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Louwers, M.L.D.

Publication date
2008

[Link to publication](#)

Citation for published version (APA):

Louwers, M. L. D. (2008). *Chromatin looping and epigenetic regulation at the maize b1 locus*. [Thesis, fully internal, Universiteit van Amsterdam].

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Chapter 2

Detection of physical chromatin interactions in plants using Chromosome Conformation Capture (3C)

Marieke Louwers¹, Erik Splinter², Roel van Driel¹, Wouter de Laat²,
Maike Stam¹

¹*Swammerdam Institute for Life Sciences, Universiteit van
Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands.*

²*Hubrecht Institute, Uppsalalaan 8, 3584 CT Utrecht, The
Netherlands.*

To be submitted for publication

Abstract

Gene regulation in higher eukaryotes frequently involves physical interactions between genomic sequence elements that are tens of kilobases apart on the same chromosome, but can also entail interactions between chromosomes. Chromosome Conformation Capture (3C) is a powerful tool to identify such interactions. 3C technology is based on formaldehyde crosslinking of chromatin, followed by restriction digestion and intramolecular ligation. Quantitative detection by PCR (qPCR) of ligation products and the evaluation of their interaction frequency provides insight into the spatial organization of a genomic region. Detailed 3C protocols have been published for yeast and mammals. These protocols can however not simply be transferred to plant tissues. In this paper we provide a plant-specific 3C protocol and present a general strategy to systematically optimize the protocol for any type of tissue. Once the technique and the appropriate controls are established, the 3C-qPCR procedure can be completed in 5-7 days.

‘INTRODUCTION’

Over the last few years it has become clear that the way chromatin is organized in the nucleus can affect gene regulation. Insulators and enhancers often are located ten to hundreds of kilobases (kb) away from the target promoter they communicate with. Several models have been proposed in the past how these interactions are established. One model involves physical contact by chromatin looping. Little is known about this level of organization and the way this is regulated, and there are no “easy” ways to study this *in vivo*.

Recently several techniques were developed that can provide information about the relationship between molecular distances, chromatin interactions and nuclear organization: Fluorescent *in situ* hybridization (FISH; Fransz et al., 2000; Gill and Friebe, 1998), RNA-Tagging and Recovering of Associated Proteins (RNA-trap; Carter et al., 2002) and Chromosome Conformation Capture (3C; Dekker et al., 2002). Each technique is based on a different principle using distinct methods, therefore these techniques can be used to complement and validate each other. Each technique has advantages and disadvantages. FISH utilizes fluorescently labeled DNA probes to detect genes or chromosomes. The probe signal is detected by fluorescent microscopy. FISH can for instance be used to observe chromosome

abnormalities, or to map molecular markers. When probing two regions in the genome, the FISH signals can be used to study the distance between the two regions. FISH has the advantage that cell to cell variations can easily be investigated. However, co-localization is not necessarily related to physical interactions, due to the limited spatial resolution of the microscope. The RNA-trap technique, based on targeted *in situ* hybridization followed by chromatin immunoprecipitation, reports on proximity: it involves targeting of horseradish peroxidase (HRP) to nascent RNA transcripts, followed by precipitation and quantitation of HRP-catalyzed biotin deposition on chromatin nearby. The fact that this method can only be used to investigate loci containing nascent RNA, does not make it generally applicable. 3C technology does not rely on active transcription, and consequently situations can be compared wherein a gene is on or off. 3C uses formaldehyde to capture physical interactions between chromatin. It does so by crosslinking proteins to proteins and proteins to DNA. Crosslinks are only made when two molecules are in close proximity. In this way, protein-mediated physical contacts between local and distant DNA sequences can be captured and analyzed. 3C is mostly used to study the organization of a single gene locus. More recently, 3C-based techniques (4C, 5C, ChIP-loop assay) were developed that allow an unbiased, high-throughput, genome-wide search for genomic interactions (reviewed in Simonis et al., 2007).

Detailed 3C protocols have been published for yeast and mammals (Dekker et al., 2002; Hagege et al., 2007; Splinter et al., 2004). These protocols can however not simply be transferred to plant tissues due to their special properties and unique chemical composition. In this paper we provide a plant-specific 3C protocol, which has been successfully applied to maize tissue (Louwers et al., chapter 3 of this thesis). Moreover, we present a general strategy to systematically optimize the protocol for any type of tissue. This paper will therefore be of interest to scientists that want to use 3C in plant research, but also to those that want to develop or optimize a 3C protocol for other organisms.

Experimental design

3C technology is a powerful technique to identify long-range chromatin interactions, but a careful experimental design and critical data analysis are crucial. Crosslinking frequencies between two single fragments do not provide information about chromatin loop formations and should always be evaluated in a broader context. A sufficient number of data points needs to be examined in order to be able to detect the presence of a possible chromosome loop. In addition, every single step of the

protocol has to work efficiently in order to avoid false or missed data points. And, as with any method, controls need to be taken into account (Dekker, 2006; Hagege et al., 2007; Splinter et al., 2004).

Although the presented protocol is based on previously published 3C protocols (Splinter et al., 2004; Hagege et al., 2007), it contains several adjustments crucial for the strategy to work in plants, in particular in maize. These adjustments to the protocol are discussed in detail below. The development of the necessary controls for the 3C method, such as for PCR efficiency, assessing the level of background interactions and an internal control for data normalization, as well as the controls necessary for a correct qPCR evaluation, have not been changed in our protocol. For these controls we refer to some excellent existing protocols and papers (Dekker, 2006; Hagege et al., 2007; Splinter et al., 2004). The outline and timing of a typical 3C experiment is depicted in Figure 1.

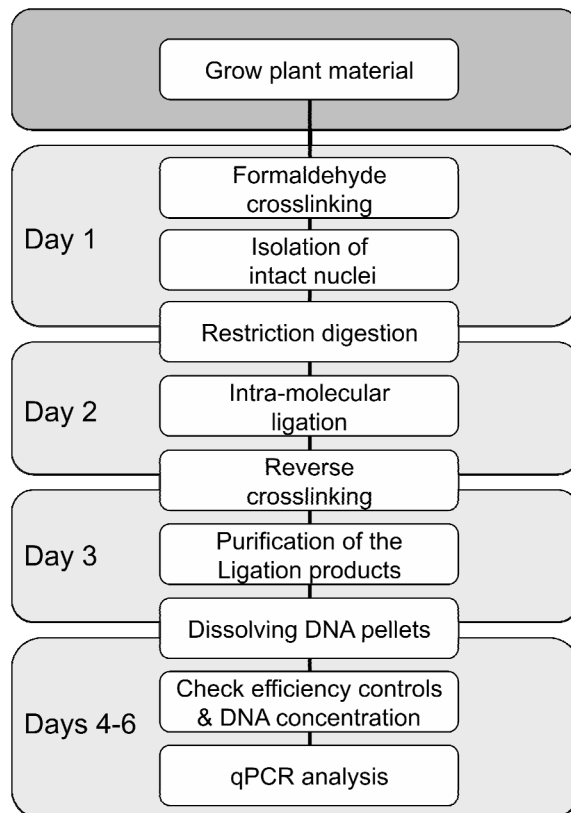


Figure 1. Outline and timing of the 3C-qPCR procedure

Fixation of plant tissue prior to the isolation of nuclei

The isolation of nuclei suitable for 3C requires a plant-specific approach. Plant tissue has a waxy surface, hampering access to the cells within the tissue. Furthermore, every cell is surrounded by a thick cell wall. These two layers make it more difficult to isolate qualitatively good and sufficient nuclei, in which the chromatin conformation is preserved. Formaldehyde fixation of the cells stabilizes chromatin structure. However, in plant research whole tissues are often used instead of cell cultures, which is the starting point of mammalian 3C protocols. This protocol was therefore optimized also for plant tissue. We noticed that the yield and the quality of the nuclei are much higher when plant tissue was first treated with formaldehyde, prior to nuclei isolation (see Fig. 2a), instead of isolation of nuclei followed by formaldehyde fixation. Due to the waxy surface and the spongy air-filled mesophyll, it is not easy for the crosslinking agent to penetrate the tissue and enter the cells. To facilitate this process, the tissue was cut into small pieces and, after the addition of formaldehyde, crosslinked under vacuum (see step 5 of PROCEDURE and TROUBLESHOOTING). To further increase fixation efficiency, the vacuum was put on and off for several times, ensuring the crosslinking agent optimally penetrated the tissue. Plant tissue that has been successfully treated with and penetrated by formaldehyde should become somewhat transparent, except for the plant nerves. Depending on what plant tissue is used, the crosslinking step should be optimized. The optimal fixation conditions must be checked experimentally. Both the length of the fixation time as well as the concentration of the crosslinking agent can be varied according to the specific research question. In general, neighboring restriction fragments require less stringent crosslinking conditions to be captured than more remote fragments.

In order for the 3C technique to be successful, the DNA within the nuclei has to be digested to near completion. To achieve this in maize tissue, it is necessary to always use freshly harvested tissue. The use of tissue stored at -80 °C results in poor digestion efficiencies. Once nuclei are fixed and isolated, we recommend proceeding immediately with the 3C protocol. Freezing and storage of the nuclei often results in partial digestion as well. We believe that long-term storage deteriorates plant nuclei or makes the chromatin less accessible, a major obstacle to 3C analysis. Many protocols available for isolating nuclei from maize or other plants are not compatible with formaldehyde solutions, because the buffers used in the isolation contain Tris. Tris contains reactive amine, which could cause crosslinking of the formaldehyde to Tris, leaving less formaldehyde to fix the DNA and proteins.

Other protocols use buffers that do not contain the correct cations to stabilize the chromatin. Still others do not yield sufficient or intact nuclei as a result of, for instance, too many centrifugation steps and not handling the nuclei gently.

Restriction digestion in intact nuclei

To determine crosslinking frequencies between various genomic sequence elements such as promoters and enhancers, restriction digestion is required to separate these elements. The restriction digestion should be as efficient as possible to successfully apply 3C. At least 60-70%, but preferentially more than 80% of the DNA should be digested by the restriction enzyme used. Partial digestion will lead to reduced amounts of specific restriction ends, which are then no longer free to ligate with other restriction fragments in the next step of the protocol. This can introduce a serious bias in the 3C analysis.

Only a limited number of restriction enzymes appear to digest DNA efficiently under 3C experimental conditions. Six-basepair cutters are to be preferred when analyzing larger loci up to several hundreds of kilobases. Three 6-base cutters have been reported to work particularly well in the context of crosslinked nuclei: *Bgl*III, *Eco*RI and *Hind*III (Splinter et al., 2004). Here we use *Bgl*III, but near-complete digestion has also been achieved using *Bam*HI, after altering some steps in the protocol (see TROUBLESHOOTING). To analyze smaller loci up to 20 kb or to fine-map interactions between larger fragments, 4-cutters such as *Dpn*II and *Nla*III may be used (Hagege et al., 2007). The enzyme of choice should preferably not generate blunt ends, as they do not ligate easily. In addition, the enzyme should not be methylation-sensitive, as that will lead to incomplete digestion of methylated DNA sites. When optimizing the 3C protocol for a certain tissue or organism, it is essential to check the digestion efficiency by DNA blot analysis or by qPCR across restriction sites. Even when optimal conditions have been established, we recommend routinely checking the digestion efficiency on gel. For more information, see ANTICIPATED RESULTS and Figures 3 and 4.

The digestion is performed on intact nuclei. In order for the restriction enzyme to access the chromatin, the nuclei have to be permeabilized. In addition, non-crosslinked proteins, in particular nucleases, should be removed to prevent DNA degradation. This is achieved by incubating the nuclei with the detergent SDS (See Fig. 2b). In maize, the standard SDS treatment is not sufficient to remove all nucleases, as apparent from frequently observed DNA degradation (see Fig. 4a and M. Stam, unpublished results). To circumvent this problem, we have introduced an

additional step that allows for the inactivation of remaining nucleases (see step 22 of PROCEDURE, ANTICIPATED results and Fig. 4a).

Purification of ligation products

To quantitatively analyze the crosslinking frequencies of certain fragments, the ligation products have to be purified after the reversal of the crosslinks. The purification starts with an RNase treatment (step 36), followed by phenol extraction (step 37). Next, the DNA is ethanol-precipitated (steps 40-42) and washed (step 43). For maize, the precipitation proved to be difficult. Precipitation of DNA is hindered by the DTT present in the ligation buffer which easily co-precipitates. To bypass this, an equal volume of MilliQ water is added prior to precipitation. Adding a carrier, such as glycogen, during the precipitation greatly improved the DNA recovery. Yields may differ depending on the brand of polypropylene tubes used; we use BD Biosciences Falcon tubes. Once the DNA has been precipitated and washed, it needs to be homogeneously dissolved. For maize DNA, dissolving the DNA by incubating at 55 °C for 20 min, as done in many other protocols, was not effective. We recommend dissolving for several hours at room temperature, followed by one night at 4 °C (step 47). Should any impurities stay on the bottom of the tube after overnight incubation, gently spinning the sample and taking the clear supernatant will increase sample purity.

‘MATERIALS’

‘REAGENTS’

- Nuclei isolation buffer (see REAGENT SETUP)
- Hepes (Sigma, cat. no. H4034)
- Sucrose (Fluka, cat. no. 84100)
- MgCl₂ Hexahydrate (Fluka, cat. no. 63072)
- KCl (Analar, cat. no. 101985)
- Glycerol (Merck, 87%, cat. no. 1.04094)
- Triton X-100 (20% (v/v); Sigma, cat. no. T8787) ‘CRITICAL’ Triton stock and work solutions are light-sensitive and should be kept in the dark; work solutions should be relatively fresh (maximum 1-2 months old).

- Phenylmethylsulfonyl Fluoride, PMSF (Sigma, cat. no. P7626) ‘CAUTION’ Toxic. The PMSF waste should be disposed of according to the regulations.
- 2-mercapto-ethanol (Merck, cat. no. 1.15433) ‘CAUTION’ Toxic and dangerous for the environment. The 2-mercapto-ethanol waste should be disposed of according to the regulations.
- Formaldehyde solution, 4% (w/v). Prepare from paraformaldehyde (ICN Biomedicals, Inc., cat. no. 150146; see REAGENT SETUP) ‘CAUTION’ Harmful (see material safety data sheet at http://www.mpbio.com/product_info.php?cPath=491_1_12&products_id=150146&catalog=150146&processTab=safety).
- Glycine, 2 M (ICN Biomedicals, Inc., cat. no. 194825)
- Liquid nitrogen
- Complete protease inhibitor tablets (Roche, cat. no. 11697498001; see REAGENT SETUP)
- Miracloth (Calbiochem, cat. no. 475855)
- Sefar Nitek filter, poresize 50 μm (Sefar Inc.) See <http://www.sefar.com> for more information.
- 4',6-Diamidine-2-phenylindole dihydrochloride, DAPI (Roche, cat. no. 10236276001) and/or Methylgrün-Pyronin ‘CRITICAL’ DAPI and Methylgrün-Pyronin solutions are light-sensitive and should always be kept in the dark.
- SuRE/Cut Buffer M (Roche, cat. no. 11417983001)
- Sodium Dodecyl Sulphate (SDS 20% (w/v); US Biological, cat. no. 85010) ‘CAUTION’ Harmful
- *Bgl*II restriction enzyme, high concentration, 40 U μl^{-1} (Roche, cat. no. 11175068001; brand critical)
- 10 x ligation buffer (see REAGENT SETUP)
- Trizma base (Sigma, cat. no. T6066)
- Dithiothreitol, 1 M (DTT; dissolved in 0.01 M NaOAc; Sigma, cat. no. D9779) ‘CAUTION’ Harmful
- Adenosine triphosphate, ATP (Sigma, cat. no. A-2383)
- Proteinase K (Roche, cat. no. 3115879001), 10 mg ml^{-1}
- T4 DNA ligase, high concentration, 5 U μl^{-1} (Roche, cat. no. 10799009001; brand critical)

- Ribonuclease A (RNase A; Roche, cat. no. 10109169001), 10 mg ml⁻¹
- Phenol, buffered with TE pH 8 (Sigma, cat. no. P4557) ‘CAUTION’ Toxic and corrosive. Phenol waste should be disposed of according to the regulations.
- Chloroform (Merck, cat. no. 1.02445) ‘CAUTION’ Harmful. Chloroform waste should be disposed of according to the regulations.
- Isoamylalcohol (ICN Biochemicals, cat. no. 1.4059) ‘CAUTION’ Harmful. Isoamylalcohol waste should be disposed of according to the regulations.
- Phenol-Chloroform-Isoamylalcohol, 25:24:1 (see REAGENT SETUP)
- Sodium Acetate (Anhydrous), 2 M pH 5.6, (NaOAc; Sigma, cat. no. S2889).
- Ethanol (Nedcalco, 96% (v/v); cat. no. 104.000.0020E)
- Glycogen, 20 mg ml⁻¹ (Fermentas, cat. no. R0561)
- Tris-HCl 10 mM pH 7.8 at 25 °C and pH 8
- Agarose (Roche, cat. no. 11388991001)
- Etidium Bromide, 10 mg ml⁻¹ (EtBr; Biorad, cat. no. 161-0433) ‘CAUTION’ EtBr is a mutagen. Always wear gloves when handling gels and solutions containing EtBr. Dispose of EtBr-containing gels and solutions appropriately.

‘EQUIPMENT’

- Speedvac (Savant Speedvac Plus SC110A; see EQUIPMENT SETUP) or exiccator
- Sorvall centrifuge with SS34 rotor (Centrifuge RC5C Meyvis; brand not critical)
- Refrigerated microcentrifuge (Eppendorf, model 5417R; brand not critical)
- Eppendorf Thermomixer (brand not critical)
- Water bath (brand not critical)
- Centrifuge for 50 ml Falcon tubes (Heraeus Labofuge 400R; brand not critical)
- Electrophoresis equipment (brand not critical)
- Nalgene Oak ridge centrifuge tubes PPCO (Nalge Company, cat. no. 3119-0050)
- Falcon tubes BD Biosciences (cat. no. 352070 – 5098512; brand critical)
- Polystyrene

‘REAGENT SETUP’

Nuclei isolation buffer 20 mM Hepes pH 8; 250 mM Sucrose; 1 mM MgCl₂; 5 mM KCl; 40% (v/v) glycerol; 0.25% (v/v) Triton X-100; 0.1 mM PMSF in isopropanol; 0.1% (v/v) 2-mercapto-ethanol in H₂O. Add PMSF and 2-mercapto-ethanol immediately before use. ‘CAUTION’ Both PMSF and 2-mercapto-ethanol are toxic.

Formaldehyde solution, 4% (w/v) Dissolve 4 gram of paraformaldehyde in 100 ml nuclei isolation buffer. Set to pH 9 to decompose the polymer into formaldehyde. Heat to 65 °C to aid dissolving. Let the solution cool down, adjust to pH 7-7.5 and store aliquots at -80 °C. Thaw only once. ‘CAUTION’ Toxic. Formaldehyde waste should be disposed of according to the regulations.

Protease inhibitors Dissolve 1 complete protease inhibitor tablet (Roche) in 2 ml H₂O. The obtained solution can be stored at 4 °C if used within 2 weeks. Store at -20 °C for longer storage (up to 3 months).

10 x ligation buffer 300 mM Tris-HCl (pH 7.8 at 25 °C); 100 mM MgCl₂; 100 mM DTT, 10 mM ATP in H₂O. Store aliquots at -20 °C. Always use freshly made ATP. The composition of this buffer is identical to the one provided by Promega (cat. no. C1263). Due to the large amounts necessary, we prepared the ligation buffer ourselves.

Phenol-Chloroform-Isoamylalcohol 25:24:1 Mix Chloroform-Isoamylalcohol 24 to 1. Mix this solution 1 to 1 with TE-buffered Phenol. Allow the mixture to equilibrate overnight at 4 °C. ‘CAUTION’ Phenol is toxic and both Chloroform and Isoamylalcohol are harmful. ‘CRITICAL’ The phenol-chloroform-isoamylalcohol solution has to be at room temperature when used. Therefore, place the solution at room temperature the day before use.

‘EQUIPMENT SETUP’

Speedvac For the fixation under vacuum conditions, we removed the rotor from the Speedvac centrifuge, and placed a box or rack that could contain 50 ml tubes into the centrifuge.

‘PROCEDURE’

This protocol is optimized for maize tissue; some adaptations may be required when this protocol is used for other plant species.

Preparation of plant tissue ‘TIMING’ 15 min

1 Use approximately 1.5 grams of plant material. When using maize husk or inner stem tissue, take 1/4th of a husk or of inner stem tissue of a maize seedling (yields 10-12 x 10⁶ nuclei). Soft plant tissue was used; tough tissue was discarded (for exact description of tissues, see Louwers et al., chapter 3 of this thesis).

‘CRITICAL STEP’ Always use freshly harvested tissue. Do not use tissue that has been stored at -80 °C; the use of such tissue hampers efficient restriction digestion (step 25).

2 Cut the tissue in small pieces (~ 1 cm³) in a petri dish (15 cm diameter) placed on ice.

3 Transfer the tissue to a 50 ml Falcon tube and add 15 ml ice-cold isolation buffer.

Formaldehyde crosslinking ‘TIMING’ 70 min

4 Add 15 ml 4% (w/v) formaldehyde solution (final 2%).

‘CAUTION’ Formaldehyde is toxic

5 Place the cap loosely on the tube and place the tube in the speedvac. Clog the tube with a few pieces of porous filter material (e.g. polystyrene) to prevent the tissue from spilling out of the tube. Vacuum infiltrate for 1 h at room temperature. At the beginning, switch the vacuum on and off for 2-3 times. Air should be able to escape through the filter material. After one hour, the material should be slightly transparent. ‘CRITICAL STEP’ Fixing plant tissue under vacuum helps the crosslinking agent to enter the cell through the cell wall, especially by putting the vacuum on and off for a few times.

‘TROUBLESHOOTING’

6 Remove the piece of polystyrene and add 1/16 volumes cold 2 M Glycine (final concentration 0.125 M). Incubate for 5 min (vacuum, room temperature) to stop the crosslinking.

Isolation of intact maize nuclei ‘TIMING’ 1-2 h

7 Pour the liquid out of the Falcon tube and rinse the tissue in the tube 3 times with MilliQ water. ‘CAUTION’ PMSF, 2-mercapto-ethanol and formaldehyde are toxic, dispose the waste according to the appropriate regulations.

8 Discard any remaining liquid and dry the tissue between paper towels.

9 Transfer the dried tissue to a chilled, clean mortar and grind it with liquid nitrogen until the tissue is pulverized into a fine powder.

‘TROUBLESHOOTING’

10 Add protease inhibitors to the isolation buffer. Add 1 tablet, dissolved in 2 ml H₂O, to 50 ml buffer.

11 Add 10 ml ice-cold isolation buffer containing protease inhibitors to the ground tissue. Gently mix the isolation buffer with the tissue and allow the frozen sample to thaw.

12 Place a Nalgene Oakridge tube on ice, put a funnel on top. Prepare a filter from one layer of Miracloth and one layer of Sefar Nitek filter, whereby the Sefar filter is on the outside. When the sample has become totally liquid, pour it through the filter. (The Sefar Nitek filters can be rinsed and re-used.) ‘CRITICAL STEP’ The filtered suspension contains nuclei. In order to keep them intact, work on ice.

‘TROUBLESHOOTING’

‘OPTIONAL STEP’

Check the quality of the nuclei by taking a 2 µl aliquot, followed by staining it with DAPI (2 µg ml⁻¹ final); check the nuclear morphology under a light microscope. There should be an abundance of round and intact nuclei in the solution (see Fig. 2a).

‘TROUBLESHOOTING’

13 Spin the nuclei suspension for 15 min at 3000g at 4 °C (Sorvall centrifuge, rotor SS34, 5000 r.p.m.).

‘CRITICAL STEP’ The isolated nuclei are quite fragile. Handle them with care, keeping them cold at all times and avoid spinning down faster than described.

14 Gently pour or pipette off the supernatant and dissolve each pellet in 1 ml ice-cold isolation buffer containing protein inhibitors. The pellet should dissolve easily by gently swirling the tubes. If not, gently pipette up and down (see ANTICIPATED RESULTS).

‘TROUBLESHOOTING’

‘OPTIONAL STEP’

Check the quality of the nuclei by taking a 2 µl aliquot, followed by staining it with DAPI (2 µg ml⁻¹ final); check the nuclear morphology under a light microscope.

There should be an abundance of round and intact nuclei in the solution (see Fig. 2a).

‘TROUBLESHOOTING’

15 Transfer the nuclei suspension to an Eppendorf tube and centrifuge 5 min at 4500 r.p.m. at 4 °C in an Eppendorf centrifuge.

16 Pipette off the supernatant and gently take up the pellet once more in 1 ml ice-cold nuclei isolation buffer containing protein inhibitors. Careful, pellet can be loose.

17 Centrifuge 5 min at 4500 r.p.m. at 4 °C in an Eppendorf centrifuge and pipette off the supernatant. Stain nuclei with DAPI or Methylgrün-Pyronin and count them in a counting chamber under a light microscope. Use 1×10^7 nuclei for a 3C experiment.

DNA digestion ‘TIMING’ 18-20 h

18 To wash the nuclei, resuspend in 400 μ l 1.2 x restriction buffer (SureCut, Roche). **‘CRITICAL STEP’** Washing the nuclei once in restriction buffer led to an increase in restriction digestion. Traces of the plant nuclei isolation buffer appear to have a negative effect on the digestion. Use freshly isolated nuclei. Freezing and long-term storage of nuclei at -80 °C systematically lead to partial restriction digestions in our hands.

19 Centrifuge for 5 min at 4500 r.p.m. at 4 °C in an Eppendorf centrifuge.

20 Remove the supernatant and resuspend the pellet in 500 μ l 1.2 x restriction buffer. Gently pipette up and down if necessary.

21 Add 7.5 μ l 20% (w/v) SDS (final 3% SDS).

22 Shake at 900 r.p.m. for 40 min at 65 °C, followed by 20 min shaking at 37 °C.

‘CRITICAL STEP’ The incubation step at 65 °C in the presence of SDS is necessary to inactivate all remaining endogenous nucleases. The SDS incubation at 37 °C is required to further permeabilize the nuclei (see ANTICIPATED RESULTS and Fig. 4a).

‘OPTIONAL STEP’

To check if the permeabilization worked well, take a 2 μ l aliquot after SDS incubation followed by staining it with DAPI (2 μ g ml⁻¹ final) and check the nuclear morphology using a light microscope. The intact nuclei as seen before should now be seriously distorted, aggregated and (partially) broken down (see Fig. 2b).

23 Add 50 µl 20% (v/v) Triton X-100 (final: 2% TX-100).

‘CRITICAL STEP’ Triton X-100 sequesters the SDS, which hampers the restriction digestion.

24 Shake at 900 r.p.m. for 60 min at 37 °C.

25 Add 400 U of the selected restriction enzyme and incubate overnight at 37 °C while shaking at 900 r.p.m.

‘CRITICAL STEP’ The brand of restriction enzymes proved to be critical in our hands.

When optimizing the protocol, check the digestion efficiency by DNA blot analysis (see ANTICIPATED RESULTS and Fig. 3).

‘TROUBLESHOOTING’

Intramolecular ligation ‘TIMING’ 7-8 h + overnight step

26 To inactivate the remaining active restriction enzyme, add 40 µl 20% (w/v) SDS (final: 1.6% SDS).

27 Shake for 20-25 min at 65 °C at 900 r.p.m.

28 Transfer the sample to a 50 ml Falcon tube.

29 Add 7 ml 1x ligation buffer (700 µl 10x Promega Ligation buffer plus 6.3 ml MilliQ).

30 Add 375 µl 20% (v/v) Triton X-100 (final: 1% Triton-100).

‘CRITICAL STEP’ Triton X-100 sequesters the SDS, which hampers the ligation.

31 Incubate for 1 hr at 37 °C.

32 To check the digestion efficiency, transfer a 300 µl aliquot to an Eppendorf tube and add 5 µl proteinase K. De-crosslink overnight at 65 °C and then continue with steps 36-47, in parallel with the ligated sample. Adjust the volumes in these steps accordingly. The result can be checked on a 0.8% agarose gel (See step 48, ANTICIPATED RESULTS and Fig. 4b + c). To process the remaining sample proceed to step 33.

‘TROUBLESHOOTING’

33 Add 100 U highly concentrated DNA ligase and incubate for 5 h at 16 °C, followed by 45 min at room temperature.

‘CRITICAL STEP’ The brand of DNA ligase and ligation buffer proved to be critical in our hands.

34 Add 30 µl proteinase K (total 300 µg).

35 Incubate at 65 °C overnight to de-crosslink the sample.

DNA purification 'TIMING' 4-5 h + overnight step

36 Add 30 μ l RNase A (total 300 μ g) and incubate 30-45 min at 37 °C.

37 Add 10 ml phenol-chloroform-isoamylalcohol and mix well by rigorously shaking the tube by hand.

'CRITICAL' The phenol-chloroform-isoamylalcohol solution should be at room temperature to allow adequate separation of the phases.

'TROUBLESHOOTING'

38 Centrifuge for 10 min at 4500g at room temperature.

39 Transfer the aqueous phase to a new Falcon tube.

'CRITICAL STEP' The brand of Falcon tubes proved to be critical in our hands.

'CAUTION' Dispose of phenol-chloroform-isoamylalcohol waste according to the existing regulations.

40 Add 7 ml sterile MilliQ, 1400 μ l 2 M NaOAc pH 5.6, 24 ml 96% (v/v) EtOH and 40 μ l glycogen and mix well by inverting the tube several times.

'CRITICAL STEP' Increasing the volume prior to the precipitation step dilutes the concentration of DTT present in the ligation sample (DTT hampers the precipitation). Adding a carrier such as glycogen greatly improves the DNA recovery during the precipitation.

41 Store at -80 °C for at least 2 h.

'PAUSE POINT' The tube can be stored at -80 °C for longer than 2 hours.

42 Centrifuge the frozen tube for 60 min at 4500g at 4 °C.

43 Discard the supernatant and wash the pellets with 10 ml cold 70% (v/v) EtOH.

'TROUBLESHOOTING'

44 Centrifuge for 15 min at 4500g at 4 °C.

45 Pipette off as much liquid as possible and air-dry the pellets for 3-5 minutes.

46 Dissolve the pellet in 150 μ l 10 mM Tris pH 7.5 (dissolve the pellet of the digestion control -aliquot taken in step 32- in 30 μ l 10 mM Tris pH 7.5).

47 To dissolve the DNA, incubate the pellets at room temperature for several hours followed by overnight incubation at 4 °C.

'CRITICAL STEP' It is crucial that the DNA is completely dissolved prior to proceeding with the quantitative PCR analysis.

48 The next day, check the digestion and ligation of the 3C sample. Load 2 μ l of the ligated 3C DNA on a 0.8% (w/v) agarose gel, next to 30 μ l DNA of the digestion efficiency control. Stain with EtBr and estimate the DNA concentration of the

ligated 3C sample, use a dilution series if necessary and/or compare to a standardized sample (See Fig. 3 and ANTICIPATED RESULTS).

‘CAUTION’ Dispose of EtBr gels and solutions according to the regulations
‘TROUBLESHOOTING’

49 The 3C DNA sample is now ready for qPCR analysis (Hagege et al., 2007).

‘PAUSE POINT’ The 3C DNA template can be stored at -20 °C for 1-2 months.

‘TIMING’

Steps 1-3, preparation of plant tissue: 15 min

Steps 4-6, formaldehyde crosslinking: 70 min

Steps 7-17, isolation of intact maize nuclei: 1-2 h

Steps 18-25, DNA digestion: 18-20 h

Steps 26-35, Intramolecular ligation: 7-8 h + overnight step

Steps 36-47, DNA purification: 4-5 h + overnight step

Steps 48-49, Check efficiency controls and qPCR analysis: 2-3 days

Total timing: 5-7 days (depending on the amount of qPCR runs)

‘ANTICIPATED RESULTS’

Isolation of intact nuclei (Step 14)

An important step in the protocol is to start with freshly isolated, qualitatively good nuclei. The nuclei should not be aggregated or ragged at the borders. Isolation of nuclei should be efficient as approximately 10^7 nuclei are needed for a 3C experiment. Up-scaling the experiment by starting with more tissue per tube is not always the best option as it makes it harder to crosslink well. Many protocols that are available for isolating maize or other plant nuclei are not compatible with formaldehyde solutions, or do not yield sufficient or intact nuclei and can therefore not be used in the 3C technology. The protocol for isolating maize nuclei presented here yields sufficient nuclei that are intact and suitable as starting material for 3C. Fig. 2a show pictures of what intact maize nuclei look like: the nuclei have a spherical shape and the chromocenters and nucleoli are easily visible (left and middle panel in Fig. 2a). The right panel in Fig. 2a is a picture from an Arabidopsis

nucleus, isolated using the nuclei isolation protocol described here. Therefore, the here presented protocol is probably suitable for other plant tissues as well.

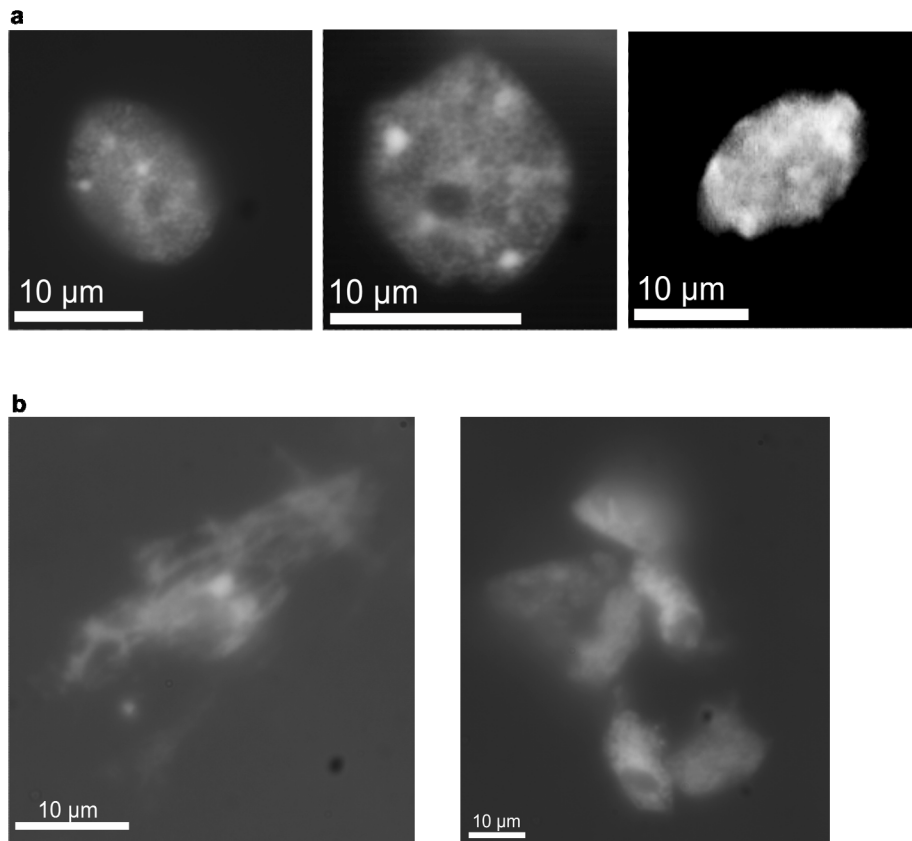


Figure 2. Plant nuclei before and after SDS treatment.

Image acquisition was carried out on a Leitz Aristoplan (Leica Microsystems AG, Wetzlar, Germany) microscope with filters for DAPI. Images were captured with a CCD camera (Apogee, Logan, UT) using ImageProPlus Software (Media Cybernetics, Silver Spring, MD). The images were digitally processed using Adobe Photoshop (Adobe, San Jose, CA).

(a) Typical example of isolated intact maize nuclei stained with DAPI (optional step in steps 12 and 14 of the procedure). Chromocenters (DAPI-dense) and nucleoli (dark regions) are visible. On the right is an example of *Arabidopsis* nucleus using the described nuclei isolation protocol.

(b) Typical example of nuclei after SDS treatment stained with DAPI (optional step in step 22 of the procedure). Nuclei are permeabilized by SDS treatment. Their round shape is lost, giving rise to distorted, partially broken down nuclei (left panel), which often tend to aggregate (right panel).

SDS permeabilization of the nuclei and possible DNA degradation (steps 21-22)

Permeabilization of the nuclei and removal of non- or badly crosslinked proteins is required to make the chromatin accessible to the restriction enzyme. It is crucial that the chromatin in all nuclei becomes accessible with a similar efficiency. In order to prevent that nuclei aggregate, they are shaken during the incubation steps and if necessary the incubation volume is increased. The treated nuclei are distorted and no longer intact. Often, the nuclei also tend to aggregate after SDS treatment (Fig. 2b).

As mentioned in the introduction, DNA degradation can occur during this SDS step at 37 °C due to the presence of nucleases (see Fig. 4a). To check for DNA degradation, nuclei that were treated with SDS at 37 °C were de-crosslinked after TritonX-100 incubation, purified and run on an agarose gel. The result was a nucleosome ladder pattern which was observed even if no restriction enzyme was added (Fig. 4a, lanes 4 and 5). Degradation was not a problem that arose during the nuclei isolation, but occurred during the 37 °C incubation. Evidence hereof is shown in Fig. 4a, lane 6, where nuclei were resuspended in a lysis mix (10 mM Tris-HCl, pH 7.5; 10 mM NaCl; 5 mM MgCl₂; 0.1 mM EGTA; 1x complete protease inhibitor), afterwards treated with proteinase K at 65 °C (de-crosslinked) and subsequently, the DNA was purified. No degradation could be observed there. To prevent non-crosslinked nucleases from degrading the DNA, we treated the nuclei with SDS and first incubated them at 65 °C for 40 minutes, followed by an additional incubation of 20 minutes at 37 °C and the standard TX-100 incubation. This resulted in a strong reduction of aspecific nuclease activity (Fig. 4a, lanes 7-9). When incubated at 65 °C for 60 min, DNA degradation was almost completely absent, but note that at this temperature, the crosslinks are also reversed.

Digestion efficiency and DNA ligation (steps 25-33)

The digestion efficiency needs to be as high as possible; preferably more than 80% of the DNA should be digested. When optimizing the digestion step, the digestion efficiency at the locus of interest should be checked in detail. To do this, part of the sample can be taken after overnight digestion and processed to de-crosslink and purify the DNA. The DNA was run on an agarose gel and hybridized to a membrane. DNA blot analysis was performed using probes specific for the locus of interest (for an example, see Fig. 3a). Panel b in Figure 3 shows the digestion efficiency when the conditions were used as described in existing 3C protocols (without the plant-specific adjustments), ranging only the enzyme (*Bgl*II) concentration. The expected band is indicated with an arrow (~1.6 kb). Many

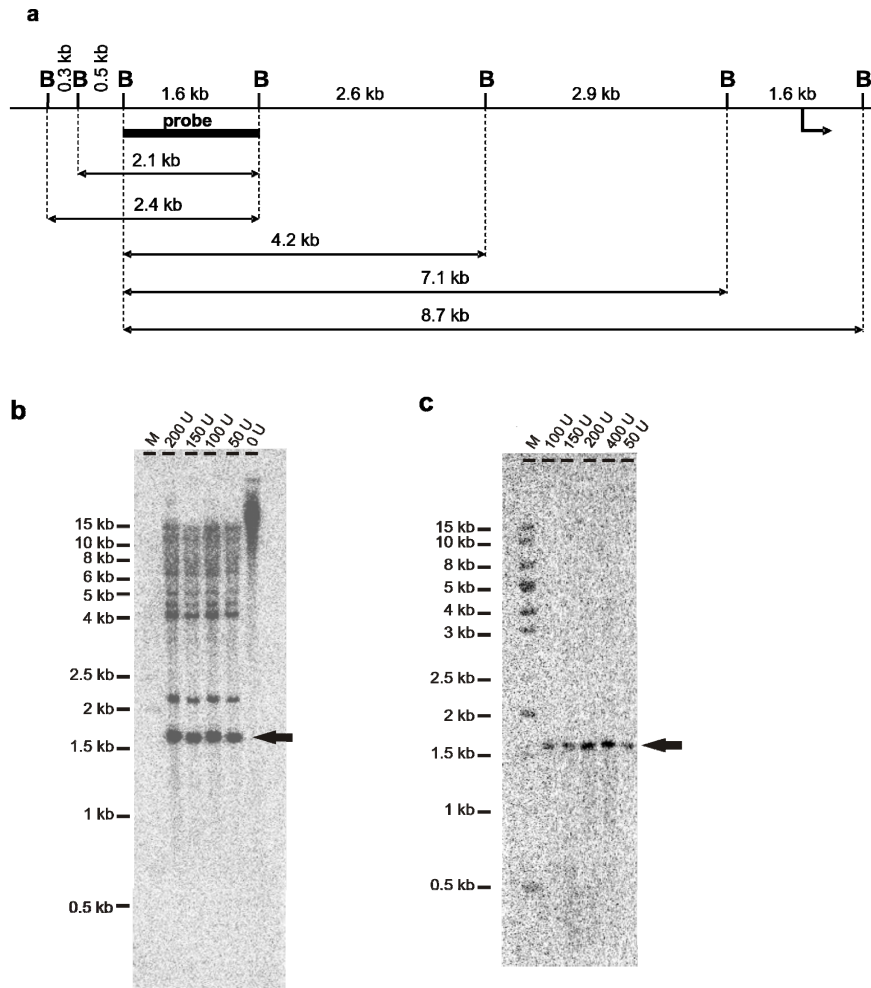


Figure 3. Optimization of the restriction digestion measured by DNA blot analysis.

(a) Restriction map of a part of the maize *b1* locus. Expected fragment sizes are indicated when *Bgl*II is used. The black bar represents the probe used in DNA blot analysis. The arrow shows the transcription start site. B = *Bgl*II restriction site.

(b) Digestion efficiency before (plant-specific) adjustments were made to the protocol. Nuclei were digested with varying amounts of *Bgl*II restriction enzyme. In case the DNA is digested completely, hybridization of the probe to the DNA blot would result in a single band of 1.6 kb in size, indicated with an arrow. Although that band is present in all lanes (except for the lane where no enzyme was added), many additional bands can be seen, due to partial digestion. U = units of *Bgl*II restriction enzyme; M = marker

(c) Digestion efficiency after (plant-specific) adjustments were made to the protocol. This DNA blot depicts a similar experiment as described in Figure 4b. In all lanes there is only one band present: the expected 1.6 kb band (arrow), indicating a high digestion efficiency. U = units of *Bgl*II restriction enzyme; M = marker

additional bands can be seen as a result of incomplete digestion. After optimizing the digestion conditions for our maize tissue, the ~1.6 kb band is practically the only band visible (Fig. 3c). Intensities of bands can be measured semi-quantitatively by scanning the blot and using Phospho-Imaging software to compare the signals on the blot. This is an easy method to evaluate every single adjustment made to the protocol to ensure the highest digestion efficiency. Alternatively, the digestion efficiency can be determined quantitatively by qPCR, as described by Hagege *et al.* in Box 2 of their paper (Hagege *et al.*, 2007).

Even if optimal conditions have been established we recommend checking the digestion efficiency in every 3C experiment prior to proceeding with the ligation step. This is done by running the digested but not ligated aliquot (taken in step 32) on an agarose gel (Fig. 4b + c). The presence of satellite bands indicated that the DNA is well digested. The digestion pattern was compared to that of a sample of genomic DNA (gDNA) digested with the same enzyme (Fig. 4a + c). If not enough DNA appears to be digested, additional restriction enzyme may be added and digestion continued for an additional period of time.

The next step in the 3C assay comprises ligation between crosslinked DNA. To achieve this, ligation is performed at a low DNA concentration, favouring intra-molecular over inter-molecular ligation. Like the digestion efficiency, the ligation efficiency must always be checked in every 3C experiment, prior to performing qPCR experiments. This can easily be done by running an aliquot (taken in step 48) of the final 3C sample on a 0.8% agarose gel. This ligation efficiency control should run as a single band at the top of the gel, and when compared to the sample prior to ligation, there should be a clear difference between the two. Figures 4b and d show examples of satisfactory and insufficient ligations. Having checked the digestion and ligation, the DNA concentration of the 3C sample can be estimated by running a serial dilution on a gel next to a reference sample (data not shown). Next, quantitative PCR analysis of the crosslinking frequencies can be carried out as described extensively in Hagege *et al.* (Hagege *et al.*, 2007).

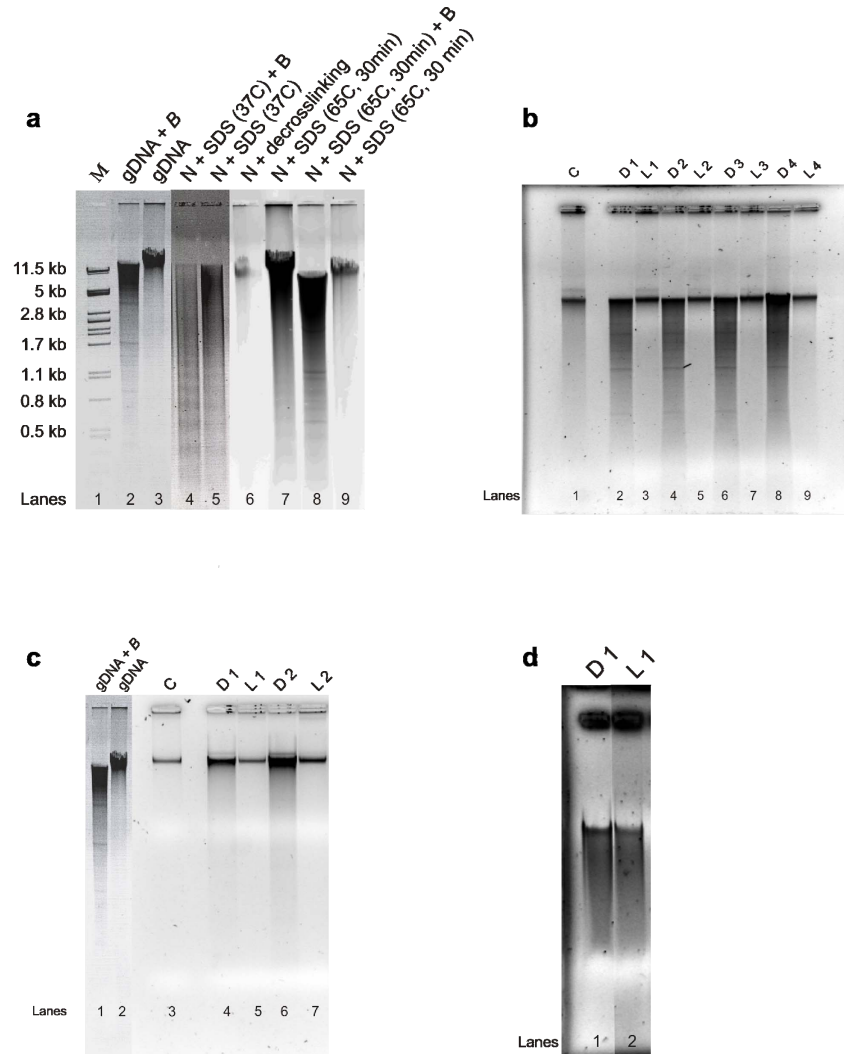


Figure 4. Digestion and ligation efficiency controls.

In all 3C experiments described in this figure, nuclei were incubated with the restriction enzyme *Bg*/III. Keys: D = digestion efficiency control; L = ligation efficiency control; M = marker; gDNA = genomic DNA; B = *Bg*/III restriction enzyme; N = nuclei; S = sample; C = control for sufficient ligation. The numbers refer to a 3C sample; from practically all 3C samples, a digestion and ligation efficiency control was analyzed.

(a) DNA degradation during SDS incubation at 37 °C. Lane 1 contains a marker indicating the sizes of the DNA fragments. Lane 2; maize genomic DNA digested with *Bg*/III as the positive control for the restriction digestion: satellite bands are clearly visible, indicating that the DNA is digested well. Lane 3; negative control: undigested gDNA. Lane 3 shows that degradation can occur during the SDS incubation step at 37 °C. Nuclei were treated with SDS for 60 min at 37 °C, followed by Triton-X incubation and

overnight (O/N) *Bgl*II digestion. The next day, the sample was de-crosslinked and purified. A clear nucleosome pattern is visible on an agarose gel. Lane 5 shows that the pattern seen on gel in lane 4 is not a result of the *Bgl*II digestion. Here, the nuclei were treated the same as in lane 4, except no enzyme was added. The same degradation pattern can be seen on gel. Lane 6 shows that the nuclei were not degraded prior to SDS incubation. Nuclei were resuspended in lysis buffer and were de-crosslinked O/N directly afterwards; no degradation was observed. Lane 7 shows nucleases can be inactivated to a large extent by SDS incubation for 30 min at 65 °C, followed by 30 min incubation at 37 °C. Next, the nuclei were treated with Triton-X and O/N *Bgl*II digestion. The following day, the sample was de-crosslinked and purified. Compared to lane 5, the majority of the DNA is now at the top of the gel and no clear nucleosome band pattern can be seen. Lane 8 shows the resulting *Bgl*II digestion (satellite bands visible!) of nuclei treated as in lane 7 (nuclease inactivation). Lane 9 shows nuclei in which the nucleases were inactivated for 60 min at 65 °C, followed by TritonX-100 inactivation and O/N *Bgl*II digestion. Even less degradation is visible, almost all DNA runs as a thick band at the top of the gel. However, 65 °C is also the temperature at which de-crosslinking occurs. To prevent the sample from de-crosslinking, nuclei were treated for 40 min at 65 °C, followed by 20 min at 37 °C, prior to Triton-X incubation and *Bgl*II digestion (see step 22 in PROCEDURE).

(b) Panel b shows a gel of satisfactory *Bgl*II digestion and following ligation for all 4 samples. Lane 1 contains a positive control for the ligation. This sample was determined as being well ligated (no smear from digested DNA visible and the DNA running as one band at the top of the gel); it was loaded on every gel for comparison. As an indication of high digestion percentages, satellite bands have to be present and the digestion pattern has to resemble the control in Fig. 4a lane 2 (gDNA + *Bgl*II). Lanes 2, 4, 6 and 8 show the digestion efficiency control in four 3C samples. Note the clear presence of satellite bands. Lanes 3, 5, 7 and 9 show the ligation efficiency control in the same four 3C samples. The ligated DNA runs at the top of the gel. These results were obtained using the protocol described in this paper, including the (plant-specific) adjustments.

(c) Panel c shows an example of poor digestion efficiency. Lanes 1 and 2 contain the positive and negative control for digestion, respectively. Lane 3 contains the positive control for the ligation. Although samples 1 and 2 look well ligated (lanes 5 and 7, respectively) compared to the control, these samples cannot be used for 3C analysis due to the low digestion efficiency (lanes 4 and 6, respectively).

(d) Panel d shows an example of poor ligation efficiency. Lane 1 shows sufficient digestion, but the sample was practically not ligated (lane 2): compare lane 1 and 2. This sample cannot be used for 3C analysis.

‘TROUBLESHOOTING’

Troubleshooting advice can be found in Table 1.

‘ACKNOWLEDGEMENTS’

The authors would like to thank Robert-Jan Palstra and Petra Klous for practical assistance in setting up 3C in plants. Laurens Bossen is thanked for providing the *Arabidopsis* picture.

Table 1. Troubleshooting guide

step	problem	possible reason	solution
5	Insufficient fixation (Check the saturation by (q)PCR signals)	Fixation buffer is not correct	Nuclei isolation and fixation buffer should not contain Tris-HCl, even though many (published) nuclei isolation/ fixation buffers do. This contains reactive amine which could cause crosslinking of the formaldehyde to Tris, leaving less formaldehyde to fix the DNA and proteins. Neither should the isolation buffer contain EDTA and EGTA; these are not compatible with the presence of Mg ²⁺ ions. The Mg ²⁺ is necessary for the stabilization of DNA in chromatin.
		Fixation time is too short, or the concentration of the crosslinking agent is not high enough	To set the optimal fixation conditions, make a time and dilutional series and check the saturation by (q)PCR
	Too much fixation (Check the saturation by (q)PCR signals)	Fixation time is too long, or the concentration of the crosslinking agent is too high	To set the optimal fixation conditions, make a time and dilutional series and check the saturation by (q)PCR
	Tissue does not look transparent after fixation	Too much tissue in the tube	Use less amount of tissue per sample. Pool samples after the fixation step if necessary.
9	Difficulties in grinding the tissue to a fine powder	Tissue is too wet	Grinding wet tissue with liquid nitrogen is difficult and less efficient. Tissue needs to be rather dry in order to grind the tissue to a fine powder and isolating sufficient nuclei.
12 (optional step)	Lots of debris surrounding the nuclei	Poor filtration of the nuclei suspension	Never squeeze the filter in order to speed up the filtration. This will result in contamination of the nuclei preparation with excess debris of for example pieces of cell wall and cytoplasm.
14	Nuclei pellet does not dissolve well		Use a cut-off P 1000 pipette tip and gently pipette the nuclei up and down. A Potter tube can also be used to dissolve big clumps of nuclei. This usually does not negatively affect the quality of the nuclei.
	Pellets of different samples have various colors	Coloring is due to the amount of starch and anthocyanins in the tissue	Pelleted maize nuclei can have different colors: usually they are green, but sometimes they can look white or purple. The white color indicates starch. The purple color indicates the presence of anthocyanins and depends on the pH value of the solution. The different colors do not seem to affect the quality of the nuclei or 3C efficiency. Do not try to wash away the color; this will only result in fewer nuclei in the end.
14 (optional step)	Broken, sheared nuclei	Centrifugation speed was too high	Do not spin down the nuclei at a higher speed than 4000-5000g in a Sorvall Centrifuge (Both 8000g and 12 000g were tested and led to breakage of the nuclei).

	Very little amount of nuclei	Too many washing steps	Limit the number of washing steps. More than two washing steps easily results in a decreased number, and a higher proportion of damaged nuclei.
25	Digestion is incomplete or does not work at all, analyzed by DNA blot analysis (Fig. 4b)	Nuclei are not dissolved well and aggregate into little clumps during the digestion	Scale up the digestion volume up to 2 ml instead of performing the digestion in 0.5 ml.
		<i>Bam</i> HI is very sensitive to SDS	The described conditions in the protocol work well in preparation of a <i>Bg</i> III, <i>Hin</i> III and <i>Eco</i> RI digestion. For a <i>Bam</i> HI digestion, the final SDS concentration to permeabilize the nuclei should be lowered to 0.15% (instead of 0.3%)
		Incubation time for restriction digestion is too short	It is important to allow sufficient time for digestion (16 h at least).
		Addition of spermidine	The use of spermidine (8 mM final concentration) is known to stimulate restriction digestions, but this was not at all the case for our 3C experiments. When spermidine was added, this had a negative effect on the restriction digestion and the DNA was not digested at all.
25 and 32	No or incomplete digestion, analyzed by DNA blot and/or gel analysis (Figs. 4b + 5c)	Triton X-100 solution too old or decayed by light	Triton X-100 is light-sensitive; it absorbs in the ultraviolet region of the spectrum. Therefore keep your solution in the dark. In addition, make a new work solution frequently. An old solution has a notable (bad) effect on the efficiency of the digestion.
32	Digestion efficiency control does not yield enough DNA to check on gel.		To check the digestion efficiency, a 30 µl aliquot can also be taken directly after step 25, followed by de-crosslinking overnight. However, in our hands, precipitation of the DNA always worked much better when we took a larger sample out of the 7.5 ml volume after the Triton X-100 incubation.
37	Insufficient separation of phenol and aqueous phases	phenol-chloroform-isoamylalcohol is too cold	Make sure the phenol-chloroform-isoamylalcohol (25:24:1) is at room temperature when you add it, even though the solution needs to be stored at 4 °C. The separation of nucleic acids from proteins occurs better at room temperature. 4 °C increases the possibility of a phase inversion. We recommend placing the phenol-chloroform-isoamylalcohol solution at room temperature the night before.

		Interphase is too large	Eppendorf's Phase-Lock Gel system for phenol extractions can be used for an easier separation (Eppendorf, PLG, Light, 50 ml, 0032.005.306)
43	Difficult to pipette off the aqueous phase No or insufficient DNA precipitation		Instead of the phenol:chloroform purification and the ethanol-precipitation of the DNA, one can also opt for purification using QIAquick PCR purification kit (Qiagen, 28104). (The number of columns needed to hold the total amount of DNA depends on the advised loading capacity by the manufacturers.) We advise using 2,5 volumes of Qiagen QG (yellow) buffer as binding buffer to reduce the total volume (QG buffer, 19063; or taken from the QIAquick Gel Extraction kit, 28704). It will take several centrifugation steps to load the entire mixture onto the columns. Qiagen's purification columns yield qualitatively good and clean DNA, but it is laborious due to the large starting volume. We suggest using this method in case the DNA does not precipitate nicely via the method presented in the protocol.
48	Ligation does not work (well) (Fig. 4d)	SDS concentration is too high for an efficient ligation	SDS reduces the ligation efficiency dramatically. If the ligation does not work well, the final SDS concentration used to inactivate the restriction enzyme can be lowered to 0,35%. Under these circumstances the restriction enzyme can still be inactivated and the ligation may work better. However, we recommend not lowering the SDS concentration unnecessarily, as the 0,35% might be very near to the limit of inactivating the restriction enzyme.
		SDS is not adequately sequestered by TX-100	The quality of the Triton X-100 has a huge effect on the ligation efficiency. It is crucial to use a relatively freshly made Triton X-100 work solution to quench all the SDS (see also troubleshooting for steps 25 and 32).
		Ligation time was not sufficient	Allow 5 h or more ligation time at 16 °C.

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