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适用于棉花荧光原位杂交的 DNA 纤维高效制备技术

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摘 要: 棉花富含酚类和多糖等高分子次生化合物, 细胞质浓厚, 且染色体形态小、数目多、制片困难, 至今还未见棉花 DNA 纤维制备方面的报道。本研究用“刀切引流法”, 在含有 Triton X-100 和 PVP_{40} 的冰冷细胞核提取缓冲液中, 用锋利的刀片切割发育一周的棉花黄化子叶以释放棉花细胞核, 所得细胞核干净完整杂质少, 不需要研磨和巯基乙醇等处理, 方便快捷无毒害, 成功率达到 100%。细胞核在室温下经温和碱裂解去除染色质上的蛋白质后, 以前端导引裂解液铺展载玻片, 即“引流法”拉伸制备 DNA 纤维, 避免了液体表面张力的影响, 消除了因载玻片推抹用力不均而导致的 DNA 纤维堆积和断裂, 所制备的 DNA 纤维平直完整、伸展程度均匀、背景清晰。用基因组和 45S rDNA 分别标记探针进行杂交, 结果表明所制备的棉花 DNA 纤维适用于荧光原位杂交。本研究探索出一套简单、高效、快捷、无毒害的适宜于棉花荧光原位杂交的 DNA 纤维制备技术, 必将为棉花基因组研究和全基因组序列的最终完成提供强有力的技术支持。

关键词: 棉花; 荧光原位杂交; DNA 纤维; 高效制备

An Efficient Method of Cotton DNA Fibers Preparation for FISH

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Abstract: Fluorescence in situ hybridization (FISH) has become the most important technique applied in molecular cytogenetics, especially in developing physical maps in plants. As a key technique, FISH on cotton DNA fibers stretched has not been reported yet, possibly owning to the difficulty in their DNA fiber preparation as well as the existence of thick cytoplasm and hard cell walls. Here we present a method of highly efficient preparation of stretched DNA fibers in cotton. Cotton cotyledons germinated in dark moisture chamber for one week were chopped with a sharp sterile scalpel in a Petri dish that contained ice-cold nucleus isolation buffer ($MgSO_4$ 10 mmol L^{-1}, KCl 5 mmol L^{-1}, HEPES 0.5 mmol L^{-1}, DTT 1 mg mL^{-1}, Triton X-100 0.25%, PVP_{40} 2%) followed by sequential filtration through 100, 50, and 30 μm nylon meshes. Nuclei were obtained by centrifuging the filtrates at 16 000× *g* for 1 min. Mixture of nucleus lysis buffer(0.5% SDS, 5 mmol L^{-1} EDTA, 100 mmol L^{-1} Tris, pH 7.0) and nuclei was incubated on the slide for 9 min, DNA fibers obtained by dragging and stretching with a clean slide edge from the end to another end on the liquid surface. After incubated at 60℃ overnight, the slides were pretreated with DNase-free RNase and then rinsed in 2×SSC. The probes and DNA fibers were denatured separately, and hybridization mixture was incubated on the slide overnight, followed by post-hybridization rinses in 2×SSC, 1×4T. The slides were blocked with 5% BSA and covered with antibody for 1h. After rinsed with 1×TNT the slides were counterstained with 4’,6-diamidino-2-phenylin-dole (DAPI) and followed by rinsing in 1×PBS. After mounting the slides with Vectashield mounting medium the hybridization signals were observed under a fluorescence microscope. Images were captured by a charge-coupled device (CCD) system and brought together to make the plate using Adobe Photoshop 7.0 software. The results indicated that it was easier to release nuclei from cells in nucleus isolation buffer by chopping cotyledon, and slowly and smoothly dragging the nuclei solution with a slide edge from the end to another end on slide treated

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with poly-L-lysine. Highly stretched and intact DNA fibers were obtained. This method is very simple and rapid, which takes only 30 min to finish the entire process, and it is also safe because poisonous mercaptoethanol is replaced by dithiothreitol. The linear or near-linear stretches of beads on-a-string signals with cotton genomic DNA and 45S rDNA as probes showed that the DNA fibers were suitable for FISH.

Keywords: Cotton; FISH; DNA fibers; Efficient preparation

荧光原位杂交(fluorescence in situ hybridization, FISH)是一种非放射性原位杂交技术，因其具有直观、安全可靠、灵敏度高、可放大信号、定量或非定量分析等优点，在高度或中度重复 DNA 序列的鉴定、低或单拷贝 DNA 序列及遗传标记的物理定位、异源染色质和非整倍体的鉴定、染色体物理图的构建、比较基因组研究、物种进化、转基因检测等方面得到广泛的应用。其中 DNA 纤维荧光原位杂交技术(DNA fiber-FISH)具有分辨率高、直观、准确等特点而成为基因组测序中不可替代的技术[1]。主要用于分析重叠克隆群[2]、检测染色体重排[3]、判断基因间距离和基因长度[4]、估算 gap 的大小、测量 DNA 位点的大小[5-7]并最终加速图位克隆[8]。然而，自 Fransz 等[9]从番茄和拟南芥中制备出 DNA 纤维进行荧光原位杂交后，该技术只在很少几种植物中得到应用[10-13]，主要是植物有细胞壁，而且多含有次生代谢物质，植物的 DNA 纤维制备技术还很不成熟。棉花基因组研究发展很快，包括基因组测序已进入实质性程序[14-16]。所以，探索棉花 DNA 纤维的荧光原位杂交技术，对于棉花分子细胞遗传学和基因组学等方面的研究均有重要意义。

棉花富含酚类和多糖等高分子次生化合物，细胞质浓厚，且染色体形态小、数目多，制片更加困难，致使棉花 FISH 研究滞后于水稻、玉米和小麦等作物[17-22]。近年来的棉花荧光原位杂交研究主要集中于有丝分裂中期染色体和减数分裂染色体[23-32]，至今还未见棉花 DNA 纤维制备方面的报道。本研究借鉴 Li 等[33]制备水稻等植物 DNA 纤维技术，结合棉花的特性，探索出一套高效的适宜于棉花荧光原位杂交的 DNA 纤维制备方法，为棉花基因组学研究和全基因组测序提供技术支持。

1　材料与方法

1.1　材料

二倍体的亚洲棉品种石系亚 1 号，来自中国农业科学院棉花研究所。45S rDNA 探针由美国 Cornell 大学的戴丝兰和吴瑞博士提供。实验试剂 HEPES 购自 Amresco 公司, DTT 购自 Bio Basic Inc., Triton X-100、SDS 购自 Sigma 公司，DAPI 购自 Roche 公司，EDTA 购自 Solarbio, Tris 购自 Bio Basic Inc，其他均为国产分析纯试剂。

1.2　黄化子叶的准备

细沙经水煮消毒后晾至紧握成团和松手即散的程度。棉花种子经约 37℃的温水中浸种 12~24 h，播种于细沙中, 30℃恒温暗室培养 2~3 d 后即长出幼嫩黄化的子叶来。

1.3　细胞核的提取

取 2 g 发育 1 周的幼嫩黄化子叶，立即置冰上直径 5 cm 的培养皿中，加 12.5 mL 细胞核提取液($MgSO_4$ 10 mmol L^{-1}, KCl 5 mmol L^{-1}, HEPES 0.5 mmol L^{-1}, DTT 1 mg mL^{-1}, Triton X-100 0.25%, PVP_{40} 2%)，用消毒无菌的锋利刀片将子叶切成细小颗粒制成悬浮液，分别以孔径为 170、100、50 和 30 μm 的尼龙膜过滤(所有操作需在冰上完成)。4℃下离心(16 000×*g*) 40 s，除去上清液，将沉淀物悬浮在 125 μL 的细胞核贮存液中($MgSO_4$ 10 mmol L^{-1}, KCl 5 mmol L^{-1}, HEPES 0.5 mmol L^{-1}, DTT 1 mg mL^{-1})，取其 1 μL 用 DAPI 染色，在荧光显微镜下检查细胞核的浓度，理想的浓度为每毫升 5×10^6 个细胞核。细胞核悬浮液可在 50%的甘油中–20℃保存数月。

1.4　DNA 纤维的制备

载玻片在 5 mol L^{-1}HCl 中煮沸 2~3 h，经双蒸水冲洗后空气干燥，再经过滤的 1×10^{-6} g mL^{-1} 的多聚赖氨酸(MW=30 000)浸没过夜，冲洗干净，备用。取 1 μL 细胞核悬浮液，置经多聚赖氨酸处理的载玻片 A 的一端，用枪头轻轻均匀地往返涂使悬浮液成平行于载玻片短边的一条线，空气中室温下晾约 3~5 min 至半干。在其上覆盖 30 μL 细胞核裂解液(0.5% SDS, 5 mmol L^{-1} EDTA, 100 mmol L^{-1} Tris, pH 7.0)，室温温育 9 min。取另一洁净载玻片 B，令其一端边沿轻轻接触细胞核裂解液，将 A 载玻片涂有细胞核的一端提起，慢慢使之与桌面成 45°角，同时令载玻片 B 拉着悬浮液涂抹整个载玻片 A，游离的 DNA 便会形成平行的 DNA 纤维。空气干燥 10 min 后，在卡诺液中固定 2 min, 60℃烘干 30 min，立即使用，或 4℃存放 5~7 d。

1.5　荧光原位杂交(FISH)

1.5.1　制片准备　　制片在 60℃烘箱中干燥过夜，

用 100 μg mL^{-1} 的 Rnase A (2×SSC 配制), 37℃温育 30 min; 2×SSC 漂去盖玻片, 2×SSC 洗 3 次, 每次 5 min; 0.10%~0.01% Pepsin(10 mmol L^{-1} HCl 配制), 37℃温育 20 min; 2×SSC 漂去盖玻片, 2×SSC 洗 2 次, 每次 5 min; 4%多聚甲醛(*W/V*, 现用现配)于 37℃下固定 10 min, 2×SSC 洗 2 次, 每次 5 min; 70%、90%、100%乙醇顺序脱水, 每次 3 min, 晾干备用。

1.5.2 探针标记 对 45S rDNA 用生物素(Biotin-dUTP)、石系亚 1 号 gDNA 用地高辛(Dig-dUTP)分别进行缺口平移法探针标记：取模板 DNA 2 μL, 加双纯水 14 μL, Biotin-Nick Translation Mix or Dig-Nick Translation Mix 4 μL, 放入 PCR 仪 15℃ 90 min 后, 加 0.5 mol L^{-1} 的 EDTA 1 μL 终止反应, –20℃保存备用。

1.5.3 原位杂交 杂交液总体积为 20 μL, 含 100%去离子甲酰胺 10 μL, 50%硫酸葡聚糖 4 μL, 20×SSC 2 μL, 10% SDS 1 μL, 荧光素标记的探针 1 μL, 用灭菌的去离子水补足 20 μL。将杂交液混匀, 于 90℃~97℃变性 8~10 min, 转入冰水中处理 10~15 min。取 20 μL 变性的杂交液滴于制片上, 加塑膜盖片, 转入 37℃水浴中杂交过夜。

1.5.4 洗脱 2×SSC, 常温洗 5 min; 2×SSC, 42℃洗 10 min; 2×SSC, 常温洗 5 min; 1×4T, 常温洗 5 min; 每张片子加 30 μL 5% BSA, 37℃温育 30 min, 1×TNT 洗 3 次, 每次 5 min; 每张片子加 30 μL 的 Avidin-fluoresecein, 37℃下温育 1 h (浓度 5 ng μL^{-1}), 1×TNT 洗 3 次, 每次 5 min; 甩干(制片不能见干), 载片用 DAPI 衬染; 用 1×PBS 洗 2 次, 每次 3 min, 晾干后用 Vectashield 封片。

1.6 染色拍照保存

在蔡司荧光显微镜下检查 DNA 纤维的质量和杂交结果, 并拍照。

2 结果与分析

2.1 细胞核的提取

采用研磨法制备棉花细胞核, 细胞核破坏严重、产率较低, 而且很多细胞质释放物难以除净(图 1-A); 如果使用刚刚发育 3 d 的子叶, 即使在提取液中加入 Triton X-100 和 PVP_{40}, 细胞核提取物中仍然含有很多其他杂质(图 1-B), 均影响细胞核的提取质量。如果采用超过 1 周以上的黄化处理, 子叶趋于老化、腐烂, 提不出细胞核。而采用锋利刀片直接切割发育 1 周的棉花黄化子叶, 很容易释放出细胞核, 不需要精细研磨, 细胞核受到破坏小, 细胞核提取成功率可达到 100% (图 1-C)。再则, 在细胞核提取液中加入 Triton X-100 和 PVP_{40} 可以去除棉酚等次生代谢物质, 不需用有毒试剂巯基乙醇等处理。此种“刀切法”, 制备棉花细胞核成功率高, 方便快捷无毒害。

2.2 DNA 纤维的制备

染色质是由 DNA 双链和蛋白质组成, 制备 DNA 纤维, 关键是除净染色质上的蛋白质。在室温条件下, 采用温和碱裂解法释放游离的 DNA 纤维, 用我们配制的裂解液浓度设计了 3、5、7、9、12 和 16 min 共 6 个温育时间, 发现蛋白质去除程度不同(图 2), 其中 9 min 蛋白质基本被除净, 释放出游离的 DNA 纤维(图 2-D)。处理时间过短, DNA 和蛋白质不能分开, 但处理时间过长, DNA 受到破坏甚至降

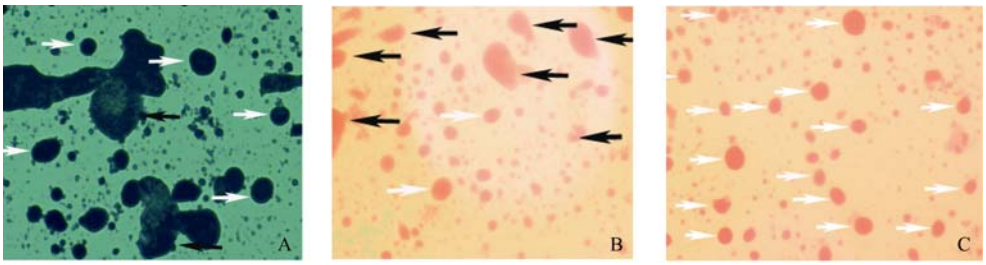

图 1 制备棉花细胞核三种方法的比较

Fig. 1 Comparison of three methods for preparing cotton cell nuclei

A: 用液氮研磨法制备细胞核; B: 用刀切法从黄化处理 3 d 的子叶中提取的棉花细胞核; C: 用刀切法从黄化处理 1 周的子叶中提取的棉花细胞核; 白色箭头指示细胞核, 黑色箭头指示杂质; 细胞核用 DAPI 染色(×400)。

A: Nuclei of cotton prepared with liquid nitrogen grinding method; B: Nuclei of cotton prepared with chopping method from fresh etiolated young cotyledon grown for 3 days; C: Nuclei of cotton prepared with chopping method from fresh etiolated young cotyledon grown for one week. The white arrows indicate the nuclei; the black arrows indicate the impurities. The cell nuclei were stained with DAPI(×400).

解, 得不到完整的 DNA 纤维。然而, 对于不同的裂解液浓度, 需进一步实验验证才能确定最佳温育时间。

伸展 DNA 纤维常用方法有两种, 一是重力自然拉伸法, 一是载玻片推动涂抹法, 两者都有一定局限性。前者因为液体表面张力的作用, 液体在下滑过程中曲折前进, 导致 DNA 纤维分布不均匀, 有时相互重叠, 制片质量很差。后者虽能使液体均匀分散, 但因推动过程中用力大小不均等因素, 易使 DNA 纤维堆积、断裂和丢失。我们采用液面前端载玻片导引, 即“引流法”来伸展 DNA 纤维, 消除了液体表面张力的影响, 也避免了涂抹法和重力拉伸法造成的 DNA 纤维堆积和断裂, DNA 纤维随着液面的铺展而伸展开来, 所制备的 DNA 纤维平直、均匀、完整(图 2-D), 完全满足荧光原位杂交的需要。

2.3　DNA 纤维荧光原位杂交

用亚洲棉石系亚 1 号自身基因组 gDNA 和 45S rDNA 作探针进行 DNA 纤维荧光原位杂交, 杂交信号均表现为典型的非连续的念珠状(图 3)。可以看出, “刀切引流法”所制备的棉花 DNA 纤维完全满足荧光原位杂交要求。

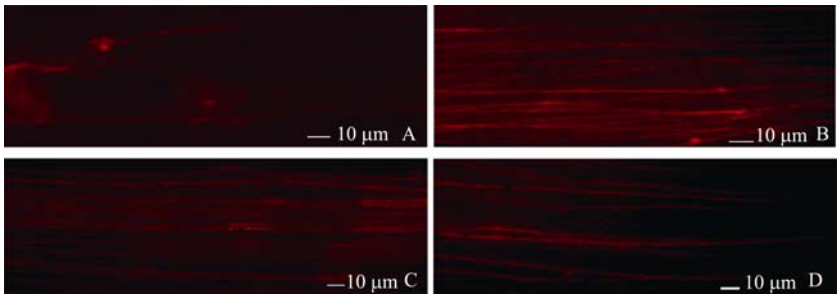

图 2　不同温育时间处理后制备的棉花 DNA 纤维

Fig. 2　Cotton DNA fibers obtained with different treated times

A: 温育 3 min 后制备的棉花 DNA 纤维(×400); B: 温育 5 min 后制备的棉花 DNA 纤维(×560);
C: 温育 7 min 后制备的棉花 DNA 纤维(×560); D: 温育 9 min 后制备的棉花 DNA 纤维(×1000)。

A: The stretched cotton DNA fibers from nuclei incubated for 3 min(×400); B: The stretched cotton DNA fibers from nuclei incubated for 5 min(×560); C: The stretched cotton DNA fibers from nuclei incubated for 7 min(×560); D: The extended cotton DNA fibers from nuclei incubated for 9 min(×1000).

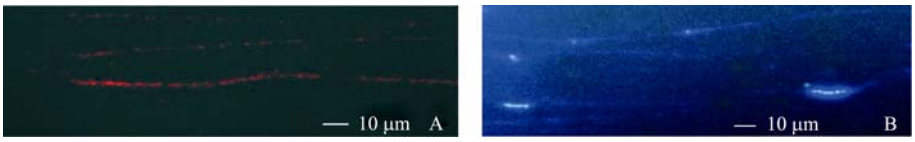

图 3　DNA 纤维荧光原位杂交

Fig. 3　Fluorescence in situ hybridization patterns of DNA fibers of cotton

A: 以地高辛标记的基因组作探针进行棉花 DNA 纤维荧光原位杂交(1000 倍);
B: 以生物素标记的 45S rDNA 作探针进行棉花 DNA 纤维荧光原位杂交(1000 倍)

A: Cotton DNA fibers hybridized with cotton genomic DNA probes labeled by Digoxigenin(×1000);
B: Cotton DNA fibers hybridized with 45S rDNA probes labeled by Biotin(×1000).

3　讨论

制备棉花 DNA 纤维, 高质量的细胞核提取是关键。首先要进行材料的黄化处理, 因为棉花细胞质浓厚, 富含棉酚、多糖、油和丹宁等次生代谢物, 细胞核的提取比较困难。我们发现, 用发育 1 周的黄化子叶提取的细胞核明显好于发育 3 d 的黄化子叶提取的细胞核, 可能是较长时间的黄化处理, 使子叶中很多次生成分被消耗, 但如果黄化处理超过 1 周以上, 子叶呈老化趋势, 容易腐烂, 不能提出细胞核。同时, 在细胞核提取液中加入 Triton X-100 和 PVP_{40}, 能有效去除棉酚等物质, 减少干扰, 所制备的细胞核杂质少、质量高。其次是如何释放出细胞核。在拟南芥、番茄等植物中, 制备 DNA 纤维用的细胞核多采用研磨法[9]。在棉花中也有用研磨法提取细胞核的报道[34], 然而, 研磨法时间长短和力度大小难以掌握, 很多细胞核被破坏, 细胞核产率较低, 而且很多细胞质释放物难以除净。我们试探成功“刀切法”, 采用锋利的刀片切割棉花子叶, 细胞核很容易释放出来而不受到破坏, 而且不需要研磨和有毒试剂巯基乙醇等处理, 方便快捷无毒害, 成功率可达 100%。

典型的 DNA 纤维 FISH 杂交信号均表现为非连

续的念珠状(beads-on-string)。对于其原因，Fransz 等[9]认为有 3 个方面，一是在制片过程中醋酸处理造成的 DNA 损伤以及载玻片表面粘附的小片段 DNA 妨碍杂交的结果。二是细胞内 DNA 总是与蛋白质结合在一起，在 DNA 纤维的制备过程中，染色体蛋白质不可能完全去除干净，残存的染色体蛋白质降低了目的序列与 DNA 纤维的接近，导致念珠间具有空隙。三是在杂交检测过程中免疫荧光抗原-抗体反应的结果，优先结合的抗原-抗体大分子对邻近的分子具有空间位阻效应，减少了荧光信号点，使其表现为强弱不等、大小不一、不连续的念珠状信号。Rijke 等[35]则认为，两念珠之间的间隙是 DNA 变性过程中靶 DNA 部分丢失的结果。念珠大小不一致是由于在杂交过程中，探针 DNA 片段与靶 DNA 随机相遇并结合，靶 DNA 有的区域得到了充分的结合，另一些区域则很少或没有结合，而念珠之间的间隙区域就是没有得到结合的部分。虽然目前形成念珠状信号的原因尚不十分确切，但是，念珠状信号代表的是 Fiber-FISH 的真实结果，所反映的是供试 DNA 序列在伸展 DNA 纤维上杂交的顺序。所以，鉴定 DNA 纤维 FISH 成功与否，或杂交信号是否真实，关键是要看荧光信号点是否连续，形状如何。本试验中杂交信号均表现为典型的非连续念珠状。

4 结论

采用刀切法提取棉花细胞核，整个过程只需 30 min 左右，不需要研磨和巯基乙醇等处理，方便快捷无毒害，成功率 100%。以引流法制备 DNA 纤维，DNA 纤维平直完整、伸展程度均匀、背景清晰，完全适用于荧光原位杂交。本研究首次在棉花中建立了简单、高效、快捷、无毒害的适宜于荧光原位杂交的 DNA 纤维制备技术，为深入进行棉花基因组研究乃至棉花基因组测序的最终完成提供强有力的技术支持。

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