, 2007).

MADS box genes encode their corresponding proteins and the proteins perform their functions by interacting with each other and forming protein complexes. According to Theißen's quartet model of flower organ identity, the MADS box proteins perform their functions by forming higher order complexes (tetramers) (Theißen, 2001). The tetramers could be fined as "dimers of dimers", but none of the putative tetramers or higher order complexes were detected (Ruokolainen et al., 2010a). Based on the studies on the structure of MADS domain, it has been defined that the N- terminal half determines the DNA-binding site while the C- terminal half is responsible for dimerization. This structure allows the formation of homo- or heterodimers composed

by different MADS box proteins (Shore and Sharrocks, 1995). In this case, most studies of the MADS box protein-protein interaction were focused on homo- or hetero-dimeriazations in order to study the formation of higher protein complexes.

1.2.2 TCP domain transcription factors

TCP domain transcription factors, which can control plant development, emerged quite early in evolution in plant cells. The *TCP* genes exist not only in angiosperms, but also in other lower life forms, which suggest that TCP transcription factors are ancient proteins (Navaud et al., 2007). The TCP proteins are named after *TEOSINTE BRANCHED 1 (TB1)* in maize, *CYCLOIDEA (CYC)* in *Anthirrinum majus*, and *PCF* in rice. The TCP proteins can be divided into two subfamilies by the primary structure of their DNA binding domain, which are *CYC/TB1 (TCP-C)* subfamily and *PCF1/PCF2 (TCP-P)* subfamily (Cubas et al., 1999). In addition, *BRANCHED1* (*BRC1*) and *BRC2* from *Arabidopsis* and *CINCINNATA* (*CIN*) gene from Antirrhinum are both important members of *TCP-C* subfamily (Crawford et al., 2004; Aguilar-Martínez et al., 2007).

The TCP domain is a region of DNA fragment encoding the basic helix-loop-helix secondary structure of these transcription factors. All the transcription factors in TCP family contain the secondary structure regions called basic helix-loop-helix (bHLH). However, these two subfamilies differ both inside and outside the TCP domain. Inside the TCP domain, the basic region of the CYC/TB1 subfamily contains a bipartite NLS (Doebley et al., 1997), while the basic region of the PCF subfamily contains only a part of a bipartite NLS. Furthermore, the helix II of the CYC/TB1 subfamily is longer than that of the PCF subfamily. On the other hand, outside the TCP domain, most members of the CYC/TB1 subfamily have an R-domain, which is predicted to form a hydrophilic a-helix and coiled coils, which are similar to those formed by leucine zippers. These coiled coils may mediate protein–protein interactions. On the other hand, all members of the PCF subfamily have the same short regions adjacent to the bHLH domain (Cubas et al., 1999).

So far, most studies have focused on the *TCP-C* subfamily and showed that they are involved in plant morphological development. To be more specific, *CYC* gene is involved in controlling floral symmetry (Carpenter and Coen, 1990). There were some *Antirrhinum* classical mutants which have fully ventralised flowers or flowers with reduced floral dorsoventral asymmetry (Cubas, 2004). In the previous experiments of

Coen and Carpenter, they generated transposon tagged mutants and identified loci involved in regulating flower dorsoventral asymmetry. With help of transposons it is possible to isolate the corresponding genes during this process (Carpenter and Coen, 1990). In their studies, they mentioned the *CYCLOIDEA (CYC)* and *DICHOTOMA (DICH)* control the *DIVARICATA (DIV)* gene, which codes for a MYB-type transcription factor that is an essential key gene regulating ventral petal identity. The *CYC* and *DICH* genes are encoding TCP proteins that control the dorsal identity of flowers and are the key point in the development of zygomorphic flowers in *Antirrhinum*. In Cubas' experiment, there are three types of mutants. The first one is *cyc* mutants, which have partially ventralised flowers, and the dorsal and lateral petals and stamens are similar to the ventral ones. Furthermore, the *cyc* flowers have one more petals and stamens than the wild-type flowers. The second mutant is *dich* mutants, of which dorsal petals are bit of internal asymmetry. The most extreme mutant is *cyc*/*dich* double mutant. They have symmetrical flowers and more and fully ventralised petals and stamens (Fig. 2).

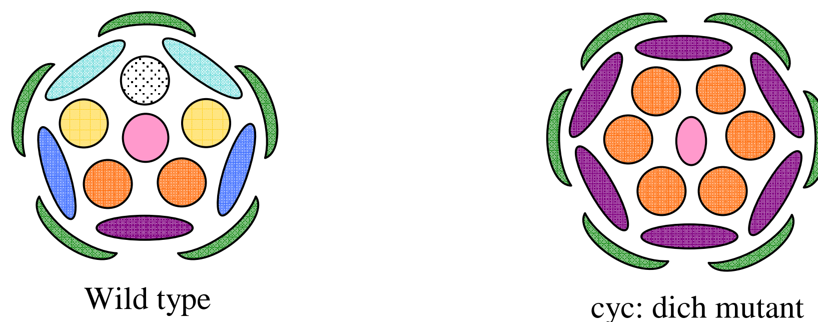


Figure 2. Wild type Antirrhinum flower and mutant flower showing that the *cyc*/*dich* double mutant have symmetrical flowers and more and fully ventralised petals and stamens which is quite different from the wild type. (Figure was modified from Cubas, 2004).

From these mutants, it can be concluded that the functions of *CYC* and *DICH* is to regulate the morphological differentiation of dorsal petals and stamens and to control their number. At the early stage of the development, *CYC* and *DICH* retard meristem growing in the dorsal part of the flower and control the number of organ primordia which will be the future petal and stamens. At the later stage, the growth patterns of

dorsal petals and stamens will be affected by them and they will also promote the formation of the dorsalmost stamen (Cubas, 2004).

Another important gene in TCP-C subfamily is *TB1*, which controls the developmental evolution of maize. The *TB1* gene is responsible for arresting the growth of some axillary buds, repressing internode elongation in branches, and arresting petal and stamen development in female flowers (Doebley et al., 1997). In Doebley's early research, he crossed maize with teosinte to create different genetic background generations in order to find out the functions of *TB1* gene in morphology (Doebley et al., 1995).

According to Doebley, there are mainly two quantitative trait loci (QTL) which control the differences in plant phenotypes and inflorescence architecture between maize and teosinte (Fig. 3). One of the loci is on chromosome arm lL, maize mutant *teosinte branchedl (tbl).* This QTL influences the inflorescence sex, the internodes number and length of the lateral branches and inflorescences, which were obvious according to the phenotypes of each generation. However, this QTL has strong effects in teosinte background but not in maize background. The second QTL is on chromosome arm X. It has the same influence as the QTL on 1L. However, the effect of this QTL on several traits is not so strong in both maize and teosinte background compared to a maize-teosinte F2 population. It can be concluded that genetic background affect gene action for both QTL. Together, these two QTL substantially affect the developmental evolution of maize family plants (Doebley et al., 1995).

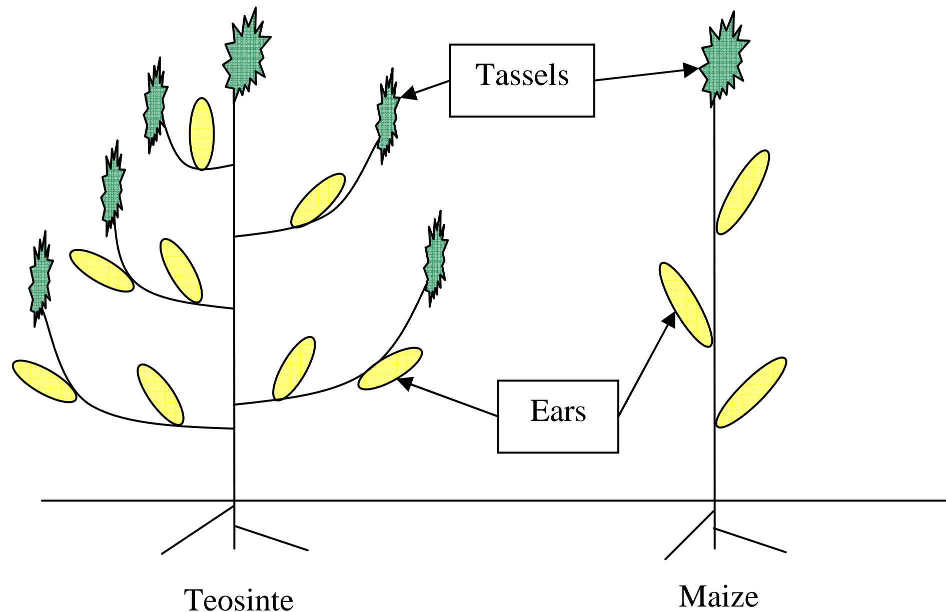


Figure 3. Pictures of maize and teosinte show the different plant architectures. There are many lateral branches in teosinte, each lateral branch terminated by a branched male inflorescence or tassel. On the contrary, maize has relatively few lateral branches, and at the end of each branch, there is an unbranched and female inflorescence (Figure was modified by the picture from Doebley et al., 1995).

In the experiment done by Takeda in Japan, the rice *TB1* gene (*OsTB1*) was isolated. In transgenic rice plants, in which the *OsTB1* gene is over-expressed, the lateral branching was inhibited. Therefore, similarly to maize *OsTB1* gene is a negative regulator for lateral branching in rice (Takeda et al., 2003).

CINCINNATA (*CIN*) genes encode the transcription factors belonging to the TCP-C family. Studies of *cin* mutants in *Antirrhinum* have showed that the leaves of *cin* mutants are larger than normal ones because of the over-growth in marginal regions. Not only in leaf growth, the *cin* mutants also have effects on petal shape, which suggests that the *CIN* transcription factors are involved in both plant growth and differentiation (Crawford et al., 2004). *BRANCHED1* (*BRC1*) and *BRC2* genes, found in Arabidopsis, are *TB1* like genes. Developing buds are the place where *BRC1* is expressed most, and the function of *BRC1* is to arrest the bud development. According to mutant experiment in Arabidopsis, *brc1* mutants have more outgrowing branches than normal plants (Aguilar-Martínez et al., 2007). Besides *BRC1*, *BRC2* is the other *tb1*-like gene in Arabidopsis. However, according to the phenotypic analysis of *brc1* and *brc2* mutants, *BRC2* gene is most likely not involved in axillary bud development (Poza-Carrión et al., 2007).

Although there are not so many studies on TCP-P subfamily, it is known that they are involved in organ border delimitation and influence cell growth. The PCF1 and PCF2 proteins were first identified from rice gene because they can bind precisely to the promoter elements for the proliferating cell nuclear antigen (*PCNA*), which is involved in meristematic cell divisions and expressed only in meristematic tissue. Therefore, it has been proposed that PCF1 and PCF2 most probably control the transcription of PCNA, and influence the cell growth and proliferation (Cubas et al., 1999).

1.3 Gerbera and MADS-box genes and TCP transcription factors in Gerbera

1.3.1 *Gerbera hybrida*

Gerbera (*Gerbera hybrida*) is a famous cut flower belonging to the *Asteraceae* family. It has been established as a new model plant to study flower organ differentiation and flower type specification in *Asteraceae*. Unlike the traditional model plants such as *Arabidopsis, Antirrhinum* or *Petunia* that only contain single types of flowers; gerbera inflorescence has more complex structure and various floral types. The inflorescence of Gerbera is composed of three different types of flowers, ray flowers, trans flowers and disc flowers (Fig. 4). The ray flowers, which are bilaterally symmetrical, are in the marginal area of gerbera inflorescence. They all have five petals, of which three petals are fused together and enlarge to form a showy ligule shape. Disc flowers are in the central area of the whole inflorescences. They have short and separate petals so they are radially symmetrical in morphology. Trans flowers are in the areas between disk and ray flowers. They are also bilaterally symmetrical, which is similar to ray flowers in shape but smaller in size (Teeri et al., 2006). The ray and trans flowers are female flowers, in which the stamens are not developed. However in disk flowers, stamens are well developed and form a fused structure covering the carpel (Kotilainen et al., 2000; Laitinen et al., 2006).

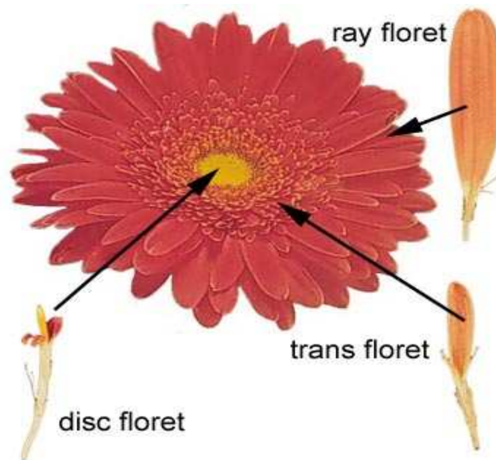


Figure 4. The inflorescence of gerbera is composed of three types of floret. Ray flowers, trans flowers and disc flowers comprise the three whorls of gerbera inflorescences. http://www.mm.helsinki.fi/mmsbl/english/research/Gerberalab/gerbera_structure.html

In order to explore the molecular mechanisms involved in regulation of the complex inflorescence structure, both MADS-box genes and TCP genes have been isolated from gerbera in the previous studies. It has been shown that the MADS-box genes in gerbera regulate the flower organ identity while TCP genes are involved in controlling flower type specification (Yu et al., 1999; Kotilainen et al., 2000; Uimari et al., 2004; Laitinen et al., 2006; Teeri et al., 2006; Broholm et al., 2008; Broholm et al., 2009; Ruokolainen et al., 2010a; Ruokolainen et al., 2010b).

1.3.2 MADS-box genes in Gerbera

MADS-box genes are encoding the A, B, C, D and E functions regulating flower organ development. Also gerbera contains MADS-box genes that have specific functions to regulate flower organ identity. *SQUAMOSA (SQUA)* is an A function gene from *Antirrhinum* while *APETALA1 (AP1)* and *FRUITFULL (FUL)* are both A function genes from *Arabidopsis* (Riechmann and Meyerowitz, 1997; Egea-Cortines et al., 1999; Litt and Irish, 2003). In gerbera, six *SQUA*-like genes (*GSQUA1-6*) have been identified, among which *GSQUA1* and *GSQUA3* belong to *SQUA*/*AP1* clade, and *GSQUA2*, *GSQUA4*, *GSQUA5* and *GSQUA6* are *FUL*-like genes. The locations of the *GSQUA* genes expression domains vary. *GSQUA1* was only expressed in the receptacle and petals of the inflorescences. The expressions of *GSQUA2* and *GSQUA5* were detected everywhere in the inflorescences. The expression levels of *GSQUA3* and *GSQUA6* were higher in gerbera leaves than in the inflorescences and the expression of *GSQUA4* was only found in the reproductive organs and the vasculature (Ruokolainen et al., 2010b).

Based on the expression patterns, none of these genes seem to control the classical A function. Instead, the functional analysis of *GSQUA2* showed that it is involved in regulation of meristem transitions (Ruokolainen et al., 2010b).

The B function genes specify the petal and stamen development. The B function genes have been found in *Arabidopsis* and *Antirrhinum*, which are two intensively studied plant species. *APETALA3 (AP3)* and *PISTILLATA (PI)* from *Arabidopsis* and *DEFICIENS (DEF)* and *GLOBOSA (GLO)* are from the *Antirrhinum* are all B function genes. Their function is mainly to promote stamen and petal identity. (Mouradov et al., 1999). There are also two paralogous lineages from *AP3/DEF* lineage, *euAP3* and *TOMATO MADS BOX GENE6 (TM6)* belonging to the B function genes (Kramer et al., 1998). Similar genes belonging to class B were isolated from gerbera, *GERBERA GLOBOSA-LIKE1 (GGLO1)* is a *PI/GLO* lineage gene, *GDEF1* is a *TM6*-like gene and *GDEF2* and *GDEF3* are *AP3* class genes. Previous studies have shown that *GGLO1* and *GDEF2* control the development of petals and stamens in gerbera, but *GDEF1* is different (Yu et al., 1999). Instead of determining the petal identity, the *GDEF1* gene is responsible for regulating stamen development (Broholm et al., 2009). According to Broholm et al. (2009), GGLO1 protein can interact with proteins GDEF1, GDEF2 and GDEF3 and form heterodimers by using GAL4 yeast two-hybrid assays (Yu et al., 1999; Broholm et al., 2009).

The C function genes control the stamen and carpel identity in flower development. *AGAMOUS (AG)* in *Arabidopsis* and *PLENA (PLE)* in *Antirrhinum* are both important genes in this clade. *GAGA1* and *GAGA2* are *AG*-like C function genes found in gerbera. They are expressed firstly in the central primordia and control the development of stamens and carpels. After the stamen and carpel was formed, in the later developmental stages, *GAGA1* and *GAGA2* were responsible for ovules formation (Yu et al., 1999). In the experiments of Ruokolainen (2010a), no interactions were detected between GAGA1 and GAGA1 were detected (Ruokolainen et al., 2010a).

E function genes are involved in development of all whorls of organs: sepals, petals, stamens, carpels and ovules. For example, the *SEPALLATA (SEP)* genes are isolated from *Arabidopsis*. According to Favaro et al (2003), the developments of ovules and seeds were disrupted or changed into other structures in *SEP1/sep1 sep2 sep3* mutant plants. Among all the *SEP* genes, *SEP3* is more efficient to promote the normal ovule development than other *SEP* genes (Favaro et al., 2003). *GRCD1* and *GRCD2* are *SEP*-like genes from *Gerbera*, but they have different functions. *GRCD1* is responsible for

stamen development, while *GRCD2* is important to carpel development (Uimari et al., 2004; Teeri et al., 2006). In addition, Ruokolainen et al. (2010a) has identified general E function genes, *GRCD4* and *GRCD5*, comparable to *Arabidopsis* SEP proteins. They show high activity in forming protein complexes with several gerbera MADS domain proteins (Ruokolainen et al., 2010a).

1.3.3 TCP genes in Gerbera.

In gerbera, *CYCLOIDEA*-like genes (*GhCYC)* were isolated and four different *GhCYC* genes were found.

Among all the *GhCYC* genes in gerbera, *GhCYC2* has been mostly studied. In the gerbera inflorescences, *GhCYC2* is mainly expressed in the showy ray flower primordia. It has been shown to regulate the flower type identity and to control the size of the gerbera inflorescences. According to the phenotypes of transgenic gerbera, overexpression of *GhCYC2* in the central disk flowers causes the disk flowers to develop similar to ray flowers, which has enlarged ventral petals and disrupted stamen development (Broholm et al., 2008).

The functions of *GhCYC1*, *GhCYC3* and *GhCYC4* have not been accomplished. According to Broholm (2008), the putative GhCYC1 amino acid sequence outside the highly conserved TCP and R region is significantly different from the GhCYC2–4 sequences. It is predicted that the amino acid sequence of GhCYC2, 3 and 4 include nuclear localization signals (NLS), which means they complete the transcription in nucleolus. However, GhCYC1 has no NLS, which implies that the GhCYC1 is not localized in the nucleus. It may occur in other places, such as chloroplasts (Broholm et al., 2008). However, the GhCYC proteins may perform their functions by forming protein complexes. The interactions among GhCYC proteins have been studied using the yeast two-hybrid system (Sari Tähtiharju, personal information). The GhCYC proteins were originated from plant; therefore, the results of protein-protein interactions in yeast should be confirmed *in planta* to avoid the drawbacks from yeast two-hybrid system.

1.4 Detecting protein – protein interactions *in planta*

1.4.1 BiFC (Bimolecular fluorescence complementation)

Protein interactions can be studied with the help of methods such as yeast two hybrid system and proteomics analyses. However, some more valid and visible methods are required to make researchers see the results of interaction (Hiatt et al., 2008). The methods, which make the protein-protein interactions visible, not only can reveal the interactions, but also show the locations where the interactions happen (Weinthal & Tzfira, 2009). This is of great importance to study the interactions among transcription factors.

Bimolecular fluorescence complementation (BiFC) is a relatively new method for direct visualization of protein-protein interactions in living cells (Kerppola, 2006). The fluorescent protein used in this method cannot be seen under visible light but can be seen under UV light. However, when the fluorescence protein is split into N and C-terminal halves, the molecule does not produce fluorescence. Based on this feature, the fluorescence protein can be used to test protein and protein interactions (Hu et al., 2005). The mostly used fluorescence protein is the enhanced yellow fluorescent protein (EYFP), but also other fluorescence proteins are also used in some studies, such as green fluorescent protein (GFP), cayan fluorescent protein (CFP) and blue fluorescent protein (BFP). (Weinthal & Tzfira, 2009). The two non-fluorescent fragments, YFP/C and YFP/N, will be fused with the two putative interacting partners, therefore, if the putative partners interact with each other, the fusion of two fragments will restored fluorescence (Fig. 6). This protein can absorb a photon of high energy and emit another photon with longer wavelength (lower energy). The energy difference between the absorbed and emitted photons will be performed in many ways, such as molecular rotations, vibrations or heat (Fig. 5).

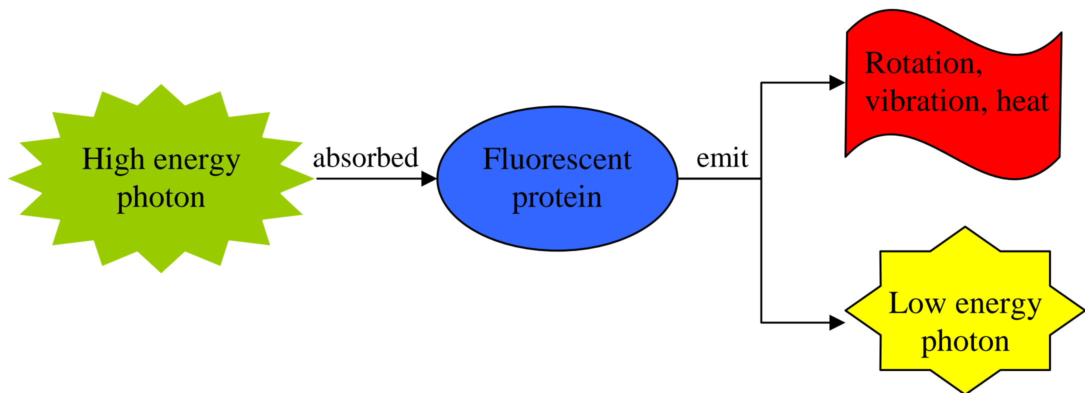


Figure 5. Light emission theory of fluorescent proteins. Fluorescent protein can absorb high energy photon and emit low energy photon. The energy differences between these two photons were released in the form of rotation, vibration or heat.

Normally, if the protein is illuminated by ultraviolet light, the emitted light will be in the visible range. From this point of view, if the interaction takes place, the emitted visible light can be seen when the proteins are under the ultraviolet light. Since the interaction take place in the living cells, the microscope can capture the image of the whole cell and the localization could also be detected.

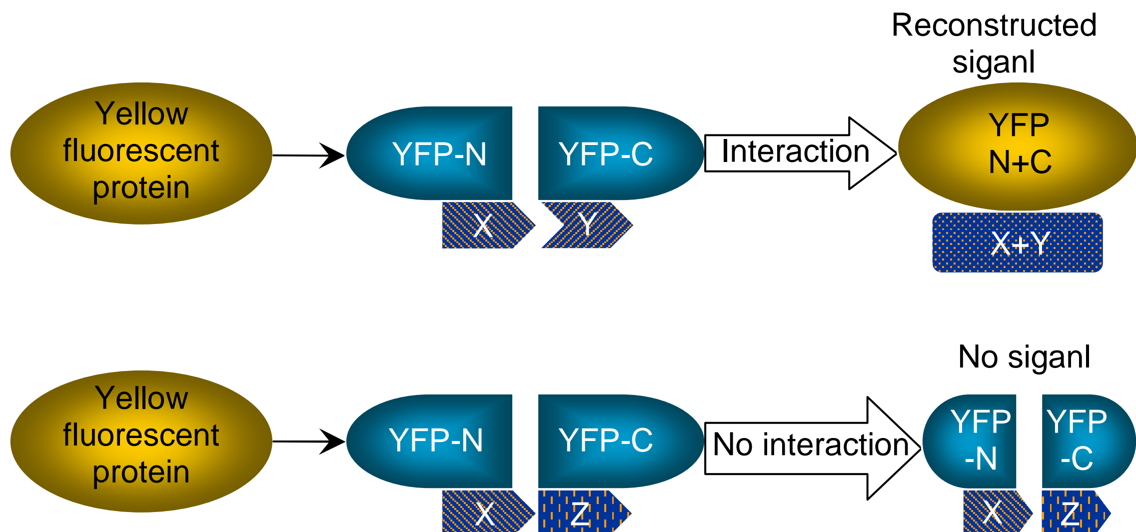


Figure 6. The theory of BiFC assay. X, Y and Z were proteins that fused to N- or C-terminal of the fluorescent protein fragments. X and Y were interacting proteins so the fluorescent protein could be reconstructed and signals could be detected. X and Z were non-interacting proteins, the N- and C- terminals could not be fused to reconstruct the fluorescent protein and no signals could be detected (Figure was modified from Weinthal & Tzfira, 2009).

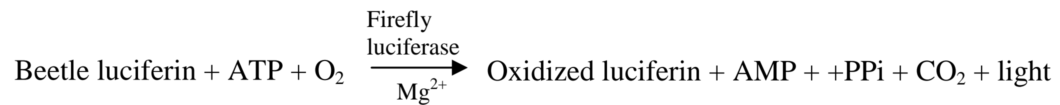
Usually, there are two places to split the YFP. The first one is between the amino acids 153 and 154 and the second option is between the amino acids 174 and 175. Many previous experiments suggested that it would be better to split YFP between amino acids 174 and 175, therefore, the YFP signal will be more consistent and stronger than splitting YFP between amino acids 153 and 154 (http://www.bio.purdue.edu/people/faculty/gelvin/nsf/index.htm). However, many experiments are still being processed with the vectors have the fragments split between amino acids 153 and 154.

It has been reported that BiFC is a relatively simple and reliable method to investigate protein-protein interactions and is technically simple in different plant systems. And it is also very sensitive as it can detect the protein-protein interaction at a low expression level (Walter, et al., 2004).

1.4.2 Split luciferase assay

Another important method to test protein-protein interactions is split luciferase assay. In this assay, the luciferase will be split to N- and C-terminal fragments, which is quite similar to BiFC assay, and these two fragments will be fused to two target genes respectively. However, the theory behind the reaction is quite different from fluorescent protein.

Luciferins can emit photons by oxidization, and then become oxidized luciferins. However, this reaction is usually very slow. Therefore, it is often catalyzed by luciferase. In the split luciferase assay, when the proteins coded by the target genes interact with each other, the N- and C- terminal of the luciferase enzyme are reconstructed and the complete luciferase can be produced. Luciferase is able to catalyze the luminescent reaction and the photons emitted by the reaction can be detected by luminometer. Various luciferins us different substrate luciferins, the most used luciferases are firefly luciferase and Renilla luciferase and the luminescent reactions are showed in figure 7 (Sherf et al., 1996).



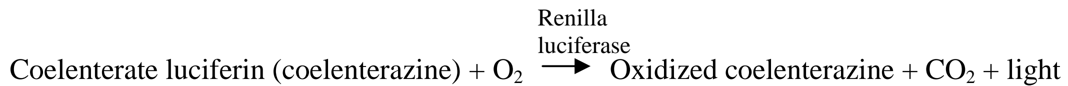


Figure 7. Luminescent reactions of beetle luciferin and coelenterazine. Each luciferin has its own corresponding luciferase. For beetle luciferin, the firefly luciferase is responsible to catalyze the luminescent reaction while Renilla luciferase is for coelenterazine (Figure was modified from Sherf et al., 1996).

The split luciferase assay method has been proved to an efficient assay of analyzing protein - protein interactions by Fujikawa and Kato in 2007. Gene H2A and H2B encoding histones, interacting with each other from *Arabidopsis*, were used in split luciferase assay (Fujikawa and Kato, 2007). According to Fujikawa and Kato, the split luciferase assay can detect protein dissociation as well, which can not be done by BiFC, besides, the split luciferase assay doesn't need any external light to measure the results which will not increase any background light in the sample (Fujikawa and Kato, 2007).

1.5 Transferring plasmid DNA into plant cells

1.5.1 Protoplast electroporation

The method of electroporation is widely used in various molecular experimental researches. Cells, including bacterial, fungal, animal, and plant cells, can be electroporated with the presence of DNA. The DNA will then be introduced into the cells and be expressed in the electroporated cells (Lurquin, 1997).

Electroporation is a membrane phenomenon. Pores are formed at the cell membrane by suddenly and significantly increasing in the electrical conductivity and permeability of the cell membrane temporarily. During this short time, the target substances will be introduced into the cells (Weaver and Chizmadzhev, 1996). If all the parameters are right, the cell membrane would heal and be still alive. However, the parameters vary in different cells types, not only in plant a cell, electroporation is used in bacterium, fungi and animal cells as well. However, even in plant cells, cells from different plant species

may have different parameters for electroporation (Lurquin, 1997). Therefore, the parameters of electroporation should be optimized to assure that the electroporation is successful.

In both BiFC and split luciferase assay, two fragments of DNA (N-terminal and C-terminal) fused with the target genes are introduced into the same protoplast and be expressed there. The protoplasts would be alive for several days and the proteins will be encoded by transformed genes, and, detected by microscope or luminometer.

1.5.2 Agroinfiltration and its application

Agrobacterium tumefaciens and other related *Agrobacterium* species were know as plant pathogens at the beginning of the 20th century. They cause crown-gall disease in natural environment by their natural ability of transferring a T-DNA from the bacterial tumor-inducing plasmid, which is known as Ti-plasmid, to the host cell. The T-DNA will then be integrated into the host genome and be expressed in the cells of the host (Tzfira et al., 2004). Based on this “natural gift” of *Agrobacterium*, *Agrobacterium tumefaciens* is intensively studied and widely used as a tool of transferring DNA into many agronomically and horticulturally important species to create transgenic plants (Gelvin S.B. 2003). However, *Agrobacterium*-mediated transformation is not successful in all plant species, there are still plant species cannot be infected. In this case, other transformation methods are needed (Chung et al., 2006).

There are various *Agrobacterium*-mediated methods of transferring T-DNA into plant, such as agroinfection and floral dip. However, Agroinfiltration is found to be a simpler and faster way to induce transient expression and analyse gene expression in plant cells (Wydro et al., 2006). Briefly, this method is to inject the suspension of *Agrobacterium*, which contain the target gene, into plant organs, e.g. leaves or petals. The agrobacterium strain containing the target gene plasmid is cultivated in liquid medium and then washed and suspended into the buffer for injection. In the natural environment, once entering through wounds of plants, the agrobacterium will be activated when surrounded by low-molecular-weight phenolic compounds and monosaccharides released from wounded plant cells, and a slightly acidic environment. In this case, acetosyringone is crucial in the suspension buffer. It is because acetosyringone can create a similar environment as plant wounding, in which situation the *vir* gene will express (Kanneganti, 2006).

The suspension of *Agrobacterium* will be injected into the airspaces of the plant organs through stomata on the underside of the leaves (Fig.8.). Sometimes, if the plant organs are not suitable for injection, a tiny incision can be made to make the infiltration easier.

Figure 8. Suspension of agrobacterium is being injected into the underside of a young *Nicotiana benthamiana* plant by the syringe without a needle.
(http://commons.wikimedia.org/wiki/File:Agroinfiltration.jpg)

Usually, there are some detectable reporter genes being transferred into agrobacterium and injected into plant. The expression of these genes can be detected two to three days after infiltration; the specific days depend on various genes (Kanneganti, 2006).

1.6 Aims of the study

The *GhCYC* genes, especially *GhCYC2*, have been proved to play an important role in regulating floral development and differentiation. However, the study from Broholm showed that at some stage, one gene is insufficient to regulate floral development, which implies that the interactions among these GhCYC proteins might exist (Broholm et al., 2008). The interactions among GhCYC1, GhCYC2, GhCYC3 and GhCYC4 have been tested by using yeast two hybrid. According to the results from Dr. Sari Tähtiharju, GhCYC1 only interact with itself, while GhCYC2, GhCYC3 and GhCYC4 have interactions with each other (Table 1.).

Table 1. The results of GhCYCs interaction by yeast two hybrids (Sari Tähtiharju, personal information).

	AD	ADGhCYC1	ADGhCYC2	ADGhCYC3	ADGhCYC4
BD	-	-	-	-	-
BDGhCYC1	-	+	-	-	-
BDGhCYC2	-	-	+	+	+
BDGhCYC3	-	-	+	+	+
BDGhCYC4	-	-	+	+	+

However, in yeast two hybrid systems, sometimes new protein interactions occur, such as 'false-positive' and 'false-negative' interactions, which are not expected, and sometimes the protein-protein interactions can not be detected (Ito, et al., 2001). In several yeast two hybrid experiments, the results did not correlate even though the authors are all dealing with the same GAL4 system (Immink, et al., 2002). Therefore, the interactions among GhCYCs should be confirmed by other methods in a more natural way.

The aim of this study is to detect and verify the protein-protein interaction in plant cells to avoid the drawbacks from yeast two hybrid systems. The interactions among GhCYCs will be confirmed interacting in tobacco protoplasts as well as in tobacco leaf epidermal cells. Electroporation and agroinfiltration are used to introduce the plasmid DNAs into the plant cells and create an appropriate environment for protein-protein interactions. Split luciferase assay and BiFC (bimolecular fluorescence complementation) were used in this experiment to confirm the results of yeast two hybrid systems and the location where the interactions take place. In addition, these two methods were compared to find out an efficient and reliable way of detecting protein-protein interactions *in planta*.

2 Materials and methods

2.1 Entry clones, destination vectors and DNA construction

For both BiFC and split luciferase assay, DNA constructs were composed of one target gene and either N-terminal or C-terminal fragment of YFP (yellow fluorescent protein) or luciferase. The target genes were cloned in the entry vectors while the N- terminal or C-terminal fragment of YFP (yellow fluorescent protein) and luciferase were in the destination vectors.

2.1.1 DNA constructs for protoplast electroporation

For protoplast electroporation, both BiFC assay and split luciferase assay were used. Two types of entry clones were used to make the DNA constructs with different destination vectors (table 2 and table 3). *GhCYC* genes (*CYC*1 to *CYC*4), isolated from gerbera (Broholm et al., 2008), were in the entry clone. pENTRCYC1 to pENTRCYC4 have stop codons at the end of the *CYC* gene. Therefore, the N or C terminal fragments should be in the upstream positions. Entry vectors pENTR2CYC1 to pENTR2CYC4 have no stop codons, the C or N terminal can be in the downstream positions (Sari Tähtiharju. Personal information).

In BiFC assay, the target genes, *CYC1, CYC2, CYC3* and *CYCY4* were in the entry vectors. The destination vectors pARC233 and pARC235 contain N-terminal YFP fragments, while vectors pARC234 and pARC236 contain C-terminal YFP fragments, and the promoters in all these vectors are CaMV35S (Welch et al., 2007). The LR recombination reaction (explained in 2.3.1) will produce the final constructs shown in table 2.

Table 2. Entry clones, destination vectors and the final constructs for BiFC assay in protoplast electroporation (All the entry clones and destination vectors were obtained form Sari Tähtiharju).

Entry clone \ Destination vector	pARC233 (N-)	pARC234 (C-)	pARC235 (-N)	pARC236 (-C)
pENTRCYC1	N-*CYC1*	C-*CYC1*	-	-
pENTRCYC2	N-*CYC2*	C-*CYC2*	-	-
pENTRCYC3	N-CYC3	C-*CYC3*	-	-
pENTRCYC4	N-*CYC4*	C-*CYC4*	-	-
pENTR2CYC1	-	-	*CYC1*-N	*CYC1*-C
pENTR2CYC2	-	-	*CYC2*-N	*CYC2*-C
pENTR2CYC3	-	-	*CYC3*-N	*CYC3*-C
pENTR2CYC4	-	-	*CYC4*-N	*CYC4*-C

In split luciferase assay, pENTRCYC1 to pENTRCYC4 and pENTR2CYC1 to pENTR2CYC4 were also used as entry clone. However, pDuEx-Dc6 and pDuEx-D7

containing C-terminal of luciferase and pDuEx-An6 containing N-terminal of luciferase are used as destination vectors. H_2A (histone 2A) and H_2B (histone 2B) are genes from *Arabidopsis* encoding histone 2A and 2B, and had been found to interact with each other (Fujikawa and Kato, 2007). Entry clone pENTRH_2A and pENTRH_2B were used as positive control. The LR recombination reactions produced the final constructs shown in table 3.

Table 3. Entry clones, destination vectors and the final constructs for split luciferase assay in protoplast electroporation.

Entry clone \ Destination vector	Bait		Prey
	pDuEx-Dc6 (-C)	pDuEx-D7 (C-)	pDuEx-An6 (N-)
pENTRCYC1	-	Cluc-*CYC1*	Nluc-*CYC1*
pENTRCYC2	-	Cluc-*CYC2*	Nluc-*CYC2*
pENTRCYC3	-	Cluc-*CYC3*	Nluc-*CYC3*
pENTRCYC4	-	Cluc-*CYC4*	Nluc-*CYC4*
pENTR2CYC1	*CYC1*-Cluc	-	-
pENTR2CYC2	*CYC2*-Cluc	-	-
pENTR2CYC3	*CYC3*-Cluc	-	-
pENTR2CYC4	*CYC4*-Cluc	-	-
pENTRH_2A	-	-	Nluc-H_2A
pENTRH_2B	H_2B-Cluc	-	-

2.1.2 DNA constructs for agroinfiltration

For agroinfiltration, different constructs were made using different entry clones and destination vectors. Destination vectors were pSPYNE and pSPYCE (Walter et al., 2004) containing the N-terminal and C-terminal of split YFP (yellow fluorescent protein) fragment respectively, and the promoter in both vectors were CaMV35S. The entry clones were pENTR2CYC1 to pENTR2CYC4, *GGLO1, GDEF1* and *GAGA1* from gerbera. The previous studies have shown that using GAL4 yeast two-hybrid assays, that *GGLO1* protein can interact with proteins *GDEF1*, while *GAGA1* cannot interact with itself (Kotilainen et al., 2000; Broholm et al., 2009; Ruokolainen et al., 2010). *FBP2* and *FBP11* from petunia were included as well and they were both found to express in the ovules and have interaction with each other (Immink et al., 2002). In this

case, the combinations of *GGLO1*+ *GDEF1* and *FBP2*+ *FBP11* were selected as positive control and *GAGA1* self-interaction was the negative control (Table 4).

Table 4. Entry clones, destination vectors and the final constructs for BiFC assay in agroinfiltration (Entry clones were from gerbera laboratory).

Entry clone \ Destination vector	pSPYNE (N-)	pSPYCE (C-)
pENTR2CYC1	N-*CYC1*	-
pENTR2CYC2	N-*CYC2*	C-*CYC2*
pENTR2CYC3	-	C-*CYC3*
pENTR2CYC4	-	C-*CYC4*
pENTRFBP2	N-*FBP2*	-
pENTRFBP11	-	C-*FBP11*
pENTRGGLO1	N-*GGLO1*	-
pENTRGDEF1	-	C-*GDEF1*
pENTRGAGA1	N-*GAGA1*	C-*GAGA1*

However, before the LR recombination reactions among pENTR2CYC1 to pENTR2CYC4 and destination vectors pSPYNE and pSPYCE were carried out, the linearization reactions of pENTR2CYC1 to pENTR2CYC4 should be performed. Because all the pENTR2CYC entry clones, and pSPYNE and pSPYCE vectors contain the same antibiotic (kanamycin) resistance gene, so that the products of LR recombination reactions contain two types of plasmid DNA, the constructs and pENTR2CYC entry clones (pSPYNE and pSPYCE vectors have *ccdB* genes which kills *E.coli*), that can make *E.coli* grow on the selected medium. It was difficult to select the right colonies containing the right constructs. Once the pENTR2CYC entry clones were linearized, it was impossible for them to express and replicate in *E.coli* so only the *E.coli* with constructs can survive.

2.2 Linearization of pENTR2CYC entry clones and DNA extraction

To linearize the entry clone, proper enzyme should be selected that does not cut the site within the attL sites or the genes of interest. The components and the volumes were shown in table 5.

Table 5. Components and volumes of linearizing different entry clones.

Components	pENTR2CYC1	pENTR2CYC2	pENTR2CYC3	pENTR2CYC4
DNA	According to concentration	According to concentration	According to concentration	According to concentration
MQ-H_2O	According to DNA volume	According to DNA volume	According to DNA volume	According to DNA volume
Enzymes	EcoRV 1 µl	Hpa1 1 µl	EcoRV 1 µl	EcoRV 1 µl
Buffers	Buffer B 2 µl	Buffer B 2 µl	Buffer B 2 µl	Buffer B 2 µl
Total	20 µl	20 µl	20 µl	20 µl

After adding the component in table 4 together, the mixtures were incubated at +37 ℃ for one hour and loaded in 1% agarose gel, electrophoresed with 0.5×TBE buffer (appendix1) for one hour at 120 V.

DNAs were extracted from the gel after electrophoresis by using GelJet™ Gel Extraction Kit (Fermentas Inc., MA, U.S.), and then tested in the agarose gel with different concentrations (5 ng/ µl, 10 ng/ µl, 25 ng/ µl and 50 ng/ µl) of λDNA to estimate the amount by comparing the black bonds of λDNA.

2.3 LR recombination reaction and DNA transfer

The fusions of entry clones and destination vectors were made by LR recombination reaction. The products of LR recombination reaction were transformed into appropriate competent cells (*E.coli* or *Agrobacterium*) to complete replication.

2.3.1 LR recombination reaction

All the DNA constructs were made by LR recombination reaction. The LR recombination reactions were done by using Gateway® LR Clonase™ II Enzyme Mix (Invitrogen Corporation, CA, U.S.). In each reaction, one entry clone and one destination vector (shown in table 2, 3 and 4) were needed. Table 6 shows the components and the corresponding volumes. All the component were added to 1,5 ml microcentrifuge tubes and then mixed with 2 µl of LR Clonase™ II enzyme. The reactions were incubated at + 25°C for 1 hour, and then, 1µl of the Proteinase K solution was added to each reaction. The reactions were incubated at + 37°C for further 10 minutes.

Table 6. LR recombination reaction components and the corresponding volumes.

Component	Volume (μl)
Entry clone (100ng/reaction)	5
Destination vector (150ng/ μl)	1
TE buffer, pH 8.0	to 8

2.3.2 DNA transformation into *E.coli* by heat shock

DHα5 *E.coli* was used as the competent cell for the DNA constructs shown in table 2 and table 3. The competent cells were stored in - 80℃ and were thawed on ice before use. 3 μl DNA products from LR recombination reaction were added to each competent cell tube. The tubes were incubated on ice for 30 minutes and then a heat shock at + 42 ℃ for 30 to 90 seconds was given and further incubated on ice for two minutes. 1000 μl of LB (Luria-Bertani) liquid medium (room temperature) was added into the tubes and they were incubated at + 37℃ for 1 hour with horizontal shaking at 200 rpm. After one hour incubation, the tubes were centrifuged at 8000 rpm for two minutes and 1000 μl supernatant was discarded. The cells were resuspended in the remaining liquid and plated on petri dishes containing L-broth medium with ampicillin and grown overnight at + 37℃.

2.3.3 DNA transformation into *E.coli* by electroporation

The constructs cannot be transferred into *E.coli* efficiently if the sizes of the constructs are too large. In this case, electroporation would be the best choice for DNA transformation.

The competent cells DHα5 *E.coli* were stored at - 80℃ and were thawed on ice before electroporation. 1.5 μl of LR recombination reaction products were added into the competent cells and transferred into electroporation cuvette. The conditions for electroporation were 200 ohms, 25 μFD and 2.5 kV. 1 ml of SOC medium (2% (W/V) Tryptone, 0.5% (W/V) Yeast Extract, 0.05% (W/V) NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose) was added into the electroporation cuvette and the mixtures were transferred into the enppendorf tubes, and then incubated in the shaker at + 37℃ for one hour. The cells were harvested by centrifuging at 8000 rpm for two minutes, and most of the supernatant was discarded. Cells were resuspended in the remaining medium (200

μl) and plated on Petri dishes containing selection medium LB (Luria-Bertani) with kanamycin 50μg/ml.

2.3.4 DNA transformation into *agrobacterium*

Agrobacterium tumefaciens (C58GV3101 (pM90)) strain stored at -80℃ was used for transferring DNA constructs in table 3. The cells were thawed on ice and then 10-100 ng products of LR recombination reaction were added into the cells, and incubated on ice for one minute. The cell- DNA mixtures were added into the pre-cooled electroporation cuvette, and electroporate at the condition of 200 ohms, 25 μFD and 2.4 kV. After electroporation, cells were transferred into an eppendorf tubes by rinsing the cuvette with 1 ml of SOC medium(2% (W/V) Tryptone, 0.5% (W/V) Yeast Extract, 0.05% (W/V) NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose) without antibiotics and incubated in the shaker at + 28℃ for two hours. The cells were harvested by centrifuging at 8000 rpm for two minutes, and most of the supernatant was discarded. Cells were resuspended in the remaining medium (100 - 200 μl) and plated on Petri dishes containing selected medium LB (Luria-Bertani) with rifampicin 100 μg/ml, gentamicin 25 μg/ml and carbenicillin 100 μg/ml (table 7). The cells were incubated for 2 - 4 days incubation at + 28℃ to obtain enough colonies.

Table 7. Antibiotics and concentrations in $LB_{rif100gen25cb100}$ selected medium.

Antibiotic stocks and concentrations	Concentrations in selected medium
Rifampicin (rif) (50mg/ml)	100 (μg/ml)
Gentamicin (gen) (25mg/ml)	25 (μg/ml)
Carbenicillin (cb) (100mg/ml)	100 (μg/ml)

2.4 Miniprep, Maxiprep harvest and enzyme digestion

2.4.1 Miniprep and Maxiprep harvest

Plasmid DNA from both *E.coli* and *Agrobacterium* were isolated by using QIAGEN® Plasmid Mini/Maxi Kit (QIAGEN, Hilden, Germany). Miniprep was for low concentrations of DNA while Maxiprep could isolate much more DNA and acquire higher concentration DNA solutions.

2.4.2 Enzyme digestion

DNAs isolated by Miniprep were digested by enzymes to test if the LR recombination reaction was successful. The components and volumes were shown in table 8. The enzyme varies according to various DNA constructs, and enzyme BsrG1 was used in this experiment.

Table 8. Components and volumes in enzyme digestion solution.

Components	Volumes (µl)
1 µg DNA	According to concentrations
MQ-water	According to DNA volumes
10 NEB2 buffer	2
10 BSA buffer	2
Enzyme BsrG1 (unit/µl)	1
Total volume	20

After adding the component in table 8 together, the digestion solutions were incubated at +37 ℃ for one hour. The agarose gel electrophoresis was used to testing the results of enzyme digestion. The gel percentage was 1%, and the buffer was 0.5× TBE (appendix1).

2.5 DNA transformation into plant cells

Two methods, protoplast electroporation and agroinfiltration, were used to transfer DNA into plant cells. However, the plant material for each method was different.

2.5.1 Plant material and growing conditions

Tobacco plants (*Nicotiana tabacum* SR1) were used as the source of protoplast, and *Nicotiana benthamiana* was the suitable tobacco species for agroinfiltration. Both two species were grown at the temperature of + 24 ℃ in the daytime and + 20 ℃ at night. The photoperiod was 16 hours and the relative humidity was 65%. Plants were watered

twice a week (every Tuesday and Friday) with soluble fertilizer SUBSTRAL® Vita plus, which contains 6% N, 1.3% P and 5% K (Oy Transmeri Ab, Finland).

2.5.2 Protoplast isolation and electroporation

Leaves of 10 to 20 cm in length from *Nicotiana tabacum* SR1 were appropriate for protoplast isolation, as the cell walls were easy to digest. During protoplast isolation, all the steps were carried out gently, because the protoplasts are fragile and easy to break.

Leaves were sterilized using 70% EtOH and diluted Na-hypochlorite, which was made by one time commercial Na-hypochlorite (NaClO < 5%) (Colgate-Palmolive Company, Finland) and three times MQ water. Leaves were cut into 1mm wide strips and dipped in 1× Man-pp solution (appendix1) for 30 minutes. Enzyme solutions (appendix1) were freshly made and leaf strips were left in enzyme solution and covered with foil for 16 hours.

After the digestion was finished, the suspensions were pipetted through 100 μm nylon net into a 50 ml tube and then centrifuge at 500 rpm for 5mins to get the pellet. The pellet will then be re-suspended in 3 ml of 50% Percoll solution (appendix1). Three ml of 50% Percoll solution with protoplast was pipetted below 8 ml 20% Percoll solution (appendix1) and 1 ml 1× Man-pp was added on the top of 20% Percoll solution (Figure 9).

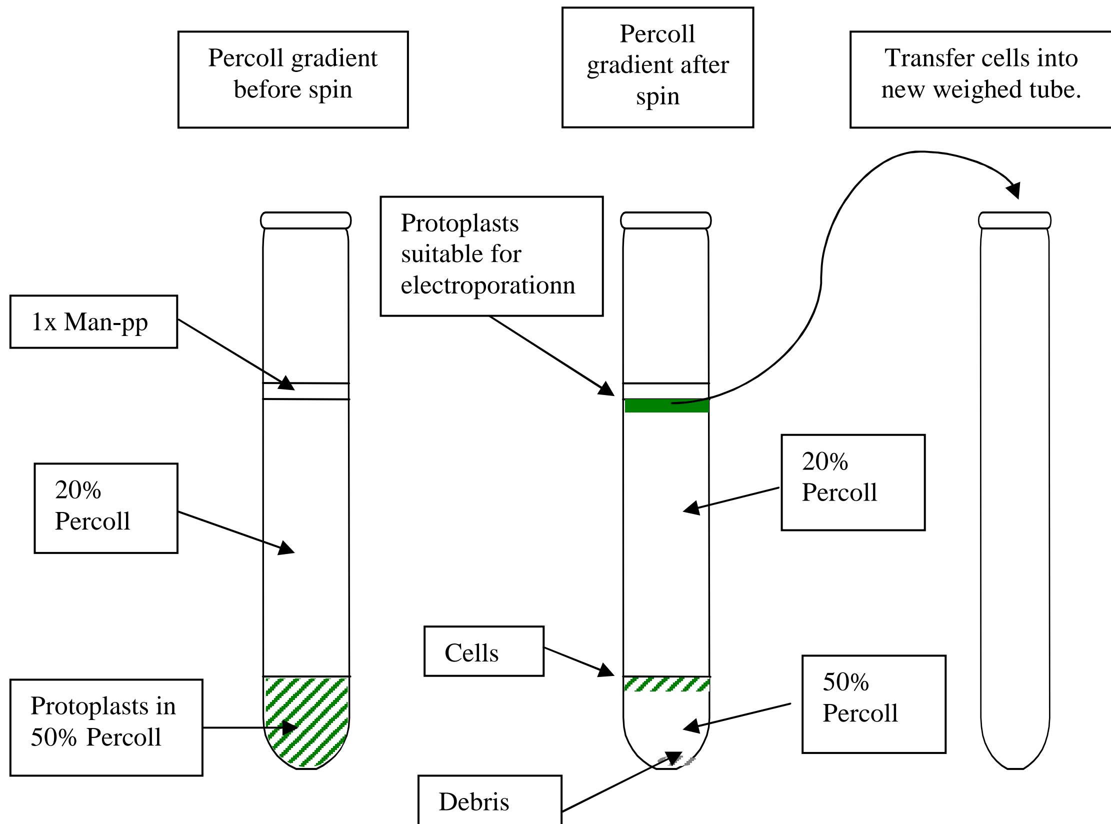


Figure 9. Schematic presentation of setting up the Percoll gradient for isolation of protoplasts.

The tube was then centrifuged at 1000 rpm for 10 min and the protoplasts on top of the 20% percoll solution were pipetted into a new weighted 15 ml tube. And the new tube was filled with Aa-buffer (appendix1) to 12 ml. The new tube was then centrifuged at 500 rpm for 10 min and supernatant was discarded. The tube was weighted again to calculate the amount of protoplast (one million protoplast weigh 45 mg). The protoplasts were used right away.

Electroporator was used to introduce DNA constructs into protoplasts. The electroporation system was crucial to transfer the plasmid DNA into plant cell successfully. Different parameters and systems were used during this experiment to optimize the system (Table 9) for the reconstructed DNA and tobacco protoplast. Plasmid DNA pHTT672 (full length Renilla luciferase) was used as a control to optimize the electroporation systems.

Table 9. Electropotation systems and parameters used for protoplast electroporation.

Voltage (V)	Capacitance (μF)	Resistance (Ω)	Cuvette (mm)
175	750	∞	4
220	250	∞	4
300	325	∞	4

DNA and protoplast densities were also important. 10 μg and 20 μg plasmid DNA were tested in electroporation respectively, and 1×10^5/200 μl protoplasts were used for every electroporation.

After electroporation, protoplasts were washed by 2 ml K3-Man-MES solution with hormones (appendix1) and incubated in the dim light for 24 to 48 hours, depending on the gene expression. If the time was too long, the protoplasts would die and the proteins would not be detected. If the time was too short, there would be not enough time for DNA expression; the protein could not be detected neither. The ideal incubation time was 24 hours, so the DNA can express and the protein would have enough time to interact with each other.

2.5.3 Agroinfiltration

Before infiltration, the *Agrobacterium* containing DNA constructs were incubated in liquid LB medium without antibiotics at +28℃ for 20 hours. OD600 values were measured and volumes of cultures needed for infiltration were calculated according to the formula:

Vconstruct=n × Vfinal × 0.5/OD600

n is the number of leaves to infiltrate, Vfinal should be at least 3 ml per leaf.

The *Agrobacterium* containing different DNA constructs, the interactions of which were investigated, were then mixed together and centrifuged at 5000g for 15 minutes at room temperature. Supernatant was discarded and the mixture was resuspended in Vfinal of activation buffer (appendix1), and then incubated for at least two hours at room temperature before infiltration. The mixture was injected into the abaxial side of 5 - 6 week old *N. benthamiana* by using a 1 ml syringe without needle. The plants will be incubated for 2 - 6 days depending on the level of protein expression (Waat and Kudla, 2008).

2.6 Methods of detecting and measuring gene expression

2.6.1 Split luciferase assay

After the incubation, 1.5 ml of K3-Man-MES solution was discarded and the protoplasts were transferred into 1.5 ml eppendorf tubes with the cut tip. 100 μl modified Lux buffer (appendix1) was added into the tubes and the protoplasts were crushed with a blue rod. Tubes were then centrifuged for 10mins at 15000 rpm at +4℃ to separate the protein and the cell residues.

The split luciferase assay was done by using Dual-luciferase® reporter assay system (Promega Corporation, Madison, WI, U.S.). 100 μl luciferase assay reagent II pre-dispensed into luminometer cuvette and 20 μl liquid from top layers of the samples were added into the luciferase assay reagent II and mixed by pipetting 2 to 3 times. The first measurement was taken immediately, and firefly luciferase was measured at this time. Then, 100μl Stop&Glo reagent was added and vortex briefly, the second measurement for Renilla luciferase should be finished within 5 seconds.

Both the first and second measurements were carried out by luminometer, luminoskan TL Plus Generation II (Thermo Lab Systems, Finland).

2.6.2 Data analysing of split luficerase assay

The significant tests of some data from split luciferase assay were analysed by SPSS software (Statistical Package for the Social Sciences, version 17.0, SPSS Inc., Chicago, IL, USA) (Fig. 15). Single sample *t* test was used to detect the significances of the results.

2.6.3 Detection of YFP under microscope fluorescent light

If the genes were expressed *in planta* and the proteins interact with each other, the C-terminal YFP protein and N-terminal YFP protein were able to reconstruct to form the entire YFP, which could be detected under the microscope in UV-light.

Camera Leica DFC 420C was used for detecting YFP in protoplast electroporation, and camera OLYMPUS DP50 was used for observing YFP in agroinfiltration.

3 Results

3.1 LR recombination reaction

Constructs for various assays were made by LR recombination reactions. They were digested with the appropriate enzymes (BsrG1 in this experiment) and tested in 1% agarose gel. Gel pictures below showed the purity of the constructs.

3.1.1 Enzyme digestions and testing of the constructs for BiFC assay with protoplast electroporation

Constructs of BiFC assay for protoplast electroporation were created by LR recombination reactions. In order to confirm the result of LR recombination reactions and the purity of the constructs, all the constructs were digested by enzyme BsrG1 and tested in 1% agarose gel. Vectors pARC233, pARC234, pARC235 and pARC236 were included in the enzyme digestion as controls (Fig. 10.).

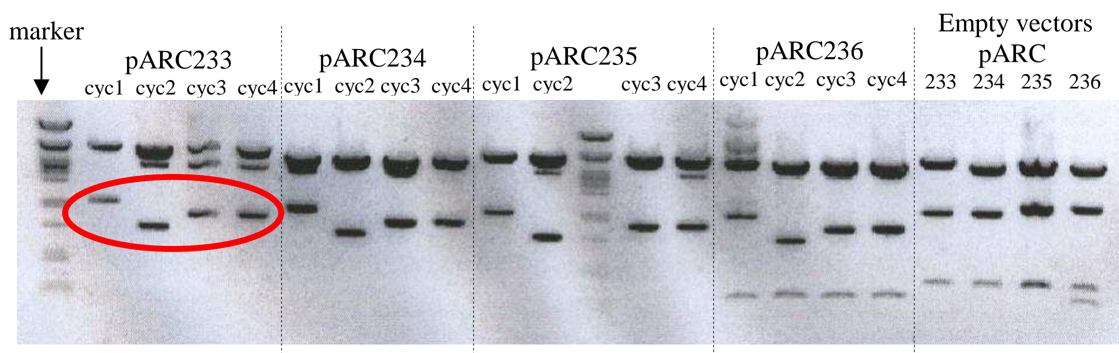


Figure 10. Gel picture of enzyme digestion of constructs for BiFC assay in protoplast electroporation. Inserts corresponding to *CYC1, CYC2, CYC3* and *CYC4*, marked in red oval, were shown clearly, which implied the LR recombination reactions were successful and the purity of the constructs was high enough.

Gene fragments shown by the red oval correspond to genes *CYC1, CYC2, CYC3* and *CYC4*, which were in entry clones before LR recombination reactions and combined with various destination vectors. In the combinations with different destination vectors, each *CYC* gene showed the same pattern which means that both the LR recombination reaction and the enzyme digestion were successful. Empty destination vectors pARC233 to pARC236 were used as controls and they showed different patterns from the constructs as expected.

3.1.2 Enzyme digestions and testing of the constructs for split luciferase assay with protoplast electroporation

The constructs for split luciferase assay were also created by LR recombination reactions. The enzyme BsrG1 digestion and 1% agarose gel test were performed to confirm the success of the LR recombination reactions and the purity of the constructs. Destination vectors, pDuEx-D7, pDuEx-An6 and pDuEx-Dc6, and entry clones pENTRH_2A and pENTRH_2B were included as controls (Fig. 11 and Fig. 12).

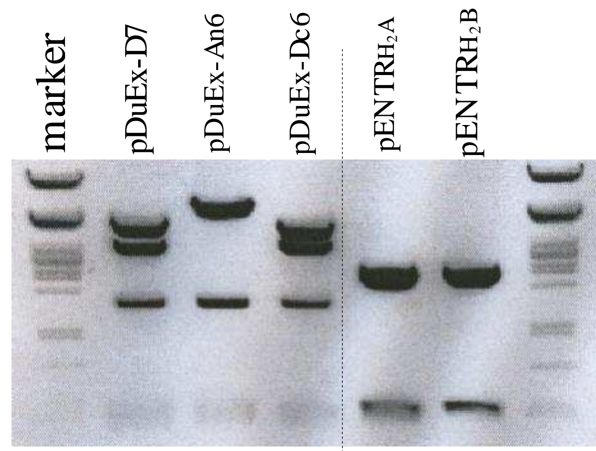


Figure 11. Destination vectors pDuEx-D7, pDuEx-An6 and pDuEx-Dc6, and entry clones pENTRH2A and pENTRH2B were digested as controls.

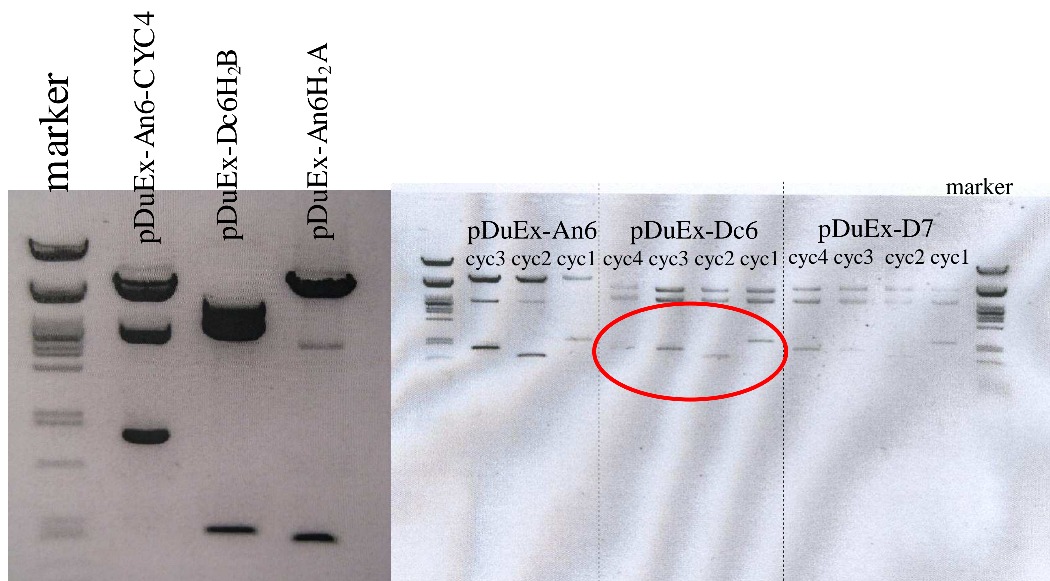


Figure 12. Gel picture of enzyme digestion of constructs of split luciferase assay in protoplast electroporation. Patterns of genes *CYC1, CYC2, CYC3* and *CYC4,* marked in red oval, were clear and showed the same pattern as in figure 10. The LR recombination reactions were successful and the constructs were pure.

CYC genes were digested from the constructs and could be seen clearly from the picture. *CYC* gene restriction patterns were the same as the patterns in figure 10, which convinced the success of LR recombination reactions. Constructs pDuEx-An6H_2A and pDuEx-Dc6H_2B were positive controls for split luciferase assay. Patterns of pDuEx-An6H_2A and pDuEx-Dc6H_2B in figure 12 showed the genes H_2A and H_2B were identical as the H_2A and H_2B patterns in figure 11, which means the LR recombination reactions were successful and pDuEx-An6H_2A and pDuEx-Dc6H_2B could be used as reliable positive controls

3.1.3 DNA constructs for BiFC assay with agroinfiltration

The DNA constructs for BiFC assay of agroinfiltration were made by LR recombination reactions. Linearization of the entry clones were completed by digesting the entry clones with selected enzymes. All the constructs were made successfully and tested by agarose gel electrophoresis (gel pictures not shown).

3.2 Optimization of conditions for protoplasts electroporation

Protoplast electroporation is one of the basic methods to introduce the plasmid DNA into plant cells. In this study, tobacco (*Nicotiana tabacum* SR1) protoplasts were used as the material for electroporation. The efficiency of electroporation is crucial and has direct impact on the results of the experiment. Therefore, the conditions of electroporation should be optimized beforehand. There are many important parameters for electroporation, such as the voltage (V) and capacitance (μF), the concentration of tobacco protoplasts and plasmid DNA and even the incubation time after electroporation.

3.2.1 Voltage (V) and capacitance (μF)

To obtain the optimal combination of voltage and capacitance, pHTT672 plasmid DNA containing the full length Renilla luciferase gene was used. The pHTT672 plasmid DNA was introduced into protoplasts by electroporation. Three sets of voltage and capacitance were tested. The space of the electroporation cuvette was 4 mm. The relative light unit (RLU) was the unit to measure the light emission from the luminescent reaction; higher RLU indicated successful expression of the luciferase.

The concentration of protoplasts for each electroporation was 1×10^5/200µl, which was also optimized. Three different concentrations, 5×10^5/200µl, 1×10^5/200µl and 0.5×10^5/200µl, were tested. The concentration of 5×10^5/200µl had the lowest RLU, while the concentration of 1×10^5/200µl had the highest RLU, which was more than ten times higher than the lowest one. This suggested that the concentration of 1×10^5/200µl protoplasts may have the highest transformation efficiency and it was selected as the proper concentration for electroporation.

The RLU obtained with the combination of 170V/ 750 µF was the highest among all the three sets of parameters (Fig.13). The mean RLU of the combination of 170V/ 750 µF was almost 500000. The combination of 300V/325 µF had the lowest transformation efficiency, the RLU of which was 23. The option of 220V/ 250 µF worked, but the RLU value was not high enough, which means the transformation efficiency was not very high in this combination (Fig 13.).

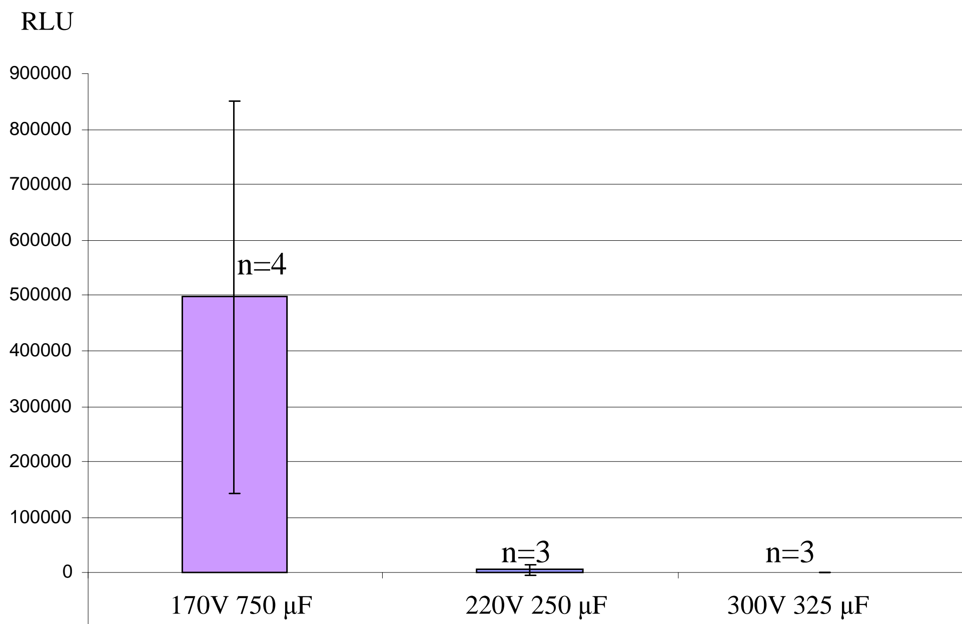


Figure 13. Means of RLU +/- SD in the Renilla luciferase assay with different combinations of voltage (V) and capacitance (µF) in electroporation. The 170V 750 µF is the best option among all the three sets of parameters. Protoplast concentration of 1×10^5/200µl was used.

3.2.2 Amount DNA (μg) and incubation times

Optimal voltage and capacitance (175V/750μF) was the key to an efficient DNA transformation by electropration. In addition, the amount plasmid DNA and incubation times would be the crucial factors for detection of protein-protein interactions. To find out the best amount of DNAs for interaction and the incubation times after electroporation, plasmid DNA pSOT1 (pDuEx-An6 H_2A) and pSOT7 (pDuEx-Dc6 H_2B) were used. pSOT1 and pSOT7 contain *H_2A* and *H_2B* genes with an N-terminal of luciferase fragment and C-terminal of luciferase fragment respectively, and these two genes encoding histone proteins in *Arabidopsis*, have been proved to interact with each other (Fujikawa and Kato, 2007). In this case, the protein-protein interactions *in planta* could be studied and high RLU value would suggest strong interactions.

Three different concentrations of DNA amounts, and two different incubation times were tested. Each treatment was performed at least three times (Fig 14.). For 24 hours incubation, the mean RLU of 10+10 μg/200μl DNA was 98.31, which was the highest among all the combinations. The mean value negative control was 23.89, which was the lowest among all the combinations. The treatment of 7.5+7.5 μg/200μl DNA also had a rather low RLU value 28.67, which was slightly higher than the negative control. Therefore, for 24 hour incubation, protein-protein interactions from the treatment of 7.5+7.5 μg/200μl DNA were not very intensive. And for the treatment of 10+10 μg/200μl DNA, protein-protein interactions were relatively stronger than other treatments.

For 48 hours incubation, the mean value of 5+5 μg/200μl DNA was 89.00, which was the highest among all the treatments. Again, the negative control had the lowest RUL value (22.67) and RLU value of 7.5+7.5 μg/200μl DNA was 36.00, which was slightly higher than the negative control. The protein-protein interaction after 48 hour incubation was quite strong in the treatment of 5+5 μg/200μl DNA, but still not strong enough with the treatment of 7.5+7.5 μg/200μl DNA.

In comparison of 24 hour and 48 hour incubation, the treatments of 5+5 μg/200μl DNA and 7.5+7.5 μg/200μl DNA in 48 hour incubations led to mean RLU values of 89.00 and 36.00 respectively. These are higher that the values obtained in 24 hour incubations, which were 68.75 and 38.67. However, for 10+10 μg/200μl DNA, the

mean RLU value in 24 hours incubation (98.31) was higher than that of 48 hour incubation (69.33), and it was the highest value among all the treatments.

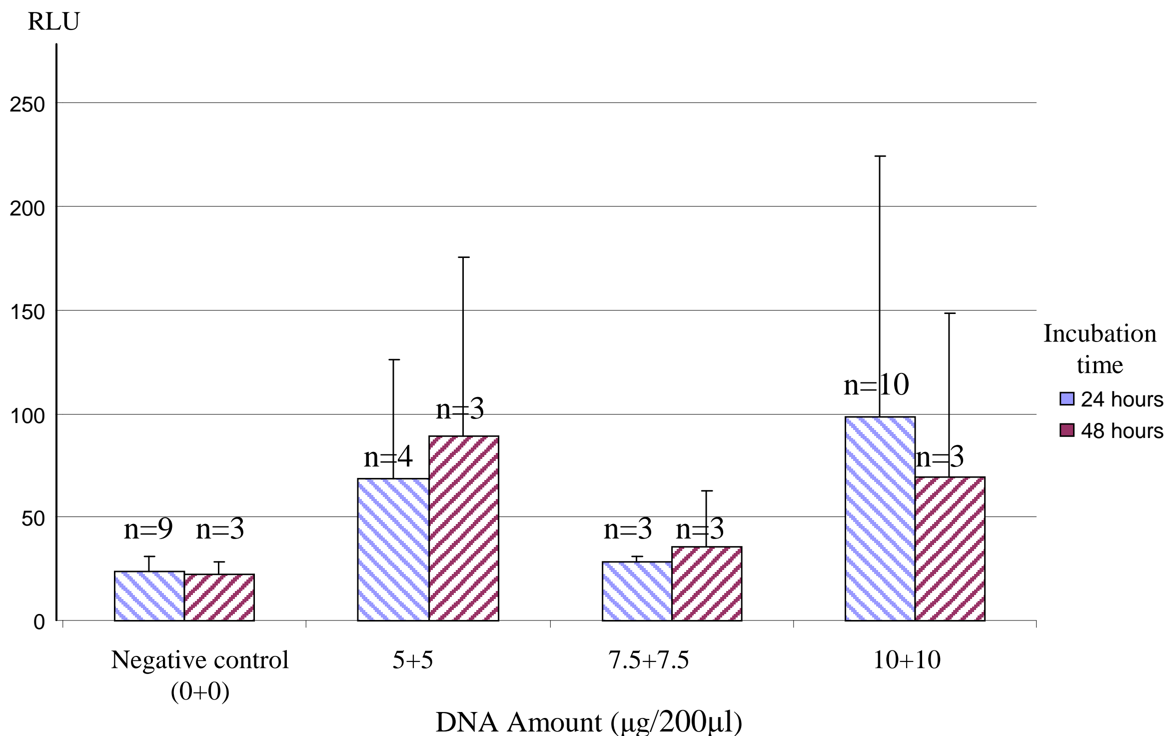


Figure 14. The average values of different treatment. The option of 10+10 μg/200μl DNA with 24 hours incubation has the highest reading, while the treatment of 7.5+7.5 μg/200μl DNA with 24 hours incubation has the lowest value among all the combinations.

However, for the combinations of 5+5 and 10+10 μg/200μl DNA, the standard deviations were very large, which indicated that the results were variable. It was difficult to determine the optimal combination by simply comparing the means. These results suggested that the protein-protein interactions could be affected by various factors, and it might be unreliable to affirm the protein-protein interactions by measuring the RLU values.

3.2.3 Interactions of *GhCYC* genes in protoplasts

In order to investigate the protein-protein interactions of GhCYC *in planta*, constructs containing *GhCYC* genes and N or C terminal of the luciferase, made by LR recombination reactions, were introduced into tobacco protoplasts by electroporation. Interactions, including self-interactions, among CYC1, CYC2, CYC3 and CYC4 were

tested, and 10 μg/200μl DNA for each *GhCYC* constructs were used for one electroporation. All the electroporations were carried out in the conditions of 170V/ 750 μF using the 1×10^5/200μl concentration of protoplasts. The measurements of RLU were taken after 24 hours incubation.

The mean RLU values in figure 15 showed that the combinations of N-CYC1 and C-CYC4, N-CYC3 and C-CYC4 as well as N-CYC4 and C-CYC4 had relatively high value, which indicated that the interactions between CYC1 and CYC4, CYC3 and CYC4 as well as CYC4 and CYC4 were relatively stronger than other combinations. The combinations of N-CYC1 and C-CYC1, N-CYC2 and C-CYC2 as well as N-CYC4 and C-CYC2 had relatively low RLU values, which were even lower that the negative controls. These suggested that there were no strong interactions between CYC1 and CYC1, CYC2 and CYC2 as well as CYC4 and CYC2.

However, the standard deviations were quite large for most of the combinations, which means the RLU values in different replicates varied a lot. The large standard deviations implied the unstable results and the interactions cannot be decided by simply comparing the means.

The significant values (P) of the combinations N-CYC1 and C-CYC4, N-CYC3 and C-CYC4 as well as N-CYC4 and C-CYC4 showed that the results from those combinations were not significant ($P>0.05$), which implies that the interactions in those combinations may not occurred. However, the significant value of the controls ($P<0.05$) suggested that the interaction between the H_2A and H_2B occurred.

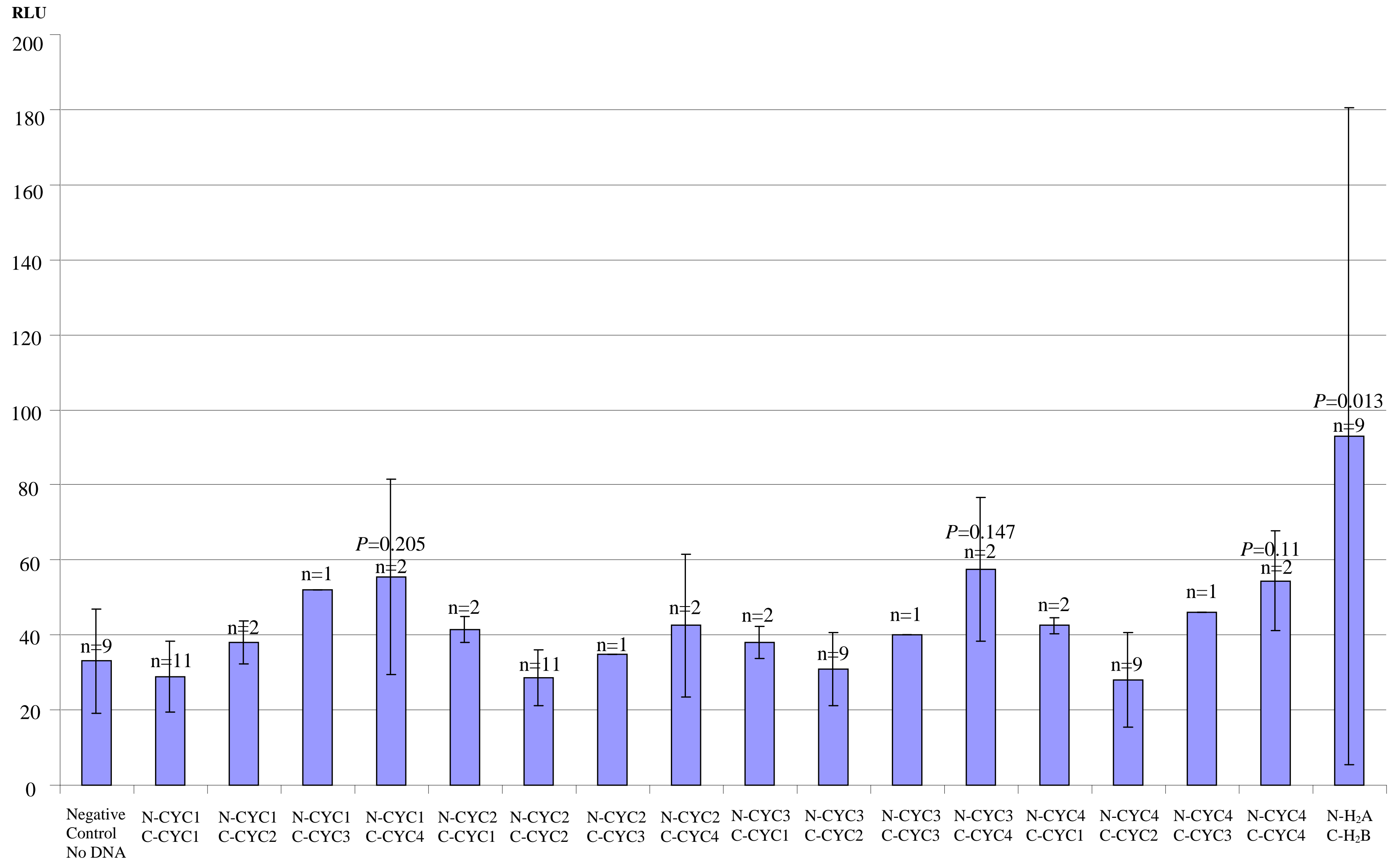


Figure 15. Protein-protein interactions between different combinations of GhCYC genes. Judged by the means, the combinations of N-CYC1 and C-CYC4, N-CYC3 and C-CYC4 as well as N-CYC4 and C-CYC4 had relatively high value. And the combinations of N-CYC1 and C-CYC1, N-CYC2 and C-CYC2 as well as N-CYC4 and C-CYC2 had relatively low RLU values. However, the large standard deviations implied the unstable interactions and the *P* values of combinations of N-CYC1 and C-CYC4, N-CYC3 and C-CYC4 as well as N-CYC4 and C-CYC4 suggest the differences were insignificant.

3.3 BiFC with protoplast electroporation

In order to compare the results from split luciferase assay, bimolecular fluorescence complementation (BiFC) was used as another method to detect protein-protein interactions *in planta*. In addition, the results from split luciferase assay were unclear, and the reactions of forming the luciferase were reversible, so it is not very easy to get clear and stable results. In this case, BiFC may provide another good choice. It is a visible method to detect the protein-protein interactions *in planta,* in addition, the reaction of forming YFP is an irreversible reaction, which means the low-level expressed protein-protein interactions could be detected by BiFC (Walter et al., 2004).

To investigate protein-protein interactions in protoplast by using BiFC assay, vectors pARC246 and pARC249 were used as positive controls. They contained the genes FBP2 and FBP11 respectively, and were made by Gateway cloning on the vector pARC235 and pARC236. *FLORAL BINDING PROTEIN (FBP)* genes are MADS box genes isolated from *Petunia.* Both FBP2 and FBP11 protein were found to be involved in ovule formation and have been proven to interact with each other (Immink et al., 2002).

The vectors pARC246 and pARC249 were introduced into protoplasts by electroporation, and after two days incubation, the fluorescent protein was observed clearly in protoplasts under the UV light but not under the normal light (Fig. 16). The fluorescent protein could be seen clearly in figure 16 (a1), which indicates that pARC246 and pARC249 were successfully transferred into protoplasts and expressed *in planta*. Proteins encoded by *FBP2* and *FBP11* were interacting and fluorescent proteins were successfully reconstructed. Figure 16 (b1) showed the same protoplast in normal light, and chloroplasts could be seen clearly. It can also be found that the fluorescent proteins can only be observed in UV light by comparing the central part of the protoplast in a1 and b1.

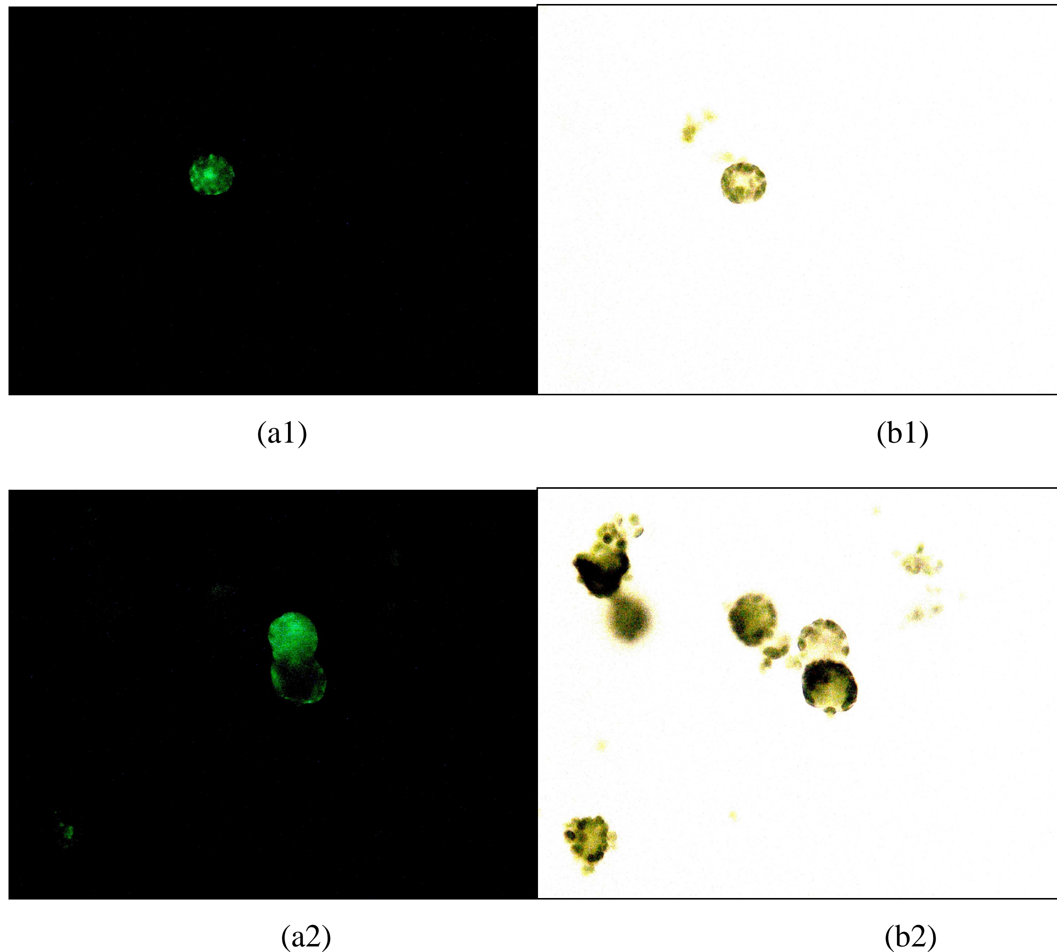


Figure 16. Photos of the same protoplasts taken under UV light and normal light. (a1 and a2) Protoplast under UV light. Fluorescent protein could be observed clearly; (b1 and b2) Protoplast under normal light. Only the chloroplasts could be observed. All the photos were taken with 25X objective, and time of exposure was two seconds.

For one electroporation, not all the protoplasts were successfully transformed with plasmid DNA. To make a comparison of the transformed and non-transformed protoplasts, photos of several protoplasts in the same field were taken (Fig. 16. (a2) and (b2)). In figure 16 (a2), under the UV light, one protoplast could be clearly observed and it was filled with fluorescent protein, which means the plasmid DNA were introduced and expressed in the protoplasts. In (b2), in the normal light, more than one protoplast was observed in the same field, and those protoplasts which cannot be seen in (a2) indicated that there were no fluorescent proteins inside.

3.4 BiFC assay with agroinfiltration

Agroinfiltration is another efficient method for transforming DNA into living plant cells and for studying protein-protein interactions *in planta*. To study protein-protein

interactions in plants' epidermal cells by using BiFC assay, agrobacterium containing the target genes were infiltrated into plants' lower epidermis and the genes will be transferred into living plant cells and expressed into proteins.

3.4.1 BiFC assay with agroinfiltration using control genes from gerbera

To confirm that the BiFC assay works efficiently and properly, MADS-box genes from gerbera were used as control genes to optimize the BiFC assay. Proteins coded by B function genes *GGLO1* and *GDEF1* in gerbera have been found to interact with each other by using GAL4 yeast two-hybrid assays (Broholm et al., 2009), and while the C function gene *GAGA1* encode proteins that do not interact with itself (Kotilainen et al., 2000). In this case, MADS-box genes *GGLO1*, *GDEF1* and *GAGA1*, were selected as control genes, and the combination of GDEF1+GGLO1 was the positive control while the combinations of GDEF1+GAGA1, GGLO1+GAGA1 and GAGA1+GAGA1 were the negative controls.

For the positive control (GDEF1+GGLO1), YFP signals were detected clearly under the UV light with both 25× and 40× magnification, especially in nuclei, the signals were much stronger than other parts of the epidermal cells (marked in white circles) (Fig.17.). The detection of the YFP signals implied that the target genes were successfully transformed into the plant cells by agroinfiltration. Strong signals from YFP indicated that the proteins coded by *GDEF1* and *GGLO1* interacted with each other and strong protein-protein interactions existed in the nucleuses of the epidermal cells.

For the negative controls (GDEF1+GAGA1 and GGLO1+GAGA1), the shape of epidermal cells could be observed under the UV light, but no YFP signals could be detected (Fig. 17). This indicated the YFP protein was not reconstructed because the protein from *GAGA1* had no interactions with the proteins from *GDEF1* and *GGLO1*. However, for the combination of GAGA1+GAGA1, weak signals could be detected under 40× magnification (marked in white circles), which suggests the potential weak interactions of the proteins from *GAGA1+GAGA1* or a false positive result.

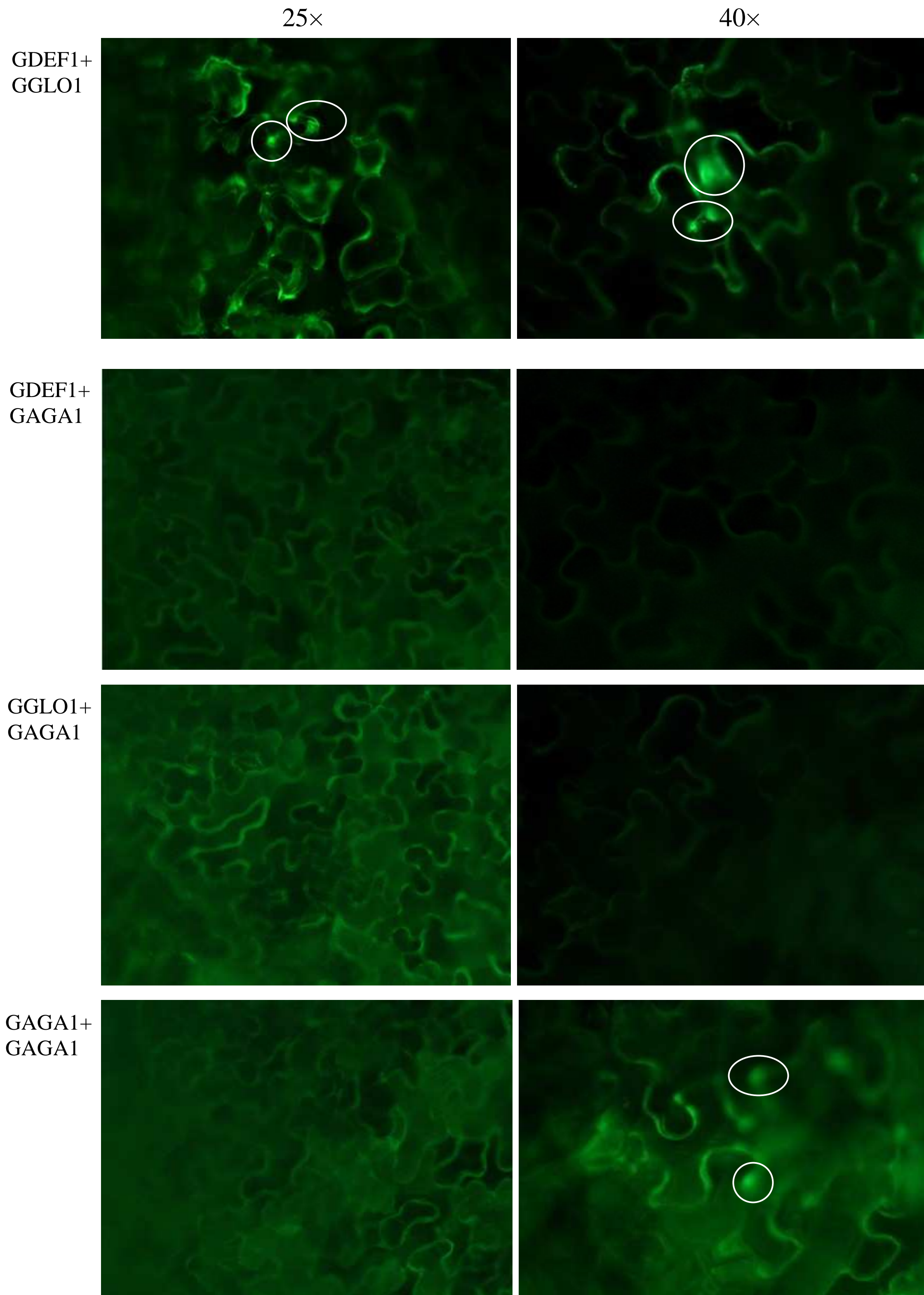


Figure 17. BiFC assays of protein-protein interactions from the gerbera control genes. For the combination of GDEF1+GGLO1, YFP signals from the nucleuses of the epidermal cells could be detected, which implies the DNAs were transformed into the plants' cells the successfully and strong protein-protein interactions existed between GDEF1 and GGLO1. For the combinations of GDEF1+GAGA1 and GGLO1+GAGA1, no YFP signals could be detected, which indicated no protein-protein interactions in the combinations of GDEF1+GAGA1 and GGLO1+GAGA1. For GAGA1+GAGA1, weak signals were found with 40× magnifier, which suggested weak interactions or a false positive result.

3.4.2 BiFC assay with agroinfiltration using *GhCYC* genes from gerbera

To study the protein-protein interactions from *GhCYC* genes, agrobacterium containing *GhCYC2*, *GhCYC3* and *GhCYC4* genes were infiltrated into tobacco (*Nicotiana benthamiana*). GhCYC1 was not included because of the failure of LR recombination reaction. Combinations of CYC2+CYC2, CYC2+CYC3 and CYC2+CYC4 were tested. For the positive control, *FBP2* and *FBP11* from petunia MADS-box gene group were used. These two genes encode proteins that were expressed in ovules and interact with each other to regulate the development of ovules (Immink et al., 2002). For the negative control, *CYC2* and *GAGA1* were used, which encoded TCP and MADS domain transcription factors in gerbera respectively and do not interact with each other.

For the positive control (FBP2+FBP11), strong signals from YFP were detected by microscope under UV light with both 25× and 40× magnification. Similar to the combination of DEF1+GGLO1, the signals from the nucleuses (marked in white circles) were stronger than other parts of the epidermal cells (Fig.18.). In addition, some small signal spots were observed in the cytoplasm (no shown in the photos). The success of detecting the signals from YFP indicated that the agroinfiltration was successful and the agrobacterium transformed the target genes into plant cells successfully. For the negative control (CYC2+GAGA1), no signals were observed under UV light, which suggested no interaction occurred between the proteins encoded by *CYC2* and *GAGA1*.

For the combination of CYC2+CYC2, no signals were detected, which indicated that no YFP were reconstructed and there were no interactions between CYC2 and CYC2 itself. However, for the combination of CYC2+CYC3, signals from YFP were clearly detected, and the signals were from nucleuses (marked in white circle) of the epidermal cells (Fig.18.). This suggested that the interactions occurred between the proteins encoded by *CYC2* and *CYC3*, and the locations of the interactions were in the nuclei. For the combination of CYC2+CYC4, signals from the YFP were observed under UV light with both 25× and 40× magnification as well, but the signals were not as strong as those found in the combination of CYC2+CYC3. It might be that interaction between CYC2 and CYC4 exist, but is not very strong.

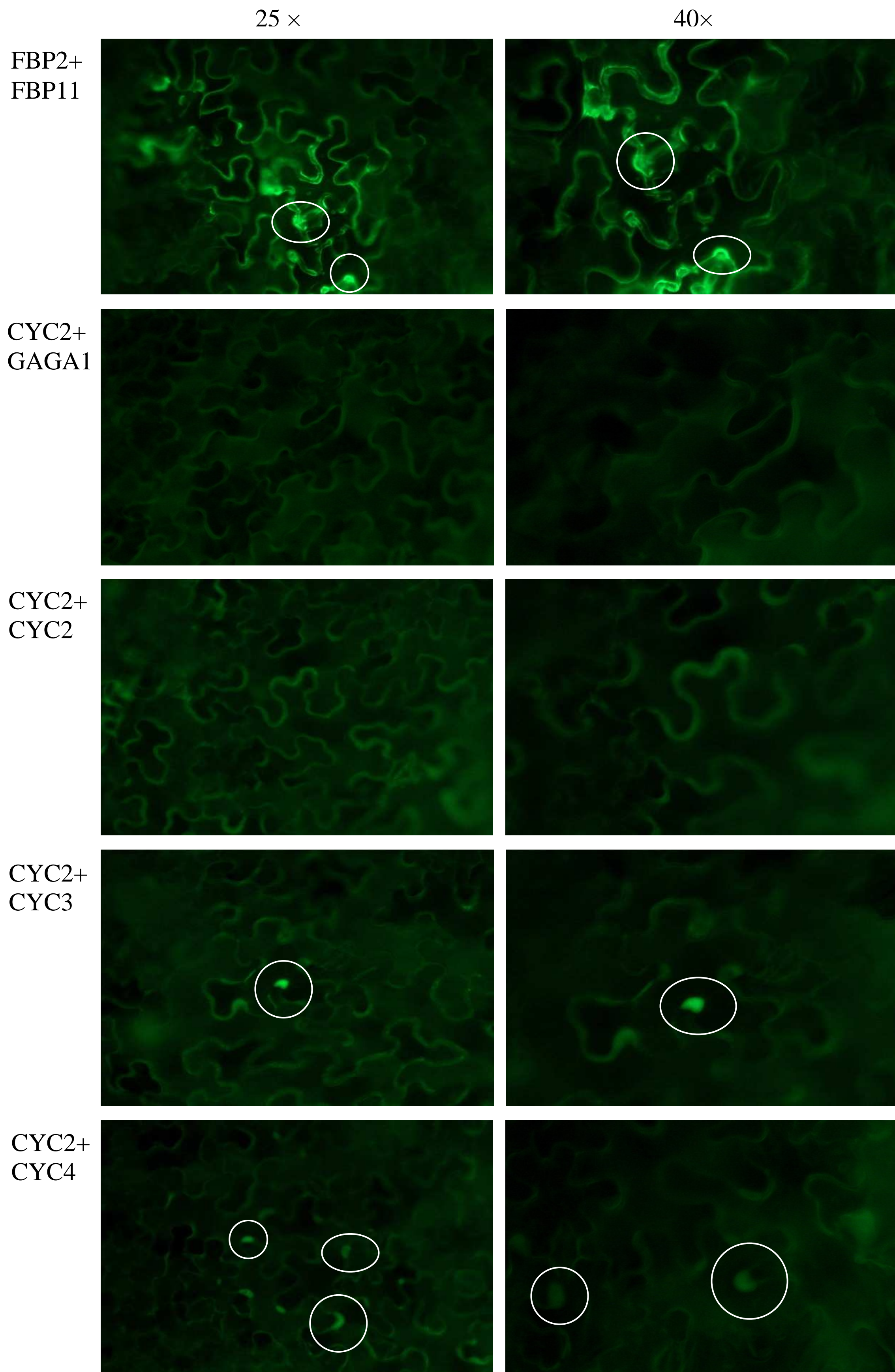


Figure 18. BiFC assays of the protein-protein interactions from *GhCYC* genes. FBP2+FBP11 was the positive control, and signals from YFP were observed from the photos. CYC2+GAGA1 was the negative control, and no signals were detected. Photos of CYC2+CYC2 have no signals detected, which indicated that no interactions between CYC2 and CYC2. In the photos of CYC2+CYC3 and CYC2+CYC4, signals were detected, but the interactions between CYC2 and CYC3 were stronger than CYC2 and CYC4.

4 Discussions

4.1 Optimization of electroporation conditions

Electroporation is a membrane phenomenon. Pores are formed at the cell membrane by a sudden and significant but temporary increase of the electrical conductivity and permeability of the cell membrane. During this short time, the target substances will be introduced into the cells (Weaver and Chizmadzhev, 1996). If all the parameters are right, the cell membrane would heal and be still alive. However, the parameters may vary in different cell types. Electroporation is used in bacterium, fungi， animal cells as well as plant cells, for which optimal parameters for electroporation varies with plant species. (Lurquin, 1997).

4.1.1 Optimization of voltage and capacitance of protoplasts electroporation

The data from optimization of voltage and capacitance showed that the highest RLU mean value was from the combination of 170V/ 750 μF. This suggested that 170V/ 750 μF was the optimal combination for tobacco protoplast electroporation among all three combinations in this experiment (170V/ 750 μF, 220V/ 250 μF and 300V/325 μF). The transformation efficiency decreased when the voltage and capacitance increased, which indicated that high voltage and capacitance were inappropriate for tobacco protoplasts in this experiment.

In Fromm’s article (1985), however, best result was obtained by using 350V electric pulse, and in his conclusion, higher voltage and capacitance led to higher transformation efficiency (Fromm et al., 1985). According to Bates (1995), the most effective transformation voltage for plant cells is between 500~1000V/cm, which means the voltage should be between 200~400V in the 0.4 cm cuvette (Bates, 1995). This is quite close to the voltage 170V used in this experiment. And according to Sagi (1994), the DNA would have the high transformation efficiency when the voltage ranged from 800 to 900 V/cm, which was 200-225 V for 4 mm cuvette (Sagi et al., 1994).

Other factors which are likely to affect the optimal range of voltage should also be taken into consideration. For example, Planckaert and Walbot (1989) found that protoplasts with different diameters have their own optimal voltages for electroporation.

In their experiment with maize protoplasts, the optimal voltage for 60 μm protoplasts was 450 V/cm, at the capacitance of 1550 μF, and for 20-50 μm protoplasts, the voltage was 600 V/cm (Planckaert and Walbot, 1989).

4.1.2 DNA amount, incubation time and electroporation solution in protoplasts electroporation

The data of RLU from optimization of DNA amount and incubation time showed that the combination of 10+10 μg/200μl DNA with 24 hours incubation resulted in the highest RLU value among all the treatments, which suggested that more DNA was helpful for the higher efficiency of the transformation and protein-protein interactions. Sagi et al. (1994) found that the optimal DNA concentration for electroporation was 60μg/ml, which was 12μg/200μl. Fewer DNA resulted in unstable and highly variable results (Sagi et al., 1994). Higher concentrations of DNA have also been shown to express more protein in living protoplasts (Fromm et al., 1985).

Incubation time, together with the electroporation buffer, affected the viability of protoplasts, which directly influenced gene expression in living protoplasts. The protoplasts had higher viability in chloride-free electroporation buffer with shorter incubation times. The number of surviving protoplasts varied irregularly in the chloride electroporation buffer, which resulted in unstable results (Sagi et al., 1994). According to Fromm (1985), however, the presence of $CaCl_2$ in the electroporation buffer increased electroporation efficiency and protoplast viability (Fromm et al., 1985).

4.2 Split luciferase assay of the interactions of GhCYC proteins in protoplasts

The data of split luciferase assay of the interaction of GhCYC proteins indicated that the interactions between CYC1 and CYC4, CYC3 and CYC4 as well as CYC4 and CYC4 were comparatively stronger than the other combinations. This confirmed the results detected by yeast two hybrid systems that interactions existed between CYC1 and CYC4, CYC3 and CYC4 as well as CYC4 and CYC4.

On the other hand, the split luciferase assay indicated no strong interactions between CYC1 and CYC1, CYC2 and CYC2 as well as CYC4 and CYC2, while the results from yeast two hybrid systems detected the existence of the interactions between CYC1 and CYC1, CYC2 and CYC2 as well as CYC4 and CYC2.

The problems of 'false positive' and 'false negative', which are inevitable in the yeast two hybrid system, have negatively influenced the reliability of the interactions detected (Ito et al., 2001). In this study, however, the standard deviations from split luciferase assay were very high, which suggested that the split luciferase assay was not able to provide stable results in this experiment. In this case, the existences of the interactions between CYC1 and CYC1, CYC2 and CYC2 as well as CYC4 and CYC2 could not be defined.

4.3 Large standard deviations of split luciferase assay

In this experiment, the high standard deviations existed in most results of split luciferase assay. The *P* values of the combinations of N-CYC1 and C-CYC4, N-CYC3 and C-CYC4 as well as N-CYC4 and C-CYC4 suggested the insignificant interactions. Based on these facts, the transformation efficiency is the foremost thing to consider. In general, electroporation can introduce DNA into protoplast with high transformation efficiency. However, there are many factors that are likely to make the transformation efficiency fluctuate. Besides the voltage and the diameter of protoplasts, the source of protoplast is another important factor. According to Planckaert and Walbot’s experiment with maize, the protoplasts isolated from suspension cells and those isolated directly from callus had different optimal voltages for electroporation, which will affect the transformation efficiency (Planckaert and Walbot, 1989).

Viability of protoplasts after electroporation, which directly affect the level of DNA expression, is another possible factor that may cause the large standard deviations DNA can only be successfully expressed into protein when the protoplasts stay alive after electroporation. As mentioned before, the electroporation buffer was crucial for the viability of protoplasts. The chloride electroporation buffer caused irregular change of viability of protoplasts (Sagi et al., 1994).

The electroporation buffer in this experiment Aa-buffer (appendix1) did not contain chloride. The incubation buffer K3-Man-MES solution with hormones (appendix1), however, contained chloride that may affect the protoplast viability and DNA expression into proteins. Studies of electroporation have shown that heat shock before electroporation may affect the transformation efficiency and gene expression. In Tautorus’ study in 1989, positive effect of gene expression was found when the protoplasts were heat shocked before electroporation (Tautorus et al., 1989).

4.4 Agroinfiltration and BiFC assay of control genes from gerbera

The pictures of BiFC assay of control genes from gerbera showed that both the agroinfiltration and BiFC assay were successful as the DNAs were expressed and successfully transferred into plant cells, while fluorescent signals were clearly detected under UV light. *GDEF1* and *GGLO1* encode proteins that have interactions with each other (Broholm et al., 2009) and signals from reconstructed fluorescent proteins were detected. The signals were from nuclei which indicated that the protein-protein interactions occurred in the nuclei and these proteins should be encoded by transcription factor genes *GDEF1* and *GGLO1*. However, in the recent meeting of the gerbera laboratory, the vectors containing *GDEF1* and *GGLO1* were found to be incomplete, which means the proteins coded by these vectors were not able to interact with each other. In this case, the signals detected from the combination of GDEF1 and GGLO1 should be a false positive result. For further studies, the interactions between GDEF1 and GGLO1 need to be confirmed again.

As for the combination of GAGA1+GAGA1, weak signals from nucleuses could also be detected which confirms the results from Kotilainen that weak interactions existed between GAGA1 and GAGA1 (Kotilainen et al., 2000). According to Ruokolainen’s study in 2010, however, no interaction exists between GAGA1 and GAGA1 (Ruokolainen et al., 2010). In this case, the fluorescent signals detected in this experiment might be from other interactions or it was a false positive result. In the combinations of GGLO1+GAGA1 and GDEF1+GAGA1, no fluorescent signals were detected which suggested no interactions occurred between the proteins.

4.5 BiFC assay with *GhCYC* genes

The photos of BiFC assay of *GhCYC* genes suggested that the agroinfiltration and gene transformation were successfully performed. The interactions of the positive control (FBP2+FBP11) in this experiment had been confirmed by many studies, yet there were still problems about the localization of the interactions between FBP2 and FBP11. Expressed individually, FBP2 is localized in nucleus while FBP11 in cytoplasm. However, the proteins encoded by FBP11 were transported into nuclei where the interactions occurred (Immink et al., 2002). The small signal spots in cytoplasm observed in this experiment might be the interactions of FBP11 proteins with other

proteins in cytoplasm. As for the negative control in this experiment, results were in accordance with the expected. CYC2, which is one of the TCP transcription factors, is not expected to interact with GAGA1, coded by the B function gene of MADS box genes in gerbera (Kotilainen et al., 2000; Broholm et al., 2008).

Photos of CYC2+CYC2 indicated that no interaction occurred in the epidermal cells, which confirmed the result of split luciferase assay that the RLU value of CYC2+CYC2 was the lowest among all combinations (even lower than the negative control). However, the unpublished results from yeast two-hybrid system showed the existence of the interaction between CYC2 and CYC2 (Sari Tähtiharju, personal information). In this case, while the possibility that the interaction between CYC2 and CYC2 in yeast two-hybrid system is a false positive result could not be safely excluded, it could also be possible that CYC2 was not even able to interact with itself properly, as shown by the result *in planta*.

From the photos of CYC2+CYC3, strong signals could be seen clearly, which suggested strong interactions between CYC2 and CYC3. The fact that both CYC2 and CYC3 have NLS and express themselves in nucleus has proved the occurrence of the interactions inside nucleus. Similarly, interactions were also observed in yeast two-hybrid system. However, the low RLU value of CYC2+CYC3 in split luciferase assay indicated the existence of the weak interaction.

Signals from the photos of CYC2+CYC4 suggested that interactions between CYC2 and CYC4, although not as strong as those between CYC2 and CYC3, occurred and were localized in nucleus. The results of yeast two-hybrid system also showed a positive result of the existence of the interactions. These two results confirmed the results from split luciferase assay that the interactions between CYC2 and CYC4 existed, but were not strong.

The protein-protein interactions of GhCYC genes were performed only once due to time limitation in the schedule of the whole experiment. In order to reach a higher stability and reliability of the results, the same infiltration should be done for at least twice and more.

4.6 Comparison of split luciferase assay and BiFC

The split luciferase assay and BiFC assay were used to confirm the protein-protein interactions of *GhCYC* genes. Judging from the results of the two assays, BiFC assay

appears to be not only easier but also more reliable. However, this does not necessarily suggest the inferiority of the luciferase assay as an analytical method, as the failures to obtain better results might be ascribed to the problems in protoplast electroporation.

Regardless of the methods of DNA transformation, both BiFC assay and split luciferase assay have in themselves advantages and disadvantages as analytical methods. The BiFC assay is a visible assay where the protein-protein interactions can be seen and visually scored. During this process, the fluorescent protein is irreversible once reconstructed. According to Walter (2004), BiFC is a very sensitive method that can detect protein-protein interactions at a very low level (Walter et al., 2004). Sometimes, the interactions occurred even when the fluorescent protein fragments were separated by a very short distance (Hu et al., 2002). In addition, false positive results can be detected if there are proteins in plant cells that interact with the proteins encoded by the foreign DNA. It was pointed out by Fujikawa and Kato (2007) that an external light is needed in the BiFC assay, which has inevitably increased the background light of plant cells and thus made it harder to distinguish the light from the fluorescent proteins when the interactions are weak (Fujikawa and Kato, 2007).

Split luciferase assay can be used to detect the protein dissociation taking the advantage of the reversibility of the luminescent reactions. However, this is made possible only when the data is collected at an exact time. Besides, failure to specify the location of the protein-protein interactions is another disadvantage of the split luciferase assay (Fujikawa and Kato, 2007).

4.7 Comparison of protoplast electroporation and agroinfiltration

Protoplast electroporation and agroinfiltration were the two methods applied in this experiment for transferring DNA into plant cells. By comparing the results of these two methods, it can be concluded that agroinfiltration is more likely to lead to stable and reliable results and it is much easier to use than protoplast electroporation.

As shown in figure 13, 14 and 15, the large standard deviations in the results of split luciferase assay indicated that protoplast electroporation is not an ideal method to obtain stable results. The problem might be that the DNAs cannot be transferred into protoplast efficiently or the DNAs were not able to express after electroporation. As discussed before, there are many factors affecting transformation efficiency of protoplast electroporation and protoplast viability after electroporation, such as voltage and

capacitance, DNA concentration, electroporation solution and even the size and source of the protoplasts (Fromm et al., 1985; Planckaert and Walbot, 1989; Sagi et al., 1994). Newell also pointed out in his review in 2000 that the protoplast electroporation is more suitable for single copy of plasmid DNA than for multiple copy of DNA, which suggested that protoplast electroporation is not an ideal method of detecting protein-protein interactions (Newell, 2000).

The problem of killing cells is one of the major disadvantages of electroporation not only in plant studies but also in animal studies. In Fenton's study with lymphoblastoid (1998), he pointed out that the survival rate of lymphoblastoid cells after electroporation was from 1% to 10%, and only 10% of the survival cells were successfully transformed with DNAs (Fenton et al., 1998).

Another disadvantage of protoplast electroporation lies in the preparation of protoplast. Successful electroporation and high transformation efficiency demand high quality of protoplasts. Newell mentioned in his review in 2000 that the protoplasts culture and preparations are highly time-consuming and many methods with protoplasts have already been replaced by faster systems (Newell, 2000).

Agroinfiltration, an *Agrobacterium* mediated transformation method, is relatively simpler and faster as compared to protoplast electroporation. It was performed directly on the plants without the procedure of preparing for protoplasts. Genes were then transferred into plant cells in a more natural way and the transformation efficiency is also higher than in electroporation.

5 Conclusions

The protein-protein interactions *GhCYC* genes were confirmed in this experiment by using two different assays. BiFC assay provided the more stable and reliable results than split luciferase assay. Based on BiFC results, no protein-protein interactions existed between CYC2 and CYC2, which was contrary to the results obtained in yeast two-hybrid. The protein-protein interactions between CYC2 and CYC3 were quite strong, and this confirmed the results from the previous yeast two hybrid assays. CYC2 and CYC4 might interact but not very strongly, since only weak signals were detected from the BiFC assay.

From this experiment, the BiFC assay seemed to be a better method of detecting protein-protein interactions *in planta*, since it detected the protein-protein interactions

successfully and specified the localizations where the interactions occurred. For split luciferase assay, the results were unstable and no specific conclusions could be made. The failures in obtaining good results may be due to the problems of protoplasts electroporation. Therefore, another new method of DNA transformation could be used together with the split luciferase assay in order to find out its reliability.

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Appendix1

<u>1 x TBE buffer</u>

90 mM	Tris
90 mM	Boric
2 mM	EDTA

The pH value should be close to pH 8.3.

<u>1 x Man-pp solution (for 500 ml)</u>

B5 salts	(sigma G-5893)	1.94 g
500 mM	mannitol (MW 182.2g/mol)	45.6 g
2%	sucrose	10 g
0.5%	MES pH5.7 (KOH)	2.5 g

Autoclave 15 min at + 120℃
2 x Man-pp solutions was prepared for Percoll solutions

<u>K3-Man-MES solution with hormones (for 10 ml)</u>

1 x	Man-pp 2 x	5 ml
1%	adjunct salts 100 x	100 µl
0.1 µg/ml	NAA (1 mg/ml is 10 000 x)	1 µl
0.2 µg/ml	BAP (1 mg/ml is 5 000 x)	2 µl

This solution should be made just before use.

<u>Aa-buffer</u>

550 mM	mannitol
35 mM	aspartic acid monopotassium salt
35 mM	glutamic acid monopotassium salt
5 mM	calsium gluconate
5 mM	MES, Ph 7.0 (KOH)

Autoclave 15 min at + 120℃

<u>Adjunct salts</u>

75 mg/ml	$CaCl_2 \cdot 2H_2O$ (510 mM)
25 mg/ml	NH_4NO_3 (312 mM)

Autoclave 20 min at + 120℃

Enzyme solution

1 x	Man-pp	
0.5%	cellulose	(750 mg/150ml)
0.2%	macerase	(300 mg/150ml)

Mix 30 – 60 min to dissolve, be made just before use, sterilize by filtration

Percoll solutions

	50%	20%
2 x Man-pp	2 ml	5 ml
100% Percoll	2 ml	2 ml
DDW	-	3 ml

Be made just before use.

Modified Lux buffer

50 mM	Na – phosphate	Ph 7.0
4%	soluble PVP (MW 360.000)	
2 mM	EDTA	
20 Mm	DTT	

The PVP should be dissolved at room temperature. DTT was added just before use.

Activation buffer

1 M $MgCl2$, autoclaved	10 mM
0.5 MES/KOH (pH5.6), filter- sterilized	10 mM
0.5 M Acetosyringone in DMSO, stored at -20℃	150 µM