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A transposon-based activation-tagging population in *Arabidopsis thaliana* (TAMARA) and its application in the identification of dominant developmental and metabolic mutations

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Abstract A population of 9471 stable activation-tagged lines was generated by transposable element mediated activation tagging mutagenesis in Arabidopsis (TAMARA) using the maize *En/Spm* transposon system. Based on DNA gel blot and flanking sequence analysis, this population contains approximately 6000 independent transposon insertions. A greenhouse-based screen identified six dominant or semi-dominant activation tagged mutants with obvious developmental alterations, among these a new *pistillata* mutant allele. In addition, a subset of 1500 lines was screened by a HPLC based high-throughput method for dominant activation tagged mutants with enhanced contents of phenolic compounds. One dominant activation tagged mutant (*hpc1-1D*) was isolated showing accumulation of a particular compound due to the upregulation of an R2R3-MYB transcription factor.

Keywords: Transposon; Activation tagging; Dominant mutation; Shoot development; Phenylpropanoid metabolism

1. Introduction

The screening for loss-of-function mutants has been a primary tool for dissecting genetic pathways in many organisms, including Arabidopsis. However, a limitation of this approach is that redundantly acting genes can hardly be identified. Sequencing of the Arabidopsis genome revealed that about 70% of the genome is made up of duplicated loci [1]. To overcome this type of genetic redundancy, gain-of-function mutants can be studied. Gain-of-function phenotypes can either be caused by mutations in the coding region that lead to constitutive activation of the corresponding protein, as is the case for dominant ethylene response mutants [2] or by activation tagging that leads to altered levels or patterns of gene expression.

A transfer DNA (T-DNA) vector, that possesses four copies of an enhancer element of the constitutively active promoter of the Cauliflower mosaic virus (CaMV) 35S gene [3] was constructed by Walden and colleagues [4]. These enhancers can cause transcriptional activation of adjacent genes, and, because activated genes will be associated with a T-DNA insertion, the method is called activation tagging. In Arabidopsis, large collections of such T-DNA lines have been described [5,6], which contain numerous gain-of-function alleles.

T-DNA insertions, however, are often complex and characterized by multiple inverted or tandem copies or truncated T-DNA inserts, which often make a molecular analysis difficult [7,8]. One possibility to overcome this problem would be the use of single copy transposon insertions as the activation tags. In addition, transposon insertions can be remobilized germinally, thereby producing revertants that can confirm the phenotypic consequences of the insertion.

The enhancer–inhibitor (*En–I*), also known as suppressor–mutator (*Spm–dSpm*), system of maize is an efficient tool for heterologous transposon tagging in Arabidopsis [9–13]. *En/Spm* elements transpose at a high frequency to unlinked locations, without a known bias towards specific regions in the genome.

The use of positive and negative selectable markers has further improved transposon technology [12,13]. The system makes use of the positive selectable marker *bar* [14] conferring resistance to the herbicide Basta, as well as the negative selectable marker *SU1* [15] that converts the pro-herbicide R7402 (DuPont) into the herbicide sulfonylurea. Both markers are carried on a single construct: the *bar* gene is placed inside the nonautonomous *I/dSpm* element, the *SU1* gene and an immobile source of transposase (an *En/Spm* element that lacks the terminal-inverted repeats) in the adjacent T-DNA. Application of R7402 allows selection against the transposase source and subsequent application of Basta allows selection for stable transposed *I/dSpm* elements [12,13]. If, in addition, a tetramer of the CaMV 35S enhancer is placed inside the *I/dSpm* element, the system can also be used to generate activation tagged inserts [13].

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Abbreviations: HPLC, high performance liquid chromatography; T-DNA, transfer DNA; CaMV, Cauliflower mosaic virus; *En–I*, enhancer–inhibitor; *Spm–dSpm*, suppressor–mutator

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In the present study, we have used the two-element transposon system to generate an activation tagged population in Arabidopsis (transposable element mediated activation tagging mutagenesis in Arabidopsis, TAMARA), which will become publicly available through the Nottingham Arabidopsis Stock Centre. This population was used to identify and isolate new Arabidopsis gain-of-function alleles affected in shoot development. In addition, an high performance liquid chromatography (HPLC)-based screening was established to identify activation tagged mutants with altered contents of phenolic substances. A first characterization of such a mutant (*hpc1-1D*) is described.

2. Materials and methods

2.1. TAMARA construct

The counterselectable marker gene *SSU-SU1* was isolated from the plasmid SLJ8241 [12,15] as a *Eco*RI + *Bam*HI fragment and ligated into the T-DNA vector pGPTV [16]. Replacement of the *Eco*RI site by an *AscI site* resulted in plasmid pGPTV-SSU. Plasmid SLJ7713 [12] was digested with *Sma*I + *Xho*I to isolate the transposase gene, which was then inserted between *Bam*HI and *Xho*I sites of pRT-Ω/Not/Asc [17] to yield pRT-TNP. Subsequently, the transposase cassette with the 35S promoter- and terminator sequences was excised from pRT-TNP using the flanking *Asc*I.

The Basta resistance gene *bar* was obtained from vector SLJ0512 [12] as *Bcl*I fragment and transferred into the *Pst*I inside the *dSpm* element in SLJ7648 [12]. The resulting intermediate was digested with *Hind*III to insert the 35S enhancer tetramer isolated as a *Bam*HI and *Bgl*II fragment from pPVICEn4HPT [4]. The final *dSpm* element was excised with *Cla*I and cloned into the *Bam*HI site of pGPTV-SSU after fill-in reactions. To complete construction of the TAMARA plasmid, the *Asc*I fragment from pRT-TNP was inserted into the *Asc*I site of pGPTV-SSU. The TAMARA construct was introduced into wild-type plants (*Arabidopsis thaliana* ecotype Col-0) by vacuum infiltration [18] using *Agrobacterium tumefaciens* strain GV3101.

2.2. Generation of stable activation tagged lines (TAMARA population)

For amplification of TAMARA starter lines, the progeny (T_2) of primary transformants (Basta resistant T_1 plants) were sown at a density of 500 plants per tray (45 × 30 cm). Only lines, which segregated approximately 75% Basta resistant seedlings, were selected as starter lines. Seeds from individual starter lines were harvested as a pool (T_3). To select for transposition events, T_3 plants were sown on soil in a temperature-controlled greenhouse at a density of 2500 plants per tray under a 16 h light/8 h dark cycle. Seedlings were sprayed with a solution of 75 μg L^{-1} R7402 (DuPont) 6 days after germination, and 14 days after germination with a solution containing 150 μg L^{-1} R7402 and 0.5 mL L^{-1} Basta (Hoechst, which contains 200 g L^{-1} glufosinate ammonium). Double resistant plants (transposants) were transferred to 77 well trays to score for developmental phenotypes and to collect seeds of single plants (T_4). In total, seeds of 9471 single plants were obtained and represent the TAMARA population.

2.3. Isolation of genomic DNA, Southern hybridisation and isolation of dSpm-Act flanking sequences

Genomic DNA was isolated as described previously [19]. For Southern blot analysis, about 10 μg of genomic DNA was digested with *Pst*I or *Eco*RI, separated by electrophoresis on 0.8% TBE agarose gels and blotted onto Hybond N^+ nylon membrane (Amersham). For hybridisation, fragments specific for the *bar* gene (1.6 kb *Bam*HI/*Bcl*I from SLJ0512) or the 5′ *dSpm* element (1 kb *Pst*I/*Cla*I from SLJ7648) were labelled in the presence of [α-^{32}P]-dCTP. Isolation of *dSpm*-Act flanking sequences was done as described previously [20] or by inverse PCR. For inverse PCR, genomic DNA (0.5 μg) was digested with four-cutting enzymes. Fragments were phenol/chloroform extracted and circularized overnight at 16 °C in a total volume of 300 μL with 5 U of T4 DNA ligase. After phenol/chloroform extraction, the ligation mix was resuspended in 10 μL of H_2O. PCR was performed in a total volume of 25 μL containing 1 μL of the above solution, 0.2 U Biotherm polymerase, and 50 pmol of each primer. PCR conditions were: 5 min, 94 °C; 40× [30 s, 94 °C; 45 s, 60 °C; 2 min, 72 °C]; 5 min, 72 °C. Flanking sequences were obtained with primer combinations as follows: 3′*dSpm* – for digestions with Sau3a I or Asn I: 5′-AGTCCATACAAAACGCAATCATAG-3′ and 5′-CTTAGAGTGTCGGCTTATTTCAGT-3′, for Rsa I: 5′-GGACCGACGCTCTTATGTTAAAAG-3′ and 5′-CAGTAAGAGTGTGGGGTTTTGG-3′; 5′*dSpm*-digestions with Sau3a I or Asn I: 5′-GCACGACGGCTGTAGAATAGG-3′ and 5′-CAAGAAGTCAAAACGCTATGTGG-3′, Rsa I: 5′-GCACGACGGCTGTAGAATAGG-3′ and 5′-CGCGCACCTCCAAGTAGC-3′. The resulting PCR products were subcloned into pCR II-TOPO (In Vitrogen). Flanking sequences were compared to the Arabidopsis genome database using the BLAST algorithm [21] at http://www.ncbi.nlm.nih.gov/ to determine the position and orientation of the *dSpm* insertion.

2.4. Extraction of metabolites and HPLC conditions

For the identification of metabolic mutants, a set of 1500 lines of the TAMARA population was screened by HPLC. Approximately 20 seeds per individual T_4 line were grown in a temperature controlled greenhouse and sprayed 7 and 10 days after germination with 1.25 mL L^{-1} Basta (Hoechst). 50 mg of leaf material was collected and extracted repeatedly with methanol. Phenolic constituents were analyzed by a HPLC system as described previously [22], consisting of a photodiode array detector (Waters, Eschborn, Germany) combined with a fluorescence detector (FP-920, JASCO, Groß-Umstadt, Germany). Data acquisition and processing were performed with the Millenium software (Waters, Eschborn, Germany). Spectra from 250 to 400 nm were recorded and chromatograms of absorbance at 280 nm were extracted from the data sets. The wavelength settings for the fluorescence detector were 300 nm for excitation and 400 nm for emission. The identification of major peaks in the UV chromatograms (280 nm) in methanolic extracts of Arabidopsis leaves was done by comparing their retention time and UV light spectra (250–400 nm) with those of known standards.

2.5. Expression analysis

Total RNA from mutant and wild-type leaves was isolated using the RNeasy Plant Kit (Qiagen). Oligo (dT)-primed cDNA from 1 μg of total RNA was synthesized using the SuperScript Reverse Transcriptase system (InVitrogen). 2 μl of the total Reverse Transcriptase reaction was used to perform PCR with gene-specific primers. For the *MYB 51* gene, primers used were 5′-GTGTTGCAAAGCTGAACTAGGGTT-3′ and 5′-TTGTTAACGGAGGAATCAGAG-AAC-3′; for *At1g18560*, primers were 5′-TGTGCTTGATTGGTGGAAGGTAA-3′ and 5′-TTCTAAACCTCCGGCAGAATTATC-3′ and the primers used for *ACTIN 1* were 5′-TAACTCTCCCGCTATGTATGTCGC-3′ and 5′-CCACTGAGCACAATGTTACCGTAC-3′. PCR conditions were 2 min, 94 °C; 26 or 40 cycles of [30 s, 94 °C; 45 s, 60 °C; 2 min, 72 °C]; 5 min, 72 °C.

2.6. Construction of 35S-MYB51 plants

The full-length cDNA for *MYB51* was amplified using 5′-CACCATGGTGCGGACACCGTGTTGCAAAGC-3′ and 5′-TCATCCAAAA-TAGTTATCAATTTCGTC-3′ as described above and subcloned into pENTR/D TOPO vector (InVitrogene). Subsequently, the cDNA was transferred into the GATEWAY destination vector pGWB2 by an LR Clonase reaction (InVitrogene). The pGWB2 vector containing the CAMV 35S promotor, was provided by Dr. Tuyoshi Nakagawa (Shimane University, Japan). The 35S-MYB51 construct was introduced into wild-type plants (*A. thaliana* ecotype Col-0) by vacuum infiltration [18] using *Agrobacterium tumefaciens* strain GV3101. Of the resulting three Kanamycin resistant lines, one line overexpressed the *MYB51* gene and was further investigated by HPLC analysis.

3. Results

3.1. Generation and establishment of an activation tagged population (TAMARA)

All elements required for the TAMARA system were integrated into a single T-DNA and introduced into Arabidopsis