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3C Technologies in plants

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ABSTRACT

Chromosome conformation capture (3C) and 3C-based technology have revolutionized studies on chromosomal interactions and their role in gene regulation and chromosome organization. 3C allows the *in vivo* identification of physical interactions between chromosomal regions. Such interactions are shown to play a role in various aspects of gene regulation, for example transcriptional activation of genes by remote enhancer sequences, or the silencing by Polycomb-group complexes. The last few years the number of publications involving chromosomal interactions increased significantly. Until now, however, the vast majority of the studies reported are performed in yeast or animal systems. So far, studies on plant systems are extremely limited, possibly due to the plant-specific characteristics that hamper the implementation of the 3C technique. In this paper we provide a plant-specific 3C protocol, optimized for maize tissue, and an extensive discussion on (i) plant-specific adjustments to the protocol, and (ii) solutions to problems that may arise when optimizing the protocol for the tissue or plant of interest. Together, this paper should facilitate the application of 3C technology to plant tissue and stimulate studies on the 3D conformation of chromosomal regions and chromosomes in plants.

1. Introduction

Long-distance intra- and interchromosomal interactions have long been implicated in the regulation of gene expression and chromosome architecture in higher eukaryotes (see e.g. Refs. [1–8]). It was however not until the development of the chromosome conformation capture (3C) technique by Dekker et al. [9] that the role of chromosomal interactions in gene expression and genome function became widely appreciated. Since the development of 3C technology, chromosomal interactions are implicated in numerous different nuclear processes, such as gene expression [10–17], Polycomb group (PcG) protein-mediated gene silencing [18–20], X-inactivation [21,22], and chromosome architecture [23–27]. It has for example been shown that regulatory elements control the expression of genes hundreds of kilobases away by means of chromosomal interactions, and that gene expression can be affected by interactions that partition the genome in different, functionally independent loops [28,29]. In fact, 3C-based technology has revealed that DNA sequences are involved in numerous interactions (e.g. [15,30]), some of which will play a role in gene regulation, while others will play a role in other processes.

In a typical 3C experiment, chromosomal interactions are crosslinked by formaldehyde, followed by digestion of the fixed chromatin with one or several restriction enzymes and intramolecular ligation (Fig. 1). As a result, fragments that were previously far apart on a linear DNA molecule, but physically close together, will ligate together with a relatively high frequency. After reversal of the crosslinks, the ligation products are purified and the relative crosslinking frequencies, a measure for the interaction frequencies, analyzed.

3C technology allows the high-resolution detection of physical interactions between chromosomal regions [31]. While the basic 3C technique allows the detection of interactions between a limited numbers of known sequence regions, there are several variations on this technique that allow a more unbiased, genome-wide detection of interactions. 4C (3C-on-chip and Circular 3C), for example, enables the unbiased, genome-wide detection of interactions between a known sequence region and the rest of the genome [15,16,30,32,33]. 5C (3C-carbon copy) detects interactions between many known sequences, usually within one chromosomal region or a relatively small genome [26,34,35], while Hi-C is the "all-versus-all" technique that detects interactions between unknown sequences on a genome-wide scale by making use of the powerful next-generation sequencing technologies [24,25]. Besides 4C, 5C, and Hi-C type methods there are yet other variations to the 3C theme, such as methods that combine 3C technology with chromatin immunoprecipitation (ChIP) to select for interactions involving a protein of interest. Examples of the latter technology are ChIP-loop ("one-versus-one" [36]), 6C (combined 3C-ChIP-cloning, "many-versus-many" [37]) and ChIA-PET

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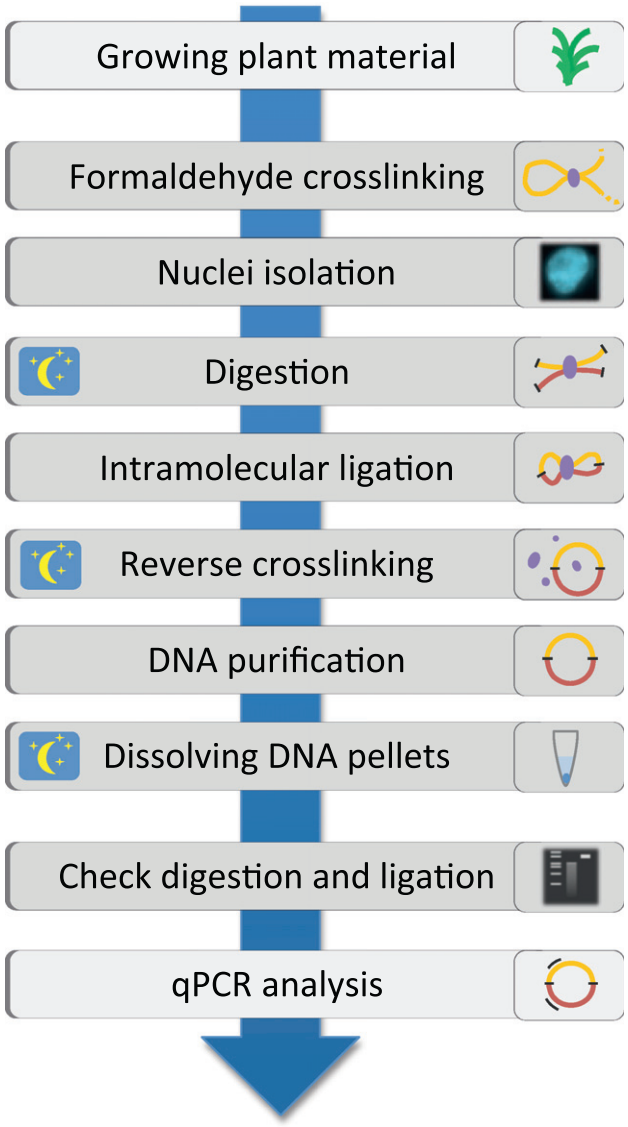

Fig. 1. Outline and timing of a 3C experiment in plant tissue. The procedure described in this paper is shown in dark grey boxes. The icons on the right side of the boxes are representative for the major steps in the protocol. The moon icons on the left side indicate overnight incubation steps.

(Chromatin Interaction Analysis Paired-End Tag, "all-versus-all" [38]) For a more elaborate discussion on all 3C-based techniques, see the recent review of de Wit and de Laat [31] and contributions to this Methods issue.

Almost all 3C protocols reported so far use yeast or animal cells as starting material. In this paper we will discuss the adjustments required to perform 3C experiments on plant tissue, and provide a plant-specific 3C protocol. Major plant-specific adjustments that will be discussed are amongst others (i) the fixation of chromosomal interactions within intact plant tissue, (ii) the isolation of nuclei from plant tissue, (iii) the inactivation of endogenous nucleases, and (iv) the recovery of ligation products.

2. Practicalities of 3C in plants

Most steps in a 3C experiment are independent of the organism or type of tissue studied. Some steps, however, need organism- or tissue-specific adjustments. Below, crucial plant-specific adjustments are discussed. In addition, the restriction digestion step and the required controls are shortly reviewed. For elaborate discussions on non-plant-specific aspects of the 3C protocol, we refer to previously published 3C protocols [39,40].

2.1. Fixation of plant tissue

Plant tissue as starting material for a 3C experiment requires several adjustments to the common procedure. Unlike mammalian tissue or cultured cells, due to its specific characteristics, plant tissue is relatively difficult to fix with formaldehyde. It is covered by a waxy surface, contains air-filled spaces between the mesophyll cells, and each cell is surrounded by a think cell wall, together hampering the fixative to enter the tissue and cells. To minimize the penetration problem, if compatible with the experiment, it is recommended to use relatively soft plant material that is rich in unexpanded cells. To increase the penetration of fixative into the tissue, the fixation is carried out under vacuum, which removes air from the tissue. To enhance the fixation efficiency further, the vacuum can be switched on and off a few times during the fixation process. The duration of crosslinking should be adjusted to the particular plant material used. The smaller the tissue area and softer the material, the shorter the fixation time should be. A good indication for successful fixation is that the tissue becomes translucent. Plant material that is rich in chlorophyll becomes in addition recognizably darker during successful fixation.

2.2. Nuclei isolation

A way to avoid the penetration problems that occur when fixing plant nuclei within intact tissues would be the prior isolation of nuclei, followed by a crosslinking step. A prerequisite of successful 3C experiments is, however, that the isolation procedure does not affect the chromosomal interactions within the nuclei. To release nuclei from plant tissue it has to be ground with liquid nitrogen, followed by various purification steps on unfrozen material. As a result, preservation of the chromatin conformation within the unfixed nuclei cannot be guaranteed. Hence, nuclei should preferably be isolated from crosslinked material, as described in the protocol presented in this paper.

The purification of nuclei involves the separation of nuclei from other plant tissue components by filtration through a nylon membrane with a defined pore size. Squeezing the filter can disrupt the membrane's pores and lead to a high amount of debris in the nuclei sample, which should be avoided. The isolated nuclei should be kept cold at any time and handled with care, as 3C experiments require a nuclei preparation of good quality. To avoid damaging the nuclei, the centrifugation steps should be limited and carried out at low speed. The nuclei should preferably be used immediately for a 3C experiment, instead of first being stored at −80 °C. In our hands, the digestion efficiency is higher in freshly isolated nuclei than in nuclei that were stored at −80 °C.

2.3. Inactivation of endogenous nucleases

In published 3C protocols [39,40], prior to adding the restriction enzyme, nuclei are treated with 0.3% SDS at 37 °C to permeabilize the nuclei and inactive remaining enzyme activities. Nuclei, such as those of maize, however, can contain relatively high concentrations of endogenous nucleases [4,41,42], degrading the DNA once the nuclei are incubated at 37 °C (step 3.5.2). Such DNA decay can be circumvented by incubating the nuclei at 65 °C prior to incubating at 37 °C. At 65 °C, however, formaldehyde crosslinks are being reversed, and as a result chromatin interactions lost. Therefore, the incubation time at 65 °C should be kept as short as possible to prevent extensive reversal of the crosslinks. The activity of endogenous nucleases may differ between different sources of nuclei. Therefore, when using other types of tissue then used to