柑橘不同倍性种质胚性愈伤组织诱导及无病毒植株再生

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摘　要：【目的】通过离体培养败育胚珠诱导柑橘二倍体和四倍体种质的胚性愈伤组织，为柑橘多倍体研究提供成对离体材料；继续诱导胚性愈伤组织再生无毒种苗实现柑橘无核品种提纯复壮。**【方法】**从柑橘成熟果实拨取败育胚珠，接种于 3 种愈伤诱导培养基，离体培养过程中诱导并驯化胚性愈伤组织；再诱导愈伤组织分化为胚状体和不定芽，将不定芽嫁接到枳橙砧木形成完整植株；通过 SSR 分子标记和病毒检测鉴定再生植株遗传来源和脱毒效果。**【结果】**离体培养诱导出 13 个柑橘种质的胚性愈伤组织，其中 8 个愈伤组织完成驯化，分别为红橘二倍体/四倍体（2x/4x）、W. 默科特橘橙 2x/4x、鄂柑 1 号椪柑 2x/4x、日辉橘 2x 和早红脐橙。基因型影响柑橘胚性愈伤组织诱导效率，‘早红’脐橙诱导率高达 74.73%，而红橘诱导率仅 6.85%；柑橘四倍体愈伤组织诱导率（0.56% - 22.61%）低于对应二倍体（6.85% - 37.75%）。3 种不同培养基的胚性愈伤组织诱导效率不同，其中 MGS 培养基对温州蜜柑在内的 5 个品种的诱导率较高（14.13% - 63.01%），MK 培养基对温州蜜柑在内的 4 个品种的诱导率较低（21.62% - 69.51%），但 MK 培养基对椪柑 2x 和红橘 2x 的诱导率分别高达 70.21%和 17.31%。分别获得早红脐橙、伦晚脐橙、国庆 1 号温州蜜柑、大分 4 号温州蜜柑和兴津温州蜜柑 5 个无核品种的愈伤组织再生苗 23、22、20、10 和 15 株；嫁接嵌合体早红脐橙的愈伤组织再生苗经 SSR 分子鉴定实为罗伯逊脐橙；国庆 1 号温州蜜柑、伦晚脐橙和早红脐橙再生苗经 PCR 检测证明未感染黄脉病和衰退病病毒。**【结论】**柑橘胚性愈伤组织诱导率受品种基因型和培养基影响；同一品种的不同倍性种质，二倍体愈伤组织诱导率和胚状体发生率均较高；胚性愈伤组织再生植株实现了病毒完全脱除，是柑橘无核品种提纯复壮的有效手段。

关键词：柑橘；胚性愈伤组织；倍性种质；无核品种；离体再生

Embryogenic callus induction from ploidy germplasm and virus-free plantlet regeneration from seedless cultivars in Citrus

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Abstract:【Objective】Citrus is the biggest fruit industry in southern China and in the world. China is the origin center of citrus, owning abundant and diverse citrus germplasm resources, and it is important to properly conserve and utilize the elite citrus germplasm. The embryogenic callus induced from the aborted ovules was genetically identical to the original explant and is capable of regeneration, making it proper citrus germplasm for in vitro conservation. The embryogenic callus also provides in vitro materials for researches of important traits, such as somatic embryogenesis, fruit quality control and stress resistance. We have induced and preserved embryogenic callus germplasm of over 100 different genotypes. However, the embryogenic calluses are all induced from the diploid germplasm, while induction of embryogenic callus from polyploid germplasm has been rarely reported. The polyploid is characterized by giant organ, dwarf plant and stronger adaptability. The polyploid embryogenic callus would accelerate basic researches and applications of citrus polyploidy, by providing in vitro materials with short

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growth cycle, stable status, and easily accessibility. In addition, the whole new plantlets regenerated from embryogenic callus are always virus-free, which are promising in resolving the problem of fruit yield and quality delineation in seedless cultivars caused by virus infection after many years propagation by grafting. In this study, the aborted ovules of diploid and tetraploid citrus germplasm were cultured to induce embryogenic callus, to provide in vitro materials for citrus ploidy research. Regeneration of virus-free plantlets from embryogenic callus achieves purification and rejuvenation of seedless cultivars like navel orange and Satsuma mandarin. 【Methods】 The aborted ovules from mature fruits were inoculated on three types of callus induction mediums (MES: MT[19] + ME 0.5 $g \cdot L^{-1}$ + SAD 40 $mg \cdot L^{-1}$; MGS: MT + ME 0.5 $g \cdot L^{-1}$ + SAD 40 $mg \cdot L^{-1}$ + GA_3 1 $mg \cdot L^{-1}$; MK: MT + KT 0.5$mg \cdot L^{-1}$) under sterile condition to induce and train embryogenic callus, and the ploidy of embryogenic callus was determined by flow cytometry. Embryogenic callus was induced to differentiate into embryoids and adventitious buds. When the regenerated buds grew to the size of 2~3 cm, they were grafted to the yellowing rootstock in test tube to form a plantlet. SSR (Simple Sequence Repeats) analysis was used to identify the genetic origin of the regenerated plantlets, and PCR was used to detect Citrus tristeza virus (CTV) and Citrus yellow vein clearing virus (CYVCV) in the plantlets. 【Results】The embryogenic calluses were induced from 13 citrus germplasms, and eight of them have been trained for subculture, including red tangerine diploid and tetraploid (2x, 4x), Nadorcott tangor (2x, 4x), Egan No.1 Ponkan mandarin (2x, 4x), Sunburst mandarin (2x) and Zaohong navel orange. The ploidy of the embryogenic calluses was consistent with the germplasm from which they were induced, as detected by flow cytometry. The embryogenic callus induction efficiencies were different among germplasm. The induction rate of Zaohong navel orange was the highest (74.73%), followed by Guoqing No.1 Satsuma mandarin, Okitsu Satsuma mandarin, Oita 4 Satsuma mandarin and Egan No.1 Ponkan mandarin, each with an induction rate over 37%. The induction rates of red tangerine 2x, Sunburst mandarin 2x, Lane Late navel orange and Nadorcott tangor 2x were lower than the other genotypes, with induction rates between 6.85% and 12.61%. Embryogenic calluses were induced from four pairs of ploidy materials, and the induction rate of diploid germplasm was 6.85%~37.75%. Among them, the callus induction rate of Egan No.1 Ponkan mandarin 2x was the highest, while that of red tangerine 2x was the lowest. The callus induction rates of the tetraploid germplasm (0.56%~22.61%) were lower than the diploids. Among them, the callus induction rate of Egan No.1 Ponkan mandarin 4x was also the highest, while that of Nadorcott tangor 4x was the lowest. As for the incidence of embryoids, they were 16.15%~58.28% for diploids and 1.13%~30.65% for tetraploids. In summary, the callus induction and embryoids incidence rates of Egan No.1 Ponkan mandarin 2x and 4x were the highest among the four pairs of 2x and 4x germplasm, while those of Nadorcott tangor 4x were the lowest among the tetraploid germplasm. Notably, the callus induction and embryoids incidence rates of the tetraploid were lower than the corresponding diploid. Besides, the induction rate of embryogenic callus on different mediums was different. The embryogenic callus induction rates of Guoqing No.1 Satsuma mandarin, Okitsu Satsuma mandarin, Oita 4 Satsuma mandarin, Nadorcott tangor 2x and Sunburst mandarin 2x on MGS medium were higher than MK and MES. The callus induction rates of Egan No.1 Ponkan mandarin 2x and red tangerine 2x on MK medium was the highest, and was significantly higher than MES and MGS. A total of 23, 22, 20, 10 and 15 plantlets were regenerated from five seedless cultivars, including Zaohong navel orange, Lane Late navel orange, Guoqing No.1 Satsuma mandarin, Oita 4 Satsuma mandarin and Okitsu Satsuma mandarin, respectively. SSR analysis showed that the regenerated

plantlets of the chimeric Zaohong navel orange were indeed Robertson navel orange. According to PCR, CTV and CYVCV were undatable in the regenerated plantlets of Guoqing NO.1 Satsuma mandarin, Lane Late navel orange and Zaohong navel orange, proving them to be virus-free. 【Conclusion】In this study, the aborted ovules of mature citrus fruits were cultured in vitro, and the diploid and tetraploid embryogenic callus of three varieties were obtained by induction and training, which provide stable and easily accessible in vitro materials for the research of citrus polyploidy. The embryogenic callus induction rates differed among different citrus germplasms and induction mediums. The embryogenic callus induction rate and somatic embryogenesis rate of the diploids were higher than the corresponding tetraploid for each cultivar. The regenerated virus-free plantlets provided materials for purification and rejuvenation of five polyembryonic and seedless citrus cultivars. The molecular marker analysis proved that the regenerated plantlets of the grafting chimera Zaohong navel orange were indeed Robertson navel orange.
Key words：Citrus; Embryogenic callus; Ploidy germplasm; Seedless cultivars; *In vitro* regeneration

柑橘是世界和我国南方第一大水果。我国是柑橘起源中心[1]，拥有丰富多样的柑橘种质资源，合理保存和利用我国优异的柑橘种质具有重要意义。柑橘胚性愈伤组织由败育胚珠诱导而来，其遗传上与亲本保持一致；且胚性愈伤组织具有细胞全能性，能通过体细胞胚发生途径再生植株，是柑橘种质资源离体保存的主要方式。本研究团队多年来诱导保存了 100 多个柑橘品种的胚性愈伤组织[2]，为利用原生质体融合、基因编辑等生物技术改良柑橘提供了丰富的起始材料[3]，为柑橘体细胞胚发生[4]、品质调控[5]和抗性[6]研究提供了离体体系。然而，柑橘胚性愈伤组织均从二倍体种质诱导获得，柑橘多倍体种质胚性愈伤组织诱导未见报道。

多倍体普遍具有器官巨大型、植株矮化和适应性广等特征。柑橘同源四倍体相比二倍体对盐胁迫、干旱、营养缺乏等非生物逆境抗性更强[7-9]，柑橘四倍体相比二倍体果实品质也发生显著变异[10-11]，为柑橘倍性育种提供了优良亲本。诱导柑橘多倍体的胚性愈伤组织，将为柑橘多倍体倍性和代谢等研究提供周期短、状态稳定、易获得的离体材料，加速柑橘多倍体基础研究和利用。

无核是柑橘鲜食品种选育的重要目标性状[12]。但无核品种常年通过嫁接繁殖，累积多种病毒，导致果实品质和产量下降。我国较常见且对柑橘生产危害较大的病毒和细菌性类病害主要有柑橘黄龙病、溃疡病、裂皮病、衰退病和黄脉病等。柑橘无核品种没有种子无法通过珠心苗实生繁殖脱毒；柑橘茎尖培养难以生根，微芽嫁接操作要求高且成活率低，脱毒效率有待提高。胚性愈伤组织可通过体细胞胚发生途径再生植株，稳定遗传亲本性状的同时，能培育无病毒植株，操作相对简单，再生苗数量多，结构完整，尤其适合多胚无核柑橘品种提纯复壮[13]。

我们利用柑橘多胚品种存在珠心细胞自然加倍的现象，从柑橘 60 多个接穗和砧木类型中发掘获得了一大批同源四倍体植株[14-16]，丰富了我国柑橘多倍体种质资源，为通过倍性杂交培育柑橘三倍体无核新种质提供了优良亲本[17]。本研究通过离体培养二倍体和四倍体柑橘的败育胚珠，诱导胚性愈伤组织，将为多倍体抗性和代谢相关研究提供倍性成对的离体材料；无核品种诱导胚性愈伤组织并通过体细胞胚发生途径再生不携带病毒的植株，将为我国主栽的脐橙和温州蜜柑等无核品种提纯复壮提供种苗。

1 材料与方法

1.1 实验材料

用于诱导胚性愈伤组织的柑橘二倍体及其同源四倍体（2x/4x）成对倍性材料，包括红橘（*Citrus. reticulata* Blanco）2x/4x、W. 默科特橘橙（*C. reticulata* Blanco × *C. sinensis* L. Osbeck）2x/4x、鄂柑 1 号椪柑（*C. reticulata* Blanco）2x/4x、日辉橘（*C. reticulata* Blanco）2x/4x；无核柑橘品种，包括早红脐橙（*C. sinensis* L. Osbeck）、伦晚脐橙（*C. sinensis* L. Osbeck）、国庆 1 号温州蜜柑（*C. unshiu* Blanco）、大分 4 号温州蜜柑（*C. unshiu* Blanco）和兴津温州蜜柑（*C. unshiu* Blanco）。

1.2 柑橘胚性愈伤组织离体诱导

将成熟果实浸泡于 75%酒精 20~30 min 后，超净工作台内用酒精灯灼烧果实表面消毒。在无菌滤纸上，将果实切开后，用镊子将败育胚珠挑出，接种于 3 种愈伤诱导培养基[18]（MES：MT[19] + ME 0.5 $g·L^{-1}$ + SAD 40 $mg·L^{-1}$；MGS：MT + ME 0.5 $g·L^{-1}$ + SAD 40 $mg·L^{-1}$ + GA_3 1 $mg·L^{-1}$；MK：MT + KT 0.5$mg·L^{-1}$）进行暗培养。离体培养约 1 个月后，待败育胚珠萌发产生胚状体或胚性愈伤组织后，将胚性愈伤组织挑出并继代于 MT 培养基，光下培养至愈伤组织状态稳定并持续增殖，即完成愈伤组织驯化。将驯化完成的愈伤组织保存在 MT 培养基中光照培养，每月继代 1 次长期保存。胚性愈伤组织诱导率 ＝ 形成胚性愈伤组织的败育胚珠数/离体培养的败育胚珠总数 × 100%，胚状体发生率 ＝ 形成胚状体的败育胚珠数/离体培养败育胚珠总数 × 100%。

1.3 胚性愈伤组织胚状体增殖、生芽和试管嫁接

新诱导获得的柑橘胚性愈伤组织在 MT 培养基上驯化和继代培养过程中，会再生胚状体，将胚状体转移至增殖培养基（MT + ME 1.5 $g·L^{-1}$ + 蔗糖 50 $g·L^{-1}$），待胚状体膨大后转移至生芽培养基（MT + 6-BA 0.5 $mg·L^{-1}$ + KT 0.5 $mg·L^{-1}$ + NAA 0.1 $mg·L^{-1}$）诱导生芽。待丛生芽长至 2~3 cm 高时，采用试管嫁接技术将其嫁接至枳橙黄化砧木；待嫁接苗成活并新长出 3~4 片真叶后，将其转移到生长室炼苗；待幼苗长至 6~7 片叶时转移至温室。

1.4 倍性测定和 SSR 分子鉴定

利用流式细胞仪（Cyflowspace, Sysmex, Japan）检测胚性愈伤组织及胚状体的染色体倍性，详情参考解凯东等[17]的方法。制备待测植株叶片混合液，比较待测样品与二倍体参照峰值横坐标位置关系，确定待测样品倍性。SSR 分子鉴定方法参考谢善鹏等[14]方法，多态性 SSR 引物[20]（表 1）由生工生物工程（上海）股份有限公司合成。用 ProFlex PCR 仪（ABI, USA）进行 PCR 反应，PCR 扩增体系为 10 μL：2 × Rapid Taq Master Mix 5 μL，ddH_2O 3.5 μL，DNA 1 μL，Primer F、R 各 0.25 μL（10 $μmol·L^{-1}$）。扩增程序：95 ℃预变性 5 min，95 ℃变性 30 s，55 ℃退火 30 s，72 ℃延伸 10 s，35 个循环，72 ℃延伸 5 min，4 ℃保存。

表 1 早红脐橙 SSR 引物序列

Table 1 SSR primer sequences for ‘Zaohong’ navel orange

引物名称	序列	文献

Primer name	Primer Sequence	Reference
InDel p1 (chr1)	F: TTACTGACCCACAGCCCAC; R: CTTACCGACGATTGCCCTA	[20]
InDel p7 (chr5)	F: AAATGTGGGACGGCAAGG; R: AATGCTAGTTGGAAGACTCGTCA	
InDel p11 (chr6)	F: ATCAGGTCGGTCAGCCAGT; R: CATTTGTACGTAACACGAGTGC	
InDel p15 (chr9)	F: TGCAGTAGAATGATTTCCATGC; R: GCTTAAACCCGTGACCTCG	

1.5 病毒 PCR 检测

取嫁接苗植株叶片，液氮冷冻后磨样。叶片 RNA 提取实验方法参照 HiPure HP Plant RNA Mini Kit（Magen）说明书；反转录实验方法参照 HiScripte II Q RT SuperMix for qPCR(+ gDNA wiper)（Vazyme）说明书。PCR 反应体系和程序同 SSR 分子鉴定，设置阴性（ddH_2O 模板）和阳性对照（感染黄脉病和衰退病的夏橙叶片 DNA）。衰退病[21]和黄脉病检测引物[22]（表 2）由生工生物工程（上海）股份有限公司合成。

表 2 病毒检测引物序列

Table 2 Primers sequence for virus detection

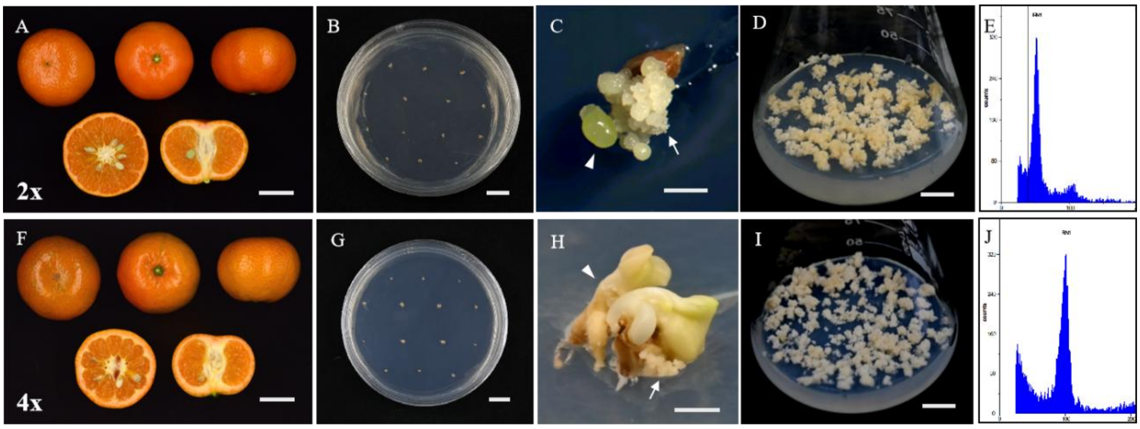
引物名称 Primer name	序列 Primer Sequence	文献 Reference
F-PM85+（CTV）	GGACAAACTTTNNTTTCTGTGAACCTTTC	[21]
R-PM86-（CTV）	GATGAAGTGGTGTTCACGGAGAACTC	
VF-1（CYVCV）	TACCGCAGCTATCCATTTCC	[22]
VR-1（CYVCV）	GCAGAAATCCCGAACCACTA	

2 结果与分析

2.1 柑橘胚性愈伤组织诱导和驯化

从柑橘成熟果实挑出败育胚离体培养，暗培养 0.7~3 月后在败育胚珠附近形成胚性愈伤组织。将胚性愈伤组织转移至 MT 培养基多次继代培养进行驯化，耗时 5~9 个月。13 个材料均诱导出胚性愈伤组织，经过多次继代培养，其中 8 个材料的胚性愈伤组织状态稳定并能持续增殖，包括红橘 2x/4x、W. 默科特橘 2x/4x、鄂柑 1 号椪柑 2x/4x、日辉橘 2x 和早红脐橙，说明这些胚性愈伤组织已完成驯化，可常规继代培养；其余 5 个材料包括日辉橘 4x、伦晚脐橙、国庆 1 号温州蜜柑、大分 4 号温州蜜柑和兴津温州蜜柑诱导出的愈伤组织胚性较强，经多次继代逐渐分化形成胚状体，虽未能获得稳定增殖的胚性愈伤组织，但通过体细胞胚发生途径可能再生植株。采用流式细胞仪对 8 份 2x/4x 胚性愈伤组织进行检测，其倍性均与其败育胚珠外植体来源母本植株一致（图 1）。

不同于其 2x 能直接从败育胚珠中长出胚性愈伤组织，W. 默科特橘橙 4x 接种的败育胚珠均未长出愈伤组织或胚状体；而是从较大的瘪种子中长出胚状体，再从胚状体下胚轴长出胚性愈伤组织（图 1 H）。相比 W. 默科特橘 2x 胚性愈伤组织诱导和驯化分别耗时 0.7 和 5.5 个月，W. 默科特橘橙 4x 的愈伤组织诱导和驯化耗时较长，分别为 3 和 5.5 个月。红橘 4x 愈伤组织诱导和驯化均相比对应的 2x 耗时也较长，但鄂柑 1 号椪柑 4x 愈伤组织诱导耗时较 2x 更短。



A-E, F-J. W. 默科特橘橙 2x/4x 胚性愈伤组织诱导过程；A, F. 2x/4x 果实；B, G. 接种培养 2x/4x 败育胚珠；C. 2x 败育胚珠直接长出胚性愈伤组织；H. 4x 瘪种子先长出胚状体，再从胚状体下胚轴长出愈伤组织；D, I. 胚性愈伤组织完成驯化；E, J. 流式细胞仪鉴定愈伤组织倍性为 2x/4x；箭头指示胚状体，带尾箭头指示胚性愈伤组织；

标尺：3 cm (A. F), 1 cm (B. D. G-I), 1 mm (C)

A-E, F-J. Nadorcott tangor 2x/4x embryogenic callus induction process; A, F. Fruit of diploid/tetraploid (2x/4x); B, G. Inoculation of 2x/4x aborted ovules; C. Embryogenic callus generated directly from 2x shrivelled seed; H. Embryogenic callus generated from the hypocotyl of the embryoid that formed from the aborted ovule; D, I. The trained 2x/4x embryogenic callus; E, J. Ploidy analysis of 2x/4x embryogenic callus; The arrow head points to the embryoid, and the arrow point to embryogenic callus;

Bar = 3 cm (A. F), 1 cm (B. D. G-I), 1 mm (C)

图 1 W. 默科特橘橙二倍体/四倍体（2x/4x）胚性愈伤组织诱导过程

Fig. 1 Induction process of embryogenic callus from diploid/tetraploid (2x/4x) of Nadorcott tangor

2.2 基因型和培养基影响胚性愈伤组织诱导效率

柑橘不同基因型的胚性愈伤组织诱导效率不同（表 3）。早红脐橙诱导率最高，达 74.73%；其次为国庆 1 号温州蜜柑、兴津温州蜜柑、大分 4 号温州蜜柑和鄂柑 1 号椪柑 2x，诱导率均＞37%；红橘 2x、日辉橘 2x、伦晚脐橙和 W. 默科特橘橙 2x 的诱导率较低，仅在 6.85%~12.61%之间。4 对倍性材料均诱导出胚性愈伤组织（其中日辉橘 4x 愈伤组织未能完成驯化获得稳定增殖的胚性愈伤组织），二倍体愈伤组织诱导率为 6.85%~37.75%，其中鄂柑 1 号椪柑 2x 愈伤组织诱导率最高为 37.75%，红橘 2x 的愈伤组织最低为 6.85%。四倍体愈伤组织诱导率相比二倍体较低，仅为 0.56%~22.61%，其中鄂柑 1 号椪柑 4x 愈伤组织诱导率仍最高，达 22.61%，而 W. 默科特橘橙 4x 的愈伤组织诱导率最低，仅为 0.56%。2x/4x 成对倍性材料中，二倍体的胚状体发生率为 16.15%~58.28%，椪柑的胚状体发生率最高，为 58.28%。四倍体的胚状体发生率为 1.13%~30.65%，椪柑仍为最高，为 30.65%，W. 默科特橘橙最低，仅为 1.13%。总结可知，椪柑 2x 和 4x 的愈伤组织诱导率和胚状体发生率均为 4 个品种 2x 和 4x 材料中最高，W. 默科特橘橙 4x 的愈伤组织诱导率和胚状体发生率均为 4 个四倍体品种中最低；同品种二倍体的愈伤组织诱导率和胚状体发生率均比对应的四倍体更高。

表 3 柑橘不同品种和配方的胚性愈伤组织诱导率和胚状体发生率

Table 3 The induction rates of embryonic callus and embryoids from different citrus cultivars and medium

注：表内“-”代表该处理未实施；未标注倍性为二倍体；愈伤组织诱导率/胚状体发生率=愈伤组织或胚状体数/接种败育胚珠

品种 Cultivars	外植体接种数 Number of explants			胚性愈伤组织诱导率(%) Induction rate of embryogenic callus				胚状体发生率(%) Induction rate of embryoids			
	MES	MGS	MK	MES	MGS	MK	平均诱导率	MES	MGS	MK	平均发生率
红橘 2x Red tangerine 2x	79	109	104	0	1.83	17.31	6.85	40.51	36.70	46.15	41.10
红橘 4x Red tangerine 4x	105	124	126	0.95	2.42	10.32	4.79	19.05	24.19	17.46	20.28
W. 默科特橘橙 2x Nadorcott tangor 2x	35	40	44	5.71	17.50	13.64	12.61	20.00	22.50	27.27	23.53
W. 默科特橘橙 4x Nadorcott tangor 4x	72	50	55	1.39	0	0	0.56	2.78	0	0	1.13
鄂柑 1 号椪柑 2x Egan No.1 Ponkan mandarin	48	56	47	25.00	21.43	70.21	37.75	52.08	44.64	80.85	58.28
鄂柑 1 号椪柑 4x Egan No.1 Ponkan mandarin	59	74	66	30.51	13.51	25.76	22.61	30.51	32.43	28.79	30.65
日辉橘 2x Sunburst mandarin	101	92	98	6.93	14.13	11.22	10.65	8.91	20.65	19.39	16.15
日辉橘 4x Sunburst mandarin	70	81	-	1.43	3.70	-	2.65	21.43	9.88	-	15.23
早红脐橙 Zaohong navel orange	83	112	82	77.11	76.79	69.51	74.73	86.75	89.29	64.63	81.23
伦晚脐橙 Lane Late navel orange	66	72	58	15.15	9.72	10.34	11.73	28.79	20.83	20.69	23.47
国庆 1 号温州蜜柑 Guoqing NO.1 Satsuma mandarin	108	73	62	42.59	63.01	37.10	47.33	47.22	68.49	41.94	52.26
大分 4 号温州蜜柑 Oita 4 Satsuma mandarin	60	58	45	36.67	44.83	33.33	38.65	46.67	72.41	35.56	52.76
兴津温州蜜柑 Okitsu Satsuma mandarin	39	39	37	51.28	53.85	21.62	42.61	33.33	61.54	21.62	39.13

数；平均诱导/发生率为 3 种培养基诱导/发生率的加权平均；MES\MGS\MK 表示 3 种培养基。

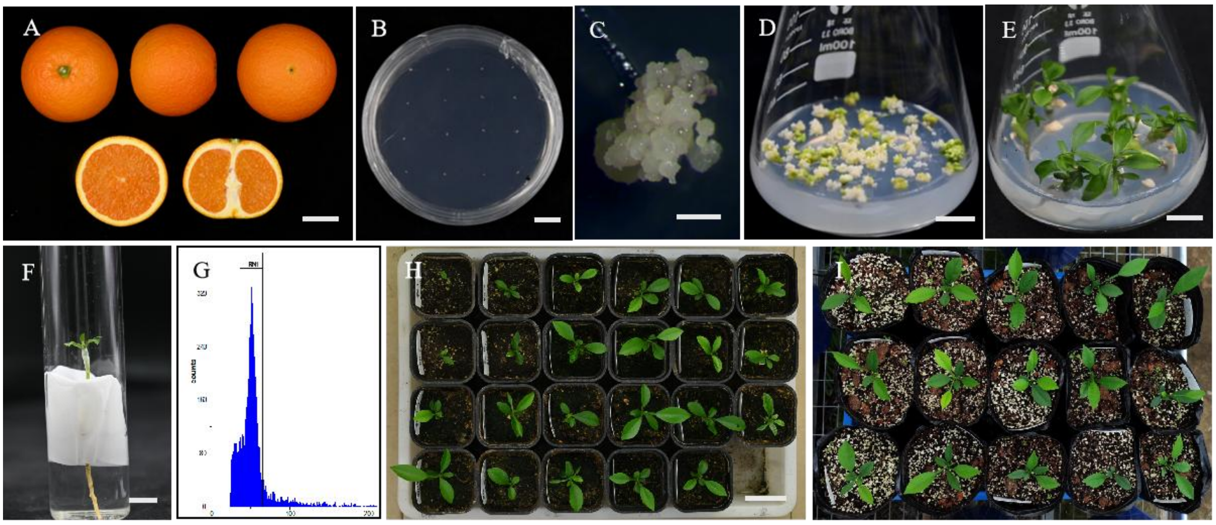
Note: ‘-’ represents that the treatment is not implemented, and the ploidy not specified is diploid. Induction rate = number of callus or embryoids / number of inoculated ovules. The average induction rate is the weighted mean of induction rates on three induction medium. MES\MGS\MK represents three medium.

不同培养基的胚性愈伤组织诱导效率不同（表 3）。国庆 1 号温州蜜柑、兴津温州蜜柑、大分 4 号温州蜜柑、W. 默科特橘橙 2x 和日辉橘 2x 共 5 个品种在 MGS 培养基的胚性愈伤组织诱导率均高于 MK 和 MES 培养基。早红脐橙、国庆 1 号温州蜜柑、大分 4 号温州蜜柑和兴津温州蜜柑这 4 个品种在 MK 培养基的愈伤组织诱导率最低；但 MK 培养基对鄂柑 1 号椪柑 2x 和红橘 2x 的愈伤组织诱导率最高，且明显高于 MES 和 MGS 培养基的诱导率。椪柑 2x 在 MK 培养基的愈伤组织诱导率高达 70.21%，约为 MES、MGS 培养基的 3 倍；红橘 2x 在 MK 培养基的愈伤组织诱导率高达 17.31%，约为 MGS 培养基的 15 倍。

2.3 柑橘无核品种胚性愈伤组织再生植株及其来源和病毒检测

早红脐橙、伦晚脐橙、国庆 1 号温州蜜柑、大分 4 号温州蜜柑、兴津温州蜜柑 5 个无核品种诱导出的胚性愈伤组织胚性较强，诱导过程产生大量胚状体；挑选胚状体进行增殖培养，胚状体膨大后转移至生芽

培养基诱导生芽。约 2 个月后再生大量丛生芽，将再生芽切下进行试管嫁接，待嫁接苗长出 3~4 片真叶移栽至基质炼苗，1~2 个月后移栽至温室。早红脐橙愈伤组织再生苗经流式细胞仪鉴定均为二倍体。分别获得无核品种早红脐橙、伦晚脐橙、国庆 1 号温州蜜柑、大分 4 号温州蜜柑和兴津温州蜜柑的离体再生苗 23、22、20、10 和 15 株。



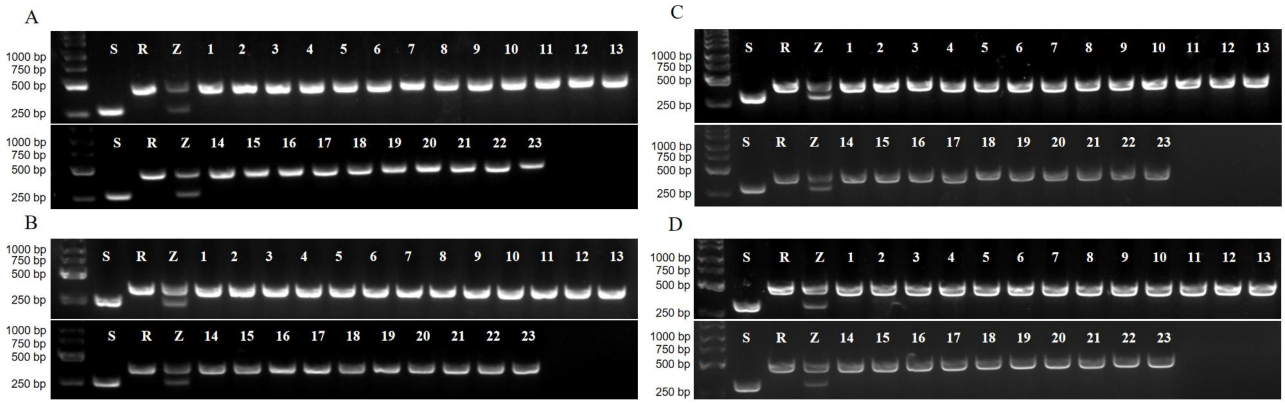
A. 早红脐橙成熟果实；B. 接种培养败育胚珠；C. 愈伤组织形成；D. 胚性愈伤组织分化胚状体；E. 胚状体生芽；F. 丛生芽试管嫁接；G. 再生苗倍性鉴定；H. 嫁接苗转移至基质炼苗；I. 移栽至温室；标尺：5 cm (H), 3 cm (A), 1 cm (B. D-F), 1 mm (C).

A. Fruit of Zaohong navel orange; B. Inoculation of aborted ovules in callus induction medium; C. Callus formation; D. The embryoid regenerated from embryogenic callus; E. Plants regenerated from the embryoids; F. The grafted plantlet; G. Ploidy analysis of the regenerated plants by flow cytometry; H. The transplanted plantlets; I. Plantlets in greenhouse; Bar = 5 cm (H), 3 cm (A), 1 cm (B. D-F), 1 mm (C).

图 2 早红脐橙胚性愈伤组织诱导及植株再生

Fig. 2 Induction of embryogenic callus from Zaohong navel orange and regeneration of plantlets

早红脐橙是罗伯逊脐橙（Robertson navel orange）高接于温州蜜柑产生的嫁接嵌合体[23]。利用前人筛选的 4 对多态性SSR标记[20]鉴定早红脐橙胚性愈伤组织再生苗的遗传组成。结果表明早红脐橙 23 株嫁接苗与其亲本罗伯逊脐橙带型相同（图 3），认为嵌合突变体早红脐橙胚性愈伤组织再生苗实为罗伯逊脐橙。



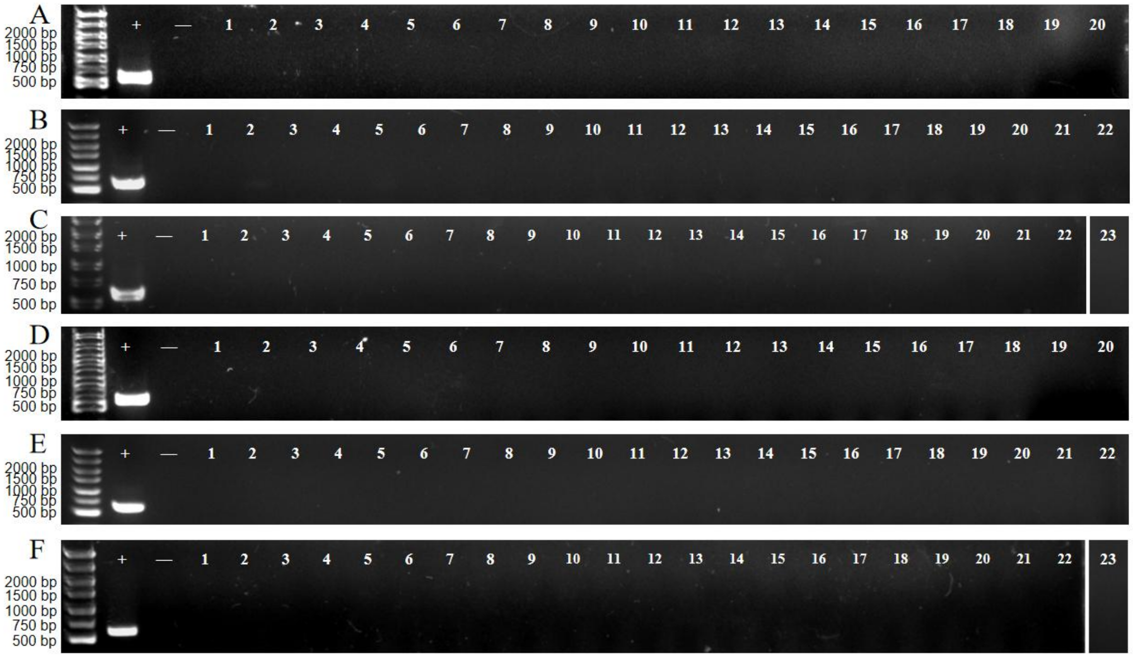
注：温州蜜柑（S），罗伯逊脐橙（R），早红脐橙（Z），早红脐橙再生苗（1-23）；引物：A InDel p1，B InDel p7，C InDel p11，D InDel p15.

Note: Satsuma mandarin (S), Robertson navel orange (R), Zaohong navel orange (Z), the regenerated plants of Zaohong navel orange (1-23); Primers: A InDel p1, B InDel p7, C InDel p11, D InDel p15.

图 3 早红脐橙再生苗 SSR 分子标记鉴定

Fig. 3 The SSR profile of the regenerated plantlets of Zaohong navel orange

柑橘衰退病(Citrus tristeza virus, CTV)和黄脉病(Citrus yellow vein clearing virus, CYVCV)是柑橘果园最常见的两种病毒。对国庆 1 号温州蜜柑、伦晚脐橙和早红脐橙再生苗进行 PCR 病毒检测，结果显示阳性样本（带有衰退病和黄脉病的夏橙叶片）泳道有明显条带，3 个品种的再生苗与阴性对照(水模板)均未扩增出目的条带（图 4），证明国庆 1 号温州蜜柑、伦晚脐橙和早红脐橙再生苗均不含可检出量的衰退病和黄脉病病毒，无毒率达 100%。



A-C, D-F. 衰退病（CTV）、黄脉病（CYVCV）病毒检测结果；A, D. 国庆 1 号温州蜜柑再生苗；B, E. 伦晚脐橙再生苗；C, F. 早红脐橙再生苗

A-C, D-F. virus detection results for CTV and CYVCV; A, D. regenerated plantlets of Guoqing NO.1 Satsuma mandarin; B, E. regenerated plantlets of Lane Late navel orange; C, F. regenerated plantlets of Zaohong navel orange;

图 4 柑橘胚性愈伤组织再生苗病毒 PCR 检测结果

Fig. 4 PCR-based virus detection of citrus regenerated plantlets

3 讨论

柑橘胚性愈伤组织诱导效率受基因型影响，9 个二倍体品种的胚性愈伤组织诱导率在 6.85%~74.73%之间。由柑橘基本种杂交而来的橙类（脐橙）和柑类（温州蜜柑）的胚性愈伤组织诱导率较古老的橘类（椪柑、红橘）更高；其中红橘的胚性愈伤组织诱导率最低，且诱导时间长，败育胚珠离体接种 3 个月后才长出少量愈伤组织。这可能是由于较古老的橘类其次生代谢更丰富，影响了胚性愈伤组织诱导。桃野生近缘种光核桃大量积累次生代谢物，其子叶外植体的愈伤组织诱导率相比一般品种也较低[24]，与本研究结果相似。

本研究发现二倍体品种的胚性愈伤组织诱导率和胚状体发生率均高于其对应的同源四倍体，如 W. 默科特橘橙 2x 的愈伤组织诱导率是其 4x 的 25 倍。以败育胚珠为外植体诱导获得的胚性愈伤组织由胚性珠心组织增殖而来，而柑橘二倍体加倍成四倍体后果实种子数减少[10]，且种子的珠心胚数减少（数据未发表），可

能是导致四倍体胚性愈伤组织诱导率及体细胞胚发生率均低于二倍体的原因。

本研究采用 3 种不同培养基对柑橘败育胚珠进行离体培养，培养基类型影响不同品种的愈伤组织诱导率。前人研究中表明柑橘 MGS 培养基广泛适用于柑橘品种的胚性愈伤组织诱导[18, 25]，本研究同样发现 MGS 培养率基诱导 7 个柑橘品种的胚性愈伤组织诱导率均为最高。除温州蜜柑外，利用 MK 培养基不经过胚状体发生途径直接诱导出胚性愈伤组织的诱导率多于其他 2 种培养基，并且愈伤组织状态更为松软，颜色偏白，可能 0.5 $mg \cdot L^{-1}$ KT 更有利于诱导柑橘形成疏松的胚性愈伤组织。对于 3 个温州蜜柑品种，MK 培养基诱导的愈伤组织结构较为致密、颜色偏黄，而 MGS 培养基诱导的愈伤组织质地疏松、颜色偏白、增殖旺盛。前人研究发现温州蜜柑愈伤组织生长状态与植物生长调节剂有密切关系，添加 GA_3 和麦芽提取物（ME）有利于温州蜜柑愈伤组织生长[26]，本研究在添加了 GA_3 和 ME 的 MGS 培养基中诱导出的温州蜜柑的愈伤组织的状态良好，与前人报道结果相似。

柑橘无核品种由于长期嫁接繁殖累积多种病害。无核品种没有种子，不能利用珠心胚实生苗脱毒，而茎尖微芽嫁接操作要求高，脱毒可能不完全[27]。本研究通过诱导胚性愈伤组织并再生国庆 1 号温州蜜柑、伦晚脐橙和早红脐橙植株均未检测出果园最常见的衰退病和黄脉病病毒，说明胚性愈伤组织通过体细胞胚发生途径再生植株能达到无毒的效果，为无核品种提纯复壮的无毒种苗。早红脐橙是由罗伯逊脐橙和温州蜜柑构成的周缘嵌合体，其L1 层来源于温州蜜柑（果肉），L2/L3 层来源于罗伯逊脐橙（果皮和生殖细胞）[23]。根据“原套—原体”学说，果实的生殖细胞由L2 层细胞衍生而来，可遗传给后代；早红脐橙愈伤组织再生苗由多胚的珠心组织发育而来，属于L2 层，因此其再生植株应为罗伯逊脐橙。SSR分子标记检测证明早红脐橙胚性愈伤组织再生植株确为罗伯逊脐橙。

4 结论

本研究通过离体培养柑橘成熟果实的败育胚珠，经诱导和驯化获得 3 个品种的二倍体/四倍体的胚性愈伤组织，为柑橘多倍体研究提供了稳定易获得的离体材料；二倍体的胚性愈伤组织诱导率和胚状体发生率均高于对应品种的四倍体。利用体细胞胚发生诱导获得 5 个多胚无核品种的愈伤组织再生苗，经鉴定不携带果园常见的黄脉病和衰退病，为品种提纯复壮提供种苗。嫁接嵌合体早红脐橙的愈伤组织再生苗经过 SSR 分子标记鉴定为罗伯逊脐橙。

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