Measuring Arabidopsis Chromatin Accessibility Using DNase I-Polymerase Chain Reaction and DNase I-Chip Assays

Huan Shu, Wilhelm Gruissem, and Lars Hennig*

Department of Biology and Zurich-Basel Plant Science Center, Eidgenössisch Technische Hochschule Zurich, CH-8092 Zurich, Switzerland (H.S., W.G., L.H.); Functional Genomics Center Zurich, University of Zurich/Eidgenössisch Technische Hochschule Zurich, CH–8057 Zurich, Switzerland (W.G.); Department of Plant Biology and Forest Genetics, Uppsala BioCenter, Swedish University of Agricultural Sciences and Linnean Center for Plant Biology, SE–75007 Uppsala, Sweden (L.H.); and Science for Life Laboratory, SE–75007 Uppsala, Sweden (L.H.)

ORCID IDs: 0000-0002-3395-2409 (H.S.); 0000-0002-6645-1862 (L.H.).

DNA accessibility is an important layer of regulation of DNA-dependent processes. Methods that measure DNA accessibility at local and genome-wide scales have facilitated a rapid increase in the knowledge of chromatin architecture in animal and yeast systems. In contrast, much less is known about chromatin organization in plants. We developed a robust DNase I-polymerase chain reaction (PCR) protocol for the model plant Arabidopsis (Arabidopsis thaliana). DNA accessibility is probed by digesting nuclei with a gradient of DNase I followed by locus-specific PCR. The reduction in PCR product formation along the gradient of increasing DNase I concentrations is used to determine the accessibility of the chromatin DNA. We explain a strategy to calculate the decay constant of such signal reduction as a function of increasing DNase I concentration. This allows describing DNA accessibility using a single variable: the decay constant. We also used the protocol together with AGRONOMICS1 DNA tiling microarrays to establish genome-wide DNase I sensitivity landscapes.

Chromatin has a major impact on genome organization and gene activity. Differential accessibility of DNA is thought to be a major consequence of locally different chromatin composition and structure (Li et al., 2007). Chromatin sensitivity to nucleases has proven to be a powerful tool to probe DNA accessibility in chromatin. Frequently used nucleases include DNase, micrococcal nuclease, and restriction enzymes. The resolution of restriction enzymes is limited by their sequence specificity, and micrococcal nuclease is more often used to determine nucleosome occupancy (Schones and Zhao, 2008). Chromatin sensitivity to DNase I has often been used to define the “openness” of chromatin relative to its higher order structures. Its applicability has been manifested by detecting regulatory elements, such as promoters, enhancers, and insulators, as DNase I-hypersensitive sites (Wang and Simpson, 2001; Crawford et al., 2004, 2006; Dorschner et al., 2004; Sabo et al., 2006; Boyle et al., 2008; Naughton et al., 2010; Pique-Regi et al., 2011). DNase I sensitivity can also be used as a measure for the general accessibility of chromatin (Weil et al., 2004).

Initially, the chromatin accessibility of local genomic regions to DNase I was probed by Southern blotting (Mather and Perry, 1983; Bender et al., 2000; Wang and Simpson, 2001; Bulger et al., 2003). However, Southern blotting is tedious and lacks sensitivity, and the interpretation of results can be challenging. Therefore, analysis methods based on PCR have been developed (Pfeifer and Riggs, 1991; Feng and Villeponteau, 1992; McArthur et al., 2001; Dorschner et al., 2004; Martins et al., 2007). In recent years, DNase I assays were coupled to high-throughput genome-wide profiling strategies such as genome tiling arrays and next-generation sequencing (Crawford et al., 2004, 2006; Sabo et al., 2004, 2006; Weil et al., 2004). While much has been learned about the accessibility of chromatin in animal and yeast systems, our knowledge of chromatin accessibility in plants is limited. Most studies have focused on selected genomic regions such as the general regulatory factor1 (GRF1) gene and the alcohol dehydrogenase1 (Adh1) and Adh2 genes in maize (Zea mays); Paul and Ferl, 1995; Paul and Ferl, 1998a, 1998b) or the GRF gene, the Adh gene, and an 80-kb genomic region harboring 30 protein-coding genes in Arabidopsis (Arabidopsis thaliana); Vega-Palas and Ferl, 1995; Paul and Ferl, 1998a, 1998b; Kodama et al., 2007). The technique used in these reports was exclusively DNase I treatment and analysis of accessibility using Southern blotting. More recently, we have combined
the DNase I sensitivity assay with whole-genome tiling arrays in Arabidopsis to generate a genome-wide chromatin accessibility profile (Shu et al., 2012).

Here, we present a robust, optimized DNase I sensitivity assay protocol for Arabidopsis tissues based on PCR. This protocol can be adapted to different samples or experimental objectives; the strategies for optimizing each step are also discussed. Analysis of relatively large fragments by PCR has proven to be highly robust as a first step in probing DNase I sensitivity in any region of the genome. We also introduce a new strategy for presenting the DNase I sensitivity of the tested regions using a decay constant calculated by fitting PCR product intensity values from a gradient digestion. In this way, the sensitivity of each region is characterized by a single value, facilitating comparisons between different regions or samples. Finally, we describe how our protocol can be combined with genomic techniques for genome-wide profiles of chromatin accessibility.

RESULTS AND DISCUSSION

DNase I PCR assays often monitor the digestion of nuclei over a time course (Feng and Villeponteau, 1992; Wang and Simpson, 2001) or in a gradient of enzyme concentrations (Vega-Palas and Ferl, 1995; McArthur et al., 2001; Martins et al., 2007). Here, we discuss the entire procedure in the following sections: (1) isolation of permeabilized nuclei; (2) DNase I digestion and DNA recovery; (3) analysis of chromatin sensitivity by PCR; and (4) data interpretation (Fig. 1).

Assays to probe chromatin accessibility should use freshly prepared nuclei. The time for isolating nuclei and the delay before digestion should be minimized to reduce unspecific damage to chromatin. The yield and reproducibility of nuclei extraction can be pretested by measuring the DNA content from identical or similar extractions. The key to reproducible DNase I sensitivity assays is to keep an invariable DNase-to-chromatin ratio between replicates. An optimal ratio has to be determined empirically for each tissue. The DNA recovered from such digests is a mixture of long and short fragments, depending on the accessibility of the chromatin. Samples must be handled with much care to minimize unspecific fragmentation. We recommend using large-pore pipette tips and gentle handling of nuclei and chromatin suspensions. Rigorous mixing (e.g. by using vortex mixers) must be avoided. To control for possible differential sensitivity of the DNA template, a digestion of isolated DNA can be included. Ideally, the DNA is extracted from material identical to the nuclei to be used in the experiment.

Isolation of Permeabilized Nuclei

Plant Material

Because cells in expanded green tissues usually contain large vacuoles, the nuclei-cell (w/w) ratio is much lower in older than in young tissues. In our experience, it is often more efficient to isolate nuclei from plant cell cultures, inflorescences, or young seedlings than from mature green tissues. Fresh samples can be used for extracting nuclei. However, it is often more practical to use frozen material. In this way, not only can plant material be stored almost infinitely at −80°C, but nuclei isolation can also be easily synchronized when multiple samples are involved.

Isolation of Nuclei

To minimize the time for nuclei isolation, we developed a one-incubation-step protocol. The short isolation step also favors a higher yield of nuclei. The entire isolation procedure should be carried out on ice or at 4°C.

Frozen plant tissue is ground to a fine powder in liquid nitrogen to disrupt cell walls and other fibrous structures. The disrupted plant tissue is then treated with a cell lysis buffer to release the nuclei. Cells are lysed by a surfactant in the buffer for disrupting membrane structures. A nonionic surfactant (e.g. Triton X-100 or Igepal CA 630) is normally used at concentrations between 0.3% and 1% (v/v) depending on the specific requirement of each experiment (Bowler et al., 2004; Wagschal et al., 2007). Normally, a 15-min treatment with cell lysis buffer containing 0.5% Triton X-100 is sufficient for most Arabidopsis green tissues. It is advisable to include Suc or hexylene glycol in the buffer (Bowler et al., 2004) to maintain a physiological osmotic pressure. Such an osmotic agent also increases the viscosity of the buffer system slightly and forms an

Figure 1. Outline of the DNase-PCR procedure.
additional protection for the nuclei. After cell lysis, the lysate is filtered through multiple layers of Miracloth (Calbiochem) or nylon mesh filters such as CellTrics (Partec) to remove cell debris. We recommend the CellTrics, as they guarantee a more reproducible nuclei yield. When Miracloth is used, the Miracloth filter should be presaturated with cell lysis buffer before filtering to minimize nuclei retention in the fabric. Furthermore, the Miracloth should not be squeezed, as this can lead to considerable organelle (and organelle DNA) contamination and, therefore, an unreliable nuclei yield estimation. After filtering, crude nuclei can be collected by mild centrifugation (for details, see “Materials and Methods”).

DNase I Digestion

DNase I time-course or gradient digestion can be used, but the latter can be more practical when taking advantage of multichannel pipettes and PCR cyclers for handling multiple samples. In this way, adding enzyme or stop buffer to the reaction mixture can be carried out in synchrony for all samples. PCR cyclers are robust instruments for providing precise temperature control for the incubation of the digestion reaction (Fig. 2). The procedure for the DNase I gradient digestion is detailed below, and steps for time course digestion need to be adjusted accordingly.

Before digestion, the crude nuclei are washed at least once with digestion buffer to remove the detergent carried over from the cell lysis buffer. Afterward, the nuclei are resuspended in digestion buffer. For easier handling, the nuclei suspension should be split into equal aliquots of the planned gradient digestion steps (Fig. 2). Because isolated nuclei sediment rapidly, equal aliquotting requires pipetting the nuclei suspension up and down gently before taking each aliquot. In parallel, aliquots of a DNase I dilution series are also transferred to PCR tubes. Once the reaction is ready to start, equal volumes of the enzyme dilutions are added to the nuclei suspensions using a multichannel pipette (Fig. 2) and mixed by pipetting up and down. The digestion mixtures should be immediately transferred to the preheated PCR cycler for incubation (Fig. 2). After the desired reaction time (typically 3–5 min), digestions are stopped by adding stop buffer (EDTA and/or EGTA; for details, see “Materials and Methods”) to all the reaction mixtures using the multichannel pipette. If the reactions are incubated at a suboptimal temperature for DNase I activity, such as 30°C (Kodama et al., 2007; Song and Crawford, 2010) instead of 37°C, incubation times can be extended to 10 to 15 min. Such an extended incubation time is advantageous because it reduces effects caused by errors in handling time.

There may be no best number of DNase I dilutions fitting all experiments. A rule of thumb is to include enough dilutions to detect smooth transitions of band intensities and differences between insensitive and sensitive control regions for the particular experiment (see below). In addition, a mock-digested (no DNase I) or minimally digested (highly dilute DNase I concentration) step should be included as an input chromatin control.

After the DNase I treatment is completed, DNA should be immediately purified to avoid further degradation. We recommend against column-based DNA purification techniques, because they usually retain large genomic DNA fragments and, therefore, introduce a digestion bias. Instead, organic solvent-based extraction and ethanol precipitation work best. Precipitation carriers such as glycogen can be added for more efficient recovery of the DNA. DNA pellets should be immediately reconstituted in 10 mM Tris-HCl buffer (pH 8.0).

After a limited DNase I digest, a large proportion of the inaccessible DNA remains in very long DNA fragments. Precipitated very long DNA can be difficult to resuspend. However, we strongly advise against high-temperature incubation, fierce vortexing, or pipetting of the pellet to facilitate dissolving. Instead, the DNA pellet should not be overly dried, and reconstitution buffer should be added to the DNA pellet when it is still moist. After adding the buffer, samples can be incubated on ice or in the refrigerator for 30 min to overnight. The DNA samples are then gently pipetted or tapped to ensure homogenous distribution in the buffer. Repeated freeze-thaw cycles should be avoided by storing the DNA samples at 4°C. We found that recovered DNA samples can be stored at 4°C for at least 1 month without detectable changes in the PCR results (data not shown).

Analysis of Digested DNA by PCR

Chromatin sensitivity to DNase I results in reduced accumulation of PCR products in treated samples in comparison with input controls (Fig. 3). There are two major factors affecting the signal reduction in such a

Figure 2. Schematic illustration of the digestion procedure. For easier handling and better synchronization, sample aliquots can be transferred using multichannel pipettes. Digestion can be performed taking advantage of PCR tube strips and PCR cyclers. [See online article for color version of this figure.]
DNase I PCR assay: (1) the ratio of DNase I to chromatin in the reaction; and (2) the length of the PCR amplicon, because the shorter the PCR amplicon, the lower the probability that cleavage events occur within the probed region. Therefore, longer amplicons are better suited to probe the DNase I sensitivity. Because quantitative PCR relies usually on very short amplicons, its power is limited in such DNase I-PCR assays. However, quantitative PCR-based strategies can be used if long stretches of genomic regions are probed by tiling amplicons (Dorschner et al., 2004) or if prior knowledge of DNase I-hypersensitive sites allows precise positioning of amplicons (McArthur et al., 2001; Martins et al., 2007). In contrast, when prior knowledge is lacking, we found conventional PCR most powerful to scan genomic regions for differential sensitivity. Usually, 1.5- to 2-kb-long amplicons (covering approximately seven to 10 nucleosomes) work best at this stage. Once a sensitive region is identified, higher resolution probing by quantitative PCR can be performed.

Here, we describe a strategy to probe the sensitivity of a genomic region by conventional PCR using decay constants calculated from gradient digestions. As an example, the chromatin sensitivity was analyzed for two silent transposable element (TE) genes (Ta2 and Cinful-like) and two active genes (GAPDHα and ACTIN7 [ACT7]) in Arabidopsis. We and others have shown that transcription is generally associated with accessible chromatin (Weil et al., 2004; Boyle et al., 2008; Bell et al., 2010; Shu et al., 2012; Zhang et al., 2012). Thus, we predict that the two TE genes should be much less sensitive to DNase I than the two active genes and, therefore, can be used as insensitive and sensitive controls in future assays. Reduced DNA accessibility is now also considered a silencing mechanisms established by Polycomb group (PcG) proteins in animals (Bell et al., 2010; Eskeland et al., 2010; Naughton et al., 2010; Bantignies et al., 2011) and plants (Shu et al., 2012). Therefore, two Arabidopsis PcG target genes (AG and FT) were included in the assays.

In the absence of particular hypersensitive sites, the probability of cleavage within a region is proportional to the length of this region. Thus, it is important to maintain similar amplicon sizes for the different regions to be probed. Because inefficient PCR and saturated PCR will strongly distort results, PCR conditions have to be carefully optimized for each primer pair by establishing at least (1) an optimal annealing temperature and (2) an optimal cycle number within the exponential phase. Cycle numbers might differ between primer pairs but should lead to comparable PCR signals for the input sample.

It is important to include insensitive and sensitive controls in a DNase I sensitivity experiment. Conventional choices for the controls are DNA for heterochromatin regions and active genes. If PCR conditions were correctly optimized but differences between insensitive and sensitive controls are not easily seen, the DNase I digestion conditions are not appropriate. Typical problems are overdigestion, underdigestion, or damaged chromatin in input nuclei. A gradient digestion with isolated DNA can also be included to control for possible differential sensitivity of the DNA template, although...
this does not seem to be a common problem (Sabo et al., 2006). DNase I digestion of native chromatin and purified DNA was carried out in triplicate (Fig. 4). For the chromatin digestion, PCR signals of the two heterochromatic TE genes remained nearly constant while PCR signals of the two active genes dropped rapidly, confirming our hypothesis of differences between heterochromatin regions and active genes. This is in agreement with genome-wide profiles (Shu et al., 2012) and establishes these regions as insensitive and sensitive controls for future assays. Interestingly, the sensitivity of the two PcG-targeted genes AG and FT seemed to be between the TE genes and the active genes. In contrast, there were no systematic differences in the DNase I sensitivity of isolated DNA: for all tested fragments, PCR signals became reduced following similar patterns. This control confirms that (1) DNA in chromatin is indeed much less accessible to nuclease digestion than after purification and (2) the observed differences in the DNase I sensitivity of chromatin reflect differences in chromatin properties and not a differential intrinsic sensitivity of the DNA template.

PCR Product Quantification and Calculation

Gel images such as in Figure 4 can reveal strong differences, but the comparison of many probed fragments is not trivial. Therefore, a quantitative description of the results is desirable. PCR products can be quantified from gel images using image-processing software such as ImageJ (Schneider et al., 2012). This does not require specialized equipment and can be implemented on any computer. However, quantification from gel images is error prone. Therefore, it is recommended to use a microfluidics-based electrophoresis system such as Bioanalyzer (Agilent) or Experion (Bio-Rad). Of note, some organic components in the PCR buffer can interfere with the measurement using such systems (for more details, see the manufacturer’s instructions). Because purification of PCR products can introduce biases, it is best to consider using PCR buffers with only basal salts.

Figure 5. Fitting curves of the DNase I-PCR results for the selected genomic regions. PCR products as shown in Figure 4 were quantified using an Agilent Bioanalyzer. The measurements were normalized to the input values and fitted to an exponential decay model. The fitted curves for all tested genomic regions are shown for the three replicates (rep. 1–3) for both digested chromatin (top panels) and digested isolated DNA (bottom panels).

Figure 6. Distribution of the decay constants of the tested genomic regions on a heat axis. The fitted decay constants were averaged over the three replicates for digested chromatin (blue text) and digested isolated DNA (red text). The results were plotted on a logarithmic axis. Error bars show the s of the decay constants. The intercept table shows a summary of the calculated results (top right corner).
DNase I sensitivity can be quantified using the PCR signal as a function of increasing DNase I concentration; the steeper the slope, the more accessible is the DNA fragment. The PCR signal \( y \) can be expressed as an exponential decay function of DNase I concentration \( x \) (units mL\(^{-1}\)):

\[
y = Y_0 e^{-\lambda x t}
\]

where \( Y_0 \) is the input PCR product intensity, \( \lambda \) (units mL\(^{-1}\) kb\(^{-1}\) min\(^{-1}\)) is the decay constant characteristic for a given DNA fragment, \( l \) is the amplicon length (kb), and \( t \) is the digestion time (min). Note that the decay constant of the curve is largely independent of primer efficiency. Nevertheless, we recommend using primer pairs with similar high efficiency when comparing different amplicons. Figure 5 shows the fitted curves for the quantified PCR products shown in Figure 4. The fitted curves show that \( Ta2 \) and \( Cinful-like \) were more resistant to DNase I than \( ACT7 \) and \( GAPDH\). 

DNA I sensitivity can be probed locally but also genome wide, where DNase I treatment can be coupled to DNA size fractionation. Depending on the profiling of hypersensitivity or hyposensitivity, different size fractions of DNA are used. For profiling hyposensitivity, the least cleaved DNA fragments should be enriched. In our hands, isolation of fragments larger than 17 kb worked well. This fraction was shown to be highly enriched for DNase I-hyposensitive DNA, including TE genes such as \( Ta2 \) from pericentric heterochromatin.

Table II. Primers and universal probes for quantitative PCR

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer Sequence (5’–3’)</th>
<th>Probe No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ta2</td>
<td>ATGAAGCCGTCCCATCA</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>CGACTGCTATCCCGTGTCC</td>
<td></td>
</tr>
<tr>
<td>ACT7</td>
<td>GGAACATCGTCTCATGATGT</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>CTGTATCTCAGCTGCTAGT</td>
<td></td>
</tr>
</tbody>
</table>

Table I. Primers for DNase-PCR

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer Sequence (5’–3’)</th>
<th>Amplicon Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ta2</td>
<td>TACAGTGGCTACCACATTGC</td>
<td>1,680 bp</td>
</tr>
<tr>
<td></td>
<td>ATGAAGCCGTCCCATCA</td>
<td></td>
</tr>
<tr>
<td>Cinful-like</td>
<td>ACCGCAATATCCGATACGAC</td>
<td>1,651 bp</td>
</tr>
<tr>
<td></td>
<td>TCAACGTCGAAGCAGTCTAG</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>GTGAAACAAATTTCTCGCAATGTC</td>
<td>1,631 bp</td>
</tr>
<tr>
<td></td>
<td>TCTAGTCGAGATTGCTTGCG</td>
<td></td>
</tr>
<tr>
<td>FT</td>
<td>TCTTTAGAAACTTTTCGCTTTTG</td>
<td>1,695 bp</td>
</tr>
<tr>
<td></td>
<td>GGTGCTAATCTGGAAACATC</td>
<td></td>
</tr>
<tr>
<td>GAPDH(\alpha)</td>
<td>GGTCGCTCTTGCTGGCTCT</td>
<td>1,648 bp</td>
</tr>
<tr>
<td>ACT7</td>
<td>GAAGCTCCTTGCCTGCTCT</td>
<td>1,646 bp</td>
</tr>
<tr>
<td></td>
<td>AGCATGTCTCTCCAGATT</td>
<td></td>
</tr>
</tbody>
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over active genes such as ACT7 (Fig. 7, right three columns). In contrast, no enrichment was seen for the undigested control (Fig. 7, left three columns). Isolated DNA can be labeled and hybridized to genomic DNA tiling arrays (Shu et al., 2012). Alternatively, isolated DNA can be subjected to next-generation sequencing. On the other hand, the hypersensitive DNA population can be enriched by minimal DNase I treatment. In this case, small-sized DNA should be enriched, and high enrichment of active genes versus heterochromatin fragments is expected (Sabo et al., 2006). Alternatively, end labeling of DNA after minimal DNase I treatment can be used to enrich for hypersensitive sites (Crawford et al., 2006; Follows et al., 2006; Sabo et al., 2006).

CONCLUSION

We have presented a robust and reproducible protocol for DNase I sensitivity assays using Arabidopsis nuclei and have discussed points to consider when adapting the protocol for specific experiments. In principle, this protocol should be easily adaptable to other plant species. We have shown that two TE genes are highly insensitive to DNase I and that two active genes are highly sensitive. These genes can serve as insensitive and sensitive controls in future chromatin DNase I sensitivity assays. We also have shown that two PcG targets are less sensitive than the active genes, supporting the model that PcG proteins employ compaction of the chromatin as a repression mechanism not only in animals but also in plants. Subsequent to assays of DNase I sensitivity by PCR, higher resolution can be achieved by quantitative PCR (Dorschnier et al., 2004) or by high-throughput DNase-chip or DNase sequencing for genomic chromatin accessibility profiling (Crawford et al., 2006; Sabo et al., 2006; Song and Crawford, 2010; Shu et al., 2012).

Because altered DNA accessibility is a major effect of many histone modifications, histone variants, and nonhistone proteins, testing of DNA accessibility will become more common in future studies on chromatin function.

MATERIALS AND METHODS

Plant Material

Two hundred milligrams of 15-d-old seedlings of Arabidopsis (Arabidopsis thaliana) accession Columbia was harvested for each replicate. Seedlings were immediately frozen in liquid nitrogen and ground to powder with precooled mortar and pestle. The powder was transferred to 15-mL tubes stored at −80°C or used for nuclei extraction immediately.

Nuclei Isolation

Frozen powder of Arabidopsis plant tissue was suspended in 10 mL of ice-cold nuclei isolation buffer (1 mM N-butyline glycol, 20 mM PIPES-KOH, pH 7.6, 10 mM MgCl₂, 1 mM EDTA, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 0.5% Triton X-100 [v/v], 10 mM β-mercaptoethanol, and 1× protease inhibitor cocktail [Roche]). β-Mercaptoethanol and protease inhibitor were added immediately before use. The mixture was incubated for 15 min at 4°C with gentle rotation. The suspension was filtered through 30-mm CellTrics, and the eluate was centrifuged for 10 min at 1,500g at 4°C. The supernatant was discarded, and the pellet was collected as crude nuclei extract.

DNase I Digestion and DNA Recovery

Nuclei extracts were washed once with 1 mL of digestion buffer (40 mM Tris-HCl, pH 7.9, 0.3 mM Suc, 10 mM MgSO₄, 1 mM CaCl₂, and 1× protease inhibitor cocktail [Roche]) and gently resuspended in 670 μL of fresh digestion buffer by pipetting until no clumps were visible. Aliquots of 80 μL were transferred to five PCR tubes and kept on ice.

A DNase I (RQI RNase-Free DNase; Promega) dilution series was prepared by step-wise dilution using digestion buffer to establish the following concentrations: 0, 0.125, 1.25, 2.5, and 5 units mL⁻¹. Aliquots of 30 μL were transferred to five PCR tubes and kept on ice.

Nuclei suspensions were preheated on a PCR cycler for 2 min at 37°C. Twenty microliters of diluted DNase I was transferred to the corresponding nuclei suspensions using a multichannel pipette and mixed briefly by pipetting. The final DNase I concentrations in the digestion mixtures were 0, 0.025, 0.25, 0.5, and 1 unit mL⁻¹. The digestion mixtures were incubated at 30°C for 15 min. Reactions were stopped by adding 10 μL of 100 mM EDTA. Reaction mixtures were then returned to ice. DNA was recovered using phenol:chloroform:isoamyl alcohol precipitation.

For digestion of isolated DNA, genomic DNA was extracted from 240 μL of the nuclei suspension using phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation. The DNA was resuspended in 240 μL of digestion buffer, and 80-μL aliquots of the extracted DNA were digested with DNase I at final concentrations of 0, 0.025, and 0.05 units mL⁻¹. Digested isolated DNA was recovered similarly.

PCR Analysis

The precipitated DNA was resuspended in 150 μL of nuclease-free water, and 5 μL was used for each PCR. PCR was performed using EuroTaq (Euroclone) in 25-μL reactions according to the manufacturer’s protocol. The PCR fragments were designed to overlap the start codons of AG, FT, ACT7, and GAPDH or to locate within the annotated sequence of Cinful-like and Ta2. For PCR primer sequences, see Table I. The absolute quantity of the PCR product was measured using the DNA 7500 kit (Agilent) on an Agilent 2100 Bioanalyzer platform. PCR measurements were averaged across the three digestion replicates. Quantitative PCR was performed as described before (Shu et al., 2012) using the Universal Probe Library system (Roche). For quantitative PCR primer sequences, see Table II.

Analysis on Tiling Arrays

Alternatively, the precipitated DNA was resuspended in 15 μL of nuclease-free water and resolved on 3% agarose gels. DNA fragments of sizes above 17 kb were extracted from the gel using the freeze/thaw method. The extracted DNA was amplified and labeled with biotin using the BioPrime DNA Labeling System (Invitrogen). Labeled DNA was hybridized to Affymetrix AGRONOMICS Arabidopsis tiling arrays as recommended by the manufacturer and as described previously (Rehrauer et al., 2010; Shu et al., 2012). Data analysis using freely available R packages has been described (Shu et al., 2012).

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