



REVIEW ARTICLE

Microgenomics: gene expression analysis at the tissue-specific and single-cell levels

Stephan P. Brandt*

Technische Universität Darmstadt, Institut für Botanik, Schnittspahnstraße 3–5, D-64287 Darmstadt, Germany

Received 25 October 2004; Accepted 28 October 2004

Abstract

Plant organs are composed of many different cell types and the analysis of 'bulk' material results in the average of all information in these cells. Therefore, this does not reflect any individuality of the tissues present in plants. This review briefly summarizes different sampling methods which provide tissue- and cell-specific samples, respectively. In addition, gene expression analysis tools that allow the analysis of transcripts in minute samples are discussed in detail. The combination of both approaches results in high resolution gene expression data, which increases understanding of plant physiology in such diverse areas as primary and secondary metabolism, plant defence or stress response.

Key words: Array hybridization, cDNA libraries, gene expression pattern, glass capillary, homomeric tailing, linear antisense amplification, microdissection, RT-PCR, single cell analysis, tissue specificity.

Introduction

Multiparallel methods such as GC-MS for metabolites or microarray hybridization for mRNA expression have provided a vast amount of information about plant physiology. With respect to gene expression, transcriptomes from different plants or organs at different physiological states can now be monitored and compared (Seki *et al.*, 2004). As a huge amount of genetic information is now available, science has entered the post-genomic era and one of the major challenges will be revealing the function of genes and their products. mRNA expression profiling, which represents the most prominent high throughput method for gene expression analysis, requires comparatively large amounts of starting material. Consequently, it is not directly applicable to tissue- and single-cell-specific samples (Brandt

et al., 2002; Meyers *et al.*, 2004). Therefore, with respect to tissue- or cell-specific gene expression only a small amount of information is available. Consequently, in this field, plant sciences are still in the infancy of the genomic era rather than in the post-genomic era.

The high degree of plant differentiation can be observed macro- and microscopically. Anatomical differentiation is accompanied by biochemical and physiological differentiation. Particular physiological processes are allocated to specialized cell types. For instance, photosynthesis is restricted to mesophyll cells. Another prominent example is that phloem loading occurs in companion cells, which are metabolically completely different from the parenchymatous cells in their vicinity. Furthermore, isoforms of genes may be allocated to different cell types (Laval *et al.*, 2002). Even 'homogenous' tissues like leaf epidermis exhibit gradients regarding ion and metabolite distribution (Fricke *et al.*, 1995).

Most experiments described in the current literature rely on 'bulk material' and, consequently, do not reveal this individuality. Homogenization and analysis of entire organs result in (i) averaged information (Levsky and Singer, 2003) and (ii) information, which cannot be assigned to particular cell types. Physiological contributions of cell types which are by far outnumbered may not be recognized (a phenomenon which also depends on the sensitivity of the assay). For example, transcripts of the potassium channel KST1 could not be detected by northern blot hybridizations of total RNA extracted from potato leaves. However, after an enrichment of guard cells prior to RNA extraction KST1 expression could be demonstrated (Kopka *et al.*, 1997).

Some reports claimed the investigation of only a small tissue biopsy. Although the fresh weight of the samples was reduced to the sub-microgram range, due to the lack of accurate cell separation such samples are often far away from any tissue specificity (Harrison *et al.*, 1997; Hertzberg *et al.*, 2001). This demonstrates the overall importance of

* Fax: +49 6151 164630. E-mail: sbrandt@bio.tu-darmstadt.de

the sampling strategy. Finally, sample specificity (instead of size) is critical in analyses with spatial resolution.

Generally, the less a method interferes with the whole plant metabolism the smaller is the resulting sample and the more delicate is the subsequent analysis. Therefore, sampling methods can be distinguished by their extent of interference. With respect to gene expression, the crucial point for small samples representing only a few cells is the amplification of mRNA prior to analysis. This can be accomplished by several specifically designed methods (adapted from 'bulk material' methods or newly developed) either on one, several or all transcripts at the same time.

Recently, reviews have been published on methods for tissue-specific analysis of metabolites and some physico-chemical parameters (Kehr, 2001, 2003; Tomos and Sharrock, 2001). A brief introduction to the different strategies for obtaining tissue-specific samples, down to individual cells, is presented here. Subsequently, the gap will be closed and methods to analyse gene expression on the tissue-specific scale will be discussed in detail. The focus will be on methods, which have already been successfully applied to plant samples. In addition, protocols will be referred to which have been exclusively used in animal or human research so far, but will have a high potential in plant science in the future.

Sampling strategies

Methods for sampling are reviewed in detail by several authors (Bligny and Douce, 2001; Kehr, 2001, 2003; Outlaw and Zhang, 2001; Tomos and Sharrock, 2001; Cornea and Mungenast, 2002). A non-comprehensive selection of current sampling strategies classified by their interference with the overall plant metabolism is listed briefly here. Figure 1 illustrates the principal strategies for

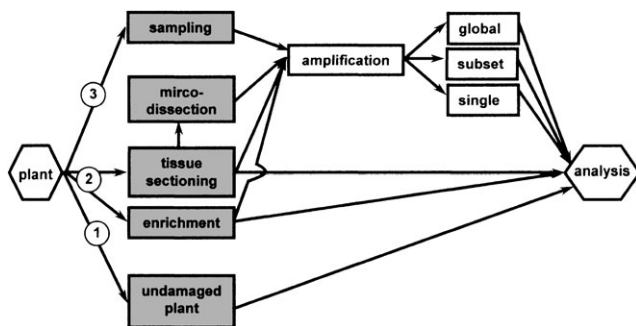


Fig. 1. This scheme shows a flow chart of different strategies for sampling and gene expression analysis. Whereas *in planta* analysis (1) usually does not need any nucleic acid amplification, sampling cells [e.g. by glass capillaries (3)] depends on the amplification of RNA prior to analysis. Tissue sectioning and enrichment of cells (2) are intermediate and can be done with or without an amplification step depending on the subsequent analysis strategy (e.g. *in situ* RT-PCR and *in situ* hybridization, respectively). Grey shaded boxes mark different 'sampling' strategies. For full details see text.

sampling and gene expression analysis. A detailed overview about the combination of sampling and analysis methods is presented in Table 1, which will be discussed in depth in the section 'Gene expression analysis'.

Non-invasive sampling

Non-invasive sampling methods do not interfere with the plant or at least do not cause any wounding. With respect to gene expression analysis, there is only one method available (Table 1), which is the generation of transgenic plants carrying a promoter-reporter gene construct. As no sampling is involved in this method, the details are discussed in the section 'Gene expression analysis' below.

Minimal invasive sampling

Sampling can be carried out with minimal invasion by means of fine glass capillaries (Table 1). The technical set-up of the method is similar to that of the pressure probing technique. Glass microcapillaries are mounted on a micro-manipulator allowing the penetration and aspiration of an individual cell under optical control. This procedure is not restricted to surface cells (Jones and Grierson, 2003), but inner cell types such as mesophyll cells (Lu *et al.*, 2002) or specific phloem cells can also be sampled (Brandt *et al.*, 1999; Raps *et al.*, 2001). Typical volumes are in the range of tens to hundreds of picolitres, depending on the cell type and plant species. Consequently, most standard protocols such as northern blot or microarray hybridizations are not feasible since they need much larger sample sizes (Brandt *et al.*, 2002). Therefore, specially designed methods are required for gene expression analysis in these samples (see below). One major advantage of this sampling method is that only one single cell is (or for inner cell types: only a few cells are) destroyed, and in terms of the whole plant this destruction is minimal. Therefore, investigations on the living specimen can be conducted to determine concentration changes and metabolite fluxes (Kehr, 2001).

Invasive sampling

Invasive sampling strategies serve the purpose of massively enriching the cell type of interest (except *in situ* methods, see below). This opens a wide range of standard protocols for cell-type-specific analysis (Table 1). By enriching cells, the physiological integrity of organs is totally abolished. For example, organs can be enzymatically digested to create protoplasts which are subsequently sorted by physical properties, such as size, presence of chloroplasts or markers (Birnbaum *et al.*, 2003; Ivashikina *et al.*, 2003). Alternatively, organ cell layers can be peeled off (Gaedeke *et al.*, 2001). Consequently, the interference with the plant's physiology is massive (e.g. the loss of a leaf has a high impact on the overall photosynthetic capacity and on the biomass of this individual). However, enrichment of cells of the same kind results in averaged information about

Table 1. A summary of combinations for different methods of sampling and analysis

Gene expression analysis methods are listed vertically, different sampling methods are listed horizontally. The respective cells give the possibility of a certain combination of a sampling protocol and a gene expression analysis method. 'Theoretically yes' indicates combinations which have not been used so far. Where applicable, references are given.

Gene expression analysis method	Non-invasive	Minimal invasive	Invasive		
	<i>In planta</i>	Glass capillary	<i>In situ</i>	Micro-dissection	Tissue enrichment
Reporter gene	de Ruijter <i>et al.</i> , 2003	Brandt <i>et al.</i> , 1999	de Ruijter <i>et al.</i> , 2003	No	Ivashikina <i>et al.</i> , 2003
cDNA library construction	No	No	No	Theoretically yes	Müller-Röber <i>et al.</i> , 1995
Hybridization	No	No	Engler <i>et al.</i> , 2001	Theoretically yes	Kopka <i>et al.</i> , 1997
Microarray hybridization	No	No	No	Theoretically yes	Leonhardt <i>et al.</i> , 2004
Amplification prior to					
<i>In situ</i> detection	No	No	Koltai and Bird, 2000	No	No
cDNA library construction	No	Karrer <i>et al.</i> , 1995	No	Asano <i>et al.</i> , 2002	Dresselhaus <i>et al.</i> , 1995
Gel electrophoresis	No	Brandt <i>et al.</i> , 1999	No	Brandt <i>et al.</i> , 2003	Richert <i>et al.</i> , 1996
Microarray hybridization	No	Brandt <i>et al.</i> , 2002	No	Nakazono <i>et al.</i> , 2003	Theoretically yes

this particular cell type. General differences between samples may be recognized, but the cell-to-cell difference within a given sample is lost (Gehwolf *et al.*, 2002). Furthermore, the extreme interference with metabolism can result in gene expression alterations (Grosset *et al.*, 1990; Titarenko *et al.*, 1997; Birnbaum *et al.*, 2003). However, this effect might be minimized by using transcription and RNA degradation inhibitors (Leonhardt *et al.*, 2004).

Other methods require a fixation step prior to sampling, which, among other things, reduces alterations in gene expression. After fixation and sectioning, cells of interest can be isolated from the tissue by micromanipulation. This can be carried out mechanically either by razor blade fragments (Outlaw and Zhang, 2001) or by vibrating steel chisels (Brandt *et al.*, 2003). More sophisticated systems use laser beams for cell isolation (as well as for transfer of isolated tissue). These techniques have been described in detail recently (Cornea and Mungenast, 2002; Kehr, 2003; Kerk *et al.*, 2003).

By contrast to the invasive methods described so far, *in situ* methods do not require any tissue enrichment. They are performed directly on fixed tissue sections which means that they are analysis rather than sampling methods. Therefore, they are discussed in the 'Gene expression analysis' section below.

Gene expression analysis

In this second section, the advantages and disadvantages of different gene expression analysis protocols which have been applied to plant samples, are discussed. However, some outstanding protocols that have great potential and

should, therefore, be applied to plant single-cell analysis in the future, will also be emphasized. Generally, the methods can be classified in two major groups: (i) methods, which do not need any amplification and (ii) those which require an amplification step prior to analysis (Table 1).

Analysis without amplification

Non-invasive analysis: The starting point is a DNA construct which consists of a reporter gene under the control of a promoter sequence, which is derived from the gene of interest. After successful plant transformation the expression of the reporter gene can be monitored in these plants or their progenies reflecting the expression pattern of the gene. Ideally, the product of the reporter gene does not need any substrates (see below) such as fluorescent proteins like GFP (or one of its derivatives; Ehrhardt, 2003). Reporter genes, which require a substrate such as GUS or luciferase, are not suitable for non-invasive experiments since the application of the substrates is a major intervention in the metabolism of cells. Furthermore, these assays often need fixation, embedding and tissue sectioning (de Ruijter *et al.*, 2003) turning them into invasive methods.

Depending on the insertion locus into the genome, the introduction of such a construct may or may not influence the metabolism of the plant. Nevertheless, this method can be termed non-invasive as the assay of fluorescent proteins itself does not interfere with any metabolic processes (see above). Therefore, the *in vivo* expression of the respective gene is accurately reflected and can often be assigned even to cell compartments (Erhardt, 2003). Recently, Mirabella *et al.* (2004) also demonstrated the use of fluorescent proteins to monitor gene activity dynamics.

The creation of transgenic plants is laborious and extremely time-consuming. Only for a relatively limited number of plant species are transformation and regeneration protocols available so far. Furthermore, it is restricted to the analysis of one gene (or a few genes) per transformation, making it even more cumbersome and time-consuming. Nevertheless, the use of reporter genes is a very useful and widespread method since it does not need any specialized equipment (such as micromanipulators or laser capture microdissection systems). In particular, it is a feasible strategy if the spatial and temporal expression pattern of the gene of interest is expected to be complex.

Analysis following invasive sampling: After enrichment of material either by tissue peeling, cell enrichment, or microdissection (see above, Table 1), northern blot hybridizations, the classical method for gene expression analysis, can be performed (Kopka *et al.*, 1997). Once a northern blot is prepared, it can be hybridized repeatedly which makes one sampling event accessible for multiple analysis. Therefore, quantitative gene expression analysis relative to housekeeping genes is possible. Furthermore, it is easy to perform, does not depend on expensive equipment, and exhibits virtually no technical bias which would compromise the results (in contrast to amplification-based protocols, see below). A big step forward towards a greater throughput is the use of array hybridizations instead of northern blot hybridizations due to its powerful multiparallel character (Birbaum *et al.*, 2003; Leonhardt *et al.*, 2004).

Much more challenging is (fluorescent) *in situ* hybridization (Table 1). After fixation and sectioning of tissue, labelled antisense probes can be hybridized to mRNA transcripts, which are present in the sections. A recent improvement to *in situ* hybridization is the so-called colour bar coding method (Levsky *et al.*, 2002). This method combines spectrally distinct fluorescent dyes to provide more (virtual) colours for detection. One transcript is represented by at least two differentially labelled probes. This increases the specificity of the readout if more than one transcript needs to be detected. Consequently, the transcription of up to 11 genes could be analysed simultaneously. Due to its parallel character, colour bar coding will doubtless speed up gene expression analysis, not only in animal and human, but also in plant research in the future.

As hybridization specifically takes place within the tissue sections, spatial information of mRNA abundance is preserved and excellent qualitative expression data are provided (Engler *et al.*, 2001). Because cell isolation can be omitted and only one hybridization is required to cover all cell types in a tissue section, the analysis is accelerated. Generally, *in situ* hybridizations are a valuable tool if the spatial expression pattern of a gene within tissues is complex. Since different cell types may exhibit different mRNA accessibility and hybridization efficiencies, quantitative

interpretation of gene expression levels might be difficult and has to be considered with caution (Shu *et al.*, 1999).

Amplification prior to analysis

If an enrichment of cells is not possible or only results in limited amounts of material, standard methods like northern blot hybridization are not applicable (see above). An amplification step is then required prior to analysis. In recent years, a couple of protocols for the amplification of single, several or all transcripts in parallel from small tissue specimens have been established (Fig. 1). These methods are very diverse, although most include a PCR-based amplification step (Table 2). However, so far not all of them have been successfully applied to plant systems. One reason for this might be the existence of cell walls in plant tissues, which make cell isolation more difficult. Furthermore, these methods are intensively used in the non-academic clinical field (e.g. in prenatal and cancer diagnostics), which provides a strong driving force for development and application of new strategies.

Single transcript amplification: Starting from 'normal' tube RT-PCR this method was adopted to work on samples even as small as individual protoplasts (Richert *et al.*, 1996) and single cell samples derived by glass capillaries (Brandt *et al.*, 1999). Furthermore, the applicability to (mechanically) microdissected tissue could be demonstrated (Brandt *et al.*, 2003).

To obtain quantitative information, Lu *et al.* (2002) have developed an assay for relative quantification. After performing two PCR reactions on a split cDNA sample, the amount of synthesized cDNA of the gene of interest is normalized to the amount of produced cDNA of a housekeeping gene. This approach allows the quantitative comparison between samples.

To avoid repeated sampling a given sample can be used several times. If an oligo(dT)-primer bound to (magnetic) beads serves as primer for reverse transcription, a bead-bound primary cDNA library can be constructed. After each PCR reaction, the cDNA is recovered (Fellmann *et al.*, 1996; Jones and Grierson, 2003). This approach decreases the number of samples required for a set of experiments thus saving time and, more importantly, avoiding multiple sampling of the same individual.

In nested PCR an 'outer' primer pair is used in a first round of PCR, which is raised against conserved regions of a gene family. This primary amplified cDNA pool provides sufficient template for several second-round PCR reactions, which make use of unique ('inner' or 'nested') primer pairs. Because the 'outer' primers have to be raised against conserved regions, the applicability of this approach is restricted to gene families. On the other hand, the sensitivity of the assay is extremely high due to two rounds of PCR making it suitable even for very low-abundance transcripts in samples of small size (Jones and Grierson, 2003).

Table 2. Different amplification strategies

A short description is given, for details refer to the text. References in italics indicate those which represent plant examples.

Method	Amplification strategy ^a	Reference
Single transcript amplification		
Specifically primed RT-PCR	Primers raised against a specific gene are used in the PCR reaction. Intron spanning primers allow to distinguish between genomic and cDNA origin of the products making DNase treatments prior to PCR obsolete.	<i>Richert et al., 1996;</i> <i>Brandt et al., 1999</i>
Real time RT-PCR	Although no application using real time PCR for plant single cell analysis has been published so far, this method would open the horizon to quantitative analysis.	<i>He et al., 2002;</i> <i>Volkov et al., 2003</i>
Nucleic acid sequence based amplification (NASBA)	cDNA is synthesized by a sequence specific 3'-primer harbouring a T7 promoter. Using a second (upstream) the complementary strand is produced. T7 RNA polymerase synthesis antisense RNA molecules which serve as templates for a second round of amplification.	<i>Deiman et al., 2002;</i> <i>Vaskova et al., 2004</i>
Amplification of transcript subsets		
Differential display RT-PCR	In the PCR a (dT) ₁₅ -primer is used in combination with an arbitrary primer which binds statistically to the cDNA. Consequently, all cDNA molecules which are bound get amplified.	<i>Liang and Pardee, 1992;</i> <i>Bauer et al., 1993;</i> <i>Brandt et al., 2002</i>
Multiplex RT-PCR	Although no plant single cell application has been published so far, this method could be transferred to minute amounts of starting material with reasonable effort. In one reaction tube, primer pairs raised against different genes are combined. This reduces the number of samples as well as the work and time needed.	<i>Recchi et al., 1998;</i> <i>Ponce et al., 2000</i>
Nested RT-PCR	In a first round of amplification, primers raised against conserved regions of e.g. a gene family are used to amplify all members. In a second round an aliquot of the first round is amplified by 'inner' primers specific for a single gene.	<i>Massengill et al., 1997;</i> <i>Jones and Grierson, 2003</i>
Global transcript amplification		
Tailing	Terminal deoxynucleotidyltransferase synthesizes homomeric ends (tails) to the 3' end of any cDNA molecule. The PCR is performed with the complementary homomeric primer and a (dT) ₁₅ -primer.	<i>Dresselhaus et al., 1994;</i> <i>Klein et al., 2002</i>
Adaptor ligation	The first strand cDNA is converted into a double strand and adaptors are ligated to the unknown sequence end of the cDNA molecules. Its sequence as well as a (dT) ₁₅ -primer serve as primers in PCR.	<i>Karrer et al., 1995;</i> <i>Gallagher et al., 2001</i>
Template switching	Some reverse transcriptases exhibit a terminal deoxynucleotidyltransferase activity, which synthesizes some Cs at the 3' end of the nascent first strand. By adding a complementary G _n -primer, the transcriptase switches over to this new template elongating the cDNA first strand with its complementary known sequence. This primer in combination with a (dT) ₁₅ -primer can be used in a subsequent PCR.	<i>Petalidis et al., 2003;</i> <i>Voelckel and Baldwin, 2003;</i> <i>Ivashikina et al., 2003</i>
Linear antisense RNA amplification	The (dT) ₁₅ -primer for the reverse transcription also harbours a T7-RNA-polymerase-promoter region. After conversion into double stranded cDNA, antisense RNA is produced by <i>in vitro</i> transcription. The amplification is linear and thought to preserve the original transcript levels far better than exponential PCR amplification.	<i>Van Gelder et al., 1990;</i> <i>Nakazono et al., 2003</i>
Terminal continuation	Terminal continuation is a mixture of template switching and linear antisense RNA amplification. During first strand cDNA synthesis either of the two primers can bear the RNA polymerase promoter. Consequently, either sense or antisense RNA is linearly amplified by subsequent <i>in vitro</i> transcription.	<i>Ginsberg and Che, 2002</i>
Multiple displacement amplification	Surprisingly, isothermal rolling circle amplification by use of random hexamer primers and ϕDNA polymerase also works with linear (genomic) DNA. However, the DNA is amplified in a cascading, strand displacement reaction.	<i>Dean et al., 2002</i>

^a Prior to the described strategies, the mRNA within the samples is reversibly transcribed into single-stranded cDNA.

Because all these protocols rely on routinely used technology they are easy to perform and are of low cost as no special reagents (e.g. enzymes, labelled nucleotides, dyes) are required. Furthermore, they are versatile and can be used for many gene-tissue-species combinations without further adaptation (Brandt *et al.*, 1999). The straightforward nature and speed of analysis make these RT-PCR protocols the method of choice for preliminary experiments and for routine analysis of gene expression, when only a few genes are aimed to be monitored.

For an absolute quantification of gene expression, real time RT-PCR has been applied to 'bulk samples' obtained in the medical, animal, and plant field. Quantitative PCR has been also successfully adopted for cell-type-specific samples of microdissected human tissue (Gjerdrum *et al.*, 2004) and for more than 100 pooled *Arabidopsis thaliana* leaf protoplasts (Ivashikina *et al.*, 2003). These comparatively 'large' sample sizes were amenable for RNA extraction and purification prior to analysis. Although real-time PCR has also been used for expression analysis in individual animal and human cells (He

et al., 2002), the adaptation of this method to single-cell samples obtained from plants is more difficult. The main reason for this is the low reproducibility (S Brandt *et al.*, unpublished data), which might be caused by secondary metabolites which are more common in plants. Unfortunately, RNA extraction and purification prior to PCR is not feasible for these extremely small samples. Therefore, certain plant-specific secondary metabolites may interfere with the dye binding or the fluorescence readout resulting in a low performance. In order to overcome this limitation the use of alternative fluorescent dyes exhibiting different binding properties and excitation/emission wavelengths will be helpful. In addition, the utilization of hybridization probes rather than dyes may answer the problem.

Following tissue fixation and sectioning, RT-PCR can be performed directly *in situ* omitting any further sampling (Fig. 1). Although this technique is much more laborious and more prone to RNA degradation than tube RT-PCR, its major advantage is the elucidation of single gene expression profiles across the entire tissue section in which the PCR is performed. Consequently, only a single reaction is necessary to gain highly spatial resolved results (Koltai and Bird, 2000) making it especially favourable when the gene activity is complicated with respect to the spatial pattern.

The last method for single transcript amplification mentioned here is nucleic acid sequence-based amplification (NASBA). Using a sequence-specific primer, which also harbours an RNA polymerase promoter, reverse transcription is performed. After synthesis of the second strand (by use of another sequence-specific primer) subsequent *in vitro* transcription amplifies one specific sequence (Deiman *et al.*, 2002). The method is particularly well suited for single-stranded RNA detection. Although it has a high impact in medical research, only a very limited number of plant-related applications have been reported (Vaskova *et al.*, 2004). Interestingly, in both medical and plant research NASBA is used in most cases for mRNA detection of pathogens. One reason for this might be the enormous sensitivity (amplification factor of up to 10^9), which is even higher than for PCR ($\sim 10^7$). One reason why NASBA is not routinely used for nucleic acid amplification might be that the created RNA is much more unstable and prone to degradation compared with cDNA. But when carefully carried out, this protocol represents a valuable complementary method for gene expression studies.

Amplification of transcript subsets: To accelerate the analysis, different transcripts can be amplified in parallel. This is highly efficient with respect to time, cost and labour. Moreover, because fewer samples are needed, the risk of contamination is reduced as well.

A straightforward further improvement of single-transcript RT-PCR was the development of multiplex-PCR. Several primer pairs, which are specific for different genes, are added in parallel to the PCR mix, containing all necessary compon-

ents for the amplification reaction. Although multiplex-PCR was adopted for expression analysis in single cancer cells (Recchi *et al.*, 1998), so far the smallest plant samples have been entire flowers and leaves, respectively (Ponce *et al.*, 2000). On the other hand, multiplex-RT-PCR has recently been applied to plant tissue sections as an *in situ* method (Pesquet *et al.*, 2004). Because multiplex-PCR is a valuable tool due to its parallel character and since there are no obvious obstacles it will represent an important method for single plant cell analysis in the future. But as multiplex-PCR is technically very demanding, particularly in terms of primer design (Henegariu *et al.*, 1997; Elnifro *et al.*, 2000) and technical setup, it has to be carefully considered if multiplex-PCR or a set of single-gene PCR reactions are to be favoured.

A milestone in gene expression analysis was the introduction of arbitrarily primed PCR/differential display (Welsh *et al.*, 1992; Liang and Pardee, 1992). Using these techniques, larger numbers of genes can be monitored in a single reaction using oligo(dT)- and arbitrary primers, which exhibit a certain probability to anneal to a subset of DNA molecules (Bauer *et al.*, 1993). Consequently, only these molecules are amplified during PCR cycling. Although several improvements have been suggested (Cheung and Nelson, 1996; Dixon *et al.*, 1998; Menke and Müller-Röber, 2001) arbitrarily primed PCR is still sensitive to misamplification mainly due to the relatively low annealing temperatures. This can lead to imbalances in the observed gene expression levels (Baldwin *et al.*, 1999). Furthermore, the number of false positives can increase unacceptably (Brandt *et al.*, 2002). On the other hand, the method exhibits some big advantages: It is easy to carry out, does not need any specialized equipment/reagents and requires no adaptations for small-scale samples.

However, when 20–50 single plant cell samples were pooled, arbitrarily primed PCR resulted in satisfying yields and quality of amplified cDNA, (Brandt *et al.*, 2002). But bearing in mind the potentially high technical bias, this method should be considered with caution.

Global transcript amplification: In order to obtain a comprehensive view on the transcriptome present in cells at a given time, multiparallel analysis is necessary. For this, global amplification of all transcripts is desirable. This decreases the number of required samples and, therefore, further reduces labour, time, and expense.

PCR-based protocols need to overcome the problem of unknown 3'-ends of cDNA first strands. To get universal 3'-sequences, which can serve as primers in combination with the oligo(dT)-stretches at the 5'-ends, different strategies have successfully been applied: (i) 'homomeric tailing', (ii) adaptor ligation, and (iii) 'template switching'.

(i) 'Homomeric tailing'. The enzyme deoxynucleotidyl-transferase can synthesize several nucleotides of the same

type to any 3'-end of first strand cDNA. The homomer's complementary sequence and the oligo(dT)-sequence can be used as primers in subsequent PCR reactions. Tailing was used not only for animal samples (Klein *et al.*, 2002), but also to amplify the mRNA from 128 maize egg cell protoplasts for generation of a cDNA library (Dresselhaus *et al.*, 1994).

(ii) Adaptor ligation. DNA linkers are ligated to the unknown 3'-ends of cDNA molecules. As a result, these adaptor sequences combined with oligo(dT)-primers serve as primers in the PCR reaction. However, the generation of cDNA libraries from as little as five pooled mesophyll cells (Gallagher *et al.* 2001) or individual epidermal and guard cells, respectively, was accomplished (Karrer *et al.*, 1995).

Tailing and adaptor ligation methods have to be employed with caution as they involve a series of washing steps, which might result in a loss of considerable amounts of starting material as well as contamination of the samples. These serious problems can be partially overcome by using oligo(dT)-primers during reverse transcription, which are bound to (magnetic) beads (Karrer *et al.*, 1995; Gallagher *et al.*, 2001). Another point to be considered is, that during PCR shorter cDNA molecules are more efficiently amplified than longer ones (Phillips and Eberwine, 1996; Luo *et al.*, 1999) which changes the transcript representation. This limitation can be compensated by restricting the length of all cDNA first strands to a few hundred nucleotides during reverse transcription (Iscove *et al.*, 2002).

(iii) 'Template switching'. This strategy is based on the attachment of some cytidine nucleotides to nascent cDNA first strands by reverse transcriptase. These act as binding sites for a second [3'-(dG)_n containing] primer, which is subsequently used as template instead of the mRNA ('template switch'). The first strand cDNA is extended to the end of the primer and its complementary sequence serves as second primer in downstream PCR reactions. The protocol has been already used for large plant samples (Voelckel and Baldwin, 2003) and also on samples as small as 145 pooled leaf protoplasts (Ivashikina *et al.*, 2003). This method overcomes the danger of losing starting material and introducing contamination because it omits purification steps with the repeated opening of reaction tubes. But the resulting cDNA molecules still have different lengths and, therefore, influence the transcript representation after PCR (see above; Phillips and Eberwine, 1996; Luo *et al.*, 1999).

It has to be pointed out, that one serious problem of all PCR-based methods cannot be overcome, which is the so called 'Monte Carlo'-effect (Karrer *et al.*, 1995). This effect is caused by small and random differences in amplification efficiency between PCR templates. It dramatically decreases the reproducibility especially when small and complex template mixtures are needed for amplification (Karrer *et al.*, 1995). Therefore, a non-PCR-based method is strongly recommended to get comprehensive, unbiased amplification of all transcripts in parallel.

Even though the method for linear antisense RNA amplification has been already reported by Van Gelder and colleagues in 1990, it took about one decade to use it in plant-related research. This excellent technique makes use of an oligo(dT)-primer for reverse transcription, which harbours an RNA-polymerase promoter region. After creation of the complementary second strands and two rounds of *in vitro* transcription the enrichment can be as high as 10⁶ (Phillips and Eberwine, 1996). One of the major advantages of antisense RNA amplification is its linear amplification character. This results in excellent preservation of quantitative information compared with PCR-based amplification. Furthermore, the need for creating known 3'-ends on all cDNA molecules is bypassed.

This method has been used to amplify mRNA of micro-dissected tissues from human cancer (Ohyama *et al.*, 2000), animal neurons (Luo *et al.*, 1999), as well as rice phloem cells (Asano *et al.*, 2002) and maize epidermis/vascular tissue (Nakazono *et al.*, 2003). Moreover, the method has already been successfully applied in animal single cell analysis (O'Dell *et al.*, 1998). The first reports on the application of this method for single cells from plants is expected soon, since this method represents by far the highest potential for tissue-specific analysis as it circumvents the inherent technical bias and limitations of global PCR-based approaches.

The other protocols mentioned in Table 2 [terminal continuation (Ginsberg and Che, 2002) and multiple displacement amplification (Dean *et al.*, 2002)] have great potential, but still need to be adapted to (pooled) single-cell analysis. Once this is done, these methods might become significant tools in single-cell gene expression analysis in all fields of research.

Analysis after amplification

Due to amplification, the transcripts of interest are enriched to a level at which they can be detected using common laboratory protocols. If only a single or a few transcripts were amplified, most often agarose gel electrophoresis and ethidium bromide staining provide sufficient mass discrimination and sensitivity. For the detection of low abundance mRNAs or when a low yielding DNA polymerase was used, direct product labelling and detection (Dresselhaus *et al.*, 1994; Brandt *et al.*, 1999) or cDNA blot hybridization (Lu *et al.*, 2002) may be necessary for visualization. Alternatively, the PCR products can be detected by highly sensitive capillary electrophoresis using a dye and laser-induced fluorescence detection (Liu *et al.*, 2004).

PCR products amplified by arbitrarily primed PCR consist of complex mass mixtures. Polyacrylamide gels are useful for their separation as hundreds of bands and mass differences of single nucleotides can be resolved. Once differences between two pools of mRNA are identified, subamplification, cloning, and sequencing of differentially

displayed bands are required to reveal the identity of the transcripts (Bauer *et al.*, 1993).

A similar situation is found when the amplified transcripts are cloned into cDNA libraries. Two (or more) libraries need to be compared by methods such as colony hybridization or subtractive library hybridization followed by cloning and sequencing of the differentially expressed clones.

The emergence of microarray hybridization has revolutionized the detection of differences in complex cDNA mixtures (e.g. after arbitrarily primed PCR or linear antisense RNA amplification). The time-consuming and cumbersome cloning/sequencing steps have become redundant. Once amplified, PCR products can be labelled, hybridized to arrays and the differentially expressed genes can be identified directly (Brandt *et al.*, 2002; Nakazono *et al.*, 2003).

Applications

In plant research, the number of publications in tissue-specific and single-cell analysis is still small compared with animal and human research. Most of the reports present introductions of new methods and proofs of their concepts. Exceptions, of course, are *in situ* RT-PCR, *in situ* hybridization as well as promoter-reporter gene studies, which have been widely used for a comparatively large number of genes and environmental conditions.

Rausch *et al.* (2004) isolated the low-affinity orthophosphate (Pi) transporter Pht2:1. To analyse its expression pattern they transformed potato and *A. thaliana* plants with the GUS reporter gene under the control of a 2 kbp fragment of the *Pht2;1* promoter. As a result the reporter gene exhibited quite a complex expression pattern. For example, expression was observed throughout leaf mesophyll, in central cylinder regions of growing lateral roots, and several tissues of flowers depending on the developmental stage and age of the plant. These findings demonstrate that reporter genes are a versatile tool for analysis of genes with complex spatial and temporal expression. In order to analyse the subcellular localization of this transporter in leaf mesophyll, plants were transformed with chimeric Pht2;1-GFP constructs, and it could be shown that the gene product was localized in plastids.

The occurrence of photosynthesis-related gene transcripts in leaves is known. However, Lu *et al.* (2002) investigated the expression of sucrose:fructan fructosyltransferase, Rubisco, a chlorophyll *a/b* binding protein, and actin in more detail. To this end, they analysed whole leaf RNA and, in parallel, pooled epidermal, mesophyll, and parenchymatous bundle sheath cells of *Hordeum vulgare* plants which were sampled by glass capillaries during the day and night. In all experiments they could clearly demonstrate the light-independent and constitutive expression of actin. Therefore they used it as a control. At the leaf level, all photosynthesis-related genes showed higher expression levels in the light

whereas the expression in the different cell types exhibited a higher complexity. Under light conditions the chlorophyll *a/b* binding protein was equally expressed in mesophyll and bundle sheath cells, but not in epidermal cells. Rubisco showed a similar expression pattern, but its expression in the bundle sheath cells was weaker than in the mesophyll. By contrast, sucrose:fructan fructosyltransferase expression was almost exclusively restricted to mesophyll cells and exhibited a stronger light induction than the other genes. The comparison between the whole leaf and cell-type-specific experiments demonstrates once more the importance of highly spatially resolved analysis as well as the potential of specifically primed RT-PCR.

In 'patch-catch' experiments, the electrophysiological properties and the gene expression of the same individual cell can be determined. Firstly, patch-clamp measurements on a protoplast are performed. After identifying a particular transporter and characterizing its properties, the protoplast is aspirated into the patch pipette, transferred to a reaction tube and subjected to mRNA amplification and analysis (Gehwolf *et al.*, 2002). This strategy combines electrophysiological with molecular biological analysis on the same individual cell. Consequently, cell-to-cell variation is excluded from the integration of the results. The general importance of single-cell analysis was illustrated by finding a relatively high variability in the expression of an H⁺-ATPase in pollen grains. If (relative) quantitative RT-PCR would be applied instead of single-gene RT-PCR, even the expression levels of the respective gene could be determined (Lu *et al.*, 2002).

Using laser capture microdissection and linear antisense RNA amplification, gene expression in epidermal cells and the vascular tissue of maize plants was compared (Nakazono *et al.*, 2003). Roughly 250 genes were identified that exhibited a differential expression pattern. Amongst the preferentially epidermis-expressed genes several were found which are involved in the shikimate and other secondary metabolites generating pathways. By contrast, in vascular tissue, genes were found with high expression levels which belong to transporters, aquaporins, metal homeostasis, and other gene families. This work combines the most powerful methods in sampling and amplification, which will play a major role in future experiments.

Conclusions and outlook

In summary, different sampling strategies are at hand which result in tissue-specific or even single-cell samples. Combined with suitable analysis methods, which have been designed to work even on sub-nanolitre/sub-picogram samples, highly spatially resolved gene expression data can be obtained. The crucial point in planning an experiment is the required spatial resolution. The finer the scale the more technically demanding is the analysis. Therefore, the extent of spatial resolution, as well as the advantages

and disadvantages of the different analysis protocols, have to be considered carefully for each type of experiment.

The future will bring not only many more applications of single-cell technologies in nearly all fields of plant physiology, but also a further development of methods to perform the analysis. One promising example is the use of capillary electrophoresis for transcriptomic studies (Zabzdyr and Lillard, 2001; Li and Yeung, 2002).

Gaining more cell-type-specific information will lead to a much better understanding of plant physiology in such diverse areas as primary and secondary metabolism, development, cell-to-cell communication, pathogen defence, or abiotic stress response. Furthermore, single cell analysis can help to target transgenes to the 'right' cell type. Consequently, unwanted (toxic) side-effects can be avoided. All this will further increase an understanding of life's complexity. The major challenge for the experimenter will be the integration of all the new information for improving understanding of how cells, tissues and plants are functioning.

Acknowledgements

I am thankful to Esther Eckert and Stela Mandelj for proofreading the manuscript. I am also thankful to Dr Ute Roessner-Tunali and Dr Alisdair Fernie for their critical comments on the manuscript. Professor Christoph Giersch is gratefully acknowledged for all his support.

References

- Asano T, Masumura T, Kusano H, Kikuchi S, Kurita A, Shimada H, Kadowaki K. 2002. Construction of a specialized cDNA library from plant cells isolated by laser capture microdissection: toward comprehensive analysis of the genes expressed in the rice phloem. *The Plant Journal* **32**, 401–408.
- Baldwin D, Crane V, Rice D. 1999. A comparison of gel-based, nylon filter and microarray techniques to detect differential RNA expression in plants. *Current Opinion in Plant Biology* **2**, 96–103.
- Bauer D, Müller H, Reich J, Riedel H, Ahrenkiel V, Warthoe P, Strauss M. 1993. Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). *Nucleic Acids Research* **21**, 4272–4280.
- Birnbaum K, Shasha DE, Wang JY, Jung JW, Lambert GM, Galbraith DW, Benfey PN. 2003. A gene expression map of the *Arabidopsis* root. *Science* **302**, 1956–1960.
- Bligny R, Douce R. 2001. NMR and plant metabolism. *Current Opinion in Plant Biology* **4**, 191–196.
- Brandt S, Kehr J, Walz C, Imlau A, Willmitzer L, Fisahn J. 1999. A rapid method for detection of plant gene transcripts from epidermal, mesophyll and companion cells of intact leaves. *The Plant Journal* **20**, 245–250.
- Brandt S, Kloska S, Altmann T, Kehr J. 2002. Using array hybridization to monitor gene expression at the single cell level. *Journal of Experimental Botany* **53**, 2315–2323.
- Brandt S, Walz C, Schad M, Pavlovic N, Kehr J. 2003. A simple, chisel assisted mechanical microdissection system for harvesting homogenous plant tissue suitable for the analysis of nucleic acids and proteins. *Plant Molecular Biology Reporter* **21**, 417–427.
- Cheung VG, Nelson SF. 1996. Whole genome amplification using a degenerate oligonucleotide primer allows hundreds of genotypes to be performed on less than one nanogram of genomic DNA. *Proceedings of the National Academy of Sciences, USA* **93**, 14676–14679.
- Cornea A, Mungest A. 2002. Comparison of current equipment. *Methods in Enzymology* **356**, 3–12.
- Dean FB, Hosono S, Fang L, et al. 2002. Comprehensive human genome amplification using multiple displacement amplification. *Proceedings of the National Academy of Sciences, USA* **99**, 5261–5266.
- Deiman B, van Aarle P, Sillekens P. 2002. Characteristics and applications of nucleic acid sequence-based amplification (NASBA). *Molecular Biotechnology* **20**, 163–179.
- de Ruijter NCA, Verhees J, van Leeuwen W, van der Krol AR. 2003. Evaluation and comparison of the GUS, LUC and GFP reporter system for gene expression studies in plants. *Plant Biology* **5**, 103–115.
- Dixon AK, Richardson PJ, Lee K, Carter NP, Freeman TC. 1998. Expression profiling of single cells using 3 prime end amplification (TPEA) PCR. *Nucleic Acids Research* **26**, 4426–4431.
- Dresselhaus T, Lörz H, Kranz E. 1994. Representative cDNA libraries from few plant cells. *The Plant Journal* **5**, 605–610.
- Ehrhardt D. 2003. GFP technology for live cell imaging. *Current Opinion in Plant Biology* **6**, 622–628.
- Elnifro EM, Ashshi AM, Cooper RJ, Klapper PE. 2000. Multiplex PCR: optimization and application in diagnostic virology. *Clinical Microbiology Reviews* **13**, 559–570.
- Engler JD, De Groodt R, Van Montagu M, Engler G. 2001. *In situ* hybridization to mRNA of *Arabidopsis* tissue sections. *Methods* **23**, 325–334.
- Fellmann F, Pretet JL, Fellmann D. 1996. Simplified protocol of solid-phase cDNA libraries for multiple PCR amplification. *Bio-Techniques* **21**, 766–770.
- Fricke W, Hinde PS, Leigh RS, Tomos AD. 1995. Vacuolar solutes in the upper epidermis of barley leaves. *Planta* **196**, 40–49.
- Gaedek N, Klein M, Kolukisaoglu U, et al. 2001. The *Arabidopsis thaliana* ABC transporter AtMRP5 controls root development and stomata movement. *EMBO Journal* **20**, 1875–1887.
- Gallagher JA, Koroleva OA, Tomos AD, Farrar JF, Pollock CJ. 2001. Single cell analysis technique for comparison of specific mRNA abundance in plant cells. *Journal of Plant Physiology* **158**, 1089–1092.
- Gehwolf R, Griessner M, Pertl H, Obermeyer G. 2002. First patch, then catch: measuring the activity and the mRNA transcripts of a proton pump in individual *Lilium* pollen protoplasts. *FEBS Letters* **512**, 152–156.
- Ginsberg SD, Che SL. 2002. RNA amplification in brain tissues. *Neurochemical Research* **27**, 981–992.
- Gjerdrum LM, Sorensen BS, Kjeldsen E, Sorensen FB, Nexø E, Hamilton-Dutoit S. 2004. Real-time quantitative PCR of microdissected paraffin-embedded breast carcinoma: an alternative method for HER-2/neu analysis. *Journal of Molecular Diagnostics* **6**, 42–51.
- Grosset J, Marty I, Chartier Y, Meyer Y. 1990. Messenger RNAs newly synthesized by tobacco mesophyll protoplasts are wound inducible. *Plant Molecular Biology* **15**, 485–496.
- Harrison J, Gallagher JA, Pollock CJ. 1997. A simple and rapid method for the analysis of water-soluble carbohydrates from small segments of cereal leaf tissue. *Journal of Plant Physiology* **151**, 654–659.
- He LP, Chinnery PF, Durham SE, Blakely EL, Wardell TM, Borthwick GM, Taylor RW, Turnbull DM. 2002. Detection and quantification of mitochondrial DNA deletions in individual cells by real-time PCR. *Nucleic Acids Research* **30**, e68.
- Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH. 1997. Multiplex PCR: critical parameters and step-by-step protocol. *Biotechniques* **23**, 504–511.

- Hertzberg M, Sievertzon M, Aspeborg H, Nilsson P, Sandberg G, Lundeberg J. 2001. cDNA microarray analysis of small plant tissue samples using a cDNA tag target amplification protocol. *The Plant Journal* **25**, 585–591.
- Iscove NN, Barbara M, Gu M, Gibson M, Modi C, Winegarden N. 2002. Representation is faithfully preserved in global cDNA amplified exponentially from sub-picogram quantities of mRNA. *Nature Biotechnology* **20**, 940–943.
- Ivashikina N, Deeken R, Ache P, Kranz E, Pommerrenig B, Sauer N, Hedrich R. 2003. Isolation of AtSUC2 promoter-GFP-marked companion cells for patch-clamp studies and expression profiling. *The Plant Journal* **36**, 931–945.
- Jones MA, Grierson CS. 2003. A simple method for obtaining cell-specific cDNA from small numbers of growing root-hair cells in *Arabidopsis thaliana*. *Journal of Experimental Botany* **54**, 1373–1378.
- Karrer EE, Lincoln JE, Hogenhout S, Bennett AB, Bostock RM, Martineau B, Lucas WJ, Gilchrist DG, Alexander D. 1995. *In situ* isolation of mRNA from individual plant cells: creation of cell-specific cDNA libraries. *Proceedings of the National Academy of Sciences, USA* **92**, 3814–3818.
- Kehr J. 2001. High resolution spatial analysis of plant systems. *Current Opinion in Plant Biology* **4**, 197–201.
- Kehr J. 2003. Single cell technology. *Current Opinion in Plant Biology* **6**, 1–5.
- Kerk NM, Ceserani T, Tausta SL, Sussex IM, Nelson TM. 2003. Laser capture microdissection of cells from plant tissues. *Plant Physiology* **132**, 27–35.
- Klein CA, Seidl S, Petat-Dutter K, et al. 2002. Combined transcriptome and genome analysis of single micrometastatic cells. *Nature Biotechnology* **20**, 387–392.
- Koltai H, Bird DM. 2000. High throughput cellular localization of specific plant mRNAs by liquid-phase *in situ* reverse transcription-polymerase chain reaction of tissue sections. *Plant Physiology* **123**, 1203–1212.
- Kopka J, Provart NJ, Müller-Röber B. 1997. Potato guard cells respond to drying soil by a complex change in the expression of genes related to carbon metabolism and turgor regulation. *The Plant Journal* **11**, 871–882.
- Laval V, Koroleva OA, Murphy E, Lu CG, Milner JJ, Hooks MA, Tomos AD. 2002. Distribution of actin gene isoforms in the *Arabidopsis* leaf measured in microsamples from intact individual cells. *Planta* **215**, 287–292.
- Leonhardt N, Kwak JM, Robert N, Waner D, Leonhardt G, Schroeder JI. 2004. Microarray expression analyses of *Arabidopsis* guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. *The Plant Cell* **16**, 596–615.
- Levsky JM, Shenoy SM, Pezo RC, Singer RH. 2002. Single cell gene expression profiling. *Science* **297**, 836–840.
- Levsky JM, Singer RH. 2003. Gene expression and the myth of the average cell. *Trends in Cell Biology* **13**, 4–6.
- Li H, Yeung ES. 2002. Selective genotyping of individual cells by capillary polymerase chain reaction. *Electrophoresis* **23**, 3372–3380.
- Liang P, Pardee AB. 1992. Differential display of eucaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967–971.
- Liu X, Ma L, Zhang JF, Lu YT. 2004. Determination of single-cell gene expression in *Arabidopsis* by capillary electrophoresis with laser induced fluorescence detection. *Journal of Chromatography B* **808**, 241–247.
- Lu CG, Koroleva OA, Farrar JF, Gallagher JA, Pollock CJ, Tomos AD. 2002. Rubisco small subunit, chlorophyll *a/b*-binding protein and sucrose:fructan-6-fructosyl transferase gene expression and sugar status in single barley leaf cells *in situ*. Cell type specificity and induction by light. *Plant Physiology* **130**, 1335–1348.
- Luo L, Salunga RC, Guo H, et al. 1999. Gene expression profiles of laser-captured adjacent neuronal subtypes. *Nature Medicine* **5**, 117–122.
- Massengill JL, Smith MA, Son DI, O'Dowd DK. 1997. Differential expression of K_{4-AP} currents and Kv3.1 potassium channel transcripts in cortical neurons that develop distinct firing phenotypes. *Journal of Neuroscience* **17**, 3136–3147.
- Menke U, Müller-Röber B. 2001. RNA fingerprinting of specific plant cell types: adaption to plants and organization of RNA arbitrarily primed PCR (RAP-PCR). *Plant Molecular Biology Reporter* **19**, 33–48.
- Meyers BC, Galbraith DW, Nelson T, Agrawal V. 2004. Methods for transcriptional profiling in plants. Be fruitful and replicate. *Plant Physiology* **135**, 637–652.
- Mirabella R, Franken C, van der Krogt GNM, Bisseling T, Geurts R. 2004. Use of the fluorescent timer DsRED-E5 as reporter to monitor dynamics of gene activity in plants. *Plant Physiology* **135**, 1879–1887.
- Müller-Röber B, Ellenberg J, Provart N, Willmitzer L, Busch H, Becker D, Dietrich P, Hoth S, Hedrich R. 1995. Cloning and electrophysiological analysis of KST1, an inward rectifying K^+ channel in potato guard cells. *EMBO Journal* **14**, 2409–2416.
- Nakazono M, Qiu F, Borsuk LA, Schnable PS. 2003. Laser-capture micro dissection, a tool for the global analysis of gene expression in specific plant cell types: identification of genes expressed differentially in epidermal cells or vascular tissue of maize. *The Plant Cell* **15**, 583–596.
- O'Dell DM, Raghupathi R, Crino PB, Morrison B, Eberwine JH, McIntosh TK. 1998. Amplification of mRNAs from single, fixed, TUNEL-positive cells. *BioTechniques* **25**, 566–569.
- Ohyama H, Zhang X, Kohno Y, Alevizos I, Posner M, Wong DT, Todd R. 2000. Laser capture micro dissection-generated target sample for high-density oligonucleotide array hybridization. *BioTechniques* **29**, 530–536.
- Outlaw WH, Zhang SQ. 2001. Single-cell dissection and microdroplet chemistry. *Journal of Experimental Botany* **52**, 605–614.
- Pesquet E, Barbier O, Ranocha P, Jauneau A, Goffner D. 2004. Multiple gene detection by *in situ* RT-PCR in isolated plant cells and tissues. *The Plant Journal* **39**, 947–959.
- Petalidis L, Bhattacharyya S, Morris GA, Collins VP, Freeman TC, Lyons PA. 2003. Global amplification of mRNA by template-switching PCR: linearity and application to microarray analysis. *Nucleic Acids Research* **31**, e142.
- Phillips J, Eberwine JH. 1996. Antisense RNA amplification: a linear amplification method for analyzing the mRNA population from single living cell. *Methods* **10**, 283–288.
- Ponce MR, Pérez-Pérez JM, Piqueras P, Micol JL. 2000. A multiplex reverse transcriptase-polymerase chain reaction method for fluorescence-based semiautomated detection of gene expression in *Arabidopsis thaliana*. *Planta* **211**, 606–608.
- Raps A, Kehr J, Gugerli P, Moar WJ, Bigler F, Hilbeck A. 2001. Immunological analysis of phloem sap of *Bacillus thuringiensis* corn and of the nontarget herbivore *Rhopalosiphum padi* (Homoptera: Aphidae) for the presence of Cry1Ab. *Molecular Ecology* **10**, 525–533.
- Rausch C, Zimmermann P, Amrhein N, Bucher M. 2004. Expression analysis suggests novel roles for the plastidic phosphate transporter Pht2;1 in auto- and heterotrophic tissues in potato and *Arabidopsis*. *The Plant Journal* **39**, 13–28.
- Recchi MA, Hebbbar M, Hornez L, Harduin-Lepers A, Peyrat JP, Delannoy P. 1998. Multiplex reverse transcription polymerase chain reaction assessment of sialyltransferase expression in human breast cancer. *Cancer Research* **58**, 4066–4070.

- Richert J, Kranz E, Lörz H, Dresselhaus T.** 1996. A reverse transcriptase-polymerase chain reaction assay for gene expression studies at the single cell level. *Plant Science* **114**, 93–99.
- Seki M, Satou M, Sakurai T, et al.** 2004. RIKEN *Arabidopsis* full-length (RAFL) cDNA and its applications for expression profiling under abiotic stress conditions. *Journal of Experimental Botany* **55**, 213–223.
- Shu GG, Baum DA, Mets LJ.** 1999. Detection of gene expression patterns in various plant tissues using nonradioactive mRNA *in situ* hybridization. *World Wide Web Journal of Biology* **4**, <http://www.epress.com/w3jbio/vol4/shu>.
- Titarenko E, Rojo E, Leon J, Sanchez-Serrano JJ.** 1997. Jasmonic acid-dependent and -independent signaling pathways control wound-induced gene activation in *Arabidopsis thaliana*. *Plant Physiology* **115**, 817–826.
- Tomos AD, Sharrock RA.** 2001. Cell sampling and analysis (SiCSA): metabolites measured at single cell resolution. *Journal of Experimental Botany* **52**, 623–630.
- Van Gelder RN, von Zastrow ME, Yool A, Dement WC, Barchas JD, Eberwine JH.** 1990. Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proceedings of the National Academy of Sciences, USA* **87**, 1663–1667.
- Vaskova D, Spak J, Klerks MM, Schoen CD, Thompson JR, Jelkmann W.** 2004. Real-time NASBA for detection of strawberry vein banding virus. *European Journal of Plant Pathology* **110**, 213–221.
- Voelckel C, Baldwin IT.** 2003. Detecting herbivore-specific transcriptional responses in plants with multiple DDRT-PCR and subtractive library procedures. *Physiologia Plantarum* **118**, 240–252.
- Volkov RA, Panchuk II, Schoffl F.** 2003. Heat-stress-dependency and developmental modulation of gene expression: the potential of house-keeping genes as internal standards in mRNA expression profiling using real-time RT-PCR. *Journal of Experimental Botany* **54**, 2343–2349.
- Welsh J, Chada K, Dalal SS, Cheng S, Ralph D, McClelland M.** 1992. Arbitrarily primed PCR fingerprinting of RNA. *Nucleic Acids Research* **20**, 4965–4970.
- Zabzdyr JL, Lillard SJ.** 2001. UV- and visible-excited fluorescence of nucleic acids separated by capillary electrophoresis. *Journal of Chromatography A* **911**, 269–276.