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# Functional Genomics and Transcriptomics in *Musa*

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## ABSTRACT

Genome analysis of multicellular organisms reveal a large number of genes for which no function is known or can be predicted. Many genetic tools are now available for investigating gene function. In general they are divided into two broad categories-forward and reverse genetics. This chapter reviews some of the techniques for gene functional analysis with special emphasis on insertional mutagenesis, targeted gene disruption by homologous recombination, RNA interference, virus-induced gene silencing (VIGS) technology, and Targeting Induced Local Lesions IN Genomes (TILLING). In addition progress in some areas of transcriptomics research in *Musa* is reviewed. Insertional mutagenesis has been widely used for cloning genes, promoters, enhancers and other regulatory sequences in the model plant *Arabidopsis*. Insertional mutagenesis provides a direct route to determining function. Most other approaches are correlative and do not necessarily prove a causal relationship between gene sequence and function. Gene targeting by homologous recombination, although cumbersome, is now feasible in rice. RNA interference (RNAi) is based on sequence-specific RNA degradation that follows the formation of double-stranded RNA (dsRNA) homologous in sequence to the targeted gene. RNAi

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*List of abbreviations after the text.*

allows silencing one, several, or all members of a multigene family or homoeologous gene copies in polyploids. Virus-induced gene silencing (VIGS) is a powerful and rapid technique for analysis of gene function in plant growth and development. To overcome the limitations of knocking out an entire gene and to expand knowledge of active gene mutations, a new process called Targeting Induced Local Lesions in Genomes or TILLING was developed. TILLING uses chemical mutagenesis to yield a traditional allelic series of point mutations for virtually all genes. Serial Analysis of Gene Expression (SAGE) is based on the massive sequential analysis of short cDNA sequence tags. The chapter also summarizes the role of Bacterial Artificial chromosomes (BACs), Expressed Sequence Tags (EST) libraries and Microarray technology in the functional genomics of *Musa*. Finally recent studies on comparative functional genomics to study drought stress in *Musa* is also addressed.

**Keywords**: gene knockout, insertional mutagenesis, RNA interference, TILLING, transcriptomics

## 9.1 Introduction

One of the most significant findings revealed by analysis of genomes of multicellular organisms is the large number of genes for which no function is known or can be predicted (Alonso et al. 2003). Functional genomics is a relatively new technology that uses high throughput methods to analyze a large number of genes at a time. It studies the function of individual or groups of genes. Functional genomics allows researchers to gain an understanding of the control of genes during normal growth and development, and in response to environmental stresses. Researchers can further investigate the complex interactions that occur in genetic and cellular networks and ideally, the technology will allow the entire genetic make-up of an organism to be studied.

Many genetic tools are now available for investigating gene function. In general, they are divided into two broad categories—forward and reverse genetics. Forward genetics refers to a process where studies are initiated to determine the genetics underpinnings of observable phenotypic variation (Tierney and Lamour 2005). In many cases, the observable variation has been induced using a DNA damaging agent (mutagen) but also may be naturally occurring. The investigator eventually ends up sequencing the gene or genes thought to be involved. Instead of going from phenotype to sequence as in forward genetics, reverse genetics works in the opposite direction—a gene sequence is known, but its exact function is uncertain. In reverse genetics, a specific gene or gene product is disrupted or modified and then the phenotype is measured (Tierney and Lamour 2005).

A number of strategies are now available to disrupt or alter a gene and observe its phenotypic effect on an organism. These include methods that target genes specifically or those that bring about non-targeted random disruptions and include:

(i) Targeted gene disruption (Oliver 1996; Kempin et al. 1997). Although this method has been used in *Arabidopsis*, it is laborious, involving the generation of hundreds or thousands of transgenic plants for every gene assayed (Kempin et al. 1997).
(ii) Gene silencing via sense or antisense suppression has been a popular method in the past (Baulcombe 1999). However, this method also requires that several independent transgenic lines to be generated for every gene.
(iii) Chemical, radiation, and transposon mutagenesis is regarded as the most versatile method for assessing gene function. Transposon and T-DNA [portion of the Ti (tumor-inducing) plasmid that is transferred to plant cells] insertions offer the additional advantage of tagging the target gene molecularly and in many cases genetically via reporter and selectable marker genes carried by the insertion (Martienssen 1998).

Transcriptomics, which is the high-throughput analysis of differential gene expression is also a powerful tool for discovering novel genes or for gaining additional information about certain biological processes on a genomic scale (Mochida and Shinozaki 2010). Genome-wide expression analysis of almost all genes has become possible, even though the profiling techniques are technically rather demanding and expensive, e.g., serial analysis of gene expression (SAGE) (Velculescu et al. 1995) and massively parallel signature sequencing (MPSS) (Brenner et al. 2000).

This chapter reviews some of the techniques for gene functional analysis with special emphasis on insertional mutagenesis, targeted gene disruption by homologous recombination, RNA interference, virus-induced gene silencing (VIGS) technology, and targeting induced local lesions in genomes (TILLING). In addition progress in some areas of transcriptomics research in *Musa* is reviewed.

## 9.2 Insertional Mutagenesis and Gene Functional Analysis

Gene suppression (deletion/mutation knockout) or over-expression permits the gene sequence to be linked to a phenotype from which the function of the gene can be deduced (Matzke and Matzke 1995).

Gene disruption is a powerful way to obtain knockout mutants that help in ascertaining biological function of the numerous uncharacterized open reading frames (ORFs) from genome sequences or from expressed sequence tags (Radhamony et al. 2005). Insertional mutagenesis has been extensively

used for cloning genes, promoters, enhancers and other regulatory sequences from *Arabidopsis* (Radhamony et al. 2005). Gene knockout systems provide a direct route to determining function. Most other approaches to dissect gene function are correlative and do not necessarily prove a causal relationship between gene sequence and function. For example, DNA chips provide a means to discover conditions under which gene expression is regulated on a genome wide scale (Becker et al. 2003; Birnbaum et al. 2003).

However, factors other than mRNA level may also determine the activity of a gene product *in situ*. Therefore, expression studies cannot prove a causal relationship. By contrast, the availability of a null mutation for the gene of interest allows one to directly monitor the effect of this deficiency on the organism's ability to function (Radhamony et al. 2005). Although methods such as gene replacement through homologous recombination work well in yeast and mice, this technique has not been easy for targeted insertion in plants. However, efficient gene targeting by homologous recombination has been reported in rice (Terada et al. 2002; Yang et al. 2010; Yu et al. 2010).

The use of insertional mutagenesis, in principle, provides a more rapid way to clone a mutated gene. DNA elements that are able to insert at random within chromosomes such as transposons (Sundaresan et al. 1995; Martienssen 1998) or the T-DNA of *Agrobacterium tumefacians* (Azpiroz-Leehan and Feldmann 1997), can be used as mutagens to create loss of function mutations in plants. Transposons are mobile genetic elements that can relocate from one genomic location to another (Hayes 2003). They are DNA sequences that can insert themselves at a new location in the genome without having any sequence relationship with the target locus (Lewin 2004). Transposon-based signature-tagged mutagenesis has been successful in identifying essential genes as well as genes involved in infectivity of a variety of pathogens. Strategies for insertional mutagenesis using transposons have been developed for a number of animal and plant models (Hayes 2003). Transposon-based methods have been used in *Arabidopsis*, maize and other plants (Stemple 2004).

In T-DNA insertional mutagenesis, use is made of the segment of the Ti plasmid of *A. tumefaciens* known as T-DNA that carries genes to transform the plant cell has also been utilized for this purpose. Since the sequence of the inserted element is known, the gene in which the insertion has occurred can be recovered, using various cloning or PCR-based strategies. One drawback of insertional mutagenesis is the low frequency of mutations, necessitating the screening of large numbers of individuals to find mutations in any given gene (Gilchrist and Haughn 2005). Insertions in essential genes will usually cause lethality, and less severe mutations must be generated in these genes in order to understand gene function (Till et al. 2003).

An advantage of using T-DNA as the insertional mutagen as compared to transposons is that the T-DNA insertions do not transpose subsequent to insertion and are chemically and physically stable through multiple generations (Radhamony et al. 2005). The T-DNA not only disrupts the expression of the gene into which it is inserted, but also acts as a marker for subsequent identification of the mutation. It is important to establish linkage between the mutant phenotype and the insertion element. Even if some linkage can be established, this does not exclude the possibility that the phenotype is caused by a nearby but independent mutation. Consequently it is necessary to identify the gene disrupted by the insertion and test for complementation of the mutant phenotype by the wild type allele in transgenic plants.

A highly efficient procedure for obtaining mutants in genes identified in sequencing programs takes advantage of the availability of large collections of plants mutagenized by an insertion element.

Mutational approaches have been successfully used for the study of genetic and molecular bases for many traits in plant biology. Access to the mutation is obtained using positional cloning strategies. This strategy is facilitated in model species such as *Arabidopsis*, for which dense genetic maps with many visible and molecular genetic markers exist, and for which the complete physical map consisting of a collection of overlapping cloned DNA fragments and total genome sequence are already available. The limiting factors for this approach are the time and effort required for creating the mapping population and the fine mapping of the mutant locus.

Besides the model plant *Arabidopsis*, insertional mutagenesis for gene functional analysis has been applied to other plants such as rice, tomato and *Brassica*. The maize Ac/Ds transposon system has been shown to be efficient for gene tagging and functional analysis in rice (Hiroyuki et al. 1999). T-DNA tagging has also been used successfully for gene discovery in rice. In tomato new tools for functional analysis based on insertional mutagenesis, with Ac/Ds system in the background of the miniature cultivar Micro-Tom has been established (Meissner et al. 2000). In addition, promoter trapping using firefly luciferase reporter gene and enhancer trapping using GUS was also developed. T-DNA tagging strategy for isolation of new promoters has been tried in *Brassica napus* employing hypocotyl transformation with a promoterless *gus*: *npt*II tagging construct.

The use of mutagenesis to find and study plant genes is increasingly being used in functional genomics. Thus T-DNA tagging in conjunction with other mutation based techniques like transposon insertion, TILLING, etc., would not only continue to provide useful information in *Arabidopsis* but is likely to prove an efficient tool for functional genomics in other plants also. Genome sequences of banana are rapidly becoming available (Cheung and Town 2007) and these techniques may be used for its gene functional analysis

by these methods. Santos et al. (2009) conducted a genome-wide T-DNA tagging strategy for the identification and characterization of novel banana promoters. The method proved valuable for the identification of novel promoters and genes in banana and for monitoring expression patterns throughout *in vitro* development and low temperature treatment.

## 9.3 Targeted Gene Disruption by Homologous Recombination

Homologous recombination is a reciprocal exchange of DNA sequences, as in between two chromosomes that carry the same genetic loci (Lewin 2004). Just as homologous recombination has been found to be mainly initiated with a double-strand break, gene targeting by homologous recombination is associated with the repair of double strand breaks. The double-strand break repair and synthesis-dependent strand-annealing models are the most generally accepted models to explain gene targeting (Iida and Terada 2004).

A reverse-genetic system using homologous recombination has recently been developed for *Drosophila*. It is promising, but is a lengthy procedure and requires generation of specific transgenic flies (Stemple 2004). Reproducible gene targeting by homologous recombination is now also feasible in rice. With the combination of site-specific recombination systems (such as Cre-lox), the future of gene targeting by homologous recombination as a routine procedure for engineering the genome of rice and presumably other plants is bright (Iida and Terada 2004).

## 9.4 Gene Silencing by RNA Interference

In contrast to insertional mutagenesis, RNA interference (RNAi) is based on sequence-specific RNA degradation that follows the formation of double-stranded RNA (dsRNA) homologous in sequence to the targeted gene (Marx 2000; Baulcombe 2004). RNAi allows silencing one, several, or all members of a multigene family or homoeologous gene copies in polyploids by targeting sequences that are unique or shared by several genes (Lawrence and Pikaard 2003; Miki et al. 2005) and may be useful in banana, which is a polyploid crop.

RNA interference (RNAi) is the process by which expression of a target gene is inhibited by antisense and sense RNAs. It works based on the ability of double-stranded sequences to recognize and degrade sequences that are complementary to them (Lewin 2004). RNA interference has recently become a powerful tool to silence the expression of genes and analyze their loss-of-function phenotype, allowing analysis of gene function when mutant alleles are not available.

RNAi has already become a useful method for gene targeting in fungi (Nakayashiki 2005), viruses (Baulcombe 2004; Wani et al. 2010), bacteria (Escobar et al. 2001) and plants (Brodersen and Voinnet 2006) as it allows the study of the function of hundreds of thousands of genes to be tested (Godge et al. 2008). It can silence a gene throughout an organism or in specific tissues (Islam et al. 2005), offer the versatility to partially silence or completely turn off genes, work in both cultured cells and whole organisms and can selectively silence genes at particular stages of the organism's life cycle (Milhavet et al. 2003). One interesting feature of RNA silencing in plants is that once it is triggered in a certain cell, a mobile signal is produced and spreads through the whole plant causing the entire plant to be silenced (Dunoyer et al. 2007).

The greatest difficulty in using RNAi as a reverse genetic technique in plants is that throughput is limited by the ability to deliver siRNAs to target loci (Henikoff et al. 2004). A number of methods for delivering double stranded (dsRNA) or siRNA into different cells and tissue has been tested and includes:

(i) transformation with dsRNA forming vectors for selected gene(s) by an *Agrobacterium*- mediated transformation (Waterhouse et al. 2001; Chuang and Meyerowtiz 2000);
(ii) delivery of cognate dsRNA of *uidA* GUS (β-glucuronidase) and TaGLP2a: GFP (green fluorescent protein) reporter genes into single epidermal cells by particle bombardment (Schweizer et al. 2000);
(iii) introducing a vector in plants by infiltration (Liu et al. 2002a);
(iv) delivery of dsRNA into suspension cells by cationic oligopeptide polyarginine-siRNA complex;
(v) infecting plants with viral vectors that produce dsRNA (Dalmay et al. 2000);
(vi) delivery of siRNA into cultured plant cells; and
(vi) gene silencing by nanosense pulsed laser-induced stress wave (LISW) (Tang et al. 2006).

RNA-mediated gene silencing (RNA silencing) has been used as a tool for gene targeting in fungi. Homology based gene silencing induced by transgenes (co-suppression), antisense, or dsRNA has been demonstrated in many plant pathogenic fungi, including *Cladosporium fulvum* (Hamada and Spanu 1998), *Magnaporthae oryzae* (Kadotani et al. 2003; Kim et al. 2009; Chen et al. 2010), *Venturia inaequalis* (Fitzgerald et al. 2004), *Neurospora crassa* (Goldoni et al. 2004), *Aspergillus nidulans* (Hammond and Keller 2005), and *Fusarium graminearum* (Nakayashiki 2005) whether it is suitable for large scale mutagenesis in fungal pathogens remains to be tested.

Antiviral RNAi technology has been used for viral disease management in human cell lines (Bitko and Barik 2001; Gitlin et al. 2002; Jacque et al.

2002; Novina et al. 2002). Such silencing mechanisms (RNAi) have been exploited to protect and manage viral infections in plants (Waterhouse et al. 2001; Ullu et al. 2002). The effectiveness of the technology in generating virus resistant plants was demonstrated in potato (Waterhouse et al. 1998), black gram (Pooggin et al. 2003), cassava (Vanitharani et al. 2003), and tobacco (Qu et al. 2007).

The origin of RNAi technology from the classical studies with the nematode *C. elegans* has shown ways and means to explore the possibilities of this mechanism for protecting plants from nematode damage (Wani et al. 2010). Two approaches have been advocated. One that relies on targeting plant genes that are involved with the infection process and the other targets the essential genes within the nematode. RNAi can be induced in *C. elegans* by feeding it dsRNA, hence it was reasoned that expressing hpRNAs containing sequences of vital nematode genes in the host plant might deliver dsRNA to a feeding nematode to incapacitate or kill it (Wani et al. 2010).

RNAi-mediated suppression of a gene plays an indispensable role in hampering the nematode development and may adversely affect the progression of pathogenesis in direct or indirect ways. There are accumulating evidences for the efficacy of RNAi in plant parasitic nematode management and a wide range of genes have been targeted for silencing in cyst and rootknot nematode species.

Ongoing research demonstrates the relevance of the targeted parasitism genes during the nematode life cycle and more importantly, suggest that a viable level of resistance in crop plants may be accomplished in the future by using RNAi technology against cyst nematodes.

RNAi and miRNA technologies of gene silencing are newly developed genomics tools that have great advantages over antisense and co-suppression due to their higher silencing efficiency and shorter time requirements for screening (Wani et al. 2010).

Future directions will focus on developing finely tuned RNAi-based gene silencing vectors that are able to operate in a temporally and spatially controlled manner. However, a better and comprehensive understanding of RNAi would allow researchers to work effectively and efficiently in order to improve crop plants nutritionally and manage various intruders of crop plants (Wani et al. 2010).

## 9.5 Virus-induced Gene Silencing (VIGS): A Tool for Plant Functional Genomics

Virus-induced gene silencing is a powerful and rapid technique for analysis of gene function in plant growth and development (Baulcombe 1999). VIGS is a RNA-mediated post-transcriptional gene silencing mechanism

that protects plants against foreign gene invasion. In addition to allowing a better understanding of how plants defend themselves against plant viruses, VIGS is also an extremely powerful functional genomics tool for knocking out gene expression of target plant genes in some plants. If the virus is used as a vector incorporating sequence from the host plant, the enzymatic pathway induced will silence the host gene (gene knock-out). Numerous methods have been employed to initiate this homology-based RNA degradation process, but all rely on the activity of double-stranded RNAs (dsRNAs) corresponding to the gene of interest. It involves creation of engineered viruses carrying sequences corresponding to the host gene to be silenced. dsRNA can be introduced into plants either as stable hairpin-RNA-synthesizing transgenes or by infection with recombinant viruses that carry segments of plant genes (Baulcombe 1999; Wang and Waterhouse 2002). This was first noticed when infection of *Nicotiana benthamiana* with tobacco rattle virus (TRV) containing a fragment of the gene encoding phytoene desaturase, an essential enzyme in carotene synthesis, resulted in a photo-bleached phenotype in photosynthetic tissues. Although many plant viruses have been used to develop VIGS vectors (Ruiz et al. 1998; Ratcliff et al. 2001; Gossele et al. 2002; Turnage et al. 2002), the TRV-based vectors have been suggested to have potential for use with a wide range of plant species due to the wide host range of TRV (Ratcliff et al. 2001). TRV provides the most robust results in terms of efficiency, ease of application, and absence of disease symptoms.

VIGS offers several advantages over existing functional genomics approaches because it does not require genetic transformation to silence a target plant gene, and phenotypes can be identified within a few weeks of virus inoculation. It is possible to silence multiple classes of closely related genes, as well as genes where true knock-outs are embryo-lethal. It is also more amenable to high-throughput studies and has been used successfully in model and non-model host systems (Liu et al. 2002c; Lu et al. 2003; Burch-Smith et al. 2004, 2006; Constantin et al. 2004; Fofana et al. 2004; Ding et al. 2006).

To date there are no research reports available for VIGS in banana and plantain. Renner et al. (2009) demonstrated that barley stripe mosaic virus (BSMV) can infect two species within the Zingiberaceae, and that BSMV–VIGS can be applied to specifically down-regulate phytoene desaturase in ginger *Zingiber officinale.* These results suggest that extension of BSMV–VIGS to other monocots such as banana many be possible in the future.

## 9.6 Tilling and Ecotilling

Traditional reverse genetic methods, such as the use of transposons to "knock out" a specific gene, can accurately determine phenotype but require

time consuming transgenic or sophisticated tissue culture methodologies (Colbert et al. 2001). Such "knockout" methods are limiting because the entire gene is knocked out—the effects of partial loss of function of an active gene cannot be observed.

To overcome the limitations of knocking out an entire gene and to expand knowledge of active gene mutations, a new process called targeting induced local lesions in genomes or TILLING was developed (McCallum et al. 2000). TILLING uses chemical mutagenesis to yield a traditional allelic series of point mutations for virtually all genes. The TILLING process is of particular value for essential genes where sublethal alleles are required for phenotypic analysis. Furthermore, because TILLING does not involve transgenic modifications, it is attractive not only for functional genomics but also for agricultural applications (Henikof et al. 2004). The value of TILLING for genetic research is enhanced by its proven viability for a rapidly growing range of organisms including *Drosophila, Arabidopsis,* zebrafish, *maize, Medicago truncatula* and others. A full description of TILLING for *Arabidopsis* is described in Henikof et al. (2004).

The high densities of EMS (ethylmethane sulfonate) mutagenesis in TILLING raise the concern about background mutations being mistaken for mutations in target genes during phenotypic analysis. However, EMS-generated mutations at densities comparable to those in TILLING lines continue to be a basic learning tool for genetics, where background mutations have not been a problem. On the one hand, mutations in genes expected to impact a phenotypic trait controlled by many genes, such as plant height or size or leaf shape, may be subject to epistatic interactions, and outcrossing to the wild type may be necessary. On the other hand, mutations in genes expected to impact a phenotype that is controlled by few genes are unlikely to produce phenotypes perturbed by background mutations, and outcrossing is not a prerequisite for analysis (Henikoff and Comai 2003).

Ecotilling an adaptation of TILLING (Colbert et al. 2001; Comai et al. 2004) is a high-throughput method for the discovery and characterization of single nucleotide polymorphisms (SNPs) and small insertions/deletions (indels). It has been shown to be an accurate, lowcost and high-throughput method for the discovery and evaluation of nucleotide diversity in humans, plants and other organisms (Gilchrist et al. 2006; Till et al. 2006a, b; Nieto et al. 2007; Weil 2009).

Till et al. (2008) were the first to apply TILLING techniques in banana. EMS mutagenized populations of the Grand Naine variety are being developed. An evaluation of utilizing gamma irradiation for TILLING different varieties of banana including "Calcutta 4" is being investigated. Mutagenized populations of diploid "Calcutta 4" AA for TILLING are currently being developed at INIVIT in Cuba as part of an FAO/IAEA

Coordinated Research Project, where mutagenesis of cell cultures is also being investigated (Jain et al. 2011).

Till et al. (2010) used EcoTILLING to identify polymorphisms in the *Musa* genepool. They discovered over 800 novel alleles in 80 *Musa* accessions. In addition two single nucleotide polymorphisms that may be deleterious for the function of a gene putatively important for phototropism were identified. Evaluation of heterozygous polymorphism and haplotype blocks revealed a high level of nucleotide diversity in *Musa* accessions. Till et al. (2010) applied this strategy for the simultaneous discovery of heterozygous and homozygous polymorphisms in diploid accessions to rapidly evaluate nucleotide diversity in accessions of the same genome type. They also concluded that Ecotilling is suitable for diversity studies in *Musa*, that it can be considered for functional genomics studies and as a tool in selecting germplasm for traditional and mutation breeding approaches. Many challenges arise when TILLING is considered for banana. Widely consumed triploid varieties are largely sterile, infertile and/or parthenocarpic, thus requiring vegetative propagation, making seed mutagenesis impractical. Mutation induction using *in vitro* material can therefore be considered. Embryogenic cell suspensions are now available in banana (Strosse et al. 2003), although the technique appears to be cultivar specific. The problem of chimerism can be reduced through successive rounds of meristem isolation, followed by cutting meristems and allowing plantlets to regenerate (Roux 2004). The development of mutagenized populations for reverse genetic screens, and the optimization of TILLING and EcoTILLING in banana, will become useful resources for *Musa* research.

## 9.7 Functional Genomics Tools for Banana Improvement

Functional genomics and systems biology research are facilitating the identification of gene networks that are involved in controlling genetic variation for agronomically valuable traits (Varshney and Tuberosa 2007). Furthermore, combining new knowledge from functional genomic research with conventional breeding methods is essential for enhancing response to banana improvement. Currently, a number of functional genomics tools have used in banana.

In this section Serial analysis of gene expression (SAGE), massively parallel signature sequencing (MPSS), bacterial artificial chromosomes (BACs) expressed sequence tags (ESTs), and microarrays and their possible use for banana improvement is presented.

### *9.7.1 Serial Analysis of Gene Expression (SAGE)*

Serial analysis of gene expression (SAGE) is based on the massive sequential analysis of short cDNA sequence tags. Each tag is derived from a defined position within a transcript. Its size (14 bp) is sufficient to identify the corresponding gene and the number of times each tag is observed provides an accurate measurement of its expression level. SAGE allows an accurate quantitative analysis of gene expression for thousands of genes at a time (Velculescu et al. 1995). SAGE is also a powerful tool for the identification of novel candidate genes and the characterization of specific metabolic or regulatory pathways (Coemans et al. 2005). SAGE in plants has mainly been limited to model organisms such as *Arabidopsis* and rice since the annotation of the tags depends mainly on the availability of cDNA libraries or EST collections (Matsumura et al. 1999, 2003a,b; Fizames et al. 2004). Various ways to increase the tag length, thereby enhancing annotation frequency has been made. These improvements include modified SAGE (18-bp tag; Ryo et al. 2000), Long SAGE (21-bp tag; Saha et al. 2002) and SuperSAGE (26-bp tag; Matsumura et al. 2003a). In banana and plantain with limited or no genomic DNA and cDNA/EST sequences available, classical SAGE yielding short tags would not be practical due to the very low chance and reliability of annotating the sequenced tags. However, non-model plant species possess numerous important traits not available for study in model plants, which emphasizes the need for high-throughput transcript profiling generally applicable to all crop plants. These traits may include different organs (e.g., fleshy fruits), special developmental processes (e.g., apomixis or parthenocarpy) and distinct quality traits such as flavor, nutrient or medicinal substances. Certain plant–pathogen interactions with serious economical consequences may also justify a large-scale functional analysis in the target organisms directly.

In banana, leaf material from the wild diploid banana *Musa acuminata* was used to generate a SuperSAGE library (Coemans et al. 2005). A total of 10,196 SuperSAGE tags were analyzed, which corresponded to 5,292 expressed genes. Although the most abundant tag was represented more than 300 times, which is close to 3% of the total transcripts, the vast majority of transcripts occurred only once. The number of unique tags steeply decreased with increasing tag copy number. For example, 4,409 tags, which correspond to 83.3% of all unique tags, occurred once and constituted 43.2% of the transcripts present in the analyzed tissue. In contrast, only 214 tags, representative of 2.1% of all unique tags, had a tag copy number ≥5. These 214 tags, however, represented 40.9% of the transcript pool analyzed

(Coemans et al. 2005). These findings are similar to a SuperSAGE study in rice leaves, where the sequencing of 12,119 tags identified 7,546 (62.3%) as being unique (Matsumura et al. 2003b). Furthermore, the 26-bp SuperSAGE tags were successfully applied as primers in 3′RACE, thus allowing the identification of unknown transcripts. Hence, SuperSAGE combined with 3′RACE and TAIL-PCR provides a powerful tool for functional genomics in non-model organisms for which supporting sequence resources are less extensive (Coemans et al. 2005).

Further studies by Sagi et al. (2005) reported SuperSAGE libraries with more than 5,000 unique 26-bp long tags generated from banana leaves, allowing a quantitative analysis of the transcriptome. Besides the identification of extremely low-abundance transcripts putative differentially expressed genes were identified after infection with *Mycosphaerella fijiensis*, the most important fungal pathogen of banana.

Extension of both types of tags to full-length genes is in progress and the functional analysis of these genes will contribute to the understanding of the banana genome.

### *9.7.2 Massively Parallel Signature Sequencing (MPSS)*

Massively parallel signature sequencing (MPSS) stands for high-throughput sequencing of millions of cDNAs conjugated to 32-mer capture oligonucleotide tags on the surface of 5 μm diameter microbeads, where each microbead harbors some 100,000 identical copies of a particular cDNA ("microbead library"), that circumvents separate cDNA isolation, template processing and robotic procedures (Brenner et al. 2000). In brief, the abundance of each and every mRNA of a cell can be estimated by counting the number of clones with identical signatures. However, the multitude of steps (e.g., combinatorial synthesis of capture oligonucleotides, cDNA synthesis and restriction, addition of capture oligos to the 3′-end of each cDNA, cloning with PCR handles, PCR, denaturing of the product, ligation of annealed sequences, fluorescence-activated cell sorting, sequencing of about 16–20 bases at the free ends of the cloned templates, flow-cell management, adaptor ligation, etc.) makes this technique cumbersome for expression profiling. Apart from these sophisticated technologies, a number of less complex techniques are available that produce enough information for an initial transcriptome analysis (e.g., random activation of gene expression, RAGE; Harrington 2001). Other variations of MPSS are described in Mochida and Shinozaki (2010).

### *9.7.3 Bacterial Artificial Chromosomes (BACs) and Expressed Sequence Tags (EST) Libraries*

ESTs are created by partial "one-pass" sequencing of randomly picked gene transcripts that have been converted into cDNA (Adams et al. 1993). Since cDNA and EST collections can be acquired regardless of genomic complexity, this approach has been applied not only to model species but also to a number of applied species with large genome sizes due to polyploidy and/or to their number of repetitive sequences (Mochida and Shinozaki 2010). The rapid accumulation of ESTs and cDNA clones have become significant resources for function al genomics.

Several thousand ESTs (gene sequences analyzed by isolation of mRNA), important for examination of gene expression, responses and differentiation of the plants, and examination of diversity, have been published (Santos et al. 2005) and many tens of thousands more are becoming available. Comparisons of EST libraries will be very valuable for identification of genes that are differentially expressed under stress conditions. Santos et al. (2005) made libraries from banana plants grown in cold (5°C) and hot (45°C) conditions, and found that about 30% of the genes in their library had been identified in other species as being involved in responses to environmental stress, and that there were substantial differences in the expression between the two libraries.

Several BAC libraries are already available in for *M. acuminata* and *M. balbisiana* (Vilarinhos et al. 2003; Safar et al. 2004; Ortiz-Vázquez et al. 2005; Piffanelli et al. 2008). Several BAC clones have been sequenced and show that a coding gene occurs every 6·4 to 6·9 kb in genomic DNA (Aert et al. 2004; Cheung and Town 2007) except in regions with large numbers of transposable elements where there are fewer genes. The application of low-depth 454 sequencing provided the largest amount of DNA sequence data, and enabled a detailed analysis of repetitive components of its nuclear genome (Hribova et al. 2010).

## 9.8 Transcriptomics of Fruit Ripening in Banana

The banana fruit undergoes several important physico-chemical changes during ripening (Godoy et al. 2010). The knowledge and understanding of the control points occurring during ripening can provide the scientific basis for the development of new post-harvest technologies, for improvement of shelf-life, but also for the enrichment of the nutritional and sensorial attributes of banana products (Lajolo 2004).

Many studies have examined fruit ripening aspects in banana, especially the genes involved in the production of ethylene. Analysis of gene expression would permit identification of important genes and regulatory elements involved in this process to also manipulate post-harvest banana ripening. The isolation of mRNA transcripts encoding proteins associated with the ripening process is a powerful tool for this purpose.

Differential screening of cDNA libraries from banana pulp at ripening stages 1 and 3 revealed that the following cDNAs were down-regulated in pulp during the ripening process: starch synthase, granule-bound starch synthase (GBSS), chitinase, lectin, and a type-2 metallothionein (Clendennen and May 1997). In contrast, the following cDNAs showed increased abundances early in ripening:

Endochitinase, [beta]-1,3-glucanase, a thaumatin-like protein, ascorbate peroxidase, metallothionein, and a putative senescence-related protein (Clendennen and May 1997).

In another study, cDNA libraries from banana pulp at different ripening stages were differentially screened using a nove1 microtiter plate method. The identified ripening-related cDNAs encoded enzymes involved in ethylene biosynthesis, respiration, starch metabolism, cell wall degradation, and severa1 other key metabolic events (Medina-Suárez et al. 1997). Liu et al. (1999) investigated the characteristics of ethylene biosynthesis associated with ripening in banana. At the onset of the climacteric period in naturally ripened fruit, ethylene production increased greatly, with a sharp peak concomitant with an increase in the accumulation of *MA-ACS1* mRNA, and then decreased rapidly. At the onset of ripening, the *in vivo* ACC oxidase activity was enhanced greatly, followed by an immediate and rapid decrease. Expression of the *MA-ACO1* gene encoding banana ACC oxidase was detectable at the preclimacteric stage, increased when ripening commenced, and then remained high throughout the later ripening stage despite of a rapid reduction in the ACC oxidase activity. The results suggested that ethylene production in banana fruit is regulated by transcription of *MA-ACS1*.

Most of the cDNAs isolated in these studies are abundantly expressed in bananas, reflecting a limitation of differential screening. Therefore to isolate genes that are differentially expressed at lower levels, a more powerful tool is suppression subtractive hybridization (SSH; Diatchenko et al. 1996). Manrique-Trujillo et al. (2007) reported the first set of unigenes differentially expressed at late stages of banana fruit ripening, obtained from a pulp cDNA subtraction library, comparing the PCI 1 and PCI 5 ripening stages. The unigene set was produced by assembling the ESTs from the subtraction library. This allowed us to improve sequences in length and quality in addition to removing redundancy. The differential expressed groups of genes were involved in processes associated with fruit ripening, such as

stress, detoxification, cytoskeleton and biosynthesis of volatile compounds. Kesari et al. (2007) used SSH approach, and identified 37 EST-unigenes from banana, which are expressed differentially during ripening. About 50% of these belonged to processes such as stress, defense and detoxification. In addition, genes, which are known to involve regulation of gene expression and other processes, were identified.

Four banana cDNAs encoding EIN3-like proteins were isolated from Cavendish banana (Mbeguie´-A-Mbeguie´ et al. 2008). Coinciding with the biochemical changes observed during banana fruit ripening, differentially expressed genes were isolated from the pulp (Clendennen and May 1997; Medina-Suarez et al. 1997) and peel (Drury et al. 1999; Liu et al. 2002b) of banana fruit after the initiation of the ripening process.

Jin et al. (2009) conducted a cDNA microarray analysis coupled with RT-PCR on banana fruit 10 days after harvest, and reported that 16 cDNAs were found to be up-regulated and six were down-regulated. The cDNAs identified are involved in signal transduction, amino acid metabolism, lipid metabolism, proteolysis, citrate biosynthesis and metabolism, and the uptake and transport of potassium. This information provides new clues that aid in understanding the regulation of ethylene biosynthesis initiation in postharvest banana ripening. Transcriptomics for fruit ripening appears to be one of the most active research areas in *Musa.*

To isolate differentially expressed genes at the early stage of postharvest banana ripening, a forward SSH cDNA library was constructed (Xu et al. 2007). SSH was performed with cDNA from banana fruit on the day of harvest as the "driver" and cDNA from banana fruit 2 days postharvest (DPH) as the "tester." A total of 289 clones in the SSH library were sequenced. BLASTX results revealed that 191 cDNAs had significant sequence homologies with known sequences in the NCBI database. Of the 191 cDNAs, 138 were singletons, and 53 belonged to divergent clusters containing 2–8 sequences. The identified cDNAs encoded proteins involved in cellular processes such as: metabolism; protein destination and storage; protein synthesis; signal transduction; transport and intracellular traffic; cell structure, growth, and division; transcription and post-transcription; and disease and defense. To characterize differentially expressed cDNAs in the SSH library, cDNA microarray analysis was conducted. A total of 26 cDNAs in the 2-DPH banana fruit were found to be up-regulated and these results were confirmed by using reverse transcriptase-PCR (RT-PCR).

Godoy et al. (2010) isolated 12 differentially expressed cDNAs, which were confirmed by dot-blots and Northern blots. Among the sequences identified were sequences homologous to plant aquaporins, adenine nucleotide translocator, immunophilin, legumin-like proteins, deoxyguanosine kinase and omega-3 fatty acid desaturase. Some of these cDNAs correspond to newly isolated genes involved in changes related to

the respiratory climacteric, or stress-defense responses. The information generated in these studies provides new clues to aid in the understanding of banana ripening.

## 9.9 Microarray Technology

Microarray analysis has emerging technologies in the last few years as a flexible method for analyzing large number of nucleic acids fragments in parallel. Its origin can be traced to several different disciplines and techniques. Microarrays can be seen as a continued development of molecular biology hybridization methods, as an extension of the use of fluorescence microscopy in cell biology, as well as a diagnostic assay using capture to solid surface as a way to reduce the amount of analysis needed. With the recent and rapid increase in the number of sequenced species in whole-genome and/or large-scale cDNA clones, a number of DNA microarrays have also been developed for transcriptome analysis in various plant species. The convergence of ideas and principles utilized in these fields, together with technological advancements in preparing miniaturized collection of nucleic acids on solid supports, have all contributed to the emergence of microarray and microchip technologies (www.crownscientific.com). Systems-wide approaches such as microarray RNA-profiling are ideally suited to the study of the gene complex overlapping responses of plants to biotic and abiotic stresses. Tiling arrays, which are high-density oligonucleotide probes spanning the entire genome in a particular organism, are a platform for analyzing expressed regions throughout a whole genome; it is an effective method by which to discover novel genes and elucidate their structure. Transcriptome analysis in *Arabidopsis* under abiotic stress conditions using a whole-genome tiling array and discovered a number of antisense transcripts induced by abiotic stresses (Matsui et al. 2008). Similar analysis could be conducted in banana.

## 9.10 Comparative Functional Genomics to Study Drought Stress in *Musa*

*Musa* spp. is mainly cultivated in tropical and subtropical regions where rainfall is often erratic and dry seasons may exist. To date there have been few comparative studies on *Musa* drought stress tolerance, but field observations suggest that B-genome confers greater tolerance to drought than the A-genome (Thomas et al. 1998). Banana and plantains display a wide range of overlapping responses to both biotic and abiotic conditions and the diversity of physiological, biochemical and molecular strategies adopted during adaptation to unfavorable environmental conditions (including drought) create problems to study the molecular level and find

out the stress responsible genes and expression profile (Dunoyer et al. 2007). However use of commercially available high-density oligonucleotide microarrays from closely related, heterologous species can be used to probe the transcriptomes of non-model plants.

Davey et al. (2009) evaluated the use of commercial, high-density Affymetrix rice and *Arabidopsis* ATH-1 Gene-Chip® microarrays to analyze complex plant responses in banana. They were able to rapidly profile the response of the *Musa* transcriptome to chronic drought stress, and to identify a range of structural and regulatory *Musa* gene-homologs previously found to be associated with the water-deficit response in other (model) plants. Further, despite the large phylogenetic difference between *Musa* and rice or *Arabidopsis* probes, comparisons to publically available transcriptome-profiling experiments identified a range of common drought-responsive genes, supporting the assigned *Musa* gene identities and descriptions. This approach outlines the potential of this strategy for the characterization of stress-resistance in banana and plantain varieties for which relatively little sequence information is currently available. Following cross-hybridization of *Musa* gDNA to the Rice GeneChip® Genome Array, ~33,700 gene-specific probe-sets had a sufficiently high degree of homology to be retained for transcriptomic analyses. In a proof-of-concept approach, pooled RNA representing a single biological replicate of control and drought stressed leaves of the *Musa* cultivar "Cachaco" was hybridized to the Affymetrix Rice Genome Array. A total of 2,910 *Musa* gene homologs with a >2-fold difference in expression levels were subsequently identified. These drought-responsive transcripts included many functional classes associated with plant biotic and abiotic stress responses, as well as a range of regulatory genes known to be involved in coordinating abiotic stress responses. This latter group included members of the ERF, DREB, MYB, bZIP and bHLH transcription factor families. Fifty-two of these drought-sensitive *Musa* transcripts were homologous to genes underlying quantitative trait loci (QTLs) for drought and cold tolerance in rice, including in two instances QTLs associated with a single underlying gene. The list of drought-responsive transcripts also included genes identified in publicly-available comparative transcriptomics experiments (Davey et al. 2009).

## 9.11 Conclusion

Banana is a good candidate for functional genomics because it has a relatively small genome size (600 Mbp) and important characteristics such as climacteric fruit, parthenocarpy or vegetative propagation, which are absent in model organisms (Sagi et al. 2005). Today, a number of advances in genetics and genomics have enhanced our understanding of structural and functional aspects of plant genomes and have included basic knowledge in

ways that can enhance our ability to improve crop plants. Combining the new knowledge from genomic research with conventional breeding methods is vital for enhancing crop improvement. Current genomic techniques can contribute to banana and other crops improvement in two ways. First, a better understanding of the biological mechanisms can lead to improved screening methods for selecting superior genotypes more efficiently. Second, new knowledge can improve the decision-making process for more efficient breeding strategies (Varsheny et al. 2005).

## Abbreviations

| | | |
|---|---|---|
| 3′RACE | : | rapid amplification of 3′ cDNA end |
| Ac/Ds | : | activator-dissociator |
| BACs | : | bacterial artificial chromosomes |
| BSMV | : | barley stripe mosaic virus |
| cDNA | : | complementary DNA |
| dsRNA | : | double-stranded RNA |
| EMS | : | ethylmethane sulphonate |
| ESTs | : | expressed sequence tags |
| GBSS | : | granule-bound starch synthase |
| GUS | : | Beta-glucuronidases |
| MPSS | : | massively parallel signature sequencing |
| PCR | : | polymerase chain reaction |
| RNAi | : | RNA interference |
| RT-PCR | : | reverse transcriptase |
| SAGE | : | serial analysis of gene expression |
| SSH | : | suppression subtractive hybridization |
| TAIL-PCR | : | thermal asymmetric interlaced PCR |
| T-DNA | : | tumor-inducing DNA |
| TILLING | : | Targeting Induced Local Lesions IN Genomes |
| TRV | : | Tobacco Rattle Virus |
| VIGS | : | virus-induced gene silencing |

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