Mini Review

Cell Type-Specific Gene Expression Profiling in Plants by Using a Combination of Laser Microdissection and High-Throughput Technologies

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Laser microdissection (LM) allows for the isolation of specific cells of interest from heterogeneous tissues under direct microscopic visualization with the assistance of a laser beam. By permitting global analyses of gene expression and metabolites in the selected cells, it is a powerful tool for understanding the biological processes in individual cell types during development or in response to various stimuli. Recently, LM technology has been successfully applied to the separation of individual plant cell types. Here, we provide an overview of applications of LM combined with high-throughput technologies including transcript analyses [microarrays, serial analysis of gene expression (SAGE) and 454-sequencing], proteomic analyses and metabolomic profiling, for cell type-specific gene expression analyses in plants.

Keywords: 454-sequencing — Laser microdissection (LM) — Microarray — Serial analysis of gene expression (SAGE).

Abbreviations: EST, expressed sequence tag; FAS, fluorescence-activated sorting; LM, laser microdissection; SAGE, serial analysis of gene expression; SAM, shoot apical meristem; SNP, single nucleotide polymorphism.

Introduction

Higher plants are multicellular organisms, which consist of >40 cell types of different functions (Demura et al. 2002). Each cell type has a unique transcriptome, proteome and metabolome. Regulatory networks coordinated by these unique gene products and other functional molecules control plant development and physiological responses to internal or external stimuli. To understand these diverse biological processes in plants, it is important to study individual cell types and regulatory networks among these cell types.

Several different technologies have been applied to isolate specific plant cells. These technologies include micropipetting, fluorescence-activated sorting (FAS) and laser microdissection (LM). Micropipetting is a technique for collecting cellular contents directly from single cells in living tissues using microcapillaries (Karrer et al. 1995, Brandt et al. 2002, Brandt 2005). However, it is not well suited for cells that are difficult to identify, such as cells located deep inside plant organs. FAS is a method to collect protoplasts that have the same properties such as size and chlorophyll content via flow cytometry. Specific types of cells labeled with green fluorescent proteins have been collected via FAS and used for subsequent analyses such as transcript profiling (Galbraith et al. 1995, Sheen et al. 1995, Birnbaum et al. 2003, Birnbaum et al. 2005). A problem with this method is that the procedures required to generate protoplasts can change the expression patterns of a small number of genes (Birnbaum et al. 2003). LM is a method for isolating specific cell types (Emmert-Buck et al. 1996), including those of plants (Asano et al. 2002, Kerk et al. 2003, Nakazono et al. 2003). To collect cells by LM, tissues are fixed, e.g. with Farmer's fixative [ethanol (75%) and acetate (25%)] or acetone, or simply frozen, embedded in paraffin or OCT medium, and cut into sections. Target cells are isolated from these sections by laser capture or laser cutting (Figs. 1, 2). Laser capture reliably targets cells for collection but may sometimes collect some surrounding cells that inadvertently remain attached to the target cells (Fig. 1A). On the other hand, laser cutting via gravity (Fig. 1B) or laser pressure (Fig. 1C) minimizes the collection of non-target cells, but is potentially (depending on weather conditions) subject to interference by factors such as static electricity. Several recent reviews describe sample preparation and cell collection methods for LM (Kehr 2003, Schnable et al. 2004, Day et al. 2005, Nelson et al. 2006). Unlike micropipetting, LM can be used in a broad range of plant organs and tissues where target cells can be identified in the tissue sections via microscopy (Nelson et al. 2006). Changes in gene expression are expected to be minimized in fixed tissues for LM-mediated cell collection (Schnable et al. 2004). Comparisons of the advantages and disadvantages

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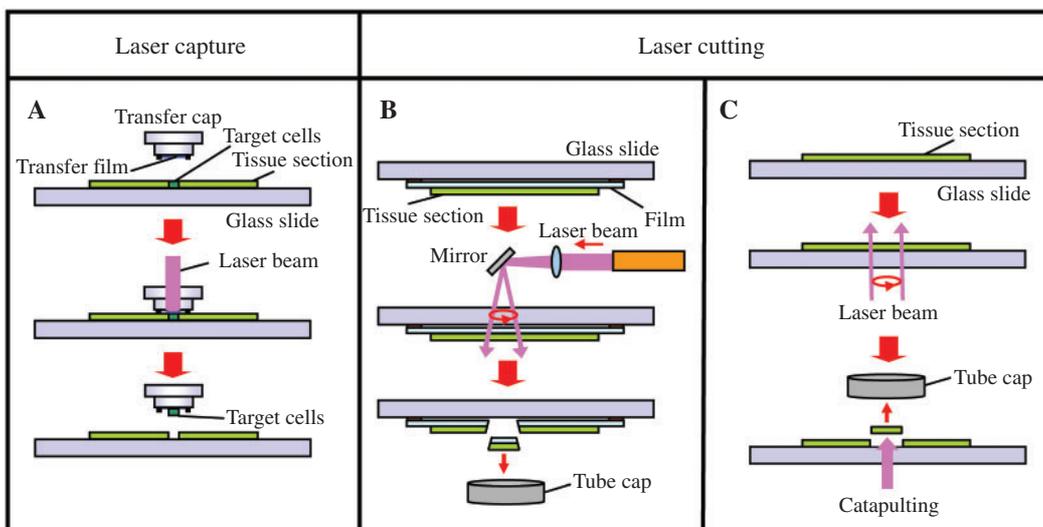
Fig. 1 Several methods for isolating plant target cells using LM systems such as laser capture (A) or laser cutting (B, C). In laser capture [i.e. Laser Capture Microdissection (LCM)], which is licensed to Arcturus Bioscience (now Molecular Devices, Mountain View, CA, USA), cells of interest from the tissue sections are captured on a transfer film with the aid of a near-infrared laser (A). In laser cutting, a UV laser is used to cut target cells from tissue sections. The methods for collecting the cut cells are different between different LM instruments. For example, in the AS LMD system (Leica Microsystems, Wetzlar, Germany), samples fall with gravity and are collected into a collection tube (B), and in the Laser Microdissection and Pressure Catapulting (LMPC) system (P.A.L.M. Microlaser Technologies, Bernreid, Germany), samples are catapulted into a collection tube with the pressure of a laser beam (C).
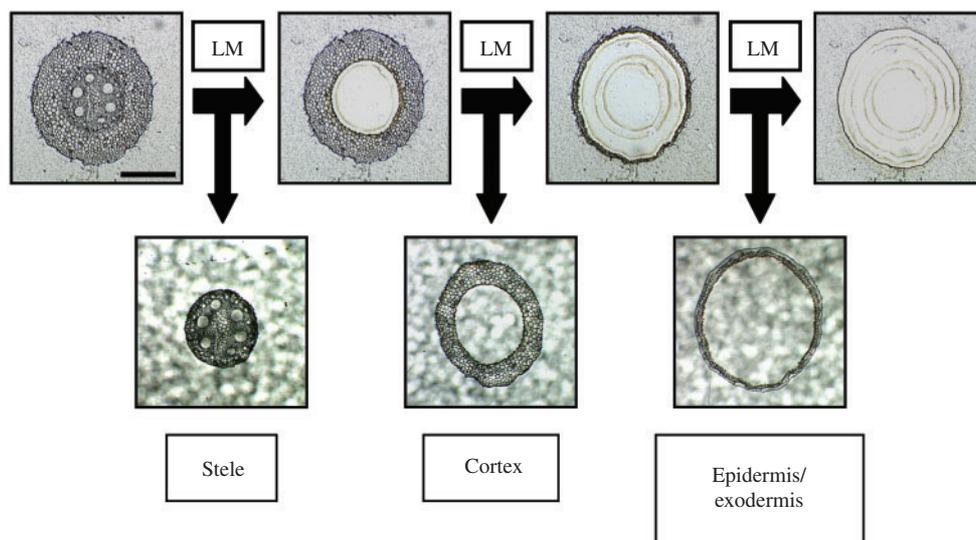
Fig. 2 Laser microdissection of plant cells from a cross-section of maize root. The section was prepared from a seminal root of a 3-day-old seedling that was fixed in Farmer's fixative and embedded in paraffin. The tissue was laser cut with a Leica AS LMD system. The first cut removed the stele, the second cut removed the cortex and the third cut removed the epidermis and exodermis, all shown in RNA extraction buffer. Scale bar, 500 μm.

of micropipetting, FAS and LM for isolating specific plant cells are given in other reviews (Kehr 2001, Brandt 2005, Lange 2005, Lee et al. 2005, Galbraith and Birnbaum 2006).

To examine the unique functions of individual plant cell types, it is necessary to perform global analyses of their gene expressions, metabolites and other functional molecules. Hence, the technologies for isolating specific types of plant cells when combined with high-throughput technologies such as microarray and proteome analyses can be a powerful means for understanding the functions of these cells. In this review, we outline recent progress in applications of LM for plant cells combined with

high-throughput technologies. Most of the progress has been achieved in transcript profiling analyses because linear RNA amplification methods [e.g. T7 RNA polymerase-based (T7-based) RNA amplification (Eberwine et al. 1992)] have made it possible to analyze very small quantities of RNA. Indeed, picogram to nanogram quantities of RNA samples isolated from LM-collected plant cells have been successfully amplified in a highly repeatable manner (Nakazono et al. 2003) and used for subsequent analyses (Kerk et al. 2003, Nakazono et al. 2003).

High-throughput transcript profiling in plants via LM

'Closed' platform-mediated approaches—microarrays

Microarray technology makes it possible to detect transcript accumulation levels of thousands of genes in a single experiment (Richmond and Somerville 2000). Nakazono et al. (2003) conducted a microarray analysis using maize epidermal cells and vascular tissues that were collected from maize coleoptiles via LM. RNA was extracted from these LM-collected tissues, linearly amplified and used for cDNA microarray hybridizations. Approximately 250 (~3%) of the cDNAs analyzed were expressed preferentially in epidermal cells or vascular tissues. Subsequently, two more studies used the LM–microarray approach in plants. Casson et al. (2005) used a cDNA microarray to analyze transcript profiles of the apical and basal domains of LM-collected *Arabidopsis* embryos at two stages. Based on their signal intensity cut-off, ~65% of the genes on the array were expressed in the developing *Arabidopsis* embryos. The tissue-specific expression of some genes was validated with promoter–β-glucuronidase (GUS) analyses and mutational analyses. Woll et al. (2005) used an LM–microarray approach to characterize the maize lateral and seminal root initiation mutant, *rootless with undetectable meristems 1* (*rum1*). In this analysis, primary root pericycle tissues were collected via LM from both the wild-type and the mutant plants. A subsequent cDNA microarray analysis revealed that 90 genes were preferentially expressed in the wild type and 73 genes were preferentially expressed in the mutant pericycle tissues. A total of 19 of these genes were predicted to encode regulatory proteins involved in signal transduction, transcription and the cell cycle. This indicates that the LM–microarray approach is feasible for identifying regulatory networks affected by gene mutations in specific cells or tissues where the genes are functioning.

Other LM–microarray studies are in progress. Ohtsu et al. are using an LM–microarray approach to analyze global gene expression in the shoot apical meristem (SAM) of maize. Thousands of genes preferentially expressed in the SAM were identified using 37K maize cDNA microarrays. It is expected that functional analyses of these genes will
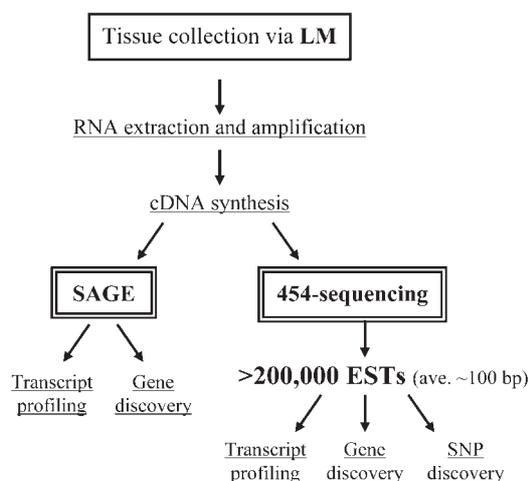
Fig. 3 Applications of SAGE and 454-sequencing combined with LM. cDNAs derived from LM-collected plant tissues are used for the SAGE or the 454-sequencing. In 454-sequencing, the cDNAs can yield >200,000 ESTs (~100 bp average length) without any subcloning steps. The resulting ESTs can be used for various types of analyses, including transcript profiling, gene discovery and SNP discovery.

enhance understanding of the SAM regulatory network. Another LM–microarray project in progress (described by Nelson et al. 2006) is the creation of an atlas of the transcript profiles of 135 different rice cell types using an oligo microarray.

'Open' platform-mediated approaches—serial analysis of gene expression (SAGE) and 454-sequencing

Microarrays are considered a 'closed' platform because only the genes spotted on the arrays can be analyzed. In contrast, 'open' platforms, such as serial analysis of gene expression (SAGE) and 454-sequencing of cDNAs, can give transcript profiles without prior knowledge of the genes to be identified and thus enable the discovery of new expressed genes.

SAGE allows comparative and quantitative expression analyses of thousands of genes by producing non-normalized short expressed sequence tags (ESTs) of 13–14 bp (Velculescu et al. 1995), 21 bp (longSAGE; Saha et al. 2002) and 26 bp (SuperSAGE; Matsumura et al. 2003, Matsumura et al. 2005). SAGE libraries have been generated from small amounts of RNA via T7-based RNA amplification in animals (Vilain et al. 2003, Heidenblut et al. 2004). This indicates that the SAGE method is a good alternative to transcriptome analyses of LM-collected plant cells, especially for non-model plants (Fig. 3). The SuperSAGE method has advantages over other SAGE methods. SuperSAGE tags (26 bp) are long enough to design primers for the 5'- or 3'-rapid amplification of cDNA ends. SuperSAGE was also combined with

microarray technology, which was called SuperSAGE array (Matsumura et al. 2006). The SuperSAGE array has 26 bp oligonucleotides directly synthesized onto the slide, which correspond to SuperSAGE tags. The SuperSAGE method is feasible to use for LM-collected plant cells such as rice epidermal cells interacting with a fungal pathogen, *Magnaporthe grisea* (Matsumura et al. 2005). In fact, a SuperSAGE library was generated from LM-collected rice cells involved in the programmed cell death of the coleoptile (M. Nakazono et al. in preparation).

454-sequencing technology is a recently developed highly parallel sequencing method (Margulies et al. 2005). This system sequences 25 million bases in one 4 h run, which is 100 times faster than the standard sequencing methods. Emrich et al. (in press) used this method to obtain >260,000 ESTs (454-SAM ESTs, ~100 bp average length) from cDNA derived from LM-collected maize SAMs (Fig. 3). Approximately 30% of the 454-SAM ESTs did not match any of the >648,000 existing maize ESTs. Despite their short lengths, the 454-SAM ESTs annotated >25,000 maize genomic sequences, >15,000 of which did not have prior evidence of expression. These results demonstrate that the combination of LM and deep sequencing enriches for rare and/or tissue-specific transcripts and demonstrate the great advantage of 'open' platform-mediated transcript profiling.

More recently, thousands of putative single nucleotide polymorphisms (SNPs) were identified by aligning 454-SAM ESTs from the B73 and Mo17 inbreds (W.B. Barbazuk et al. in preparation). Over 85% of a sample of these putative SNPs could be validated and converted into accurate, high-throughput genetic markers. These results demonstrate that the combination of LM and 454-based transcriptome sequencing is an efficient method by which to generate gene-associated SNPs.

Other high-throughput technologies combined with LM

Plant development and responses to various stimuli are regulated at multiple levels such as the transcript, protein and metabolite levels. To understand the biological processes in individual cell types, it is necessary to perform global analyses of proteins and metabolites as well as transcript profiling. It is technically challenging to prepare a sufficient amount and quality of proteins or metabolites from LM-collected tissues for proteome or metabolome analyses. However, by modifying sample preparation methods, Kehr and co-workers (Schad et al. 2005a, Schad et al. 2005b) were able to use LM to collect ~20,000 cells from the vascular bundles of *Arabidopsis* stems for protein analysis and ~5,000 cells for metabolite analysis. They were able to identify the proteins in these samples by high-efficiency liquid chromatography in conjunction with tandem mass spectrometry (LC–MS/MS)

(Schad et al. 2005a) and to identify the metabolites by gas chromatography-time of flight mass spectrometry (GC–TOF MS) (Schad et al. 2005b). Although further technical optimization is required to apply these methods to a wider range of plant cell types, these two studies have opened a path to cell type-specific protein and metabolite profiling of plants.

Perspectives

The combination of LM and high-throughput technologies is a promising approach to understand molecular events in individual plant cell types. Nonetheless, there have been only a limited number of reports so far in which this approach was used for plants. One possible reason is that sample preparation from some plant tissues can be technically challenging. Inada and Wildermuth (2005) reported a new microwave paraffin preparation method that was used for LM collection of *Arabidopsis* leaf tissue. This method resulted in higher integrity of RNA than the conventional paraffin preparation method (N. Inada, personal communication). Further optimizations of the sample preparation will accelerate the use of the LM–high-throughput technology approach for plants.

To understand the diverse biological processes in plants, it is necessary to integrate information about transcript, protein and metabolite profiles in individual cell types to construct a 'map' of the cellular systems or the cell-to-cell networks. The first step is to accumulate data about individual cell types, as is being done by the rice cell-type atlas project (Nelson et al. 2006). After sufficient transcript, protein and metabolite data are accumulated for a particular cell type, a reasonable choice for the next step would be to conduct functional analyses of the cells using methods such as the reverse genetic approach. High-resolution comparisons of individual cell types in mutants and the wild type at the transcript (Woll et al. 2005), protein and metabolite levels will provide important clues about the regulatory networks of these cell types or among different cell types. In the case of reverse genetics, it is particularly important to determine which genes are the best to knock out. For example, in maize, nearly 400 454-SAM ESTs were orphans (Fu et al. 2005, Emrich et al. in press). These would be excellent candidates for reverse genetic analyses to study regulatory networks in the SAM. Another important regulatory factor in plants is the epigenetic status of chromatin. Lippman et al. (2004) determined the epigenetic status of a 1.5 Mb region containing the heterochromatic knob (*hk4S*) on *Arabidopsis* chromosome 4 using a ChIP-chip assay. A combination of LM and the ChIP-chip assay would help to integrate

information to understand better the regulatory networks involved in plant development.

Acknowledgments

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