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Investigation of False Positive Issues in Plant Single-Chromosome Sequencing (Postprint)

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Abstract

Single-cell sequencing technology is currently widely applied in research on humans, animals, microorganisms, and other species; however, in the plant kingdom, the presence of cell walls has made its application extremely challenging. If single plant chromosomes can be isolated and subsequently amplified and sequenced using single-cell sequencing technology, interference from cell walls can be avoided, which holds significant application value. Researchers have continuously attempted to sequence single plant chromosomes, yet to date, no successful reports have been published, nor have any articles discussed the reasons for failure. This study employs hexaploid Chinese Spring wheat as material to investigate and discuss the false-positive issues arising during single-chromosome amplification using single-cell sequencing technology, and analyzes the primary causes of amplification failure. Furthermore, using high-throughput sequencing technology, we examined the potential sources of exogenous contamination, demonstrating that under conditions of strict control over laboratory equipment, consumables, and environmental contamination, human contact may constitute the primary contamination source. Additionally, we hypothesize that single-chromosome amplification failure may result from the supercoiled state of chromosomes themselves, which hinders primer binding and replication, and propose feasible recommendations to address these issues. This study provides valuable insights for future successful single-chromosome sequencing.

Full Text

Preamble

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Title: Investigating the Problem of False Positives in Plant Single Chromosome Sequencing

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Abstract

Single-cell sequencing technology is widely used in research on humans, animals, microorganisms, and other species. However, its application in plants has been challenging due to the presence of cell walls. If single plant chromosomes can be isolated and then amplified and sequenced using single-cell sequencing techniques, the interference from cell walls can be avoided, which would hold significant application value. Researchers have long attempted to sequence individual plant chromosomes, yet to date there have been no successful reports, nor have any articles discussed the reasons for failure. This study used hexaploid Chinese Spring wheat as material to investigate and explore the problem of false positives that occur during single-chromosome amplification using single-cell sequencing technology. We analyzed the main causes of failure in single-chromosome amplification and used high-throughput sequencing to investigate possible sources of exogenous contamination. Our results indicate that, based on strict control of experimental equipment, consumables, and environmental contamination, human contact may be the primary source of contamination. We also hypothesize that single-chromosome amplification failure may be caused by the superhelical state of chromosomes themselves, which prevents primer binding and replication. Finally, we propose feasible suggestions to address these issues. This study provides valuable lessons for future successful single-chromosome sequencing.

Keywords: single chromosome, single-cell sequencing, exogenous contamination, false positives, MDA, MALBAC

Introduction

Single-cell sequencing technology represents one of the most active areas in life sciences research. This technique enables high-throughput sequencing analysis of genomes, transcriptomes, and epigenomes at the single-cell level using extremely limited template material, thereby capturing cellular heterogeneity information that cannot be obtained through conventional tissue samples. It has been widely applied in human disease diagnosis, early cancer detection, developmental biology, microbiology, and neurobiology (Huang et al., 2015; Navin et al., 2011; Lasken, 2007). Currently, the most commonly used techniques for single-cell whole-genome sequencing include Multiple Displacement Amplification (MDA) (Hosono et al., 2003) and Multiple Annealing and Looping-Based Amplification Cycles (MALBAC) (Zong et al., 2012). While single-cell sequencing technology has been widely applied in humans, animals, and microorganisms, its use in plant research remains limited, primarily due to the presence of cell

walls that make isolation and lysis difficult using conventional methods. Yan Jianbing' s team (Li et al., 2015) developed a simple and feasible method to successfully isolate maize microspore tetrads and performed whole-genome sequencing using single-cell sequencing technology, achieving several innovative scientific discoveries. This marked the first successful application of single-cell sequencing technology in plants. However, there have been no successful reports of its application at the chromosome level.

Plant chromosomes represent the superhelical form of chromatin during mitosis or meiosis, forming a higher-order structure that is easily distinguishable and isolable. This raises the question of whether single-cell sequencing technology can be used for in vitro amplification and sequencing of individual plant chromosomes. Since single-cell sequencing technology can sequence single microbial cells, and most bacterial genomes are smaller than ten megabases—considerably smaller than many individual plant chromosomes—theoretical feasibility exists. Isolating chromosomes for in vitro amplification can avoid cell wall interference, and several successful cases of isolating and amplifying single chromosomes have been reported, though these primarily used PCR-based whole-genome amplification techniques such as Degenerate Oligonucleotide-Primed PCR (DOP-PCR) (Telenius et al., 1992) and Ligation-Mediated PCR (LA-PCR) (Chen & Armstrong, 1995). However, these techniques suffer from issues including non-specific amplification, amplification bias, and low genome coverage. If single-cell sequencing technology could be applied to amplify individual plant chromosomes, these problems could be fundamentally resolved, offering promising application prospects. Unfortunately, few studies have reported on this approach. Yin Hui et al. (2015) attempted to apply single-cell sequencing technology for in vitro amplification of single chromosomes in rubber trees, but no follow-up reports have been published.

In vitro amplification of single plant chromosomes presents challenges beyond working with extremely limited templates; preventing exogenous contamination is also a critical aspect of the technology. Effective contamination control is a key factor ensuring correct amplification of trace templates and subsequent sequencing quality. However, due to the high sensitivity of MDA and MALBAC-based amplification, even trace amounts of exogenous nucleic acid templates can cause contamination amplification and escalation, leading to false-positive phenomena and amplification failure. Exogenous contamination during amplification may originate from the experimental environment, reagents, water, various buffers, consumables such as pipette tips and centrifuge tubes, and glass slides and needles used during chromosome preparation and isolation (Wang et al., 2010). Additionally, aerosol contamination in laboratory environments cannot be ignored (Du et al., 2015). Therefore, strict control of exogenous contamination at every step of plant single-chromosome in vitro amplification is essential to ensure high-quality amplification.

Currently, no successful reports of single-chromosome sequencing exist. Through communication with sequencing companies and researchers in related

fields, we found that false-positive phenomena are widespread while target chromosomes fail to amplify effectively. No systematic studies or feasible suggestions have been proposed to address this issue, particularly regarding insufficient understanding of the causes of false positives and main sources of contamination, which hinders the development of targeted contamination control strategies and experimental improvements.

This study attempted to isolate and amplify single wheat chromosomes in vitro using single-cell sequencing approaches. Under strict contamination control, we investigated the commonly encountered false-positive problems during experiments, explored their possible sources and causes, and proposed feasible suggestions to address the main issues in single-chromosome sequencing. Our aim is to provide experience and reference for future plant single-chromosome sequencing endeavors.

Materials and Methods

1.1 Plant Materials

Seeds of hexaploid Chinese Spring wheat (2n=6×=42) were provided by Professor Wang Daowen' s research group at the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences.

1.2.1 Preparation and Isolation of Wheat Metaphase Chromosomes

Chinese Spring wheat seeds were soaked in cold water at 4°C for one day, then cultured in darkness at 25°C. When root tips reached 1.5–2.0 cm in length, they were excised with a sharp blade and pretreated with 0.1% colchicine for 3 hours. After washing with sterile water, root tips were fixed in Carnoy's fixative (glacial acetic acid:ethanol = 1:3) at low temperature for 5 minutes, then transferred to 70% ethanol and stored at 4°C. For use, required root tips were removed with forceps, rinsed three times with sterile water, and drained. Twenty microliters of cellulase and pectinase (4% and 3% v/v, respectively) were added, and samples were digested at 37°C for 45 minutes. After digestion, samples were washed three times with sterile water, drained, and 10 L of carbol fuchsin stain was added. Root tips were crushed with a pipette tip, briefly incubated, and then squashed. Prepared slides were placed at -70°C for 30 minutes, coverslips were quickly removed, and slides were examined under an inverted microscope for microdissection using fine glass needles (Nikon Ti-S research-grade inverted micro-manipulation system). Single wheat chromosomes were isolated, and simultaneously, single intact cells containing all metaphase chromosomes were isolated as controls.

1.2.2 Chromosome Amplification Using MALBAC

Isolated single intact cells containing all chromosomes and single wheat chromosomes were amplified using the MALBAC Single Cell Whole Genome Amplifica-

tion Kit (KT110700324) from Yikon Genomics. The procedure followed the manufacturer's instructions (http://www.yikongenomics.com/upload/2017/1124/KT1107003_MALBACweiliangjiy 40c9e.pdf). According to the manual, amplification cycles were set to 21 cycles for single-chromosome amplification and 17 cycles for conventional amplification. Amplification products were detected using 1% agarose gel electrophoresis.

1.2.3 Chromosome Amplification Using MDA

Isolated single intact cells containing all chromosomes and single wheat chromosomes were amplified using Qiagen' s REPLI-g Single Cell Kit (Cat No./ID: 150343). The procedure followed the manufacturer' s instructions (https://www.qiagen.com/cn/resources/resourcedetail?id=38faca1c-64b0-4281-aab3-aa8324bbd181&lang=en). Amplification products were detected using 1% agarose gel electrophoresis. In parallel with in-house amplification using the kit, some single chromosomes were isolated and entrusted to Annoroad Gene Technology Co., Ltd. for in vitro amplification using their lysis buffer.

1.2.4 Pollution Source Exclusion Experiments

To investigate false-positive phenomena during chromosome amplification and exclude possible sources of exogenous contamination, we commissioned Yikon Genomics to test sterile water, PBS solution, centrifuge tubes, and the kit itself using MALBAC technology. The experimental design is shown in .

Table 1 Experimental design for screening pollution source

Test	Materials	Details
Test 1	The suspected PBS, water and blank control, each repeated twice	0.2 ml PCR tubes, Qualified kit (1806B)
Test 2	The suspected PBS, water and blank control, each repeated twice	Qualified 0.2 ml PCR tubes, Qualified kit (1806B)
Test 3	Qualified nuclease-free water and blank control, each repeated twice	Test sample of 0.2 ml PCR tubes, Qualified kit (1806B)
Test 4	Qualified nuclease-free water and blank control, each repeated twice	Qualified 0.2 ml PCR tubes, The suspected kit (1702A)

Note: Comparison between Test 1 and Test 2 determines whether 0.2 ml PCR tubes cause contamination; comparison between Test 1 and Test 3 determines whether submitted samples (water and PBS) cause contamination; blank controls in Test 2 and Test 4 determine whether reagents cause contamination.

1.2.5 SCoT Molecular Marker Detection

SCoT (Start Codon Targeted) markers detect polymorphism at translation initiation codons and can therefore determine whether exogenous contamination originates from foreign species genes or from primer dimer formation. SCoT primers were synthesized according to sequences published by Collard & Mackill (2009) and Luo et al. (2011). The SCoT primers used in this study for PCR detection of single-chromosome amplification products are listed in .

Table 2 SCoT primers used for checking single-chromosome amplification products

SCoT Primer	Sequence (5 -3)
P17	ACCATGGCTACCACCGAG
P21	ACCATGGCTACCACCGCG
P34	ACGACATGGCGACCCACA

PCR reactions were performed in 20 L volumes containing 10 L 2×Taq PCR Mix, 1.0 L amplification product (diluted 1,000-fold, ~30 ng · L ¹), 0.8 L 10 mol · L ¹ primer, and 8.2 L ultrapure water. The thermal cycling program was: 94°C for 5 min; 35 cycles of 94°C for 45 s, 50°C for 1 min, 72°C for 2 min; and a final extension at 72°C for 10 min. Eight microliters of amplification product were analyzed using 2% agarose gel electrophoresis.

1.2.6 Southern Hybridization Identification

Wheat whole-genome DNA digests, chromosome amplification products, and negative controls were electrophoresed together and transferred to positively charged Hybond nylon membranes via blotting. Wheat whole-genome DNA was digested, labeled, and used as a probe for hybridization. The procedure followed the manufacturer’ s instructions for the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche).

1.2.7 High-Throughput Sequencing

False-positive amplification products from wheat chromosomes were selected for single-cell genome MDA library construction. After quality control, libraries were sequenced on the Illumina platform with PE150 read length. High-quality filtered data were randomly sampled for 10,000 reads, which were blasted against the NT database using BLAST software to analyze potential contamination sources.

Results

2.1 Preparation and Microdissection of Wheat Metaphase Chromosomes

Under 40× magnification, well-dispersed and clear wheat metaphase chromosome spreads were identified. Fine glass needles were prepared, and single wheat chromosomes were accurately picked using a micromanipulator. Chromosomes were adsorbed onto the needle tip via electrostatic forces and quickly transferred to 200 L centrifuge tubes containing lysis buffer, briefly centrifuged at 8,000 rpm for 20 seconds, and used for subsequent amplification experiments. [Figure 1: see original paper]

2.2 False-Positive Phenomena in MALBAC and MDA Amplification of Wheat Chromosomes

Amplification results using the MALBAC Single Cell Whole Genome Amplification Kit (KT110700324) and the REPLI-g Single Cell Kit (Cat No./ID: 150343) based on MDA technology showed that amplification bands from intact single cells containing all chromosomes (Lane 1) were essentially identical to those from single chromosomes (Lane 2). However, both sterile water negative controls (Lane 3) and PBS negative controls (Lane 4) also produced bands clearly similar to target samples in both methods. Both single-cell sequencing kits exhibited false-positive phenomena, suggesting possible exogenous DNA contamination during amplification. [Figure 2: see original paper]

2.3 Exclusion of Possible Contamination Sources

To exclude potential causes of false positives, we commissioned Yikon Genomics to test water, PBS solution, centrifuge tubes, and the kit itself using MALBAC single-cell amplification technology. The results showed no detectable amplification or bands in water, reagents, or consumables used for in vitro amplification [Figure 3: see original paper]A, while the positive control showed clear bands [Figure 3: see original paper]B, indicating contamination was within controllable limits. However, we learned that the company used 17 amplification cycles in the second round rather than the 21 cycles used in our single-chromosome amplification experiments. When we repeated amplification with 17 cycles, neither chromosomes nor negative controls produced bands, demonstrating that higher cycle numbers in MALBAC technology readily generate non-specific false-positive amplification.

2.4 Preliminary Validation of Amplification Results Using SCoT Molecular Markers

If reagents and consumables did not produce obvious exogenous contamination, why did increasing cycle numbers in MALBAC or using MDA technology produce clear false-positive phenomena? What are the possible causes? We first

used SCoT molecular markers to determine whether exogenous contamination originated from foreign species genes or from primer dimer formation. Amplification results showed that negative controls and MALBAC-amplified samples produced no clear polymorphic bands, while MDA-amplified samples using primers P17 and P21 showed clear bands in two samples: one containing all chromosomes from a single cell (Sample 3) and one single chromosome (Sample 5), suggesting these might represent genuine amplification of foreign genes. [Figure 4: see original paper]

2.5 Validation Using Southern Blotting

To further verify whether Samples 3 and 5 represented true amplification of wheat chromosomes or amplification of foreign species genes, we performed Southern blot validation. The MDA amplification products from these two samples were hybridized with probes labeled from wheat genomic DNA. Results showed no hybridization signals in either sample or negative controls, except in the genomic positive control [Figure 5: see original paper]. This confirmed that the amplification products were likely caused by exogenous species DNA contamination.

2.6 High-Throughput Sequencing and Sequence Alignment to Validate Contamination Type and Source

To further confirm the source and type of contamination, we performed whole-genome sequencing on amplification products from Samples 3 and 5, along with two samples (MDA01 and MDA02) amplified by Annoroad Gene Technology Co., Ltd. Sequence alignment of all four samples showed no mapping to the wheat genome, but instead mapped to human (*Homo sapiens*), microorganisms (*Propionibacterium*, *Cutibacterium acnes*), and chimpanzee (*Pan troglodytes*) . Notably, *Cutibacterium acnes* (formerly *Propionibacterium acnes*) and *Propionibacterium phage* are microorganisms that inhabit human skin (Brigitte et al., 2018; Lood & Collin, 2011).

Table 3 Results of samples blasted to NT database

Samples	Hit 1	Hit 2
MDA01	*Homo sapiens*	Unknown
MDA02	*Homo sapiens*	*Cutibacterium acnes*
Sample 3	*Propionibacterium phage*	*Homo sapiens*
Sample 5	*Propionibacterium phage*	*Pan troglodytes*

Discussion

Isolating single chromosomes for in vitro amplification to establish single-chromosome DNA libraries holds important application value for constructing high-density molecular marker linkage maps, isolating important genes,

physical mapping, and chromosome evolution studies. In high-throughput whole-genome sequencing, complex genomes with polyploidy, high heterozygosity, or abundant repetitive sequences create difficulties in subsequent assembly and alignment, resulting in low quality and efficiency. If single chromosomes could be isolated, amplified, and sequenced, the difficulty of physical map construction would be substantially reduced.

In recent years, numerous institutions have attempted single-chromosome sequencing using single-cell sequencing technology, but no successful reports have emerged, primarily due to severe false-positive amplification from exogenous contamination. Based on strict contamination control, this study investigated false-positive problems in single-chromosome sequencing, explored possible contamination pathways and sources, and offers the following perspectives:

First, false-positive phenomena are difficult to completely avoid. Single-chromosome isolation and in vitro amplification involve multiple steps, each requiring numerous reagents, consumables, and different operating spaces, making absolute contamination control challenging. Second, when using MALBAC technology, higher cycle numbers in the second round increase the probability of false positives, while MDA technology often produces false positives in negative controls (Yilmaz et al., 2010; Marcy et al., 2007). Both single-cell sequencing technologies exhibit high sensitivity to templates, and false positives may result from amplification of trace exogenous DNA when target samples are absent, or from primer dimer formation. When target samples are present and amplify preferentially, exogenous contamination or dimer formation is suppressed. Additionally, sequencing analysis of false-positive products revealed that contaminants were closely related to the human body, with relatively few from other sources, suggesting that environmental, reagent, and consumable contamination are relatively controllable. However, human-derived exogenous DNA cannot be degraded or destroyed using conventional methods. Therefore, direct contact between any part of the body and experimental reagents, consumables, or even the operating environment must be strictly avoided. Disposable shoe covers, masks, hats, and lab coats should be worn throughout the procedure to minimize exogenous contamination.

Second, single-chromosome amplification failure may not result from insufficient template quantity but rather from ineffective amplification of the chromosomes themselves. In this study, amplification of all 42 chromosomes from a single intact cell also failed to obtain target genome amplification information, despite the wheat genome size being 15.4–15.8 Gb (Appels et al., 2018), larger than most species successfully sequenced using single-cell technology. We therefore hypothesize that the main reason for failure in wheat single-chromosome amplification using single-cell technology may be chromosome morphology. The highly condensed superhelical state of chromosomes may hinder histone removal and DNA denaturation, and conventional lysis conditions may be insufficient to convert DNA from this superhelical state to a linear form that can effectively bind primers and initiate replication. Additionally, residual fixatives and stains from

chromosome preparation may affect histone digestion and DNA polymerase reactions.

To successfully apply single-cell sequencing technology for in vitro amplification of single chromosomes, we recommend the following approaches: (1) Ensure a completely sterile environment throughout the operation. All instruments and vessels must be strictly sterilized by autoclaving and UV irradiation. Commercial kits that have undergone rigorous sterilization should be selected for in vitro amplification. Experimental water and other reagents must be strictly sterilized and quality-tested to ensure absence of exogenous DNA contamination. Strict personal protection should be maintained to minimize exposure of body parts to the environment. Sterile water negative controls should be included throughout. Ideally, newly established laboratories and clean benches should be used, with chromosome isolation and amplification performed in separate laboratories to prevent aerosol contamination. (2) Remove histones as thoroughly as possible using high-salt buffers, proteinase K digestion, and extended digestion times. Combine these with biological enzymes (such as topoisomerase) and mechanical centrifugal force to convert chromosomes to a loose linear state. (3) Evaluate the impact of different reagents used in chromosome preparation on subsequent experiments, select the most appropriate chromosome preparation and staining methods, and reduce or eliminate these effects through shortened operation time, neutralization reactions, and solvent washing. We recommend using gentler methods to obtain chromosome suspensions, followed by direct isolation using fine glass needles (or tubes) without staining before subsequent lysis and amplification reactions.

Based on our analysis and in-depth investigation of the causes of single-chromosome sequencing failure, our research team has successfully isolated and amplified single plant chromosomes from wheat by improving chromosome suspension preparation and isolation methods and adding proteinase K digestion steps. Southern hybridization validation has been performed, showing relatively weak hybridization signals. Quality assessment is currently underway, and the results will be reported in a subsequent article.

This study specifically analyzed and discussed the common problems of amplification failure and false positives encountered during single-chromosome in vitro amplification and sequencing using single-cell sequencing technology, providing feasible suggestions and valuable references for further improvement of plant single-chromosome amplification technology.

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