Transposons as tools for functional genomics

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Abstract – Transposons have been used extensively for insertional mutagenesis in several plant species. These include species where highly active endogenous systems are available such as maize and Antirrhinum majus, as well as species where heterologous transposons have been introduced through transformation, such as Arabidopsis thaliana and tomato. Much of the past use of transposons has been in traditional ‘forward genetics’ approaches, to isolate and molecularly characterize genes identified by mutant phenotypes. With the rapid progress in the genome projects of different plants, large-scale transposon mutagenesis has become an important component of functional genomics, permitting assignment of functions to sequenced genes through reverse genetics. Different strategies can be pursued, depending upon the properties of the transposon such as the mechanism and control of transposition, and those of the host plant such as transformation efficiency. The successful use of these strategies in A. thaliana has made it possible to develop databases for reverse genetics, where screening for the knockout of a gene of interest can be performed by computer searches. The extension of these technologies to other plants, particularly agronomically important crops such as rice, is now feasible. © 2001 Éditions scientifiques et médicales Elsevier SAS

gene tagging / reverse genetics / transposons

Ac, Activator element / Ds, Dissociation element / dSpm, defective Suppressor-mutator element / En, Enhancer / FST, flanking sequence tag / I, Inhibitor / Mu, Mutator / Spm, Suppressor-mutator

1. INTRODUCTION

The advent of large-scale sequencing projects in several plants, together with the anticipated completion of whole genome sequences for Arabidopsis thaliana and possibly rice as well, has resulted in an explosion of gene sequence information in plants. As more and more sequences are available in the databases, it becomes critical to assign functions to the thousands of new genes identified. There are several approaches being attempted to tackle this problem.

The most direct approach to determine the functions of the sequenced genes in an organism is to disrupt or generate mutations in the genes and analyse the consequences. Methods that have been developed in plants for this purpose include gene replacement, sense and anti-sense suppression, and insertional mutagenesis. Although recently it has been shown that targeted gene replacement by homologous recombination is possible in A. thaliana, the frequency is very low [39], which makes the method laborious for generating large numbers of gene knockouts. Recently, a nuclear gene of Nicotiana tabaccum has been mutated by site-specific base substitution using self-complementary chimeric oligonucleotides [9] but this method is also too labour intensive for use in functional genomics. Strategies for sense and anti-sense suppression have been developed for the inactivation of known genes [8, 37] but as these strategies require generation of several independent transgenic lines for each gene they are currently limited to the study of single genes. Currently the most widely used approach for large-scale gene function analysis in plants is random insertional mutagenesis. Either T-DNA (reviewed in [2, 42]) or transposons (reviewed in [50, 77, 84]) can be used as insertional mutagens in plants.

Insertional mutagenesis using Agrobacterium mediated T-DNA integration into plant genomes (primarily in A. thaliana, and more recently in rice) has proved to be very successful [2, 34, 42]. This approach has the advantage of simplicity as each transformant yields a stable insertion in the genome and does not need additional steps to stabilize the insert. Several groups have used this approach in A. thaliana to generate tens of thousands of independent lines that can be used for reverse genetics. Recently, a National A. thaliana Knockout Facility has been established at the Univer-
sity of Wisconsin, USA, with access to 60 480 insertion lines [42]. Modified T-DNA insertions have been used in A. thaliana as gene [3], promoter traps [46] and in activation tagging [85]. Recently, Jeon et al. [34] have also been using T-DNA insertions for functional genomics in rice. Despite the extremely successful use of T-DNA in A. thaliana, there are however a few disadvantages to this approach. The integration of the T-DNA is generally complex, resulting in tandem direct and inverted repeats and deletions in one or more borders. Such rearrangements can make the subsequent molecular analysis difficult in many cases, and adversely affect the success of large-scale strategies such as flanking sequence databases (described later). Secondly, complex and multiple insertions are more likely to lead to artefactual patterns of reporter gene expression when using entrapment vectors such as gene and enhancer traps. Finally, while the T-DNA approach is extremely useful for plant species where quick and efficient transformation methods are available, it may not be feasible in those plant species where the transformation methods are slow or labour intensive.

For these reasons, insertional mutagenesis using transposable elements offers some advantages over T-DNA mutagenesis. The insertions generated by transposons are generally single intact elements, which lend themselves easily to molecular analysis. Such insertions are also less likely to result in artefactual patterns of expression if the transposon is being used as a gene trap or enhancer trap. An additional advantage is that many transposons can be excised from the disrupted gene in the presence of transposase. Such excisions can result in phenotypic reversion to the wild type or give rise to alleles with weaker phenotypes. This property of many transposons provides ready confirmation that the mutation was really tagged by the transposon, as well as the possibility of generating an allelic series. In addition, another property of several transposons to preferentially insert into genetically linked sites [7, 33, 36], can be used to perform local mutagenesis in a particular region of interest by re-mobilizing the transposon [31, 35, 69]. Finally, transposons can be used for insertional mutagenesis in plant species where transformation is inefficient, since the generation of new insertions occurs through crossing or propagation rather than through transformation.

In this review, we discuss the most widely used transposons and how these elements have been exploited successfully in heterologous plant species as an insertional mutagen and as a tool for ‘reverse genetics’ for functional genomics.

| Table I. Endogenous transposons in different plants. |
|-----------------|-----------------|-----------------|-----------------|
| Element | Plant | Similar to | Reference |
| Activator, Ac | Maize | – | [15, 16, 52] |
| Tam3 | Antirrhinum majus | Ac of maize | [14, 28] |
| Tph1 | Petunia hybrida | Ac of maize | [23] |
| Tgl1 | Arabidopsis thaliana | Distantly related to Ac of maize | [19, 79] |
| Slide | Tobacco | Ac of maize | [25] |
| SpmlEn | Maize | – | [53, 63, 64, 65] |
| Tam1 | A. majus | SpmlEn | [58] |
| Tnr3 | Rice | SpmlEn | [56] |
| Psl | P. hybrida | SpmlEn | [72] |
| Mutator | Maize | – | [67] |

2. ENDOGENOUS TRANPOSABLE ELEMENTS IN DIFFERENT PLANT SPECIES

Transposable elements like Activator (Ac), Suppressor-mutator/Enhancer (SpmlEn) and Mutator (Mu) were originally discovered and molecularly characterized in maize (table I [15, 16, 52, 53, 63, 64, 67]). Subsequently, a number of endogenous transposable elements in other plant species that are similar or distantly related to the maize Ac element have been identified including Tam3 in Antirrhinum majus [14, 28], Tag1 in A. thaliana ecotype Landsberg [79] and slide1 in tobacco [25]. Also SpmlEn-like transposable elements have been reported in A. majus (Tam1 [58]), rice (Tnr3 [56]) and in Petunia (Psl [72]).

Endogenous transposable elements have been used to clone genes in maize, A. majus and Petunia hybrida. In maize, all three families (Ac, Spm and Mu) of transposable elements have been widely used as ‘tags’ to isolate genes (reviewed in [24, 83]). The Tam elements from A. majus (reviewed in [24]) and the dTph elements from P. hybrida [73] have proved similarly valuable in their host species. Some families of endogenous elements are present in high-copy numbers in their hosts. For example elements of the Mu family in maize and dTph family in P. hybrida exist in more than 100 copies per genome. This is very advantageous for large-scale mutagenesis, as a few thousand lines will be sufficient to cover the whole genome. On the other hand, there are a few disadvantages in using high-copy number endogenous transposons. First, since there is a number of insertions per line, there will be a continuous transposition of elements. If there was a significant frequency of germinal excisions, this would result in mutations that are due to the excision footprints, and therefore not tagged.
Secondly, confirmation that a mutation is indeed caused by a particular copy of the transposon may require several out-crosses to remove other elements, which is a time consuming process. Finally, the use of gene/enhancer trap elements is not feasible, as the patterns of expression due to several insertions of the elements per plant will be additive and therefore inaccurate.

One of the most successfully used high-copy number transposon families is the \textit{Mu} transposable element family consisting of the autonomous element \textit{MuDR} and the non-autonomous elements \textit{Mu1} to \textit{Mu8} (reviewed in [83]). These elements have been used to clone several genes in maize [10]. The \textit{Mu} elements have been particularly effective tool for gene tagging for the following reasons: (a) higher forward mutation frequency when compared to \textit{Ac} or \textit{Spm} element (20 to 50 \% higher) (reviewed in [83, 84]); (b) equal transposition to linked and unlinked sites when compared to other transposable elements [47]; (c) characteristically late excisions which stabilizes new alleles in sibling progeny and reduces the probability of foot-print mutations. However, transfer of the \textit{Mu} system into heterologous plant species has not yet been successfully accomplished.

Retrotransposons, which transpose through a RNA intermediate, have been shown to generate spontaneous mutations in maize [82] and are the major class of transposable elements in most plants (reviewed in [11]). Retrotransposons are potentially very useful for gene tagging as these elements generate stable insertions, and unlike many DNA transposons they integrate into unlinked sites. However, the relatively low frequencies of transpositions of most plant retrotransposons have restricted their uses for large-scale gene tagging. Recently however, the \textit{Tos17} retrotransposon has been developed as a promising system for rice [29]. The endogenous rice \textit{Tos17} retrotransposons appear to be inactive during normal growth conditions, but they are reactivated by tissue culture, resulting in high transposition frequencies suitable for insertional mutagenesis [29].

### 3. TRANSPOSONS IN HETEROLOGOUS SYSTEMS

The first report that maize transposable element \textit{Ac/Ds} can be active in a heterologous system came from studies with transgenic tobacco (\textit{Nicotiana tabacum}) by Baker et al. [4, 5]. Since then, the maize \textit{Ac/Ds} elements have been shown to transpose actively and been exploited in tagging studies in a number of heterologous species including \textit{A. thaliana}, rice, tomato, \textit{P. hybrida}, flax, carrot, lettuce, potato, etc. (table II). Similarly maize \textit{Spm/En} (\textit{Enfl}) has been utilized successfully in tobacco, potato and \textit{A. thaliana} (table II). Apart from maize transposable elements, the \textit{A. thaliana} transposon \textit{Tag1} which is distantly related to the maize \textit{Ac}, has been shown to be active in tobacco [19] and in rice [48].

In order to better regulate the transpositional events and obtain stable insertions, two-component systems are preferred. In a typical two-component system, a transgenic line will be generated which harbours an immobilized (‘wings clipped’) autonomous element (\textit{Ac} or \textit{Spm/En}) that will provide the transposase source. Second line transgenic for the non-autonomous element (\textit{Ds} or \textit{dSpm/En}), which cannot transpose unless the transposase source is available, will be generated. Selectable markers such as antibiotic resistance or herbicide resistance markers can be engineered in the non-autonomous elements to select for the presence of transposed elements. In order to monitor the transposition events, the non-autonomous transposon can be inserted between a promoter and the marker gene so that excision results in expression of the selectable marker gene (reviewed in [76]). Plants homozygous for transposase and for the dependent element are used as starter lines and crossed to facilitate transposition. Lines carrying stable single insertions can be obtained subsequently by segregation of the transposase source, which can be simplified by the use of negative selection markers [76] (figure 1B).

The ability of \textit{Spm/En} elements to amplify through continued propagation in \textit{A. thaliana} has been exploited to increase the number of insertions per plant [74, 88].

<table>
<thead>
<tr>
<th>Plant of origin</th>
<th>Transposon type</th>
<th>Activity in heterologous plants</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>\textit{Ac/Ds}</td>
<td>\textit{A. thaliana}</td>
<td>[7, 81]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rice</td>
<td>[32, 57, 70]</td>
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<tr>
<td></td>
<td></td>
<td>Tomato</td>
<td>[35, 54, 90]</td>
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<tr>
<td></td>
<td></td>
<td>\textit{Petunia}</td>
<td>[13, 26, 66]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flax</td>
<td>[17, 44]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tobacco</td>
<td>[5, 18, 86]</td>
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<tr>
<td></td>
<td></td>
<td>Carrot</td>
<td>[81]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lettuce</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Potato</td>
<td>[40]</td>
</tr>
<tr>
<td>Maize</td>
<td>\textit{Spm/En}</td>
<td>\textit{A. thaliana}</td>
<td>[1, 12]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Potato</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tobacco</td>
<td>[51, 62]</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>\textit{Tag1}</td>
<td>Rice</td>
<td>[48]</td>
</tr>
</tbody>
</table>

Reference Table II. Transposons activity in heterologous plants.
In one strategy, the autonomous Spm/En element was transformed into A. thaliana and propagated for five generations by a single seed descent. This yielded 15,000 insertions in 3,000 lines [88]. Alternatively, a two component (in-cis) En-I (Spm/dSpm) system has been introduced into A. thaliana, where a mobile I (dSpm) element and an immobilized trans-active En (Spm) transposase are contained in the same T-DNA. This in-cis element system was used to generate 2,592 A. thaliana lines containing multiple insertions (approx. twenty inserts/line) of mobile I element [74]. This approach of amplifying the number of insertions per plant has the very significant advantage that it permits near-saturation mutagenesis of a plant genome with a relatively small number of plants. However, there are also some disadvantages in using this approach, arising from the continuous nature of the transposition events. First, the PCR strategies to identify a gene knockout are complicated by somatic insertion events that will result in the detection of insertions which are not transmitted through the germ line. Second, the presence of footprints in genes due to imprecise excisions will lead to mutations that are not tagged.

Most transposable elements, including Ac/Ds and Enl/Spm, have a tendency to preferentially transpose to genetically linked sites [7, 33, 36]. This feature can be advantageous for directed tagging of a specific target gene or for performing local (regional) insertional mutagenesis in a selected region of a chromosome when a transposable element is inserted close to the target gene or within the derived chromosomal region (figure 1A) [31, 35, 69]. In another instance, Ac/Ds transposons and cDNA scanning methods were used together to perform regional insertional mutagenesis on genes from CIC7E11/8B11 and 5CIC5F11/CIC2B9 loci on A. thaliana chromosome V [31, 69]. This allows cloning of cDNAs from a small region in the genome. The flanking sequences of insertions showed that 14–20% of the transpositions were located in about 1 Mb of genomic DNA surrounding Ds donor sites.

When random saturation mutagenesis is desired, the high fraction of closely linked site transpositions poses a serious limitation. In principle, this limitation can be overcome by using many starter lines, with Ds/dSpm insertions distributed evenly over different chromosomes. Alternatively, in the Ac-Ds system of Sundaresan et al. [77], the propensity of Ds elements to transpose to linked sites is overcome by selection against the donor site using a negative selection marker. By simultaneous selection against the transposase source and the donor site, a population of stable, unlinked, transposon insertion lines can be generated (figure 1B) [61, 77]. In the modified Spm/En system designed by Tissier et al. [78], the Spm transposase and the mobile dSpm elements are contained within the same T-DNA transformed into A. thaliana. This system eliminates the need for crossing, and also reduces the number of progeny required for the selection by a factor of four, as the negative selection is applied against only a single locus as opposed to two loci when the transposase is introduced separately. However, the maintenance of starter lines becomes problematic, as the dSpm elements will continually transpose in the pres-

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**Figure 1.** Schematic diagram of different approaches to transposon tagging. In both A and B, the Ds element is mobilized in the presence of Ac transposase. A, Directed tagging; B, random tagging. 1, 2 and 3 represent different chromosomes, and X, Y, Z different genes. C, Insertion of gene trap or enhancer trap element. SA, splice acceptor; TA, minimal promoter; E, enhancer sequence in the genome; X, unknown gene and GUS, glucuronidase. D, Insertion of activation tagging element. X denotes an unknown gene. TE, transposable element; LB, left border of T-DNA; RB, right border of T-DNA; PSM, positive selection marker; NSM, negative selection marker.
4. SPECIALIZED TAGGING SYSTEMS

4.1. Gene and enhancer trap elements

Insertional mutagenesis by transposon tagging is useful when disruption of a gene leads to an obvious phenotype. But in eukaryotic systems, disruptions of genes frequently do not result in visible phenotypes due to functional redundancy, or they may result in early lethality that obscures late-acting functions when the same gene has multiple functions in development. To overcome these difficulties, modified transposons called gene trap or enhancer trap transposable elements have been developed (reviewed in [43, 75, 76]). Such modified elements were first used in Drosophila melanogaster and mouse [71, 87], and were subsequently extended to plant transposon systems [75–77].

Gene/enhancer trap elements carry a reporter gene whose expression pattern reflects the activity and regulation of the disrupted genes (figure 1C). The enhancer traps contain a reporter gene driven by a minimal or weak promoter in the dependent element. The reporter gene expression is achieved (by utilizing the endogenous enhancer sequences) only when the transposition occurs within or close to a gene (figure 1C). A variation of this vector called a promoter trap contains a promoterless reporter gene, which will express only when the transposon is inserted downstream of an active endogenous plant promoter. In contrast, gene trap vectors are designed to contain one or more splice acceptor sequences preceding the reporter gene. This allows expression of the reporter only when it inserts in a transcribed region. The splice donor sites from the intron of the endogenous gene and the splice acceptor sequences, which precede the reporter gene, will be spliced to generate a fusion transcript (reviewed in [50, 75, 76]). Thus far only the Ac/Ds transposon system has been modified for enhancer and gene trap approaches.

4.2. Activation tagging elements

Gene disruptions through insertional mutagenesis nearly always generate recessive loss-of-function mutations. Such mutations do not always produce an obvious phenotype due to various factors such as functional redundancy (see next section). In such cases, increasing the expression level or ectopically expressing a gene can provide dominant gain-of-function mutations that produce informative mutant phenotypes. A strategy to generate mutants as a consequence of increased or expanded expression of a tagged gene is known as ‘activation tagging’. The principle involves using strong enhancers in T-DNA or a transposon resulting in ectopic expression or over-expression of nearby genes through transcriptional activation. The first successful activation tagging in plants was reported by Hayashi et al. [27], who introduced four copies of enhancer elements from the cauliflower mosaic virus 35S promoter (CaMV 35S) into T-DNA, generating gain-of-function mutations and novel phenotypes. This approach has been used to generate a large-scale tagging population by Wiegel et al. [85]. The principle of activation tagging has also been adapted for transposable elements by Wilson et al. [87]. They used the Ds element with a complete CaMV 35S promoter pointing outwards from the element (figure 1D), and generated a population of insertions through crosses with Ac transposase. This approach was used to identify dominant mutations at various loci in A. thaliana, including TINY, Late Elongated Hypocotyl (LHY), and Short Internodes (SHI) [22, 68, 87]. A limitation of this approach is that the observed frequency of dominant mutations through activation tagging has been significantly lower than that of recessive mutations arising from insertional inactivation [85], suggesting that many genes may be over-expressed without resulting in observable phenotypes. Nevertheless, activation tagging has proven to be a valuable complementary approach for the identification of gene functions.

5. FORWARD VS. REVERSE GENETICS

Cloning of genes that produce mutant phenotype or function forms the basis of ‘forward genetics’ (i.e. phenotype or function to gene). On the other hand, if the gene sequence is known and the biological function of that gene is not known, a knockout mutant can be generated and analysed to determine its function. This forms the basis for ‘reverse genetics’, i.e. from gene to phenotype or function (reviewed in [42, 50, 60]). Since genome sequencing projects for various plant species are progressing rapidly, more and more sequences encoding predicted genes are available in the databases. Reverse genetics strategies will be of great importance for the purpose of assigning functions to predicted genes. As discussed earlier, gene disruption by transposons constitutes a powerful tool for reverse genetics.
6. STRATEGIES FOR REVERSE GENETICS SCREENING

In order to perform reverse genetic screens efficiently, it is necessary to generate a large population of transposon tagged mutants. The number of lines to be screened is dependent on the genome size and the number of genes of a given plant species, the type of transposon used (single or multi-copy) etc. A population containing insertions with a reasonable probability of finding at least one insertion in any given gene is important for the success of this approach.

In order to screen for an insertion in a particular gene, a PCR-based strategy has been applied in D. melanogaster for site-specific selection of insertions using P elements [6, 38]. In this approach, a gene-specific primer and an insertion-specific primer are used for PCR amplification. Several insertion lines are pooled together and the DNA extracted is then used as a template for PCR reactions. Samples of 20 to 100 insertion lines are pooled to extract genomic DNA, and a gene-specific primer and an insertion-specific primer were used for PCR [55, 78]. Any pool showing a positive signal is re-screened using DNA from individual lines, to identify the line carrying the insertion of interest. Several pools can be combined to form a super pool if the number of insertion lines in the population is very high [78]. Alternatively, a three-dimensional matrix pooling strategy has been tested for P. hybrida lines carrying the multi-copy dTph1 transposon [41]. The leaf material from a population of 1000 plants were pooled according to a three-dimensional matrix (columns, rows and blocks). DNA samples were extracted from ten blocks of 100 lines. Similarly DNA is extracted from ten columns and ten rows each of them containing 100 lines. Altogether thirty DNA samples were used to perform PCR, which can identify a single plant that contains the insertion in the specific gene. This method is advantageous as it requires fewer amplifications, and directly identifies the single plant with insertion (figure 2 [41]).

An alternate approach to identify the genes that have been tagged in a population of insertion lines involves random amplification of the DNA flanking the insertions. Several different methods can be followed within this approach. Transposon display [80] and amplification of insertion mutagenized sites (AIMS) [20] are techniques used to identify tagged genes in families with multi-copy transposons like dTph1 in Petunia and Mu in maize. For example, the maize Bx1 gene and the Fbp1 gene fragment from Petunia were isolated by this method [20, 80]. The transposon display and AIMS techniques utilize adapters that are ligated to the genomic DNA, which has been digested with restriction enzyme(s). The appropriate restriction enzyme(s) are selected which recognizes one site in the insertion element and the other in the flanking region. A chimeric adapter and transposon primer is used from one end and an adapter primer from the other end to amplify the flanking region in the transposon display technique. On the other hand, adapter primers are used as forward and reverse primers for amplification in AIMS approach. Flanking regions obtained by amplifications are displayed which can be cloned and sequenced subsequently ([20, 80] and reviewed in [50]).

A method called inverse display of insertions (IDI) has been developed by Tissier et al. [78] to isolate the flanking sequences spanning the dSpm1 element. The inverse PCR (iPCR) method [59] has been adapted for

![Figure 2. Flow chart for the use of insertional mutants in a reverse genetics approach to functional genomics. The steps involved from the identification of an insertion in a gene of interest to the phenotypic characterization of the mutant are detailed.](image-url)
this strategy. Since the selection of restriction sites (not too close or too far from the insertion site) is a critical factor for a successful iPCR, three different restriction enzymes were used to greatly increase the probability of amplifying the flanking region. DNA pools from insertion lines were used as template and iPCR reactions were performed. These products were mixed and spotted in grid arrays on a nylon membrane, and hybridized with labelled probes from the genes of interest [78].

Alternatively, the DNA flanking the insertions can be amplified and sequenced individually to catalogue the insertions by chromosomal location [61, 78]. This strategy is especially useful when substantial genome sequence information is available, as in the case of A. thaliana. A protocol such as iPCR, ligation-mediated PCR or thermal asymmetric interlaced PCR (TAIL-PCR; [49]) can be used to amplify and sequence the flanking region of single- or low-copy insertion lines. With an appropriately constructed database of such flanking sequences, it will be feasible to identify insertions in a particular gene by simple computer searches, removing the necessity for the more tedious pooling and hybridization protocols. In addition, even if no ‘hit’ is found, the availability of a transposon insertion close to a gene of interest can be quickly ascertained by a computer search. As described previously, transposons from the Ac/Ds and Spm/En families are very useful for mutagenesis of closely linked genes, and a collection of sequenced insertions provides launch pads for tagging most of the genes within the genome.

7. GENE SEQUENCE TO FUNCTIONAL ANALYSIS

Once a knockout in a desired gene is identified through reverse genetics strategies among the population of insertional mutants, it becomes necessary to functionally characterize the mutant (figure 2). The first step in the characterization process is to obtain a mutant that has a single insertion in the gene of interest. If a two-component transposable element system (Ac/Ds for example) has been used in generating a population of insertion mutants, then obtaining single insertion line is rather straightforward. On the other hand, if multi-copy transposable elements are used to perform the reverse genetics screen, then it is necessary to do several out-crosses to make sure that only a single insertion is in the gene. The next step for characterization is the identification of phenotypes caused by gene knockout. If there is an observable phenotype caused by the single stable insertion, then the gene functions may be deduced through detailed analysis of the phenotype (figure 2). However, there are many instances, where a gene knockout does not show an observable phenotype. This could be due to the fact that several genes may be required and expressed only under specific conditions, such as pathogen infection or environmental stresses. For mutations in such genes, in order to detect a phenotype it will be necessary to subject the plants to conditions in which the gene is required. These conditions may include challenges with pathogens and the use of specific growth media or growth conditions. For example, a T-DNA insertion in the AKT-1 potassium channel gene of A. thaliana was identified by a reverse genetics approach using pooled PCR and hybridization. On most nutritional media, the akt-1 mutant plants were indistinguishable from wild type plants, but on media containing a low potassium concentration of 100 µM or less, the growth of the mutant plants was defective [30].

Another reason for not observing a clear phenotype when a gene is knocked out is functional redundancy with other genes. In such cases, creation of double or triple mutants of the functionally redundant genes will uncover the phenotype and permit characterization of their functions (figure 2). For example, A. thaliana genes ‘SHATTERPROOF1’ (SHP1) and ‘SHATTERPROOF2’ (SHP2) regulate fruit dehiscence or pod shatter. Both genes are functionally redundant, as neither single mutant produces a novel phenotype. However, shp1 and shp2 double mutants produce fruits or pods that do not shatter [45]. Since the A. thaliana genome-sequencing project is almost complete and the sequences are available, it is feasible to identify all the closely related members of a gene family in this species. Together with computer searches of flanking sequence databases, it will soon be possible to construct combinations of mutants to reveal novel functions that have gone undetected using the current genetics methodologies.

8. CONCLUSION

In the post-genome era, sequences of many plant genomes will be available and functional assessment of the genes identified will depend on many approaches including insertional mutagenesis, polymorphism analysis, expression microarrays, and bioinformatics. A large population of insertional mutants generated by transposon mutagenesis can be used to dissect out
gene functions in combination with the sequences obtained from the EST and genome sequencing projects in many plant species, through various strategies such as pooled PCR screening and the construction of flanking sequence databases. Recent advances demonstrate the feasibility of using transposon-based functional genomics approaches for the study of any species of flowering plant.

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