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兔眼蓝莓离体叶片再生组织细胞学观察

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摘 要:离体叶片再生是兔眼蓝莓离体快繁和遗传转化的重要途径。为了探明兔眼蓝莓离体叶片再生途径,以兔眼蓝莓杰兔品种的试管苗叶片为外植体,对离体叶片再生途径进行细胞学观察。结果表明,离体叶片不定芽在 30 d 内基本完成其发生、发育和形成的全过程。离体叶片以直接再生途径发生不定芽,且属于多起源,其分生组织起源于离体叶片切口附近与维管束相邻的上表皮细胞、维管组织薄壁细胞及周围薄壁细胞,通过细胞分裂和分化形成分生细胞团,直接形成芽,再发育成苗。此外,兔眼蓝莓离体叶片不定芽的形成具有特定的时空特性,多个不定芽先后在离体叶片上形成单芽或丛生芽。

关键词:兔眼蓝莓;外植体;离体叶片培养;不定芽再生;细胞学观察
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Histological and Cellular Observations of Regeneration from Leaf Explants of Rabbiteye Blueberry (*Vaccinium ashei* Reade) *in vitro*

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Abstract: The leaf regeneration *in vitro* is an important way of rapid propagation and genetic transformation of rabbiteye blueberry. The regeneration pathway for leaf explants of rabbiteye blueberry were firstly clarified by tissue culture and paraffin section techniques, in which the tube plantlet leaves of rabbiteye blueberry "Premier" were used as explants. The results showed that the adventitious buds on the leaf explants could basically be formed within 30 d *in vitro* culture, going through the processes of occurrence, development and formation. The adventitious buds were regenerated from different tissues of leaf explants of rabbiteye blueberry by direct regeneration way. The meristems originated from the epidermal cells, the parenchyma ones of vascular tissues, and the surrounding ones, which formed meristematic cells by cellular division and differentiation and then formed buds and shoots directly. What's more, the adventitious buds were successively produced with single buds or multiple shoot clumps on leaf explants, which indicates that the formation of adventitious buds had some particular space-time characteristics.

Key words: rabbiteye blueberry; explant; leaf *in vitro* culture; adventitious shoot regeneration; cytological observation

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蓝莓属于杜鹃花科 (Ericaceae) 越桔属(*Vaccinium*)多年生落叶或常绿灌木类果树植物,果实风味醇美、营养丰富,富含多种维生素和其他果品中少有的特殊营养成分[1-5],具有预防心脏疾病、明目、延缓衰老及抗癌等保健功能[6, 7],被誉为世界水果之王。蓝莓是 20 世纪初首先在美国发展起来的一种小浆果类果树,基于其特殊的营养保健功能,已被多个国家和地区引种栽培。

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建立高效的离体叶片培养再生体系, 既是采用叶盘法进行外源基因遗传转化的重要基础, 又有利于植物的离体快繁。蓝莓离体叶片培养研究起步较晚, 始于 1998 年。随着生物技术的发展, 有关蓝莓叶片离体培养技术的研究进展较快, 现已建立了高丛蓝莓多个品种的叶片离体再生培养体系[8~13], 并进行了相关组织学观察[11, 14]和遗传转化研究[15~19]。此外, 矮丛蓝莓的离体叶片再生培养也有相关报道[20, 21]。兔眼蓝莓与高丛蓝莓和矮丛蓝莓并称为蓝莓的三大主要类型, 因果实成熟前颜色红如兔眼而得名。其品质高, 口感好, 经济价值高, 生态适应性广, 抗病虫性较强, 适宜于各种类型土壤栽培。在之前工作中我们已经对兔眼蓝莓离体叶片再生条件进行了研究[22], 为了进一步探明兔眼蓝莓离体叶片再生途径, 本研究以兔眼蓝莓杰兔品种的离体叶片为实验材料, 对不定芽再生过程进行了组织细胞学观察, 以期为兔眼蓝莓遗传转化育种提供细胞学依据。

1　材料与方法

1.1　材料

供试植物材料为湖南师范大学生命科学学院植物园种植的兔眼蓝莓品种杰兔, 以无菌培养获得的试管苗叶片为外植体，选用 WPM 为基本培养基。

1.2　方法

1.2.1　离体叶片的培养

选取继代培养 1~2 个月的试管苗上部第 3~6 片颜色嫩绿的叶片切下, 切除叶柄, 在 1/2 叶片处横断叶片, 以远轴面(叶背面)朝下接种于 WPM+1.7 mg/L ZT+20 g/L 蔗糖+9 g/L 琼脂粉(pH=5.2)的培养基中, 每瓶内放置 10 个外植体, 重复 6 次。先暗培养 (25±2 ℃) 20 d, 然后转移到光照环境下培养, 培养条件为: 温度 25±2 ℃; 光照强度 1 200 lx 左右; 光照周期 14 h/d。

1.2.2　石蜡切片制作及组织细胞学观察

离体叶片接种后每隔 1~3 d 取样 1 次, 共取样 10 次, 将样品在 FAA 固定液(乙醇∶醋酸=3∶1, v∶v)中固定 24 h, 然后换入 70%乙醇中, 于 4 ℃保存备用。用苏木精染料于室温条件下染色 54 h, 然后用蒸馏水冲洗材料表面染色剂, 依次进行浓度梯度酒精脱水、二甲苯透明、浸蜡、包埋, 常规石蜡切片(切片厚度为 6 μm), 将贴好的切片放于 42 ℃恒温箱中烘干, 经二甲苯脱蜡和中性树胶封片后, 置于光学显微镜(Olympus-bx 51, 日本)下进行观察拍照。

2　结果与分析

2.1　离体叶片培养过程中不定芽形态特征

横向切断叶片形成的叶尖段和叶基段在离体培养过程中, 其不定芽诱导率的差异均未达显著水平。离体叶片不定芽在 30 d 内基本完成其发生、发育和形成的全过程。离体暗培养 3 d 后, 叶片出现卷曲加厚现象; 暗培养 10 d 后, 在切口边缘有愈伤组织形成; 暗培养 18 d 时, 可观察到叶腹面叶脉上出现多个透明状的分生细胞团, 靠近切口边缘的叶脉位置容易早形成不定芽, 且主脉是形成不定芽的高频区(图 1a); 暗培养 20 d 后转到光下培养, 在叶基段和叶尖段的叶片腹面近切口处叶脉上均有分化形成的绿色小芽(图 1b, c); 随着叶片的继续培养, 小芽长势茂盛, 幼叶展开, 茎伸长, 形成健壮的小苗(图 1d)。

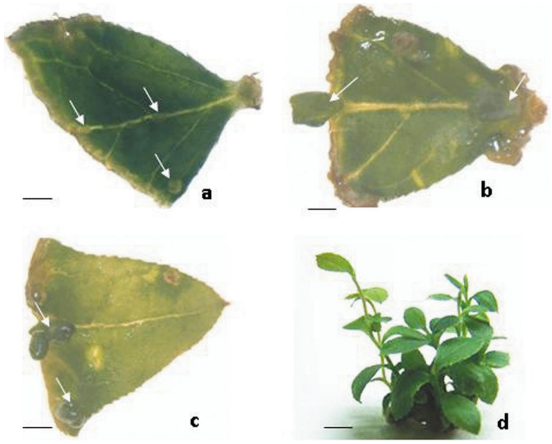

图 1　离体叶片培养过程中再生不定芽形态

a. 离体培养 18 d 叶片的叶基段。叶片腹面叶脉处出现透明状的分生细胞团(箭头所示),bar=1 000 μm;b. 离体培养 30 d 叶片的叶基段。叶片腹面近切口叶脉处分化形成小芽(箭头所示),bar=1 000 μm;c. 离体培养 30 d 叶片的叶尖段。叶片腹面近切口叶脉处分化形成小芽(箭头所示),bar=1 000 μm;d. 离体叶片培养 50 d 后再生形成的丛生小苗, bar=125 μm。

Fig.1　Morphology of adventitious buds from leaf explants during culturing *in vitro*

a. Transparent meristematic cells (arrow) were formed at the veins of leaf abdomen in the leaf petiole after culturing *in vitro* for 18 d, bar=1 000 μm; b. Young buds (arrow) grew at the veins of leaf abdomen in the leaf petiole approaching the cutting after culturing *in vitro* for 30 d, bar=1 000 μm; c. Young buds (arrow) grew at the veins of leaf abdomen in the leaf apex approaching the cutting after culturing *in vitro* for 30 d, bar=1 000 μm; d. A clump shoots were formed from the leaf explants after culturing *in vitro* for 50 d, bar=125 μm.

2.2 离体叶片不定芽发生的组织细胞学特征

通过组织切片技术, 从叶片横切面可以观察到, 叶片由上表皮、下表皮、栅栏组织、海绵组织和维管组织组成。表皮为单层细胞组成, 细胞排列整齐; 栅栏组织呈柱状排列, 细胞体积大, 排列较紧密; 海绵组织细胞形状不规则, 排列疏松, 细胞间隙发达; 栅栏组织和海绵组织的界限明显, 两者间有维管束分布; 维管束周围的薄壁细胞均较其他部位小且排列紧密。在维管组织中, 木质部居于近轴面, 由导管、管胞、木薄壁细胞等组成; 韧皮部位于远轴面, 由筛管、伴胞、韧皮薄壁细胞等组成; 形成层居于木质部与韧皮部之间, 其分裂活动有限(图 2a)。

培养 2~5 d, 离体叶片略有膨大。在切口处的细胞核染色深, 同时一些靠近维管束的栅栏组织细胞及其维管组织薄壁细胞开始分裂(图 2b, c); 在切口附近靠近维管束的薄壁细胞和维管束薄壁细胞染色加深, 细胞核大, 细胞质浓厚, 表明细胞脱分化并开始启动分裂(图 2d)。

培养 6~9 d, 整个叶片组织明显增大, 叶片切口处膨大增厚。在切口处的细胞体积小, 排列紧密, 细胞染色深, 分裂活跃(图 2e, f)。在切口附近的维管组织也增大, 其中维管组织及其周围的脱分化薄壁细胞分裂活跃, 并分化形成染色深的分生细胞团, 其细胞体积小, 细胞核大且位于细胞中央, 细胞质浓厚。这时, 由于维管薄壁细胞活跃的分裂活动, 使维管组织细胞被分散开, 不能保持原有的维管束结构(图 2g, h)。

培养 12 d 时, 切口处有少许愈伤组织形成, 这些愈伤组织不能继续分化形成有效的分生组织细胞团; 同时切口附近紧邻维管束的上表皮细胞染色加深, 细胞核大, 细胞质浓厚, 处于脱分化状态(图 2i)。培养 15 d 时脱分化的上表皮细胞多次分裂, 形成染色深的分生细胞团并突出叶片表面(图 2j)。培养 18 d 时形成圆形排列的分生细胞团(图 2k)。此外, 在切口附近来源于维管组织薄壁细胞和周围薄壁细胞的分生细胞保持旺盛的分裂活动, 它们进一步分化形成多个成束排列的染色较深的分生细胞团(图 2l), 且这些分生细胞在叶肉组织中连成一个染色较深的闭合环形结构(图 2m)。需要注意的是, 分生细胞团在叶片中所处的位置并不相同: 有的离上表皮较近, 随着细胞的分裂很快突出表皮; 有的离上表皮较远, 随着细胞分裂穿过重重叶肉细胞, 最终突破上表皮(图 2m)。这些突出上表皮的分生组织基部与叶片中连成环形的分生细胞相连, 在分生组织两侧分化出叶原基并发育形成两个小叶片, 最后形成小芽(图 2n)。起源于上表皮细胞的分生组织基部与表皮相连, 也能发育成小芽(图 2o)。在蓝莓叶片离体培养过程中, 分生细胞的启动分裂和分生组织的形成具有特定的时空特点: 首先在时间上具有不同步性, 有些已分化形成小芽, 有些则刚启动分裂(图 2m, n); 其次在空间上具有不确定性, 有些相隔一定距离形成单生苗, 有些则聚集在一起形成丛生苗(图 2m~p)。

3 讨论

大量的研究表明, 植物离体叶片培养过程中可通过器官发生和体细胞胚胎发生的直接或间接途径再生植株[23, 24]。有关高丛蓝莓和矮丛蓝莓叶片离体培养的研究认为, 大部分的再生植株是通过器官发生途径进行的[8, 13, 20, 25]。最近有研究者通过组织学观察证实了高丛蓝莓品种‘Aurora’离体叶片再生不定芽是通过直接器官发生途径进行的[14]。在本研究中, 通过组织学观察发现,兔眼蓝莓离体叶片再生不定芽起源于靠近叶片切口处的表皮细胞(图 2i), 以及维管束薄壁细胞和周围薄壁细胞(图 2b~d), 它们通过旺盛的细胞分裂和分化形成分生组织(图 2j~l), 进而形成芽(图 2n, o)。虽然叶片切口处细胞脱分化, 分裂形成愈伤组织, 但是不能进一步分化形成不定芽, 首次证实了兔眼蓝莓离体叶片再生不定芽属于直接器官发生的方式。直接再生器官发生不经过愈伤组织阶段, 而是在外植体上直接诱导产生不定芽, 可以减少或避免因组织培养引起的遗传突变。因此, 本研究所获得的高效稳定离体叶片再生体系, 有助于兔眼蓝莓离体快繁和遗传转化。

直接再生不定芽的分生组织起源存在三种形式: 第一种是外起源, 起源于外植体表皮细胞和亚表皮细胞[26, 27]; 第二种是内起源, 起源于外植体维管束周围薄壁细胞[28~31]; 第三种是多起源, 起源于外植体表皮细胞和维管束周围薄壁细胞[14, 32]。在本研究中, 兔眼蓝莓叶片再生不定芽属于多起源, 起源于离体叶片表皮细胞(图 2i)和维管束及周围的薄壁细胞(图 2b~d), 且主要发生在靠近切口的叶脉上(图 1a~c)。外源生长调节剂通过调节外植体内源激素水平及不同激素之间的平衡来控制植物离体培养的器官发生[32, 33]。维管束是激素等外

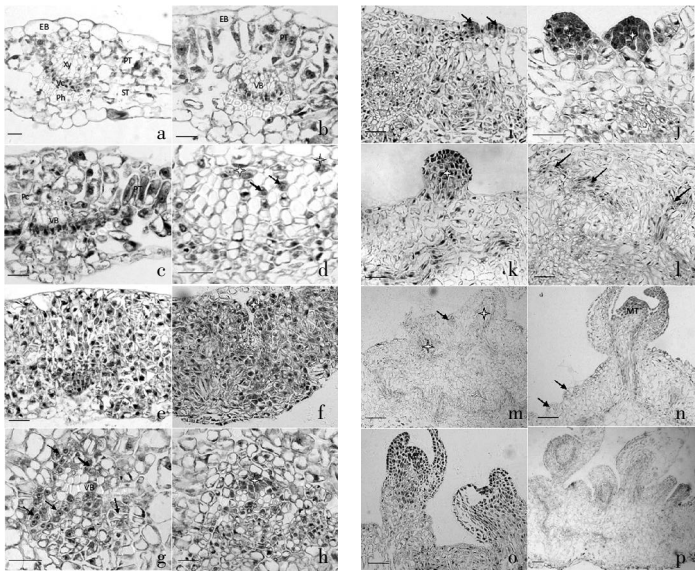

图 2　离体叶片不定芽再生过程的组织学观察(Bar=50 μm)

a. 叶片横切面,可见叶表皮(EB)、栅栏组织(PT)、海绵组织(ST)以及由木质部(Xy)、韧皮部(Ph)和形成层(Vc)组成的维管组织;b. 培养 4 d 叶片切口处,靠近维管组织(VB)的栅栏组织(PT)细胞核染色深;c. 培养 5 d 叶片切口处,靠近维管组织(VB)的薄壁细胞(Pc)和栅栏组织(PT)细胞处于分裂中;d. 培养 3 d 叶片近切口处,维管组织薄壁细胞(箭头)和周围薄壁细胞(星号)染色加深,细胞核大,细胞质浓厚;e. 培养 6 d 叶片切口处,细胞体积较小,排列较紧密,染色深;f. 培养 9 d 叶片切口处,细胞体积小,排列紧密,染色深,分裂活跃,且分化形成染色深的分生细胞团(星号);g. 培养 6 d 叶片近切口处,维管组织(VB)及其周围的分生细胞(箭头)分裂活跃;h. 培养 9 d 叶片近切口处,维管组织及其周围的分生细胞分裂活跃,并进一步分化成分生细胞团(星号);i. 培养 12 d 叶片近切口处,上表皮细胞(箭头)染色深,细胞核大,胞质浓厚;j. 培养 15 d 叶片近切口处,由脱分化的表皮细胞分裂多次形成的分生细胞团(星号);k. 培养 18 d 叶片近切口处,起源于表皮细胞的圆形排列的分生细胞团(星号);l. 培养 21 d 叶片近切口处,叶肉组织中形成成束排列的染色较深的分生细胞团(箭头);m. 培养 24 d 叶片近切口处,叶肉组织中的分生细胞连成一个染色较深的闭合环形,分裂活跃的分生细胞团(星号)在叶片中所处的位置和发育时期不同,箭头所示为起源于表皮细胞的分生细胞团;n. 培养 30 d 叶片近切口处形成的小芽,分生组织(MT)基部与叶肉中的环形分生细胞相连, 在分生组织两侧分化形成两个小叶片, 箭头所示为起源于表皮细胞的分生细胞团;o. 培养 27 d 叶片近切口处形成的小芽,分生组织(MT)基部与表皮相连,两侧分化形成两个小叶片;p. 培养 30 d 叶片近切口处分化形成多个分生组织。

Fig.2　Histological observation of the process of adventitious bud regeneration from leaf explants (Bar=50 μm)

a. The leaf cross-section, showing leaf epidermis (EB), palisade tissue (PT), sponge tissue (ST) and vascular tissue containing xylem (Xy), phloem (Ph), and cambium (Vc); b. The cutting of leaves after culturing *in vitro* for 4 d, showing the cells with the deeply stained nuclei close to the vascular tissue (VB) of palisade tissue (PT); c. The cutting of leaves after culturing *in vitro* for 5 d, showing the dividing parenchyma cells (Pc) and palisade tissue (PT) ones close to the vascular tissue (VB); d. The near cutting of leaves after culturing *in vitro* for 3 d, showing the parenchyma cells (arrow) in the vascular tissue and the surrounding parenchyma ones (asterisk) with deeply stained large nuclei and dense cytoplasm; e. The cutting of leaves after culturing *in vitro* for 6 d, showing the small-sized and closely packed and deep staining cells; f. The cutting of leaves after culturing *in vitro* for 9 d, showing the small-sized and closely packed and deep staining cells, which had active division and differentiated into deeply stained meristematic cells (asterisk); g. The near cutting of leaves after culturing *in vitro* for 6 d, showing the meristematic cells (arrow) in the vascular tissue and the surrounding ones with active division; h. The near cutting of leaves after culturing *in vitro* for 9 d, showing the active division cells in the vascular tissue and the surrounding ones differentiated into meristematic cells (asterisk); i. The near cutting of leaves after culturing *in vitro* for 12 d, showing the upper epidermis cells with the deeply stained large nuclei and dense cytoplasm (arrow); j. The near cutting of leaves after culturing *in vitro* for 15 d, showing the meristematic cells (asterisk) formed by multiple division of dedifferentiation epidermis cells; k. The near cutting of leaves after culturing *in vitro* for 18 d, showing the meristematic cells (asterisk) originating from epidermis cells in a circular array; l. The near cutting of leaves after culturing *in vitro* for 21 d, showing the deeply stained meristematic cells (arrow) originating from mesophyll tissue in a bundle arrangement; m. The near cutting of leaves after culturing *in vitro* for 24 d, showing the meristematic cells (asterisk) in mesophyll tissue into a closed ring with deep staining. The meristematic cells were in the different position with different development period. The arrow shows the meristematic cells originating from epidermis cells; n. The near cutting of leaves after culturing *in vitro* for 30 d, showing the small buds, whose meristem (MT) connected to ring meristematic cells in mesophyll and formed two small blades on both sides. The arrow shows the meristematic cells originating from epidermis cells; o. The near cutting of leaves after culturing *in vitro* for 27 d, showing the small buds, whose meristem (MT) connected to epidermis cells and formed two small blades on both sides; p. The near cutting of leaves after culturing *in vitro* for 30 d, showing multiple meristems.

源物质运输的主要通道，有利于周围细胞脱分化和再生[28, 34-36]。因此，维管束在兔眼蓝莓离体叶片薄壁组织细胞脱分化过程中起了重要作用。

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