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Plant Transformation: Needs and Futurity of the Transgenes

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Abstract: To produce transgenic plants which have various applications in agricultural and non-agricultural fields, a marker gene is necessary to recover a viable transgenic plant. To express or transcribe of transgenes, utilization of promoters is also unavoidable. Analysis of transgenes includes copy number, insertion site, integration stability, expression and it's pattern and variability is immensely important in order to develop a successful transgenic event. This review presents the necessities for better recovery of transgenic plants, transcription or expression of transgenes, as well as methods to analyze transgenes.

Key words: Expression, integration site, marker gene, promoter, reporter gene

INTRODUCTION

Presently, transgenic crops are increasingly becoming a dominant feature of the agricultural landscapes of developed countries. Since the introduction of Genetic Modified (GM) crops in 1996, the average growth in area has been more than 10% per year. This growth represents one of the highest adoption rates for any crop technology (Dunwell, 2004; James, 2005). Transgenic plants have also great potential impacts in non-agricultural fields, e.g., production of the medically useful products (pharmaceutical farming) or edible vaccine for oral virus immunization (Richter *et al*., 2000). Introduction of desired genes into the plants consist of several steps. One of the steps of gene delivery to plant is the stably integration of the transgene into the host cell's genome in a manner that will result in proper expression of the gene. To establish an efficient expression from quantity and frequency points, it is unavoidable that up-stream promoting sequences are inserted exactly before the gene without any frame shift. Selection of a promoter depends on some factors, e.g., type of plant, target tissue(s), inducible or non-inducible expression, etc. Insertion region effects strongly the expression. In addition, in order to occur any gene knockout or disruption, it is very important to analysis of the insertion region. Others factors such copy number and its variation also effect expression. Therefore, quantification of expression for few generations is indispensable. Expression pattern and time are the others which must be analyzed. Marker genes are essential to recover viable transgenic plants because the low transformation efficiency. Then, it must be inserted into the cassette or co-transformed. At present, different methods developed to deletion of the marker due to concerns about widespread occurrence in novel ecosystems, horizontal gene transfer or vertical gene transfer (Darbani *et al*., 2007; Holger, 2003). Substitution of the new marker genes without any risk after spreading is the other way (Darbani *et al*., 2007; Mentewab and Stewart, 2005).

In this study, we will discuss about promoters and markers as the necessities to produce transformed plants as well as different points of transgene analysis, e.g. copy number, expression and integration site.

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NEEDS FOR BETTER PLANT TRANSFORMATION

Theoretically, it is possible to produce transgenic plant using a coding region transformation. But it is not applicable due to the low chance of a proper integration (exactly after a promoter) and paucity of the transformation occurrences which caused to need a costly and time consuming process. However, these hindrances could be obviated via promoters and marker genes.

Promoters: Expression cassettes are comprised of three segments of DNA that includes up-stream regulatory region (promoters and enhancers), multiple cloning sites with the gene of interest and down-stream regulatory sequences. Up-stream and down-stream regions must be compatible with plants of interest. Therefore, efficient expression of foreign genes in transformed plants requires that they are placed under control of an active promoter in plant cells. Promoters are sequences that bind the RNA polymerase enzymes as cis-acting regulatory region, which is responsible for RNA transcription. Promoters can be generally divided into constitutive promoters, chemically or physically inducible promoters and tissue-specific or development-stage-specific promoters.

The *Cauliflower mosaic virus* (CaMV) 35S promoter is the most widely used constitutive promoter in dicots (Guilley *et al*., 1982). The 35S promoter has a high level of transcription in nearly all plant tissues (Kay *et al*., 1987). Other viral promoters have been characterized and cloned for use in transformation experiments (Mor *et al*., 2003; Carrillo-Tripp *et al*., 2006) such as the wheat dwarf virus (WDV) promoter. This promoter is particularly effective in monocots (Hofer *et al*., 1992). Also, Subterranean Clover Stunt Virus (SCSV) promoter has been used either in dicots and monocots with similar pattern to those resulted with the actin (Act-1) or ubiquitin (Ubi) promoters (Petra *et al*., 2003). Plant Ubi (Christensen and Quail, 1996), Act-1 (McElroy *et al*., 1990, 1991) and alcohol dehydrogenase 1 (Adh-1) (Dennis *et al*., 1984; Ellis *et al*., 1987; Zhang and Wu, 1988) are also some of the monocot constitutive promoters that are widely used in plant expression cassettes.

Inducible expression is importance not only because of transgene properties, for example, if they are developmentally lethal, but it is often advantageous to obtain conditional expression to address research goals. Some inducible promoters include those induced by tetracycline, ecdysteroid agonist and glucocorticoid (Reynolds, 1999), as well as heat-shock promoters such as Gmhspl7.5-E gene promoter (Ainley and Key, 1990), wound based inducible promoters, stress induced promoters (Reynolds, 1999) and a light induced RUBISCO small subunit gene (rbcs) promoter (Schardl *et al*., 1987).

Also, gene expression in a tissue-dependent manner and according to the developmental stage of the plant is achievable using tissue specific promoters such as root specific promoter of tobacco TobRB7 gene (Yamamoto *et al*., 1991), tuber specific promoter of potato patatin B33 gene (Rocha-Sosa *et al*., 1989), flower specific promoter of the petunia chsA gene (Koes *et al*., 1989, 1990), endosperm-specific promoter from multigene family of monocot glutenin subunits (Roberts *et al*., 1989) and seed specific napin promoter of *Brassica napus* (DeLisle and Crouch, 1989; Stayton *et al*., 1991; Stalberg *et al*., 1993). In addition, it was demonstrated the ability of T7 promoter to promote expression in plants even with tissue specificity and inducibility expression which it can be useful in both dicot and monocot plants (Nguyen *et al*., 2004; Dunn *et al*., 1988; Gatz *et al*., 1992).

Marker genes: A selectable marker gene is used to recover transformants which permits the preferential growth of transformed cells. Also, the stable introduction of genes into plants through genetic engineering normally necessitates the use of a selectable marker, especially when the transformation frequency is low, 10^{-3} to 10^{-6} (Flavell *et al*., 1992).

Several types of selectable marker genes exist, which could be separated into five groups including antibiotic-resistance, herbicide-resistance and metabolic pathway-based and positive and negative selectable marker genes. Some of well-known antibiotic selectable marker genes are: neomycin phosphotransferase gene (nptII) from *Escherichia coli* transposon Tn5 as the first used selectable marker for plant transformation (Herrera-Estrella *et al*., 1983; Bevan *et al*., 1983), hygromycin phosphotransferase gene (hpt or aphIV) from *E. coli* (Waldron *et al*., 1985; Van den Elzen *et al*., 1985; Lulsdorf *et al*., 1991; Walters *et al*., 1992), streptomycin resistance gene (spt) from transposon Tn5 (Jones *et al*., 1987), aminoglycoside-3"-adenyltransferase (aadA) (Svab *et al*., 1990), dihydrofolate reductase (dhfr) mouse gene (Hauptmann *et al*., 1988; Eichholtz *et al*., 1987) and bacterial kanamycin-resistant nptII gene and an *Arabidopsis thaliana* ATP Binding Cassette (ABC) transporter (Atwbc19) gene (Mentewab and Stewart, 2005). It is worth mentioning that while most marker genes are from bacterial origin, there is a renewed emphasis on using marker genes from plants, which might have higher biosafety. Herbicide resistance markers include a bialaphos resistance gene (bar) from *Streptomyces hygroscopicus* (Thompson *et al*., 1987; Somers *et al*., 1992; Gordon-Kamm *et al*., 1990; Schroeder *et al*., 1993), chlorsulfuron resistance gene (csr1-1) from *Arabidopsis* (Haughn *et al*., 1988; Fromm *et al*., 1990), sulfonamide resistance gene from plasmid R46 (Guerineau and Mullineaux, 1989) and a mutant petunia epsps gene (Shah *et al*., 1986).

There still remain biosafety concerns about marker genes in transgenic plants. Application of the metabolic pathway based markers, such as bacterial dihydrodipicolinate synthase (dhps) (Perl *et al*., 1993) as aspartate family metabolic pathway based marker, betaine aldehyde dehydrogenase (badh) gene from spinach (Daniell *et al*., 2001), *E. coli* derived phosphomannose isomerase (PMI) (Wright *et al*., 2001), xylose isomerase (xylA) gene of *Streptomyces rubiginosus* (Jaiwal *et al*., 2002) and trehalose-6-phosphate synthase, AtTPS1, (Leyman *et al*., 2004), without any concerns about horizontal or vertical gene transfer is one of the ways to reduce the marker gene effects from horizontal or vertical transfer points. By the way, insertion of any additional sequence into the host genome is unfavorable. Therefore, expelling of the marker gene could be the ideal which applicable via different approaches, e.g., co-transformation especially using P-DNA, site-specific recombinase-mediated marker deletion (e.g., Cre/loxP, FLP/FRT and R/RS site-specific recombination systems), transposon-based expelling systems (e.g., Ac transposon) and intrachromosomal recombination based excision (Holger, 2003; Scutt *et al*., 2002; Darbani *et al*., 2007).

Cyanamide hydratase (Cah) gene (Weeks, 2000) as positive selectable markers and nitrate reductase gene (nia) (Nussaume *et al*., 1991), cytosine deaminase (codA) (Perera *et al*., 1993) and human herpes simplex virus thymidine kinase type 1 gene (HSVtk) (Czako and Marton, 1994) as negative selectable marker genes and dao1, encoding D-amino acid oxidase as either positive and/or negative marker gene (Erikson *et al*., 2004) are some of the other markers.

The mentioned segregation or expelling based marker gene deletion approaches could be more efficient using a positive-negative selection system (Holger, 2003; Scutt *et al*., 2002; Darbani *et al*., 2007).

TRANSGENE ANALYSIS

The development of efficient crop transformation systems has necessitated the development of efficient methods for detailed molecular characterization of putative transgenic events. Transgene analysis has different aspects such as transgene quantification, position and expression assays will be discussed in this section.

Copy number and integration site: Standard Polymerase Chain Reaction (PCR) methods could be utilized to detect the presence of foreign DNA in transformed plants, but this presence/absence assay is problematic because of the chance of trace DNA contamination or residual *Agrobacterium*. It is more useful to determine of the number of inserted copies of transgenes. Traditionally, researchers have applied nucleic acid blotting techniques to provide an estimate of transgene copy number and organization (Southern, 1975; Sabelli and Shewry, 1991, 1995a). Although Southern blot analysis is the gold-standard for determining loci number and intactness of the insert, this method is unwieldy to apply in high-throughput screening of putative transformants. Recently, the development of quantitative PCR (Q-PCR) for determining transgene copy number has overcome the limitations of standard PCR and Southern blot analysis (Ingham *et al*., 2001; Mason *et al*., 2002) which it is high-throughput approach using small amounts of sample with capacity of selection as soon as at tissue culture phase, is faster and less expensive than southern blot analysis (Mason *et al*., 2002). It is important to use proper technical and statistical techniques for robust estimations of transgene copy number.

Chromosomal site of transgene can be detected via radioactive based *in situ* hybridization (Gall and Pardue, 1969) or via fluorescence *in situ* hybridization (FISH) methods (Ten Hoopen *et al*., 1996; Snowdon *et al*., 2001; Choi *et al*., 2002; Bourdon *et al*., 2002). In order to precisely studying of integration site of transgenes, thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) is usable. TAIL-PCR and high-throughput TAILPCR (HT-TAILPCR) are fast and efficient methods to amplify unknown sequences adjacent to known insertion sites (Liu and Whittier, 1995; Budziszewski *et al*., 2001; Parinov *et al*., 1999; Tissier *et al*., 1999; Singer and Burke, 2003).

Variation of transgene expression: Various factors are thought to influence transgene expression in plants, including transgene copy number, transgene insertion site and RNA silencing in the host plant. Also, regulatory sequences could be effect on expression level and its variation. For example the mannopine synthase promoter yields less variable but also lower transgene expression than that of 35S (De Bolle *et al*., 2003). It is determined that the transgene copy number may depends on the transformation method (Butaye *et al*., 2005). Gene targeting into a predefined chromosomal site could be achieved by homologous recombination. To date, however, the moss *Physcomitrella patens* is the only (lower) plant that has shown a high rate of homologous recombination. Integration of transgenic DNA through homologous recombination is so infrequent in higher eukaryotes and therefore, targeting transgenes to a specific position is nearly infeasible (Kumar and Fladung,

2001). A more promising method to generate single-copy transgenic plants is the recombinase-mediated resolution of multiple insertions, which allows site-specific integration of the transgene (Ow, 2002). However, this method has only been partially successful and efficiency is poor. Also, the implementation of Matrix Attachment Regions (MARs) in transgene constructs might be considered when low variation of transgene expression is desired (Allen *et al*., 2000). MARs can increase the expression of transgenes in whole organisms or cells in culture and is necessary for the proper formation, maintenance, or regulation of chromatin structure. MARs have been isolated from diverse range of eukaryotes, including mammals, birds, insects and plants varying from 50-200 kb in length (Schöffl *et al*., 1993; Allen *et al*., 1993, 1996; Vain *et al*., 1999; Chinn and Comai, 1996; Mlynarova *et al*., 1994; Liu and Tabe, 1998; Van der Geest *et al*., 1994; Holmes-Davis and Comai, 1998). Experimental data indicated that MARs enhance transgene expression from 2- to 60-fold in different plant systems (Holmes-Davis and Comai, 1998; Allen *et al*., 2000; James *et al*., 2002; Nowak *et al*., 2001; Ülker *et al*., 1999; Halweg *et al*., 2005). It has been recently shown that the RB7 MAR, a 1.2 kb fragment isolated from the 3' flanking region of the RB7 root-specific gene of tobacco, caused a significant increase in the activity of highly active promoters (CaMV 35S, nopaline synthase promoter and octopine synthase promoter) as compared to controls. However, the presence of RB7 MAR did not significantly increase the activity of weak promoters. Importantly, most transgenes flanked by RB7 MAR showed a large reduction in the number of low-expressing transformants relative to control constructs without MARs, suggesting that these elements can reduce the frequency of gene silencing in primary transformants (Mankin *et al*., 2003; Halfhill *et al*., 2003). Also, MARs play a role in reducing variability of that expression between independent transformants (Liu and Tabe, 1998; Mlynarova *et al*., 1995, 1996).

Expression assays: The study of transgene expression is of vital importance whenever transgenic plants are produced. Many techniques exist to analyze of transgene expression such as western blot analysis and Enzyme-Linked Immunosorbent Assay (ELISA) at the protein level (Towbin *et al*., 1979; Burnette, 1981; Yalow and Berson, 1959; Engvall and Perlman, 1971). *In situ* hybridization is a method to assay patterns of protein expression (Angerer *et al*., 1987; Long and Rebagliati, 2002). Gene expression can also be assayed at the transcript level. Several methods include northern blotting (Alwine *et al*., 1977; Kumar and Lindberg, 1972; Sabelli and Shewry, 1995b), nuclear run-on (or run-off) transcription assays (Kafatos, 1972; Ganguly and Skoultchi, 1985; Gatehouse and Thompson, 1995), RNase A/T1 mapping (Brown and Waugh, 1989; Simpson and Brown, 1995a), primer extension assays (Hershberger and Culp, 1990; Simpson and Brown, 1995b), reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative or real-time RT-PCR (Robinson and Simon, 1991; Ferre, 1992; Freeman *et al*., 1999; Gause and Adamovicz, 1994; Page and Minocha, 2004) have been developed for expression quantification at RNA level which real-time RT-PCR allows a highly sensitive quantification of transcriptional levels of the gene of interest in a few hours with minimal handling of the samples (Kammula *et al*., 2000; Mandigers *et al*., 1998; Tichopad *et al*., 2003; Valasek and Repa, 2005). RT-PCR can detect low-abundant mRNAs, but it cannot quantify them, which is achievable using real-time RT-PCR (Deprez *et al*., 2002; Bustin, 2000). Some advantages of real-time PCR quantification include a large dynamic range, tremendous sensitivity, highly sequence-specificity, minimum post-amplification processing, which make it a good technique also for large scale screening (Wong and Medrano, 2005).

Reporter genes: Many studies on plant promoters and on the regulation of gene expression have been made possible using reporter genes as well as monitoring transgenes under field conditions. Reporter genes allows for direct visual detection of transformed tissue, thus avoiding the need for more time-consuming biochemical characterization. Widely used reporter genes include the B-glucuronidase gene (uidA or gus), which originates from *E. coli* (Jefferson *et al*., 1986), firefly luciferase gene (luc) (De Wet *et al*., 1985; Ow *et al*., 1986; Mullineaux *et al*., 1990; Riggs and Chrispeels, 1987; Bolivar, 1978; Alton and Vapnek, 1979), chloramphenicol acetyltransferase gene (cat) originates from transposon Tn9 (Alton and Vapnek, 1979; Davis *et al*., 1992), lacZ gene encoding B-galactosidase (B-GAL) in *E. coli* (Alton and Vapnek, 1979) and the green fluorescent protein (GFP) gene from jellyfish (Prasher *et al*., 1992). GFP is especially useful as an *in vivo* whole-plant marker for field-level applications (Stewart, 1996; Richards *et al*., 2003). Also, the R genes of *Zea mays* are useful as a scorable marker in mature and differentiated cells which stimulate endogenous anthocyanin accumulation in the vacuoles of plant tissues (Kloti *et al*., 1993). There are additional fluorescent protein markers that have recently been described that will enable multi-color labeling in transgenic plants (Stewart, 2006).

Most researchers are familiar with GUS assays that have good sensitivity that an enzymatic-based system

can deliver. GUS requires, however, the destructive harvest of the tissues to be assayed. Therefore, researchers have increasingly adopted GFP and other fluorescent proteins to in vivo image and quantify gene expression in plants (Stewart, 2001, 2006).

CONCLUSIONS

Even though plant transformation can no longer be described as a new technology (it is over 20 years-old), it continues to evolve. In addition, tools such as marker genes continue to expand at an astonishing rate, leading to more efficient transformation. Old tools are also applied to new plants, which opens up the number of traits for crop improvement. Despite controversies, worldwide adoption of transgenic plants is expanding every year. Technological advances, such as those provided by plant biotechnology are needed to better feed the world's people.

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