Isolation and cloning of genes from tuberizing and non-tuberizing in vitro potato explants

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During *in vitro* tuberization, two contradictive phenomena have been found, potato explants could produce micro-tuber and another one could not generate micro-tuber. To elucidate such observable fact, isolation and cloning of important genes are effort should be undertaken. The aims of this research were to isolate and to clone genes, which differentially expressed between *in vitro* tuberizing and non-tuberizing potato explants. cDNA-AFLP (complementary DNA-amplified fragment length polymorphisms) technique was employed for such purpose. The cDNA-AFLP fragments labeled with γ^{33}P-ATP were excised from the polyacrylamide gel.

Eight TDFs were isolated while only seven TDFs were successfully cloned into p-GEMT-easy vector; three of these TDFs were isolated from non-tuberizing samples and the other, four bands were cut off from tuberizing samples. Seven plasmids containing DNA insert have been successfully introduced into *Eschericia coli*. Analysis of transformant bacteria, recombinant plasmid-DNA followed by digestion analysis using *Eco*RI showed that some of plasmid vector contained TDFs or DNA insert of our interest.

Keywords Gene cloning . I*n vitro* tuberization . cDNA-AFLP . Potato

Potatoes (*Solanum tuberosum* L.) are bred and produced for three main purposes: for the fresh food market, for the potato food processing industry (potato chips and snacks, French fries or Pommes, for example) and for non-food industrial uses. Research on potato has many advantages in that they are easily transformable and therefore amenable to genetic manipulation, and can be propagated rapidly both in tissue culture and through cuttings. The main drawback to the use of potatoes in research is the fact that most potato species are polyploid, which means that classical genetic experiments cannot be performed (Jackson, 1999), however many efforts have been reached especially in breeding, by using conventional and modern tools to develop new potato cultivars.

In potato breeding, yield potential, tuber quality traits, range of adaptation (or degree of stability in performance over a wide range of environments), and disease and pest resistance are the most important criteria for selection (Celis-Gamboa, 2002). Tuber quality traits, e.g. glycoalkaloid level, sugar content, taste and texture are common selection traits used in potato improvement. Therefore, research into potato tuber initiation and development, which enables our understanding and possible manipulation of these processes, is of great relevance, such as for improving potato tuber starch yield by producing a more efficient pathway of sucrose degradation.

Tuberization is a complex multi-stage process, involving stolon formation, initiation of tuberization, tuberization and resource storage. Each stage is likely to be regulated and controlled by a large set of interacting expressed genes throughout the plant (Bachem *et al*., 2000). A wide range of plant hormones and environmental factors has been shown to be involved in the tuberization stimulus (Prat *et al*., 1990; Jackson, 1999). These tuber-inducing conditions are: genotype, photoperiod (length of the dark period), temperature (cool night), low nitrogen level, level of cytokinin and gibberellic acid.

In vitro tuberization method developed by Hendriks *et al*. (1991) has been proved to be effective method to analyze the process of tuberization over time. *In vitro* tubers, usually called micro-tubers because of its small in size, have the same physiological and anatomical characteristics as tubers that are formed under field or greenhouse conditions (Appeldoorn *et al*., 1997; Veramendi *et al*., 1999). These systems essentially consist of a single nodal stem explant that will result in the differentiation of the axillary bud into a tuber instead of a leafy shoot when placed on tuber-inducing medium (Veramendi *et al*., 1999).

During *in vitro* tuberization, two contradictive phenomena have been found, potato plant cv. Bintje could produce micro-tuber and another potato cv. Bintje could not generate micro-tuber. Although these explants have been induced to tuberize by growing on tuber-inducing medium, characterized with high sucrose content (8%), low nitrogen level, and placed in the darkness. Such finding is interesting to be explored, for instances, which genes are developmentally expressed/regulated by means of up-regulated, down-regulated, or transient-induced expression on non-tuberizing explants compared to tuberizing potato explants are very remarkable to be explored.

To elucidate such observable fact, isolation and cloning (production of many identical copies) of genes provide the first set of molecular tools to dissect the differentiation of two contradictive phenotypes. To achieve such objectives, cDNA-AFLP fingerprint (technique for analyzing gene expression) was applied for doing this experiment. Efforts in gene isolation and cloning would have many advantages for optimizing utilization of our plant genetic resources through breeding programs, since useful cloned genes can be used for conducting genetic engineering via DNA recombinant technology, as result, it would significantly contribute to plant genetic improvement/variability since the useful cloned gene, which is transformed can alter phenotypic appearance. For instances, by isolating the key gene(s) with significant effect in changing metabolic pathway, particularly in potato starch biosynthesis, it would therefore be able a more rational design of metabolic engineering strategies for improving starch yield or other objectives related to potato breeding.

The aims of this research were to isolate and to clone genes, which differentially expressed between *in vitro* tuberizing and non-tuberizing potato explants.

Materials and methods

The experiment was conducted in Laboratory of Plant Breeding, Wageningen University and Research Centre, the Netherlands, from June to November 2002. RNA isolation was done according to the standard protocol developed in this laboratory. cDNA-AFLP template preparation was done according to Bachem *et al*. (1998), consist of four steps: (1) synthesis of cDNA using a poly-dT oligonucleotide, (2) production of primary template by digestion and restriction with two restriction enzymes and ligation to anchors to their termini, (3) pre-amplification with primers corresponding to anchors from the secondary template, and (4) selective restriction fragment amplification with primers extended with two specific bases. Final fingerprint is produced by labeling of one of the primers, allowing visualization of the amplification products. For PCR conditions and the sequence of primer used for template preparation and cDNA-AFLP fingerprint can be found in Carsono and Bachem (2003).

Gene Isolation and Cloning

Promising bands/transcript derived fragments (TDF) from cDNA-AFLP fingerprint were isolated using polyacrylamide gel, in which the primer complementary to one of the anchors was labeled using γ^{33}P-ATP and used the same PCR condition as for the LI-COR gel. Fragment radioactive labeled gels were selected based on their differential expression patterns, between *in vitro* tuberizing and non-tuberizing samples during six days observation.

Isolation of interest bands used several procedures as follows: the film and the gel from cDNA-AFLP fingerprint labeled by radioactive were marked as to orient it while in the cassette. The film was developed normally. The dried film was placed onto the gel, lined up the orientation marks and identified the bands to be isolated and then cut the bands carefully. Each band was put in eppendorf tube containing 150 μl water and was resuspended for over night at 4^0C (in the fridge). Two micro liters from the upper layer of this DNA fragment were taken and then were reamplified using the same primers as described for preamplification of template. The PCR results were checked on agarose gel 1.1% after stained the gel with ethidium bromide and visualization of the bands of interest using a UV transilluminator. For cloning procedures, the reamplified TDFs were cloned into plasmid pGEM-T Easy® Vector System I (see Figure 1). Ligation of plasmid vector and insert DNA using mixed reaction as the following: 1 μl pGEMT-easy vector, 2 μl DNA fragment (PCR product), 5 μl 2x ligation buffer, 1 μl T4 DNA ligase, and 1 μl H_2O. This ligation was performed for over night at 4^0C (in the fridge). For the positive control, it was used DNA fragment from the company, while for the negative control; only uncut vector was performed for determination of the transformation efficiency of the competent cells.

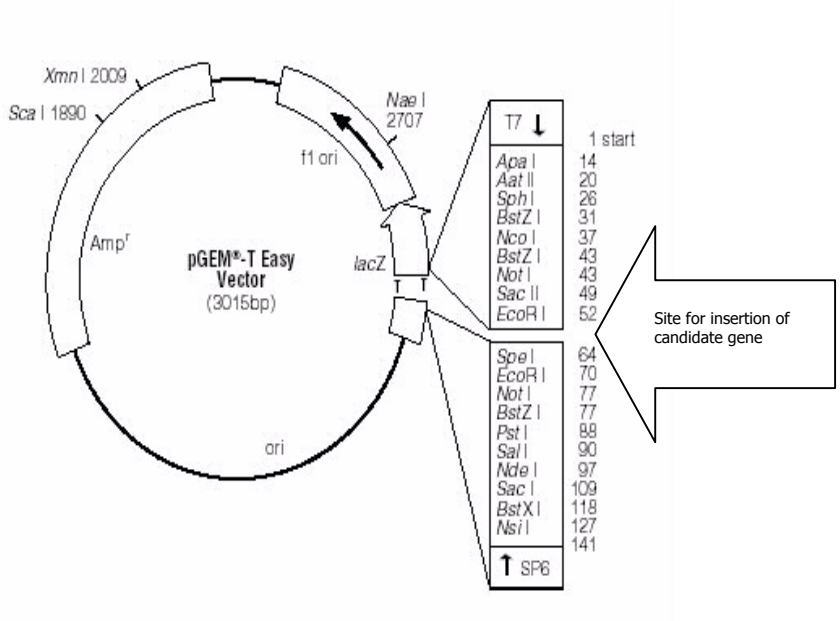

Figure 1. pGEM®-T Easy Vector circle map and sequence reference points (Source: Promega, 1999).

Transformation of DNA into bacteria

From the ligation mix, 7.5 μl was taken and then added with 50 μl competent cells containing *Escherichia coli*. This mix was placed in ice for 30 minutes and for heat shock the cells, it subsequently put on water bath (37^0C) for 2 minutes and then immediately returned this mixture to ice for 2 min. To the ligation mix, it was added 950 μl LB liquid medium. LB liquid medium was prepared previously by mixing 25 g LB, 1000 ml H_20 and sterilized by autoclaving. The ligation mix was incubated for one hour at 37^0C with shaking (225 rpm), and then centrifuged at 13.000 rpm for 5 min to get the pellets. Around 900 μl of each supernatant was discarded.

To select the transformants, 100 μl of each transformation culture was spread on the plate with LB medium containing ampicillin, X-gal and IPTG. The plates were incubated over night at 37^0C.

Minipreps isolation of recombinant plasmid DNA

The white colony assumed carrying recombinant plasmid was selected from blue-white colonies grown on the plate. The selected colony was picked with toothpaste, and put in 3 ml LB liquid medium containing ampicillin, and then incubated at 37^0C with vigorous shaking for over night. Around 1.5 ml of each culture was transferred into new tube, and for the remained was stored in the fridge. The overnight cultures were then centrifuged at 13000 rpm for 3 min. The medium was

discarded, and the pellet was dried using vacuum machine for about 15 min. The pellet was completely resuspended with 200 μl resupension solution. The mixture was then added with 200 μl cell lysis solution and mixed by inversion. 200 μl potassium acetate solution were added to the mixture for neutralizing the lysate and mixed by inversion. The mixture was subsequently centrifuged at 13000 rpm for 10 min. The supernatant was transferred into a new effendorf tube and white precipitate was discarded. To the supernatant, it was added 0.6 volume cold 2-propanol and centrifuged at 13000 rpm for 20 min. The pellet was collected and supernatant was removed. The pellet was then dissolved in 50 μl of water.

Digestion of plasmid vector

In order to confirm our DNA fragment of interest, the digestion reaction was performed using the reaction mix as the following: 2 μl DNA-plasmid (from minipreps), 1 μl 10X Buffer React 3, 1 μl EcoRI (digestion enzyme), and 6 μl H_2O. The tubes were incubated in water bath (37^0C) for 1 hour.

The DNA fragment was checked on agarose gel electrophoresis. The band of our interests should appear on agarose gel as single band that separated from the band of vector. Plasmid DNA was prepared and analyzed by restriction analysis with *Eco*RI for the presence and for size confirmation of the DNA insert.

Results and discussion

Thirty different primer combinations (using *AseI and TaqI* and combinations with plus two additional selective bases) were analyzed and around 17 primer combinations, based on their expression profile were selected for further analyses. From these primer combinations, 45 bands were selected. Of these, 53.33% TDFs present only on non-tuberizing samples, and 46.67% appear on tuberizing samples. In terms of gene expression, both samples produce relatively similar TDFs. This result is not as expected, since it appeared likely that template derived from tuberizing samples would have many differential TDFs compared with non-tuberizing samples. Since tuberization is a quantitative character, which assumed a large number of genes are involved (Struik *et al*., 1999; Hannapel, 1994), and a wide range of plant hormones and environmental factors, as previously mentioned, has influence in tuber formation (Prat *et al*. 1990; Jackson, 1999). However, Hannapel (1994), using Southern blots techniques, showed that patatin genes and the cathepsin D 22 kDa proteinase inhibitor are also present in non-tuberizing plant genome. This finding indicates that non-tuberizing explants produce TDFs in similar number and these may correspond to the genes, which are expressed in house-keeping function rather than being directly involved in tuber initiation, formation and development.

To isolate genes which showing differential expressions between tuberizing and non-tuberizing potato explants, fourteen TDFs from a cDNA-AFLP polyacrylamide radioactive labeled gel

were isolated. These isolated bands were ranging in size, which were estimated from 106 bp to 325 bp, as shown in Figure 2.

However, after intensive study in a cDNA-AFLP radioactive labeled gel, eight bands were chosen for further analysis in order to ligate the interest bands with pGEM-T easy vector and subsequently clone them into competent cells (*E. coli*).

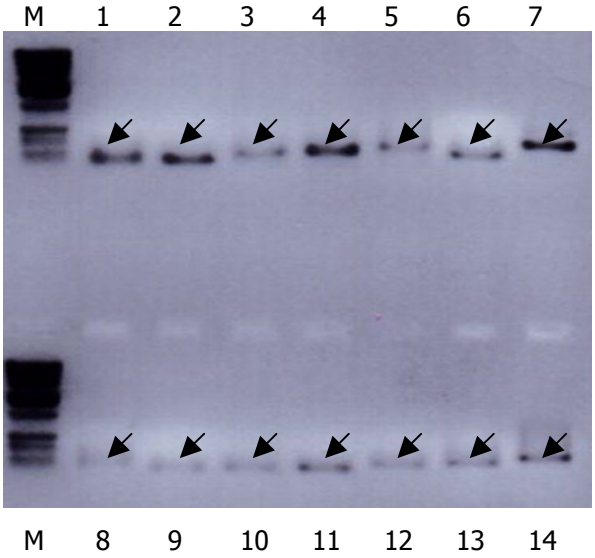

Figure 2. Gel electrophoresis of fourteen isolated fragments. M = marker (1 kb ladder), and numbers and arrows correspond to the bands that were isolated.

Seven, out of eight, of recombinants plasmid-DNA have been successfully introduced into *E. coli* as shown from a lot of number of white colonies formed in the LB medium containing ampicillin, X-gal and IPTG. These white colonies might be resulted from ligation reaction either between vector and vector or between vector and DNA inserts. By digestion with enzyme restriction (in this case by using *Eco*RI) and subsequently check them in the electrophoresis, such problem can be easily investigated. In addition, band of DNA insert and vector will be different in size, and it will be separated according to their size in base pairs (bp).

To isolate recombinant plasmid-DNA, minipreps procedures were applied. This procedure was conducted twice. After digestion with *EcoRI*, first procedure resulted only four candidate bands that were ready for sequencing, while the second generated eight bands. Promising TDFs

categorized as fragments that were separated from the band of vector. As an example, Fig. 3 presented promising bands of DNA insert and p-GEM-T easy vector bands after digestion with *Eco*RI.

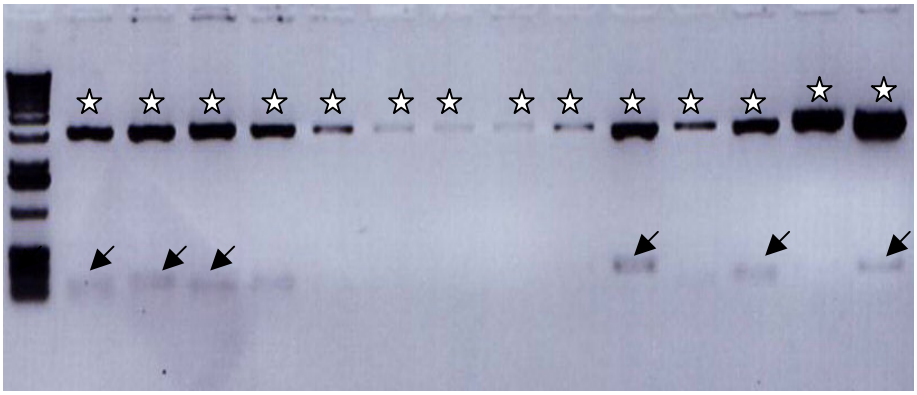

Figure 3. Gel electrophoresis of recombinant plasmid DNA after digested by *Eco*RI. Stars are symbol for bands of p-GEMT easy vector and the arrows correspond to the bands of DNA insert.

From Figure 3, it can be seen that *Eco*RI did not digest seven recombinant plasmid-DNA samples or it could be no DNA insert was exist in p-GEMT easy vector. A probable cause for this result is that ligation reaction between DNA insert and the vector did not work so well, so that DNA insert could not perfectly integrate into p-GEMT easy vector. From this experiment, in summary, it can be concluded that eight TDFs were isolated, however only seven TDFs successfully cloned into p-GEMT easy vector, three of these isolated from non-tuberizing samples and the other, four bands isolated from tuberizing samples. Information regarding on the characterization and functional classification of seven TDFs of this experiment have been published (Carsono and Bachem, 2003).

Conclusion

Eight TDFs were isolated, while only seven TDFs were successfully cloned into p-GEMT easy vector, three of these isolated from non-tuberizing samples and the other, four bands isolated from tuberizing samples. Seven plasmid containing recombinant plasmid-DNA have been successfully introduced into *Eschericia coli*. Analysis of transformant bacteria showed that some of bacteria contained TDFs or DNA insert of our interest.

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