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Protocols

Transcript Imaging with cDNA-AFLP: A Step-by-Step Protocol

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Abstract. The method of cDNA-AFLP allows detection of differentially expressed transcripts using PCR. This report provides a detailed and updated protocol for the cDNA-AFLP procedure and an analysis of interactions between its various parameters. We studied the effects of PCR cycle number and template dilution level on the number of transcript derived fragments (TDFs). We also examined the use of magnetic beads to synthesise cDNA and the effect of $MgCl_2$ concentration during amplification. Finally, we determined the detection level of the cDNA-AFLP method using TDFs of various sizes and composition. We could detect TDFs corresponding to a single copy per cell of a specific transcript in a cDNA-AFLP pattern, indicating high sensitivity of the method. Also, there was no correlation between concentration of detectable TDF and the fragment size, stressing the high stringency of the amplification reactions. Theoretical considerations and specific applications of the method are discussed.

Key words: cDNA-AFLP, plant development, RNA-fingerprinting

Introduction

Biological responses and developmental programming are controlled by the precise regulation of gene expression. To gain insight into these processes, it is necessary to study patterns of gene expression. A variety of molecular techniques are now available to identify and clone differentially expressed genes. The most recent additions include the PCR-based approaches for selective amplification of cDNAs, such as RNA-fingerprinting by arbitrarily primed PCR (RAP-PCR; Welsh et al., 1992) and differential display (DD/RT-PCR, Liang and Pardee, 1992), collectively referred to as RNA-fingerprinting. These methods, however, have important limitations such as problems with reproducibility, difficulty in representing very rare messages, and generation of

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false positives (Bauer et al., 1994). Such problems arise primarily from the use of random oligonucleotides for PCR priming and the relatively low annealing temperatures necessary to produce amplification products. Various modifications have been attempted, such as improvement of primer design (Zhao et al., 1995; Diachenko et al., 1996) and the combination of various methods with RNA-fingerprinting (Ivanova and Belyavsky, 1995; Kato, 1996). However, all methods still suffer problems of repeatability and uncertainty in the identification of specific fragments. The cDNA-AFLP method (Bachem et al., 1996) largely overcomes these limitations and makes a simple and rapid verification of band identity possible. In addition, the systematic screening of nearly all transcripts in a given biological system using small quantities of starting material is possible. cDNA-AFLP consists of four steps: (1) synthesis of cDNA using a poly-dT oligonucleotide, (2) production of primary template by restriction digestion with two restriction enzymes and ligation of 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 one or more specific bases. The final fingerprint is produced by radioactive labelling one of the primers, allowing visualisation of the amplification products.

In this paper, we present recent advances in cDNA-AFLP technology including an investigation of a wide range of parameters and their interactions on the resolution and reproducibility of the method. In addition, we have tested the detection level of several TDFs, and have drawn conclusions about the sensitivity of the procedure for visualising very rare transcripts. Together, the results provide evidence that the cDNA-AFLP method serves as a robust and reproducible method for the routine detection of differentially expressed transcripts in a wide range of experimental systems.

Material and Methods

Plant material and RNA preparation

Total RNA was isolated from axillary buds of potato nodal stem cuttings cultured on a tuber-induction medium. Plant material was ground to a fine powder under Liquid-N_2, and then mixed vigorously in hot extraction buffer: a 1:1 mixture of equilibrated phenol and RNA-buffer (100.0 mM Tris-HCl pH 8.0; 100.0 mM LiCl; 10.0 mM EDTA; 1.0% LiDS, 85 °C). After at least two chloroform extractions, the RNA was differentially precipitated using a 1/3 volume of 8M LiCl. Tissue samples were taken individually from ten days of tuber development (Bachem et al., 1996). In general, around 200 mg of plant tissue was used for each sample preparation. Other tissues used for

Table 1. Sequence of the primers used for template preparation and fingerprinting. The two selective bases are represented by NN in the selective primers, the (N) denotes the third selective base used for band verification

Primer for cDNA synthesis	5′-AAA AAA AAA AAA AAA AAA AAA AV-3′
Ase I anchor; top strand	5′-CTC GTA GAC TGC GTA CC-3′
Ase I anchor; bottom strand	5′-TAG GTA cgC AGT C-3′
Taq I anchor; top strand	5′-GAC GAT GAG TCCT GAC-3′
Ase I anchor; bottom strand	5′-CGG TCA GGA CTC AT-3′
Ase I standard primer 1	5′-CTC GTA GAC TGC GTA CCT AAT-3′
Taq I standard primer 2	5′-GAC GAT GAG TCC TGA CCG A-3′
Ase I selective primer	5′-GAC TGC GTA CCT AAT NN(N)-3′
Taq I selective primer	5′-GAT GAG TCC TGA CCG ANN(N)-3′

RNA isolation and template preparations include potato roots, stems, young and mature leaves, flower buds, open flowers, young, medium and mature fruit, swelling stolon tips and growing stolon tips.

Paramagnetic streptavidin coated beads (Dynal, Oslo, Norway) were prepared as follows: 1 mg of beads (in 10 μl) per sample was incubated at 22 °C for 30 min with 200 ng of biotinylated d[T]25V oligonucleotide (circa 1.5 excess, Table 1) and gentle agitation. The beads were washed three times with an equal volume of STEX buffer (1.0 M NaCl; 10.0 mM Tris-HCl pH 8.0; 1.0 mM EDTA 0.1% Triton X-100) to eliminate unbound oligonucleotide. After the determination and equalisation of the RNA concentration, 100 μg of total RNA was ethanol precipitated and resuspended thoroughly in 500 μl STEX buffer and denatured for 5 min at 65 °C. Ten μl of poly(A)$^+$ beads, prepared previously, were added and incubated at 22 °C for 10 min, and on ice for 5 min. The bead/poly(A)$^+$ mRNA mixture was washed 3 times in STEX buffer and finally resuspended in 20 μl of H_2O. To elute the poly(A)$^+$ mRNA, the bead suspension was incubated at 65 °C for 5 min and the supernatant was transferred to a new tube. Poly(A)$^+$ mRNA yield from 100 μg total RNA was generally 500 ng.

Template preparation

Double stranded cDNA was synthesised using protocols described by Sambrook et al. (1989) using AMV reverse transcriptase (Life Technologies B.V. Breda, NL). For the priming of cDNA synthesis, a 'V' nucleotide (G or A or C) is added at the 3′-end of the oligonucleotide $d[T]_{25}$ to enhance docking at the 5′-terminus of the poly(A$^+$) tail of the mRNA. Generally, the yield of poly(A)$^+$ mRNA is around 50–100 ng. When the cDNA was synthesised

after elution from the beads, an aliquot of the double-stranded cDNA was routinely quality-controlled on a 1% agarose gel and the remaining sample was purified by extraction with phenol/chloroform/isoamylalchohol (1:1:24) and then ethanol precipitated. The cDNA was resuspended in a reaction mix for restriction enzyme digestion. When cDNA synthesis was carried out with the poly(A)$^+$ RNA attached to the beads, additional d[T]25V oligonucleotide (10 ng) was added prior to cDNA synthesis. After cDNA synthesis, the buffer was exchanged for RL-buffer (restriction-ligation buffer: 10 mM Tris-acetate pH 7.6, 10 mM Mg-acetate, 50 mM K-acetate and 5 mM DTT) for restriction digestion. In all cases, the DNA was digested with 10 U of two restriction enzymes for 3 h in RL-buffer. For the *Taq* I/*Ase* I enzyme combination, incubation was carried out separately at 65 °C and 37 °C, respectively. However, for other enzyme combinations, double digestions were carried out simultaneously. Paramagnetic beads were eliminated from reaction mixtures before ligation of the anchors. Prior to ligation, both strands of the anchor oligonucleotides (Table 1) were heated to 65 °C and allowed to cool to room temperature. The digestion mix was then supplemented with the two annealed anchors carrying complementary sticky ends for restriction fragments used previously (Figure 1) and ligated with 1 U of T_4-DNA ligase (in RL-buffer supplemented with ATP to a final concentration of 1 mM). The product of the ligation reaction is termed primary template. In the pre-amplification of primary template, an aliquot (generally 1/5 volume), was subjected to PCR using primers complementary to the ligated anchors (standard primers 1 and 2; Table 1). Pre-amplification PCR protocols were carried out with varying cycle numbers using the following profiles: 94 °C, 30 s; 52 °C, 30 s; 72 °C, 1 min (standard profile). The product of this reaction yields the secondary template. The secondary template was checked on a 1% agarose gel and gave visible fragments in a range between 50 bp to 600 bp and a yield of about 500 ng. Secondary templates were diluted to various concentrations and subjected to a second round of amplification using primers complementary to the anchors and the modified restriction sites within the TDF and extended by two selective bases at the 3′ end (selective primers, Table 1). In this amplification reaction, the primer complementary to one of the anchors, was labelled using γ-^{33}P-ATP and polynucleotide kinase (Pharmacia, Uppsala, Sweden). PCR profiles were as follows: 11 cycles: 94 °C, 30 s; 65 °C [−0.7 °C/cycle], 30 s; 72 °C, 1 min and 30 cycles: 94 °C, 30 s; 56 °C, 30 s; 72 °C, 1 min. The resulting products were size-fractionated on a 5% polyacrylamide sequencing type gel at 80 Watts for around 1.5 h. The size resolution on these gels is from around 50–1000 bp. Figure 1 shows a diagrammatic overview of the procedure for cDNA-AFLP template production.

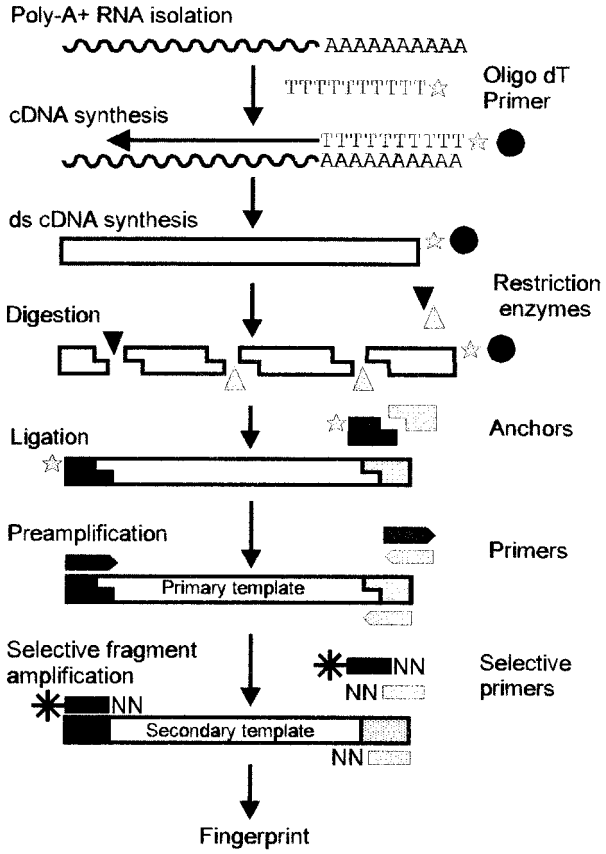

Figure 1. Diagram of the cDNA-AFLP method. The steps of the cDNA-AFLP procedure are labelled on the left of the figure and the key components are shown on the right. The star symbol on the oligo-dT primer and on the rare cutting anchor denotes a biotin group. Filled circles represent paramagnetic streptavidin-coated beads. For the restriction enzymes (triangles), anchors and primers, rare cutter specific symbols are solid and frequent cutter specific symbols are shaded grey. The asterisk represents a ^{33}P label on the 5′-end of the appropriate primer. Further details of the procedure are given in the Materials and Methods and Results sections.

Fragment isolation

After running, polyacrylamide gels were dried on Whatman 3MM paper and routinely exposed to X-ray film overnight. Bands of interest were excised and electroeluted directly onto DEAE (DE-31 Schleicher and Schühl, Dassel, Germany) ion exchange paper in pockets of a 1.5% agarose gel. DNA was recovered from the DEAE paper according to the manufacturer's recommendations. The eluted TDFs could then be re-amplified by PCR using the

standard profile (30 cycles) and the standard primers 1 and 2 (Table 1). PCR products were checked on 1.5% agarose gels and subsequently sequenced using standard primer 1 (Table 1).

Clone verification

To verify the identity and expression pattern of the isolated fragment, the sequence data from the TDF in question was used to choose a primer pair with the appropriate three selective bases (Table 1) to repeat the active PCR. This generally yielded a pattern in which 90% of the bands seen on the original gel had disappeared revealing the same target band with the same expression pattern as previously.

Results and Discussion

Improvement of primary template production

The quantity and quality of RNA extracted from different origins varies widely and depends on the fraction of cytoplasmically rich, actively dividing cells and the nature and quantity of interfering compounds (polysaccharides, polyphenolics, etc.) in the tissues of interest. These parameters determine the amount of tissue required for RNA extraction in order to obtain sufficient material for cDNA synthesis and cDNA-AFLP template preparation. Using young plant tissues, 100–200 mg fresh weight yields around 100 μg of total RNA, and that serves as the starting material for the poly(A)$^+$ RNA isolation using paramagnetic beads (as described in Materials and Methods). In older plant tissues, such as mature, field-grown potato tubers or cassava root tubers, significantly more plant material should be processed. Also, when working with plant tissues (such as potato tubers) that are rich in starch, the high levels of polysaccharides inhibit efficient template preparation and produce templates that appear as high molecular weight smears. To alleviate this problem we have used a procedure where the mRNA remains attached to the magnetic beads via the streptavidin/biotin linked $[T]_{25}V$ during first and second strand cDNA synthesis. Due to the incorporation of an additional washing step prior to restriction enzyme digestion, the contaminating compounds can be eliminated and template with an improved quality can be produced (Figure 2). This method also obviates the need for the phenol/chloroform extraction step after double-stranded cDNA synthesis, thereby considerably shortening the template production procedure. Since the restriction digestion releases the desired TDFs from the beads, the immobilised fraction containing the 3′-ends of all cDNAs and those cDNAs without restriction sites for either enzyme, can

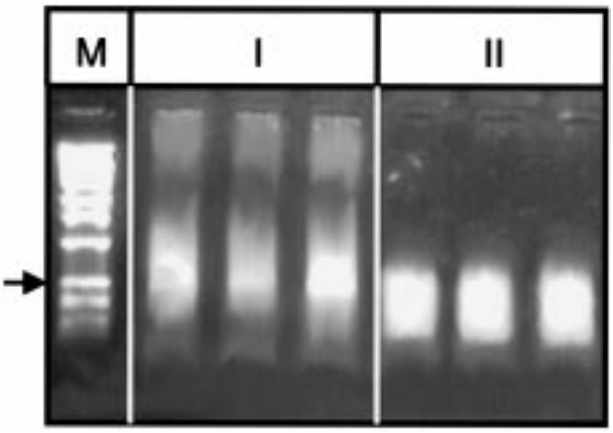

Figure 2. Elimination of template smearing by paramagnetic bead purification of cDNA. Panel I shows three samples of secondary template which were produced from RNA isolated from micro-tubers where the poly(A)$^+$ RNA was released from the paramagnetic beads before cDNA synthesis. Panel II shows secondary template stemming from the same tissues and RNA isolation where the RNA remained attached to the beads during cDNA synthesis and released after restriction digestion. Panel M shows a molecular marker (1 kb ladder) with the 506/517 bp band indicated by an arrow.

be discarded along with the beads. In this way, a reduction in the complexity of the DNA mixture in the primary template is achieved and this significantly improves the performance in later amplification steps. Furthermore, we routinely include a biotin residue on the upper oligonucleotide of one of the anchors. This allows the isolation of all fragments that have sites for one of the restrictions enzyme used in the template production on streptavidin coated paramagnetic beads eliminating unwanted restriction fragments. Although the presence or absence of such additional fragments seems not to affect the fingerprint (data not shown), their elimination reduces the chance of mis-cloning TDFs isolated from denaturing polyacrylamide gels where the non-labelled fragments may underlie the targeted TDF.

In AFLP procedures, restriction enzymes are used to tag sites in DNA, to shorten DNA fragments to appropriate sizes, and to produce acceptor sites at their termini. As in genomic AFLP (12), the cDNA-AFLP protocol prescribes two restriction enzymes for template production, allowing discrimination between the two ends of the TDFs. The enzymes should represent a so-called rare-cutting type with a recognition site of six bases, while the second enzyme used, is a frequent-cutting enzyme with a four bp recognition site. Optimally, the rare-cutting restriction enzyme, or DNA-tagging enzyme, will have one site in each cDNA. In practice, however, such restriction enzymes have sites in only about half of all cDNAs. The frequent-cutting restriction enzyme

should have a site adjacent to the rare-cutter site and produce fragments of a size that can then be resolved on a 5% polyacrylamide gel (between 50 and 1000 bp). Some other considerations can be taken into account for the choice of restriction enzymes: (i) Enzyme combinations can be chosen which have sites in cDNAs of particular interest for the research to be undertaken and which will give TDFs of predictable sizes when amplified with the appropriate primers. This allows the targeted analysis of gene expression and/or the inclusion of a control for the integrity of the system when the expression of the targeted gene is known or can be predicted. (ii) A preliminary search may be carried out on cDNA sequences available in the data banks for a given experimental system, to determine which enzyme combinations come closest to the optimal parameters described above. (iii) Since the 5′ and 3′ untranslated regions of cDNAs tend to be richer in A and T nucleotide pairs than the protein coding regions, base composition of the recognition site may be used to target specific sections of the cDNAs.

Considering the above, we have used the enzymes *Ase* I and *Taq* I for template preparation. Template produced from RNA isolated from 10 stages during tuber formation (Bachem et al., 1996) is referred to hereafter as the tuberisation template. This enzyme combination has yielded a very high information content in the 200 differentially expressed TDFs isolated from around 220 primer combinations we have tested on this template series. About 95% of these TDFs apparently originate from within structural genes and 60% show significant homology (probability factor of $<1 \times 10^{-5}$ and score of >40 as calculated by the BLAST programmes; Altschul et al., 1993) to sequences from the data banks.

We have used a series of restriction enzyme pairs for making template from cDNA (*Eco*R I, *Bam*H I and *Pst* I in combination with *Taq* I or *Mse* I; data not shown). All templates produced approximately the same number of TDFs (50–70 scorable bands) in the final fingerprint. Although the information content of these TDFs was not assessed, the indication is that these restriction enzyme combinations have an equal potential for the identification of differentially expressed genes.

Effect of cycle numbers during pre-amplification and dilution of secondary template

After the production of the primary template, three further steps are carried out to produce the cDNA-AFLP fingerprint: (i) pre-amplification, (ii) dilution, and (iii) labelled amplification of secondary template. Key parameters in these steps are the cycle numbers used in the pre-amplification, the dilution level of the secondary template, and the cycle number used in the labelled amplification. To determine the effect of varying these parameters

on the information content of the fingerprint and to optimise the cDNA-AFLP protocol, a number of experiments were performed on primary template produced from the tuberisation template. In the standard cDNA-AFLP protocol, 15 cycles for pre-amplification of primary template was recommended, followed by a 20-fold dilution of the secondary template before the active PCR. Figure 3 shows the effects of 10, 15, and 20 cycles of pre-amplification (A, B and C, respectively) combined with a dilution of 10, 50, and 100-fold (1, 2 and 3, respectively) on the profile of the fingerprint of 3 consecutive days during tuber development (Panels I, II and III). DNA quantities in the diluted secondary template represent approximately 1 ng, 200 pg and 100 pg of starting material. Although the influence of both the dilution and the PCR cycle numbers can clearly be seen, it is noteworthy that the pattern of the fingerprint remains broadly similar and also the proportionality of intensities of the individual TDFs is maintained within one day. Furthermore, the expression dynamics during the three-day developmental period also remain largely unaltered, independent of the conditions chosen. This can be seen in the TDF marked **b** which decreases in expression over the period and the TDF marked **c** which increases in expression over the three days. Using 20 cycles and a 10-fold dilution, a significant increase in high molecular weight background is produced (particularly in Panel III, A1). This background smear is also present in other variants with higher DNA concentrations, however, it appears in the mid-range molecular weight in the other dilutions (B1, all panels). In some cases the appearance of individual TDFs is adversely effected at high DNA concentration (Arrow **a**). Both the higher background and the adverse effects on amplification of individual TDFs at higher DNA input concentrations are likely due to competitive inhibition between fragments during PCR. The general conclusion that may be drawn is that the proportion of the input DNA is reflected in the final fingerprint over a wide range of conditions. Furthermore, one can conclude that the differences in the amplification efficiency of individual fragments do not seem to be a major factor within our conditions.

Although it can not be excluded that the intensity of the TDFs in the fingerprint may, in some cases, not reflect the abundance or the corresponding transcript in the steady-state mRNA population, the results do indicate that cycle number and dilution levels do not unduly influence the proportionality. This result, together with the fact that the TDFs of genes with known expression in this developmental system are also represented as predicted (Bachem et al., 1996), allows for a high level of confidence in the fingerprinting results.

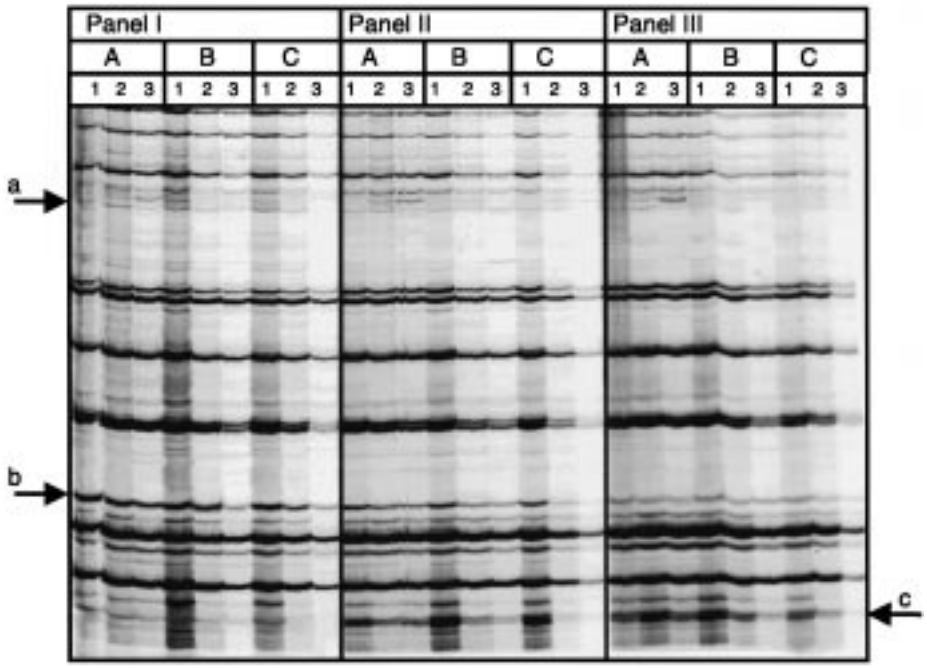

Figure 3. Effect of cycle number and secondary template dilution on the cDNA-AFLP fingerprint. Panels I, II and III show template produced from three consecutive days during tuber development. Lanes under A, B and C show amplification of template using 10, 15 and 20 cycles, respectively. Individual lanes (labelled 1, 2 and 3) represent different dilutions (10-, 50- and 100-fold, respectively) of the secondary template. Arrow **a** indicates a band which can only be visualised in certain cycle number/template dilution combinations. Arrows **b** and **c** highlight bands that are differentially expressed over the three day period.

Effects of varying Mg^{2+} concentration

Mg^{2+} ion concentration greatly affects the efficiency of PCR reactions. In most cases, it is efficacious to optimise Mg^{2+} concentrations for each individual fragment to be amplified (Du-Toit et al., 1993). Although in multiplex PCR, this strategy is not feasible, the general influence of changes in Mg^{2+} concentrations was tested to determine whether substantial changes in the fingerprint would result. Figure 4 shows that, using different $MgCl_2$ concentrations (1 mM, 2 mM, 2.5 mM, 3 mM and 4 mM $MgCl_2$, shown in Panels I-V, respectively), an increase in Mg^{2+} concentration during PCR generally results in an increase of band intensities. Furthermore, it appears that, although the expression pattern does not change dramatically, some TDFs are sensitive to the $MgCl_2$ concentration, appearing only in certain combinations of $MgCl_2$, cycle number, and dilution (arrow; Figure 4, Panel II, lane A3). By comparing multiple cDNA-AFLP patterns, each generated with different

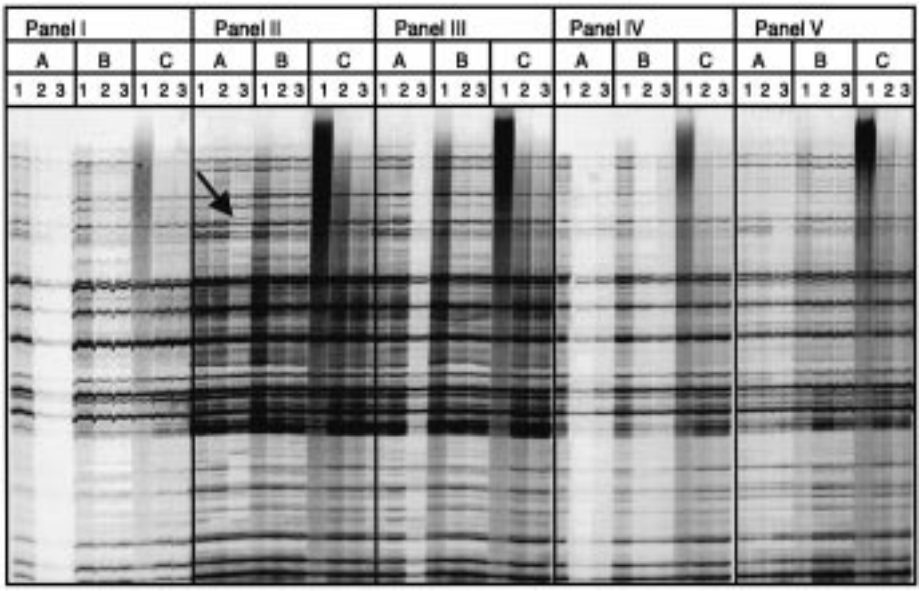

Figure 4. Interaction between Mg^{2+} concentration, cycle number and secondary template dilution on the cDNA-AFLP fingerprint. Panels I, II, III, IV and V represent fingerprints produced from template of potato micro-tubers using 1.5, 2, 2.5, 3 and 4 mM $MgCl_2$ during amplification. The lane annotations A, B, C and 1, 2, 3 are as in Figure 3, referring to number of cycles and the dilution levels of the templates. The arrow indicates a band in lane 3A, Panel II which is only visible under conditions specific for this variant (2 mM $MgCl_2$, 10 cycles of pre-amplification and 100-fold dilution).

$MgCl_2$ concentrations, it is shown that at standardised PCR conditions (15 cycles pre-amplification followed by a 50-fold dilution, Panel III, B2) the use of 2.5 mM $MgCl_2$ results in a pattern that has increased resolution and clarity.

Detection limits of individual TDFs

To test the detection level of the cDNA-AFLP method, primary template was produced under standard conditions (15 cycles pre-amplification and 50-fold dilution). To this template, three TDFs of different sizes and compositions (Figure 5), were then individually added. The chosen fragments were not normally detectable in the chosen template but were compatible with the selective primers to be used for the generation of the fingerprint. The TDFs were separately amplified, their concentration determined, and added to 200 pg of template. Various dilutions of added TDFs were calculated to give between 1×10^2 to 8×10^9 molecules before the active amplification reaction. Results obtained in this experiment showed that around 1.000 molecules (10^{-8} dilu-

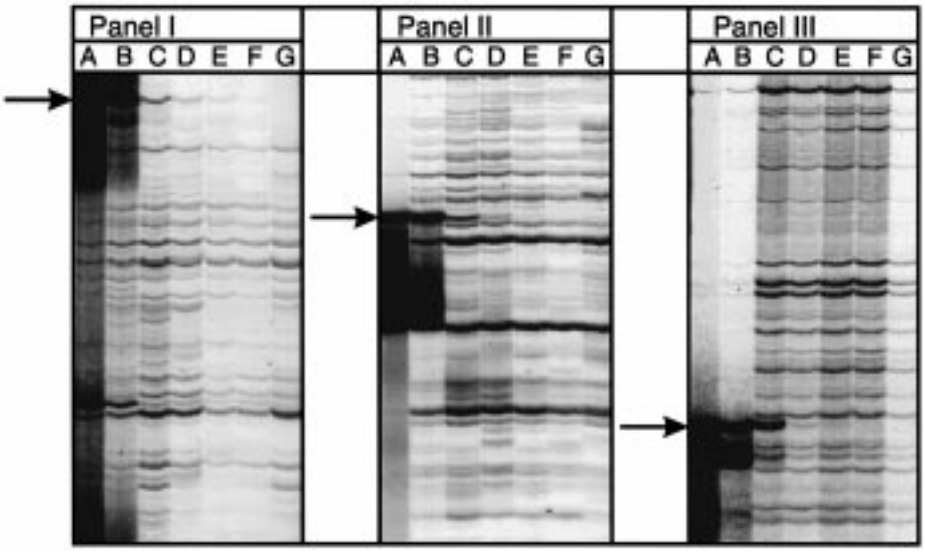

Figure 5. Detection limits of three TDFs in a background template. Panels I to III show 7 dilutions of 3 TDFs corresponding to the patatin b gene (Panel I; 838 bp, selective extensions *Ase* I: [AA], *Taq* I: [AC]) the AGPase b gene (Panel II; 360 bp, selective extensions *Ase* I: [CA], *Taq* I: [AC]) and a potato homologue for ascorbate free radical reductase (Panel III; 182 bp, selective extensions *Ase* I: [GA], *Taq* I: [TT]). All these TDFs are expressed after day five in our tuber development system. The DNA corresponds to dilutions of 10^{-2} (lane A), 10^{-4} (lane B), 10^{-6} (lane C), 10^{-8} (lane D), 10^{-9} (lane E), 10^{-10} (lane F), and 10^{-11} (lane G).

tion) of a specific TDF can still be detected using the cDNA-AFLP method (Figure 5, lane E in all panels) in a multiplexed PCR of around 10^9 molecules.

A comparison of the detection limits of the different TDFs (Figure 5), also shows that there is no correlation between the concentration at which the TDF could still be detected and its size, base composition, or the selective extension. The concentration in which the TDF is present, however, does influence the rest of the fingerprint. This can be seen in the patterns where a very high amount ($> 1 \times 10^6$ molecules, lanes A and B in all panels) of the TDF is added, which results in competitive inhibition of the other TDFs in the fingerprint. In the highest concentration of the added fragment, no other bands can be detected which are larger than the added TDF.

To determine whether the input DNA conscientiously reflects the output band intensity during other steps in the cDNA-AFLP procedure, TDF was also added before pre-amplification or an appropriate restriction fragment was added prior to anchor ligation. The results were identical to those described above for the addition of TDF to the secondary template (data not shown). Together these results show that very low levels of a transcript can

be detected using cDNA-AFLP corresponding to less than one copy per cell in the original tissue.

Cloning of multiple bands

One of the major problems encountered with recovery of DNA fragments from multiplex PCRs is that a band in a gel can correspond to a single DNA fragment or a mixture of several fragments. The problem is further compounded with denaturing polyacrylamide gels since the possibility of contamination with an unlabeled (invisible) DNA strand exists. Northern blotting is generally used to verify that the expression profile of the isolated band is the same as the fingerprint (Liang et al., 1995). However, it is quite conceivable that the transcript of an individual gene will have an expression pattern different from that observed by Northern analysis, particularly when the gene belongs to a highly homologous gene family. When using cDNA-AFLP, it is possible to verify the identity of a TDF (after isolation and sequence determination) by amplifying the source template, using an additional base corresponding to the determined sequence at either end of the selective primers (Table 1). The use of the third selective base at either end of the TDF will result in a 16-fold reduction in the number of bands and generally allows the unequivocal identification of the targeted band. Alternatively, interesting TDFs from a fingerprint with two selective bases can be rerun directly with all of the 16 possible triple selective base-primers and the profile containing the band of interest can then be used as the source for isolation and sequence determination. This significantly reduces the chance of mis-cloning.

It should be noted, however, that mis-cloning still remains a problem inherent to DNA isolation from denaturing polyacrylamide gels. We suggest a verification protocol involving the following steps: (i) sequence determination of the target band using direct fragment sequencing after re-amplification, (ii) PCR with the appropriate triple selective base primers, or (iii) when sequence data is ambiguous due to double signal or when re-amplification of the TDF does not give a clear unique band, direct use of the appropriate 16 triple selective base primers and isolation of the band(s) of interest from the resultant gel(s).

Theoretical considerations

In PCR reactions, the efficiency of the reaction depends on a wide variety of factors such as the type of polymerase, the base composition of the template and primers, the composition of the buffer and the temperature profile of the PCR. The amount of the product is, furthermore, determined by the number of cycles that can be represented by the formula X^n where X is

the polymerisation factor and **n** is the number of cycles. In order to maintain the proportionality of input and output during the amplification reaction, the value of **n** should remain constant independent of X. Moreover, in a multiplex PCR, the constancy of X should hold for any template molecule independent of its initial concentration. In practice it has, however, been demonstrated that X decreases as the **n** value increases beyond a threshold level and in multiplex PCR, each template molecule may even have an individual X value, depending on its initial concentration and on other factors such as its base composition. For cDNA-AFLP, this should result in a change in the pattern of the fingerprint as the cycle numbers increase both during pre-amplification and active PCR. Using the conditions described, we find, however, very limited changes in the fingerprint under widely varying conditions of cycle numbers and template dilutions. This suggests that under the relatively high stringency PCR conditions used, the X value remains stable for most of the TDFs independent of cycle number and initial DNA concentration. However, we could also show that when a TDF is present in very high concentrations, rather than reaching a plateau as described by Mathieu-Daudé et al. (1996), for DD/RT-PCR, the abundant fragment will continue to be amplified at the expense of other fragments in the mixture. In our experiments, this competitive inhibition only becomes significant when the added template fragment is present at a concentration of more than 2×10^8 molecules (dilution 10^{-2}). In 200 pg of standard template, this is equivalent to a level where the added number of fragments is equal to the number of compatible molecules in the rest of the template. Although in our screening, we have encountered some competitive inhibition (Bachem et al., 1996), it is relatively infrequent as it requires a gene to be expressed at more than 3% of total mRNA. The calculation is based on bands derived from transcripts with known expression levels, such as patatin.

We have tested 220 of the 256 available primer combinations on template derived from developing potato tuber tissue. This screening has allowed us to visualise about 15,000 TDFs (220×70 bands/primer combination). From these 15,000 TDFs, approximately 8%, display an unchanged expression pattern during development of potato tubers. Interestingly, most of these constantly expressed TDFs are also detectable in at least 9 of the 11 potato plant tissues that were used in parallel as controls. Our results also indicate that the proportion of transcripts detectable in 4 or fewer of the tested potato tissues is 75% indicating that tissue specificity and developmental regulation are generally linked and that constitutive expression is a relatively rare phenomenon in actively developing issues.

From all the differentially displayed TDFs in our large-scale screen, we have now cloned and analysed around 200. From these, we find only 2% that

appear to be derived from the same transcript. If this can be considered to be representative, then around 17,600 TDFs are available when using *Ase* I and *Taq* I ([256 primer combinations $\times$70 bands/primer combination] minus 2%). Although large fragments are encountered in cDNA-AFLP, the vast majority of TDFs do fall within the size window that is resolvable on a polyacrylamide gel when using *Taq* I and *Ase* I enzyme combinations. However, as this enzyme combination only digests around 45% of all cDNAs, the estimate for the number of genes expressed in potato tuber tissues during tuber formation is thus around 40,000. This relatively high figure may, in part, be due to the sensitivity of the cDNA-AFLP process that visualises transcripts not specific for this tissue or the tuberisation process but which are visualised due to expression at very low levels.

Several strategies can be employed to visualise virtually all transcripts using cDNA-AFLP. The first would be the use of additional enzyme combinations for screening. Additional enzymes will enhance the detection level to about 75% with two restriction enzyme combinations if it is assumed that the second restriction enzyme combination also has sites in half of the cDNAs and half of this population has sites for both rare-cutters. The yield of retrieval of new sequences is likely to be further reduced with every further enzyme combination. Another method would be to incorporate a site for the rare cutter as part of the cDNA synthesis primer (5′-[restriction enzyme site]-poly-dT-3′; Money et al., 1996). When template is prepared from such a cDNA, the restriction on transcript visualisation would only be limited by the site frequency of the second frequently cutting restriction enzyme. Here, however, a bias to the 3′-end of the transcript is re-established which is one of the major disadvantages of DD/RT-PCR and has been circumvented by cDNA-AFLP.

Finally, using restriction enzymes with a four-base recognition sequence would significantly increase the number of cDNAs visualised (Habu et al., 1997). The disadvantage of this approach is that there will be a proportionally greater risk of multiple bands being produced from each cDNA and, due to the large number of bands appearing per lane, less discrimination can be made between individual bands. However, this approach may prove useful when attempting to visualise transcripts in highly homologous systems where only small differences in expression are expected.

When cDNA-AFLP is applied using the amendments described in this paper, the procedure serves as a rapid and reliable method for isolating tissue- or process-specific genes in a wide range of biological systems. As such, cDNA-AFLP may be a valuable complement to the large-scale, random cDNA sequencing projects as a tool for the identification of novel process-related genes. In addition to gene isolation, cDNA-AFLP may also prove to be a powerful tool for fingerprinting tissues from a developmental stage, for ex-

ample during early stages of pathogenesis. Thus cDNA-AFLP might also be used for diagnosis.

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