International Journal of Plant Genomics

Wet Laboratory Tools Widely Used in Plant Genomics

Guest Editors: Hikmet Budak, Hongbin Zhang, Pushpendra K. Gupta, Boulos Chalhoub, Andrew James, and Chunji Liu

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Hindawi Publishing Corporation
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Editorial

Wet Laboratory Tools Widely Used in Plant Genomics

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Received 30 December 2009; Accepted 30 December 2009

The availability of laboratory tools is essential for advanced research in all areas of biological sciences. The recent development of genomic tools has made it possible to deeply investigate and to continuously improve agronomically important traits such as crop yield, quality, and biotic and abiotic stress tolerances. Integrating the newly advanced or developed wet laboratory tools that are widely used in modern genomics research and making them readily accessible will be greatly helpful for research of plant genomics and other disciplines of plant biology. Furthermore, the compilation of the tools will also facilitate scientists to advance the existing tools or develop new tools to address complicated or new questions that were previously intractable in plant genomics and biology.

In this special issue of the International Journal of Plant Genomics, "*Wet lab tools widely used in plant genomics*", we present the current status of widely used genomics tools and update them with their new advances. Articles published in this special issue cover tools for structural, functional, and comparative genomics and proteomics. The issue also summarizes the advances of genome technology in the past decades and synthesizes the current status of knowledge of new tools with an extension of suggestions. By covering up-to-date genomics tools, this special issue provides a reference for studying the structural and functional organization and evolution of plant genomes. We aim that this special issue will become useful material for teaching and research in plant genomics and biology.

There are 8 articles in this special issue, starting with articles on tools for studying plant authopagy (Mitou et al.) and microRNA identification (Unver and Budak), cloning of small RNA (Eric et al.), and the use of virus-induced gene silencing techniques (Unver et al.) for functional analysis of genes and QTLs in plants. Included is also a comprehensive article on heterologous gene expression techniques given by Filiz and Sayers. An up-to-date protocol of Agro-mediated gene transfer in cereal crops is presented by Hensel et al. Additionally, Dechyeva and Schmidt reported one of the critical tools, the molecular cytogenetic mapping of chromosomal fragments and immunostaining of kinetochore proteins, which will greatly help cytogeneticists for identifying and tagging genes in plants, thus promoting plant molecular breeding.

Acknowledgments

We thank all reviewers who helped us in reviewing these articles. We also would like to thank staff of the Editorial Section of The International Journal of Plant Genomics (IJPG), who cooperated at all stages of the production of this special issue.

Hikmet Budak
Hongbin Zhang
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Hindawi Publishing Corporation
International Journal of Plant Genomics
Volume 2009, Article ID 262463, 11 pages
doi:10.1155/2009/262463

Review Article

Review of Current Methodological Approaches for Characterizing MicroRNAs in Plants

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Received 17 February 2009; Revised 19 June 2009; Accepted 16 July 2009

Recommended by Andrew James

Advances in molecular biology have led to some surprising discoveries. One of these includes the complexities of RNA and its role in gene expression. One particular class of RNA called microRNA (miRNA) is the focus of this paper. We will first briefly look at some of the characteristics and biogenesis of miRNA in plant systems. The remainder of the paper will go into details of three different approaches used to identify and study miRNA. These include two reverse genetics approaches: computation (bioinformatics) and experimental, and one rare forward genetics approach. We also will summarize how to measure and quantify miRNAs, and how to detect their possible targets in plants. Strengths and weaknesses of each methodological approach are discussed.

1. Introduction

1.1. MicroRNA Description. With the advent of more advanced molecular techniques, a newly discovered phenomenon of gene expression involving RNA has been characterized. MicroRNAs (miRNAs) are small endogenous, noncoding regulatory RNA sequences. They have been found to play key roles in regulatory functions of gene expression for most eukaryotes [1–3]. These endogenous RNA sequences are the interest of intensive research in various model organisms, ranging from plants to mammals. In plants, miRNAs are involved in a number of biological mechanisms including plant growth, development, and defense response against abiotic stress. Evidence indicates that miRNAs regulate gene expression at posttranscriptional levels in various organisms [3–6]. Further research has shown that miRNA sequences in plants are deeply conserved [5] and have near perfect complementarities with their specific messenger RNA (mRNA) targets. As a result of this complementarity, a plant miRNA guides the cleavage, degradation, or translational inhibition of its target mRNA, thereby affecting gene expression.

Figure 1 and the following text illustrate the biogenesis of miRNAs. MicroRNAs are 21–24 nucleotides long and are processed in the nucleus from longer stem-loop structures called pre-miRNAs, that are approximately 70 nucleotides in length. miRNA genes are transcribed by RNA polymerase II, with some miRNA genes residing in intron sequences. After transcription, the 5′ end of pri-miRNA is capped and the 3′ end is polyadenylated. Based upon sequence homologies, the stem and loop structures are formed with base pairings. Pri-miRNA is thought to be longer than conserved stem-loop structures (pre-miRNA), therefore some processing takes place next.

Pri-miRNA is processed by an enzyme complex called *microprocessor* which includes Dicer like-1 nucleases (members of an RNase III endonuclease family) and HYL1, a dsRNA binding protein, bound to the pri-miRNA complex. In *Arabidopsis thaliana* all of the miRNA biogenesis steps are processed by one of the four Dicer-like RNase III endonucleases [7]. Among these, Dicer-like-1 is specialized for miRNA processing, while other Dicer-like enzymes are involved with another type of small RNA biogenesis and accumulation known as short interfering RNAs (siRNAs) [8]. However, Dicer like-4 dependence for the accumulation of several plant miRNAs has also been detected [9]. MicroRNAs are structurally and functionally similar to

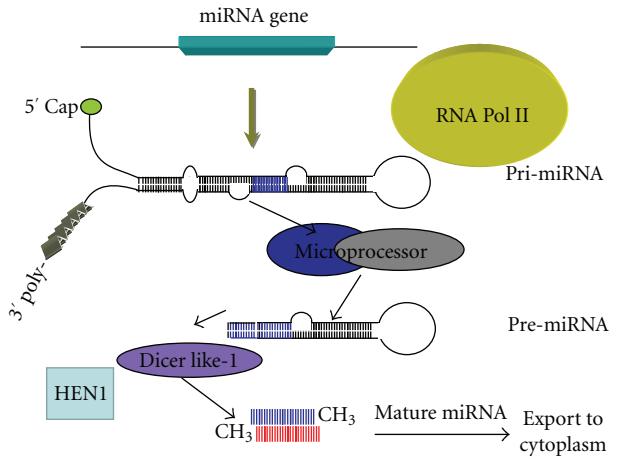

Figure 1: MicroRNA biogenesis.

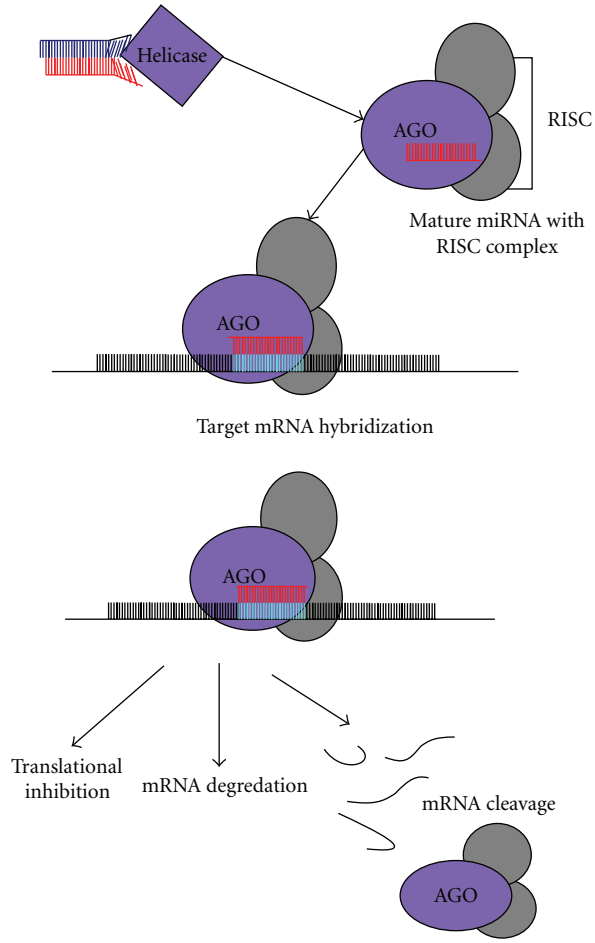

Figure 2: MicroRNA mechanism in plants.

siRNA, but miRNAs originate from long stem loop double stranded RNAs (dsRNAs) [10].

At this point, the structure is now referred to as "pre-miRNA" and has one final step remaining. The pre-miRNA complex is methylated with HEN1 enzyme and then exported into the cytoplasm with the help of HASTY, an miRNA transporter. After methylation, the structure is called "mature miRNA". The HASTY protein might be a plant ortholog of an animal enzyme known as Exportin-5, which has been shown in animal systems to also transport mature miRNAs to the cytoplasm [2, 11].

The mature miRNA structure (miRNA) is next loaded into an RNA-induced ribonucleoprotein silencing complex (RISC) to cleave its specific target mRNA or to inhibit the translation of its target transcript (Figure 2) [12]. The RISC complex includes Arganoute (AGO1) (PAZ, RNA-binding domain and RNaseH-like P-element induced wimpy testis (PIWI) domain containing protein) and miRNA* is degraded. The AGO protein family is the most important and key component of the miRNA-RISC complex [7]. Their ability to suppress protein synthesis and association with miRNAs was demonstrated in human [13]. Single-stranded miRNA in the RISC is able to target a specific mRNA sequence, having sequence complementarity and by the Piwi domain. The AGO component can then cleave the miRNA-mRNA duplex (or siRNA-mRNA duplex) [7], thereby slowing gene expression of that particular mRNA.

On the other hand, translational inhibition and mRNA degradation are also other ways gene expression is regulated by miRNA. This can occur via deadenylation of the 3′ poly (A) tail and decapping of the 5′ end in mRNAs, which leads to progressive mRNA decay and degradation [14]. It has been demonstrated that in *Drosophyla melanogaster* S2 cells, the P-body protein GW182, which is a key component marking mRNAs for decay, interacts with the AGO1 [15]. Furthermore, RNA directed-DNA methylation revealing epigenetic regulation of gene expression has been demonstrated in *Arabidopsis*, reviewed in [16]. This process is initiated with RNA signal through cleavage of dsRNA by Dicer Like family 3 (DCL3) proteins. These signals target DNA methyltransferases to catalyse methylation of DNA [17]. Histone H3 lysine 9 is an important target for epigenetic modifications in plants. H3K9 methylation is associated with epigenetic regulation of gene expression and heterochromatin modification [18].

To summarize the basic principles of miRNA biogenesis, although we see many similarities between plant and animal systems, there are also ample differences between plant and animal miRNA characteristics and biogenesis.

First, it is known that plant miRNAs are mostly generated from noncoding transcriptional units [5] in contrast with some of the animal miRNAs which are processed from introns and protein coding genetic sequences [19]. Compared to animals, plants have a more complex small RNA population in their transcriptomes. Due to the abundance of plant-specific RNA Polymerase IV and RNA Polymerase V-dependent siRNA and *trans*-acting siRNAs, plant miRNAs are represented in the pool of small RNAs. By contrast, animal small RNA populations are generally filled with miRNAs in their transcriptomes [8]. Plant miRNAs have a unique 5′ end which differs from animal miRNA 5′ end sequences. To repress translation, plant miRNAs tend to bind to the protein-coding region of target mRNAs [20–22], but

animal miRNAs bind to the 3′ untranslated region (3′ UTR) of their target mRNA transcripts [23].

To date, 1763 miRNAs have been identified in plants, including 187 from *Arabidopsis*, 377 from rice, 234 from Populus, 98 from maize, 72 from sorghum, 230 from Physcomitrella, 38 from *Medicago truncatula*, 78 from soybean, 37 from *Pinus taeda*, 58 from *Selaginella moellendorffii*, 44 from *Brassica napus* and 16 from sugar-cane (miRBase release 13.0, March, 2009, http://microrna.sanger.ac.uk/sequences/). These plant miRNAs have been identified via computational (bioinformatics) and/or experimental methods. For instance, through sequence homology analysis, 30 potential miRNAs were predicted from cotton [24] and an additional 58 wheat miRNAs have been identified by Yao et al. [25]. The majority of plant miRNAs studied to date negatively regulate their target gene expression at the posttranscriptional level. They are involved in regulating developmental processes [3, 26, 27] responding to environmental stresses [27, 28] and play a variety of important biological and metabolic processes [29–31]. Some examples of these processes include the regulation of plant development (miR172, floral organ specification, and miR166, leaf polarity), root initiation and development (ath-miR164), signal transduction (i.e., miR159, miR160, miR164, and miR167), and also plant environmental response (miR391 and miR395), as reviewed by Zhang et al. [32].

1.2. Strategies for miRNA Identification and Characterization. In reverse genetics strategies, researchers are utilizing known sequences to discover functions or phenotypes. miRNA identification largely relies on two main reverse genetics strategies: (1) computer-based (bioinformatics) and (2) experimental approaches. A third identification approach, forward genetics, is rarely used in miRNA discovery. Forward genetics is the classical approach where researchers have a known phenotype, but the DNA sequence (genotype) coding for that particular phenotype is unknown.

miRNA identification using bioinformatics tools is one of the most widely used methods, contributing considerably to the prediction of new miRNAs in both animal and plant systems. This is largely due to the low cost, high efficiency, fast and comprehensive methodology of bioinformatics. The main theory behind this approach is finding homologous sequences of known miRNAs both within a single genome and across genomes of related organisms [33, 34]. Sequence and structure homologies are used for computer-based predictions of miRNAs. Computational strategies provide a valuable and efficient manner to predict miRNA genes and their targets. The software-based approach is applied to animals, human, fungi, and plants [35–38]. For example Zhang et al. identified 338 new possible miRNAs in 60 different plant species [31] and Adai et al. have predicted 43 new miRNAs in *Arabidopsis* [39].

In contrast, cloning and sequencing of small RNA libraries represents an experimental approach to identify and characterize miRNAs. However, in contrast with bioinformatics, such approaches for miRNA identification also have limitations. First, most of the miRNAs are tissue and time specific, and generally their expression level is low. In addition, they mostly express in response to specific environmental stimuli. They also coexist with their cleaved and degraded target mRNAs, hence cloning small RNAs (miRNA and siRNA) is difficult, whereas computational approaches are effective because of no need for cloning. Since forward genetics, or the genetic screening approach, is time consuming, expensive, and less efficient, it is rarely used for plant miRNA identification. Next generation massive sequencing techniques such as pyrosequencing and Illumina are also applied to identify new miRNAs in plants [40, 41].

Here we summarize the main approaches to each strategy for identifying plant miRNAs, starting with computational (bioinformatics) approaches.

2. Computational Approach

2.1. Sequence and Structure Conservation in miRNAs. Once potential miRNA sequences have been cloned and sequenced, the sequence data can be imported into a variety of software programs for computational analysis. These bioinformatics tools search for sequence and structure conservation of miRNAs [42] using homology searches with previously known/identified miRNAs. To date a number of computational methods have been reported for the identification of plant miRNAs [5, 27, 33, 39, 43, 44]. Research in plants has revealed that short length sequences of mature miRNAs are conserved and have high complementarities to their target mRNAs [27]. Hence, candidate miRNAs can be detected using the conserved complementarities of miRNA to target mRNA, if the mRNA target sequence is known. On the other hand, it has also been shown that the secondary structures of miRNA precursor (pre-miRNA) are relatively more conserved than pri-miRNA sequences (precursor of pre-miRNA) (Figure 1) [45]. Recent bioinformatics tools were used to identify miRNA utilizing both sequence and secondary structure alignments; one of these tools is miRAlign in which more properties of miRNA structure conservation are considered (http://bioinfo.au.tsinghua.edu.cn/miralign) [43, 45]. Since the characteristic patterns of the conservation of miRNAs are searched by algorithms, the major challenge is finding miRNAs which are species specific and unrelated to previously known organisms.

2.2. Bioinformatics Tools Used for Identifying miRNA and Its Target mRNA. Several programs have been designed for the identification of miRNAs and their targets. Here we summarize five of the most commonly and widely used software tools for identifying miRNAs and miRNA targets. In this section, the softwares and databases are exemplified and their use in identifying plant miRNAs is described. The first one, miRBase, is currently a database of all known miRNA sequences. Following the description of miRBase, the plant miRNA-mRNA target finder called miRU will be explained. The secondary structure for a given pre-miRNA sequence can be predicted with appropriate criteria using

a third software, RNAmFold. Another program, micro-HARVESTER, can be applied to find homology of a given miRNA in one plant species with a candidate miRNA in another plant species. Also mentioned in this section is findmiRNA, which is used for finding possible miRNAs in a given precursor miRNA sequence. After each of these software tools (described below), an example will be given.

2.2.1. miRBase (http://microrna.sanger.ac.uk/). miRBase is a central online database for all (plant, animal, virus, fungus to date) miRNAs including sequences, nomenclature, and target mRNA prediction data from all species. Currently the 13.0 version of the online database (March, 2009) consists of 8619 miRNA total entries from 103 species. These entries represent 9539 hairpin precursor miRNAs, expressing 9169 mature miRNA products with 1763 plant miRNAs. The database has three main functions. *miRBase::Registry* is where individual data is uploaded to the database prior to publication of novel miRNAs. *miRBase::Sequences* provides miRNA sequences, nomenclature, and references. *miRBase::Targets* provides the prediction of the mRNA target from all published animal miRNAs [46].

2.2.2. miRU (http://bioinfo3.noble.org/miRNA/miRU.htm). miRU is known as a potential plant mRNA target finder. Using this database, a mature miRNA sequence from a plant species is uploaded. The miRU system searches for potential complementary target sites in miRNA-target recognition with acceptable mismatches. Specifically, the user enters a mature miRNA sequence in the 5′ to 3′ direction (entered sequence can be in a range of 19–28 nucleotides long) then the dataset should be selected for prediction of mRNA target in the intended organism of interest. The allowable complementary mismatches between the target mRNA and the uploaded miRNA sequence can be adjusted or limited by the user. The output report provides information for each predicted miRNA target including gene identifier, target site position, mismatch score, number of mismatches, and target complementary sequence with color highlighted mismatches [31]. miRU is a very useful software for identifying mRNA targets of specific plant miRNAs. However not all plant species are available at this time.

2.2.3. RNA mFold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). The algorithm utilized in the RNAmFold bioinformatics tool predicts secondary structures of single stranded RNA or DNA sequences. It is currently packaged in the Vienna RNA websuite, a collection of tools for folding, designing, and analyzing of RNA sequences [47]. The package also provides additional analysis of folding parts using the barriers program and structural RNA alignments. The package includes basic programs such as *RNAFold* for structure prediction of single sequences, *RNAalifold* for consensus miRNA structure prediction on a set of aligned sequences, *RNAinverse* for seqence design, *RNAcofold* and *RNAup* for RNA-RNA interaction analysis, *LocARNA* for the generation of structural alignment and barriers, and *treekin* for folding kinetics analysis. The RNAmFold tool is used for predicting the minimum free energy (MFE) secondary structure using the algorithm originally proposed by Zuker and Stiegler [48]. Equilibrium base-pairing probabilities of MFE structures are calculated via McCaskill's partition function (PF) algorithm [49].

2.2.4. micro-HARVESTER (http://www-ab.informatik.uni-tuebingen.de/brisbane/tb/index.php). micro-HARVESTER is a computational tool that searches for miRNA homologs in a given miRNA sequence query. Due to sequence similarity, the search step is followed by a set of structural filters. This method is a sensitive approach to identify miRNA candidates with higher specificity. The approach uses a BLAST search to generate the first set of candidates and then the process continues with a series of filters based on structural features specific to plant miRNAs to achieve the desired specificity [50].

2.2.5. findmiRNA (http://sundarlab.ucdavis.edu/mirna/) (A Resource of Predicted miRNA and Precursor Candidates for the Arabidopsis Genome). findmiRNA algorithm is used for predicting potential miRNAs in a given set of candidate precursor sequences which have corresponding target sites in the transcriptome. Generally the algorithm is based on the complementarity existing between plant miRNAs and their mRNA targets to identify initial putative miRNA. Then the software analyzes the candidate miRNA precursor sequence with regard to forming a stem-loop structure [39]. Since the tool identifies any sequence with the potential to form hairpin structures, it has limitations such as the possibility of identifing tRNAs, foldback elements, and retrotransposons [39].

2.2.6. MiRCheck (http://web.wi.mit.edu/bartel/pub/software.html). MiRCheck is an algorithm designed to identify 20 mers which encode potential plant miRNAs [27]. Entries should be (1) putative miRNA hairpin sequences, (2) putative hairpin secondary structures, and (3) 20-mer potential plant miRNA sequences within the hairpin for MIRcheck algorithm. This software requires data of miRNA complementarities that should be conserved between homologous mRNAs in *Arabidopsis* and *Oryza sativa*. Researchers use this software to check their candidate miRNAs if they have potential to encode miRNA.

To briefly summarize, the largest limitation of most bioinformatic methods is the need to start from a known homologue and depend heavily on conservation of secondary structure and mature miRNA sequences. More advanced methods using hidden Markov models can overcome this limitation. As an example, Kadri et al. [51] have developed a novel approach, Hierarchical Hidden Markov Model (HHMM) that utilizes region-based structural information of miRNA precursors. They used this model for computational miRNA hairpin prediction in the absence of conservation in human [51].

2.3. EST Database Analysis Used for miRNA Prediction. Now we will describe how the above tools are utilized in the search

of miRNAs. One such example is with Expressed sequence tags (ESTs). ESTs are partial sequences of complementary DNA (cDNA) cloned into plasmid vectors [52]. RNA is the starting material from which the cDNA clone is made, using *reverse transcriptase*. Many important plant genes using EST databases have been cloned [53, 54]. It is well known that miRNAs are deeply conserved from species to species, which allows researchers the ability to predict orthologues of previously known miRNAs by utilizing EST databases. Availability of ESTs in databases for identifying new plant miRNAs increases with coverage of the genome and number of sequences. Currently, GeneBank release 171.0 April 2009 (http://www.ncbi.nlm.nih.gov/Genbank/) contains 103 335 431 EST sequences, representing more than 1370 different organisms. The number of ESTs available for a specific organism can be found at http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html. This particular website is the best to utilize because conserved candidate miRNAs and their precursors can be predicted using this resource. The largest number of plant ESTs is from maize (*Zea mays*) (2 018 530), thale cress (*Arabidopsis thaliana*) (1 527 298), soybean (*Glycine max*) (1 386 618), rice (*Oryza sativa*) (1 248 955), wheat (*Triticum aestivum*) (1 064 111), oilseed rape (*Brassica napus*) (596 471), and barley (*Hordeum vulgare*) (525 527).

To identify homologous miRNAs across plant species, EST analysis approaches have been developed using sequence conservation of known miRNAs. An extra filtering which provides structure prediction (secondary structure), such as the "Zuker folding" algorithm with RNAmFold software [55], has also been applied [27, 31, 39].

Zhang et al. [31] reported an EST database analysis for predicting new plant miRNA genes using the BLAST algorithm to search known plant miRNAs (taken from miRBase 3.1 April, 2004). Their additional filter was the Zuker folding algorithm (mFold 3.1) to predict the secondary structure of putative miRNA sequences. The Zuker algorithm outputs used to analyze the results included the number of structures, free energy (ΔG kcal/mol), miRNA-like helicity, the number of arms per structure, size of helices within arms, and size and symmetry of internal loops within arms. Then hairpin stem-loop structures of predicted putative miRNAs were analyzed for structure filtering with known miRNAs using computational strategies [42]. The highest score of stem-loop structures was considered as new miRNAs and then the ESTs (with high similarity, *E* value less than e-100) were assigned as miRNA clones.

Jones-Rhoades and Bartel [10] have also analyzed EST databases to look for possible miRNAs in other plant species using known *Arabidopsis* miRNAs. They also revised the computational strategy with possible miRNA targets to increase sensitivity of the approach. Their first step for identifying miRNAs in the genome was detecting genomic portions containing imperfect inverted repeats using the "EINVERT" algorithm. Then they used the RNAmFold software to predict secondary structures of miRNA candidates. They checked all 20 mers within the inverted repeats against MiRCheck. After MiRCheck analysis they applied Patscan to identify 20 mers in *At*Set1 that matched at least one 20 mer in *Os*Set1 with 0–2 base substitutions. The Patscan algorithm was used in consideration of 20 mers on the same arm of their putative hairpins.

Bonnet et al. [35] also applied computational approaches to detect miRNAs and then applied EST analysis to confirm 91 newly identified miRNAs in *Oryza sativa* and *Arabidopsis thaliana*. Zhang et al. [31] have taken previously all-known *Arabidopsis* miRNAs (miRBase Release 3.0, April 2004) and searched the EST databases (using Basic Local Alignment Search Tool for nucleotide analyzes (BLASTn) 2.2.9 (May 1, 2004)) to find ESTs matched with miRNAs. They found a total of 18 694 BLAST hits in the databases and removed the EST sequences with high numbers (more than 2) of mismatched hits. Their result came to a total of 812 ESTs with 0 to 2 mismatches. They used those ESTs to predict secondary structures with RNA mFold software. Finally they identified 338 new potential miRNAs in 60 plant species.

2.4. Sample Method to Identify miRNA in a Plant Species Using Computational Approach. In this section, we work through an example of using ESTs and the software tools discussed previously, to identify plant miRNAs. In order to predict the plant miRNAs, EST sequences should be downloaded from the GenBank database (http://www.ncbi.nlm.nih.gov/) (Figure 3(a)). Searching for miRNA-like sequences includes two major procedures: searching pre-miRNA-like sequences and identifying pre-miRNAs and miRNAs. First, the RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) [47] program or mFold program (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi) [55] can be used to find potential miRNA hairpin structures from the databased EST sequences [56]. According to Weibo et al. [57], strict criteria should be adopted in the identification of pre-miRNA-like sequences from hairpin structure sequences. These are

(a) 60 nucleotides is the minimum length of pre-miRNA sequences;

(b) the stem of the hairpin structure (including the GU wobble pairs) includes at least 17 base pairs;

(c) −15 kcal/mol should be the maximum free energy of the secondary structure;

(d) the secondary structure must not compromise multi-branch loops;

(e) the GC content of pre-miRNA should be between 24 and 71%.

Following these criteria assures that the processed sequences are similar to real pre-miRNAs, according to widely accepted characteristics. Second, the real pre-miRNAs might be identified from a large number of pre-miRNA-like sequences using a program such as GenomicSVM (http://geneweb.go3.icpcn.com/genomicSVM/). This program was developed using the "Support Vector Machine" model [57]. Alternatively, miRCheck (http://web.wi.mit.edu/bartel/pub/softwareWebTools.html) can be applied (Figure 3(a)) for confirming sequences to identify, if

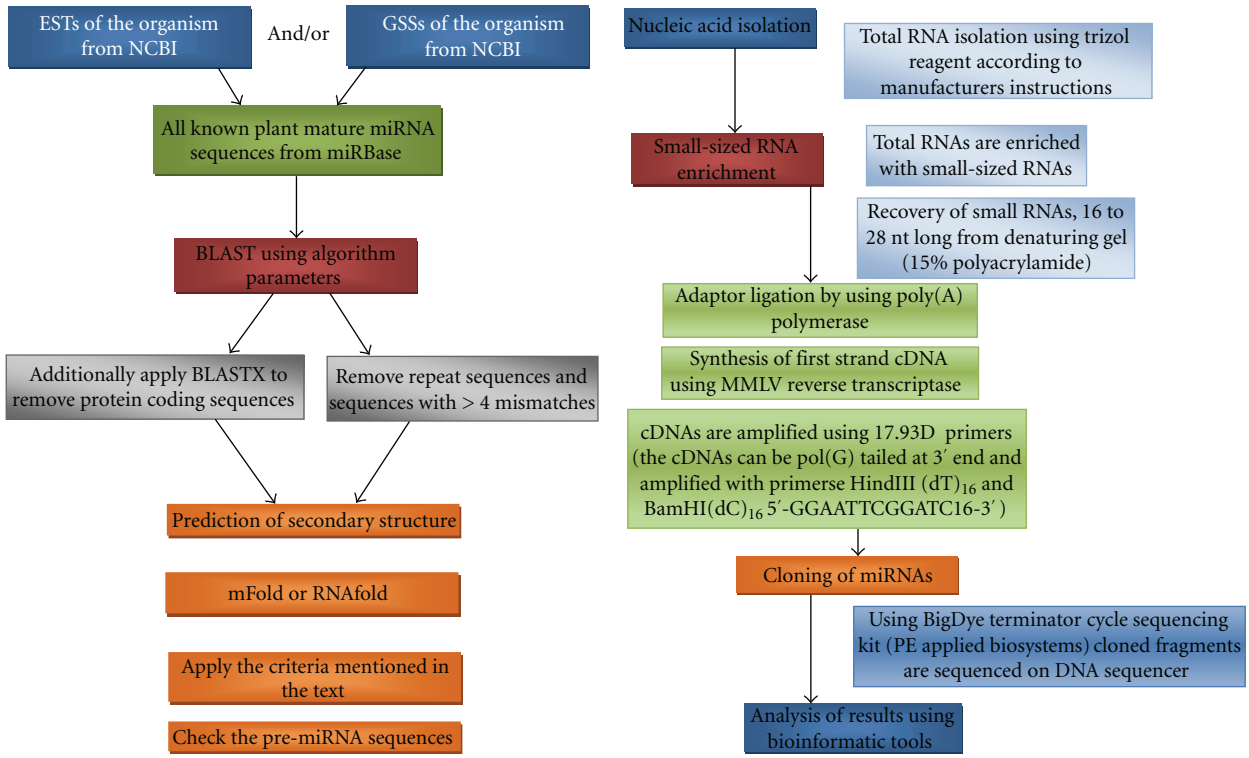

Figure 3: (a) Flow chart for computational approaches in identifying plant miRNAs. (b) Flow chart for experimental approaches in identifying plant miRNAs.

the entries contain 20 mers which encode potential plant miRNAs.

3. Experimental Approaches

Computational methods for identifying miRNAs in plants are rapid, less expensive and relatively easy compared with experimental procedures. However, these bioinformatic approaches can only identify conserved miRNAs among organisms and DNA or RNA sequence information is required in order to run the softwares. On the other hand, the computationally predicted miRNAs should also be confirmed via experimental methods. These experimental method options are described next.

3.1. Direct Cloning and Sequencing of Small RNA Libraries. Direct cloning of small RNAs from plants is one of the basic approaches of miRNA discovery. Scientists have used this methodology to isolate and clone small RNAs from various plant species such as *Arabidopsis* and rice [5, 20, 21, 58, 59]. Identification of miRNAs using the direct cloning approach basically involves the creation of a cDNA library and includes six steps: (1) isolation of total RNA from plant tissue, (2) recovery of small RNAs from an acrylamide gel, (3) adaptor ligation, (4) reverse transcription, RT-PCR, (5) cloning, and (6) sequencing methods. Expression of several miRNAs is broad but many of them are detected in certain environmental conditions, at different plant developmental stages and tissues. Therefore specific time points, tissues, and/or biotic and abiotic stressed induced plant samples are used for miRNA cloning. The most common plant species used for direct cloning are *Arabidopsis thaliana* [5, 58, 59], *Oryza sativa* (rice) [60], (cottonwood) [61], and *Triticum aestivum* (wheat) [24].

3.2. An Example of the Direct Cloning Experimental Approach. Total RNA is extracted from the organism of interest [40, 41]. Next, small RNAs approximately 16–28 nucleotides long are selected from the total RNA and excised from a polyacrylamide gel. Next, these small RNAs are ligated with an adaptor and reverse transcribed [62]. Resulting cDNAs are amplified with Real-Time PCR (RT-PCR) using primers designed for adaptor sites. Finally, the RT-PCR products are concatamerized and cloned [63]. Selected clones are sequenced and the sequence data is then analyzed (Figure 3(b)). These experimental procedures for identifying miRNA have been successfully applied and detailed by Elbashir et al. [63], Lau et al. [62], and Park et al. [58]. Alternatively, new high-throughput technologies such as 454 pyrosequencing and Solexa sequencing can be used for identification of plant miRNAs [40, 41].

The most important advantage of high-throughput deep sequencing technology compared to computational approaches is the opportunity for finding nonconserved and

species specific miRNAs. To identify conserved and nonconserved miRNAs in tomato, Moxon et al. (2008) used the pyrosequencing approach [40]. On the other hand, Szittya et al. (2008) successfully used Solexa sequencing to find new miRNAs in barrel medic (*Medicago truncatula*) [41]. They have identified 25 conserved and 26 novel nonconserved miRNAs using 1 563 959 distinct sequences and 2 168 937 reads [41]. Experimental approaches also provide to detect and measure the specific miRNAs expressed in plants. Using the following methods plant miRNAs are efficiently detected and quantified.

3.3. miRNA Detection and Quantification Methods. Efficient and suitable miRNA detection and quantification are essential to understand miRNA function in specific conditions, cell and tissue types. Northern hybridization, cloning, and microarray analysis are widely used to detect and quantify miRNAs in plants, but these techniques are less sensitive and are not high throughput compared with quantitative real-time reverse transcription PCR (qRT-PCR) and end-poind PCR. Effective and sensitive qRT-PCR detection can circumvent these limitations. Several methods have been developed to detect and quantify miRNA for mammalian cells [64–66]. Recently Varkonyi-Gasic et al. [67] described a protocol for an end-point and real-time looped RT-PCR procedure. Their approach includes two steps. In the first step, a stem-loop RT primer is designed, following the strategy developed by Chen et al. [68] and is hybridized with the candidate miRNA. The second step includes the specific amplification of the miRNA, using a forward primer specific for the miRNA and a universal reverse primer, which is designed for the stem-loop RT primer sequence. The clues for designing the reverse RT primers and miRNA specific forward primers are that the specificity of stem-loop RT primers for a certain miRNA is conferred by a six nucleotide extension at the 3′ end. This extension is the reverse complement of the last six nucleotides at the 3′ end of the miRNA. Forward RT primers are specifically designed for individual miRNA sequences. At the primer's 5′ end 5–7 random and relatively GC-rich nucleotides are added to increase the template's melting temperature [67].

To perform end-point and real-time looped RT-PCR miRNA quantification experiments, total RNA is isolated from a plant sample using the TRizol reagent according to manufacturer's protocol (Invitrogen, Carlsbad, CA). Then the stem-loop RT PCR reaction is performed by mixing the component as follows: 0.5 μL 10 mM dNTP mix, 11.15 μL nuclease-free water, 1 μL of appropriate stem-loop RT primer (1 μM), and the mix is heated at 65°C for 5 minutes and chilled on ice for 2 minutes. Additional components are added to the mixture, 4 μL 5× First-Strand buffer, 2 μL 0.1 M DTT, 0.1 μL RNaseOUT (40 units/μL), and 0.25 μL, SuperScript III RT (200 units/μL). Finally the pulsed RT reaction incubation is set up as 30 minutes at 16°C, followed by pulsed RT of 60 cycles at 30°C for 30 seconds, 42°C for 30 seconds, and 50°C for 1 second. RT products can be used to detect and quantify individual miRNAs in plants via three different strategies, end-point PCR, SYBR Green I assay, and TaqMan UPL procedure. Each of these strategies is described next.

3.3.1. End-Point PCR. A nontemplate control should be included with each experiment to insure the expected banding pattern for specific cDNA of miRNA amplification. A PCR master mix is prepared and the following components added to nuclease-free eppendorf tubes: 15.4 μL nuclease-free water, 2 μL 10 × PCR buffer, 0.4 μL 10 mM dNTP mix, 0.4 μL forward primer (10 μM), 0.4 μL reverse primer (10 μM), and 0.4 μL Advantage 2 Polymerase mix. Then 19 μL of the PCR master mix should be aliquot into different tubes and 1 μL RT product is added to reaction mixtures. After that, the thermal cycler is set up as 94°C for 2 minutes, followed by 20–40 cycles of 94°C for 15 seconds and 60°C for 1 minute. Finally the PCR reaction products are analyzed by electrophoresis on a 4% agarose gel in 1× TAE gel.

3.3.2. SYBR Green I Assay. SYBR Green I master mix is prepared according to real-time qPCR system (5 × LightCycler for Roche Diagnostics or 2X Master mix for Stratagene Mx3005p) by adding 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM), 12 μL nuclease-free water (for 5 × LightCycler for Roche Diagnostics) or (6 μL nuclease-free water for 2X Master mix for Stratagene Mx3005p), 4 μL SYBR Green I master mix for 5 × LightCycler for Roche Diagnostics or (10 μL SYBR Green I master mix for Stratagene Mx3005p). Using nuclease-free eppendorf tubes for each qPCR reactions, 18 μL prepared mixtures containing master mix and primers are pipetted into each tube. Adding 2 μL RT products to tubes, the reaction is started. qPCR machine is set up as 95°C for 5 minutes, followed by 35–45 cycles of 95°C for 5 seconds, 60°C for 10 seconds, and 72°C for 1 second. For melting curve analysis samples are denaturated at 95°C, and then cooled to 65°C at 20°C per second. Fluorescence signals are collected at 530 nm wavelengths continuously from 65°C to 95°C at 0.2°C per second. Finally results are analyzed using the LightCycler or Stratagene software.

3.3.3. miRNA TaqMan UPL Probe Procedure. To perform a TaqMan assay for miRNA detection and quantification using Universal Probe Library (UPL) probes, first a 5× LightCycler TaqMan master mix (Roche Diagnostics) is prepared according to manufacturer's instructions. Next the following components are added to a nuclease-free eppendorf tube: 11.8 μL nuclease-free water, 4 μL TaqMan master mix, 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM), 0.2 μL UPL probe no. 21 (10 μM). Then real-time qRT-PCR is performed with cycling temperatures and resulting data analyzed as described above with the SYBR Green I assay protocol. An educational eLesson and animation further describing the real time PCR technique can be found at the Plant and Soil Sciences eLibrary (http://plantandsoil.unl.edu/croptechnology2005/pages/index.jsp?what=topicsD&topicOrder=1&informationModuleId=1057077340).

3.4. Forward Genetics. miRNAs were first discovered via mutant analysis [64] in animals. However to date, there is only one example using a forward genetics experimental approach to identify miRNA in plants. Baker et al. [69] identified an miRNA loss of function allele by a transposon insertion upstream of the predicted *MIR164c* stem-loop. The miRNA mutant resulted in a flower phenotype with extra petals. Since highly conserved plant miRNAs are encoded by gene families, functional redundancy restricts the loss of function of an miRNA gene, making mutation searches highly inefficient. Overexpression of miRNA genes and precursors or construction of miRNA resistant transgenic plants have the potential to better provide a clear assessment of overlapping functions of other miRNA family members. If this comes to fruitition, then forward genetics approaches may become more viable in identifying miRNAs.

4. Identification of miRNA Targets

So far we have described computational (bioinformatics) and experimental approaches used to identify miRNA sequences. Now we will describe methods utilized to identify their targets, mRNA sequences cleaved or targeted by miRNA. Specific miRNA targets in plant genomes and transcriptomes have been identified with both experimental and computational approaches. Predicting miRNA targets in plants is much easier due to the high and significant complementarities to miRNA-mRNA targets [34]. The ability for plant miRNA to target mRNA with perfect sequence complementary matches was first shown with miR171 [59]. It was shown that miR171 has perfect antisense complementarity with three SCARECROW-like (SCL) transcription factors in the *Arabidopsis* genome. Additionally, this particular miRNA is transcribed from an intergenic locus and lacks a stem-loop structure [5, 21]. Predicting conserved miRNA targets in different organisms has revealed that homologous mRNAs are targeted by conserved miRNAs within an miRNA family, yet allowing more gaps and more mismatches between an individual miRNA and its target [27]. Next we will summarize some of the bioinformatic and experimental methods utilized to find mRNA targets of known miRNAs in plants.

4.1. Computer-Based Procedures for Predicting mRNA Sequences Targeted by miRNA. Several algorithms are used for predicting putative miRNA-mRNA targets in plants; for this purpose mirU is one of the widely used softwares. The mirU system using given miRNA sequences searches for potential mRNA targets with tolerable mismatches [70]. Additionally, Jones-Rhoades and Bartel [10] have developed a more refined method by using the MIR check algorithm to predict miRNA targets specifically in *Arabidopsis* and *Oryza sativa*. The MirCheck software allows for more mismatches and gaps in miRNA-mRNA complexes in these two species. This software also needs miRNA complementarities that should be conserved between homologous mRNAs in *Arabidopsis* and *Oryza sativa* [10]. They have scored the miRNA complementary sites as 0.5 for G:U wobble pairs, 1 for non-G:U wobble pairs, 2 for each bulged or loop nucleotide in the miRNA or target site. They have reported scored complementary results of ≤ 2 in conserved miRNA target mRNA sites in both *Arabidopsis* and *O. sativa*.

4.2. Experimental Approaches for Prediction of miRNA Targets. As with computational approaches, experimental approaches have been utilized widely to predict plant miRNA-mRNA target sites. Genome-wide expression profiling to search for miRNA targets can be applied on expression arrays. In one example, array data showed that five transcripts encoding *TCP* genes were downregulated via overexpression of miR319a (miR-JAW) in *Arabidopsis*. Those five TCP transcription factor mRNAs show up to five mismatches, or four mismatches when G:U wobble counts 0.5 mismatch [71]. Additionally Schwab et al. [22] overexpressed four different miRNAs in each *Arabidposis* plant and examined each expression profile to experimentally establish parameters for target cleavage guided by plant miRNAs. However, they found no new target mRNAs other than previously identified by computational approaches. Two new targets, not found through bioinformatics, were detected, but their cleaved products were not confirmed via 5′ RACE experiments.

4.3. 5′ RACE Experiment. At present, the most powerful method to confirm miRNA-mRNA targets is the 5′ RACE procedure (Random Amplification of cDNA Ends). 5′ RACE has been used by many researchers to identify miRNA targets in plants [3, 21, 71, 72]. Cleaved mRNA products in plants have two diagnostic properties. One is that the 5′ phosphate of a cleaved mRNA product can be ligated to an RNA adaptor with T4 RNA ligase. Second, in general, the precise target cleavage position is that mRNA target nucleotides pair with the tenth nucleotide of miRNA [21, 73]. Cleaved mRNA products by miRNA guided activity can be amplified with ligation of an oligo-nucleotide adaptor to the 5′ end, followed by reverse transcription and PCR amplification with a gene specific primer [21]. A modified 5′ RACE procedure can be applied as follows. Total RNA is isolated and polyA mRNA is prepared (Qiatex mRNA midi kit, Qiagen, CA) and directly ligated to an RNA oligo adaptor (supplied by GeneRacer kit, Invitrogen, CA). Oligo dT is used to synthesize the first strand of cDNA with reverse transcriptase. This first cDNA strand is amplified with GenRacer 5′ and 3′ primers for nongene specific amplification (according to manufacturer's procedures, Invitrogen, CA, USA or Clontech, RL, USA). Then the 5′ RACE PCR and 5′ nested PCR are performed using specific primers supplied with kits. RACE products are gel purified, cloned, and sequenced.

5. Concluding Remarks

miRNA studies in plants have already explained a number of biological events in response to both biotic and abiotic stresses. Improved understanding of molecular mechanisms of miRNA in plants will lead to the development of novel and more precise techniques that will help better understanding some posttranscriptional gene silencing in response to both

biotic and abiotic stresses. Accumulating knowledge on the roles of plant miRNA's in molecular biology is leading to the development of more efficient and reliable tools for their characterization. Building new algorithms and models will overcome drawbacks and limitations of current softwares used for miRNA and target mRNA identification. New bioinformatic tools should be generated to separate miRNA hairpin structures from tRNAs and retrotransposons and to differentiate miRNAs from siRNAs. New algorithms or new models to help cleaved mRNA product analysis for target identification also require improvement. Considering the discovery that miRNAs play important roles in numerous biological events, greater research in this area is being stimulated.

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Hindawi Publishing Corporation
International Journal of Plant Genomics
Volume 2009, Article ID 915061, 13 pages
doi:10.1155/2009/915061

Review Article

Methodologies for In Vitro Cloning of Small RNAs and Application for Plant Genome(s)

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Received 17 February 2009; Accepted 30 March 2009

Recommended by Chunji Liu

The "RNA revolution" that started at the end of the 20th century with the discovery of post-transcriptional gene silencing and its mechanism via RNA interference (RNAi) placed tiny 21-24 nucleotide long noncoding RNAs (ncRNAs) in the forefront of biology as one of the most important regulatory elements in a host of physiologic processes. The discovery of new classes of ncRNAs including endogenous small interfering RNAs, microRNAs, and PIWI-interacting RNAs is a hallmark in the understanding of RNA-dependent gene regulation. New generation high-throughput sequencing technologies further accelerated the studies of this "tiny world" and provided their global characterization and validation in many biological systems with sequenced genomes. Nevertheless, for the many "yet-unsequenced" plant genomes, the discovery of small RNA world requires in vitro cloning from purified cellular RNAs. Thus, reproducible methods for in vitro small RNA cloning are of paramount importance and will remain so into the foreseeable future. In this paper, we present a description of existing small RNA cloning methods as well as next-generation sequencing methods that have accelerated this research along with a description of the application of one in vitro cloning method in an initial small RNA survey in the "still unsequenced" allotetraploid cotton genome.

1. Introduction

In the 1990s two independent discoveries opened up the previously unsuspected world of noncoding RNAs (ncRNAs). The phenomenon of RNA interference (RNAi) was being uncovered as cosuppression in plants [1, 2], quelling in fungi [3, 4], and RNAi in nematodes [5] through the 1990s and at least the broad strokes of the mechanism were elucidated by the turn of the 21st Century [6]. At the same time, another curious phenomenon was being observed by Victor Ambros, Gary Ruvkun, and colleagues in nematodes [7, 8]. Like RNAi, this phenomenon, initially called short temporary RNA (stRNA), was at first regarded as a one-off curiosity but, again like RNAi, persistence paid off with the explosive validation of the microRNA (miRNA) [9–12]. The two worlds of RNAi and miRNAs merged when it was observed that both RNAi and miRNAs employed the same mechanism to carry out their mission of regulating eukaryotic gene expression [13].

Over the past several years RNAi has become a powerful tool for understanding the role played by dozens of plant and animal genes in a wide range of cellular processes, both normal and pathogenic [14]. Moreover, RNAi is proving to be a potentially powerful tool in attacking pathogenic cellular processes [15]. Similarly, the world of miRNAs has grown from the two original nematode "genes" to now number more than one thousand loci in plants and animals and their role in regulating cellular processes has expanded to a point where virtually all normal and pathogenic cellular processes are affected at some point by one or more of these tiny entities. Hence, the discovery of miRNAs represents a hallmark in RNA science for understanding RNA-dependent

regulation of many complex biological processes such as development, function of metabolic pathways, cell fate and death [16].

In addition, the universe of small RNAs has expanded to include not only miRNAs but new classes including endogenous small interfering RNAs (siRNAs), 21U RNAs, and Piwi-interacting RNAs (piRNAs) [17]. Of these small RNA classes, only miRNAs form a characteristic thermodynamically stable hairpin structure. That stable hairpin makes miRNA prediction in sequenced genomes a relatively tractable exercise. On the other hand, de novo finding of miRNAs in species whose genomes have yet to be sequenced and discovering new classes of small RNAs must still rely upon in vitro cloning from purified cellular RNAs. Thus, reliable and reproducible methods for cloning small RNA species are of paramount importance and will remain so into the foreseeable future. Here, we present a compilation of extant small RNA cloning methods, options for sequencing, and some of the small RNA results that we have obtained in the "still unsequenced" allotetraploid cotton genome.

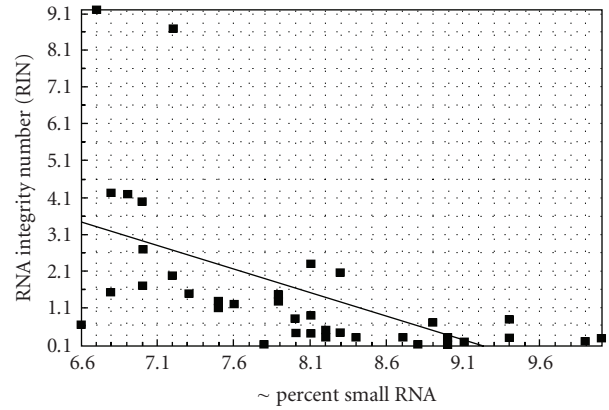

Figure 1: Linear regression of total RNA quality (RIN) and the relative mass of the small RNA population determined for forty human, mouse and rat tissues. A significant negative correlation coefficient, $r = -0.58$, $P < .01$, df = 38, derived from the regression indicates that total RNA quality is an essential component of small RNA cloning in that higher quality RNA retains a more pure small RNA fraction but, as a corollary, that enrichment of the small RNA fraction prior to cloning is crucial to success.

2. Small RNA Cloning Strategies

There are a number of strategies that have been proposed for cloning small RNAs. Before discussing these, however, there is one factor common to all of them that is essential to be aware of. Small RNAs, whether from plant cells, animal cells, or other sources, represent a small fraction of the total RNA mass present. Agilent Technologies quantifies the quality of cellular RNA in the form of their RNA Integrity Number (RIN). Very high quality intact RNA has a RIN of 10.0 and the lower the RIN, the more degraded the RNA. RIN values between 6.5 and 10.0 represent a continuum of acceptable to excellent RNAs. Using RIN as the point of departure, Agilent assessed the relative fraction of total RNA that is within the small RNA size range in forty tissues from human, mouse, and rat [18].

The results, summarized in Figure 1, show two important features. First, for all but five tissues, the relative mass of small RNAs is below 3% and, second, there is a significant negative correlation ($r = -0.58$; $P < .01$, df = 38) between overall RNA quality as assessed by RIN value and relative small RNA mass. Clearly, increasing amounts of RNA degradation will introduce a greater mass of small fragments that lie in the true small RNA zone. This will result in a greater mass of competing RNA that will make it more and more difficult to see the real small RNAs that are the targets of interest even if the majority of the degraded RNAs are themselves unclonable by some of the methods discussed below. While there will be variation from RNA source to RNA source, it is clear that larger RNA components like mRNAs, rRNAs, and tRNAs, comprise by far the bulk of the total RNA and that the relative mass of the true small RNA fraction should and will be the smallest in very high quality RNA. A generalized RNA mass profile for high RIN RNA is presented in Figure 2. As can be seen, the true miRNA region is indeed a very small part of the total mass. Given this, it is essential to the small RNA cloning process that RNA quality, as assessed by measures like RIN, be as high as possible and that as much of the competing RNA mass as possible be removed so that a "target-rich" small RNA component can be purified prior to starting the cloning process.

Small RNA enrichment can be accomplished in a number of ways. One of the simplest ways is to simply run a sample of total RNA on a denaturing polyacrylamide gel (dPAGE) and excise the area of the gel containing the small RNA fraction (see the appendix). The problem with this method is that the enriched small RNAs must be removed from the gel and purified for further manipulations and this routinely results in a substantial loss of what is already a small amount of mass to begin with. There are ways to minimize this loss of material and we will discuss one of these in the next section. Other methods for enriching the small RNA fraction have been developed including column capture and release methods like the mirVana protocol from Ambion and the timed size exclusion method, represented by the flashPAGE fractionator system, also from Ambion. The point is that, whatever method is employed, the small RNA fraction of total cellular RNA must be enriched to increase the likelihood of successfully cloning small RNAs.

Once the small RNA fraction is enriched and purified, there are several ways to proceed to clone the individual small RNAs contained in the fraction. Berezikov et al. [19] reviewed the basic small RNA cloning methods. In all cases the target species for direct cloning is an RNA varying in size between 18 and 25 nucleotides (nt) having a free 3′ hydroxyl group and a free 5′ phosphate group. Although some variation exists [20], the universal initial step in the cloning process is first to ligate a 3′ adaptor sequence through the free 3′ hydroxyl. The 3′ adaptor will serve as the site for later annealing of an oligonucleotide primer for reverse transcription. As seen in Figure 3, there are several possible ways to accomplish this adaptor joining. In one option, the

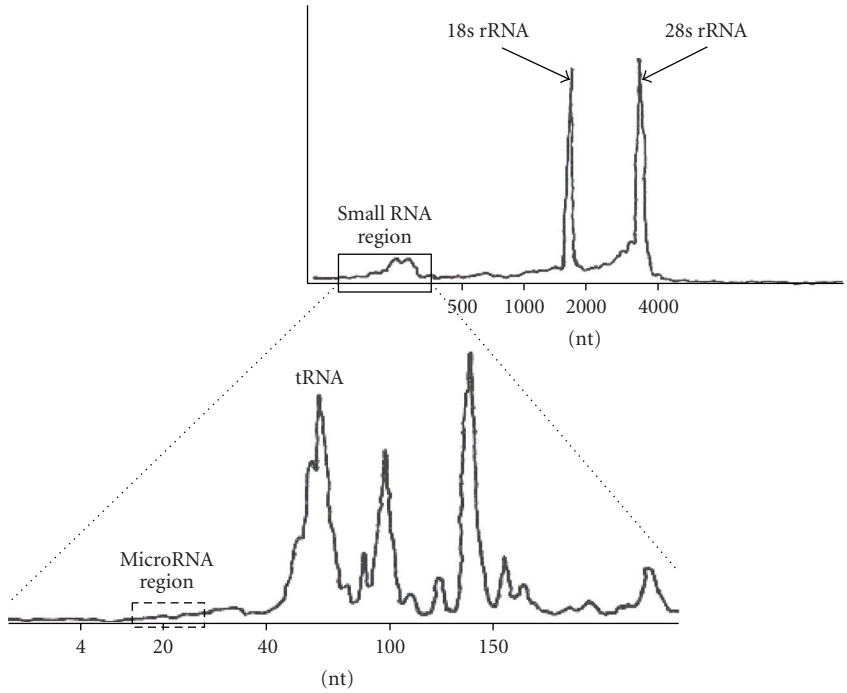

Figure 2: Mass profile of human RNA. Here, the absolute mass fractions of RNAs up to 4000 nt in length are shown. The position and composition of the small RNA region, defined as that portion of the total RNA mass that is between 0 and 200 nt long are highlighted. It can be seen that, even within the small RNA region, the microRNA region lying between 18 and 26 nt, is a very small fraction of the total. Figure adapted, with permission, from Agilent Technologies.

small RNA species are polyadenylated creating a 3′ extension [21]. However, as many small RNA species in plants have been shown to contain 2′-O-methyl modifications on their 3′ ends, this method may be of only limited utility since such modifications block polyA polymerase extension [22]. Both of the other 3′ adaptor joining options are designed to prevent later circularization of the linkered RNAs. In one variation, the RNAs are dephosphorylated prior to adaptor ligation and then rephosphorylated for subsequent processing [23, 24]. In the other variation, the 5′ end of the adaptor is preadenylated and the 3′ end blocked by a nonstandard group such as a dideoxynucleotide [10, 25]. Preadenylation of the adaptor obviates the need to dephosphorylate the target RNAs because the adaptor joining via T4 RNA Ligase can be carried out in the absence of ATP. Given the obvious advantage that this method confers by reducing the number of operations required to process target RNAs, New England BioLabs (NEB) has introduced a truncated T4 RNA Ligase that specifically reacts with preadenylated 3′ linkers [25–27]. Regardless of the method chosen, however, producing a stable and reactive 3′ linkered small RNA population is the goal of the first step in cloning.

The next phase of cloning is to join a second adaptor to the small RNA population. This time, the adaptor is joined to the 5′ end. As shown in Figure 3, there are now but two ways to do this and the choice is dictated by the methods chosen for 3′ adaptor joining. If the method chosen is the polyadenylation route, then the 5′ adaptor joining method is to carry out a template switch. This method relies on the property of a number of reverse transcriptases to add a small number of nontemplated nucleotides to the 3′ ends of cDNAs. Since the nontemplated nucleotides tend to be mostly deoxycytidines, an adaptor containing a poly-G 3′ run can be used to switch the template from the miRNA to the adaptor [19]. The other path is to use a 5′ adaptor with a 3′ hydroxyl group that will ligate to the 5′ phosphate of the target RNAs. This is carried out with a T4 RNA Ligase in the presence of ATP and is followed by a reverse transcription using a primer complementary to the 3′ linker. In both cases, the resulting cDNA population is PCR amplified in preparation for cloning and/or sequencing.

PCR amplicons can be directly cloned using any one of several PCR cloning vectors or the amplicons can be processed to form concatamers which are then cloned. Concatamer formation from amplicons is a direct descendant of the Serial Analysis of Gene Expression (SAGE) methodology developed in the 1990s by Velculscu and colleagues [24, 28]. The obvious advantage of concatamer cloning is that individual clones will contain more small RNAs than the ones that will be present if the PCR amplicons are simply shot-gun cloned. This is a consideration for conventional Sanger dye-terminator sequencing but, as will be discussed later, new generation deep sequencing methods have circumvented the need for concatamers and, indeed, for cloning at all.

One aspect of the cloning methods shown in Figure 3 is that small RNAs will all contain a 5′ phosphate group following 3′ adaptor joining. This constant feature that

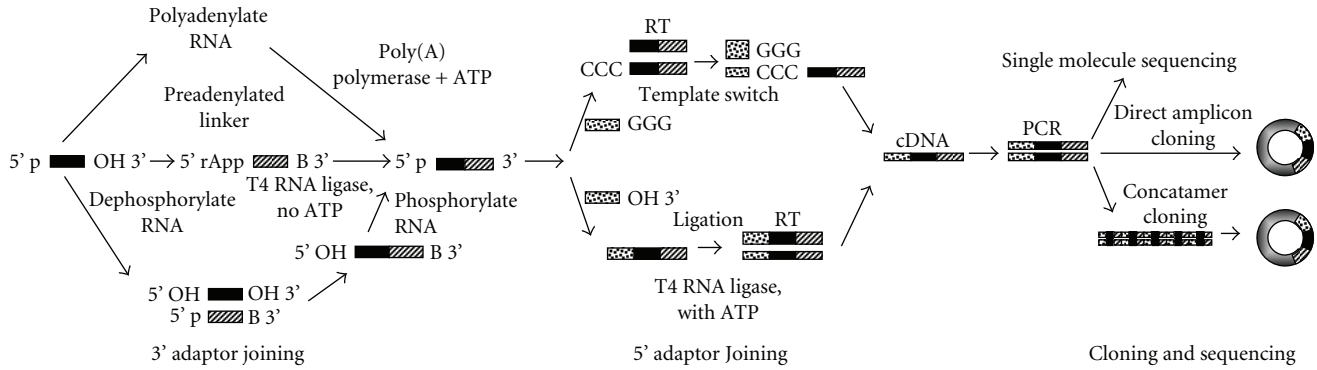

Figure 3: Diagram of extant small RNA cloning strategies. Following small RNA enrichment, all strategies share the same outline of first placing an adaptor on the 3′ end of the target RNAs, then placing a second adaptor on the 5′ end of the RNAs, followed by reverse transcription, amplification and cloning. Recent advances in next generation high throughput single molecule sequencing platforms have eliminated the actual cloning step but still relie to a greater or lesser extent on the same upstream methods. Figure adapted, with permission, from Berezikov et al. [19].

allows for subsequent 5′ adaptor joining was believed to represent the universal state of small RNAs in vivo. In 2007, Pak and Fire [29] announced that this is not the case. Attempts to clone a specific small RNA in *C. elegans* called Cel-1 repeatedly failed even though there was ample evidence that it existed. Their persistence in uncovering the reason for Cel-1 being refractory to conventional small RNA cloning methods paid off in their discovery that Cel-1, and, now, other small interfering RNAs, was tri-phosphorylated on its 5′ end [29]. They developed an alternative method for cloning troublesome RNAs featuring the use of *two* 3′ ligations with the reverse transcription step in between the two ligations. This alternative method, named by them 5′ Ligation Independent Cloning, is completely indifferent to the state of the 5′ end of the target RNAs. The reverse transcription step following the initial 3′ adaptor ligation makes the initial 5′ end the new 3′ end with a hydroxyl group ready for a second 3′ ligation step regardless of what may or may not have been present on that initial 5′ end. The 5′ Ligation Independent Cloning option revealed that a secondary pool of small RNAs was being produced in *C.elegans* via a completely different pathway from conventional miRNAs [29].

3. Cloning with Adenylated Linkers

While each small RNA cloning strategy has its own strengths and weaknesses, the method employing a preactivated, adenylated 3′ linker sequence, pioneered by David Bartel [10], has proved to be a readily accessible and flexible method. The adenylation of the 5′ end of a DNA oligonucleotide provides a preactivated linker that will specifically ligate to the 3′ hydroxyl group of RNA in the presence of the enzyme T4 RNA Ligase. This reaction proceeds in the absence of ATP, which is known to promote circularization of the target RNAs in solution. The 3′ end of the preactivated linker is blocked with a nonstandard base, such as dideoxycytidine (ddC), to prevent circularization of the linker. The synthesis and ligation reactions are shown in Figure 4. The synthesis reaction begins with an deoxyoligonucleotide synthesized with a 3′ block, such as ddC, and a 5′ phosphate. Adenylation at the 5′-end of the oligonucleotide is achieved through the introduction of adenosine 5′-phosphorimidazolide in the presence of magnesium chloride as the catalyst.

Once purified, the linker, with the form rApp-(dNTP)n-ddC, will react with the free 3′ hydroxyl of an RNA in the presence of T4 RNA Ligase and the absence of ATP to create a 3′-linkered RNA plus AMP. This reaction is quite efficient so long as a relatively small mass of T4 RNA Ligase is used. Aravin and Tuschl [26] showed that the enzyme itself in commercial preparations of T4 RNA Ligase is adenylated and that this can cause circularization of the target RNA species and other unwanted side reactions that severely reduce production of the desired ligation product. A truncated T4 RNA Ligase called T4 RNL-2 truncated, that specifically and efficiently ligates adenylated linkers to RNAs in the absence of ATP without producing side reactions is available from New England BioLabs [25–27]. A number of preadenylated 3′ linkers are now commercially available. New England BioLabs offers one with a 3′ amino block and Integrated DNA Technologies (IDT) offers three linkers, each with a 3′ ddC block.

Once the target small RNAs are 3′ ligated, any unligated linkers are removed by a denaturing polyacrylamide gel electrophoresis (dPAGE) purification of the ligated material. As with initial small RNA enrichment, gel purification of the ligated RNAs is subject to substantial loss of material. One way to significantly reduce this loss is to process the acrylamide gel slice containing the RNAs using a column originally developed by Edge Biosystems for cleaning up Sanger dye terminator cycle sequencing reactions. Called Performa Columns, these spin columns will retain the acrylamide gel, salts, and urea while passing as much as 95% of the RNA into the collection tube (see the appendix). The 3′-linkered RNAs so recovered will have a 3′ end block courtesy of the linker but will retain their 5′ phosphate

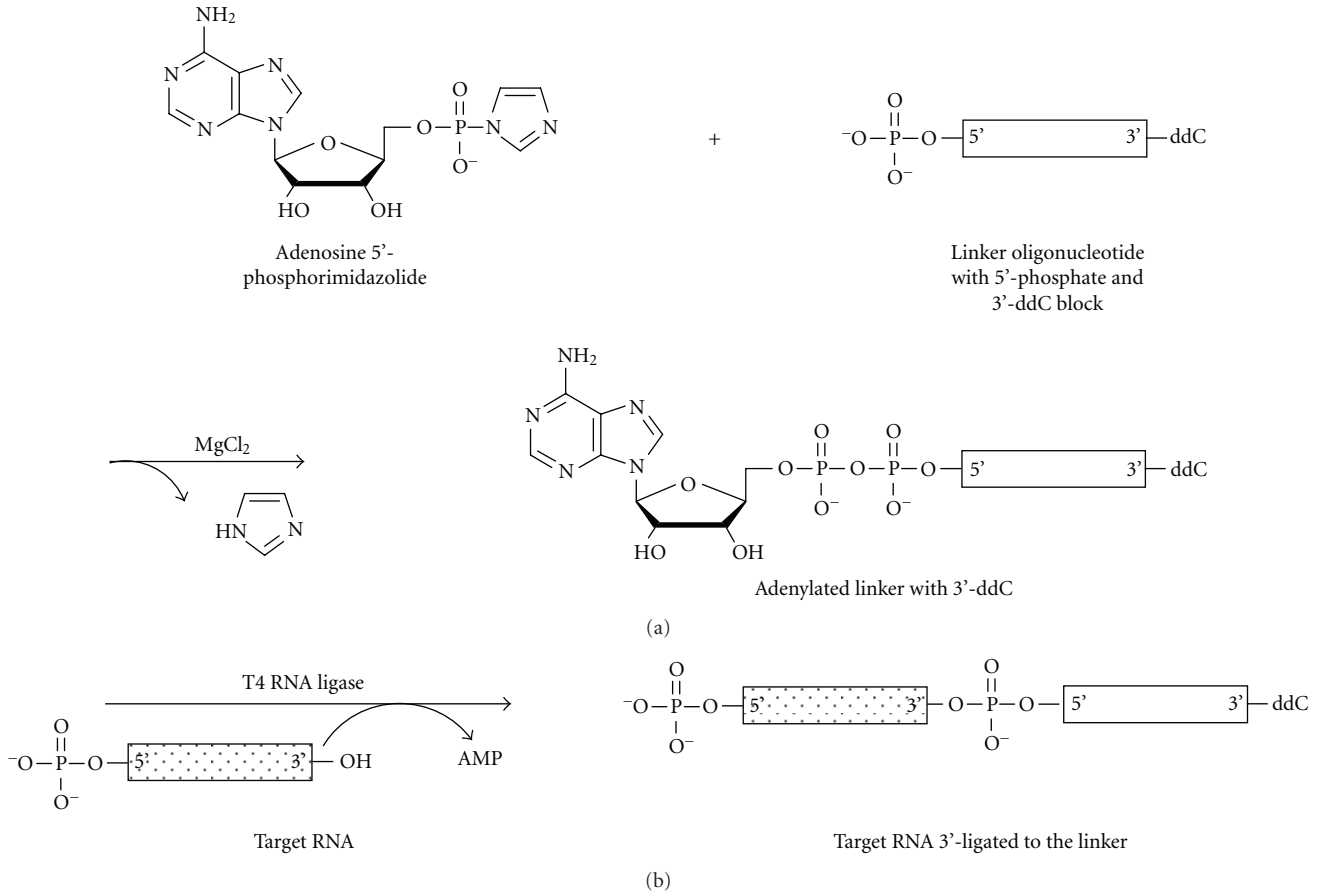

Figure 4: Synthesis and ligation of high efficiency 3′ adenylated cloning linkers. (a) An adenosine 5′-phosphorimidazolide is attached, in the presence of magnesium chloride, to a synthetic deoxyribo-oligonucleotide bearing a dideoxycytidine (ddC) block on its 3′ end and a free, reactive phosphate group on its 5′ end. (b) The synthetic, preactivated 3′ linker is ligated to target small RNAs in the presence of T4 RNA Ligase. This reaction is carried out with high efficiency in the absence of ATP to prevent circularization of the target RNA species prior to ligation. Reaction energy is provided by the phosphorimidazolide at the 5′ end of the linker.

groups. This provides a coupling group for ligation of an oligonucleotide composed of a few 5′ DNA bases and a run of 3′ RNA bases that will ligate to the target RNAs in the presence of T4 RNA Ligase and ATP. Again, a commercial 5′ linker, called 5′ MRS, is available from IDT that is compatible with each of their 3′ linkers as well as the NEB 3′ linker.

Doubly-ligated RNAs are converted into an all DNA substrate by reverse transcription using an RT primer complementary to the 3′ linker. These cDNAs are then amplified in a PCR reaction that uses the RT primer as the reverse PCR primer and a forward PCR primer compatible with the 5′ linker. Thus, all target RNAs can be amplified for subsequent cloning using a universal PCR primer pair. Following PCR amplification the target-containing amplicons can be cloned with any one the vector systems designed for PCR cloning.

4. Sequencing Strategies

The generally accepted criteria for adding a new miRNA to the ever growing catalog being ably curated in miRBase [30, 31] are that the sequence of the mature 21 to 23 nt candidate is not already present among extant miRNAs, that the sequence is expressed, and that there is flanking sequence ranging in size from 60 to more than 100 nt that, with the mature sequence inside, forms a thermodynamically stable hairpin secondary structure [19, 32]. Direct cloning and sequencing from an enriched pool of small RNAs satisfies the first two of these three criteria at the same time. For this reason, sequencing is obviously a crucial part of miRNA cloning and, given that there are usually hundreds of small RNAs being expressed at various levels in tissues of interest, the more efficiently that clones can be sequenced, the better the chances of discovering new candidates. In the world of Sanger-type, dye terminator sequencing a solution is available. This solution makes use of the simultaneous sequencing capabilities of multi-capillary platforms like the GE Healthcare MEGABACE or the ABI 3730xl 96-capillary machines. On these platforms small RNAs can be sequenced either as single insert shot-gun clones (e.g., [33]) or as concatamers as shown in Figure 3. This is clearly an improvement over any previously available method but

Table 1: Examples of Roche (454) fusion primer sequences and a set of simple bar-coded Roche (454) fusion primer sequences based upon the **3′** and **5′** linkers in the IDT miRCat Small RNA Cloning Kit.

miRCat linker-specific PCR primers:	
Forward	5′-TGGAATTCTCGGGCACC-3′
Reverse	5′-GATTGATGGTGCCTACAG -3′
Roche (454) fusion primers:	
Forward adaptor A	**5′**-GCCTCCCTCGCGCCATCAGTGGAATTCTCGGGCACC-**3′**
Reverse adaptor B	**5′**-GCCTTGCCAGCCCGCTCAGGATTGATGGTGCCTACAG-**3′**
Simple fusion primer bar-coding scheme (6 of 256 possible sequences):	
5′-GCCTCCCTCGCGCCATCAGGTACTGGAATTCTCGGGCACC-3′	
5′-GCCTCCCTCGCGCCATCAGCGATTGGAATTCTCGGGCACC-3′	
5′-GCCTCCCTCGCGCCATCAGTCGATGGAATTCTCGGGCACC-3′	
5′-GCCTCCCTCGCGCCATCAGATGCTGGAATTCTCGGGCACC-3′	
5′-GCCTCCCTCGCGCCATCAGCCTCTGGAATTCTCGGGCACC-3′	
5′-GCCTCCCTCGCGCCATCAGTGGATGGAATTCTCGGGCACC-3′	

one of the most important technological advances of the post-genome era is the development of several Massively Parallel Signatures Sequencing (MPSS) [34] systems that not only produce several orders of magnitude with more quality sequences per run but also allow researchers to skip the actual cloning steps in Figure 3 altogether.

The first of the massively parallel sequencing systems to arrive on the scene was the Roche pyrosequencing platform originally developed at 454 Life Sciences [35]. This platform utilizes the phenomenon of pyrophosphate release that accompanies nucleotide incorporation to initiate a light detection reporting system based on the cleavage of oxyluciferin by luciferase [36]. The nucleic acids to be sequenced are sequestered in micron-sized emulsion PCR "reactors" following ligation of 5′ and 3′ adaptors that serve as the universal templates for clonal amplification inside the reactors. Universal adaptor ligation and subsequent clonal amplification provide an ideal opportunity to feed 5′ and 3′ ligated small RNAs directly into the sequencing flow by making "fusion primers" that incorporate both the RNA linker and Roche (454) adaptor sequences. These fusion primers would be 40-mers composed of the Roche (454) 5′ adaptor plus the 5′ linker sequences on one end and the 3′ linker plus the Roche (454) 3′ adaptor sequences on the other end (Table 1). These primers would then be used to amplify directly from the reverse transcript cDNAs. In addition, these primers can be "barcoded" so that mixed RNA populations could be simultaneously sequenced and the sequences deconvoluted later based upon the barcodes (Table 1). Similar models have already been successfully used [37, 38]. The performance obtained by the Roche 454 Life Science commercial system Genome Sequencer (GS-FLX) platform of 99.5% accuracy and average read lengths of over 250 bp resulting in outputs exceeding 200 000 reads with acceptable Phred values (a DNA sequence quality score) is ideal for searching genomes for new small RNAs and, indeed, such studies have already resulted in the discovery of the curious 21U RNA class of small RNA in *C. elegans* [39]. According to the latest updates, current 454 FLX platform is capable of sequencing 400–600 million high-quality bases in ten hours with an average of ~ 400 bp long reads and a raw base accuracy of 99% (http://www.454.com/products-solutions/system-features.asp; [40]). This makes the 454 FLX platform with several hundred times higher throughput compared to the current state-of-art Sanger-based capillary sequencing system. However, current limitations of this platform compared to Sanger system are relatively shorter read length as well as challenges with sequencing of homopolymer regions. The latter limitation is due to nonterminating chemistry during pyrosequencing that introduces nucleotide substitution errors [41].

Another of the next generation sequencing platforms, based on a four-color DNA sequencing-by-synthesis (SBS), introduced by Illumina/Solexa (http://www.solexa.com/), also incorporates the use of oligonucleotide adaptor ligations to produce millions of short, ligated nucleic acid fragments that are then covalently bound to a solid surface and ultimately interrogated by reversible fluorescent terminator synthesis reactions [36, 41, 42]. In comparison with the current 454 FLX platform, Illumina/Solexa platform has a higher throughput sequencing capability that equals to 1–1.5 billions of 35 bp reads per run [41]. The read length is well suited to the 21 to 31 nt size range of the so-far known small RNA classes. Although 454 FLX and Illumina/Solexa platforms utilize the same SSB sequencing principle, the sequencing chemistries (pyrosequencing versus fluorescent-based solid phase) and consequently the limitations of two systems are substantially different [41]. The major limitation of the Illumina/Solexa platform with regard to small RNA applications is also the potential for nucleotide substitution errors though the use of fluorescent-based solid phase dye terminators makes homopolymeric runs less problematic [41].

Also in the small RNA size range of read lengths is the Applied Biosystems' Sequencing by Oligo Ligation and Detection (SOLiD) platform. SOLiD is the combination

of MSSP and polymerase colony (polony) sequencing [41, 42, 44, 45] that creates emulsion PCR generated clonal amplicons on 1 μm magnetic bead from genomic fragments. Sequencing-by-ligation is carried out on enriched beads through the repeated cycles of ligation of mixture of sequencing and 8-mer fluorescently labeled oligonucleotide probes to the amplicons and detecting the color [36, 42, 45]. The SOLiD system delivers 1–3 billion bases read per run or 200–300 million bp sequence data per day with 25 to 35 bp lengths and a raw base accuracy of 99% [41, 42]. This comparatively higher throughput level of SOLiD system is achieved by using smaller beads and random array format compared to 454FLX system (26 μm and ordered format). However, similar to the Illumina/Solexa system, there is a potential for incorporating substitution errors and with the shorter read lengths these can be misleading when sequencing small RNAs [41].

Although yet-unavailable for many small scale molecular biology laboratories with limited funding constraints, these new generation sequencing platforms are already being widely used by plant researchers to characterize plant small RNAs. A pioneer MPSS effort has revealed more than 2 million small RNAs from flower and seedling tissues of model plant *Arabidopsis thaliana*, yielding over 75 thousand distinct sequence signatures [46]. The small RNAs in various Arabidopsis [47, 48] and maize [49] mutant backgrounds were deep sequenced and characterized. Recently, small RNA/miRNA pools in rice were characterized using these next generation sequencing platforms [50, 51]. Chellappan and Jin [52] published an excellent review of small RNA cloning and discovery methodology in plants and have compared the deep parallel sequencing of small RNA libraries using aforementioned 454, Illumina/Solexa, and SOLiD technologies.

In general, all of the next generation sequencing technologies offer unprecedented sequencing depth in a very short time. The power of these platforms is that they are only capable of finding all or nearly all of the small RNAs expressed in a particular tissue but they can do so in a quasiquantitative manner due to the enormous number of sequence reads generated, dramatically reducing the cost. However, since next generation sequencing platforms are still under development and most likely will be improved for higher throughput and accuracy at reduced cost, at present, the suitability of any particular platform for small RNA sequencing comes down to study objectives and the availability of the platforms.

5. Application: Cotton Small RNAs

There are many excellent methods available that utilize known microRNA sequences for the purpose of determining both absolute and relative expression levels in various tissues and under various conditions. These methods primarily focus upon either quantitative, or real-time, PCR or microarray hybridizations. However, as noted above, the primary objective of small RNA cloning is different, it is discovery of both new miRNAs and new classes of small RNA. In this final section, we will briefly present results that we have obtained using an adenylated cloning linker strategy (refer to [33, 53] for detailed protocol) to investigate the pool of small RNA signatures and discover plant small RNAs in root tip and developing ovule tissues of a widely grown Upland cotton *G. hirsutum* L. These results are initial surveys, but the first effort of "wet-bench" works toward studying the small RNA world for a complex "still unsequenced" allotetraploid cotton genome.

The genus *Gossypium* L. includes approximately 45 diploid A-G to K genomic groups [54] and 5 allotetraploid (AD_1–AD_5 lineages formed by A- and D-genome hybridization about 1-2 million years ago) species [55]. The genomes of allotetraploid cottons have a chromosome complement of $2n = 4X = 52$, a haploid genome size of 2200–3000 Mb DNA, and a total recombination length of approximately 5200 cM (an average of 400 kb per cM) [56]. Accordingly, allopolyploid cotton genomes are one of the largest plant genomes with its complex nature, and are an important model system to study fundamental biological studies in plants [57]. Furthermore, cotton fiber is regarded as a unique single-celled model system to study cell growth initiation, elongation, differentiation and cellulose biosynthesis in plants [57–59].

As of February 2009, a search of the GenBank nucleotide database for *Gossypium* revealed a total of 452, 634 nucleotide sequences, corresponding to an 8, 239 core subset of nucleotide, 375, 447 Expressed Sequence Tag (EST), and 68948 Genome Survey sequence (GSS) records (http://www.ncbi.nlm.nih.gov; searched on February 16, 2009). Efforts toward sequencing entire cotton genome(s) are in progress [55] and the smallest genome, *G. raimondii* (D_5), will soon be completely sequenced and available for researchers [60]. Nevertheless, one of the major present sources of cotton genomic sequences, available through GenBank, only corresponds to an 11.4 Mb of cotton genome [57]. This is a serious obstacle for systematically searching the cotton genome for small RNA/microRNA signatures although several investigators have reported initial efforts to identify these tiny elements in cotton using *in silico* bioinformatics analysis [61–63]. This underlies the necessity for wet laboratory cloning of cotton small RNA sequences for de novo discovery of unique small RNAs and microRNAs from various tissues in cotton, which then subsequently will be validated with availability of a complete DNA sequence of cotton genome(s) [33].

Using the adenylated cloning linker strategy outlined above, we have conducted an initial survey of small RNA content in the 3–5 days old root tip tissue of Texas-Marker-1 (*G. hirsutum* standard line) and sequenced ~300 individual colonies with the **3′** and **5′** specific linker ligated small RNA inserts [64]. Our sequencing efforts have confirmed 20 microRNA signatures from 8 families including miR-156 (7), miR-156* (1), miR-166 (4), miR-167 (1), miR-168 (1), miR-169 (2), miR-171 (2), miR-396 (1), and miR-457 (1), suggesting their involvement during early root development of cotton seed germination process (Figure 5). These very abundant micro-RNAs have known targets including transcription factor and stress response genes in other plants, and miR-156 and miR-166 are considered two

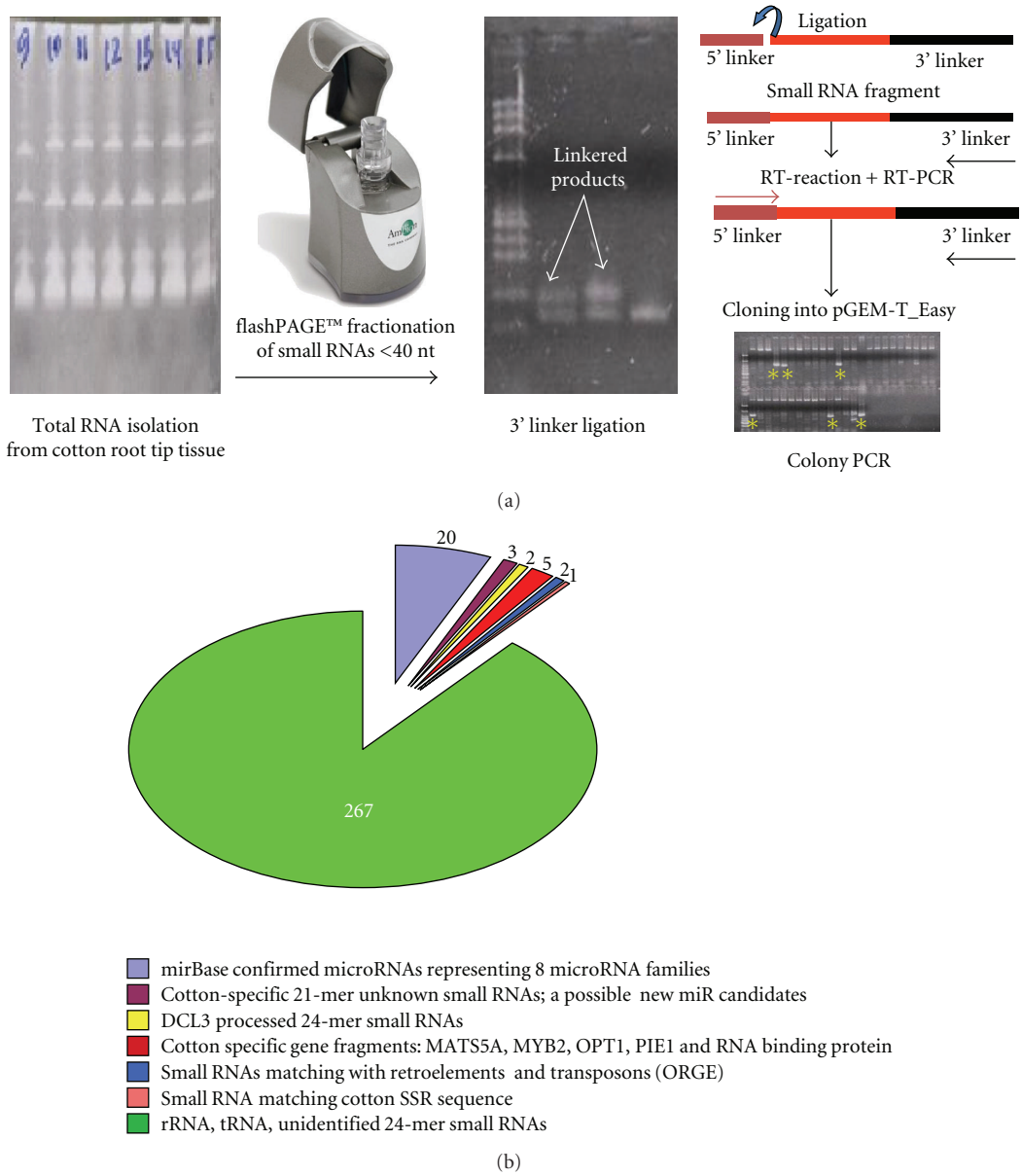

Figure 5: Size-directed cloning of small RNAs from cotton root tips: (a) cloning procedure stages from a total RNA isolation, small RNA fractionation, 3′ and 5′ linker ligation, and sequencing; (b) annotation of cotton root tip small RNA pools where specific group of small RNAs is color-coded for simplicity.

of the largest and oldest miRNA families in plants [65]. In addition, we found several unidentified 21-mer small RNAs that possibly have a potential to be cotton-specific microRNAs. We also have several 24-mers that match DCL3 processed small RNAs in Arabidopsis and many unidentified 24-mers that might also be DCL3 processed small RNAs in cotton. Moreover, we found several gene-specific fragments. Two (+/−) gene hits that are notable are the Ashbya gossypii OPT1 gene and a hit on MYB2. Thus, the results of our initial attempts using size-directed small RNA cloning strategy demonstrated that the cloning method does work for finding small RNAs/microRNAs in cotton. They also confirmed the difficulty of finding plant microRNAs since we only have 20 microRNAs, representing only 8 loci, in more than 300 sequenced clones from cotton root tissue small RNA library.

Recently, using the same size-directed small RNA cloning strategy with adenylated linkers, we have characterized [33] the small RNA sequence signatures in eleven postanthesis (DPA) periods of fiber development (0–10 DPA) (Figure 6). Sequencing more than 6500 individual colonies from 11 ovule small RNA libraries, we identified nearly 2500 candidate small RNAs comprising of 583 unique sequence signatures of 21–24 nt size range. As reported by Abdurakhmonov et al. [33], results showed (1) the presence of only a few mirBase-confirmed plant microRNAs (miR172, miR390 and ath-miR853-like), and these were differentially represented

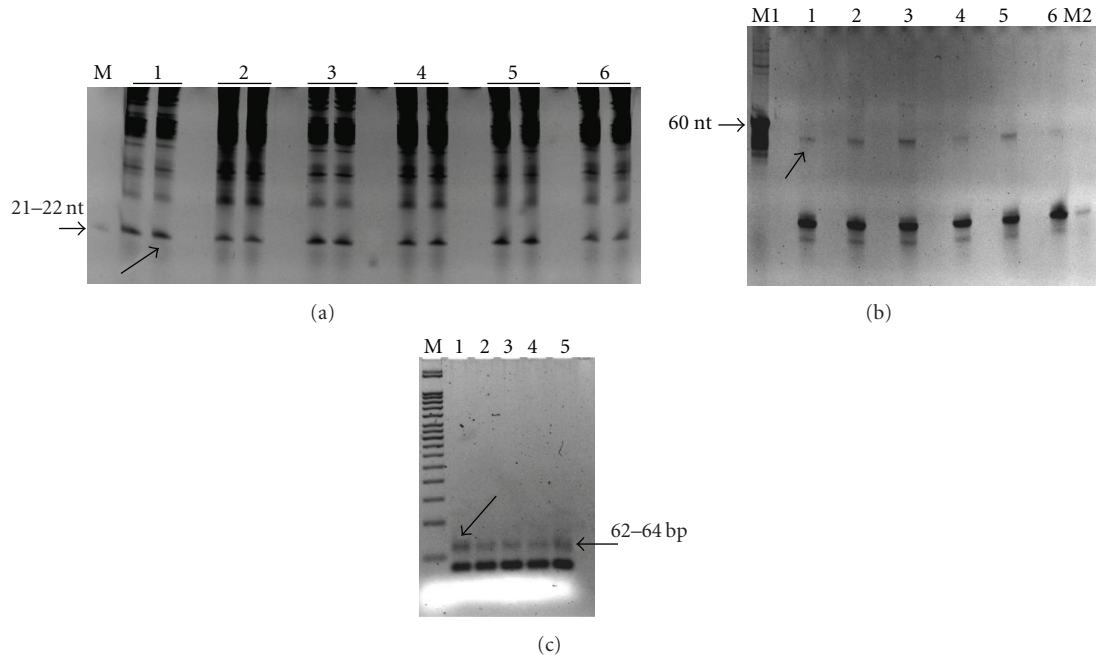

Figure 6: Isolation and cloning of small RNAs from cotton ovule tissue libraries [33]: (a) the example of 15% denaturing PAGE electrophoresis of total RNA from developing ovules at different DPA (0 to 6), spiked with 10 pmoles of the miSPIKE (Integrated DNA Technologies) 21-mer control RNA, M-21 nt RNA size control; (b) the example 15% denaturing PAGE electrophoresis of 3′ end linkering reaction for small RNAs from developing ovules at different DPA (0 to 6), M1–62 nt RNA size control, M2–21 nt small RNA size control; (c) 2% high-resolution agarose gel picture where RT-PCR product of 3′ and 5′ end linker ligated small RNAs of ovules was loaded, M-50 bp size ladder. Arrows indicate the small RNA fraction in (a), and linker ligated small RNA products ((b) and (c)).

in specific DPA periods of ovule development. (2) The vast majority of sequence signatures were expressed in only specific DPA period and this included nearly all of the 24 nt sequences, Further, they showed (3) the existence of specific pattern of sequence diversity and abundance between 0–2 to 3–10 DPA periods, possibly corresponding to the transition of fiber initiation to elongation phase of fiber development. Further, target predictions *in silico* using ovule-derived small RNA sequences putatively indicated their involvement in numerous important biological processes including processes involving previously reported fiber-associated proteins (Figure 7). Results collectively demonstrate that the initiation and elongation stages of cotton fiber development are at least partially regulated by specific sets of small/microRNAs [33]. However, to get a better picture of cellular mechanisms of small RNA network during fiber development process, there is urgent need for so-called "deep sequencing" efforts of small RNA pools using next generation sequencing platforms [36, 49] that will undoubtedly increase multi-DPA representation of small RNAs.

6. Conclusions

The discovery of the world of small, regulatory RNAs has provided geneticists with a phenomenal array of opportunities as well as questions. This discovery has also led to the development of a powerful set of new molecular tools that can be used to answer those questions and take full advantage of those opportunities. The techniques built around RNA interference, real-time PCR, and microarrays allow an unprecedented level of precision in unraveling the mechanisms of gene expression and regulation. So, too, have the developments in small RNA cloning and next generation DNA sequencing discussed here opened previously barred windows on genome organization that will continue to feed into the functional genomics pipeline. The size-directed small RNA cloning strategy using adenylated linkers, highlighted with its application for the "yet-unsequenced" cotton genome small RNA characterization, is an efficient methodology for studying these tiny molecules in various plant genomes, especially suitable for the "small-scale" plant genome laboratories worldwide, that lack access to the still-expensive next generation sequencing platforms.

Appendix

A. RNA Recovery from Denaturing PAGE Using DTR Columns

(1) Run total RNA spiked with 10 pmoles of the miSPIKE (Integrated DNA Technologies) 21-mer control RNA on a 12% to 15% denaturing PAGE (7 M Urea) for 90 minutes at 275 V (be sure to monitor the gel so that the small fragments do not run off).

(2) Stain the gel with GelStar nucleic acid stain (Lonza Cat. No. 50535) and place on uV light box.

FIGURE 7: Annotation of biological processes targeted by abundant copy (>5 copies) candidate siRNAs of developing ovules in cotton. To better visualize the specific and overlapping putatively targeted proteins at 0 to 10 DPA ovules, Cytoscape [43] was used to generate genetic interaction networks of putative targets at different DPA stages of ovule, where each node (DPA) and its edges (targeted proteins) were colored. The interaction networks were depicted using Cytoscape's "spring embedded layout algorithm" for both full protein target dataset and protein groups targeted by only abundant copy candidate siRNAs, importing the "simple interaction format (SIF) files" into Cytoscape. SIF files were created based on specific and overlapping target protein information for 0–10 DPA ovule stages [33].

(3) Select RNA fragment(s) to be purified and cut it (them) from the gel as shown in Figure 8.

(4) Place the gel slice in a 1.5 mL tube and crush with a glass rod. (Note: we have had very good results using the 1.5 mL tubes and disposable pestles from Kontes Glass Company.)

(5) Add 200 μL IDT sterile, nuclease-free water and continue to crush the gel into a fine slurry. Place the tube at 70°C for 10 minutes.

(6) Following manufacturer's recommendations, prepare a Performa DTR column for each gel slice.

(7) Vortex the gel slurry, transfer the entire volume onto the column and spin at 3000 rpm for 3 minutes.

(8) Discard the DTR column.

(9) Add 3 μL 10 mg/ml glycogen, 25 μL of 3M NaOAc (pH5.2), and 900 μL ice cold 100% EtOH to the eluent. Mix by inversion and place at −80°C for 20 minutes.

(10) Spin tubes at full speed (≥10 000 rpm) for 10 minutes to pellet the RNA. Pour off the supernatant and dry the pellet.

(11) Proceed to next procedure/application (e.g., miRCat protocol).

This protocol successfully removes the Urea and other salts with substantially less loss of RNA than is seen with conventional crush and soak methods followed by NAP-5 column desalting or by dialysis methods. Detail list of small RNA cloning products and protocol for miRCat can be found from IDT product manual at (http://www.idtdna.com/Support/Technical/TechnicalBulletinPDF/miRCat_User_Guide.pdf).

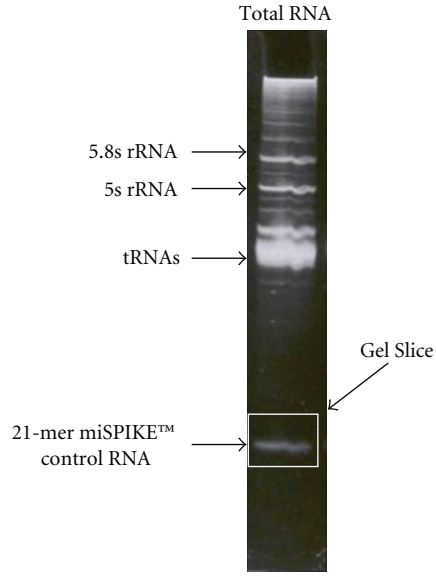

Figure 8

Acknowledgments

Cotton small RNA characterization research was funded by Academy of Sciences of Uzbekistan under research Grant 4F-P-149. The authors are grateful to the United States Department of Agriculture/Agricultural Research Services (USDA/ARS)—Former Soviet Union (FSU) Scientific Cooperation Program, Office of International Research Programs, USDA/ARS for financial support of cotton genomics research in Uzbekistan. The authors thank anonymous reviewers of the manuscript for valuable suggestions.

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Hindawi Publishing Corporation
International Journal of Plant Genomics
Volume 2009, Article ID 198680, 8 pages
doi:10.1155/2009/198680

Review Article

Virus-Induced Gene Silencing, a Post Transcriptional Gene Silencing Method

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Received 1 December 2008; Accepted 30 March 2009

Recommended by Chunji Liu

Virus-induced gene silencing (VIGS) is one of the reverse genetics tools for analysis of gene function that uses viral vectors carrying a target gene fragment to produce dsRNA which trigger RNA-mediated gene silencing. There are a number of viruses which have been modified to silence the gene of interest effectively with a sequence-specific manner. Therefore, different types of methodologies have been advanced and modified for VIGS approach. Virus-derived inoculations are performed on host plants using different methods such as agro-infiltration and in vitro transcriptions. VIGS has many advantages compared to other loss-of-gene function approaches. The approach provides the generation of rapid phenotype and no need for plant transformation. The cost of VIGS experiment is relatively low, and large-scale analysis of screening studies can be achieved by the VIGS. However, there are still limitations of VIGS to be overcome. Nowadays, many virus-derived vectors are optimized to silence more than one host plant such as TRV-derived viral vectors which are used for *Arabidopsis* and *Nicothiana benthamiana*. By development of viral silencing systems monocot plants can also be targeted as silencing host in addition to dicotyledonous plants. For instance, Barley stripe mosaic virus (BSMV)-mediated VIGS allows silencing of barley and wheat genes. Here we summarize current protocols and recent modified viral systems to lead silencing of genes in different host species.

1. Introduction

Gene silencing at posttranscriptional level, posttranscriptional gene silencing (PTGS), is an RNA-mediated systemic silencing mechanism which was described as quelling in fungi [1] and RNA interference in animals [2]. To specifically silence or knock down the expression of targeted gene in plants several approaches of PTGS have been developed. Virus-Induced Gene Silencing (VIGS) is one of these tools to suppress expression level of the gene of interest in plants [3, 4]. The term VIGS was first coined by A. van Kammen to describe the resistance event against viral infection [5]. Plants infected by many viruses induce RNA-mediated defense which targets viral RNAs and any transgene RNA products inserted into it [6]. As a gene silencing method VIGS has several advantageous such as fast, transient suppression of gene expression, and it involves cloning of short sequence fragments of targeted gene to be silenced. As a reverse genetic approach VIGS provides silencing of target gene in sequence specific manner. RNA-induced gene silencing mechanism is also acting on VIGS in which 21–25 nucleotide sequence of small interfering RNAs (siRNAs) guides specific cleavage or suppression of target mRNAs at posttranscriptional level [2, 7]. siRNAs which are processed from long double-stranded RNAs (dsRNA) by DICER, an RNAse-like enzyme, are then incorporated into RNA-induced silencing complex (RISC). This complex with siRNA targets specific mRNA transcripts having sequence complementarity with the specific siRNA. In other words the antisense strand of the siRNA associates with the RNAi silencing complex (RISC) to target homologous RNA for degradation [8]. dsRNAs may be originated in infected plant during cytoplasmic replication of positive-sense single-stranded (ss)RNA viruses and in the case of replicative form and replicative intermediates may represent the pool of dsRNAs [6]. For transgenes dsRNA may be generated by host RNA dependent

RNA polymerases (RdRp) [9]. To be a PTGS inducers transgenes also designed and constructed to produce dsRNA [10].

2. Development of VIGS Methodology

Some virus species were previously modified and used for silencing the gene of interest (Table 1). Tobacco mosaic virus (TMV) is one of the modified viruses which was used for effective *pds* gene silencing in Nicotiana benthamiana plants [11]. TMV is the first modified virus for application of VIGS methods to plants. The viral delivery leads downregulation of transcript of target gene through its homology dependent degradation so potential of VIGS for analysis of gene function was easily recognized [3]. Thomas et al. detected the minimum length of RNA for PTGS. A minimum of 23 nucleotide possessing 100% homology to the target gene was observed to be required but not enough for efficient PTGS, and longer identical sequence is needed to initiate silencing [12, 13]. Tobacco rattle virus (TRV) was also modified to be a tool for gene silencing in plants. VIGS has been effectively applied in *N. benthamiana* [14] and in tomato [15] by using TRV vectors. The significant advantage of TRV-based VIGS in *Solanaceous* species is the ease of introduction of the VIGS vector into plants. The VIGS vector is placed between Rigth Border (RB) and Left Border (LB) sites of T-DNA and inserted into *Agrobacterium tumefaciens* [15, 16]. Another property of TRV is the more vigorous spreading all over the entire plant including meristem, and infection symptoms of TRV are mild [15]. Modified TRV vectors such as pYL156 and pYL279 have strong duplicate 35S promoter and a ribozyme at C-terminus for more efficient and faster spreading. These vectors are also able to infect other plant species [13, 14]. TRV-based vector has been used by Liu et al. for gene silencing in tomato [14]. Dalmay et al. have also used TRV-based VIGS to silence gene in *A. thaliana* [9]. Burch-Smith et al. [17] have developed an efficient TRV-based VIGS method to silence the *A. thaliana* genes with minimal modification of widely used TRV-based VIGS technique. Very recently, Pflieger et al. [18] have shown that a viral vector derived from Turnip yellow mosaic virus [TYMV) has the ability to induce VIGS in *Arabidopsis thaliana*. VIGS of *N. benthamiana* using Potato virus X (PVX) was also achieved [19]. PVX-based vectors have more limited host range (only three families of plants are susceptible to PVX) than TMV-based vectors (nine plant families show susceptibility for TMV) but PVX-based vectors are more stabile compared to TMV [20].

Geminivirus-derived vectors can be used for VIGS studies especially to study function of genes involved in meristem function. Tomato golden mosaic virus (TGMV) was used to silence a meristematic gene, proliferating cell nuclear antigen (PCNA) in *N. benthamiana* [34]. The TGMV-based silencing vector had been used for also silencing of nonmeristematic gene silencing [39]. Satellite-virus-based vectors are also used for efficient gene silencing in plants only with the help of other helper viruses. This two-component system is called Satellite-virus-induced silencing system, SVISS. In a study Tomato yellow leaf curl China virus being helper and a modified satellite DNA ware used to silence gene in *N. benthamiana* [38]. There are other viruses modified for silencing of dicotyledonous plants such as *African cassava mosaic virus* in cassava [37], *Pea early browning virus* in pea [29], and *Bean pod mottle virus* in soybean [28].

Previously barley stripe mosaic virus (BSMV) was developed for efficient silencing of *pds* gene in barley [26]. This system was then used for silencing of wheat genes [27]. BSMV is a positive sense RNA virus containing a tripartite (α, β, γ) genome. The modified γ of BSMV genome replaced by DNA vector was used for plant gene cloning. β genome has been deleted for viral coat protein production defect. Each of the modified DNAs is used to synthesize RNAs by in vitro transcription. Recently, Brome mosaic virus strain has been modified for VIGS of *pds*, *actin,* and *rubisco activase*. These genes were also silenced in important model plants such as rice [33].

3. Methods Used in VIGS

3.1. PVX (Potato Virus X)-Derived VIGS for Potato Silencing. PVX is RNA virus and infects broad range of solanaceous plants. A PVX derivative vector, an agroinfection vector, pGR106, has been previously constructed for gene silencing [19]. The vector was also used for the PVX-mediated VIGS in leaves and tubers of potato plants [21].

3.1.1. Construction of PVX-Derived Vectors. PVX.GFP and PVX.PDS_{AS} can be constructed via PCR-based cloning using specific oligonucleotide primers incorporating *Asc*I and *Not*I restrictions sites, respectively, at the 5′- and 3′-termini into pGR106, a PVX derivative vector (Sainsbury Laboratory, Norwich, UK).

3.1.2. Agrobacterium Tumefaciens Transformation. Transformation procedure can be followed as outlined previously [40]. *A. tumefaciens* strains (such as LB4404 and GV3101) should be prepared, and 500 mL of SOB medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl) in a flask should be inoculated with 1.0 mL of an overnight culture of bacteria for 6 hours at 28°C with shaking till OD550 reaches 0.7. The culture then chilled on ice for 30 minutes. The cells should be harvested at 6000 rpm for 10 minutes at 4°C. The pellet will be washed four times with 200 mL 10% glycerol (90% sterile water). The final re-suspension can be made with 0.5 mL in ice cold 10% glycerol. The prepared competent cells can be used immediately or stored at −80°C in small aliquots. Transformation of electrocompetent *A. tumefaciens* cells is performed by an electroporator. A prechilled electroporation cuvette is filled with 20–30 μL electrocompetent cells and up to 5 μL ligation products and should be treated with recommended 330 μF capacitance, 4000 Ω resistance, and 380 V[1] voltage. Cells are then put into 0.5 mL of SOC medium and incubated for 1 hour with shaking (100 rpm). The transformed cells are selected via antibiotic selection on spread plates with supplemented selection [40].

Table 1: Viruses used for silencing of genes and their hosts with targeted genes are listed.

Viruse/viruse type	Silencing host species	Group	Genes silenced	Natural host species	Reference
Tobacco mosaic virus (TMV)/RNA virus	*Nicotiana benthamiana, nicotiana tabacum*	Tobamovirus	*pds*	Tomato, squash, potato, tobacco	[11]
Potato virus X (PVX)/RNA virus	*Nicotiana benthamiana, Arabidopsis*	Potexvirus	*pds*	Potato, oilseed, rape	[21, 22]
Tobacco rattle virus (TRV)/RNA virus	*Nicotiana benthamiana*, tomato, *Arabidopsi,*solanum species, chilli pepper, opium poppy, *Aquilegia vulgaris*	Tobravirus	*Rar1, EDS1, NPR1/NIM1 pds, rbcS,*	Spinach, beet, potato, tobacco	[14, 16, 23–25]
Barly stripe mosaic virus (BSMV) RNA virus	Barley	Hordeivirus	*pds, Lr21, Rar1, Sgt1, Hsp90*	Barley, wheat	[26, 27]
Bean pod mottle virus (BPMV)/RNA virus	*Glycine max*	Comovirus	*pds*	*Phaseolus vulgaris,glycine max*	[28]
Pea early browning virus (PEBV)/RNA viruse	*Pisum sativu, Medicago truncatula, Lathyrus odorata*	Tobravirus	*pspds, uni, kor, pds*	*Pisum sativum, Phaseolus vulgaris*	[29, 30]
Satellite tobaccomosaic virus (STMV)/Satellite virus	*Nicotiana tabacum*	RNA satellite virus	*pds, rbcS, rbcL* and various genes	*Nicotiana glauca*, pepper	[31]
Poplar mosaic virus (PopMV)/RNA virus	Poplar	Carlavirus	*gfp*	*Nicotiana benthamiana*	[32]
Brome mosaic virus (BMV)/RNA virus	Barley, rice, maize	Bromovirus	*pds, actin 1, rubisco activase*	Barley	[33]
Tobacco golden mosaic virus (TGMV)/DNA virus	*Nicotiana benthamiana,*	Begomovirus	*su*	Tomato	[34]
Tomato bushy shunt virus (TBSV)/RNA virus	*Nicotiana benthamiana,*	Tombusvirus	*gfp*	*Lycopersicon esculentum*	[35]
Cabbage leaf curl virus (CaLCuV)/DNA	*Arabidopsis*	Begomovirus	*CH42, pds*	Cabbage, broccoli, cauliflower	[36]
African cassava mosaic virus (ACMV)/DNA virus	*Nicotiana benthamiana, Manihot esculenta*	Begomovirus	*pds, su, cyp79d2*	*Manihot esculenta*	[37]
Tomato yellow leaf curl China Virus (TYLCV)/DNA virus	*Nicotiana benthamiana, Lycopersicon esculentum, N. glutinosa, N. tabacum*	DNAbeta satellite DNA	*pcna, pds, su, gfp*	Tomato	[38]

3.1.3. Agrobacterium Infection of Plants. *Agrobacterium tumefaciens* strain possessing helper plasmid pSoup is generally transformed with PVX.GFP or PVX.GOI using procedure described above. Agroinfiltration of *N. benthamiana* and Solanum species should be performed as follows. PVX.GOI construct containing *A. tumefaciens* culture will be grown overnight at 28°C, harvested at 3000 rpm for 20 minutes, and resuspended in the same volume of 10 mM $MgCl_2$, with 100 μM acetosyringone and 1 mM Mes, pH 5.6. The culture should be infiltrated into leaves by a syringe at lower face [40].

3.2. TRV-Derived VIGS for Arabidopsis Silencing. The most widely used viral delivery vectors are Tobacco rattle viruses (TRV, 16] because introduction of virus into plant including is easy in meristematic tissue [16]. TRV-mediated gene silencing was applied to many plants from diverse genera such as *Nicotiana benthamiana* [14, 16], tomato [15], pepper (*Capsicum annuum*; 32), potato (*Solanum tuberosum*; 33), and petunia (*Petunia hybrida*; 34) from Solanaceae family, opium poppy (*Papaver somniferum*) from Papaveraceae [23], and *Arabidopsis thaliana being* a model organism [17]. The TRV silencing in plants is usually mediated by *Agrobacterium tumefaciens.* TRV vectors pTRV1 and pTRV2 are placed between LB and RB sites separately. One of these vectors pTRV1, is constructed with GOI for targeted gene silencing (Figure 1).

3.2.1. Construction of TRV Vectors and Agrobacterium-Mediated Infiltration. The TRV vectors pTRY1 (pYL192) and pTRY2 (pYL156) have been described earlier [14], and the procedure can be followed described by Birch-Smith et al. [17]. *Xba*I-*Eco*RI-cut pTRV2 vector is ligated with *Xba*I-*Eco*RI-engineered PCR fragment of GOI and then transformed into *A. tumefaciens* GV3101 strain which is made electrocompotent (described in Section 3.1.2). The *Agrobacterium* culture transformed with both pTRV1 and pTRV2-GOI (grown in 50 mg/L gentamycin and 50 mg/L kanamycin overnight culture) and infiltrated into *Arabidopsis* leaves by pressing a syringe (described in Section 3.1.3, Figure 1).

3.3. "One-Step" TYMV-Derived Arabidopsis Silencing. Turnip yellow mosaic virus is a positive strand of RNA virus from the genus Tymovirus and infects many *Brassicaseae* including *Arabidopsis* [41]. Recently, Pflieger et al. [18] have developed a TYMV-derived vector to induce VIGS in *Arabidopsis.* The TYMV-derived vector for efficient silencing includes inverted repeats of target gene fragments. The system has ability to silence the gene even expressed in meristem and contains only a single vector. The other advantage of the TYMV mediated VIGS system that allows direct delivery of plasmid DNA to plant cells using rub-inoculation is the precluding of in vitro transcription, biolistic, and agroinfiltration steps [18].

3.3.1. Cloning of Plasmid DNAs. The plasmid pTY has been generated by Pflieger et al. [18] using full-length TYMV

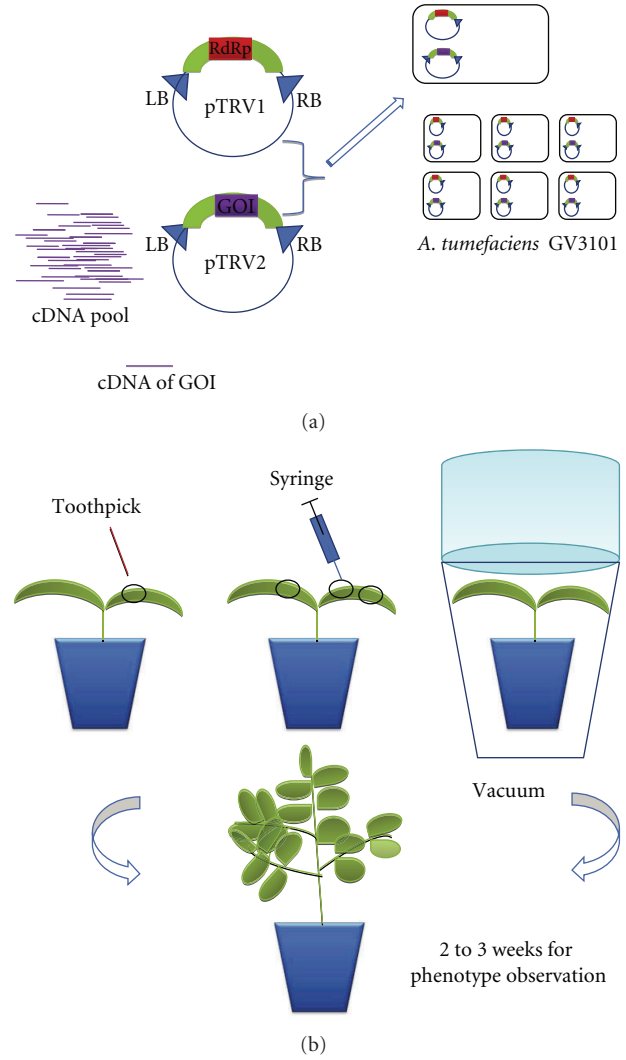

Figure 1: TRV-mediated VIGS in *N. benthamiana*. TRV-based virus induced gene silencing assay covers many steps; the gene with known sequence is first selected and then genetically engineered for cloning into pTRV2. pTRV1 consists of a TRV1-based cassette (RNA-dependent RNA polymerase gene, movement protein, etc.), LB and RB site for plant transformation. The plasmids are transformed into *A. tumefaciens*, and then agro-inoculation is applied. *Agrobacterium* can be inoculated on plant into seedling by a toothpick, a syringe and a vacuum infiltration as shown in the picture.

cDNA clone under the control of the duplicated CaMV 35S promoter and terminator. This vector can be used for efficient gene silencing by cloning the gene(s) of interest into the vector. For example, pTY-PDS52-IR can be obtained by cloning the self-hybridized palindromic oligonucleotides PDS52 into the *Sna*BI site of pTY-S.

3.3.2. Preparation and Transfection of Protoplasts. Protoplasts of *A. thaliana* can be prepared from cell suspension culture using the procedure described by [42]. A total of 106 protoplasts are transfected DNA plasmids (prepared as

in Section 3.1), using the quantities indicated. Transfected protoplasts are incubated at 24°C in the dark for 48 hours (18).

3.4. Barley Stripe Mosaic Virus (BSMV)-Mediated Silencing. The pγ.bpds4As can be used to make construction as pγ.(gene of interest, GOI)As by replacing pds4 insert with short GOI fragment applying restriction digestion. The same procedures can be followed for pγ.(gene of interest, GOI)S silencing using pγ.bpds4S as template [26] (Figure 2).

3.4.1. Barley and Wheat Pds Gene Silencing and Measurement of Silencing Levels

Linearization of Plasmids. For linearization, pα, p$\beta\Delta\beta$a, pγ, pγ.bpds4S, and pγ.bpds4As plasmids should be digested with following restriction enzymes. pα plasmid DNA is digested with *Mlu*I enzyme. To perform digestion, 10 μg purified pα plasmid DNA, 1X RE buffer, 10 U *Mlu*I enzyme, and PCR grade water are combined in a sterile eppendorf tube to a final volume of 50 μL. Mixture is incubated at 37°C for 2 hours. *Bcu*I enzyme can be used for p$\beta\Delta\beta$a plasmid DNA digestion. For digestion, 10 μg purified p$\beta\Delta\beta$a plasmid DNA, 1X, 10 U *Bcu*I are combined in a sterile tube to reach a final volume of 50 μL PCR water is used. Mixture is incubated at 37°C for approximately 2 hours. pγ plasmid can be digested with *Bss*HII enzyme. To generate linearization of pγ vectors 10 μg pγ plasmid DNA, 1X enzyme buffer, 10 U *Bss*HII enzyme, and PCR grade water are combined in a tube to handle a final volume of 50 μL. Mixture is generally incubated at 50°C for 2-3 hours. After the incubation samples should be observed on 1% agarose gel. Linearized plasmids should then be excised and purified [26, 27, 43].

In Vitro Transcription. In vitro transcription is performed for the silencing of selected target gene. It requires at least three separate in vitro transcription reactions which are the transcription of α, $\beta\Delta\beta$a, and γ linearized genomes. According to manufacturer's procedure mMessage mMachine T7 in vitro transcription kit (cat no: 1344, Ambion, Austin, TX) transcriptions are performed. Components are mixture in a sterile tube: separately for each linarized plasmids (*Mlu*I digested pα-*Bcu*I digested p$\beta\Delta\beta$a, *Bss*HII digested pγ- or *Bss*HII digested pγ.bpds4S and *Bss*HII digested pγ.bpds4As) 80 ng template is used per one silencing reaction (linearized plasmid DNA), 1X Buffer (Ambion), 1X nucleotide mix with NTP Cap (Ambion), 0.3 μL of T7 RNA polymerase mix (Ambion), and sterile distilled water are combined up to 3 μL. Mixture is incubated at 37°C for 2 hours and stored at −80°C until use [26, 27, 43].

BSMV Transcript Inoculations on Plants. Barley and wheat plants can be used for BSMV-mediated PTGS. The second leaves (approximately 7–10 days upon germination) should be inoculated with BSMV for silencing. All BSMV transcripts which are α, $\beta\Delta\beta$a, and γ will be mixed in a 1 : 1 : 1 ratio (1.0–1.5 μg of each transcript concentration is observed on spectrophotometer, Figure 2, Table 2). Transcription mix is combined with 50 μL FES. 50 mL FES requires GP solution (10X GP: (18.77 g glycine, 26.13 g K2HPO4, dd$H_2$0 upto 500 mL, sterilized by 20 minute autoclaving) which is then combined with 2.5 g sodium pyrophosphate, 2.5 g bentonite, 2.5 g celite with dd$H_2$0 up to 250 mL and re-autoclaved [44], and directly applied to the second leaf (when it is 5–7 cm long) from the bottom of leaf to the tip. After 7–10 days post inoculation (dpi), appearance of mosaic symptoms on leaves should be observed showing systemic spread of the virus. Leaves from inoculated plants are collected after approximately 14-15 day postinoculation (dpi) in order to check *pds* gene silencing level by qRT-PCR [26, 43].

4. Improvements of Virus-Induced Gene Silencing

Gene specific silencing via VIGS system is now used for diverse monocot and dicot plant species. Therefore, a number of viral-derived vectors have been developed (Table 1), and many procedures have been optimized by the researchers. TRV system was efficiently optimized for efficient silencing of Solanaceous plants [14, 15], and the system was also applied for tomato to study role of fruit ripening genes [45]. TRV-mediated VIGS has been modified for robust and effective gene silencing in a model organism, *Arabidopsis* by [17]. The emerging model plant columbine *Aquilegia vulgaris* has been efficiently silenced via TRV-mediated VIGS [24]. Many economically important plants were studied to optimize TRV-derived VIGS silencing such as opium poppy [23]. Efficiency of the TRV-derived viral vector used VIGS system on tomato fruit *via* agro-injection has been improved up to 90% silencing compared to agro-infiltration of cotyledons and first leaves of plants (66%) [46]. Lacomme et al. [47] have described a method to enhance the robustness of the VIGS phenotype by increasing the level of dsRNA by incorporation of 40–60 base direct inverted-repeats into a plant viral vector. Cheapness and easiness of *Arabidopsis* silencing have been improved *via* "*one-step*" *TYMV*-derived VIGS [18]. Monocot plants are also subjected to be silenced *via* VIGS. For this propose, Holzberg et al. [26] developed a BSMV-mediated VIGS system for barley, and Scofield et al. [27] have applied the system to wheat. BMV has also been used to silence genes in monocot plants. Ding et al. [33] efficiently silenced the genes in barley, rice and maize.

5. Comparison of VIGS with Other Gene Silencing Methods

VIGS has many advantages and disadvantages compared to other techniques used for functional analysis of plant genes. Generally, the method is chosen for its reliability, low cost, easiness, and rapidness. Several tools have been used for identification of loss-of-function of gene(s) such as, TILLING, chemical and physical mutagenesis, T-DNA, and transposon insertion techniques. However, VIGS presents an intended potential for the researchers working with

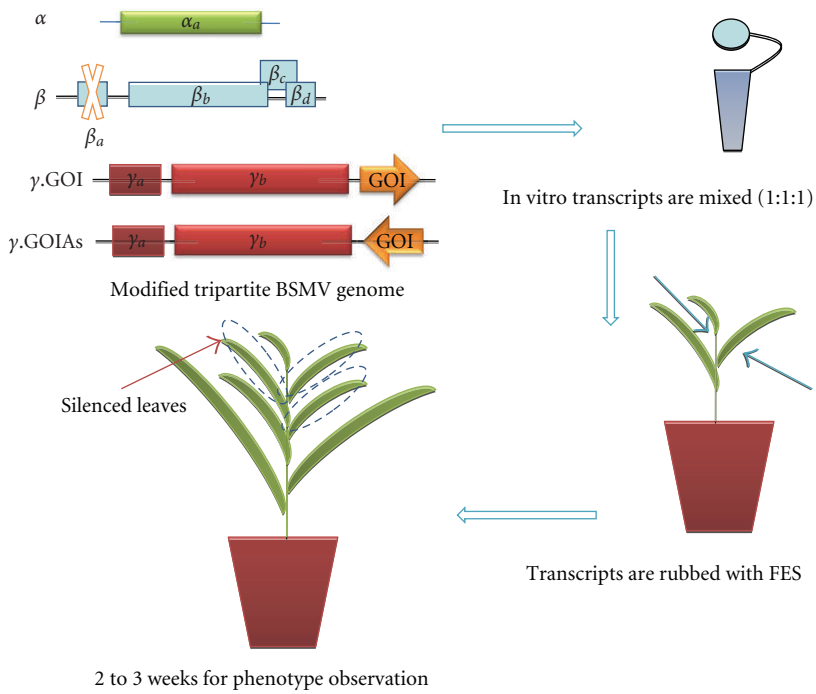

Figure 2: BSMV-mediated VIGS in barley. Barley stripe mosaic virus has a tripartite genome, and it has been modified to specific VIGS in barley plants [26, 27, 43].

Table 2: Construction of transcripts for the BSMV inoculation [26, 27, 43].

Inoculation for silencing	pα transcript	pβΔβ a transcript	pγ transcript	pγ.GOIS transcript	pγ.GOIAs transcript	FES solution
BSMV:00 (viral control)	1.0–1.5 μg	1.0–1.5 μg	1.0–1.5 μg	—	—	50–55 μL
BSMV:GOIS (sense version)	1.0–1.5 μg	1.0–1.5 μg	—	1.0–1.5 μg	—	50–55 μL
BSMV:GOIAs (anti-sense version)	1.0–1.5 μg	1.0–1.5 μg	—	—	1.0–1.5 μg	50–55 μL
FES (non silencing control)	—	—	—	—	—	50–55 μL

functional genomics due to the fact that it avoids many of limitations of the above approaches. Its main advantage is the generation of rapid phenotype and that there is no need for plant transformation. The cost of VIGS experiment is relatively low; *Agrobacterium* or in vitro transcription mediated VIGS assays do not cost effectively. VIGS method also provides a large-scale screening of genes for functional analysis. Moreover, there is no need to screen large populations to detect the function of a specific gene; only a single plant is enough to follow phenotype with targeted silencing. Therefore, repeating the experiment is easy and time effective. Host range wideness of viral vectors is the other versatility of the approach. For instance TRV can infect spinach, beet, potato, and tobacco naturally. Hence TRV-based VIGS is applied to *Nicotiana benthamiana*, tomato, *Arabidopsis,* chilli pepper, opium poppy, and *Aquilegia vulgaris*(Table 1). Since it does not require plant transformation, VIGS is particularly useful on plants which are difficult or impossible to transform. Therefore, VIGS system can be applied to the genes associated with embryonic development or essential housekeeping functions in plants [33, 38]. Functional redundancy problem is overcome by VIGS application using most conserved region of the gene family [26, 27]. Despite the valuable advantages of VIGS approach, there are also limitations. One of the most important limitation is that complete loss-of-function by VIGS might not be achieved. Generally 75–90% downregulation in the expression level of the targeted gene is accomplished [18, 43, 46]. Unfortunately the low level of gene expression can be enough to produce functional protein and phenotype in silenced plant. Some of viral infections can cause symptoms on plant that might mask the phenotype caused by the phenotype. This problem might be minimized as TRV-VIGS system because of mild symptoms [14, 16]. VIGS aims to silence the specific gene, which can only be achieved by sequence specific manner so the system relays on sequence information. The approach also depends on pathogen-host interaction, so the disadvantage is that pathogen infection may manipulate host function and alter development and morphology. There should be positive control in all VIGS assays to mark the effect of viral inoculation on silenced plant. Lastly, VIGS might suppress nontargeted gene in silenced plant cell or tissue [17]. This response should be addressed before the next genomic era.

6. Concluding Remarks

VIGS as a reverse genetics tool for functional genomics studies presenting many advantages promises rapid generation of functional genomics even proteomics. By the progressing and completing whole genome sequencing of many important crops, VIGS approach will be widely and mostly used. Despite its great potential to extensively use, many limitations remains to be overcome. Firstly host range of viral vectors will become wider; the VIGS assays and viral vectors for model organisms such as *Arabidopsis* and rice should be well optimized. As mentioned sequence information is crucial for VIGS approach so the whole genome sequence databases and EST databases will be add great contribution of VIGS usage. With the whole genome sequence availability, *Brachypodium distachyon* (L.) Beauv., a model temperate grass species, should also be used in application of VIGS system for generation of genomics information to improve temperate crops. Large-scale screening *via* VIGS-based method to detect important and fascinating phenotypes should be performed.

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Hindawi Publishing Corporation
International Journal of Plant Genomics
Volume 2009, Article ID 765367, 13 pages
doi:10.1155/2009/765367

Methodology Report

Reproducible RNA Preparation from Sugarcane and Citrus for Functional Genomic Applications

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Received 9 April 2009; Revised 12 September 2009; Accepted 13 October 2009

Recommended by Hongbin Zhang

High-throughput functional genomic procedures depend on the quality of the RNA used. Copurifying molecules can negatively impact the functionality of some plant RNA preparations employed in these procedures. We present a simplified, rapid, and scalable SDS/phenol-based method that provides the high-quantity and -quality RNA required by the newly emerging biotechnology applications. The method is applied to isolating RNA from tissues of two biotechnologically important crop plants, sugarcane and citrus, which provide a challenge due to the presence of fiber, polysaccharides, or secondary metabolites. The RNA isolated by this method is suitable for several downstream applications including northern blot hybridization, microarray analysis, and quantitative RT-PCR. This method has been used in a diverse range of projects ranging from screening plant lines overexpressing mammalian genes to analyzing plant responses to viral infection and defense signaling molecules.

1. Introduction

Preparation of high-quality RNA is critical for functional genomic studies. Isolating high-quality RNA from biotechnologically important crops such as sugarcane and citrus is complicated by the presence of high concentrations of intrinsic polysaccharides, polyphenols, and other secondary metabolites. Levels of these compounds increase in plants under biotic and abiotic stresses, such as pathogen infection or drought [1, 2]. These metabolites tend to copurify with the RNA, interfering with downstream applications that are highly sensitive such as sequence expressed tag-marker-assisted polymorphism, cDNA library construction, and microarray hybridization. In addition, variability in purity from sample to sample will impact physiological and biochemical studies [3].

Several methods exist for isolating RNA from tissues of species with a high content of polysaccharides or polyphenols. These methods mainly use denaturing agents such as guanidine- and phenol-based extraction buffers in combination with isopropyl alcohol precipitation [4–6], detergents such as sodium dodecyl sulfate (SDS) [7] or cetyltrimethylammonium bromide (CTAB) [8, 9], followed by lithium chloride (LiCl) precipitations [8, 9]. Some improved methods combine guanidine and CTAB [10] or SDS and phenol [11, 12], with additional use of the antioxidant polyvinylpyrrolidone (PVP) [13] or benzyl chloride for cell wall degradation [14] during extraction. Other methods include an additional precipitation step using ethanol or 2-buthoxyethanol [15, 16]. Although these procedures produce high-quality RNA from specific species, most of them are time consuming or result in low yield. Thus, there is a need to improve methods for problematic plant species to increase speed of RNA preparation and provide both high quality and high quantities of RNA required by the new high-throughput biotechnological applications.

We have developed a simple, rapid, and scalable procedure for isolation of high-quality RNA from sugarcane and citrus to facilitate the application of functional genomic studies in these crops. The procedure is a simplified

SDS/phenol extraction method with sequential steps of purification from polysaccharides and polyphenols using 2-mercaptoethanol/PVP-binding, chloroform partitioning, and sodium acetate/ethanol- and LiCl–mediated precipitations. It relies on two extraction steps using automated homogenization from small amounts of tissue and extraction buffer and on two rounds of precipitation. In citrus, only one extraction step is needed. High yields and quality of RNA are consistently obtained from multiple samples. Low and high molecular weight-RNA as well as low- and high-abundant RNA isoforms can be recovered.

The simplified RNA isolation method was compared with other RNA extraction methods used for functional genomic studies in sugarcane and citrus, namely, those based on guanidine thiocyanate [17–21], TRIZOL reagent (phenol and guanidine isothiocyanate) [22–26], and SDS/phenol [27–29]. The present study illustrates that the use of this method considerably accelerates the screening of transgenic plants containing high amounts of polysaccharides and secondary metabolites as well as the transcriptome analysis of genetically complex crops such as sugarcane in response to stress.

2. Material and Methods

2.1. Genetic Constructs and Plant Transformation. Constructs carrying a synthetic mammalian gene (0.455 kilobase [kb]), codon optimized for expression in either monocots or dicots, were generated. The sugarcane construct consisted of the mammalian gene cloned into the *Bam*HI-digested vector-pZero (Invitrogen Life Technologies, Carlsbad, CA) and placed under the control of the maize *ubiquitin 1* (*Ubi1*) promoter and the 35S cauliflower mosaic virus terminator, yielding pZero*Ubi1*:mammalian gene. The construct for citrus transformation was generated by cloning the mammalian gene into the binary vector pBIN34S [30] to produce pBIN34S:mammalian gene.

Embryogenic callus was established from young leaf bases and immature flowers of commercial sugarcane (*Saccharum* spp. hybrid, cultivar CP72-1210) and transformed with pZero*Ubi1*:mammalian gene together with the *Ubi1*:*BAR*-pUC8 (pAHC20) plasmid [31] as described previously [32, 33]. RNA was isolated from leaves of 4-month-old control and transformed plants for northern blot analysis. Citrus transformation with pBIN34S:mammalian gene was carried out using seedling-derived epicotyl segments of the Hamlin orange cultivar (*Citrus sinensis* Pers.) and *Agrobacterium tumefaciens* strain EHA105 [30, 34].

The presence and expression of the mammalian gene was confirmed on both citrus and sugarcane plants by Southern (data not shown) and northern blot analyses. Leaf tissues from 3- to 4-month-old control and transformed plants, grown in a controlled-environment greenhouse (28°C with 14-hour-light/10-hour-dark), were used for northern blot analysis.

2.2. Plant Growth and Treatment Conditions. Sugarcane (*Saccharum* spp. hybrid, cultivar CP72-1210) was grown in potting mix (Redi-earth mix, Scotts, Hope, AR) in a controlled-environment greenhouse (28°C with 14-hour-light/10-hour-dark) for stress-induction experiments. Four-month-old plants were treated with the stress-regulated hormones, salicylic acid (SA) or methyl jasmonate (MeJA) (Sigma-Aldrich, Saint Louis, MO). Treatments were conducted by spraying plants till run-off with a 5 mM SA solution in water and 0.05% (v/v) Tween-20 and kept at 80% humidity in the greenhouse. MeJA (a volatile form of jasmonates) treatment was carried out by placing a cotton swab containing 500 μL of 100 μM solution in 0.1% (v/v) ethanol and 0.05% (v/v) Tween-20 at the soil surface, close to the main stem of plants kept in clear plastic bags inside the greenhouse. Control plants were treated identically except without the addition of SA or MeJA. Stems, leaves, and roots of treated and control plants were collected at 0, 24, and 48 hours of treatment. At least three plants were tested for each time point of treatment and pooled to produce a biological sample. Two biological replicates were used.

Viral infection of sugarcane was carried out by inoculating with sap extract from sorghum (*Sorghum bicolor* cultivar Rio) infected with a compatible strain of *Sorghum mosaic virus* (SrMV) (described by Yang and Mirkov [35]) according to Ingelbrecht et al. [33]. Control plants were not inoculated but were otherwise treated identically. Leaf RNA from six SrMV-infected sugarcane plants was used for the microarray analysis.

Table 1: The TENS-PCI protocol used for RNA isolation.

(1) Extraction	(a) TENS[(a)] and PCI[(b)] (equal volume)
	(b) PCI
(2) Precipitation	3 M sodium acetate (pH 5.2) (0.1x volume)
	Ethanol (3.0x volume)
(3) Re-precipitation	4 M lithium chloride

[(a)]TENS: 10 mM hydroxymethyl aminomethane (Tris-HCl) (pH 7.5), 1 mM ethylenedinitrilo-tetraacetic acid (sodium EDTA) (pH 8.0), 0.1 M sodium chloride (NaCl), 1% (w/v) sodium dodecyl sulfate (SDS), 2% (w/v) polyvinylpyrrolidone (PVP)-40, and 7% (v/v) 2-mercaptoethanol.
[(b)]PCI: phenol (pH 4.3): chloroform: isoamyl alcohol (1.0: 0.8: 0.2).

2.3. RNA Extraction Protocol. A simple scalable protocol (TENS-PCI) was developed for the isolation of RNA from sugarcane and citrus tissues. The protocol steps are outlined in Table 1.

2.3.1. Isolation of RNA by the TENS-PCI Method

Extraction. For small-scale RNA isolation, tissue (0.1-0.2 g, snap frozen in liquid nitrogen) was homogenized in 2 mL screw-cap microcentrifuge tubes for 30 seconds at 5000 rpm with the Precellys 24 homogenizer (MO BIO Laboratories, Carlsbad, CA) in the presence of a ceramic spherical bead (0.64 cm-diameter). Up to 24 samples could be processed at a time with this homogenizer. A mixture (600 μL total volume) containing equal volumes of TENS extraction buffer and phenol (pH 4.3): chloroform: isoamyl alcohol (1.0: 0.8: 0.2) (PCI) reagent was added to the homogenate with thorough

mixing by hand. The TENS buffer consisted of 10 mM hydroxymethyl aminomethane (Tris-HCl) (pH 7.5), 1 mM ethylenedinitrilo-tetraacetic acid (EDTA) (pH 8.0), 0.1 M sodium chloride (NaCl), 1% (w/v) sodium dodecyl sulfate (SDS), 2% (w/v) polyvinylpyrrolidone (PVP)-40, and 7% (v/v) 2-mercaptoethanol. The homogenate was centrifuged at 10,000 g for 20 minutes at 4°C, and the supernatant was re-extracted with an equal volume of PCI. In citrus, only one extraction (TENS/PCI) was performed.

Precipitation. The supernatant was mixed with 0.1x volume of 3 M sodium acetate (NaOAc) (pH 5.2) and 3.0x volume of ice-cold 100% (v/v) ethanol and incubated at −20°C for 1 hour. The precipitate was collected by centrifugation at 10,000 *g* for 20 minutes at 4°C, air-dried briefly, and dissolved in 100 μL of nuclease-free water. A second round of precipitation was performed, using an equal volume of 4 M LiCl (100 μL) at −20°C overnight. The RNA pellet was recovered by centrifugation at 10,000 g for 20 minutes at 4°C, washed with 70% (v/v) ethanol, air-dried, and dissolved in 50–100 μL of nuclease-free water.

Large-scale RNA preparations from 60 g of stem tissue, 1 g of leaf, or 1 g of root tissue followed the same protocol as above by proportionally scaling up the volumes of the buffers.

Total RNA was treated with RNase-free DNase I (Applied Biosystems/Ambion, Austin, TX) prior to use.

2.3.2. Comparing the TENS-PCI Method with Standard RNA Isolation Methods. The TRIZOL reagent (phenol and guanidine isothiocyanate [36]) (Invitrogen Life Technologies) as well as two standard commercial laboratory kits, the RNeasy Plant Mini kit (Qiagen, Valencia, CA) that uses guanidine isothiocyanate or guanidine hydrochloride, and the FastRNA Pro Green kit (MP Biomedicals, LLC, Solon, OH), which is phenol-based, were tested in parallel with the TENS-PCI method for RNA extraction.

2.4. RNA Yield and Integrity. RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). RNA integrity (size and distribution of the extracted RNA molecules) was determined using an Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA). The ratio of the peak areas of the plant large subunit (LS) (about 25S) to the small subunit (SS) (18S) ribosomal (r) RNAs was assessed by the Bioanalyzer software.

2.5. RNA Amplification. Amplified RNA (aRNA) was prepared from sugarcane total RNA by the Eberwine method [37] using a MessageAmp aRNA kit (Applied Biosystems/Ambion) following the manufacturer's instructions. One to five μg of total RNA from sugarcane tissue was used for each amplification, and 50–100 μg of aRNA was obtained after one round of amplification.

2.6. Northern Blot Analysis. Total RNA (10 μg each lane) from leaf tissues of sugarcane or citrus lines transgenic for the mammalian gene was fractionated on denaturing formaldehyde agarose gel and blotted onto nylon membranes (Nytran^R SuperCharge, Schleicher and Schuell BioScience, Inc., Keene, NH) in 10x SSPE buffer [38]. RNA blots were hybridized with probes amplified by PCR using primers derived from the full-length coding region of the target gene. PCR products were labeled with [^{32}P]α-dATP by random priming using Klenow Exo$^-$ DNA polymerase (New England BioLabs Inc., Ipswich, MA). Hybridization and washes were conducted in accordance with the method of Church and Gilbert [39]. Hybridized blots were visualized and quantified with the BAS-5000 scanning system (Fujifilm Life Science USA, Stamford, CT). RNA loading and transfer efficiency was normalized relative to the band intensity of the sugarcane constitutive *ubiquitin* gene [40].

2.7. cDNA Microarray Preparation and Analysis. A set of 229 stem-expressed cDNA clones were initially identified by the differential hybridization of a sugarcane stem cDNA library (13,824 clones) [40] and used to construct a cDNA microarray. The identity of the arrayed clones was confirmed by cycle sequencing and Blastx search. Information on the nucleotide sequence of these clones can be found at http://enterprise.bio.tamu.edu/. cDNA inserts in the pCR2.1 vector (Invitrogen Life Technologies) were amplified and printed on PL-100C poly-L-lysine-coated glass slides (CEL Associates, Pearland, TX) as described [41].

Randomly primed fluorescent probes were produced from aRNA samples using the 3DNA Array 350RP expression array detection kit (Genisphere, Hatfield, PA) as recommended. The fluorescent dye on probes derived from the experimental aRNA was Cy5, whereas the dye on control probes was Cy3. Hybridizations and washings followed Genisphere's suggestions.

Labeled arrays were scanned with an Affymetrix 428 array scanner (Affymetrix, Santa Clara, CA). Resulting images were analyzed with GenePixPro (Axon Instruments, Union City, CA). Data files were further analyzed using GeneSpring (Agilent Technologies) to facilitate normalization, parameter assignment, and filtering. Experimental values were divided by the control values and further normalized relative to the positive control genes: *glutathione-S-transferase*, *G protein-coupled receptor*, *histone deacetylase*, *ribulose epimerase*, *tubulin,* and *ubiquitin* [40]. Regulated genes were defined as those with a two-fold or higher amplitude change in their normalized ratios and a *t*-test *P*-value of .05 or less. Two biological samples were used for each tissue type or time point. Each biological sample was used for three hybridizations. Six microarray hybridizations were conducted per sample. A total of six microarray hybridizations with dye swaps were carried out per tissue or treatment.

2.8. Quantitative RT-PCR Analysis. First-strand cDNAs were synthesized from DNase I-treated aRNA (2 μg) using the TaqMan^R reverse transcription kit (Applied Biosystems, Foster City, CA). qRT-PCR was performed on an ABI PRISM 7700 (Applied Biosystems) with the SYBR^R Green

PCR master mix (Applied Biosystems) according to the manufacturer's instructions. Primers were designed with the Primer Express1.5 software (Applied Biosystems). qRT-PCR was performed twice in triplicate with two biological repeats of aRNA. Results were analyzed with SDS1.7 software (Applied Biosystems) and recorded as C_T (threshold cycle) values. Each transcript was quantified relative to that of the sugarcane *ubiquitin* gene, using the comparative C_T method (user bulletin 2, ABI Prism 7700 sequence detection system; Applied Biosystems). A threshold of a two-fold difference in relative mRNA levels was used to designate genes as induced in one tissue type or one treatment time. Primer pairs used for some representative genes were as follows: *OMT* (5′-agattcggcaagctcttcgac-3′ [F] and 5′-ttgccacgatgtccatgatg-3′ [R]), *DIR1* (5′-cattcggcaaaacaacagaca-3′ [F] and 5′-gcgtccaaagaaacagatga-3′ [R]), *DIR11* (5′-atcaatcaagcacaatataa-3′ [F] and 5′-agatcgtgaaaagatacatt-3′ [R]), *DIR12* (5′-gcttgatcgactagcgc-3′ [F] and 5′-gcacaagaagcagctg-3′ [R]), *DIR16* (5′-cctgggcgcttctaccaac-3′ [F] and 5′-acacttgtcgatcaagcgtcg-3′ [R]), and *ubiquitin* (5′-ccaaaccccgacgatcc-3′ [F] and 5′-tctcgtacttgtgccggtcc-3′ [R]).

3. Results and Discussion

We are interested in carrying out functional genomic studies on sugarcane and citrus, two biotechnologically important crops that are rich in fiber, polysaccharides, and secondary metabolites. The handful of such studies with these crops employed RNA extracted using TRIZOL reagent [22–26], guanidine thiocyanate [17–21], or SDS/phenol [27–29]. We tested the TRIZOL reagent, the standard SDS/phenol method, as well as two commercially available RNA extraction kits, one based on guanidine (RNeasy) and the other on phenol (FastRNA Pro Green), to isolate RNA from sugarcane. We found that these methods did not perform well across multiple experiments. The RNA obtained produced a high background or a very weak signal when used in northern blot or microarray analysis. The major aim of the current study was to develop a simple, rapid, and scalable method that yields high-quantity and -quality RNA from sugarcane and citrus.

3.1. Developing the TENS-PCI Method for Preparation of High-Quantity and -Quality RNA. The TENS-PCI method is a simplified RNA isolation technique that uses SDS and phenol with a high concentration of antioxidants (PVP-40 and 2-mercaptoethanol) for extraction and two rounds of precipitation to yield high-quantity and -quality RNA from 100 mg to 60 g of tissue in an efficient manner. The method is simplified in terms of requiring less manipulation, with only two extraction steps and two rounds of precipitation. It is also considered as a rapid isolation method due to the reduced time needed for automated tissue homogenization (30 seconds) and RNA precipitation (one precipitation for one hour and another for 16 hours).

Optimization of the TENS-PCI micro-scale isolation method was achieved by reducing the leaf tissue weight from 1.0 g usually required for grasses [12] to 0.1 g. This is very similar to the amounts generally harvested from succulent tissues of dicot species. For the macro-scale method, only one gram of leaf or root tissue was used for extraction as opposed to 4 g of sugarcane leaf roll used in the SDS-phenol method of Carson and Botha [27, 28].

Automated tissue homogenization allowed the extraction of 24 samples in a very short period of time (30 seconds) in small tubes (2 mL), using a reduced extraction volume (600 μL). Higher-throughput preparations could be easily achieved with this method by adapting the use of a higher-capacity homogenizer.

An important component of the TENS-PCI extraction buffer was the presence of high levels of NaCl (0.1 M) used to increase the solubility of polysaccharides, reducing their coprecipitation with RNA in subsequent steps [42]. Higher concentrations of the antidoxidant 2-mercaptoethanol (7%), together with PVP (2%), were also used into the extraction buffer, as compared to earlier methods (0% to 5% 2-mercaptoethanol or 0% to 2% PVP) in woody plants and grasses [6, 10, 12, 16, 27]. This was done to improve the sequestering of phenolic compounds that are released during tissue homogenization [43, 44].

Two precipitation steps were included in the TENS-PCI method as opposed to the SDS-phenol method of Carson and Botha [27, 28] that used three rounds of precipitation, with two of them performed overnight. The first precipitation step of the TENS-PCI method, with NaOAc and ethanol for one hour, precipitates both nucleic acids and polysaccharides. The second, with a high-molarity LiCl solution (4 M) for 16 hours, differentially precipitates RNA from DNA and residual polysaccharides, thereby increasing RNA purity [45]. This significantly reduced the time needed to recover the RNA.

Incorporation of high amounts of antioxidants (7% of 2-mercaptoethanol and 2% of PVP) into the TENS-PCI extraction buffer as well as the adoption of only two rounds of selective precipitation proved to be efficient in removing polysaccharides and phenols. The TENS-PCI method yielded RNA with low levels of polysaccharides/phenols as indicated by the spectrophotometric ratios of A_{260} : A_{230} that are close to 2.0 (Table 2). Ratio values of 1.8 to 2.0 are usually considered an acceptable indicator of high-purity RNA [38, 46]. However, the RNA isolated by the RNeasy-guanidine hydrochloride and the FastRNA Pro Green methods tested in this study displayed low A_{260} : A_{230} ratio averages of 1.20 and 1.17, respectively, (Table 2), suggesting high levels of contamination by polysaccharides and/or polyphenols. These results are in agreement with previous reports on the presence of polysaccharides/phenols in RNA extracted from grapevine [44], plum [44], lemon [44], and London plane tree [6] using the RNeasy, the guanidine, and the standard SDS/phenol and CTAB methods (Table 2).

Previous reports showed that stress treatments such as pathogen infection or water and nutrient deficiencies can enhance the levels of accumulation of polysaccharides and secondary metabolites, particularly phenols, in plants [1, 2]. Our results indicate that the quality of RNA isolated by the TENS-PCI method was not affected by infection with

the *Sorghum mosaic virus* (SrMV) pathogen or treatment with the stress-regulated hormones, salicylic acid (SA), and methyl jasmonate (MeJA). For example, average A_{260} : A_{230} ratios of 2.37 ± 0.12 and 1.98 ± 0.06 were obtained with RNA extracted with TENS-PCI from plants treated with MeJA for 24- and 48-hours, respectively, compared to those of RNA extracted from the control plants at 24- and 48-hour treatment with water/ethanol, which were 2.21 ± 0.11 and 2.25 ± 0.14, respectively.

The TENS-PCI method was also the most efficient in removing proteins, with RNA samples from sugarcane and sweet orange repeatedly exhibiting A_{260} : A_{280} ratio values around 2.0 (Table 2). The phenol-based FastRNA Pro Green kit yielded a lower purity RNA, as detected by the slightly lower A_{260} : A_{280} ratio value, that is, 1.79 (Table 2). Low values were also reported for RNA extracted from London plane tree [6] with the SDS/phenol, guanidine, or CTAB method (Table 2).

In the case of the RNeasy-guanidine isothiocyanate kit, acceptable A_{260} : A_{230} and A_{260} : A_{280} ratios were obtained, but yields were low (Table 2). This is similar to what has been reported for the rapid CTAB method in grapevine, plum, and lemon [44] (Table 2). An improvement in RNA yield was achieved with the TENS-PCI micro-scale method (5.2 μg/mg tissue) by 4.7-fold in leaf as compared to the two tested kits, the RNeasy (0.11 μg/mg tissue) and FastRNA Pro Green (0.11 μg/mg tissue) (Table 2), although the quantity of the starting tissue was the same for all tested methods. The low RNA yield could be attributed to the presence of polysaccharides, saturating the binding capacity of the resin columns supplied with the kits during the RNA isolation step. A significant increase in the RNA yield was observed with the TENS-PCI micro-scale method compared to non-kit based RNA isolation methods that have been used for other woody plants. The increase in yield was on average of 368.8-fold for leaves and 366.7-fold for stems as opposed to the standard SDS/phenol, guanidine, and CTAB methods used for grapevine [44], lemon [44], plum [44], and London plane tree [6] (Table 2).

We further assessed the performance of the TENS-PCI method across tissues and stress-related treatments, by checking the integrity of the RNA isolated from different sugarcane tissues such as leaf, stem and root, as well as from plants treated with the SrMV pathogen or the stress-regulators, SA and MeJA, using chip-based microcapillary electrophoresis. We similarly tested RNA obtained with the two kits, FastRNA Pro Green and RNAeasy Mini Plant (guanidine isothiocyanate). RNA degradation was evaluated using the ratios of the area under the curve of the peaks corresponding to the signal intensity of the large subunit to that of the small subunit (LS : SS) rRNA (based primarily on checking the quantity and quality of the ribosomal subunits). Values of LS : SS rRNA ratios of about 2.0 are considered to be indicators of good RNA integrity, depending on the tissue analyzed and the biological system used [47, 48].

Elaborate measures designed to improve RNA purity can increase the risk of RNA that is degraded in the process. The simplified TENS-PCI method produced a high-purity intact RNA from leaves, stems, and roots, showing a non-significant variation among these tissues, as reflected by the ratios of the LS : SS rRNA peak areas of the RNA profiles of the tested samples (Figure 1). RNA of high integrity was also isolated from plants infected with the SrMV pathogen or treated with stress-regulated hormones. No significant RNA degradation occurred as shown by the intact rRNA peaks and the LS : SS ratios (Figure 2). The overall values across tissues and treatments for the LS : SS ratios ranged from 1.71 to 2.15 (Figures 1 and 2).

Low-integrity RNA was obtained with the FastRNA Pro Green kit as shown by the low LS : SS ratios for the three tested tissues, leaf, root, and stem (Figure 1). The RNeasy-guanidine isothiocyanate yielded a better purity RNA, especially from stem and root, although the LS : SS ratio values were significantly lower than those obtained with the TENS-PCI method, indicating that RNA degradation had occurred (Figure 1). Thus, this method produced RNA of good purity but with a lower yield and lower quality than did the TENS-PCI (Table 2).

3.2. RNA Isolated with the TENS-PCI Method is Suitable for Northern Blot Analysis. Over 350 transgenic sugarcane and citrus plants were generated that constitutively expressed a mammalian gene. These were screened by Southern blot hybridization for presence and copy number of the gene (data not shown). To demonstrate the suitability of TENS-PCI for detecting specific transcripts, we tested the expression of the mammalian gene in leaves of transgenic sugarcane and citrus lines using northern blot hybridization. RNA isolated from the same lines using the RNeasy Plant Mini and the FastRNA Pro Green kits was also tested. Binding of the probe was the strongest and the most specific with RNA extracted by the TENS-PCI method, resulting in an intense band corresponding to the expected mammalian transcript size, of about 1.20 kb (Figures 3(a) and 3(b)), indicating that the RNA is intact and non-degraded. Binding of the mammalian probe to the RNA obtained with the RNeasy (GB-ICT and GB-H) and the FastGreen Pro (PB) methods was of low specificity since multiple bands were detected (Figure 3 (a)). Furthermore, this binding was greatly inhibited (Figure 3 (a)), probably due to the presence of secondary metabolites that were copurified with the RNA. This shows that the RNeasy and FastGreen Pro kits did not produce RNA adequate for northern blot hybridization of sugarcane and citrus. Previous studies have reported the inefficiency of these tested kits in extracting high-quality RNA from tissues rich in polysaccharides or polyphenols [13, 16, 44].

The inconsistency in RNA quality provided by the tested kits can impede the quantitation of the signal strength across multiple samples. Using the TENS-PCI micro-scale method, we were able to measure the transcriptional levels of multiple transgenic lines in a short time to identify high expressers of a mammalian gene. Our results were corroborated by Western analysis of protein levels and measurement of enzyme activity (data not shown). Thus, the TENS-PCI method represents a good option when limited amounts of

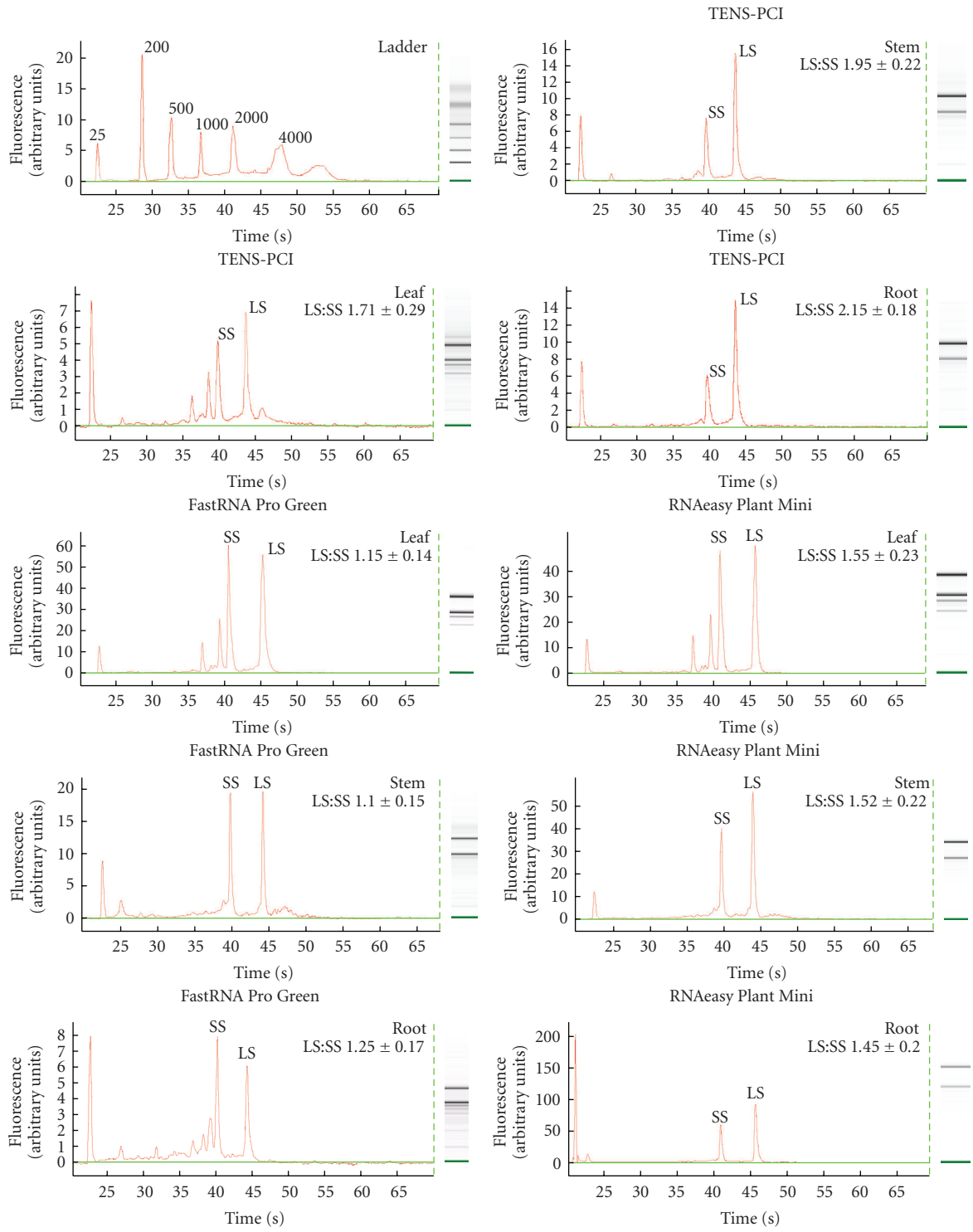

Figure 1: Assessment of the integrity of total RNA isolated by the TENS-PCI method, and the two kits, FastRNA Pro Green and RNeasy Plant Mini, from sugarcane leaf, stem, and root tissues, using chip-based electrophoretic separation with a bioanalyzer. A total of 100 ng of RNA was loaded per well. Representative RNA profiles corresponding to the three tissues are shown. Gel-like images of RNA from each tissue, generated from the RNA profiles, were included for clarity on the right side. The Agilent RNA 6000 Nano ladder is included for sizing. SS and LS represent the small and large ribosomal subunits, respectively. Numeric values represent mean and standard error of two biological replicates and at least three technical repeats.

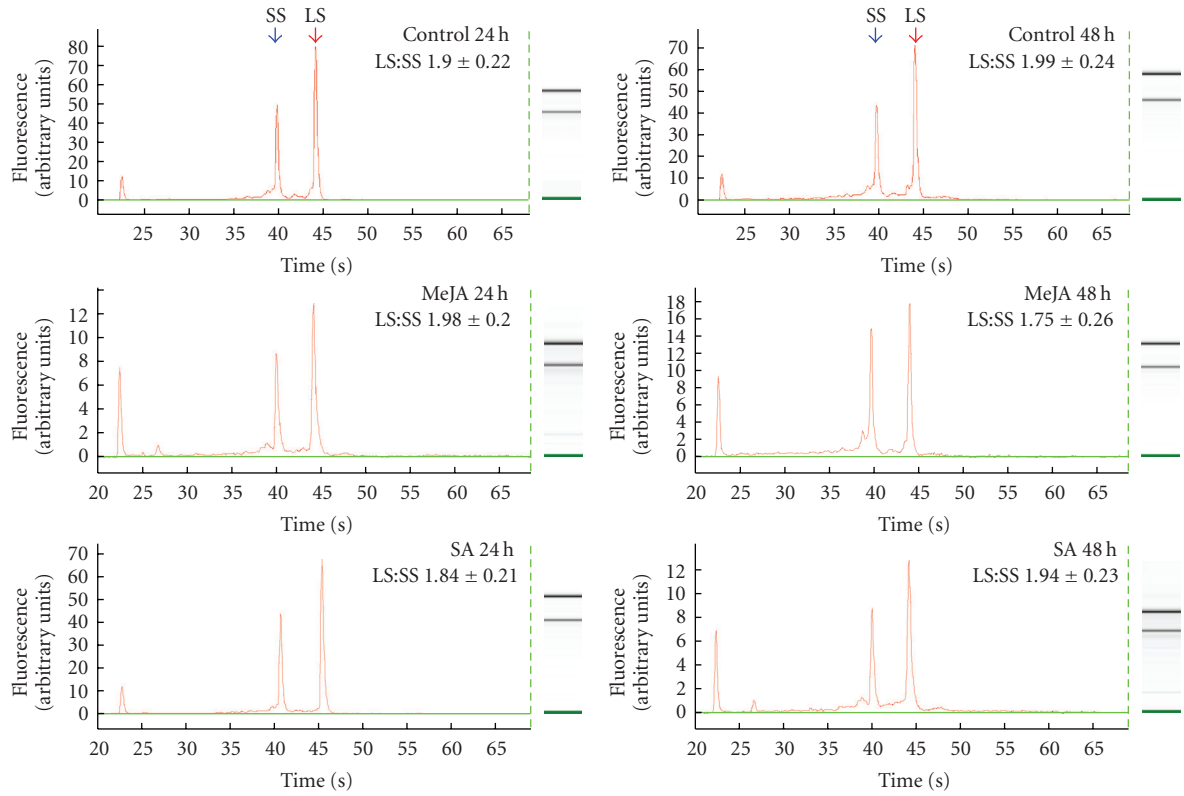

Figure 2: Assessment of the integrity of total RNA isolated by the TENS-PCI method from sugarcane stem tissues of plants treated with stress-regulated hormones, using chip-based electrophoretic separation with a bioanalyzer. A total of 100 ng of RNA was loaded per well. Representative RNA profiles corresponding to stem tissues of plants after treatment with SA (5 mM) or MeJA (100 μM) at 0, 24, and 48 hours (h) are shown. Gel-like images of RNA from each treatment, generated from the RNA profiles, are included for clarity on the right side. SS and LS represent the small and large ribosomal subunits, respectively. Numeric values represent mean and standard error of two biological replicates and at least three technical repeats.

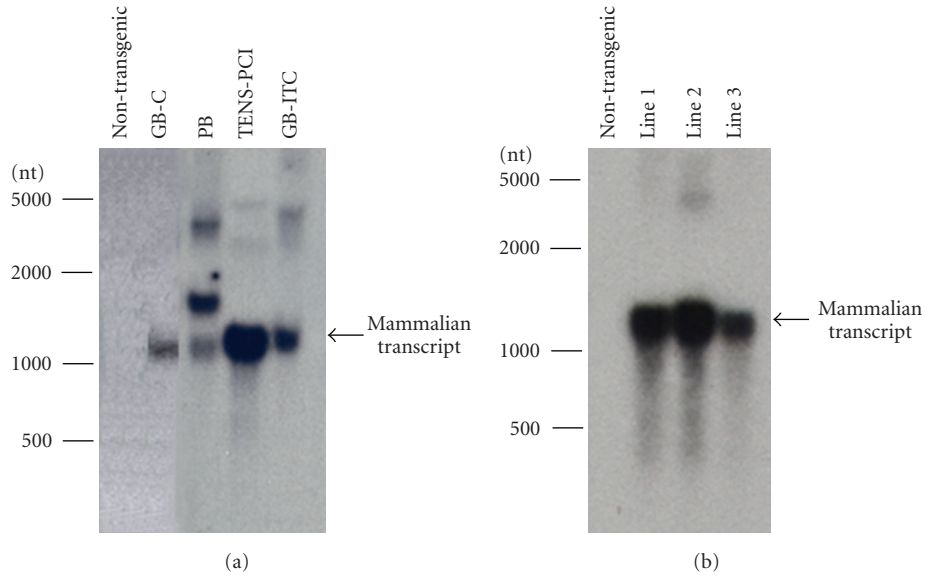

Figure 3: Northern analysis of the expression levels of sugarcane (a) and sweet orange (b) lines overexpressing a mammalian gene. Total RNA was isolated from leaf tissues by the TENS-PCI, the RNeasy guanidine-based (GB), and phenol-based (PB) methods. GB-ITC refers to guanidine isothiocyanate and GB-C to guanidine hydrochloride. RNA (10 μg each lane) was fractionated, blotted, hybridized, washed, and imaged as described in Material and Methods.

Table 2: Comparison of the TENS-PCI method with other RNA isolation procedures used for plant species with high levels of secondary metabolites.

Method	RNA yield[(a)] (μg/mg tissue)			RNA purity[(a)] Leaf	
	Leaf	Stem	Root	$A_{260} : A_{230}$	$A_{260} : A_{280}$
*Sugarcane (*Saccharum *spp. hybrid) (present study)*					
TENS-PCI: Micro-scale	0.59 ± 0.07	0.44 ± 0.05	0.45 ± 0.03	1.99 ± 0.07	1.97 ± 0.09
Macro-scale	2.42 ± 0.15	3.92 ± 0.27	0.99 ± 0.08	2.01 ± 0.07	1.98 ± 0.06
RNeasy Plant Mini Kit					
Guanidine isothiocyanate	0.10 ± 0.04	NA[(b)]	NA	2.30 ± 0.08	1.95 ± 0.10
Guanidine hydrochloride	0.11 ± 0.04	NA	NA	1.20 ± 0.06	1.92 ± 0.11
FastRNA Pro Green Kit (Phenol-based)	0.11 ± 0.03	NA	NA	1.17 ± 0.05	1.79 ± 0.07
*Sweet orange (*Citrus sinensis *Pers.) (present study)*					
TENS-PCI: Macro-scale	2.29 ± 0.11	NA	NA	2.13 ± 0.01	2.07 ± 0.01
Woody plants					
(1) Grapevine (*Vitis* spp.) [44]					
Rapid CTAB	0.11 ± 0.05	0.11 ± 0.03	NA	2.29 ± 0.12	1.89 ± 0.03
RNeasy kit	0.04 ± 0.01	0.04 ± 0.01	NA	0.36 ± 0.05	1.07 ± 0.04
Guanidine	0.12 ± 0.02	0.10 ± 0.02	NA	1.11 ± 0.08	1.77 ± 0.10
(2) Lemon tree (*Citrus limon* Burm.f.)	0.14 ± 0.03	0.06 ± 0.01	NA	2.28 ± 0.14	2.01 ± 0.03
Rapid CTAB [44]					
(3) Plum (*Prunus domestica* L.)	0.18 ± 0.01	0.21 ± 0.04	NA	2.23 ± 0.11	2.03 ± 0.02
Rapid CTAB [44]					
(4) London plane tree (*Platanus acerifolia* Willd.) [6]					
Guanidine	0.20 ± 0.004	NA	NA	1.49 ± 0.07	1.35 ± 0.11
SDS/phenol	0.12 ± 0.004	NA	NA	1.47 ± 0.06	1.49 ± 0.89
CTAB	0.26 ± 0.007	NA	NA	1.61 ± 0.07	1.62 ± 0.79

[(a)]Data represent mean and standard error of at least 6 biological replicates and 6 technical repeats.
[(b)]NA: data not available.

tissue samples are available, since it consistently produces high-quantity and -quality RNA and does not interfere with the detection of specific transcripts.

3.3. RNA Extracted with the TENS-PCI Method Is Successfully Amplified for the Generation of Reproducible Microarray Data. Important sources of variability in genomic data include variations in tissue processing, RNA preparation and assay protocols, as well as inherent tissue heterogeneity [49, 50]. Of particular interest is the effect of the quality of the starting RNA. Jahn et al. [48] have observed that low-quality RNA samples did not show a significant difference in relative transcript expression ratios for a protein when RNA from mutants deficient in the gene for that protein was compared to RNA from the wild-type organism. This illustrates the importance of using high-quality RNA for reproducibly detecting significant differential gene expression data from transcriptome analyses.

Feldman et al. [51] have described the advantages of amplifying RNA for improving microarray analysis. RNA amplification is dependent on the quality of the input RNA. The RNA obtained with the TENS-PCI method from different tissues and hormonal treatments was successfully amplified to provide sufficient amounts for high-throughput transcript profiling studies. The yield and size of the aRNA produced (Figure 4) were in the range expected from good-quality input RNA. This provides a functional proof of the quality of the RNA isolated by the TENS-PCI method.

Microarray analysis performed with aRNA derived from the TENS-PCI method revealed significant differences in relative gene expression ratios, as reflected by the two-fold up- or down-regulation of the identified genes (Table 3, see Figure 1S in Supplementary Material available online at doi: 10.1155/2009/765367). No such differences were detected in the microarray data generated with aRNA derived from the RNeasy-guanidine isothiocyanate method (Supplementary Figure 1S). Furthermore, high correlation for the relative gene expression ratios was obtained among the replicate experiments performed with the TENS-PCI RNA (Pearson correlation coefficient [r] = 0.89–0.96; t-test $P \leq .05$) (Supplementary Figure 1S). Lower correlations (r = 0.65–0.70) were obtained from comparisons between replicate experiments that used RNA extracted with the RNeasy-guanidine isothiocyanate method (Supplementary Figure 1S). The detection of differential gene expression as well as the small variation between replicate experiments shows that the TENS-PCI RNA is of high quality to generate biologically significant and reproducible microarray data.

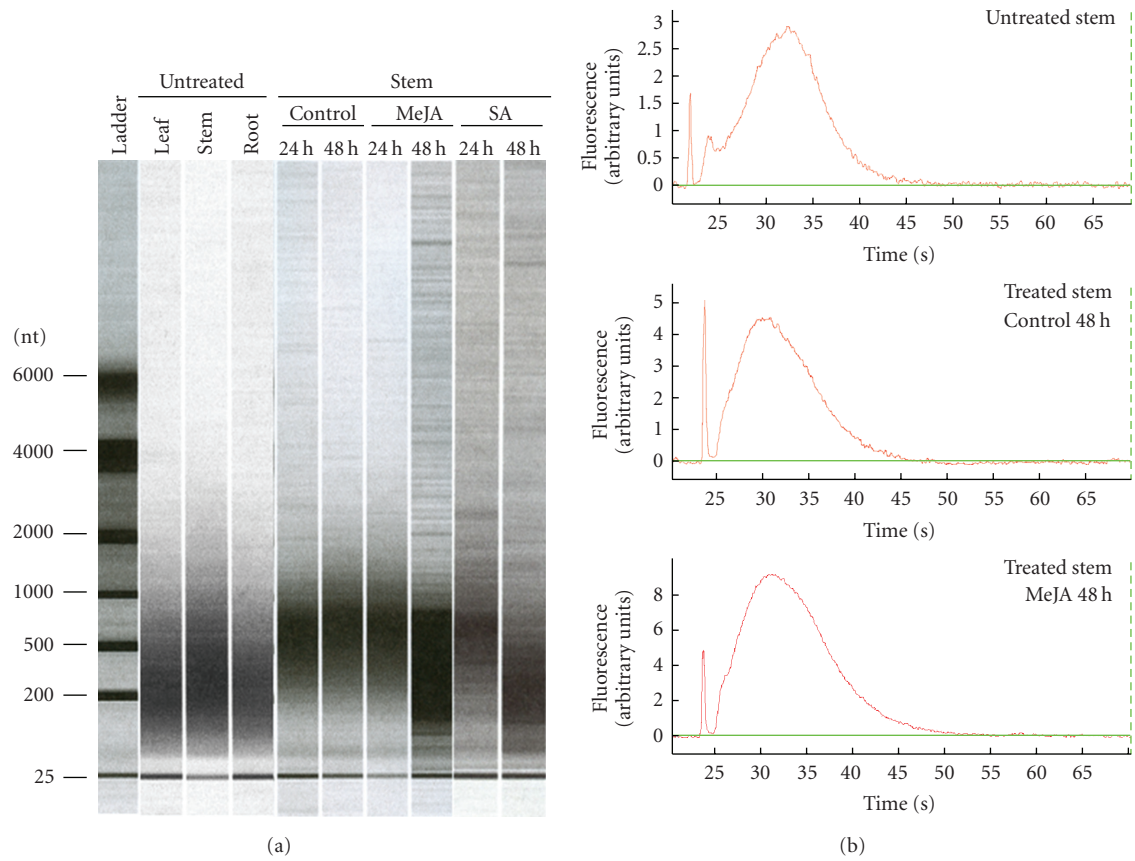

Figure 4: Chip-based electrophoretic separation of amplified total RNA (aRNA) isolated by the TENS-PCI method. aRNA was analyzed using an Agilent 2100 Bioanalyzer. A total of 20 ng of aRNA was loaded per well. (a) Densitometry simulation for aRNA from stem, leaf and root tissues of untreated plants as well as from stem tissues of plants after treatment with SA (5 mM) or MeJA (100 μM) at 0, 24 and 48 hours (h). (b) Representative profiles of aRNA corresponding to stems of untreated plants, as well as to stems of control (mock: water and 0.1 % ethanol, 48 hours) and treated plants (MeJA at 48 hours). The Agilent RNA 6000 Nano ladder is included for sizing.

3.4. Defense- and Stress-Responsive Marker Genes Are Identified in the Transcript Profiling Analysis. Plant stress responses are largely mediated by phytohormones that trigger conserved defense mechanisms, with intricate signaling pathways leading to protection. Salicylic acid (SA) and the jasmonates (including methyl jasmonate (MeJA)) are major signaling molecules that regulate such protective responses via synergistic and antagonistic actions, referred to as signaling cross-talk [52–55]. To further demonstrate the functional quality of the TENS-PCI RNA, we performed a transcriptome analysis, looking specifically at defense- and stress-responsive genes. We did a focused microarray analysis to monitor the mRNA expression profiles of 229 sugarcane stem-regulated cDNAs in response to biotic and hormonal stresses. The cDNA microarray was hybridized to cDNA probes synthesized from RNA isolated by the TENS-PCI method from stem, leaf, and root tissues and from stem of plants treated with SA, or MeJA, or infected with the compatible pathogen *Sorghum mosaic virus* (SrMV).

The micorarray analysis identified three major transcript profiling groups (Table 3) that consist of (a) stem-expressed genes that are co-induced by SA and MeJA, (b) genes that are induced by SA and repressed by MeJA, and (c) genes that are induced by MeJA and repressed by SA. The microarray data were confirmed by qRT-PCR analysis (Table 3). The genes in these groups are predominantly implicated in defense response, secondary metabolism, and fiber biosynthesis. They include the defense-related and fiber biosynthesis *dirigent* (*DIR*) family [17, 56] and *O-methyltransferase* (*OMT*) [57] genes as well as an antimicrobial *chitinase* gene [58] (Table 3). These results are consistent with earlier studies. Casu et al. [17] reported on the abundance of *DIR* genes in sugarcane maturing stems. MeJA has previously been shown to induce a number of *DIR* genes in sugarcane root [59]. *OMT* has been reported to be expressed in mature sugarcane stems [17]. Previous work has shown *OMT* to be induced in barley by MeJA [60] and in sorghum by SA and MeJA [61]. Cooperative regulation of chitinase by SA and MeJA has been observed in sorghum [61].

In addition, many of the genes that are regulated by SA and/or MeJA were two-fold down-regulated upon SrMV infection in our microarray analysis (Table 3). Such genes encode several DIR proteins and a chitinase. This down-regulation of gene expression upon viral infection is in agreement with Shi et al. [62, 63] who observed

Table 3: Relative mRNA levels of representative defense- and stress-responsive genes that are two-fold up-regulated in sugarcane after treatment with the pathogen *Sorghum mosaic virus* (SrMV) or the stress-regulated hormones, salicylic acid (SA) and methyl jasmonate (MeJA), as detected by microarray and qRT-PCR analyses.

		Fold induction or repression									
				SA (h)				MeJA (h)			
Putative function	Accession no.	Stem/Leaf	SrMV	Microarray		qRT-PCR		Microarray		qRT-PCR	
				24	48	24	48	24	48	24	48
(1) Genes coding for antimicrobial proteins											
Chitinase	AW746272	1.48	0.98	1.2	**9.28**	1.82	**12.36**	1.27	0.31	1.87	1.56
(2) Genes coding for proteins involved in secondary metabolism											
O-methyltransferase	NM_001155649	**3.2**	1.03	0.67	**2**	1.01	3.52	**3.99**	**2.53**	**2.53**	0.3
Dirigent-SoDIR1	AY421731										
DIR1		**2.4**	1.1	1.23	**17.21**	1.84	**32.41**	1.68	0.62	1.99	1.18
DIR3		**2.54**	1.02	1.24	**9.09**	1.79	**14.05**	**2.25**	0.34	**4.26**	1.75
DIR8		**2.18**	0.98	0.99	**5.27**	1.89	**8.89**	1.75	0.64	1.96	1.61
DIR9		**2.3**	1.11	1.42	1.02	1.97	1.97	**3.05**	0.79	**7.98**	1.79
DIR10		**3.58**	0.96	1.43	**21.66**	**2.56**	**30.33**	**2.17**	0.29	**3.65**	0.97
DIR27		**2.62**	1.1	0.97	**6.93**	1.75	**10.06**	1.42	0.54	1.98	1.36
DIR32		**2.96**	1.03	1.16	**7.73**	1.99	**11.04**	1.14	0.43	1.96	1.06
DIR34		**2.3**	1.04	1.4	0.7	1.88	1.09	**3.08**	1.06	**6.27**	1.97
DIR44		**7.84**	0.89	1.24	**19.22**	1.94	**29.18**	**4**	0.79	**9.03**	1.74
Dirigent-SoDIR2	AJ626722										
DIR11		**4.83**	0.91	1.33	**20.96**	1.08	**5.3**	**6.45**	1.98	**7.21**	**2.23**
DIR37		**2.62**	1.01	1.04	**11.32**	**2.12**	**19.24**	1.32	0.35	1.98	0.88
Dirigent-like	AY781903										
DIR12		**3.97**	0.99	1.01	**15.81**	1.97	**26.09**	**3.16**	0.2	**6.04**	1.45
DIR13		**3.25**	1.13	1.25	**18.19**	**2.02**	**30.56**	**2.25**	0.24	**5.12**	1.87
DIR16		**3.08**	0.94	1.01	**19.64**	**2.02**	**19.86**	**8.67**	1.16	**9.97**	**2.99**

Relative abundance of mRNA transcripts of the cDNAs was determined in sugarcane stem, leaf, and root as well as in stem of plants infected with SrMV or treated with SA or MeJA. Values of each transcript were normalized to that of the sugarcane constitutive *ubiquitin* gene. Values represent the average normalized ratios of transcripts obtained: (1) from stems to those obtained from leaves, and (2) from stems at the indicated time of treatment to those obtained from the untreated (0 time or no infection). Data are representative of two biological samples and three technical repetitions. Values for cDNAs that are two-fold up-regulated are in bold.

a differential expression of defense- and stress-responsive genes, including those coding for chitinases, in near-isogenic maize lines challenged with the *Sugarcane mosaic virus*.

In summary, transcript profiling analysis of the sugarcane stem in response to the defense-inducing and stress-regulated hormones, SA and MeJA, has enabled the identification of marker genes that are associated with defense and stress responses. Such genes, specifically *OMT* and *DIRs*, were of particular interest to us in relation to their relevance as stem-regulated and stress-induced type markers reflecting both, the stem- and the stress-regulated origin of the RNA extracted by the TENS-PCI method.

4. Conclusions

We have developed a simple, rapid, and scalable protocol enabling an efficient and robust extraction of RNA from sugarcane and citrus on a micro- and macro-scale, reducing significantly the cost of RNA extraction per sample. Compared to other protocols, the presented TENS-PCI method is a simplified method that consists of two extraction steps for sugarcane and one for citrus, using SDS, phenol, and a high concentration of antioxidants (2-mercaptoethanol and polyvinylpyrrolidone-40) as well as two rounds of precipitation (sodium acetate/ethanol and lithium chloride). This method represents a good option since it combines the advantages of high RNA recovery (especially

when limited amounts of tissue are available), high RNA integrity, reproducibility among biological and experimental replicates, and applicability to a wide range of tissues. We have demonstrated that this method accelerates the screening of transgenic plants with tissues rich in polysaccharides and secondary metabolites, using northern blot analysis. We have further shown that the high-quality RNA obtained by the TENS-PCI method can be easily amplified to generate reproducible and biologically significant gene expression data. We provide evidence of the utility of the RNA extracted by the TENS-PCI method in sensitive assays by showing that several defense- and stress-responsive marker genes are differentially regulated during the transcript profiling of part of the sugarcane transcriptome in response to pathogenic and hormonal stresses. These data corroborate with previously reported findings on the signaling pathways governing the plant stress response. We anticipate that the application of the TENS-PCI method in novel high-throughput functional genomic technologies such as next generation DNA sequencing will shed more light into the cross-talk signaling in biotechnologically important crops with complex genomes, such as sugarcane.

Acknowledgments

The authors would like to acknowledge Dr. Andrew Tag and Rick Hammer for bioinformatics support, Hyun Park-Kang and Denise Rossi for excellent technical support, and Ginger Stuessy for plant maintenance. This work was supported by grants from the Texas Grain and Grass Gene Initiative Program and the Rio Grande Valley Sugar Growers Cooperative.

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Hindawi Publishing Corporation
International Journal of Plant Genomics
Volume 2009, Article ID 835608, 9 pages
doi:10.1155/2009/835608

Research Article

Agrobacterium-Mediated Gene Transfer to Cereal Crop Plants: Current Protocols for Barley, Wheat, Triticale, and Maize

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Received 8 April 2009; Accepted 17 April 2009

Recommended by Hikmet Budak

The development of powerful "omics" technologies has enabled researchers to identify many genes of interest for which comprehensive functional analyses are highly desirable. However, the production of lines which ectopically express recombinant genes, or those in which endogenous genes are knocked down via stable transformation, remains a major bottleneck for the association between genetics and gene function in monocotyledonous crops. Methods of effective DNA transfer into regenerable cells of immature embryos from cereals by means of *Agrobacterium tumefaciens* have been modified in a stepwise manner. The effect of particular improvement measures has often not been significantly evident, whereas their combined implementation has resulted in meaningful advances. Here, we provide updated protocols for the *Agrobacterium*-mediated generation of stably transgenic barley, wheat, triticale and maize. Based upon these methods, several hundred independent transgenic lines have been delivered, with efficiencies of inoculated embryos leading to stably transgenic plants reaching 86% in barley, 10% in wheat, 4% in triticale, and 24% in maize.

1. Introduction

Cereals such as barley, wheat, triticale and maize play a pivotal role for the nutritional intake of humans, being such via direct utilisation as food or through livestock breeding. For 2007, the FAO estimated a global production of some 700 Mt of maize, over 600 Mt of wheat, 137 Mt of barley and 13 Mt of triticale [1]. This data may explain why these crops are a focus of research and biotechnological development.

Over the past centuries improvement of cereals was achieved mostly by conventional breeding. However, due to the ever-growing world population, limited availability of water, increasingly exhausted fossil energy resources, and the changing climatic conditions, new technologies are urgently required to cope with future challenges. Since the mid 1990s, genetic engineering of cereals has provided a novel field of opportunities for faster and more directed modification or introduction of agronomically useful traits [2–6]. While the first successful genetic transformation events in cereal species had been based on direct gene transfer, which was associated with a number of disadvantages, the pioneering study of Hiei et al. [7] on *Agrobacterium*-mediated transformation of rice represents another milestone. They generated many independent transgenic plants, with T-DNA being stably integrated in the nuclear genome, and the transgenes were shown to be expressed. Ishida and colleagues [8] were then the first to publish a protocol for the generation of transgenic maize, which also relied on *A. tumefaciens*. In the following years, similar protocols for all major cereal crops including barley [9] and wheat [10] were published.

The ability to efficiently form shoots originating from single totipotent cells is indispensible for successful genetic transformation of plants. In contrast to dicotyledonous plants, cereal crops are hardly able to regenerate plants from leaf tissue. However, other gene transfer target explants, for example, immature embryos [4], embryogenic pollen cultures [11] and isolated ovules [12] have proven useful in cereals. *Agrobacterium*-mediated genetic transformation of cereals has been largely confined to particular genotypes that combine the amenability to gene transfer by *Agrobacterium*

with adequate regeneration potential. Besides the most suitable lines used as models in routine transformation, namely, the cv. "Golden Promise" in barley [9], Hi II hybrids in maize [4, 13], and the breeding line "Bobwhite 26" in wheat [10], some other genotypes have turned out to be useful for *Agrobacterium*-mediated transformation, albeit with significantly lower efficiency [3, 5, 14–18]. In triticale, the winter type cv. "Bogo" was shown to perform exceedingly well in plant regeneration from immature embryo explants [19–21]. However, no study has yet provided ample evidence of genomic integration of recombinant DNA by means of *A. tumefaciens* in this cereal crop species.

It is not surprising that protocols efficiently used for cereal transformation generally rely on the use of hypervirulent *Agrobacterium* strains such as EHA101 and EHA105 in maize [22], AGL-0 and AGL-1 in barley and wheat [9, 18, 23–26] as well as hypervirulent derivatives of LBA4404 in maize, barley and wheat [11, 18, 27–29]. Hypervirulence can be mediated by accessory *Vir* genes that are either contained on particular Ti-plasmids [30], on so-called superbinary vectors [31], or on an additional plasmid present in the *Agrobacterium* clone employed [11].

Particular attention has to be paid to the binary vectors used for cereal transformation. Many binary vectors that had been developed for dicot species turned out not to be suitable for cereals, which is mainly due to inappropriate promoters and selectable marker genes. Moreover, an exceedingly high stability of the plasmids in *Agrobacterium* appears to be vital so as to provide an adequate proportion of transformation-competent bacteria throughout the entire episode of co-cultivation, in which there are no selective conditions in terms of the bacterial resistance mediated by the binary vector. In this regard, pVS1-based vector backbones proved particularly valuable [32]. More recently, the IPKb vector series was developed that features a number of useful plasmid elements such as pVS1, monocot-compatible promoters and selectable marker genes combined with GATEWAY-cassettes for either over-expression or RNAi-constructs. Moreover, convenient modularity is provided in terms of the selectable marker expression unit and the promoter that directs candidate gene expression [33].

A successful interaction of *A. tumefaciens* with the gene transfer recipient cells depends on many particular conditions. In cereals, which are at best untypical *Agrobacterium* hosts, deviations from optimal conditions are hardly tolerated. Influencing variables which are thought to be most crucial for gene transfer events to occur during co-cultivation include nutrient concentrations, temperature, pH, presence and concentration of *Acetosyringone* and antioxidants as well as duration.

Here, we present updated *Agrobacterium*-based transformation protocols for barley, wheat, triticale and maize, which have been developed and successfully employed to produce hundreds of independent transgenic lines.

2. Materials

2.1. A. tumefaciens Strains. Transformation of *barley*, *wheat,* and *triticale* was mediated by a hypervirulent derivative of *A. tumefaciens* strain LBA4404 [34] harbouring the binary vector pSB187 that contains the *Hpt* selectable marker gene driven by a 400 bp *CaMV35S*-promoter, the *sgfp* (S65T) reporter gene [35] driven by the maize *Ubi-1*-promoter [36] and the vector backbone from pLH vectors [37] with its borders derived from a nopaline Ti plasmid.

In *maize*, gene transfer was conducted with the *A. tumefaciens* strain EHA105 [22] containing the binary vector pGH218 with the *Pat* gene as selectable marker and a *Gus*-intron reporter gene under the control of a doubled enhanced *CaMV35S*-promoter [38]. The vector backbone of pGH218 is the same as in pSB187.

The vector plasmids were introduced into *Agrobacterium* by electroporation.

2.2. Growth of Donor Plants. Germination of *barley* (*Hordeum vulgare* L.) spring type cv. "Golden Promise", *wheat* (*Triticumaestivum* L.) winter type cv. "Certo" and *triticale* (*x Triticosecale* Wittmack) winter type cv. "Bogo" grains was conducted in trays filled with a substrate mix (Spezialmischung Petuniensubstrat, Klasmann, Germany) (see Note 1) in a growth chamber (14/12°C day/night, 12 hours light, 136 μmol s^{-1} m^{-2} photon flux density). After 3 weeks small plantlets were either incubated for additional eight weeks in a vernalisation chamber with 4°C and 8 hours light per day, or transferred into 18-cm pots (2.5 L). At the beginning of tillering stage 15 g Osmocote (Scotts, Netherlands) was applied per pot. Further fertilization was conducted by watering the plants fortnightly with 0.3% Hakaphos Blau (Compo, Germany). When the stems started to elongate the plants were transferred to a controlled glasshouse (18/16°C day/night, 16 hours light, and 170 μmol s^{-1} m^{-2} photon flux density). There, they were fertilized only once with 0.3% Hakaphos Grün (Compo, Germany) when the heading commenced (see Note 2).

Maize (*Zea mays* L.) line "Hi II" grains were grown in 9-cm pots (0.25 L) containing a cultivation substrate (Substrat 2, Klasmann, Germany) in a growth chamber (22/20°C day/night, 13 hours light, 170 μmol s^{-1} m^{-2} photon flux density). Three weeks later plantlets were transferred to a controlled glasshouse cabin (25/17°C day/night, 16 hours light, 170 μmol s^{-1} m^{-2} photon flux density) in 35-cm pots (20 L) with a substrate mix and 60 g Osmocote Pro (Scotts, Netherlands) per pot for fertilization (see Notes 3, 4).

2.3. Plant Tissue Culture Media. The nutrient media used are summarised in Table 1. According to the protocols, precultivation media (PCM), pretreatment medium (PTM), infection medium (IM), liquid or solid co-culture media (CCM) as well as solid media for callus induction (CIM) and regeneration (RM) are required. PCM, PTM, CCM and CIM used in *barley*, *wheat* and *triticale* are based on MS mineral salts [39] supplemented with additional components as shown in Table 1. The RM medium is based on K4N medium which was published elsewhere [11]. In *maize* IM, CCM and CIM are based on Chu N6 mineral salts [40], and RM is based on MS mineral salts [39] supplemented with additional components as shown in Table 1. The pH was adjusted prior to filter sterilisation of the solutions.

Table 1: Details on the transformation procedures and the materials needed in barley, wheat, triticale and maize. MS (Murashige and Skoog, for example, Duchefa no. M0221), K4N [11], B5 (Gamborg B5 Vitamin Mixture, e.g., Duchefa no. G0415), Hygromycin (Hygromycin B, e.g., Roche no. 10843555001), IEs—immature embryos. In cases where it is necessary to distinguish different medium compositions, the generic abbreviations of media (PCM, CCM, CIM and RM) are preceeded by a capital letter (B for barley, W for wheat, T for triticale and M for maize) representing the species for which a particular medium has been initially developed.

Treatment/Step	Barley	Wheat	Triticale	Maize
Embryo precultivation	—	Scutellum directed up, 5 d on WPCM (4.3 gL^{-1} MS minerals, 5 μM $CuSO_4$, 103.1 mgL^{-1}MS vitamins, 0.5 gL^{-1} Glutamine, 8 mgL^{-1}Dicamba, 40 gL^{-1} Maltose·H_2O, 0.1 gL^{-1} Casein hydrolysate, pH = 5.8, 2.5 gL^{-1} Phytagel), 24°C, dark. Incubate 50 IEs per well for 2–4 hours in 6-well plate with 2.5 mL PTM (4.3 gL^{-1} MS minerals, 5 μM $CuSO_4$, 103.1 mgL^{-1} MS vitamins, 0.5 gL^{-1} Glutamine, 2 mgL^{-1} 2,4-D, 63.75 gL^{-1} Mannitol-D, 40 gL^{-1} Maltose·H_2O, 0.1 gL^{-1} Casein hydrolysate, pH = 5.8), RT, dark	Scutellum directed up, 5 d on TPCM (4.3 gL^{-1} MS minerals, 103.1 mgL^{-1}MS vitamins, 0.5 gL^{-1} Glutamine, 6.6 mgL^{-1} Dicamba, 15 gL^{-1} Glucose, 15 gL^{-1} Sucrose, 200 μM Acetosyringone, 0.1gL^{-1} Casein hydrolysate, pH = 5.2, 2.5 gL^{-1} Phytagel), 24°C, dark	—
Inoculation	30–50 IEs in a 6-well plate with 2.5 mL BCCM (4.3 gL^{-1} MS minerals, 1 mgL^{-1} Thiamine HCl, 0.8 gL^{-1} L-Cysteine, 0.69 gL^{-1} L-Proline, 2.5 mgL^{-1} Dicamba, 30 gL^{-1} Maltose·H_2O, 500 μM Acetosyringone, 1 gL^{-1} Casein hydrolysate, 0.25 gL^{-1} Myo-inositol, pH = 5.8) each. Remove BCCM and add 600 μL *Agrobacterium* OD_{600} = 2–2.5, 1 minute 500 mbar, 10 minutes resting at RT, wash for 15 minutes, BCCM	Remove PTM and add 400 μL *Agrobacterium*, OD_{600} = 2–2.5, 30 minutes resting at RT, wash 2x for 5 minutes, WCCM (4.3 gL^{-1} MS minerals, 103.1 mgL^{-1} MS vitamins, 0.8 gL^{-1} L-Cysteine, 0.5 gL^{-1} Glutamine, 6 mgL^{-1}2,4-D, 15 gL^{-1} Glucose, 15 gL^{-1} Sucrose, 500 μM Acetosyringone, 0.1 gL^{-1} Casein hydrolysate, pH = 5.8)	Collect 25 precultivated IEs to 2.5 mL BCCM (see barley for media composition). Remove BCCM and add 600 μL^{-1}*Agrobacterium* OD_{600} = 2.5–3, 1 minute 500 mbar, 10 minutes resting at RT, wash 1-2x for 5 minute, BCCM (see barley for media composition)	Collect up to 200 IEs in 1 mL IM (4 gL^{-1} Chu N6 salt mixture, 4 mgL^{-1}Chu N6 vitamins, 0.7 gL^{-1} L-Proline, 1.5 mgL^{-1} 2,4-D, 36 gL^{-1} Glucose, 68.4 gL^{-1} Sucrose, 100 μM Acetosyringone, pH = 5.2), wash 1x, remove IM, add 1ml IM with *Agrobacterium* OD_{600} = 0.7, 5 minutes resting at RT, blot IEs dry on 4 filter papers (ø 4.5 cm)
Co-cultivation	48–72 hours in 2.5 mL BCCM (see inoculation for composition), 21°C, dark	48–72 hours, 25 IEs as stack on filter paper (ø 4.5 cm) soaked with 400 μL WCCM (see inoculation for composition) + 100 mgL^{-1}Larcoll, in petri dish (ø 5.5 cm), 21°C, dark	48–72 hours, 25 IEs as stack on filter paper (ø 4.5 cm) soaked with 300 μL BCCM (see barley for composition), in petri dish (ø 5.5 cm), 21°C, dark	48–72 hours, 40 IEs on MCCM (2 gL^{-1} Chu N6 salt mixture, 2 mM $CaCl_2$, 112 mgL^{-1} B5 vitamins, 0.4 gL^{-1} L-Cysteine, 2.9 gL^{-1} L-Proline, 4.4 mgL^{-1} Dicamba, 37.6 gL^{-1} Maltose·H_2O, 100 μM Acetosyringone, 1 mM DTT, 0.5 gL^{-1} MES, pH = 5.8, 4 gL^{-1} Phytagel), 21°C, dark

Table 1: Continued.

Treatment/Step	Barley	Wheat	Triticale	Maize
Callus induction	10 IEs each for 2x 14 d on BCIM (4.3 gL^{-1} MS minerals, 5 μM $CuSO_4$, 1 mgL^{-1} Thiamine HCl, 0.69 gL^{-1} L-Proline, 2.5 mgL^{-1} Dicamba, 30 gL^{-1} Maltose·H_2O, 1 gL^{-1} Casein hydrolysate, 0.25 gL^{-1} Myo-inositol, pH = 5.8, 3 gL^{-1} Phytagel, 150 mgL^{-1} Timentin) + 50 mgL^{-1} Hygromycin, 24°C, dark	25 IEs each for 10 d on WCIM (4.3 gL^{-1} MS minerals, 5 μM $CuSO_4$, 103.1 mgL^{-1} MS vitamins, 0.5 gL^{-1} Glutamine, 2 mgL^{-1}2,4-D, 40 gL^{-1} Maltose ·H_2O, 0.1 gL^{-1} Casein hydrolysate, pH = 5.8, 3 gL^{-1} Phytagel, 150 mgL^{-1} Timentin), 24°C, dark, 25 IEs each for 7 d on WCIM + 20 mgL^{-1} Hygromycin, 24°C, dark	10 IEs each for 14 d on BCIM (see barley for composition) without Hygromycin, 24°C, dark, 14 d on BCIM + 25 mgL^{-1} Hygromycin, 24°C, dark	40 IEs each for 7 d on MCIM (4 gL^{-1} Chu N6 salt mixture, 2 mM $CaCl_2$, 5 μM silver nitrate, 112 mgL^{-1} B5 vitamins, 2.9 gL^{-1} L-Proline, 4.4 mgL^{-1} Dicamba, 34.2 gL^{-1} Sucrose, 0.1 gL^{-1} Casein hydrolysate, 0.5 gL^{-1} MES, pH = 5.8, 4 gL^{-1} Phytagel, 150 mgL^{-1} Timentin), 20 IEs each for 14 d on MCIM + 1.5 mgL^{-1} Bialaphos, 4–7x 14 d on MCIM + 3 mgL^{-1} Bialaphos, 24°C, dark
Shoot formation	3x 14 d on BRM (K4N minerals, 112 mgL^{-1} B5 vitamins, 146 mgL^{-1}L-Glutamine, 0.225 mgL^{-1}6-BAP, 36gL^{-1}Maltose·H_2O, pH = 5.8, 3 gL^{-1}Phytagel, 150 mgL^{-1} Timentin) + 25 mgL^{-1} Hygromycin, 24°C, 16 hours light (136 μmol s^{-1} m^{-2})	see barley	see barley	6–10 calluses for 7 d on MRM (4.3 gL^{-1} MS minerals, 2 mM $CaCl_2$, 103.1 mgL^{-1} MS vitamins, 60 gL^{-1}Sucrose, 0.1 gL^{-1}Myo-inositol, pH = 5.8, 3 gL^{-1}Phytagel, 75 mgL^{-1}Timentin) + 1.5 mgL^{-1} Bialaphos, 24°C, dark, 2x 14 d on MRM + 1.5 mgL^{-1}Bialaphos, in high petri dishes (100 × 20 mm), 24°C, 16 hours light (170 μmol s^{-1} m^{-2})
Plantlet formation	Each plant for 14–28 d on BRM + 25 mgL^{-1} Hygromycin, in culture vessels (see maize), 24°C, 16 hours light (136 μmol s^{-1} m^{-2})	see barley	see barley	6 plants for 7–14 d on MRM (half strength sucrose compared to shoot formation), in culture vessels (107 × 94 × 96 mm), 24°C, 16 hours light (170 μmol s^{-1} m^{-2})
Plant establishment in soil	5-6 weeks in substrate mix (Spezialmischung Petuniensubstrat, Klasmann, Germany), 40g fertiliser "Osmocote" (Scotts, Netherlands) per 7.5 L pot, 14/12°C day/night, 12 hours light (136 μmol s^{-1} m^{-2})	see barley	see barley	2–4 weeks in "Substrat 2" (Klasmann, Germany), 22/20°C day/night, 16 hours light (170 μmol s^{-1} m^{-2})

For the preparation of solid media, one volume of fourfold concentrated solution was mixed with three volumes of adequately concentrated Phytagel (Sigma, Germany) that had been autoclaved with the respective proportion of distilled water. If not stated otherwise standard 9-cm petri dishes (Greiner, Germany) were used.

2.4. Isolation of Immature Embryos and Co-cultivation with A. tumefaciens. For the isolation of immature embryos (IEs) and their subsequent co-cultivation with *A. tumefaciens*, the following materials are needed.

(1) Forceps, scalpel, spatula, and preparation needles.

(2) Preparation microscope.

(3) 6-well cell culture plates (Greiner, Germany).

(4) Petri dishes (ø 5.5 cm, Greiner, Germany).

(5) Pipettes and disposable tips (200–1000 μL and 1000–5000 μL, autoclaved).

(6) Eppendorf tubes (2 mL, autoclaved, Eppendorf, Germany).

(7) Filter paper (several sizes, autoclaved, Millipore, Germany).

(8) Exsiccator and vacuum pump.

(9) Magnetic stirrer.

3. Procedures

3.1. Isolation of Immature Embryos. In *barley*, *wheat,* and *triticale*, developing caryopses were harvested 12–16 d post pollination, immersed for 3 minutes in 70% ethanol, incubated in 5% sodium hypochlorite supplemented with 0.1% Tween for 15 minutes and washed five times in sterile, distilled water.

Barley IEs were excised from the caryopses by using forceps and a lanzet needle (see Note 5). The embryo axes of the IEs were removed. The IEs were transferred into 2.5 mL liquid BCCM (Table 1) in a 6-well plate with up to 50 IEs per well (see Notes 6–8).

Triticale and *wheat* IEs were excised as described for *barley*, yet without removal of the embryo axes. Fifty IEs were placed per petri dish with the scutellum facing up on TPCM or WPCM, respectively (Table 1).

Maize ears were harvested 10–14 d after pollination when IEs were of 1.5–2.5 mm in length. For surface sterilization the ears were first incubated 5 minutes in 70% ethanol, then in 2.4% sodium hypochlorite supplemented with 0.1% Tween for 20 minutes and finally washed 4 times in sterile distilled water for 5 minutes each. After removing the abaxial top of the kernels with a scalpel, IEs were dissected with a lancet and up to 200 collected in a 2-mL tube containing 1 mL IM (Table 1).

3.2. Growth of Agrobacterium and Co-cultivation of Immature Embryos. *A. tumefaciens* strain LBA4404 was grown in 10 mL of antibiotic-free CPY medium [34] overnight at 28°C in 100-mL Erlenmeyer flasks with shaking at 180 rpm (see Note 9). A glycerol stock (200 μL from a growing culture with an OD_{600} of 2.0 and 200 μL of 15% glycerol) stored at −80°C was thawed and added to the medium so as to start the culture. In case of *maize* transformation, CPY medium was solidified with 8 g L^{-1} bacto agar prior to autoclaving supplemented with spectinomycin thereafter.

In *barley* BCCM (Table 1) was completely removed and 600 μL *A. tumefaciens* culture was added per well. The plate was placed in an exsiccator and vacuum infiltrated for 1 minute at 500 mbar. Then it was kept for 10 minutes inside the laminar hood without agitation followed by a washing step using 2.5 mL of BCCM. For co-cultivation the embryos were left in 2.5 mL of BCCM per well and the plates were incubated at 21°C in the dark for 48–72 hours without agitation.

In *wheat* 50 precultivated IEs were collected into one well of a 6-well plate and treated with 2.5 mL liquid PTM (Table 1) for 2 to 4 hours at RT. After removal of PTM 600 μL *A. tumefaciens* culture was added, and the plate kept for 30 minutes inside the laminar hood. After washing twice with 2.5 mL WCCM (Table 1) IEs were placed in two stacks of 25 IEs each in a small petri dishes (ø 5.5 cm) on 4.5-cm sterile filter paper disks soaked with 400 μL WCCM containing 100 mg L^{-1} Larcoll and incubated at 21°C in the dark for 48–72 hours (see Note 10).

In *triticale* 25 precultivated IEs were transferred into liquid BCCM (Table 1) right prior to co-cultivation (see Note 11). The following steps were conducted as described for *barley* except that the washed IEs were placed in stacks onto filter paper disks soaked with 300 μL of BCCM as described for *wheat*.

For *maize* transformation *A. tumefaciens* was precultivated for 2-3 d on solid CPY with 100 mg L^{-1} spectinomycin at 21°C in the dark. On the day of transformation the *Agrobacterium* colonies were collected from the plate with a spatula, resuspended in IM (Table 1) and incubated 2-3 hours at 23°C and 100 rpm. OD_{600} was adjusted to 0.7. For inoculation the collected IEs were washed once with 1 mL IM. Then 1 mL of *Agrobacterium* suspension was added and mixed by inverting the tube. After incubation of 5 minutes at room temperature the IEs were transferred to four dry 4.5-cm filter paper disks to remove excess solution. Subsequently 40 IEs each were placed with the scutellum side up onto petri dishes containing MCCM (Table 1).

3.3. Callus Development, Regeneration, and Rooting. In *barley* 10 IEs were cultivated per petri dish containing BCIM (Table 1) (see Note 12). The IEs were placed onto the medium with the scutellum side facing down. Sealed petri dishes were incubated in the dark at 24°C for two weeks followed by a subcultivation on fresh medium for another two weeks.

In *wheat* 25 IEs per petri dish were cultivated containing WCIM (Table 1) and incubated at 24°C in the dark for 10 d the scutellum facing upwards. Next the IEs were incubated for another week on WCIM containing 20 mg L^{-1} hygromycin under the same conditions (see Note 13).

After co-cultivation, *triticale* embryos 10 each were transferred to petri dishes containing solid BCIM (Table 1) and cultivated for 2 weeks followed by subcultivation on fresh medium additionally supplemented with 25 mg L^{-1} hygromycin for another 2 weeks (see Note 14).

In *maize* 40 IEs were incubated first on MCIM (Table 1) at 24°C in the dark for 7 d. For the first selection of two weeks they were transferred to MCIM containing 1.5 mg L^{-1} bialaphos (Molekula, Germany). In the second selection step 20 embryos were cultivated per dish on MCIM supplemented with 3 mg L^{-1} bialaphos. The medium was replaced every 14 d for up to three months until white, rapidly growing type II calluses emerged (see Note 15).

Four weeks after gene transfer, the *barley* and *triticale* calluses were plated onto BRM (Table 1) (see Note 16). The plates were incubated at 24°C under illumination at 136 μmol s^{-1} m^{-2} photon flux density for 16 hours per day. BRM was replaced fortnightly until regenerants emerged. Plantlets with a leaf length of 2 to 3 cm were then individually

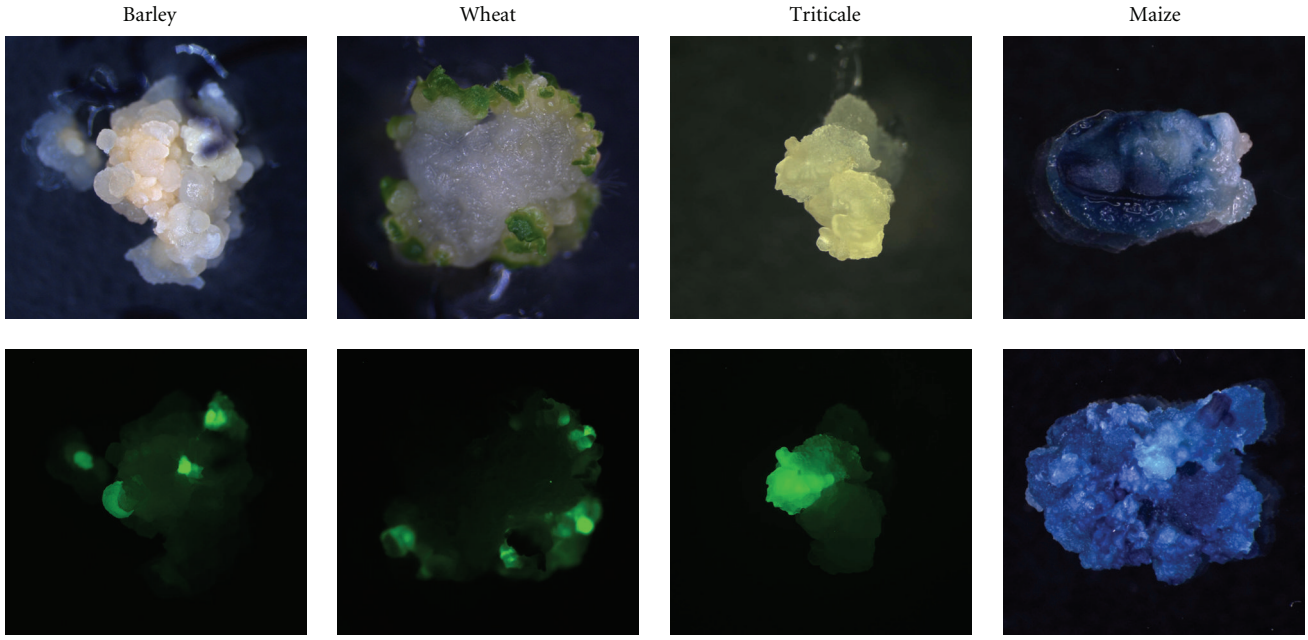

Figure 1: Reporter gene expression in immature embryo-derived calluses for three weeks (barley), four weeks (wheat), two weeks (triticale), 12 d (maize, upper picture) and 8 weeks (maize, lower picture) after co-culture. On the lower pictures of barley, wheat and triticale, the same objects are shown as above, but being exposed to far blue light and recorded with a GFP-filter set. The calluses of maize are shown following histochemical GUS assay [41].

grown in glass tubes (100 mm, ø 25 mm; Schütt, Germany) containing 4.5 mL of BRM (see Note 17). Rooted plants were transferred to the glasshouse where they grew to maturity under the same conditions as described for the donor plants.

The regeneration step for *wheat* was performed with 10 embryo-derived calluses per petri dish containing BRM (Table 1) (see Note 18) supplemented with 25 mg L^{-1} hygromycin for two weeks at 136 μmol s^{-1} m^{-2} photon flux density for 16 hours per day at 22°C. The calluses showing green tissue were selected and again transferred to BRM and incubated under identical conditions for another 2x 14 d until shoot formation. Plantlets with a leaf length of 2 to 3 cm were treated like *barley* plants.

Segments of *maize* calluses with immature somatic embryos were placed first on MRM (Table 1) with 1.5 mg L^{-1} bialaphos for one week in the dark followed by another week incubation in the light with 16 hours photoperiod of 170 μmol s^{-1} m^{-2} photon flux density at 24°C. Matured somatic embryos were removed from the callus under a preparation microscope and incubated in high petri dishes (100 × 20 mm, Greiner, Germany) containing MRM supplemented with 1.5 mg L^{-1} bialaphos for a further two weeks until plantlets were formed. These plantlets were grown in culture vessels (107 × 94 × 96, SteriVent high, Duchefa, The Netherlands) with MRM (Table 1) for up to 14 d until they reached a size of approximately 10 cm. Then they were potted into soil (Substrat 2, Klasmann, Germany) and cultivated as described for the donor plants.

3.4. Analysis of Transgenic Material. In order to facilitate the evaluation of the gene transfer and regeneration process, reporter genes were used instead of effector genes during the period of method establishment (Figure 1). For PCR analysis, genomic DNA from approximately 100 mg of leaf material stored in liquid nitrogen was isolated by means of commercially available extraction kits (e.g., DNAzol, Invitrogen, Germany) according to the manufacturer's instructions. Standard PCR reactions with the appropriate primers (Table 2) were performed using 100 ng genomic DNA per candidate plant. The PCR products were visualised following gel electrophoresis (Figure 2).

Table 2: PCR-Primer used for the analysis of transgenic plants.

Primer	Sequence 5′–3′
GH-Hpt-F1	GAT CGG ACG ATT GCG TCG CA
GH-Hpt-R2	TAT CGG CAC TTT GCA TCG GC
GH-Gfp-F1	GGT CAC GAA CTC CAG CAG GA
GH-Gfp-R1	GAC CAC ATG AAG CAG CAC GA
GH-Gfp-R2	TAC GGC AAG CTG ACC CTG AA
GH-Gus-F1	CCG GTT CGT TGG CAA TAC TC
GH-Gus-R1	CGC AGC GTA ATG CTC TAC AC
GH-Ubi-F1	TTC CGC AGA CGG GAT CGA TCT AGG

Plants which had proven PCR-positive were further analysed by Southern blot for transgene integration and copy number (data not shown). To this end, high quality DNA was prepared as described by Pallotta et al. [42]. Twenty five μg genomic DNA was digested with the appropriate restriction enzyme and the obtained fragments were separated by gel electrophoresis and blotted onto a hybond N membrane

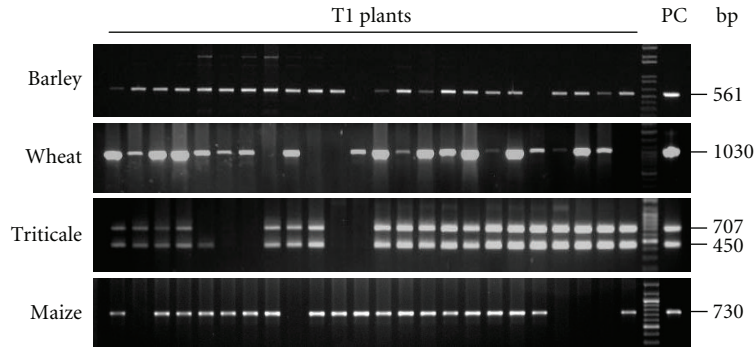

Figure 2: PCR analysis of progenies of primary transgenic plants. Twenty four plants of each T1 family were analysed for the presence of s*gfp* (barley, wheat, triticale, lower bands), *Hpt* (triticale, upper bands) or *Gus* (maize).

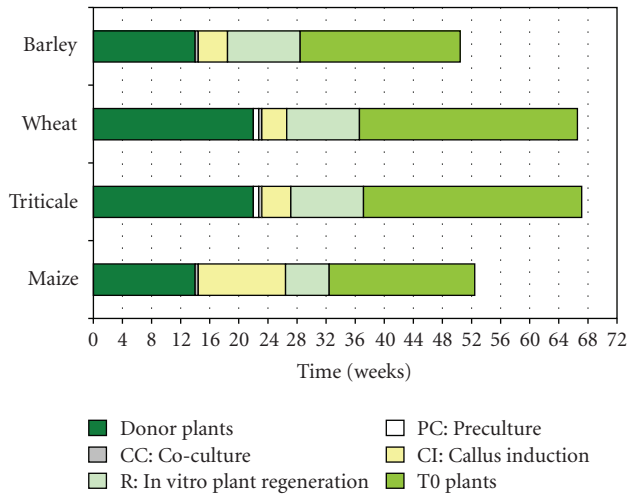

Figure 3: Time lines covering the entire transformation process from growing the donor plants until mature grains can be harvested from primary transgenic plants.

(Roche, Germany). Hybridisation of the blotted DNA with a gene-specific probe was done by labelling with DIG following the manufacturer's instructions (Roche, Germany).

Notes.

(1) The substrate mix is a special white peat substrate plus clay to ensure adequate pH buffering.

(2) Osmocote is a general long-term fertilizer that contains 19% N, 6% P and 12% K. Hakaphos Blau is a general fertilizer that contains 15% N, 10% P and 15% K. Hakaphos Grün is a general fertilizer that contains 20% N, 5% P and 10% K.

(3) Substrate 2 consists of black and white peat. After germination plants are transferred to a substrate mix (compost, sand and white peat).

(4) Osmocote Pro is a general long-term fertilizer that contains 19% N, 7% P and 10% K.

(5) Notably, the developmental stage of the IEs is more crucial than their size. For the protocols described here, transition stage IEs that are about to turn from translucent to white colour are suited best.

(6) Contradictory results have been published regarding the effect of Acetosyringone on *Agrobacterium*-mediated transformation of immature barley embryos [9, 43, 44]. The addition of Acetosyringone results in increased transformation efficiency under the conditions described here.

(7) L-Cysteine supplemented to the co-culture medium was reported to prevent embryos from browning upon inoculation with *Agrobacterium* and to increase the transformation efficiency in soybean [45].

(8) In general, there is a risk to drop a plasmid when *Agrobacterium* is grown in the absence of antibiotics. However, in the protocol described here there was not any loss of vector detected, although *Agrobacterium* used for transformation was repeatedly checked via plasmid preparation. The advantage of growing *Agrobacterium* without antibiotics prior to inoculation is that the grown suspension can be directly used and the recipient cells are not exposed to any residual antibiotics.

(9) In barley, co-cultivation in liquid medium permits a substantially increased number of immature embryos to be processed at once, which results in a remarkable improvement in terms of efficiency [18].

(10) According to our experience, wheat IEs do not tolerate co-culture in liquid medium. On the other hand, it was shown earlier that wheat transformation efficiency can be improved through slight desiccation of IEs [3]. In the protocol presented here, gene transfer to wheat IEs is conducted on filter discs soaked with co-culture medium.

(11) Triticale IEs do not tolerate liquid co-culture as is the case in wheat.

(12) The increased $CuSO_4$ concentration [46] results in improved formation of green plants compared to the conditions described by Tingay et al. [9].

(13) In wheat a resting period without selection following co-culture turned out to be crucial for the generation of transgenic lines.

(14) Although a comparatively low hygromycin concentration was used for cv. "Bogo", all regenerants obtained proved transgenic.

(15) Depending on the genotype different callus types are recommended for manual selection [17].

(16) FHG medium has been successfully used for plant regeneration in a number of published experiments [7, 9, 44, 47]. Yet, a direct comparison conducted in our lab revealed that BRM (Table 1) is superior to FHG.

(17) Alternatively, as many as 16 plants can be grown per culture vessels (see maize) containing BRM. However, glass tubes are preferred to minimize the risk of cross contamination.

(18) Several media have been described for the selective development of transgenic wheat regenerants [3]. In our experiments selection worked best on BRM supplemented with hygromycin (Table 1).

4. Conclusion

In this paper, effective and reproducible protocols for the generation of stably transgenic barley, wheat, triticale and maize plants are presented. In comparison with the earlier reports several improvements have been implemented. The selection regimes utilized for all four species proved to ensure an almost exclusive regeneration of transgenic plants, which is valid for both hygromycin-based selection in barley, wheat and triticale as well as selection of transgenic maize which relies on bialaphos. The period of time needed for the entire process from growing donor plants until the harvest of mature grains from primary transgenic lines is between 51 weeks in spring barley and maize up to 66 weeks in winter wheat and triticale (Figure 3). The transformation efficiencies obtained by the methods described have been 20–86% in barley, 2–10% in wheat, 2–4% in triticale and 0.5–24% in maize. The presented protocols are suitable for comprehensive functional analyses of recombinant nucleotide sequences on a large scale. Furthermore, they constitute a powerful fundament for applied research aiming to improve, for example, disease resistance, tolerance towards abiotic stresses as well as product quality of cereal crops.

Acknowledgments

The authors thank C. Marthe, H. Büchner, S. Wolf and E. Grützemann for their excellent technical assistance. They also thank Dr. Sylvia Broeders for providing the vector pSB187. This work was in part funded by the German Research Foundation (Project KU 2265/1).

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Hindawi Publishing Corporation
International Journal of Plant Genomics
Volume 2009, Article ID 296482, 16 pages
doi:10.1155/2009/296482

Review Article

Heterelogous Expression of Plant Genes

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Received 30 January 2009; Accepted 24 May 2009

Recommended by Pushpendra Gupta

Heterologous expression allows the production of plant proteins in an organism which is simpler than the natural source. This technology is widely used for large-scale purification of plant proteins from microorganisms for biochemical and biophysical analyses. Additionally expression in well-defined model organisms provides insights into the functions of proteins in complex pathways. The present review gives an overview of recombinant plant protein production methods using bacteria, yeast, insect cells, and *Xenopus laevis* oocytes and discusses the advantages of each system for functional studies and protein characterization.

1. Introduction

Heterologous expression involves identification of genes and transfer of the corresponding DNA fragments to hosts other than the original source for synthesis of the encoded proteins. Protein isolation, especially from plant sources, can be costly, cumbersome and lengthy, and heterologous expression provides a convenient alternative. This methodology allows large-scale production of plant proteins in microorganisms to study their biochemical and biophysical features. Foreign hosts may also provide a simpler system for studies on functions of proteins and for elucidation of their roles in complex mechanisms such as metabolic reactions and membrane transport. Recombinant plant proteins and peptides produced by heterologous expression are also used in industrial applications. Examples are provided by the synthesis of a medicinal peptide from ginseng as potential drug against diabetes [1] or production of plant lectins [2] in both cases in yeast.

The present review covers the recent literature on plant gene expression in bacteria, yeast, insect cells and *Xenopus* oocytes and presents the comparative advantages and disadvantages of each system. It also provides a survey of recent examples of application of heterologous expression technology to plant proteins. A comprehensive list of plant proteins expressed heterologously is given in Table 1. Factors influencing the choice of hosts, including the stability and folding characteristics of the protein, requirement for posttranslational modifications, efficiency of the expression system, as well as simplicity and cost are discussed in the following sections.

2. Principal Components of Heterologous Expression

Basic principles of heterologous cloning and expression are summarized in Figure 1. Major parameters that affect choices at different stages are also indicated. The choice of the expression system and vector is a critical step in this procedure and, as indicated, advantages and disadvantages of several factors have to be considered. Expression systems are selected depending on whether the purpose of study is production of large quantities of protein or investigation of functional features of the cloned protein. The physicochemical properties of the investigated protein also play a role in this choice. A general review of frequently used expression systems is provided by Yin et al. [3].

A comprehensive survey of commercially available expression vectors has recently been published [4]. The most commonly used vectors are fusion systems that link additional amino acid sequences (tags) to the protein through a recognition site for a specific protease. Tags may consist of a short peptide sequence or a full protein which can be cleaved from the protein when desired. Presence of tag sequences facilitates solubility, purification, quantification, identification, localization, and assaying of the

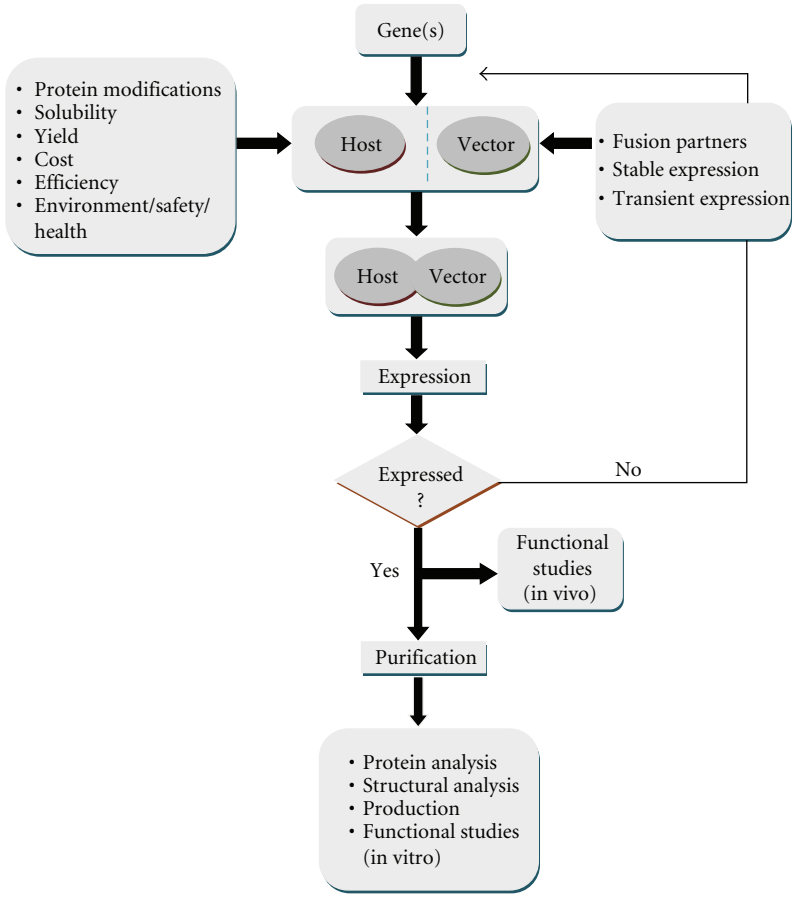


Figure 1: Flow chart for heterologous expression.

expressed protein. Frequently used fusion partners include glutathione-S-transferease (GST), his-tag (poly-histidines), maltose binding protein (MBP), thioredoxin (TrxA), FLAG epitope-tag, c-Myc epitope-tag, disulfide isomerase I (DsbA), polyarginine-tag (Arg-tag), calmodulin-binding peptide, cellulose-binding domain, poly-histidine affinity tag (HAT-tag), N-utilizing substance-A (NusA), S-tag, streptavidin-binding peptide (SBP-tag), strep-tag, fluorescent proteins (e.g., green fluorescent protein (GFP)) and ubiquitin [4]. MBP and NusA are specifically used to increase the solubility. MBP is considered to be much more effective for enhancing solubility than GST and thioredoxin [5]. The major disadvantages of fusion protein systems are the requirement of expensive proteases for cleavage from the recombinant protein and the low yield of cleavage reactions [6].

Depending on the host system, vectors for transient or stable expression can be chosen as indicated below.

3. Expression Hosts

3.1. Prokaryotic Expression Systems

3.1.1. Escherichia Coli. *Escherichia coli* (*E. coli*) is the first and most extensively used prokaryotic expression system for heterologous protein production [7]. It remains generally the first choice due to its simplicity, rapid growth rate, and relatively low cost. Almost all commercially available inducible cloning vectors are compatible with *E. coli* and extensive biochemical and genetic information is available.

One of the disadvantages of using *E. coli* as an expression host arises from its inability to perform post-translational modifications, which are often required for correct folding and functional activity of the recombinant protein. This applies particularly to some membrane proteins and enzymes [3]. Another disadvantage is that *E. coli* is generally not suitable for proteins which contain many disulfide bonds or require glycosylation, proline cis/trans isomerization, disulfide isomerization, lipidation, sulphation, or phosphorylation [8]. Some eukaryotic proteins that retain their full biological activity in the nonglycosylated form have, however, been produced in *E. coli*. The unglycosylated human growth hormone (hGH) binding protein secreted from *E.coli* retains the same binding affinity and specificity as the wild-type hGH binding protein suggesting that recombinant protein is properly folded and glycosylation is not required for binding [9].

Production of proteins that are stabilized by disulfide bonds in *E.coli* often results in proteolytic degradation or misfolding and formation of inclusion bodies [6]. One strategy developed to improve this situation is to target these proteins to the periplasm where the nonreducing environment allows formation of disulfide bonds [10, 11]. In

addition, the *E.coli* periplasm contains chaperone-like disulfide-binding proteins (DsbA, DsbB, DsbC, and DsbD), folding catalysts, and peptidyl-prolyl isomerases (SurA, RotA, FklB, and FkpA) that support disulfide bond formation and are important for correct folding of periplasmic proteins [12–14]. Disulfide bond formation is achieved via fusion to DsbA or DsbC [15, 16] and periplasmic secretion results in the functional production of a variety of recombinant proteins [17]. In a recent study, the rescue of unstable lipase B from *Pseudozyma antarctica* (PalB), with periplasmic folding factors was demonstrated [18]. Another strategy involves the use of the *trx*B *gor* double mutant lacking thioredoxin reductase and glutathione reductase genes [19, 20]. This double mutant was used for heterologous expression of barley oxalate oxidase (HvOXO) in *E.coli* [21]. The gene for an osmotin-like cryoprotective protein from *Solanum dulcamara* was expressed in *E.coli* and directed to periplasmic localization using an expression vector containing the *pelB* signal sequence [22]. This resulted in high concentrations of soluble protein with cryoprotective activity, whereas expression in the bacterial cytoplasm only yielded large amounts of insoluble and aggregated protein.

Some of the plant proteins accumulated in insoluble inclusion bodies in *E.coli* can be solubilized and refolded to restore activity after purification from the host. Examples include Arabidopsis thaumatin-like protein (ATLP3) which was purified from inclusion bodies and the refolded form displayed activity against some pathogenic fungi [23]. To validate the potential antifungal activity of *Solanum nigrum* osmotin-like protein (SnOLP) was overexpressed in *E.coli* and the recombinant protein was refolded using reduced:oxidized gluthatione redox buffer and its in vitro activity was demonstrated [24]. The soybean RHG1-LRR domain protein was solubilized from inclusion bodies using urea and refolded by removing the urea in the presence of arginine and reduced/oxidized glutathione [25].

Many plant enzymes are expressed in insoluble inclusion bodies but it is still possible to obtain high yields of active forms for structural studies [26]. The mature polypeptide of FatB thioesterase from the developing seed tissues of *Madhuca butyracea* was characterized by heterologous expression in *E.coli* [27]. The functionality of the MbFatB in the heterologous system was revealed by the altered growth behavior and cell morphology of the bacteria due to the changes in the fatty acid profile. The maize chloroplast transglutaminase (TGZ) [26] and glutamatecysteine ligase (GCL) [28] were efficiently overexpressed in *E.coli*. Recently, DELLA proteins from both Arabidopsis and *Malus domestica,* which are involved in regulation of plant growth in response to phytohormonal signals, were isolated and expressed in *E. coli* [29].

Examples of functional expression of plant proteins in *E.coli* are provided mostly by studies on membrane proteins. A mutant with very low K^+ uptake was used as host for studies on the K^+ transporters AKT2 [30], AtKUP1-2 [31], AtHKT1 [32] from Arabidopsis and EcHKT1 and EcHKT2 from *Eucalyptus camaldulensis* [33]. In another example, *E.coli* C43 strain, which is suitable for expression of membrane proteins was used for functional characterization of chloroplast ATP/ADP transporter from Arabidopsis [34].

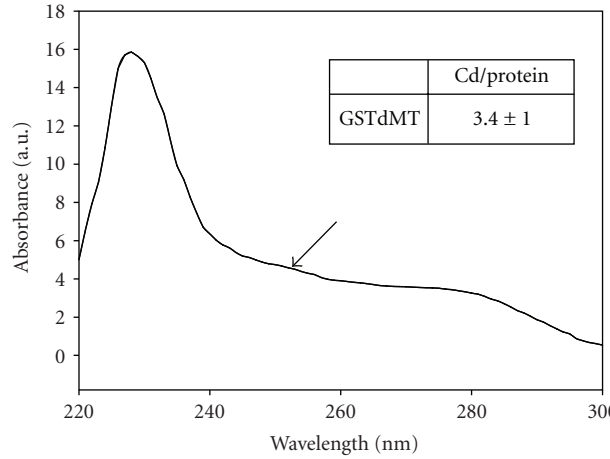


Figure 2: UV-visible absorption spectrum of GSTdMT at 2.7 mg/mL concentration in 20 mM HEPES buffer at pH 8.0. The charge transfer band between 240 and 260 nm due to Cd-S interaction is indicated by the arrow. The Cd/protein ratio is given in the inset.

The seagrass HAK K^+ transporters, CnHAK1 and CnHAK2 were also overexpressed in *E.coli* and it was found that CnHAK1, but not CnHAK2, mediated very rapid K^+ or Rb^+ influxes [35]. Using a dicarboxylate uptake-deficient *E.coli* mutant, a peptide transporter, AgDCAT1 from alder, was shown to be a dicarboxylate, including malate, succinate, fumarate, and oxaloacetate, transporter [36].

E.coli has also been used for expression of small plant proteins with a fusion partner. Metallothioneins (MTs), which are difficult to purify from natural sources because of their small molecular weight (7 kD), unusual amino acid sequences containing a large number of cysteins and their proteolytic susceptibility belong to this class. Several MTs including a Cd^{2+} binding Type 1 durum wheat metallothionein (dMT) [37], fava bean Type 1 and Type 2 MTs [38], Arabidopsis MT1, MT2 and MT3 proteins [39], Type 3 MT3-A from the oil palm [40], Type 2 MT, QsMT from *Quercus suber* [41] have been produced in *E.coli* mainly for structural analyses. Since the fusion constructs of durum MT with GST (GSTdMT) can be purified in well-defined oligomeric states they are used as model systems for studies on metal-binding and for structural analyses. Figure 2 illustrates that Cd-binding to GSTdMT can be detected by UV-visible spectroscopy. The metal content of GSTdMT was shown to be the same as that expected from dMT alone. An example of the shape models generated from X-ray solution scattering data for GSTdMT is shown in Figure 3, together with the fit to experimental data. The models support a fold for dMT similar to that expected for the free molecule [42, 43]. These results are in agreement with earlier work suggesting independent folding of GST and its fusion components [44] and indicate that recombinant fusion complexes are useful as model systems for structural studies.

3.2. Eukaryotic Expression Systems. Eukaryotic expression systems offer the possibility of posttranslational modifications and are often used for investigations of protein

Table 1: Heterologous expression of plant proteins grouped according to the host cells.

Protein expressed	Plant	Reference
Escherichia coli		
Lipase B (PalB)	*Pseudozyma antarctica*	[18]
Oxalate oxidase	*Hordeum vulgare, Triticum aestivum*	[21]
Osmotin-like cryoprotective protein	*Solanum dulcamara*	[22]
Thaumatin-like protein (ATLP3)	*Arabidopsis thaliana*	[23]
Osmotin-like protein (SnOLP)	*Solanum nigrum*	[24]
RHG1-LRR domain	*Glycine max*	[25]
Chloroplast transglutaminase (TGZ)	*Zea mays*	[26]
FatB thioesterase	*Madhuca butyracea*	[27]
Glutamatecysteine ligase (GCL)	*Arabidopsis thaliana*	[28]
DELLA proteins	*Arabidopsis thaliana , Malus domestica*	[29]
K^+ transporters; KAT1, AKT2-3, AtKUP1/AtKT1/AtPOT1, AtKUP2/AtKT2/AtPOT2, AtHKT1	*Arabidopsis thaliana*	[30–32]
K^+ transporters, EcHKT1, EcHKT2	*Eucalyptus globulus*	[33]
ATP/ADP transporter	*Arabidopsis thaliana*	[34]
HAK K^+ transporters, CnHAK1,CnHAK2	*Cymodocea nodosa*	[35]
Peptide transporter family member, AgDCAT1	*Alnus glutinosa*	[36]
Type 1 MT, dMT	*Triticum durum*	[37]
Type 1 and Type 2 MTs	*Vicia faba*	[38]
MT1, MT2, and MT3	*Arabidopsis thaliana*	[39]
Type 3 MT3-A	*Elaeis guineensis*	[40]
Type 2 MT, QsMT	*Quercus suber*	[41]
Soybean seed ferritin	*Glycine max*	[126]
Saccharomyces cerevisiae		
H^+-amino acid symporter and K^+ channel, KATl	*Arabidopsis thaliana*	[47]
Phosphate transporters; AtPT1 and AtPT2	*Arabidopsis thaliana*	[48]
K^+transporter, HvHAKI	*Hordeum vulgare*	[49]
K^+ transporters, AtKT1, and AtKT2, AtKUP1	*Arabidopsis thaliana*	[50, 51]
K^+ transporter, HKT1	*Triticum aestivum*	[52, 53]
Sulfate transporters, LeST1-1 and LeST1-2	*Lycopersicon esculentum*	[54]
Copper transporters, (COPT1–5)	*Arabidopsis thaliana*	[55]
Peptide transporter, AtPTR1	*Arabidopsis thaliana*	[56]
K^+/H^+ antiporter, *At*Chx17	*Arabidopsis thaliana*	[57]

Table 1: Continued.

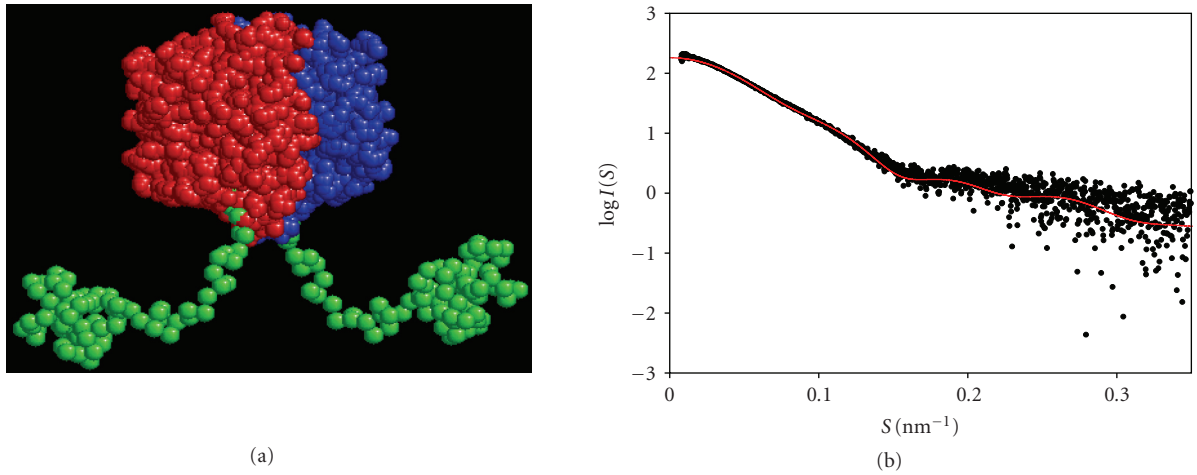
Protein expressed	Plant	Reference
Hexose transporters, VvHT4 and VvHT5	*Vitis vinifera*	[58]
Plasma membrane-localized H^+/inositol symporter, AtINT2	*Arabidopsis thaliana*	[59]
High affinity GABAtransporter, AtGAT1	*Arabidopsis thaliana*	[60]
Tonoplast Intrinsic Proteins, AtTIP2;1 and AtTIP2;3	*Arabidopsis thaliana*	[61]
Sorbitol transporters, PmPLT1 and PmPLT2	*Plantago major*	[62]
Pichia pastoris		
Nitrate reductase	*Spinacia oleracea,Zea mays*	[69]
Invertase	*Ipomoea batatas*	[70]
α1,6-galactosyltransferase	*Trigonella foenum-graecum*	[71]
α1,6-xylosyltransferase	*Arabidopsis thaliana*	[72]
Glycosyltransferases	*Arabidopsis thaliana Bos taurus, Drosophila melanogaster, Caenorhabditis elegans, Leucopersicon esculentum*	[73]
β-D-fructofuranosidase	*Oryza sativa*	[74]
Apyrase	*Solanum tuberosum*	[75]
Oxalate oxidases, HvOXO, TaOXO	*Hordeum vulgare, Triticum aestivum*	[76, 77]
Lectin	*Canavalia brasiliensis, Nicotiana tabacum*	[2, 79]
Low-affinity cation transporter (LCT1)	*Triticum aestivum*	[80]
2S albumin storage proteins (AL1 and AL3)	*Glycine max*	[127]
Baculovirus-mediated insect cell		
Patatin	*Solanum tuberosum*	[81]
Reductase isoforms, AR1 and AR2	*Arabidopsis thaliana*	[82]
Peroxisomal short-chain acyl-CoA oxidase A	*Arabidopsis thaliana*	[83]
Cyclin-dependent kinase A (CDKA)	*Arabidopsis thaliana*	[84]
NADH-cytochrome (Cyt) b5 reductase	*Arabidopsis thaliana*	[85]
Geranylgeranyltransferase-I (GGT-I)	*Arabidopsis thaliana*	[86]
Acyl-CoA synthetase	*Arabidopsis thaliana*	[87]
Homogentisate phytyltransferase	*Arabidopsis thaliana*	[88]
(+)-Abscisic Acid 8'-Hydroxylase	*Arabidopsis thaliana*	[89]
β1,2-xylosyltransferase	*Arabidopsis thaliana*	[90]
Ethylene-inducing xylanase	*Nicotiana tabacum*	[91]

Table 1: Continued.

Protein expressed	Plant	Reference
ADP-glucose pyrophoshorylase (AGPase)	*Hordeum vulgare*	[92]
K^+ channels, AKT1, KAT1, and KCO1	*Arabidopsis thaliana*	[93–95]
K^+ channels KST1, SKT1, and KST1	*Solanum tuberosum*	[96, 97]
Transporter AUX1	*Arabidopsis thaliana*	[98]
β-phaseolin polypeptides	*Phaseolus vulgaris*	[128]
Ac-specific ORFa protein,	*Zea mays*	[129]
Cysteine protease papain	*Carica papaya*	[130]
Mitochondrial protein URF13	*Zea mays*	[131]
LAT52 protein	*Lycopersicon esculentum*	[132]
Auxin-binding protein (ABP1)	*Zea mays, Nicotiana tabacum*	[133, 134]
Calreticulin and auxin binding protein	*Zea mays*	[135]
Cinnamate 4-Hydroxylase	*Arabidopsis thaliana*	[136]
Cryptochrome-1	*Arabidopsis thaliana*	[137]
Phototropin 2	*Arabidopsis thaliana*	[138]
Histidinol dehydrogenase	*Brassica oleracea*	[139]
Putative soluble epoxide hydrolase (sEH)	*Solanum tuberosum*	[140]
lmidazoleglycerolphosphate dehydratase	*Arabidopsis thaliana*	[141]
Phytone synthase, Phytoene desaturase	*Narcissus pseudonarcissus*	[142, 143]
4-coumarate:coenzyme A ligase (4Cl)	*Populus trichocarpa, Populusdeltoides*	[144]
Xenopuslaevisoocytes		
$Na^+ - K^+$ cotransporter HKT1	*Arabidopsis thaliana*	[39]
AgDCAT1 nodule-specific transporter	*Alnus glutinosa*	[43]
AtNAR2.1/AtNRT2 Nitrate Transport System	*Arabidopsis thaliana*	[102]
HKT Constructs, AtHKT1_HKT1 chimeras	*Triticum aestivum*, *Arabidopsis thaliana*	[103]
HKT1 superfamily of K^+/Na^+ transporters	*Eucalyptus camaldulensis*	[104]
Ammonium transporter, LeAMT1	*Lycopersicon esculentum*	[105]
Ammonium transporter, AtAMT1;2	*Arabidopsis thaliana*	[106]
Sucrose transporters, AtSUC2, AtSUC9, LjSUT4	*Arabidopsis thaliana,Lotus japonicus*	[107–109]
Al-activated malate transporter, BnALMT1,BnALMT2, ALMT1	*Brassica napus, Triticum aestivum*	[110, 111]
Polyol transporters, AtPLT5, PmPLT1	*Arabidopsis thaliana, Plantago major*	[112, 113]

Table 1: Continued.

Protein expressed	Plant	Reference
Inositol transporter2, AtINT2, AtINT4	*Arabidopsis thaliana*	[114, 115]
Amino acid transporter, AtCAT6,	*Arabidopsis thaliana*	[116]
Cation–Cl- cotransporter, CCC	*Arabidopsis thaliana*	[117]
Anion-selective transporter, ZmALMT1	*Zea mays*	[118]
K^+channel, SIRK	*Vitis vinifera*	[145]
K^+ channel, KZM1	*Zea mays*	[146]
K^+ channel, ZMK1	*Zea mays*	[147]
K^+ channels, SKT1 and LKT1	*Solanum tuberesum*, *Lycopersicon esculentum*	[148]
AKT2-KAT2 subunitits	*Arabidopsis thaliana*	[149]
K^+ channel, KAT1	*Arabidopsis thaliana*	[150]
Cyclic nucleotide-gated ion channels AtCNGC2, AtCNGC1, -2	*Arabidopsis thaliana*, *Nicotiana tobacum*	[151, 152]
Putative transporter (GmN70)	*Glycine max*	[153]
Al-activated malate transporter, TaALMT1	*Triticum aestivum*	[154]
High affinity γ-aminobutyric acid transporter, AtGAT1	*Arabidopsis thaliana*	[155]
Aquaporins, ZmPIP1a, ZmPIP1b, ZmPIP2a, PIP1, ZmPIP2;1	*Zea mays*	[124, 156, 157]
Aquaporin, PIP1	*Lycopersicon esculentum*	[158]
Aquaporin, PIP2	*Juglans regia*	[159]
Tonoplast intrinsic protein, AtTIP2;1	*Arabidopsis thaliana*	[160, 161]
Aquaporin, McTIP1;2	*Mesembryanthemum crystallinum*	[162]
Aquaporin, HvPIP1;6	*Hordeum vulgare*	[163]
Tonoplast intrinsic protein, PgTIP1	*Panax ginseng*	[164]
Nodulin 26 intrinsic protein, AtNIP2;1	*Arabidopsis thaliana*	[165]
PIP-1-type; NtPIP1;1, NtAQP1; PIP-2-type; NtPIP2;1	*Nicotiana tabacum*	[166]
CjMDR1, ATP-binding cassette protein	*Coptis japonica*	[167]
GlpF-like intrinsic protein (GIP1;1),	*Physcomitrella patens*	[168]
Metal tolerance protein1, AtMTP1	*Arabidopsis thaliana*	[169]
AtTPK4 tandem-pore K^+channel	*Arabidopsis thaliana*	[170]
FRD3, multidrug and toxin efflux (MATE)	*Arabidopsis thaliana*	[171]



(a) (b)

FIGURE 3: A: a low resolution shape model for GSTdMT. The GST dimer (red and blue) is located at the center from which the dMT molecules extend (green). B: the scattering curve expected from the model (−) agrees well with the experimental data (...). I(S) is the scattering intensity and S the scattering vector given by $S = 4\pi\sin\theta/\lambda$, where 2θ is the scattering angle and λ= 1.5 Å is the wavelength of X-rays. The model and the expected scattering pattern were calculated using the programs in the ATSAS package (EMBL Hamburg Outstation).

function. Processing reactions such as *O*-and *N*-linked glycosylation, tyrosine, serine, and threonine phosphorylation, addition of fatty acid chains, processing of signal sequences, disulfide bond formation, and correct folding can all be readily performed in eukaryotic hosts. The most commonly used eukaryotic systems are yeast, insect, mammalian, and plant cells.

3.2.1. Yeast. As a single cell eukaryotic organism, yeast has molecular, genetic, and biochemical characteristics which are similar to those of higher eukaryotes, and is useful for heterelogous protein production. Yeast cells can grow rapidly with high cell densities, and are easy to manipulate and yeast cultures are cost effective. The two most commonly used organisms are *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Pichia pastoris (P. pastoris)* [7].

Saccharomyces Cerevisiae. Baker's yeast, *S. cerevisiae*, is widely used as a host organism for heterologous expression of proteins. Its genetics and physiology are well documented and proteins are posttranslationally modified through the mechanisms similar to those found in plants. The limitations of this host system are low yields, cell stress due to the presence of the foreign gene and hyperglycosylation of secreted foreign proteins. Lack of a strong inducible promoter can be circumvented using *P. pastoris* [45].

Earlier work on heterelogous expression for screening of plant cDNA libraries by complementation in *S. cerevisiae* null mutants was reviewed by Frommer and Ninnemann [7]. The *S. cerevisiae* mutants provide a convenient system for functional and kinetic studies of transporters [46]. The electrophysiological properties of membrane transporters, H^+-amino acid symporter and K^+ channel, KAT1 [47] and phosphate transporters; AtPT1 and AtPT2 of Arabidopsis were characterized using *S. cerevisiae* [48]. Recently, functional expression of transporters such as an HvHAKI from barley [49], AtKT1 and AtKT2 [50], and AtKUP1 from Arabidopsis [51] also utilized *S. cerevisiae* mutants. Another K^+ transporter characterized in this system is HKT1 from wheat [52, 53]. Kinetic uptake analyses of tomato sulfate transporters, LeST1-1 and LeST1-2 were carried out using the *S. cerevisiae* sulfate transporter mutant [54]. The five members of the copper transporter family COPT1–5 from Arabidopsis were characterized using a copper transport null mutant [55]. A peptide transporter *AtPTR1* gene from Arabidopsis was isolated and complemented in a peptide transport-deficient mutant [56]. A putative K^+/H^+ antiporter, *At*Chx17 was heterologously expressed and characterized in an *S. cerevisiae kha1* deletion mutant [57]. To test their functional activity, the grapevine hexose transporters VvHT3, VvHT4, and VvHT5 were expressed in the *S. cerevisiae* mutant EBY.VW4000, which is deficient in glucose transport due to concurrent knock-out of 20 endogenous transporter genes [58]. Growth-based complementation assays were used to demonstrate function of the transporters but resulted in inadequate rates of glucose uptake. A more sensitive assay based on direct measurement of radioactively labelled glucose uptake revealed that this mutant expressing VvHT4 and VvHT5 accumulated labelled glucose at higher rates than yeast transformed with the empty vector, demonstrating the functionality of the glucose transporters. Although VvHT3:GFP (green fluorescent protein) fusion protein was targeted to the plasma membrane in plant cells, VvHT3 was found not to be functional in the yeast system [58].Yeast expression studies were, in several instances, complemented by studies in other organisms to verify functional and kinetic properties of recombinant proteins. The plasma membrane-localized H^+/inositol symporter AtINT2 of Arabidopsis was studied by expression in an inositol uptake/inositol biosynthesis double mutant in *S. cerevisiae* and in *Xenopus* oocytes [59]. In this study, the amount of AtINT2 protein in yeast plasma membrane was sufficient for complementation, but not for functional and kinetic analyses. In oocytes, however, it was possible to show that AtINT2 mediated the symport

of H^+ [59]. Expression and functional characterization of Arabidopsis AtGAT1 in *S. cerevisiae* and *Xenopus* oocytes revealed that AtGAT1 mediates H^+-dependent, high affinity transport of high affinity γ-aminobutyric acid (GABA) and GABA-related compounds. Properties of this protein could be examined in more detail in *Xenopus* oocytes [60]. Heterologous expression of AtTIP2;1 and AtTIP2;3 from Arabidopsis in both ammonium uptake-defective yeast and oocytes indicated that these TIPs transport both ammonium and methyl-ammonium in addition to water and urea [61]. The kinetic characteristics of the sorbitol transporters, PmPLT1, and PmPLT2 from common plantain (*Plantago major*) were investigated by functional expression in *S. cerevisiae* and in *Xenopus oocytes*. In the yeast system, both proteins were characterized as low-affinity and low-specificity polyol symporters. These data were confirmed in the *Xenopus* system, where PmPLT1 was analyzed in detail and characterized as an H^+ symporter [62].

The major disadvantages of using *S. cerevisiae* mutants in transporter studies are the hyperpolarization of the membrane, mislocalization of membrane proteins and recruitment of non-K^+-transporters into K^+-transporters [63].

Pichia Pastoris. P. pastoris, methylotrophic yeast, is considered a valuable tool for high yield heterologous expression of various proteins. The possibility of obtaining posttranslational modifications, high level expression of foreign proteins in either intracellular or extracellular forms, simplicity of genetic manipulations, and availability of various *P. pastoris* strains and vectors make this expression system highly popular [64]. Molecular manipulations such as gene targeting, high frequency DNA transformation, and cloning for functional complementation are similar to those in *S. cerevisiae* [64]. Tightly regulated promoters, easy integration of heterologous DNA into the host chromosome and the capacity to generate more posttranslational modifications make *P. pastoris* the preferred system compared to *S. cerevisiae*.

The wide use of *P. pastoris* expression system for recombinant plant proteins can be seen from recent reviews [64, 65]. *P. pastoris* is particularly well suited for studying plant enzymes since glycosylation of the foreign proteins is expected to be closer to that in plants [66, 67] and glycosylated proteins have shorter glycosyl chains in *P. pastoris* than in *S. cerevisiae* [68]. This expression system has the potential to produce high levels of recombinant proteins [67], up to 400 mg/L of culture [69]. Several plant enzymes have been produced in *Pichia.* Two examples are cytosolic expression of nitrate reductase from spinach and corn at high levels needed for detailed biochemical studies [69] and expression of a sweet potato invertase in milligram quantities [70]. Enzymatic activity of the membrane-bound α1,6-galactosyltransferase was shown through overexpression in *P. pastoris* [71]. The hypothesis that α-xylosyltransferase is involved in xyloglucan biosynthesis was tested by overexpressing the corresponding genes and identifying the gene product that displayed activity [72]. *P. pastoris* has been used for production of a number of glycosyltransferases involved in the biosynthesis of *N*- and *O*-linked oligosaccharides [73]. To confirm that *Osβfruct3* from rice encoded a vacuolar type β-D-fructofuranosidase, the *Osβfruct3* cDNA was expressed in this host [74]. A recombinant potato apyrase was expressed and purified in the hyperglycosylated form at 1 mg/L protein concentration [75]. The catalytically active barley oxalate oxidase, HvOXO was produced with a yield of 50 mg/L culture and biochemically characterized [76].

High-level expression of wheat germin/oxalate oxidase was achieved in *P. pastoris*as an α-mating factor signal peptide fusion to increase secretion of the protein of interest into the culture medium. Approximately 1 g (4×10^4 U) of TaOXO was produced in 5 L fermentation cultures following 8 days of methanol induction, demonstrating the possibility of large-scale production of oxalate oxidase for biotechnological applications. Glycosylation of the recombinant protein was evidenced by mass spectrometry [77]. Another application using *P. pastoris* is the expression of the α-subunit of heterotrimeric G-proteins, GPA1, from Arabidopsis. Several attempts had previously failed to produce this protein in *E. coli*, whereas in the yeast system the protein could be expressed with a his_6-tag and purified by affinity chromatography with a yield up to 20 mg from 700 mL culture [78].

Several allergens including, Cyn d 1 from Bermuda grass, Bla g 4 from *German cockroach*, Amb a 6 from *Ambrosia artemisiifolia,* and Ole e 1 from *Olea europaea* have also been produced in *P. pastoris* (see list in 64).

This system was also used for the expression of a number of plant lectins such as *Canavalia brasiliensis* lectin (ConBr) [2] and the *Nicotiana tabacum* lectin [79]. In a recent study, the low-affinity cation transporter (LCT1) from wheat was also expressed and functionally characterized using *P. pastoris* [80].

3.2.2. Insect Cells. Baculoviruses have been used for the synthesis of a wide variety of eukaryotic recombinant proteins in insect cells. In this expression system one of the nonessential viral genes is replaced with the target protein through homologous recombination. The resulting recombinant baculovirus is used to infect cultured insect cells and the heterologous genes can be expressed under the control of the extremely strong pPolh, polyhedron promoter in the late phase of infection.

The most common baculovirus used for expression studies is *Autographa californica* multiple capsid nucleopolyhedrovirus (*Ac*MNPV) and the most frequently used host insects are *Spodoptera frugiperda* and *Trichoplusia ni*. This expression system produces high levels of recombinant proteins which are soluble, post-translationally modified, biologically active, and functional [81]. The virus is not pathogenic to vertebrates or plants. The main drawback of this system over the bacterial and yeast systems lies in the noncontinuous expression of the heterologous gene; every round of protein production needs reinfection [3].

This heterologous expression system is mainly used to investigate enzymatic mechanisms in plants. The most recent examples include the Arabidopsis reductase isoforms, AR1 and AR2 [82], peroxisomal short-chain acyl-CoA oxidase A [83], cyclin-dependent kinase A [84], NADH-cytochrome b5 reductase [85], geranylgeranyltransferase-I [86], acyl-CoA

synthetase [87], homogentisate phytyltransferase [88], (+)-abscisic acid 8'-hydroxylase [89], β1,2-xylosyltransferase [90], tobacco ethylene-inducing xylanase [91], and barley ADP-glucose pyrophoshorylase [92]. The overall yield of heterelogous proteins obtained with this system is usually lower than with *P. pastoris*.

Baculovirus-infected insect cells have been used as an alternative system to *Xenopus* oocytes for expression and characterization of plant channel proteins. Several channel proteins which were not functional in oocytes could be characterized in baculovirus-infected insect cells such as the K^+ channel proteins AKT1 [93], KAT1 [94], KCO1 [95] from Arabidopsis, and KST1 [96] and SKT1 [97] from potato.

To investigate the interaction between AUX1 and its transport substrate indole-3-acetic acid (IAA) from Arabidopsis, an epitope-tagged version of AUX1 was expressed at high levels in a baculovirus expression system and suitable membrane fragments were prepared from baculovirus-infected insect cells for direct measurement of IAA binding to AUX1. AUX1-IAA interactions were determined using a radio-ligand binding assay to confirm that AUX1 was able to bind IAA with an affinity (Kd) of 2.6 mM, comparable with estimates of the Km for IAA transport [98].

The main disadvantages of using baculovirus-infected insect cells are difficulties in constructing the expression vectors, requirements for more complex laboratory facilities and skills, and the short expression periods after infection.

3.2.3. Xenopus Laevis Oocytes. The oocytes of the South African clawed frog, *Xenopus laevis*, are also used for heterelogous expression of eukaryotic genes. The mRNA for the target protein, introduced by microinjection into the cytoplasm, is translated and the protein is posttranslationally modified by the oocyte [99]. Direct injections of DNA into the nucleus are also possible, but the manipulations are difficult as the nucleus can easily be damaged in the process.

Investigations on membrane transport proteins can be readily performed on oocytes where techniques for electrophysiological measurements are well established. Although, a high proportion of cells express the foreign gene after injection variations in the quality of oocytes and in the ability of individual cells to produce the heterelogous protein can cause problems. Oocytes are not suitable for preparing large quantities of proteins and the short expression period often leads to technical difficulties. The system can also not be sustained over long periods of time and is not suitable for stable expression [99, 100].

Xenopus oocytes have, however, provided a powerful heterologous expression system for animal as well as plant genes.The possibility of using *Xenopus* oocytes as heterologous expression systems for the identification of plant transporters was first demonstrated by the expression of the H^+/glucose transporter STP1 from Arabidopsis [101]. It has, since, been mainly used for production of transporters including potassium channels, H^+/hexose cotransporters, aquaporins, and chloride channels [99]. In addition, functional expression of a nitrate transporter [102], a K^+/Na^+ transporter [39, 103, 104], ammonium transporters [105, 106], sucrose transporters [107–109], Al-activated malate transporters [110, 111], polyol transporters [112, 113], inositol transporters [114, 115], an amino acid transporter [116], a cation–Cl-cotransporter [117], and an anion-selective transporter [118] in *Xenopus* oocytes were investigated. Cases where channel proteins expressed in oocytes were not functional have also been reported. These include the K^+ channels AKT1 from Arabidopsis [93, 119, 120], TaAKT1 from wheat [121], DKT1 from carrot [122], and OsAKT1 from rice [123]. The causes for the lack of function of these recombinant proteins are not clear.

Several studies have used expression of a wild type and its mutant forms in *Xenopus* oocytes to confirm the in vivo functions of plant proteins, especially transporters and plasma membrane intrinsic proteins (PIPs or aquaporins). To demonstrate whether or not the plant K^+ channels form multimers, the wild type and a mutant were coexpressed in *Xenopus* oocytes [120]. Coexpression of tomato ammonium transporter (LeAMT1;1) and its mutant in *Xenopus* oocytes inhibited ammonium transport, suggesting homooligomerization [105]. In another study, the role of phosphorylation in the water channel activity of wild-type and mutant ZmPIP2;1 was studied in *Xenopus* oocytes [124].

In recent studies, the *Xenopus* oocyte expression system was used to investigate structure-function relationships. In one example, differences in the function of two cation transporters, wheat HKT1 and Arabidopsis AtHKT1, were investigated using a series of AtHKT1/HKT1 chimeras with point mutations [103].

4. Conclusions

Heterologous expression of plant genes in other host organisms has two main applications: (1) overexpression of the encoded protein, for biochemical and biophysical characterization and (2) expression of foreign genes for determination of the function of the encoded protein by complementing in a mutant host. Overexpression of recombinant proteins is usually carried out with a cleavable tag to simplify purification in large quantities. In contrast, complementation studies are carried out in null mutants to restore a missing activity in vivo.

Decisions on which expression vectors to use and the choice of the expression host depend on the particular application. In general *E.coli* is the first choice as host because of its simplicity, availability of expression vectors, cost effectiveness, and availability of extensive genetic information on this host. Alternative expression systems are used only if the recombinant protein is inactive due to lack of essential posttranslational modifications and when detailed studies on the recombinant protein function are planned. Yeast systems have the advantage of ease of manipulation and short generation time. *S. cerevisiae* has been extensively used for functional complementation, biochemical, and electrophysiolagical characterization of plant membrane and transporter proteins. *P. pastoris* is the preferred host for overexpression of several plant enzymes. Baculovirus-mediated insect cell expression offers the possibility for detailed investigations of plant enzymes and transporters. The oocyte from *Xenopus laevis* is often used for monitoring activity and biochemical

and electrophysiological characterization of plant plasma membrane transporter and pump proteins.

Heterologous expression is a powerful tool for functional and biochemical analyses of genes and gene families isolated from various organisms. It is particularly important for plants where the whole genome sequence is not available. This system will also provide *denovo* analysis. Its limitations, however, should be kept in mind, especially when interpreting the results in terms of the native structure and function of proteins. Major problems arise from misfolding and mislocalization of recombinant proteins in foreign hosts. Strategies developed to avoid misfolding of recombinant proteins include expression in periplasmic space, expression with a tag, and utilization of different hosts. Mislocalization, on the other hand, may occur because the recombinant protein may take over the function of the missing host protein [125]. Conclusions on function need to be tested in alternative hosts and eventually in the plant itself.

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Hindawi Publishing Corporation
International Journal of Plant Genomics
Volume 2009, Article ID 848145, 8 pages
doi:10.1155/2009/848145

Research Article

Heterologous Expression of a Membrane-Spanning Auxin Importer: Implications for Functional Analyses of Auxin Transporters

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Received 22 January 2009; Revised 9 March 2009; Accepted 27 April 2009

Recommended by Hikmet Budak

Biochemical studies of plant auxin transporters in vivo are made difficult by the presence of multiple auxin transporters and auxin-interacting proteins. Furthermore, the expression level of most such transporters in plants is likely to be too low for purification and downstream functional analysis. Heterologous expression systems should address both of these issues. We have examined a number of such systems for their efficiency in expressing AUX1 from *Arabidopsis thaliana*. We find that a eukaryotic system based upon infection of insect cells with recombinant baculovirus provides a high level, easily scalable expression system capable of delivering a functional assay for AUX1. Furthermore, a transient transfection system in mammalian cells enables localization of AUX1 and AUX1-mediated transport of auxin to be investigated. In contrast, we were unable to utilise *P. pastoris* or *L. lactis* expression systems to reliably express AUX1.

1. Introduction

Auxin (indole-3-acetic acid) and several related compounds are key hormones in plants and have a multitude of effects on plant physiology, regulating, amongst other process, tropic responses to light and gravity, organogenesis, and senescence. The polarised transport of auxin into and out of cells is essential to control cellular auxin levels and the generation and maintenance of auxin gradients required for these processes. The AUXIN RESISTANT 1 (AUX1) gene encodes an auxin influx carrier belonging to the AUX/LAX family of auxin influx transporters [1]. Loss of AUX1 function results in reductions in growth and response to gravity [2]. The AUX1 protein comprises a polypeptide of 485 amino acids, with a predicted molecular mass of 54 kDa. The protein is localised to the plasma membrane, with a predicted topology of 11 transmembrane helices, a cytoplasmic N-terminus, and apoplastic C-terminus [3] (Figure 1). Its transport substrate/ligand (auxin, indole-3-acetic acid) is a weak organic acid (pKa of 4.8), structurally similar to the amino acid tryptophan. AUX1 is proposed to function as a proton:auxin symporter since the protein (and its sequence homologues LAX1-3) shares a high degree of sequence identity with the amino acid auxin permease (AAAP) family of transporters [4]. Detailed biochemical characterization of AUX1 and other auxin transporters is critical to understand their contribution to plant development [5].

Several constraints prevent the characterization of the biochemistry of auxin transporters in plants. Firstly, a large number of additional auxin interacting proteins exist (both in cellular membranes as well as intracellularly), including auxin receptors, influx and efflux transporter proteins. Secondly, the expression level of most membrane proteins is relatively low in their natural membrane. For these reasons we attempted to express the AUX1 auxin importer in several heterologous systems. Of these, some are compatible with high level expression, essential for longer term strategies aimed at purification and reconstitution.

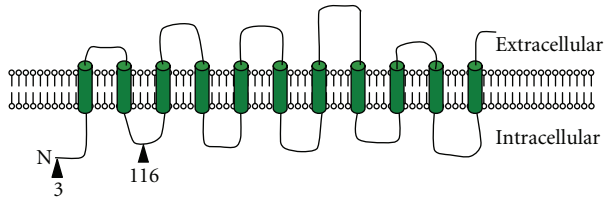

Figure 1: Epitope-tagged forms of AUX1. Diagrammatic representation of AUX1 constructs. The predicted membrane topology of AUX1 is shown with TM helices represented as cylinders. The epitope sequences for the $HIS_6$3xFLAG and HA tags are shown with the sites of insertion represented as triangles, with the specific residue number for the insertion site below.

When selecting a system for heterologous protein expression it is important to consider the capabilities of the system which best suit the downstream applications. Among the important considerations are time investment, cost, fidelity of posttranslational modification, compatibility with functional assay, and expression level scalability. For functional studies a system that has similar posttranslational modification machinery would be highly desirable for conservation of function (be that binding of ligand, or transport per se), whereas for purification a system that lends itself to the production of large quantities (mg of protein) is more desirable. We describe the approaches and our experiences with four heterologous expression systems for AUX1. One of these (*Lactococcus lactis*) is a prokaryotic expression system, whereas the other three (*Pichia pastoris*), baculovirus infected insect cells, and transfected mammalian cells are eukaryotic. Of the four systems two enabled us to pursue functional analysis of AUX1 and one will be able to support further studies on the purified and reconstituted protein.

2. Materials and Methods

2.1. DNA Constructs. AUX1 cDNAs encoding appropriate epitope tags (Figure 1) were generated: an N-terminal haemagluttinin tagged AUX1 construct (N-HA-AUX1) and two constructs with different locations of an insertion comprising a hexahistidine stretch followed by three repeats of the FLAG epitope (N-His_6-3xFLAG-AUX1 and L2-His_6-3xFLAG-AUX1). For $His_6$3XFLAG cDNAs the template DNAs were two pBluescript derived vectors pSK_AUX1_NYFP and pSK_AUX1_L2YFP described previously [3]. The YFP gene in each vector was removed by Asp718 digestion and replaced by insertion of an Asp718 digested double stranded oligonucleotide (top strand primer sequence: GGGGTACCCACCATCATCATCATCATATCG-ACTACAAAGACCATGACGGTGATTATAAAGATCATG-ATATCGATTACAAGGATGACGATGACAAGGGTACCG-G). HA-AUX1 cDNA was generated by RT-PCR from *Arabidopsis thaliana* HA-AUX1 transgenic plants [3]. Briefly, RNA was extracted from 7 day old (At) seedlings using a Qiagen RNeasy kit following the manufacturer's instructions, and 1 μg of this was reverse transcribed (2 hours, 37°C) using Superscript II (Invitrogen). The reaction was heat inactivated (70°C for 10 minutes), and the resultant cDNA was amplified by PCR with primers (5′ GGGAATTCTCTAATAGAA-AACATCTA and 5′ GGACTAGTTCAAAGACGGTGG-TGTAAAGCGGA). Tagged cDNAs were then shuttled into expression vectors (pcDNA3.1(+), pPICZB, pNZ8048, and pFastBac1, all from Invitrogen, except pNZ8048 [6]) using restriction digest and ligation. All plasmids were fully sequenced to ensure in-frame insertion of any epitope tags.

2.2. Expression of Epitope-Tagged AUX1 in Mammalian Cells. HEK293T [7] or U2OS [8] cell monolayers were propagated in complete high glucose DMEM (4.5 g/L glucose; GIBCO) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS; GIBCO), and 50 units/mL penicillin/streptomycin (GIBCO) at 37°C, 5% CO_2 and maintained by passaging when approximately 80% confluent. For transfection, cells were seeded at densities of either 400 000 per well (HEK293T) or 100 000 cell per well (U2OS) in a 6-well dish (Falcon) 27 hours before transfection. U2OS cells were transfected with Fugene (Roche) according to the manufacturer's instructions, whereas polyethyleneimine (PEI) was used to transfer DNA into HEK293T cells [9]. For PEI transfection, media was replaced with a lower serum percentage media (2% v/v) 3 hours prior to transfection. At the time of transfection 10 mM linear PEI (Polysciences, Inc.), pH 7, was added to plasmid DNA containing 5% w/v glucose (4–8 μg of DNA per well) to achieve a nitrogen : phosphorus molar ratio of 8 : 1 (the N : P ratio refers to PEI-derived nitrogen : DNA-derived phosphorus [9]). Following brief mixing, this was added dropwise to the cell monolayers. Twenty-four hours posttransfection the media was removed and replaced by 10% v/v serum-containing media supplemented with 2 mM butyric acid. Cells were harvested 48–88 hours posttransfection by repeat pipetting into ice cold phosphate buffered saline (PBS) supplemented with 2 mM EDTA and centrifuged at 300 g for 10 minutes. Cell pellets were resuspended in 250 μL PBS containing protease inhibitors (Complete EDTA-free Protease Inhibitor, Roche) and lysed by 3 × 10 seconds bursts of sonication.

2.3. Expression of Epitope-Tagged AUX1 in Sf9 Insect Cells. *Spodoptera frugiperda* (Sf9) cells were grown as orbital cultures at 27-28°C in InsectXpress medium (Lonza) supplemented with 10% v/v foetal calf serum and 50 units/mL penicillin and streptomycin. AUX1 was expressed in Sf9

cells following infection with recombinant baculovirus. Recombinant bacmid DNA was constructed using the Bac-2-Bac system (Invitrogen), following the manufacturer's instructions. After PCR screening of the bacmid DNA to ensure correct insertion of AUX1 cDNA, recombinant virus was generated by Cellfectin-mediated transfection of Sf9 cell monolayers. Baculovirus was amplified and titred using standard methodologies [10]. AUX1 expression was induced by infecting suspension cultures of Sf9 cells at 2.0×10^6/mL at varying multiplicities of infection (MOI), and cells were incubated for 24–96 hours after infection. Cells were harvested by centrifugation (500 g, 5 minutes at 4°C), resuspended in 10 times the pellet volume in 10 mM Tris pH7.4, 250 mM sucrose, 0.2 mM $CaCl_2$ with protease inhibitors (as above) and passed twice through a pressure disruptor (Constant Systems) at 5000 psi to lyse.

2.4. Expression of Epitope-Tagged AUX1 in Lactococcus lactis. Epitope-tagged AUX1 cDNAs were inserted in the pNZ8048 vector [6] via the *Nco*1 and *Spe*1 restriction sites. Following electroporation of the recombinant plasmids into electrocompetent *L. lactis* NZ9000 cells, chloramphenicol resistant colonies were picked from selective plates and grown at 30°C in M17 medium (Oxoid) supplemented with 0.5% w/v glucose and 5 μg/mL chloramphenicol. Cultures were grown to an A_{600} of 0.6 then induced by adding culture media supernatant from the nisin-producing strain NZ9700 grown to an OD_{600} of 0.9 at a range of dilutions between 1 : 250 and 1 : 20,000 v/v. Cells were harvested at 1 and 2 hours postinduction by centrifugation (4000 g, 15 minutes at 4°C), washed and resuspended in ice cold 100 mM potassium phosphate buffer, pH 7 with protease inhibitors (as above) and passed through a pressure disruptor at 20 000 psi to lyse.

2.5. Expression of Epitope-Tagged AUX1 in Pichia pastoris. Expression of epitope-tagged AUX1 was performed using the EasySelect *Pichia* Expression Kit (Invitrogen) following the manufacturer's instructions. In brief, epitope-tagged AUX1 cDNAs were inserted in the pPICZB vector and zeocin resistant colonies picked from selective plates (low salt LB agar with 25 μg/mL zeocin). Electrocompetent *P. pastoris* KM71H cells were transformed with linear pPICZB_AUX1 constructs by lithium chloride transformation following the manufacturer's instructions. Zeocin resistant colonies were picked from selective plates and used to inoculate small-scale expression cultures of minimal glycerol medium (1.34% w/v yeast nitrogen base, 1% w/v glycerol, 4×10^{-5}% biotin w/v) containing histidine (0.004% w/v) and grown in an orbital incubator (250 rpm) at 28–30°C. Cultures were induced when an A_{600} of 10 was reached by replacing the medium with minimal methanol medium containing histidine (0.5% v/v methanol replacing the glycerol) and cultured for 48 hours with a further addition of methanol (0.5% v/v) 24 hours postinduction. Cells were harvested by centrifugation (2500 g, 15 minutes at 4°C), washed with ice cold H_2O and resuspended in three times the pellet volume of ice cold YeastBuster (Merck) with protease inhibitors, incubated for 90 minutes with agitation and cell debris removed by centrifugation (500 g, 1 minute at 4°C).

2.6. Immunoblot Analysis of AUX1 Expression. Cell lysates were quantified by a detergent compatible protein assay (BioRad) and (10 μg) aliquots were resolved on 10% w/v SDS-PAGE gels, electroblotted and recombinant epitope-tagged AUX1 protein (ca. 45–50 kDa) identified by Western blotting with rabbit anti-HA or rabbit anti-FLAG monoclonal antibodies (both from Axxora; 1 : 2000 to 1 : 5000 dilutions) as appropriate. Following removal of the primary antibody, AUX1 expression was determined using horseradish peroxidase conjugated secondary antibody (goat anti-rabbit-HRP, DAKO, 1 : 2000) and enhanced chemiluminescence (SuperSignal West Pico, Pierce).

2.7. Confocal Microscopy of AUX1-Expressing Mammalian Cells. HEK293T cells were grown on cover slips (100 000 cells per coverslip in a 35 mm dish) and transfected using PEI as described above. After washing with ice cold PBS cells were fixed and permeabilized in methanol:acetone (1 : 1) for 20 minutes at −20°C and washed again. Nonspecific binding was blocked by incubating in 3% (w/v) BSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$ for 1 hour. Cells were washed in 0.3% (w/v) BSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and probed with anti-FLAG primary antibody at 1 : 1000 (v/v) then anti-rabbit-GFP (Sigma) secondary antibody at 1 : 200 (v/v). Cells were imaged using a Leica SP2 confocal laser scanning microscope. The green fluorescence of GFP was excited using the 488 nm laser line and the DAPI staining using the 405 nm laser. Optical (z) sections were collected at intervals of 0.25 microns and displayed as maximum intensity projection using the associated LCS software.

2.8. Blue Native Gel Polyacrylamide Gel Electrophoresis of AUX1. AUX1 containing membranes were solubilised at a protein concentration of 1 mg/mL for 3 hours on ice in 0.1% *n*-dodecyl-β-D-maltoside (Calbiochem) in the presence of 20 mM HEPES-KOH, pH 7.4, 250 mM NaCl, 10% v/v glycerol, 2.5 mM $MgCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol and protease inhibitors. Insoluble material was removed by centrifugation at 100 000 g for 1 hour at 4°C and 10× sample buffer (0.5% w/v G250 Coomassie brilliant blue, 0.75 M 6-aminocaporic acid, 100 mM Bis-Tris-HCl, pH7) added at a volume ratio of 1 : 10 prior to electrophoresis. The solubilised proteins were analyzed by blue native electrophoresis on 6–16% linear polyacrylamide gradient gels as previously described [11].

2.9. Interaction of IAA with AUX1 Containing Membranes. AUX1 containing membranes were isolated by ultracentrifugation following cell lysis, and the interaction of [^{3}H]-indole-3-acetic acid was determined as described previously [12]. The ability of auxin analogues to displace auxin binding was determined by incubation with 1 mM of displacing compound [12].

2.10. Detergent Solubilisation of HA-AUX1. Detergents were investigated for their ability to extract/solubilize HA-AUX1 from insect cell membrane preparations. AUX1 containing membranes (100 μg at a protein concentration of 1 mg/mL)

and detergent (between 3 and 8-fold critical micelle concentration) were combined in solubilization buffer (20 mM MOPS, 200 mM NaCl, 1.5 mM $MgCl_2$, 20% (v/v) glycerol, pH 7.4) and incubated at 4°C for 60 minutes with end-over-end mixing. Insoluble material resistant to detergent extraction was pelleted by ultracentrifugation at 100 000 g for 1 hour at 4°C and resuspended in 100 μL 10% SDS (w/v). Equivalent percentages of the solubilised (i.e., in the supernatant following detergent extraction) and insoluble material were analysed by SDS-PAGE and immunoblotting with anti-HA antibodies. Solubilisation in the presence of a strong ionic detergent (SDS) served as a positive control as 100% of the HA-AUX1 is extracted under these conditions.

2.11. Transport of IAA into Mammalian Cells. Auxin transport assays [13] were performed in AUX1 expressing HEK293T and U2OS cells. Following transfection cells were incubated at 37°C for 30 minutes in Ringers buffer, (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $NaHCO_3$, 10 mM HEPES-NaOH, 1 mM MgCl, pH 6.4), containing 2 μM [^{3}H]-IAA. Transport was terminated by several rapid washes with ice cold Ringers buffer containing 5 mM IAA, and the cells were then resuspended in 100 μL buffer, and lysed by addition of SDS to a final concentration of 2.5% w/v added and incubated at 37°C for 30 minutes to allow complete cell lysis. An aliquot was removed for protein assay and Western blot to confirm protein expression and the remainder used to determine radioactive content of the cells using a liquid scintillation counter. Each sample was performed in at least duplicate and corrected values expressed as the rate of transport in fmoles IAA/minute/mg protein. To enable comparison of data from different transfections, activity was normalised such that the accumulation observed in pcDNA_L2_$His_6$3XFLAG transfected cells was set to 100%.

3. Results and Discussion

We investigated several different nonplant expression systems for their ability to express the AUX1 protein. The heterologous systems selected included mammalian cell lines (HEK293T—an embryonic kidney cell line [7], and U2OS—an osteosarcoma cell line [8]), baculovirus-mediated infection of insect cells, a yeast expression system, and a bacterial expression system. The relative merits of these systems in terms of expression level, reliability of expression, AUX1 protein function (binding or transport), and scalability are discussed below. Our studies involved three epitope-tagged versions of AUX1 to enable identification of expressed protein and also to facilitate longer-term purification strategies. Three differently tagged versions were employed (see Methods), and these are shown schematically in Figure 1.

Expression in HEK293T cells (Figure 2) was dependent on both the epitope tag and its location as well as the quantity of DNA transfected (Figure 2(a)) and the time to harvesting posttransfection (Figure 2(b)). Optimal expression was obtained 88 hours posttransfection with 8 μg of DNA per well, with N-$His_6$3XFLAG AUX1 expression being greater than that of L2-$His_6$3XFLAG AUX1. Protein was effectively trafficked to the cell membrane as observed with confocal microscopy (Figure 2(c)). Analysis of function of AUX1 was determined using whole-cell radioisotope accumulation assays with [^{3}H-IAA], which demonstrated that transfection of vector encoding L2- $His_6$3XFLAG AUX1 was capable of causing accumulating of IAA in transfected mammalian cells to a significantly greater degree than empty vector controls ($P < .01$, paired T-test, $n = 9$; see Figure 2(d)), although function was tag-position dependent as the N-$His_6$3XFLAG construct was not transport competent (data not shown). In spite of these studies, which have been paralleled in the expression and characterisation of other auxin transporters, including the efflux transporters PIN2 and PIN7 and AtABCB1 in HeLa cells [13, 14], the limited ability to scale up the expression capacity precludes attempts to use this system for purification of AUX1.

The baculovirus expression system is well documented for its ability to express high levels of recombinant functional heterologous proteins [15]. The cells can grow in suspension making scale up facile and cost-effective, once recombinant baculoviruses are generated. For optimised expression in insect cells we compared both *Spodoptera frugiperda* Sf9 and *Trichoplusia ni* HighFive cell lines and identified Sf9 as the best line for AUX1 expression (comparison not shown). Maximum expression in Sf9 cells was obtained with infection at a multiplicity of infection (MOI) of 1 (Figure 3(a)), with higher MOIs not producing any increase in protein expression, and subsequent culture for 72 hours posttransfection for all three recombinant viruses.

All AUX1 constructs expressed in insect cell membranes were functional at least in terms of their interaction with the transport substrate indole-3-acetic acid, and the effective displacement of IAA by auxinic compounds but not by unrelated weak acids (Figure 3(b)). Further details of these initial step in transporter's catalytic cycle have been given recently [12]. Further interpretation of some of these data demonstrates the feasibility of using the insect cell systems for future purification studies of AUX1. Our published maximal binding of IAA to AUX1 is ca. 12 pmoles of IAA/mg membrane protein [12]. Using a demonstrated 1 : 1 stoichiometry [12] this equates to 6 μg AUX1/mg membrane protein. The ease of scaling of insect cell culture enables us to produce 1 g of total membrane protein per litre culture (equivalent to 2×10^9 cells), equivalent to approximately 6 mg of AUX1. Secondly, preliminary solubilisation of AUX1 from insect cell membranes indicates that a range of nonionic detergents including dodecyl-maltoside and decyl-maltoside is able to extract the protein from membranes (Figure 3(c)), underpinning future purification strategies. The disadvantage of the insect cell system is that transport studies are not possible due to viral induced loss of cell integrity 2-3 days after infection. Compromising the expression levels with shorter infection times and reduced MOIs might ameliorate this issue.

In both mammalian and insect cell expression systems we made three comparable observations. Firstly, the expression level of the L2-His_6-3xFLAG constructs was much lower than that for N-His_6-3xFLAG AUX1 construct reflecting that the tag position and identity are likely to be critical

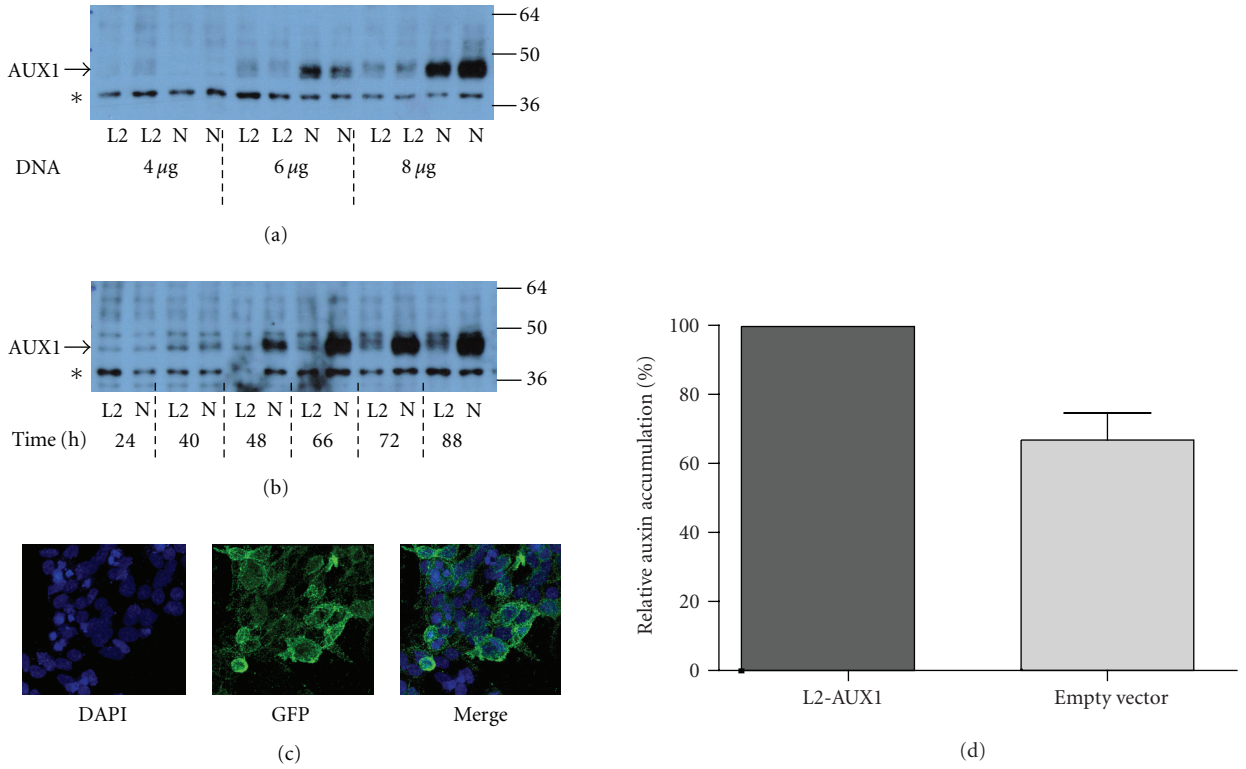

Figure 2: Expression of epitope-tagged AUX1 in mammalian cells. (a) HEK293T cells were transfected using polyethyleneimine with the indicated amounts of recombinant L2-His$_6$3xFLAG or N-His$_6$3xFLAG epitope-tagged AUX1 DNA (L2 and N, resp., below each lane), and harvested 72 hours posttransfection. (b) HEK293T cells were transfected using 8 μg of either L2-His$_6$3xFLAG-AUX1 or N-His$_6$3xFLAG-AUX1 epitope-tagged AUX1 DNA and harvested at the indicated times posttransfection. Cells were lysed by sonication, and 10 μg of lysates were resolved by SDS-PAGE and identified by western blotting with anti-FLAG antibodies. The asterisk identifies a nonspecific protein reacting with the anti-FLAG antibody. Molecular weight (kDa) of marker proteins is denoted at the right-hand side of the figure. (c) HEK293T cells were transfected on coverslips in 6-well dishes with cDNA encoding N-His$_6$3XFLAG-AUX1 and were visualised 48 hours later by confocal microscopy after immunoblotting with an anti-FLAG primary antibody (1 : 100 dilution) and a GFP-conjugated secondary antibody (1 : 200). Cell nuclei were counter stained with DAPI. (d) Transport of [^{3}H]-IAA into U2OS cells transiently transfected with L2-His$_6$3XFLAG-AUX1 compared to transport into cells transfected with empty vector. Data is expressed as a percentage of the transport rate into AUX1-transfected cells and represents the mean (± standard error) of 9 independent experiments with 2–4 determinations of transport in each transfection.

determinants of the success of a heterologous expression system (lane-by-lane comparison is shown in Figure 2 for mammalian cell expression). Similar data was obtained for insect cell expression of AUX1 (not shown). Secondly, despite a predicted molecular weight of 54 kDa, AUX1 constructs routinely migrated at just less than 50 kDa on SDS-PAGE. Native AUX1 from root cultures also runs with an apparent faster mobility than predicted, a phenomenon not unusual for highly hydrophobic membrane proteins [3]. Thirdly, we observed that HA-AUX1 migrated as multiple bands on SDS-PAGE, with apparent dimerization and higher-order oligomerization, resistant to SDS and reducing agent denaturation (see, e.g., Figure 3). In order to analyse this further (and rule out detergent-induced aggregation of the protein as being responsible), we performed blue native PAGE (BN-PAGE) on HA-AUX1 containing membranes, where separation of the protein and determination of the native molecular weight are driven by the pore size of the gradient acrylamide gel [11]. With this analysis we showed that HA-AUX1 is expressed as a trimer in insect cells with a molecular weight close to 150 kDa (Figure 4). The natural oligomeric state of the protein remains unknown but members of related transport families (i.e., of ammonium) are known to assemble into trimeric species [16].

The methylotrophic yeast *Pichia pastoris* should combine the advantages of a microorganism-based expression system (high yield and facile scale up), whilst also retaining the eukaryotic translation and trafficking machinery associated with membrane protein processing. The commercially available *P. pastoris* expression system takes advantage of this by using the alcohol oxidase promoter, which is methanol induced and tightly regulated, to drive heterologous expression of the protein of interest. It has successfully been used to express a wide range of membrane integrated transport proteins [17, 18]. Two *P. pastoris* strains GS115 and KM71H were transformed with AUX1 containing plasmids, and integration of the transgene was confirmed by PCR analysis (data not shown). However,

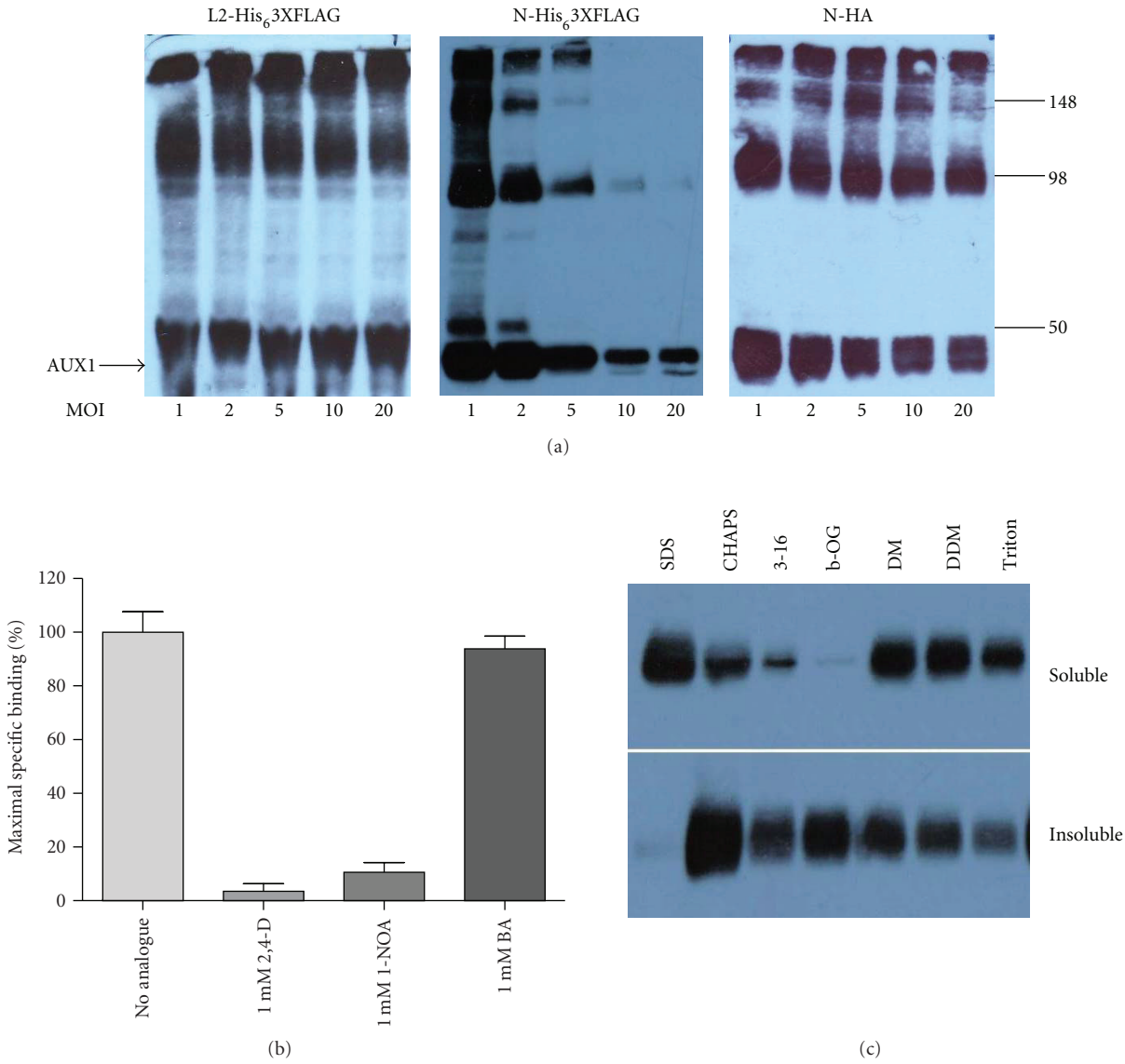

Figure 3: Expression of epitope-tagged AUX1 in insect cells. (a) Sf9 cells at densities of 2×10^6 cells/mL were infected with recombinant baculoviruses expressing epitope-tagged AUX1 at a range of multiplicity of infections (MOIs) and harvested at 24–96 hours postinfection (hpi). Cell lysates (10 μg) were resolved by SDS-PAGE and identified by western blotting with appropriate antibodies directed towards the epitope tags. Panels show data for a 72-hour postinfection only. Molecular weight (kDa) of marker proteins is denoted at the right-hand side of the figure. (b) Auxin binding to AUX1-containing membranes (72 hours postinfection) was assessed by a centrifugation-based radioisotope binding assay [12]. AUX1 displacement could be observed when membranes were incubated with 1 mM auxin analogues such as 2,4-D and 1-NOA but not when the unrelated weak acid benzoic acid (BA) was applied. (c) Solubilisation of HA-AUX1 from insect cell membranes. 100 μg of membranes were incubated for 60 minutes at 4°C with detergents at greater than 2X critical micelle concentration. Solubilised material was separated from insoluble material by ultracentrifugation and equal percentages of the two fractions resolved by SDS-PAGE and immunoblotting. Detergent abbreviations: 3–16: Zwittergent 3–16 (Calbiochem); β-OG: β-octyl-glucoside; DM: *n*-decyl-β-D-maltoside decylmaltoside; DDM: *n*-dodecyl-β-D-maltoside.

expression analysis of 16 independent transformants from both strains with N- and L2-His$_6$3xFLAG constructs identified only a single strain/construct combination which expressed AUX1 (Figure 5). Due to this unreliability—discussed in more detail in relation to the different promoters in *Pichia* expression systems [18]—we did not attempt to perform any functional analysis of Pichia expressed AUX1. Notwithstanding this, other yeast systems have been used successfully for the expression and characterisation of a number of auxin transporters, notably PIN transporters PIN2 and PIN7, and the ATP binding cassette (ABC) transporter AtABCB1. For *S. cerevisiae* based studies this has required the use of strains deficient in numerous confounding transporters [13, 14]. Most recently, an *S. pombe* expression system has been used which is able to show functional expression of 3 different classes of auxin

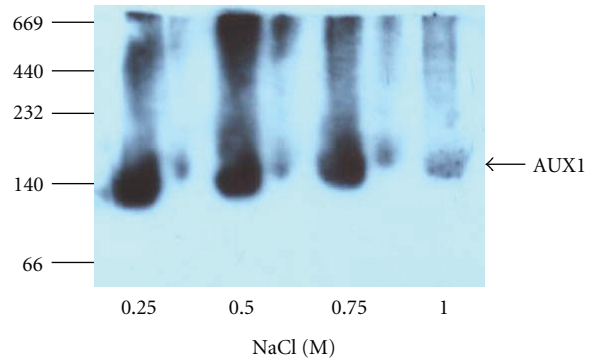

Figure 4: Blue native PAGE analysis of AUX1 expression in insect cells. N-HA-AUX1 membrane fractions from insect (Sf9) cells were solubilised in 0.1% (w/v) DDM in the presence of increasing concentrations of NaCl and were resolved by BN-PAGE on 6–16% gradient gels, transferred to PVDF and identified by immunoblotting with anti-HA antibodies. Molecular weight (kDa) of marker proteins is denoted at the left-hand side of the figure.

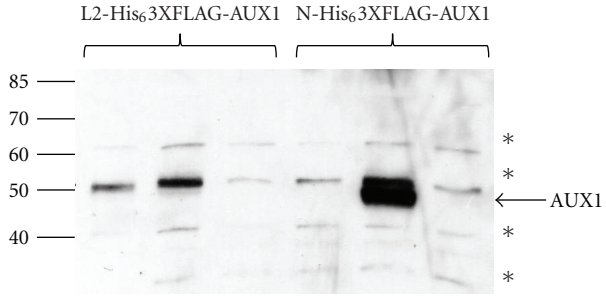

Figure 5: Expression of epitope-tagged AUX1 in *P. pastoris*. *P. pastoris* strain KM71H transformed with plasmids encoding L2-$His_6$3xFLAG-AUX1 or N-$His_6$3xFLAG-AUX1 was grown, induced, and harvested as described in the Methods. Of the 32 cell lysates analysed by Western blotting (anti-FLAG antibody), only 6 are shown here for clarity, the other 26 showing no AUX1 expression. AUX1 was observed in a single N-$His_6$3xFLAG-AUX1 culture (lane 5). A number of nonspecific bands reacting with the anti-FLAG antibody are denoted with asterisks, including one migrating just higher than the AUX1 band.

transporter, namely, AUX-LAX, PIN, and ABCB, and this seems likely to supersede other yeast systems for auxin transporter investigation [19].

The gram positive lactic acid bacterium *Lactococcus lactis* was selected as a candidate for a prokaryote-based expression of AUX1 because of reports on its successful use to express and functionally characterize a range of prokaryotic and eukaryotic membrane transporter proteins (reviewed in [20]). *L. lactis* is the model lactic acid bacterium, and a controlled inducible protein expression system for heterologous proteins has been described [21]. Although the insertion of the AUX1 gene into the pNZ8048 vector was confirmed by DNA sequencing no expression was observed for any of the tagged AUX1 constructs under the range of conditions used, in contrast to a control expression vector for an unrelated ATP binding cassette (ABC) protein. It has previously been shown that the success of expression in the *L. lactis* system is influenced by the N-terminal region of the transport protein [22]. It can therefore be hypothesised that N-terminal 50 amino acids of AUX1 prior to the first predicted TM helix is unsuitable for expression in *L. lactis*. Modifications to these regions (as described by Monné and colleagues for a range of mitochondrial carriers including the ADP/ATP carriers ACC1 and ACC2 [22]) may eventually lead to expression of AUX1 in this *L. lactis*.

4. Conclusion

We have examined four systems for their suitability to express the plant hormone transporter AUX1. The position and the nature of the epitope tag are an important consideration, as is the desired outcome, that is, transport studies, purification of protein, and so forth. Of the four systems the prokaryotic *L. lactis* was unsuccessful in our hands, with no expression observed. The methylotrophic yeast *P. pastoris* gave limited and unreliable success. In contrast, transiently transfected mammalian cells and a baculovirus infected insect cell systems enabled us to express AUX1 and assess functionally competence of the protein. These two heterologous systems for AUX1 complement the use of *Xenopus* oocytes and *S. pombe* for studies of auxin transporters [19, 23, 24]. The oocyte system, although technically beyond many research laboratories, has enabled determination of Michaelis-Menten parameters for AUX1 and LAX3. The accessible baculovirus expression system has enabled detailed analysis of binding affinities of auxin transport substrates and inhibitors [12], and this combination of approaches opens the way for similar studies on a range of these proteins. Determination of the biochemistry of auxin importers and exporters will lead to more realistic models of auxin transport, with greater power to predict responses to changes in auxin concentration [25].

Abbreviations

AUX1:	AUXIN RESISTANT 1
ABC:	ATP binding cassette
PVDF:	Polyvinylidene fluoride
SDS-PAGE:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
BN-PAGE:	Blue native polyacrylamide gel electrophoresis
2,4-D:	2,4-dichlorophenoxyacetic acid
1-NOA:	1-naphthylphthalamic acid
BA:	Benzoic acid
PBS:	Phosphate buffered saline
DMEM:	Dulbecco's modified Eagle medium
DM:	*n*-decyl-β-D-maltoside
DDM:	*n*-dodecyl-β-D-maltoside.

Acknowledgments

D. J. Carrier was supported by the Schools of Biomedical Sciences and Biosciences, and by the Biomedical Research Committee (all University of Nottingham). The BBSRC

(BB/C514958/1) and Wellcome Trust (077212/Z/05/Z) supported this research through project and equipment grant funding, respectively. J. M. Dorrian is a BBSRC funded Ph.D. student. A. Haider thanks the Government of Kuwait for a research scholarship and N. T. Abu Bakar thanks the Malaysian Government for funding.

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Hindawi Publishing Corporation
International Journal of Plant Genomics
Volume 2009, Article ID 451357, 14 pages
doi:10.1155/2009/451357

Review Article

Techniques to Study Autophagy in Plants

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Received 7 December 2008; Revised 15 May 2009; Accepted 18 June 2009

Recommended by Boulos Chalhoub

Autophagy (or self eating), a cellular recycling mechanism, became the center of interest and subject of intensive research in recent years. Development of new molecular techniques allowed the study of this biological phenomenon in various model organisms ranging from yeast to plants and mammals. Accumulating data provide evidence that autophagy is involved in a spectrum of biological mechanisms including plant growth, development, response to stress, and defense against pathogens. In this review, we briefly summarize general and plant-related autophagy studies, and explain techniques commonly used to study autophagy. We also try to extrapolate how autophagy techniques used in other organisms may be adapted to plant studies.

1. Introduction

Autophagy, literally meaning self (auto) eating (phagy), is an evolutionarily conserved and highly regulated catabolic process that leads to the degradation of cellular components using lysosomal/vacuolar degradation machinery of the same cell. Depending on the mechanism of transport to lysososome/vacuole, at least three forms of autophagy have been described: "Macroautophagy" is characterized by the engulfment of long-lived proteins and organelles in de novo formed double-/multimembrane vesicles called autophagosomes or autophagic vesicles. These vesicles subsequently deliver their cargo to the lysosome or vacuole for degradation. In another form of autophagy, called "microautophagy," lysosome/vacuole directly engulfs cytosolic components through an invagination of its membrane [1, 2]. A third common form of autophagy is called "chaperone-mediated autophagy" (CMA). CMA is a very selective process during which proteins with a KFERQ consensus peptide sequence are recognized by a chaperone/cochaperone complex and delivered to the lytic compartment in an unfolded state [3, 4].

Macroautophagy is the most studied form of autophagy. Macroautophagy ("autophagy" hereafter) occurs at basal levels in growing cells, allowing them to recycle long-lived proteins and organelles [3]. The cargo is degraded into its building blocks (i.e., proteins to amino acids), helping the cell to economize its resources, eliminate old/damaged organelles, and survive nutrient and other types of stress. For example, in plants under conditions causing cellular and organismal stress such as starvation, drought, and other abiotic stress, autophagy is upregulated [5–8]. Autophagy is also involved in physiological phenomena including plant development, senescence, and immune response [9–11]. In some cases, autophagy can function as a nonapoptotic and alternative programmed cell death mechanism, and its role in plant cell death was explored [12–15]. As a consequence of its involvement in several important physiological and pathological phenomena, autophagy became one of the fastest expanding fields of molecular biology in recent years.

In this review, we will briefly summarize the mechanisms of autophagy in general and particularly plant autophagy, list commonly used techniques to detect and quantify autophagy, and finally discuss their utility in plant autophagy detection. An exhaustive summary of the autophagy mechanisms is beyond the scope of this review. The readers may find an in-depth discussion of the mechanistic aspects of autophagy in recently published reviews [5, 9, 16].

2. General Autophagy Mechanisms

So far, nearly 30 autophagy-related genes (depicted by the acronym *ATG*) were identified using yeast mutants [17].

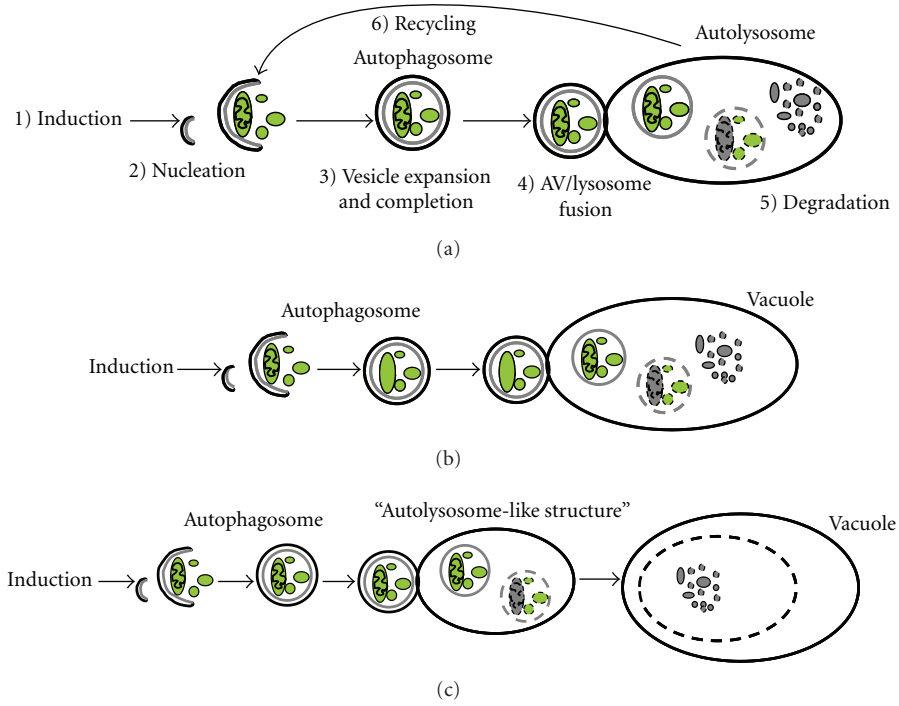

Figure 1: Autophagy mechanism and alternative pathways for autophagosomes in plants. (a) Following an upstream stimulus, such as starvation, double membrane vesicles, autophagosomes, appear and engulf portions of cytosol, long-lived proteins, and organelles such as mitochondria. Autophagosomes eventually fuse with lysosomes, endosomes, or vacuole. Autophagosomes are degraded together with their cargo and the building blocks are pumped back into the cytosol for reuse. (b) Autophagosomes may fuse directly with the vacuole (observed in *A. thaliana*) (c) or, may first transform into lysosome-like acidic and lytic structures and, fusion with the central vacuole may occur as a secondary event (observed in tobacco plant).

Plant and mammalian orthologues of most of these genes and proteins are now characterized. Data obtained from these studies underline the fact that the basic machinery of autophagy is preserved from yeast to higher eukaryotes. Autophagy proceeds through five distinct phases: namely, induction, nucleation, vesicle expansion and completion, autophagosome/lysosome fusion, and cargo degradation [9, 18] (Figure 1(a)).

2.1. Induction. This is the phase where upstream signaling mechanisms leading to autophagy activation are turned on. Many of these pathways are integrated by the "Target of rapamycin (Tor)" protein [19–21]. Tor is a serine/threonine kinase regulated in response to variation in amino acids, ATP, and growth factors. Downregulation of Tor activity correlates with autophagy stimulation [22]. Tor pathway and its effect on autophagy were preserved in plants. Yet, structural differences exist between Tor proteins in plants and other eukaryotes, therefore, rapamycin, a widely used specific inhibitor of Tor, cannot be used to study autophagy in plants [23, 24].

Tor inactivation induces autophagy at least by two mechanisms in yeast. The first involves activation of transcription factors called GLN3 (nitrogen regulatory protein) and GCN4 (General Control Nondepressible), leading to transcriptional upregulation of some of the *ATG* genes (e.g., *ATG1* and *ATG13*) [25, 26]. Second mechanism is related to the modification by Tor of an autophagy protein complex containing Atg1 and Atg13. Active Tor induces hyperphosphorylation of Atg13 inhibiting its association with Atg1 (AtAtg1 in *A. thaliana* and ULK1 (Unc-51-like kinase1) in mammals), a serine/threonine kinase required for autophagy [27]. Tor inactivation leads to rapid dephosphorylation of Atg13 and an increase in the affinity of this protein for Atg1. Atg1-Atg13 association induces autophosphorylation and activation of Atg1, promoting autophagy [27–30]. Recent evidences indicate that Atg1-13 complex regulates recycling of Atg proteins such as Atg9 and Atg23 functioning at the autophagy organization site called PAS (for the preautophagosomal structure) [31].

2.2. Nucleation. While the origin of the lipid donor membranes in autophagy is still obscure, endoplasmic reticulum, Golgi, and a so far undetermined organelle called "the phagophore" were suggested as lipid providers to autophagosomes. Whatever is the origin, autophagosomal membranes are build up de novo as crescent-shaped structures in PAS. In yeast, PAS is a prominent structure next to the vacuole, but in higher eukaryotes, several sites are involved. Nucleation of autophagosomes is initiated by a protein complex including Vps34, a class III phosphatidylinositol 3-OH kinase (PI3K), and Atg6/Vps30 (Beclin1 in mammals). Together with other regulatory proteins such as UVRAG (UV

radiation Resistance Associated Gene), Bif-1, and Ambra, Atg6-containing complex plays a role in the regulation of Vps34 activity. PI3K activity of Vps34 leads to the accumulation of phosphatidylinositol 3-phosphate (PI3P). PI3P produced by Vps34 serves as a landing pad on PAS for proteins involved in autophagosome formation such as Atg18 and Atg2 [16, 32, 33].

2.3. Vesicle Expansion and Completion. Two ubiquitination-like conjugation systems play a role in autophagosome biogenesis. In the first reaction, Atg12 is conjugated to Atg5 in a covalent manner [34]. The conjugation reaction starts with the activation of Atg12 by an ubiquitin-activating enzyme (E1)-like protein Atg7. Atg12 is then transferred to Atg10, an ubiquitin-conjugating-like enzyme (E2)-like protein [35, 36]. Finally, Atg12 is covalently conjugated to Atg5. The conjugation allows the formation and stabilization of a larger complex containing Atg12, Atg5, and Atg16 [37]. This protein complex is necessary for the second ubiquitination-like reaction to occur and to allow autophagosome membrane elongation. Atg12/5/16 complex localizes to the outer membrane of the forming autophagosome, and, dissociates from it as soon as the vesicle is completed, underlining the fact that its role is regulatory rather than structural [38].

The second ubiquitination-like reaction involves Atg8 protein (microtubule-associated protein light chain-3 or shortly LC3 in mammals). E1-like protein Atg7 activates Atg8 and transfers it to Atg3. While Atg7 is common to both conjugation reactions, E2-like protein Atg3 is specific for Atg8 conjugation to a lipid molecule (phosphatidylethanolamine, PE) [39]. Prior to conjugation, Atg8 has to be cleaved at its carboxy-terminus by Atg4, allowing the access of the lipid molecule to a Glycine residue on Atg8. Lipidation reaction is reversible since Atg4 can also cleave the conjugated lipid, enabling recycling of Atg8. Recent data provide evidence that together with Atg3, Atg12/5 complex is directly responsible for Atg8-PE conjugation [40]. The yeast Atg8 has several orthologues and isoforms in plants [41–43]. In the model plant *Arabidopsis thaliana*, at least 9 Atg8 proteins were described [44].

2.4. Autophagosome/Lysosome Fusion and Degradation. Autophagosomes fuse with late endosomes or lysosomes to form autolysosomes. Specific factors have been implicated in this step. A Vps complex and Rab GTPases proteins are involved in the organization of the fusion site. Then, SNAREs proteins (SNAP as soluble NSF attachment protein receptor) [45] form a complex which serves as a bridge between the two organelles [46, 47].

2.5. Recycling. In the lumen of lysosome/vacuole, lipases such as Atg15 first degrade the remaining autophagic membrane and the cargo is then catabolized by lysosomal lytic enzymes [48]. Following the degradation of the vesicle, building blocks are carried to cytosol for further use. Specialized lysosome membrane proteins play a role in this process including lysosomal-associated membrane proteins LAMP-1 and LAMP-2.

3. Plant Autophagy

Both microautophagy and macroautophagy are functional in plants [5]. Mechanisms of these pathways are similar to those described in other model organisms.

In plant microautophagy, the target material is directly engulfed by an invagination of the tonoplast. Cargo-containing vesicle pinches off to be released inside the vacuole and degraded within the lumen. Microautophagy was involved in accumulation of storage proteins, lipids, and degradation of starch granules in developing plants [49, 50].

As in other organisms, the macroautophagy (hereafter "autophagy") in plants is a process that starts with the formation of cup-shaped membranes in the cytoplasm. After completion, autophagosomes have at least two destinations in plants. They may fuse with the tonoplast and be directly delivered to the lumen of the vacuole as seen in *Arabidopsis*. Alternatively, autophagosomes may first transform into lysosome-like acidic and lytic structures and, fusion with the central vacuole may occur as a secondary event (Figures 1(b) and 1(c)) [51, 52].

In the model plant *Arabidopsis thaliana*, 25 orthologs of 12 yeast *ATG* genes were identified [44, 53–55]. Some exist as a single copy (i.e., Atg3 and Atg5) and others as multiple copies (i.e., Atg1 and Atg8). Functional domains of these *Arabidopsis* proteins were well conserved during evolution, indicating preservation of basic autophagy mechanisms in plants. Indeed, complementation tests in *ATG* mutant yeast strains using some of the plant Atg proteins confirmed the preservation of their function [43]. Moreover, gene targeting studies in whole plants demonstrated that plant genes of all tested autophagy proteins (i.e., for Atg7, Atg9 and Atg5-Atg12) were necessary for autophagosome formation following various types of stress [44, 53, 55]. Furthermore, some *ATG* genes were upregulated under stress conditions stimulating autophagy [7, 56–61]. A list of *Atg* genes identified in *Arabidopsis* and the phenotypes caused by their modification are depicted in Table 1.

3.1. Basal Autophagy in Plants. Autophagy is constitutively active in plant cells as in other organisms. Indeed, incubation of root tips with vacuolar enzyme inhibitors led to the accumulation of autophagic vesicles as autolysosome-like structures and in the vacuole. When cysteine protease inhibitor, E64d, was used to inhibit autophagy, autophagic vesicles accumulated inside vacuoles in *Arabidopsis* cells [13]. Similarly, growth of tobacco cells in the presence of E64d led to the accumulation of autolysosome-like structures outside the vacuole [52]. Autophagy-specific inhibitor 3-MA blocked the accumulation of autophagosomes and autolysosomes, demonstrating that autophagy is responsible for vesicle accumulation [52, 62]. Expression of a GFP fusion construct of *Atg8f* (an autophagy marker in *Arabidopsis*) resulted in the accumulation of this marker protein in the vacuole lumen. Atg8f accumulation was also detected in the presence of concanamycin A (a Vacuolar H(+)-ATPase inhibitor blocking vacuolar degradation) [57].

The role of constitutive autophagy in the degradation of damaged or oxidized molecules was confirmed using

mutants of *AtAtg18a*. These mutants produced greater amounts of oxidized proteins and lipids in comparison to wild-type plants. Increased amount of oxidized protein and lipid generation in *Atg18a*-silenced plants underlined importance of autophagy for the degradation of oxidized molecules in plant cells [8, 63]. Therefore, as in other organisms, plant basal autophagy seems to function to eliminate damaged organelles (e.g., chloroplast, a source of reactive oxygen species in plants) and to clear damaged/abnormal proteins that accumulate in the cytoplasm [64].

3.2. Autophagy in Plant Development. The role of autophagy for plant development was studied using several autophagy gene mutants. Under nutrient-rich conditions, autophagy-defective plants achieve normal embryonic development, germination, shoot and root growth, flower development, and seed generation [44, 53, 54]. When these plants are grown under carbon- or nitrogen-deficient conditions, accelerated bolting, increased chlorosis, dark-induced senescence, and a decrease in seed yield were observed. Therefore, autophagy seems to be a major mechanism of nutrient mobilization under starvation conditions in plants.

Autophagy plays a role during vacuole biogenesis as well. In a recent study, Yano et al. [65] proposed that formation of vacuoles from tobacco BY-2 protoplasts involved an autophagy-like process. However, this process could not be inhibited by classical autophagy inhibitors such as 3-MA and wortmannin, suggesting that autophagy during vacuole formation differs from constitutive autophagy taking place under normal conditions or autophagy induced by stress.

3.3. Autophagy, Stress, and Cell Death. When organisms including plants are exposed to adverse environmental conditions, they develop responses to cope with stress and to survive. One of the major processes exploited by plant cells for this purpose is autophagy. Stress conditions inducing autophagy include sucrose, nitrogen, and carbon starvation, as well as oxidative stress and pathogen infection [8, 62, 66, 67]. For example, sucrose starvation has been reported to induce autophagy in rice [68], sycamore [6], and tobacco-cultured cells [69], and carbon starvation induced autophagy in maize plants [70]. Furthermore, autophagy participates in the formation of protein storage vacuoles in seeds and cereal grains [71, 72], prolamin internalization to vacuole in wheat [73], biogenesis of vegetative vacuoles in mature meristematic cells [74, 75], and degradation of proteins in protein storage vacuoles in mung bean [49, 76].

Since plants have a rigid cell wall and they lack typical caspase proteases, apoptosis is not the mechanism utilized by plants to degrade cellular components before cell death. During programmed cell death (PCD) in plants, vacuole and cell size increase, organelles are taken up by vacuole and subsequently degraded, and finally vacuole lyses resulting in cell death. These events overlap with the major characteristics of autophagy in plants [15, 77]. In the light of these observations, the role of autophagy in plant programmed cell death needs to be further investigated.

To avoid spread of infection, plants developed an innate immune response, called the hypersensitive response programmed cell death (HR-PCD). The innate immunity is achieved through limitation of the infection with the death of cells surrounding the infected area [78]. Studies using autophagy gene mutant plants showed that an autophagy defect is associated with a failure to contain cell death at the infection site, leading to its spread into uninfected tissue [79–81]. Therefore, paradoxically, autophagy also plays a role in limiting cell death initiated during plant innate immune responses. Indeed, as seen in plants, autophagy is involved both in cell survival and cell death in various other organisms [12].

4. Techniques to Study Autophagy

Various techniques and tools were used to monitor and evaluate autophagy. While transmission electron microscopy (TEM) analysis remains "the golden standard," with the recent advances in the field, several new molecular tools are being introduced. The possibility of their usage in plant autophagy research will be discussed.

4.1. Electron Microscopy. Transmission electron microscopy (TEM) is one of the earliest tools used to characterize autophagy [82], and it is still one of the most reliable methods to monitor autophagy in cells and tissues. Yet, interpretation of the TEM data requires special expertise and there are several criteria to describe autophagosomes and autolysosomes with precision. The hallmark of autophagosomes is their double or multimembrane structures containing electron dense material with a density similar to that of the cytoplasm. Presence in autophagosomes of organelles such as mitochondria, chloroplasts, and endoplasmic reticulum (ER) strengthens the conclusion (Figure 2(b)). Autolysosomes contain darker, degenerated, or degraded material and some of them are reminiscent of lysosomes/vacuole.

Other cytoplasmic figures may be erroneously described as autophagosomes and autolysosomes. Degenerated mitochondria, folds of ER, or nuclear membrane may be mistaken for autophagosomes [83–85]. Sometimes the typical double membrane structure of autophagosomes may be disrupted (e.g., following infection with some pathogens) [86]. Therefore, unbiased and clear identification of autophagosomes using TEM requires extreme precaution. Combination of electron microscopy with immunogold-labelling of autophagosome-specific markers such as Atg8/LC3 may allow a more objective and reliable interpretation depending on the experimental needs [87]. Transmission electron microscopy was successfully used to detect autophagy in plants [61, 79].

4.2. Molecular Markers. Proteins that are involved in the autophagy process or that are degraded specifically through autophagy have been used to monitor autophagic activity. Several of them are already in use in plants. Plants knockout and transgenic for these markers are useful tools to study autophagy-related phenotypes under different experimental conditions (see Table 1). Molecular techniques, such as Atg8/LC3 dot formation, were successfully used for high-throughput screens of autophagy in various systems [88].

Table 1: Phenotypes caused by ATG gene modifications in *Arabidopsis thaliana*. E64d, inhibitor of lysosomal/vacuolar hydrolases; Concanamycin A, inhibitor of vacuolar (V-type) ATPase, preventing lysosomal/vacuolar degradation:HR-PCD (hypersensitive response programmed cell death).

Genotype	Phenotype	Reference(s)
Atg2-deficient	No autophagic inclusions in root tips upon E64d treatment.	[52]
Atg4a-/ Atg4b-deficient	Upon nitrogen starvation, no autophagosome formation and no delivery of GFP-Atg8 to the vacuole.	[54]
	Inhibition of rubisco containing body formation.	[90]
Atg5-deficient	No autophagic vesicles in root tips after E64d treatment.	[52]
	No formation of Atg5/12 complex. Defective in autophagy induced by concanamycin A treatment.	[151]
	Senescence upon light and carbon or nitrogen limitation.	[55]
Atg6-deficient	Male sterility.	[152]
	HR-PCD sensitive. Early senescence.	[80]
	Developmental defects and impaired pollen germination.	[153]
Atg7-deficient	Hypersensitive to nutrient-limitation. Senescence.	[44]
Atg8-transgenic	Expression induced by starvation. Stress leads to premature aging.	[57, 66]
Atg9-deficient	Under carbon and nitrogen starvation, accelerated chlorosis.	[53]
	Seed germination impaired and leaf senescence accelerated.	
	Weak decrease of autophagic vesicle accumulation following E64d treatment.	[52]
Atg10-deficient	Hypersensitive to nitrogen and carbon starvation. Early senescence and PCD.	[89]
	No formation of Atg5/12 complex. Defective in autophagy induced by concanamycin A treatment.	[151]
Atg18a-transgenic	Hypersensitivity to sucrose and nitrogen starvation. Premature senescence.	[154]

4.2.1. Atg8/LC3 Dot Formation and Accumulation of Its Lipidated Form. Atg8/LC3 is covalently conjugated to a lipid molecule as a result of an ubiquitination-like reaction and, its lipidation is required for autophagic membrane elongation (see Section 2.3). In plants, several isoforms of Atg8/LC3 seem to be functional during autophagy mechanisms [57]. During autophagy, Atg8/LC3 lipidation and recruitment to autophagic membranes changes its localization from diffuse cytosolic to punctuate (Figure 2) [51, 54, 89, 90]. Moreover, in SDS-PAGE protein gels, the molecular weight of Atg8/LC3 changes from 18kDa (free cytosolic form, free Atg8, or LC3-I) to 16kDa (lipidated form, Atg8-PE (or LC3-II)) [41, 54, 57]. Soon after the discovery of its autophagy-related lipidation, Atg8/LC3 had become one of the main tools to monitor autophagy. The localization change of an Atg8/LC3-fluorescent protein fusion construct (such as GFP-Atg8/LC3) is commonly used to detect autophagy in cells (Figure 2(a)) and in whole organisms including transgenic *Arabidopsis* and tobacco plants [38, 51, 54, 55, 57].

When working with isolated cells, quantification of GFP-Atg8/LC3 signal using FACscan/flow cytometer may be used as an autophagy evaluation tool [91]. In this system, induction of autophagy led to a decrease in GFP-Atg8/LC3 signal. Conversely the fluorescent signal increased following the usage of autophagy inhibitors. This method is a good quantitative tool to monitor activity in living cells by FACscan/flow cytometer [92–94], especially using cells derived from Atg8 transgenic plants.

Nevertheless some precautions must be taken even when using this popular molecular marker. Free Atg8 (or LC3-I) to Atg8-PE (or LC3-II) ratio differs among tissues, depending on stimuli and antibodies that are used, therefore, reliable controls must be added [95]. To avoid misinterpretations due to kinetics of autophagy, it is highly advised to check Atg8/LC3 lipidation at several time points after signal application rather than using only one point in time [95]. The use of vacuolar/lysosomal degradation inhibitors will help to confirm that accumulation of the lipidated form is indeed due to the canonical autophagy pathway.

Atg8/LC3 lipidation and cytosolic dot formation may not always reflect activation of autophagy. It has been reported that high level GFP-Atg8/LC3 expression may also lead to dot formation even in nonautophagic cells [96] and in autophagy mutants [97]. Furthermore, Atg8/LC3 was found to associate with protein aggregates marked with p62/SQSTM1 (see Section 4.2.7) in an autophagy-independent manner [98]. Importantly, Atg8/LC3 lipidation reflects an early stage in autophagosome formation and it

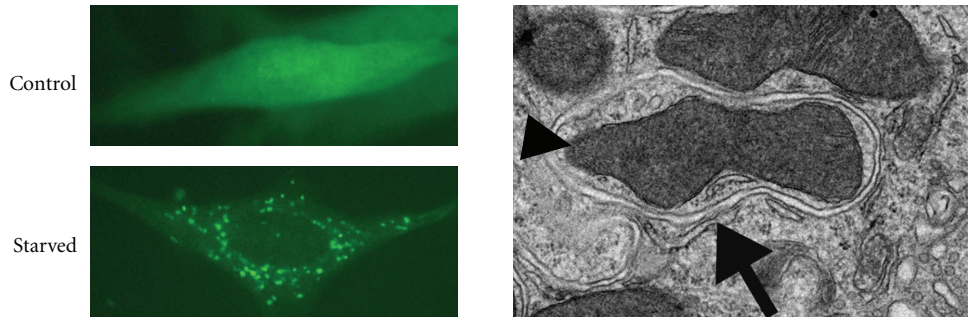

Figure 2: GFP-Atg8/LC3 dot accumulation and TEM method to detect autophagic activity. (a) LC3 dot formation upon starvation in fibroblasts isolated from GFP-Atg8/LC3 transgenic mice. The green dots are autophagic vesicles labelled by GFP-Atg8/LC3. (b) Transmission electron microscopic picture of an autophagic vesicle (arrow) in kidney of tunicamycin injected mouse. Note that in addition to cytoplasmic material, a mitochondrium (arrowhead) is also engulfed inside the double membrane vesicle.

cannot be interpreted as autophagic activity *per se* [99, 100]. Hence, this method should not be used as the only technique to monitor autophagy and it has to be complemented with other autophagy detection techniques including TEM analysis [95].

4.2.2. Atg6 and Phosphatidyl Inositol 3-Phosphate Detection. The role of Atg6 in autophagy has been extensively studied. As stated before, Atg6 regulates Vps34 class III phosphoinositide-3 kinase (PI3K) complex producing PI3P that is involved in autophagic vesicle nucleation. Similar to Atg8/LC3, intracellular localization change of a fluorescent protein fusion of Atg6 (and leading to its colocalization with PI3P) was observed upon autophagy induction [101, 102]. PI3P may be labelled in cells using a PI3P-binding peptide, FYVE fused to GFP [103]. Quantification of the accumulation of GFP-FYVE-labelled dots may also be used as a tool to quantify autophagy activation upon starvation in mammalian cells (Yamaner Y. and Gozuacik D. unpublished data). Adaptations to the plant system may be possible since orthologues of Atg6 and Vps34 are present in plants including *Arabidopsis* [104].

4.2.3. Atg5 and Atg16. Atg5 as well as Atg16 was used as a selective marker to recognize autophagosome organization centers (PAS). Since Atg5 dissociates after vesicle completion, it will not label autophagosomes or lysosomes. The signal could be detected as fluorescent dots under microscope [38, 97]. A recent study used Atg16L as a new marker to detect autophagosome formation [105]. Like Atg5, Atg16L transiently associates with the surface of autophagosomes during their formation and forms punctate structures [106]. Therefore, as Atg8/LC3, Atg5 and Atg16L, coupled with a fluorophore or detected by immunofluorescence using specific antibodies, can be used to monitor autophagosome formation. As homologues of Atg5 and Atg16 exist in plants (e.g., *Arabidopsis, Z. mays*) this technique might be useful in plants studies as well.

4.2.4. Atg18. A mammalian orthologue of the yeast Atg18, WIPI-1, was proposed as a marker for autophagy as well [107]. WIPI-1 is a WD (Tryptophan and aspartic acid) repeat protein [108] and as such, it may interact with PI3P and accumulate in dot-like structures (upon autophagy induction by amino acid starvation other stimuli). WIPI-1 dots were shown to colocalize with Atg8/LC3 [107, 109] in human cells lines. Whether plant Atg18 protein might be used as an autophagy marker has to be tested as homologues are found in plants such as *Arabidopsis*.

4.2.5. Atg4 Activity. Cleavage of Atg8/LC3 by Atg4 cysteine protease is a crucial step before its lipidation. Recently, monitoring Atg8/LC3 cleavage by Atg4 was proposed as a technique to detect autophagy [110]. The assay is based on the cleavage by Atg4 of a luciferase protein fused to Atg8/LC3 which, itself, is fixed on actin cytoskeleton. In this system, actin-associated luciferase has a secretion signal and, upon cleavage of Atg8/LC3 by Atg4, it is released from the cell. Luciferase activity can then be quantified in cellular supernatants reflecting Atg4 activity. Free luciferase can also be visualized in protein blots. Homologues of Atg4 are present in plants including *Arabidopsis* and rice; therefore, this technique could be adapted to monitor Atg4 protease activity in plants.

4.2.6. Atg1 Activity. Atg1 is a serine/threonine kinase. Its activity correlated with autophagy induction [22, 27, 111–113]. In *S. cerevisiae*, Atg1 autophosphorylation is dramatically reduced upon starvation leading to autophagy [28]. In mammals, the function of Atg1 orthologues Ulk1 and Ulk2 seems to be controlled by autophosphorylation as well [113, 114]. Hence, Atg1 kinase activity and phosphorylation status could be used as a new test of the autophagic activity in cells, tissues, and extracts. In *Arabidopsis thaliana* genome, orthologues of the yeast genes coding for Atg1 kinase and Atg13 have been identified [53, 115]. Therefore, measuring Atg1 activity could serve as a tool to monitor autophagy in plants.

4.2.7. p62/SQSTM1. Sequestosome 1 (SQSTM1), also named ubiquitin-binding protein p62 (shortly p62), is a

stress-induced adaptor/marker protein that is a common component of protein aggregates [116]. p62 was shown to bind Atg8/LC3 proteins through its N-terminal region [117]. p62/Atg8 interaction triggered degradation of protein aggregates by autophagy during which p62 itself was also degraded [118, 119]. This observation led to the use of p62 degradation as a molecular tool to detect autophagic activity [119–121]. As LC3 lipidation appears prior to p62 degradation, existence of a lag phase should be considered during the design of the experiments [95]. Of note, it is still not known whether p62 is a general marker for autophagy and caution should be taken when using this technique with new autophagy-inducing stimuli. Our preliminary analyses revealed that there are no p62 orthologues in *Arabidopsis*. Yet, we cannot exclude the possibility that p62-like proteins exist in plants.

4.3. Tests of Lysosomal/Vacuolar Activity

4.3.1. Lysotracker. Weakly basic amines selectively accumulate in cellular compartments with low internal pH and can be used to visualize acidic compartments such as lysosomes/vacuoles. Lysotracker is a fluorescent acidotropic probe used for labeling acidic organelles in live cells. It consists of a fluorophore linked to a weak base. Labelling of acidic compartments by lysotracker is likely due to its protonation and retention in the membranes of these organelles. Lytic compartment labelling methods such as lysotracker staining must be used in combination with more specific markers of autophagy in order to discriminate autophagic activity from other events increasing lysosome/vacuole activity. Lysotracker staining method has been used to monitor autophagy in various organisms including *Arabidopsis*, tobacco, and barley [79, 80, 122].

4.3.2. Acridine Orange (AO). AO is a fluorescent basic dye that has the ability to cross biological membranes. AO accumulates in acidic compartments, such as lysosomes and vacuole, and becomes protonated and sequestered in their lumen. In acridine orange-stained cells, cytoplasm and nucleolus emit bright green fluorescence, whereas acidic compartments fluoresce in bright red. Therefore, quantification of the red fluorescence reflects the degree of acidity and the volume of the cellular acidic compartments. Comparison of the ratio of green/red fluorescence in cells, using fluorescent microscopy or flow cytometry, enables quantification of the extent of autophagic degradation [123, 124]. So far, to our knowledge, no study used AO as a plant autophagy marker.

4.3.3. Monodansylcadaverine (MDC). The autofluorescent substance monodansylcadaverine is commonly used to detect autophagy in plants and in other organisms [67, 125–127]. MDC is a weak base that is capable of crossing biological membranes and concentrating in acidic compartments [128]. Although MDC was originally proposed to label autophagosomes and autolysosomes, recent studies on mammalian autophagy brought out that it is not an autophagy-specific marker. These publications revealed that MDC-positive structures colocalized only partially with autophagosome markers in cells [129]. Furthermore, in autophagy-defective Atg5 knockout cells, MDC-positive dots were still observed [130]. The figures labelled by MDC seem to be endosomes, lysosomes, and lamellar bodies [125]. Therefore, MDC associates with acidic and lipid-rich compartments and it does not discriminate between autophagosomes/autolysosomes and the aforementioned vesicular organelles. Hence, MDC staining has to be combined with other techniques to avoid misinterpretations. Whether MDC is also labelling nonautophagic structures in plants needs careful investigation.

4.4. Biochemical Methods

4.4.1. Long-Lived Protein Degradation. Since autophagy is involved in the degradation of long-lived proteins, determination of their turnover appears to be an efficient method to monitor autophagy levels in cells. In the commonly used technique, following metabolic labelling, degradation of all long-lived proteins is measured. A radioactively labelled amino acid such as valine or leucine can be used to label newly synthesized proteins. Then cells are incubated with cold amino acids to allow short-lived proteins to be degraded. Finally, release of labelled amino acids resulting from the degradation of long-lived proteins is monitored [131].

One major weakness of this technique is that autophagy is not the only mechanism of long-lived proteins degradation. Autophagic and nonautophagic degradation of long-lived proteins should be distinguished by the use of autophagy inhibitors such as 3-mehyladenine (3-MA) [132]. An alternative nonradioactive method uses chromatography to monitor the amount of released unlabeled amino acids [133].

Usage of metabolic labelling in plants was hindered by high compartmentalization of protein substrates and by the fact that metabolite pools in plant cells are generally highly dynamic [134]. Recently developed techniques allowing metabolic labeling of whole plants and plant cell cultures may overcome these difficulties and allow quantification of autophagy by long-lived protein degradation in plants [135–137].

4.4.2. Sequestration of Sugars. Radio-labelled sucrose or raffinose, delivered to cytosol through electropermeabilization, is sequestered in autophagic vesicles together with engulfed cytosolic fragments. Accumulation of radioactivity in autophagic membrane fractions was used to measure autophagic activity [138, 139]. This method has its limitations as well. For example, it cannot be used in yeast due to fast metabolism [140]. Furthermore, injection of the labelled molecule can disturb cellular homeostasis, therefore, precautions and extracontrols including determination of the metabolic equilibrium of the cell prior to the measurement are required. Sugar sequestration technique might be useful in plant cell cultures studies and it needs to be tested.

Table 2: Advantages and disadvantages of techniques used to study autophagy.

Technique	Advantages	Disadvantages
Electron microscopy	Golden standard.Morphological characterization of autophagosomes, autolysosomes and their cargo.	Equipment and expertise required.Difficult to make quantitative analyses.
Atg8/LC3 conjugation to lipid	Rapid detection and quantification of autophagy.Amenable to high throughput techniques.Used to create transgenic organisms for in vivo study of autophagy.	Dots do not always reflect autophagic activity.Molecular weight shift tests need careful interpretation.
Other molecular markers (Atg5, Atg6, Atg16 and Atg18 detection)	Detection of various stages of autophagic vesicle formation.	Most of them need further evaluation.
PI3P detection	Reflects the activity of Vps34 kinase.Quantitative analysis possible.	PI3P accumulation in phenomena not directly related to autophagy (vesicular transport).
Atg1 and Atg4 activity	Determination of enzymatic activity.	So far no clear kinetic studies were published.
P62/SQSTM1 degradation	Activated especially by protein aggregates.	Not all stimuli activate its degradation.Orthologue in plants?
Lysotracker and acridine orange staining	Determination and quantification of autophagy-related lytic activity (lysosomal/vacuolar).FACscan analysis possible.	Autophagosomes are not detected.Lytic activity induced by other conditions as well.
MDC staining	Determination and quantification of autophagy-related lytic activity (lysosomal/vacuolar).	Not all autophagosomes are detected.Lytic activity induced by other conditions as well.
Long-lived protein degradation	Measures autophagic degradation of proteins.Kinetic measurements possible.	Nonspecific degradation of proteins by mechanisms other than autophagy. Radioactive technique.
Sequestration of sugars	Measures autophagic sequestration phase.Quantification may be possible.	Sugars may be metabolized.
Phosphorylcholine accumulation	Promising plant autophagy technique.Quantification may be possible.	Quantification requires special equipment (NMR spectroscopy).
Nonselective and selective degradation of proteins	Promising techniques for plant autophagy.Detection of both sequestration and degradation phases.Quantification may be possible.	Autophagy target proteins need further characterization.
Test of mitophagy or chloroplast autophagy	Detection of autophagy target organelle degradation.Various organelle-specific proteins or organelle-tagged may be used.	Quantification not always possible.

4.4.3. Phosphorylcholine Accumulation. An assay to monitor autophagy in plants is based on the followup of phosphorylcholine accumulation in cells. The technique was developed in sycamore suspension cells cultures undergoing autophagy upon sucrose starvation [6]. Carbon starvation-activated degradation of membrane lipids led to the accumulation of phosphorylcholine in the cytoplasm. Phosphorylcholine accumulation correlated well with autophagy-induction and its quantification by 31P-NMR spectroscopy was proposed as a novel way of autophagy detection in plant cells.

4.5. Other Techniques

4.5.1. Nonselective Degradation of Cytosolic Proteins. One of the yeast techniques developed to monitor autophagy makes use of an N-terminal truncated mutant of the yeast alkaline phosphatase Pho8 [141]. In contrast to the ER-localized wild-type enzyme, the mutant form of pho8 lacking the N-terminal signal sequence (Pho8δ60), is delivered to the vacuole by way of autophagy. Following entry to the vacuole, Pho8δ60 is cleaved at its C-terminus to produce the active alkaline phosphatase. Measurement of alkaline phosphatase activity and/or protein immunoblotting to check the shift between precursor and mature enzyme allows the detection of autophagic activity in yeast cells. Nonselective degradation of marker proteins (especially those with an enzymatic activity) might also be used in plants as autophagy detection methods.

4.5.2. Selective Autophagic Degradation of Proteins. Although autophagy is generally considered as a nonselective phenomenon, some proteins appear to be selectively degraded by autophagy. A GFP or DsRed construct, targeted to the chloroplast, and a GFP fusion of rubisco were transported to the vacuole through autophagy [90, 142]. Rubisco is allocated most of the plant nitrogen and functions in carbon-fixation in chloroplasts. It is released from the chloroplasts in structures called rubisco-containing bodies (RCBs) in order to provide nitrogen from the leaves to others organs. RCB seem to overlap with autophagic vesicles, indicating that rubisco is engulfed in autophagosomes and eventually delivered to the vacuole. The process was dependent on *ATG* genes underlining the autophagic character of the transport. Therefore, targeted GFP-DsRed constructs or GFP-Rubisco may be used as tools to study selective autophagy in plants.

Another specific target of autophagy is betaine homocysteine methyltransferase. Accumulation of this protein in autophagosomes and its cleavage in the lysosome was observed [143]. Another study proposed measurement of neomycin phosphotransferase II accumulation by flow cytometry as an autophagy detection method [144, 145]. Whether the plant orthologue betaine homocysteine methyltransferase shares the same faith and whether neomycin phosphotransferase follows the same path in plants has to be determined.

4.5.3. Tests of Mitochondrial Autophagy (Mitophagy). Since autophagy is a general process for the quality control of organelles, mitochondria are common targets of autophagic degradation. The term mitophagy was coined to describe the selective degradation of mitochondria by autophagy [146]. In yeast, a technique of mitophagy detection was recently developed. This method is based on the use of a GFP-tagged mitochondrial protein and monitorization of the vacuolar release of green fluorescent protein after the degradation of chimera [147]. Indeed, degradation of mitochondrial proteins was previously used to monitor autophagy [148]. Similarly, during autophagy activated by sucrose starvation in plants, a gradual decrease in the number of mitochondria per cell was observed, indicating that techniques based on mitochondrial degradation may be developed to study autophagy in plants [149].

5. Concluding Remarks

Due to its role in fundamental biological phenomena in various organisms including humans and plants, interest in autophagy field is growing exponentially [150]. Accumulation of the knowledge on autophagy molecular mechanisms stimulated the discovery of more efficient and reliable molecular tools to study autophagy. Despite the fact that some of these methods and tools seem to be more suitable for use in specific model organisms, adaptations should be possible in many cases. Plant autophagy studies already benefit from the adaptation of various general autophagy detection techniques used in other model organisms, such as Atg8/LC3 localization tests. Main disadvantages or difficulties of available tools to study autophagy are depicted in Table 2. A better understanding of the biological phenomena involving autophagy in plants and its molecular mechanisms and targets will lead to the development of novel and more precise techniques that will allow the measurement of autophagy in plants with increasing precision and will further accelerate studies in this field.

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Hindawi Publishing Corporation
International Journal of Plant Genomics
Volume 2009, Article ID 721091, 12 pages
doi:10.1155/2009/721091

Research Article

Molecular Cytogenetic Mapping of Chromosomal Fragments and Immunostaining of Kinetochore Proteins in *Beta*

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Received 13 May 2009; Accepted 8 August 2009

Recommended by Pushpendra Gupta

By comparative multicolor FISH, we have physically mapped small chromosome fragments in the sugar beet addition lines PRO1 and PAT2 and analyzed the distribution of repetitive DNA families in species of the section *Procumbentes* of the genus *Beta*. Six repetitive probes were applied, including genotype-specific probes—satellites pTS4.1, pTS5, and pRp34 and a dispersed repeat pAp4, the telomere $(TTTAGGG)_n$, and the conserved 18S-5.8S-25S rRNA genes. Pachytene-FISH analysis of the native centromere organization allowed proposing the origin of PRO1 and PAT2 fragments. Comparative analysis of the repetitive DNA distribution and organization in the wild beet and in the addition lines allowed the development of a physical model of the chromosomal fragments. Immunostaining revealed that the PRO1 chromosome fragment binds α-tubulin and the serine 10-phosphorylated histone H3 specific for the active centromere. This is the first experimental detection of the kinetochore proteins in *Beta* showing their active involvement in chromosome segregation in mitosis.

1. Introduction

The characterization of the genome architecture of higher plants is an important scientific task. One of the most unequivocal approaches to reach this aim is to visualize distinctive chromosomal domains directly by fluorescent-in situ-hybridization (FISH). This method is of supreme efficiency to reveal the physical organization of DNA on plant chromosomes at high resolution. It allows the detection and precise localization of repetitive or single-copy sequences on interphase nuclei, chromosomes in mitosis and meiosis or chromatin fibers. After the first application in wheat [1], FISH was used in plants molecular cytogenetics for the localization of genes, karyotyping and analysis of the physical genome organization [2–4].

A large portion of plant genomes accounts for repetitive DNA [5–8]. Repeats are present in form of sequence duplications up to hundreds of thousands copies [9]. They evolve rapidly in copy number resulting in species-specific variants and/or novel sequence families [10] and are thus crucial for genome evolution [11]. On the other hand, members of many repetitive families show a remarkably high conservation; this ambivalence is a key feature of repeats in genome evolution [12]. The fast evolution leads to a characteristic distribution of the satellites in genomes of closely and distantly related species. While some of these sequences occur in a wide range of plant taxa, others are highly specific. This peculiarity makes repeats a useful tool for comparative studies of plant genomes and for the investigation of evolutionary relationship between plant species [13–16].

Centromeres are essential functional domains of plant chromosomes. They are detectable as primary constrictions or heterochromatic blocks and are responsible for the segregation of the sister chromatids during cell division. The centromere composition was analyzed to different extent in yeast, *Drosophila*, *humans*, *Arabidopsis* [17], rice [18, 19], partially for maize [20] and barley [21]. Plants have regional centromeres, spanning several megabase pairs and are generally composed of species-specific satellite DNA interspersed with retrotransposons, predominantly Ty3-*gyps* [22, 23], but may also contain several genes [24, 25].

The proteins interacting with plant centromere have also attracted attention [26–29]. The nucleosomes of centromeres

are characterized by a special H3-like histone CENH3 [30]. The centromere-associated proteins such as CENH3 (mammalian CENP-A), CENP-C and CENP-E in plants, animal and fungi have highly conserved domains [30]. To fulfill its function in cell division, a kinetochore complex is build at the centromeres where the microtubuli of the spindle apparatus are attached [31].

Eukaryotic chromosomes are terminated by specific nucleoprotein complexes—the telomeres. They are important domains responsible for the maintaining of genome stability. Telomeres permit cells to distinguish chromosome ends from double-strand breaks, thus preventing chromosome degradation and fusion [32]. They also participate in the establishment of the synaptonemal complex during meiosis [33]. The first plant telomere was cloned from *Arabidopsis* by Richards and Ausubel [34]. This sequence is highly conserved, consisting of the short repeat motif $(TTTAGGG)_n$ arranged in tandem arrays many hundreds of units long [35]. Most dicots have *Arabidopsis*-type telomere, while *Asparagales* possess variant sequences instead [36–38].

Beta species provide a suitable system for the comparative study of the nuclear genome composition and evolution. The genus *Beta* contains 14 closely and distantly related species and is subdivided into the sections *Beta*, *Corollinae*, *Nanae* and *Procumbentes* with all cultivars (sugar, fodder and table beet, Swiss chard) exclusively belonging to the section *Beta* [39]. The sugar beet has a genome size of approximately 758 Mbp DNA [39] with at least 63% repetitive sequences [5, 40], and a basic chromosome number of $n = 9$. It is a relatively young crop which origin could be traced back to a few crosses in the 18th century [41]. Therefore, sugar beet has limited genetic variability, and wild beets may provide a valuable pool of genetic resources [42]. To improve the resistance of cultivated beet to biotic and abiotic stress, triploid hybrids were generated by crossing a tetraploid sugar beet with *B. procumbens* ($2n = 18$) and *B. patellaris* ($2n = 36$) belonging to the section *Procumbentes*. After back-crossing with diploid *B. vulgaris*, *nematode-resistant* fragment addition lines PRO1 and PAT2 [43, 44] were selected among offspring. Although resistant to pests, the sugar content and biomass production of those hybrids are low. However, these addition lines are a valuable resource for genomic studies.

In this paper, we analyzed the physical organization of the small wild beet chromosome fragments in the two sugar beet mutant lines, PRO1 and PAT2, by multicolor FISH. Pachytene-FISH on meiotic chromosomes was applied to resolve the structure of the wild beet centromeres. Modification of the proteins in the active kinetochore on *B. vulgaris* and PRO1 centromeres was demonstrated by immunostaining.

2. Materials and Methods

2.1. Plant Material. Plants were grown under greenhouse conditions. The following wild beet species were included in this study: *Beta procumbens* (JKI 35336) and *Beta patellaris* (JKI 54753). The seeds were obtained from Federal Research Centre for Cultivated Plants—-Julius Kühn Institut, Braunschweig, Völkenrode, Germany, and are now available from Genebank of the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany. The *Beta vulgaris* fragment addition lines PRO1 [43] and PAT2 [44] were obtained from C. Jung (Institute of Crop Science and Plant Breeding, Christian-Albrechts University of Kiel, Germany).

2.2. Chromosome Preparation. Mitotic chromosomes were prepared from the meristem of young plants according to Schwarzacher and Heslop-Harrison [45] with some modifications [46] in enzyme solution in citrate buffer containing 6% cellulase *Aspergillus niger*, 0,77% cellulase Onozuka, and 3,0% pectinase *Aspergillus niger*.

Meiotic chromosomes were prepared from anthers by a squashing method [47] with modifications. The buds from the apex of a flower spike having young anthers at early meiosis were used. The 0.45–0.70 mm long anthers were isolated directly from fresh flower buds without fixation or pretreatment and immediately squashed onto a glass slide in 60% acetic acid. Preparations were checked individually for the presence of chromosomes at pachytene and fixed in fresh Carnoy's fixative (methanol : glacial acetic acid = 3 : 1 v/v).

2.3. DNA Probes. A set of six repetitive DNA probes representing *Procumbentes*-specific sequences, characterized by different types of organization on plant chromosomes—centromeric, terminal, or dispersed, and an rDNA probe, was used. The satellite probes were clones pTS4.1, pTS5(accession numbers Z50808, Z50809 [48, 49]), and pRp34 (accession number AM076752 [50]), while the clone pAp4 is a dispersed repeat (accession number AJ414552 [51]).

The clone pLt11 consisting of TTTAGGG repeats was used as a telomeric probe [52], and pTa71 from *T. aestivum* was used as an 18S-5.8S-25S rDNA probe (accession number X07841 [53]).

2.4. Probe Labeling and FISH. Cloned probes shorter than 3 kb were labeled with biotin-16-dUTP or digoxigenin-11-dUTP by PCR using universal primers. The rDNA probe and the telomeric probe pLt11 were labeled with DIG- or BIO-Nick Translation kits (Roche) following the manufacturer's instructions.

Fluorescent in situ hybridization (FISH) was performed according to Heslop-Harrison et al. [54] modified by Schmidt et al. [55]. The microscopy slides were incubated overnight at 37°C and pretreated with 10 ng/l μL of RNase A for 1 hour at 37°C and 50 ng/l μL pepsin for 5 minute at 37°C. Afterwards, the preparations were fixed in freshly prepared 4% formaldehyde solution for 15 minute, dehydrated in ethanol series, and air-dried. However, 30 l μL of the hybridization solution with a stringency of 76% and containing 50% formamide, 20% dextran sulphate, 0.2% SDS, 50 ng/l μL blocking DNA, and 10–100 ng of labelled probes in 2 x SSC were applied. The preparations were covered with plastic cover slips, denatured, and stepwise cooled down in an in situ thermocycler Touchdown (ThermoHybaid) and hybridized overnight at 37°C in a humid chamber. Signals were detected using antibodies

Table 1: Repetitive probes used for the characterization of the fragment addition lines PRO1 and PAT2.

Probe	Origin	Length, bp	Sequence type	Accession	Reference
Satellite					
pTS4.1	*B. procumbens*	312	*Sau*3AI restriction satellite	Z50808	Schmidt et al. 1990 [48]
pTS5	*B. procumbens*	153–160	*Sau*3AI restriction satellite	Z50809	Schmidt and Heslop-Harrison 1996 [49]
pRp34	*B. procumbens*	352–358	*Rsa*I restriction satellite	AM076755	Dechyeva and Schmidt 2006 [50]
Dispersed					
pAp4	*B. procumbens*	1353-1354	*Alu*I repeat	AJ414552	Dechyeva et al. 2003 [51]
Telomere					
pLT11	*A. thaliana*	not tested	telomeric repeat	not entered	Vershinin et al. 1995 [52]
Ribosomal genes					
pTa71	*T. aestivum*	4642	25S-18S gene fragment with spacer	X07841	Barker et al. 1988 [53]

coupled to fluorochromes Cy3 (red) or FITC (green). Chromosome preparations were counterstained with DAPI (4′, 6′-diamidino-2-phenylindole) and mounted in antifade solution (CitiFluor).

2.5. Immunostaining. Immunostaining was performed according to Houben et al. [56] with modifications for *Beta* species. Root tips were fixed in 4% formaldehyde in microtubule stabilizing buffer (MTSB), washed in MTSB, and treated with enzyme mix consisting of 2.5% pectinase, 2.5% cellulase Onozuka R 10 and 2.5% pectolyase for 30 minute at 37°C. The material was macerated and centrifuged onto a glass slide with a Cytospin 3 (Shandon) at 2000 rpm for 5 minute. The preparations were prefixed in 4% formaldehyde in PBS and blocked with 3% BSA in MTSB/0.2% Tween. Also, 50 l μL of the antibodies solution containing anti-α-tubulin (raised in mouse against rabbit, Amersham) and anti-H3 phosphorylated at Ser 10 (polyclonal rabbit, Upstate) were applied to the preparations. After overnight incubation at 37°C, the slides were washed in MTSB three times and the probes were detected with the fluorochrome-conjugated secondary antibodies anti-mouse-FITC (Roche) for anti-α-tubulin and anti-rabbit-rhodamin red (Roche) for anti-H3. Unspecific binding of antibodies was removed by washing in MTSB. The preparations were counterstained with DAPI (4′, 6′-diamidino-2-phenylindole) and mounted in antifading solution (CitiFluor).

2.6. Image Processing. Examination of slides was carried out with a Zeiss Axioplan2 fluorescence microscope equipped with Filter 01 (DAPI), Filter 02 (Cy3), Filter 09 (FITC), and Filter 25 (DAPI/Cy3/FITC). Photographs were taken on Fujicolor SUPERIA 400 colour print film and negative films were digitized on a Nikon LS-1000 scanner. Alternatively, the images were acquired directly with the Applied Spectral Imaging v. 3.3 coupled with the high-resolution CCD camera ASI BV300-20A.

Immunostaining images were acquired directly with a cooled CCD camera. After three-dimensional deconvolution, the resulting data subsets were merged through the *Z*-axis (DeltaVision).

The images were contrast optimized using only functions affecting the whole image equally and printed using Adobe Photoshop 7.0 software.

3. Results

3.1. High-Resolution FISH-Mapping of B. vulgaris Fragment Addition Lines. To analyze the physical organization of the chromosome fragments in the addition lines PRO1 and PAT2, multicolor FISH with *Procumbentes*-specific and heterologous repetitive sequences was applied. Six repetitive probes (Table 1) were hybridized in situ to chromosomes of PRO1 and PAT2 as well as to the respective donor species of the chromosome fragments *B. procumbens* and *B. patellaris*.

The in situ hybridization of *B. procumbens* with the pericentromeric probe pTS4.1 and the centromeric pTS5 demonstrated that the satellite pTS5 resides on 12 centromeres out of 18, where it is flanked with pTS4.1 (Figure 1(a), exampled by arrowheads). However, pTS4.1 occupies pericentromeric loci of all chromosomes. In contrast to pTS5 strictly confined to the centromeres, it produced weak signals on some intercalary and subterminal sites (Figure 1(a), arrows).

In PRO1, both satellites are detectable only on the chromosomal fragment (Figure 1(b)). pTS5 shows one pair of clear signals on the acrocentric fragment and is bordered by pTS4.1 from one side (Figure 1(c)).

The two centromeric satellites pTS5 and pTS4.1 were hybridized simultaneously to the tetraploid *B. patellaris* (Figure 1(d)). The satellite pTS5 labeled only twelve centromeres out of 36 (Figure 1(d), red). The pTS4.1 hybridization signals of different intensity were detectable in pericentromeric loci of all *B. patellaris* chromosomes (Figure 1(d), green) and on some chromosome ends (arrows).

In PAT2, the *B. patellaris* fragment barely visible after DAPI staining was clearly distinguished after fluorescent in situ hybridization with the genome-specific probes pTS5 and pTS4.1 (Figure 1(e), arrows). Figure 1(f) shows that the centromeric pTS5 (red) is flanked by pTS4.1 (green) producing two pairs of signals. This is strikingly in contrast with the hybridization pattern on the PRO1 chromosomal fragment, where the stretch of pTS4.1 is detectable only on one side of the centromeric pTS5 array (Figure 1(c)).

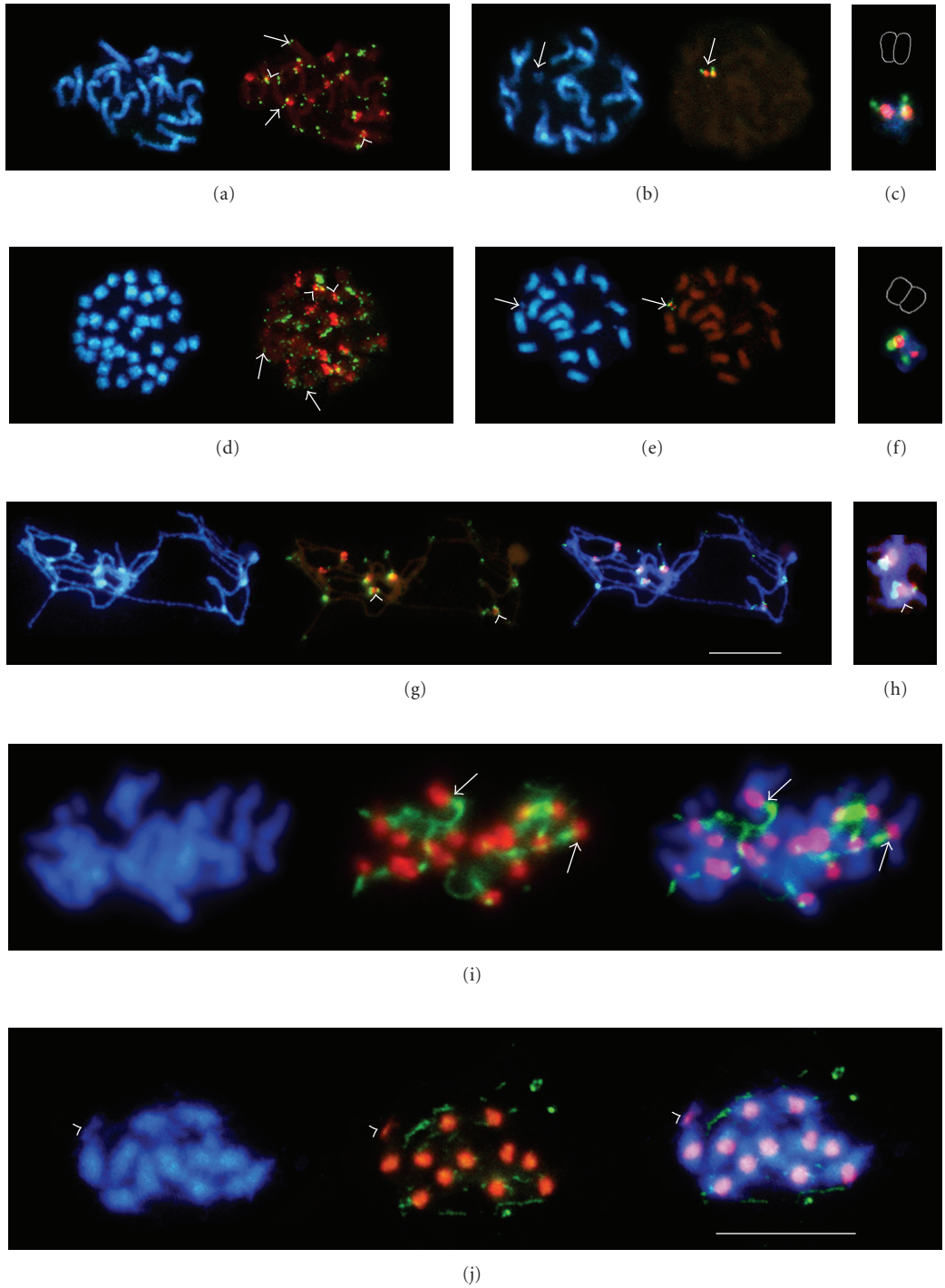

Figure 1: Blue fluorescence in each panel shows the chromosomes stained with DAPI. The scale bar in (G) for the panels A-G and in (J) for the panels I and J represents 10 ¯m. The chromatids of the chromosome fragments are schematically contoured in panels C and F. FISH with *Procumbentes*-specific satellites pTS4.1 (green) and pTS5 (red) on (A) *B. procumbens*; (B) PRO1; (C) the closed-up overlay of the panel (B); (D) *B. patellaris*; (E) PAT2; (F) the PAT2 fragment. (G) Simultaneous localization of the centromeric probes pTS5 (red) and pTS4.1 (green) on the *B. procumbens* meiotic chromosomes. (H) Close-up from the panel (G). (I-J) Localization of kinetochore proteins in *B. vulgaris* and PRO1 by immunostaining. Microtubuli are visible as green threads. Serine 10-phosphorylated histone H3 produces red signals. The right images are overlays. Microphotographs of the three-dimensional preparation were taken in different focal planes and overlaid. (I) Serine 10-phosphorylated histone H3 labels all centromeres of *B. vulgaris* in mitosis. The sites where the microtubuli of the spindle apparatus are attached to the centromeres are exampled by arrows. (J) PRO1 chromosomal fragment shows a H3S10- phosphorylated signal (arrowhead), thus indicating that its centromere is active in mitosis.

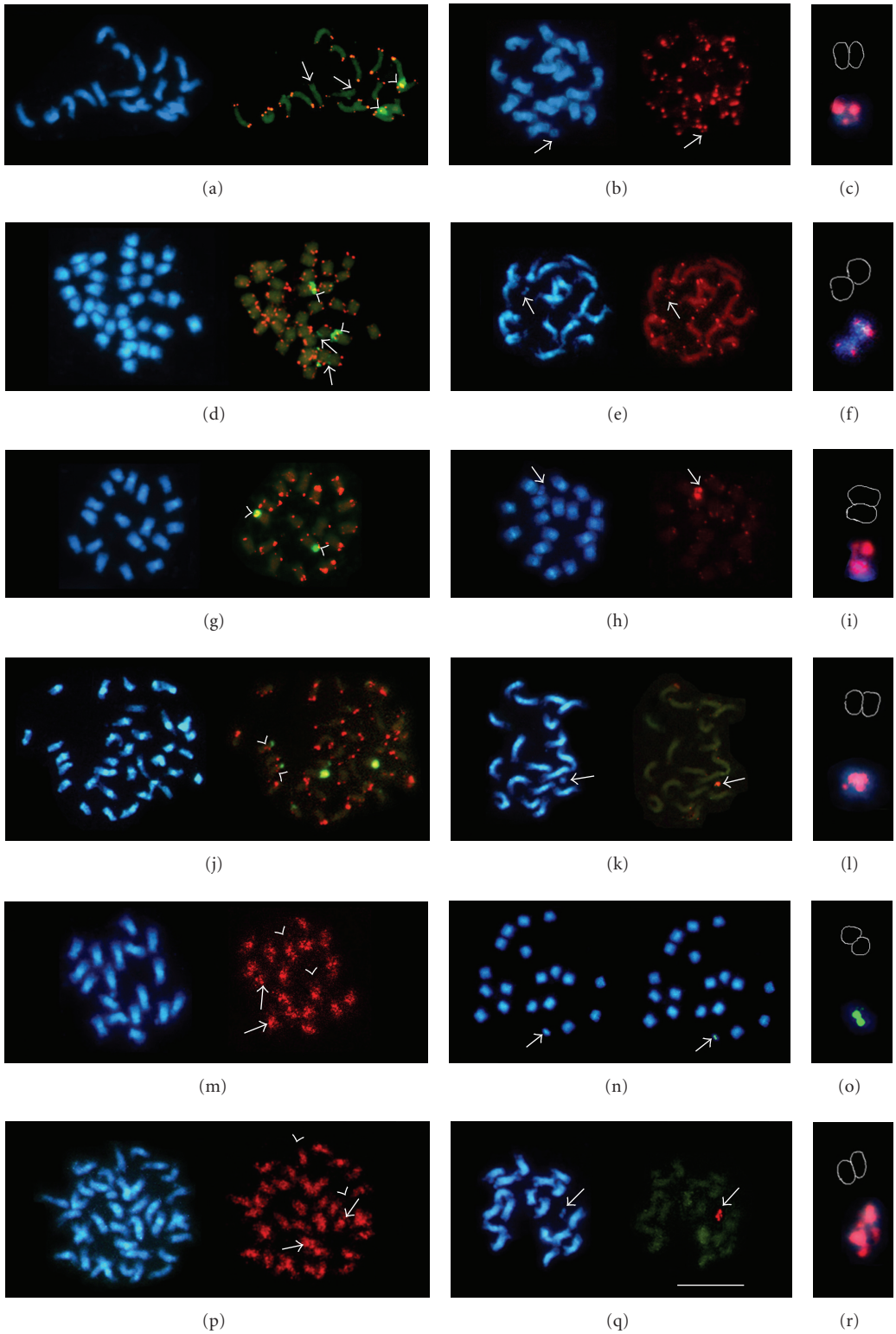

Figure 2: Blue fluorescence in each panel shows the chromosomes stained with DAPI. The scale bar for left and central panels in (Q) represents 10 ¯m. Green fluorescence shows hybridization of the ribosomal gene probe pTa71. The chromatids of the chromosome fragments are schematically contoured in right panels. FISH with the telomeric probe (TTTAGGG)n (red) and on (A) *B. procumbens*; (B) PRO1;. (C) PRO1 chromosome fragment; (D) *B. patellaris*; (E) PAT2; (F) PAT2 chromosome fragment. The subtelomeric satellite repeat pRp34 (red) cloned from *B. procumbens* is found on (G) *B. procumbens*; (H) PRO1 and its chromosome fragment (I); (J) *B. patellaris*; (K) PAT2; (L) close-up of the panel (H). The dispersed repeat pAp4 specific to the *Procumbentes* localized by FISH on (M) *B. procumbens*; (N) on the PRO1 chromosome fragment; (O) close-up of the panel (N); (P) *B. patellaris*; (Q) on PAT2 is limited to the chromosome fragment, where it shows a weak dispersed distribution (R).

To achieve a higher resolution of the physical organization of the two centromeric satellites, a double-target in situ hybridization with pTS4.1 and pTS5 was performed on meiotic chromosomes of *B. procumbens* (Figure 1(g)). The chromosomes at pachytene are far less condensed than at mitosis, but still preserve their morphology. The chromatin at this stage of the cell cycle enables a higher resolution and is especially suitable for the simultaneous detection of adjacent sequences. The experiment on meiotic spreads showed that on some centromeres the satellite pTS5 is flanked by the pTS4.1 (Figures 1(g) and 1(h), arrowheads), while on the others the pTS4.1 borders pTS5 only from one side.

The hybridization of a *B. procumbens* prometaphase spread with the telomeric probe pLT11 produced clear double signals with variable intensity on all chromosome ends (Figure 2(a), red) with the exception of a pair of chromosomes where signals were detected on one arm only (Figure 2(a), arrows). The 25S-18S ribosomal gene fragment pTa71 was used as a second probe (Figure 2(a), green). The rDNA in *B. procumbens* forms a clearly visible distal secondary constriction [57]. In this species, the rDNA array is adjacent to the telomere (Figure 2(a), arrowheads) as has been shown previously (Dechyeva and Schmidt [50]).

On the PRO1 metaphase spread, the telomeric DNA was detectable on all sugar beet chromosomes as well as on both ends of the chromosome fragment (Figure 2(b), red) where it produced a strong pair of signals on one end and a very weak one on the opposite arm (Figure 2(c), red).

The telomeric probe pLT11 labeled the ends of all *B. patellaris* chromosomes relatively uniformly (Figure 2(d), red). The simultaneous hybridization with the ribosomal gene fragment pTa71 produced two strong signals, two weak and two barely visible additional signals, all at the subterminal positions (Figure 2(d), green). The telomeres found adjacent to the larger rDNA array (Figure 2(d), arrowheads). The only chromosome, arms where pLT11 was not detectable, were those with minor pTa71 site (Figure 2(d), arrows).

As expected, all PAT2 chromosomes demonstrated telomeric signals (Figure 2(e), red). The signals on the wild beet added fragment were nearly as intense as those on sugar beet chromosomes (Figure 2(e), arrows). The chromosomal fragment has two pairs of clear signals, evidently on its both ends (Figure 2(f), red).

The localization of the telomeric sequences on the chromosome fragments was complemented by FISH with the subtelomeric satellite pRp34. The probe labeled all but two chromosomal ends of the wild beet *B. procumbens* (Figure 2(g), red), including those harboring rRNA genes (Figure 2(g), green, arrowheads). In PRO1, the sugar beet chromosomes were labeled with this *B. procumbens*-derived satellite much weaker than the wild beet fragment (Figure 2(h), red, arrow). The two pairs of the pRp34 signals on the fragment have different strengths (Figure 2(i), red).

The subtelomeric probe pRp34 was detected on one or both arms of all except two *B. patellaris* chromosomes in subtelomeric positions producing signals of various intensities (Figure 2(j), red). It is noteworthy that pRp34 signals were also found adjacent to the minor sites of the ribosomal genes (Figure 2(j), green, arrowheads). Both ends of the PAT2 chromosomal fragment showed the subtelomeric satellite signals, one weaker than the other (Figure 2(k), arrow, and Figure 2(l)).

The *Procumbentes*-specific repeat pAp4 was dispersed over all *B. procumbens* chromosomes (Figure 2(m), red). The repeat is amplified in intercalary and pericentromeric heterochromatic regions (Figure 2(m), arrows), but mostly excluded from distal euchromatic regions (Figure 2(m), arrowheads). In PRO1, the dispersed repeat pAp4 is not detectable on sugar beet chromosomes and is only found on *B. procumbens* fragment (Figure 2(n), green, arrows), where it labels both chromatids (Figure 2(o)).

The dispersed repeat pAp4 was scattered over all *B. patellaris* chromosomes (Figure 2(p), red). Examples of the reduction of the repeat in some centromeres are indicated with arrows, while examples of the exclusion from euchromatin are shown by arrowheads (Figure 2(p)). On PAT2, the repeat produced 3-4 pairs of relatively weak signals exclusively along the chromosome fragment (Figure 2(q), arrow, and Figure 2(r)) forming a dispersed pattern similar to that on intact *B. patellaris* chromosomes.

3.2. Detection of Phosphorylation in Histone H3 at Centromeres of B. vulgaris and PRO1 by Immunostaining. To get an insight into the structure and function of the kinetochore, the proteins characteristic for active centromeres were observed on metaphase preparations of *B. vulgaris* and the fragment addition line PRO1.

Mitotic beet cells were immunostained with polyclonal antibody against serine 10-phosphorylated histone H3 raised in rabbit [56]. This N-terminal modification of histone H3 is only found in functional pericentromeric chromatin [58]. The antibody against α-tubulin raised in mouse against rabbit allows visualizing the microtubuli [59] which attach as important functional parts to the active kinetochore.

Proteins in immunostaining probes should preserve their structure resulting in a three-dimensional shape of the nuclei. Such preparations cannot be successfully analyzed by conventional epifluorescence microscopy. Consequently, computational deconvolution microscopy was applied. The immunostaining with the antibody against histone H3 phosphorylated at serine 10 demonstrated that the centromeric histones of *B. vulgaris* (Figure 1(i)) and PRO1 (Figure 1(j)) in metaphase are modified by phosphorylation at the N-terminal serine 10. The sites appeared as bright red signals localized at the DAPI-positive centromeric regions. The microtubuli were detectable as green threads. It was clearly visible at some loci that the microtubuli are attached to the centromeric sites (Figure 1(i), arrows). Remarkably, the PRO1 acrocentric chromosomal fragment also showed a clear H3 S10-phosphorylated signal (Figure 1(j), arrowhead).

4. Discussion

Sugar beet is an important agricultural crop, and the results of genome research in this species might be important to the practical implementation in green biotechnology.

Currently, a fine-resolution physical map is under construction and a genome-sequencing project is carried out in the framework GABI–Genome Analysis in Biological System Plant (http://www.gabi.de/) aiming to unravel the genome composition of this crop species. Interspecific hybrids and addition lines of *B. vulgaris* are a valuable starting material for plant breeders and an interesting object for fundamental studies on plant genome composition and evolution [60–62]. The application of genome-specific repetitive probes isolated from the wild beet *B. procumbens* in combination with repetitive DNA sequences conserved among plant species enabled to map the chromosome fragments of the *B. vulgaris* addition lines PRO1 and PAT2.

4.1. Molecular Cytogenetics of the Wild Beet Species. Hybridization of *B. patellaris* with pTS5 (Figure 1(d), red) suggests that this species might be an allopolyploid: the pTS5 gave 12 signals of different intensity similar to the pattern in *B. procumbens*. It is tempting to assume that one haploid set of chromosomes of the *B. patellaris* genome is indeed derived from *B. procumbens*, while the remaining 18 chromosomes originate from another, yet unidentified species. When probed with the subtelomeric pRp34, *B. patellaris* (Figure 1(j) , red) in contrast to *B. procumbens* (Figure 1(g), red) did not produce visible signals on the chromosome ends carrying the rDNA genes (Figure 1(j) , green). On the contrary, the subtelomeric pRp34 signals were detectable proximal to weaker pTa71 signals (Figure 1(g) , arrowheads), most likely caused by an inversion of the rRNA gene array. This is another indication that *B. procumbens* is not the only species that participated in *B. patellaris* polyploidization. Similarly, hybridization of the allopolyploid *Nicotiana rustica* with the satellite NUNSSP specific to the parental U-genome (*N. undulata*) allowed distinguishing chromosomes originating from the different tobacco species, *N. paniculata* (P-genome) [63]. Recent studies on allotetraploid *Gossypium hirsutum* in comparison to the model diploid progenitors, *G. arboreum* and *G. raimondii*, revealed possible mechanisms of "genomic downsizing" in polyploids [64].

Ribosomal DNA genes in eukaryotes are tandemly arranged in thousands of copies. They reside at the chromosomal loci known as nucleolus organizer regions (NORs) [65]. These genes are highly conserved in plants and other eukaryotes. Therefore, it was expected that the heterologous 18S-5.8S-25S rDNA probe pTa71 isolated from wheat [53] produced strong hybridization signals at the secondary constriction of *B. procumbens* (Figure 1(i), arrowheads). The domains harboring rRNA genes are recognizable as prominent DAPI-positive structures located distally on two chromosomes [49, 57]. For polyploids, it has been reported that only one set of parental rRNA genes is preferentially functional: expression of rDNA of rye origin is suppressed in amphiploid triticale, and only the 1B- and 6B-rDNA from wheat is functional [66]. Similar rRNA gene silencing was observed in the natural allotetraploid *Arabidopsis suecica* and the synthetic hybrid of its progenitors *A. thaliana* and *A. arenosa* (*Cardaminopsis arenosa*) [67]. This epigenetic phenomenon, observed in many animals, like *Drosophila* and *Xenopus* [68], and plants, like *Crepis* [69], *Aegilops* x *Triticum* hybrids [70], *Brassica* [71], is known as nucleolar dominance [72]. Not only most natural polyploids possess one predominant 18S-5.8S-25S nuclear ribosomal DNA homolog in their genome; the studies on artificial interspecific hybrids suggested that in some plants, like *Glycine*, most or all repeats at one homeologous locus have been lost [73]. It can be speculated that in the tetraploid species *B. patellaris*, the two strong hybridization signals most likely correspond to functional rDNA loci (Figure 2(d), arrowheads) which can be shown by silver staining. The weak green crosshybridization signals (Figure 2(d), arrows and Figure 2(j), arrowheads) may correspond to the rDNA loci from the repressed copies of the chromosome set, which is still recognizable by the heterologous probe used in this experiment. No pTa71 signals were detectable either on PRO1 or on PAT2 chromosomal fragments (not shown).

4.2. Development of a Physical Model of the PRO1 and PAT2 Chromosomal Fragments. The addition line PRO1 [43] has a 6–9 Mbp large fragment of the *B. procumbens* chromosome [61]. The chromosome mutant PAT2 has a smaller fragment originating from *B. patellaris* [44]. Both chromosome fragments are stably transmitted in mitosis and hence should have a functional centromere. FISH-mapping of such small chromosomes is challenging, for comparison, the chromosomes of *A. thaliana* have a size of approximately 25 Mbp (TAIR, http://www.arabidopsis.org/) and are regarded as a difficult subject for conventional FISH.

Previous analysis of the long-range organization of centromeres in the wild beet *B. procumbens* allowed the development of a structural model of a plant centromere [74, 75]. According to this model, the centromeric satellite pTS5 forms a large array which is flanked by the arrays of a nonhomologous pericentromeric satellite pTS4.1. These arrays, representing the majority of centromeric and pericentromeric DNA, are interspersed with Ty3-*gypsy*-like retrotransposons *Beetle*1 and *Beetle*2 and remnants or rearranged copies thereof as shown by BAC analysis and FISH on *B. procumbens* chromosomes [23, 61].

To reveal the possible origin of the PRO1 and PAT2 chromosomal fragments, the organization of the centromere-specific satellites pTS4.1 and pTS5 was studied in detail. Hybridization of pTS5 and pTS4.1 on chromosomes of *Procumbentes* species resulted in a unique pattern on each centromere, thus allowing to classify the centromeres in those having (a) only pTS4.1, (b) both satellites present with signals of equal intensity, and (c) where pTS5 was much stronger than pTS4.1 (Figures 1(a) and 1(d)).

In PRO1, pTS5 labels one end of the acrocentric fragment, bordered by adjacent pTS4.1 array from one side only (Figure 1(c)). Gindullis et al. [75] suggested that the PRO1 fragment may be a result of a chromosome breakage within the centromeric pTS5 block which is flanked with pTS4.1 from both sides (examples of this type of centromere are indicated with arrowheads in Figures 1(a), 1(g), and 1(h)). Alternatively, the PRO1 fragment could originate from one of the chromosomes where the centromeric satellite pTS5 region is bordered by pericentromeric pTS4.1 only from one side (Figure 1(h)). In PAT2, the pTS5 block on the wild beet

chromosomal fragment is flanked with pTS4.1 arrays from both ends (Figure 1(f)), which implies that two breaks have occurred within the pericentromeric region. Hence, the most likely donators of the PAT2 chromosome fragment are one of the chromosomes exampled on Figure 1(d) by arrowheads.

The telomeres protect the chromosome ends from degradation. Both fragment addition lines PRO1 and PAT2 arose spontaneously in the offspring of *B. vulgaris* x *B. procumbens* or *B. vulgaris* x *B. patellaris* triploid hybrids back-crossed with diploid *B. vulgaris* [76]. Such chromosome fragments resulting from chromosomal breakage usually have instable ends which tend to fuse. Alternatively, breakpoints may be stabilized by the phenomenon known as "healing of broken ends," first described and discussed by McClintock [77]. However, healing of broken ends by de novo telomere formation is mostly restricted to meristematic tissue or undifferentiated cells, but is low or undetectable in mature differentiated tissues [78]. Moreover, such newly synthesized telomeres contain a considerable amount of atypical sequence units, as was shown for wheat [79].

Thus, it was important to find out whether PRO1 and PAT2 chromosome fragments indeed possess telomeres ensuring their stability. Therefore, the next probes tested on the sugar beet hybrids PRO1 and PAT2 were those located at the chromosome ends: the *Arabidopsis* telomeric probe pLT11, the subtelomeric satellite pRp34-179 originating from *B. procumbens*, and the 25S-18S rDNA probe pTa71 from wheat. The telomere and the pRp34 were clearly visible as pairs of fluorescent signals on both ends of the PRO1 (Figures 2(c) and 2(i)) and PAT2 (Figures 2(f) and 2(l)) fragments. The subtelomeric satellite pRp34 was found on both ends of the fragments PRO1 (Figure 2(i)) and PAT2 (Figure 2(l)). The two signals on the PRO1 and PAT2 chromosomal fragments indicate that not only the telomeres of the added fragments seem to be intact but also other components of the terminal chromosomal regions are present. Since not only the *Arabidopsis*-type telomeric sequence but also the subtelomeric satellites are preserved, it is possible to assume that the fragments evolved by deletions resulting in the loss of considerable amount of intercalary chromatin.

Additional signals of the subtelomeric satellite pRp34-179 on sugar beet chromosomes (Figures 2(e) and 2(h)) are due to cross-hybridization with the homologous subtelomeric satellite pAv34 from *B. vulgaris* belonging to the same repetitive DNA family as pRp34 [50]. However, these signals are relatively weak because of the relatively low sequence similarity between pAv34 and pRp34 (58.9%). The stronger signals on *B. vulgaris* chromosomes may correspond to the pAv34 sequence subsets more similar to pRp34, thus indicating chromosome-specific amplification of the subtelomeric satellite variations. The reason was most likely the divergence between the pRp34 from *B. procumbens* and pAv34 from *B. vulgaris* which share 56.8–60.7% similarity [50]. In this in situ experiment, hybridization stringency was 76%, which was too high to detect all the copies of pAv34 on *B. vulgaris* chromosomes with pRp34-179 as probe. Emergence of chromosome-specific DNA is known for human alpha satellite, where ancestral sequences have evolved into a number of chromosome-specific families, presumably by cycles of interchromosomal transfers and subsequent amplification leading to intrachromosomal sequence homogenization [80]. Similar divergence of satellite subfamilies into chromosome-specific subsets have been observed in plants. Subtelomeric repeats with chromosome-specific distribution may play a role in the recognition of homologous chromosome ends and have been suggested to be part of a complex chromosome end structure [52]. The analysis of telomeres and adjacent sequences on rye chromosomes showed that they are able to evolve in copy number rapidly [81]. Despite the fact that maize centromeres are all composed of the same related elements, the differences in composition and mutual arrangements of those elements provide each centromere with a unique molecular structure [82]. Similarly, representatives of the *Sau*3AI satellite family I of *B. procumbens* also form the subfamily pTS6 which has a different chromosomal position [49].

The presence of the dispersed repetitive family pAp4 specific for the *Procumbentes* genomes in PRO1 and PAT2 chromosome fragments in well-detectable numbers allows to conclude that the chromosomal fragments also posses intercalary chromatin, which must be essential as a lateral support for the centromeric activity and hence ensure stability of the chromosomal fragments [83].

The allocation of repetitive probes on PRO1 and PAT2 by FISH enabled to propose origins and to develop physical models of the chromosome fragments (Figure 3). The wild beet fragments in PRO1 and PAT2 contain most major types of repetitive DNA characteristic for intact chromosomes of the wild beet species *B. procumbens* and *B. patellaris*. They include arrays of two centromeric and pericentromeric satellites dispersed repetitive sequences and are terminated by subtelomeric satellite and the telomere protecting their ends from degradation.

The experiments performed in this study demonstrated that the PRO1 fragment is acrocentric. The size of the centromeric pTS5 satellite array has been estimated by fiber FISH to be 115 kb [61]. Wild type *B. procumbens* chromosomes are metacentric, submetacentric, or acrocentric (Figure 1(a)), their centromeric satellite arrays spanning 157–755 kb [74]). In metacentric or submetacentric PAT2 fragment, the centromeric array is even smaller—-only about 50 kb estimated by pulsed-field gel electrophoresis [61]. The telomere and the subtelomeric satellite pRp34 were also detected on the ends of the chromosomal fragments although amplified to a different extent. However, the transmission rate of the monosomic PRO1 and PAT2 fragments in meiosis is lower than expected 50%, reaching 34.8% maximal for PRO1 [44]. The fact that the chromosome fragment of PAT2 contains a centromeric pTS5 satellite block flanked by pericentromeric satellite pTS4 arrays as well as the telomere and the subtelomeric satellite repeat (Figure 3) leads to the conclusion that there might be other factors effecting stable transmission of this wild beet fragment in meiosis. It was shown that barley chromosomal fragments can be normally transmitted through meiosis in wheat genetic background even without typical centromeric repeats [84]. On the other hand, the field bean minichromosome

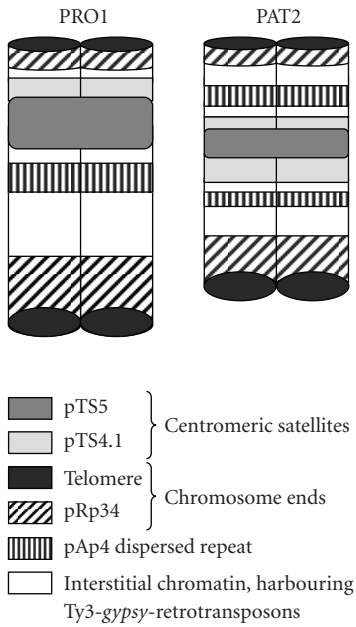

Figure 3: Structural model of the PRO1 and PAT2 chromosomal fragments. Both chromosome fragments are represented according to the distribution patterns of the repetitive DNA sequences mapped by FISH.

DI-VI containing a wild-type centromere and comprising approximately 5% of the haploid metaphase complement suffered loss during meiosis (66% loss in hemizygous condition, which is similar to PRO1 and PAT2), while the minichromosomes comprising approximately 6% of the genome were already stably segregating [83]. However, PRO1 and PAT2 chromosomal fragments are estimated to comprise only 0.8–1.2% of the 758 Mb of sugar beet haploid genome. Field bean minichromosomes of similar proportion of 1% of the haploid genome size were stably transmitted through mitosis, but not meiosis. It is supposed that lack of additional genomic DNA serving as lateral support of centromeres or insufficient bivalent stability due to the incapability of chiasma formation could be the reasons of lower transmission of the very small chromosome fragments, even though they possess the centromere and the telomeres [83]. The data generated by these experiments demonstrate that FISH is a unique method in genome analysis including comparative studies giving an insight into details of the physical organization of DNA sequences on chromosomes as small as 6–9 Mbp.

4.3. Kinetochore Proteins in the B. vulgaris Hybrid PRO1. There is no conserved DNA sequence responsible for the centromeric function in higher plants [11, 17, 21, 75, 84]. Recently, it has been shown that barley isochromosomes lacking typical centromeric sequences were normally transmitted through mitosis and meiosis [83]. However, the establishment of a centromere must involve the deposition of the centromeric histone H3 variant designated CENH3. This protein is generally viewed as the core of the centromeric complex linking centromeric DNA with the proteins of inner kinetochore. In maize, CENH3 binding to centromeric retrotransposons (CRMs) and satellite repeats (CentC) was shown by immunoprecipitation [85]. A study of rice chromosome 8 centromere (*Cen*8) indicated also that the transcribed genes are interspersed with CENH3 binding sites [25]. Although kinetochores differ in morphology from species to species, recent data have established that an important group of kinetochore proteins is conserved from *Saccharomyces cerevisiae* to humans [58, 86]. It was shown by indirect immunofluorescence that the kinetochore elements of higher plants are conserved even between very distantly related taxa like monocots and dicots [28]. Antibodies against the partial human proteins CENP-C, CENP-E, and CENP-F and against maize CENP-C recognized the centromeric regions of mitotic chromosomes of *Vicia faba* and *Hordeum vulgare* [87].

An important step during the formation of a functional kinetochore is the phosphorylation of the pericentromeric histone H3 [29]. This posttranslational chromatin modification is evolutionarily conserved in plants and animals [58]. The changes in the level of phosphorylation of serine 10 in CENH3 correspond to changes in the cohesion of sister chromatids in meiosis in maize [88]. In *Secale cereale*, *Hordeum vulgare*, and *Vicia faba*, the phosphorylation of the pericentromeric histone H3 at serine 10 correlates with the chromosomes condensation in mitosis [56].

The fluorescent immunostaining of centromere-associated proteins in the fragment addition line PRO1 allowed the comparison of the histone H3 phosphorylation patterns of *B. vulgaris* chromosomes and the *B. procumbens* chromosome fragment in mitosis, elucidating the behavior of the centromeres originating from different species in a single dividing cell. The results demonstrated that the heterologous antibody against serine 10-phosphorylated histone H3 recognized sugar beet kinetochores (Figures 1(i) and 1(j), red). The visualization of the microtubuli with anti-α-tubulin (Figures 1(i) and 1(j), green) demonstrated that the chromosomes are attached to the spindle apparatus during mitosis (Figure 1(i), arrows). For oat-maize chromosome addition lines it was shown that the introgressed maize chromosomes do not express their own CENH3 but rather utilize CENH3 available from the host genome [89]. Although species-specific CENH3-antibodies are not yet available for *Beta*, the heterologous antibody against Ser10-phosphorylated centromeric histone H3 readily immunolabelled the centromere of the PRO1 chromosome fragment at mitosis (Figure 1(j), arrowhead). Since mitotic transmission of the PRO1 wild beet fragment is normal, and meiotic transmission is only slightly reduced [76], it is reasonable to assume that the kinetochore proteins expressed by *B. vulgaris* must recognize also the centromere of the added fragment and that the fragment may utilize CENH3 similarly to maize chromosomes in oat background.

The immunostaining experiment with the antibodies against serine 10-phosphorylated histone H3 and α-tubulin gave the first insight into the centromeric function in the *B. vulgaris* fragment addition line PRO1. Further studies on this unique material combining the functional centromeres with the different molecular composition from two distantly

related species would shed light on the conservation of centromeric components in higher plants.

Acknowledgments

The authors are grateful to Andreas Houben for the opportunity to perform immunostaining and to Cordula John for assisting with the part of the FISH experiments. This work was funded by BMBF (*BioFuture* grant 0311860 and GABI START grant 0315070).

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