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植物酶的组织和细胞化学定位研究方法

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摘要: 酶是参与植物体内生化反应的特殊蛋白质。在保持活组织和细胞结构完整性的条件下, 利用组织化学、细胞化学、免疫学和显微检测等技术研究酶的即位定位, 是了解酶在组织、细胞和亚细胞中的分布、活性动态与定量及酶功能等的重要途径。对植物体中酶定位的组织化学和细胞化学方法的概念、原理与研究进展进行了综述, 并根据国际酶化学分类编号顺序, 分别介绍了 25 种酶的组织化学染色定位所用的反应介质和染色方法及 46 种酶的细胞化学定位方法的参考文献。

关键词: 植物酶; 组织定位; 染色方法; 细胞化学定位
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Methods of *In situ* Histochemical and Cytochemical Localizations of Plant Enzymes

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Abstract: Enzymes are special proteins that catalyze and regulate a series of life processes in plants. Studies on *in situ* histochemical, cytochemical, immunological and microscopical localizations of metabolism enzymes in intact plant tissues and cells are important approaches in understanding the distributions, activity dynamics and quantifications, and functions of enzymes within tissues, cells and organelles. The basic concepts, principles and research progresses of histochemical and cytochemical localizations of enzymes were reviewed. According to the international enzymology classification and taxis, the reaction media and staining method of histochemical localizations on 25 plant enzymes and references of cytochemical localizations on 46 plant enzymes were introduced, respectively.

Key words: Plant enzyme; Histochemistry localization; Stainning method; Cytochemistry localization

酶是广泛分布于生命有机体的组织细胞内具有催化活性的特殊蛋白质, 参与体内几乎所有的细胞功能活动进程。有些酶类还是不同亚细胞器的标志酶, 如酸性磷酸酶是溶酶体的标志酶, 过氧化氢酶是过氧化物酶体的标志酶, 葡萄糖-6-磷酸酶是内质网的标志酶, 氨肽酶是微粒体的标志酶, 琥珀酸脱氢酶和细胞色素氧化酶是线粒体的标志酶[1–2]。目前, 由国际生物化学酶学委员会(Enzyme Commission, EC)认定并注册登记编号的酶约有 2000 多种。按照酶对底物的特异性、催化性和来源, 酶的命名可分为 6 大类: 氧化还原酶(Oxidoreductase, EC 1)、转移酶(Transferase, EC 2)、水解酶(Hydrolase, EC 3)、裂解酶(Lyase, EC 4)、异构酶(Isomerase, EC 5)和合成酶(Synthetase, EC 6), 这些酶中已知能被现有的组织化学和细胞化学技术原位检测显示的酶仅有 200 多种[3]。1980 年, 简

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令成认为已知的酶总数接近1000种,其中约有100种可在光学显微镜上通过组织化学的定位方法得以证实[2],但他未明确指出这些均是植物酶抑或只是当时的技术水平所能检测的包括动物等在内的酶总数。由于植物的组织和细胞结构有别于人类和动物的,通常对植物酶的原位组织化学与细胞化学检测技术的研究水平和数量均显著比对人类和动物的要低。目前还缺乏较系统地涉及植物的组织化学与细胞化学方面的研究报道,故本文的目的在于初步收集与此相关的文献,为有兴趣从事此类研究的人员提供参考。

研究酶在植物组织和细胞内的即位(*in situ*)定位、活性动态和调节是了解酶功能的有效途径[4]。活细胞中酶活性定位研究包括组织和细胞水平的定位技术,这两种定位技术均要求操作过程中不能破坏目标酶的正常分布、酶与底物反应有高度的专一性、反应产物不可扩散移位。因此,保持活细胞和组织结构的完整性是获得一个酶在体内的活性数据的最重要前提[1,5]。两种定位法的差别在于:前者利用组织化学技术在酶存在的组织中专一生成反应产物,并在光学显微镜下观察酶的组织定位分布,而后者则借助于细胞化学技术在细胞的酶活性位置形成不溶性的高电子密度沉淀物或免疫标记产物,再利用电子显微镜或荧光显微镜原位检测酶的亚细胞定位,并可定性和定量[2]。

酶的组织定位方法是利用组织学及理化方法使目标酶作用于特定底物,再通过反应产物与染色剂发生作用产生有色的不溶性沉淀,通过显微镜检测或肉眼观察来判断目标酶在组织中的分布位点,还可利用专一的电脑软件进行相对的定量分析[3]。为了获得最佳的定位结果,组织化学定位研究必须仔细选择反应条件并保持组织和其细胞器完整性,并设对照实验,避免出现假象[6]。组织化学定位的流程包括新鲜材料的取材、固定、切片、温育反应和定位观察等,操作技术则因不同组织、不同酶而有所差别。组织化学定位的优点在于易操作,低成本,主要用于酶的分布特性研究。但是,由于具有专一性的染色剂种类不多,使组织化学定位的应用受到一定的限制[7]。

本文简要综述酶定位的组织化学和细胞化学方法的概念、原理与研究进展,并依酶学命名分类的排序,分别介绍已知的25种植物酶类的即位组织定位染色检测方法。此外,还提供了46种酶的细胞化学定位方法的参考文献,共88篇。

1 酶的组织化学定位研究方法

常用的酶的组织定位的染色法[2,5,8]主要有:(1)联苯胺(Benzidine)法。DAB (3-3-Diaminobenzidine, 3′,3-二氨基联苯胺)在POD或偶联POD的催化下脱氢聚合,再氧化出现棕褐色的阳性产物。(2) 四唑盐(Tetrazole)法。酶催化底物氧化释出质子,用NBT (Nitroblue tetrazolium, 氮蓝四唑)作最终的电子受体,阳性反应产物为蓝色。此染色法用于脱氢酶、氧化酶、还原酶和转移酶的定位研究。(3) 靛蓝(Indigo blue)形成法。以酯型吲哚酚(Indoxyl)为底物,在酶的催化下分解出吲哚酚,再氧化生成蓝色靛蓝。此法常用于酯酶和磷酸酶的定位研究。(4)金属沉淀法。利用铜、铁、铅、银等在酶的催化下生成有色的沉淀,常用于磷酸化酶和ATP酶的组织定位研究。下面按照酶化学分类及其编号顺序对已知的酶组织化学定位方法简述如下:

1.1 氧化还原酶类

乙醇脱氢酶 (Alcohol dehydrogenase, ADH, EC 1.1.1.2) 以NBT作为人工电子受体,酶促反应产生不溶性的蓝色甲臜(Formazane)。新鲜切片放于反应介质中40℃保温1 h,然后用蒸馏水清洗,照相。介质包含3 mmol L^{-1} $MgCl_2$,1% 蔗糖,0.6 mmol L^{-1} NBT,1 mmol L^{-1} 吩嗪硫酸甲酯(Phenazine methosulfate, PMS),0.5 mmol L^{-1} NADP,2% PVP360K,0.01% Tween-20,0.5 mmol L^{-1} 乙醇的二甲胂酸钠(Na-cacodylic acid)缓冲液,pH 7.4。ADH将乙醇转化为乙醛时将NBT还原生成不溶性的蓝色甲臜[9]。对照不加乙醇,或加15 mmol L^{-1} 的吡唑(Pyrazole)抑制ADH活性。另一种相似的方法是:反应缓冲液含100 mmol L^{-1} 磷酸钠(pH 7.5),400 μmol L^{-1} NAD,100 μmol L^{-1} NBT,3% 乙醇。切片在缓冲液中于30℃暗下保温10~15 min,至深蓝色的甲臜出现,蒸馏水冲洗切片,照相。以叶片为试材时需延长染色时间至数小时[10]。

乳酸脱氢酶 (Lactate dehydrogenase, LDH, EC 1.1.1.27) 以底物乳酸(Lactate)作电子供体,铁氰化钾(K-ferricyanide)作电子受体,Cu^{2+} 作捕获剂形成亚铁氰酸铜沉淀。反应过程同时加酒石酸钠钾(Na-K-tatrate)作为螯合剂阻止 Cu^{2+} 与反应介质其

他成分形成沉淀。反应介质含 50 mmol L^{-1} 磷酸钾缓冲液(pH 7.2), 5 mmol L^{-1} Na-K-tartrate，5 mmol L^{-1} $CuSO_4$，20 mmol L^{-1} 铁氰化钾，0.5 mol L^{-1} 乳酸(D-Lactate)[11]。

苹果酸脱氢酶 (Malate dehydrogenase, MDH, EC 1.1.1.37)　利用NBT还原的色素形成法和酶的专一底物产生有色沉淀物进行组织定位。8 μm厚的冷冻切片置于含 160 mmol L^{-1} 苹果酸钠(Na-malate)，0.66 mmol L^{-1} NAD，0.084 mmol L^{-1} PMS及 0.77 mmol L^{-1} NBT 的 63 mmol L^{-1} 磷酸钾缓冲液(pH 7.6)中，37℃温育 60~120 min。随后于 4%中性福尔马林中固定 15 min,重蒸馏水洗,甘油-白明胶封片,镜检。对照的保温介质中不加底物苹果酸钠[12]。Fatelli 等[13]提出将手切切片在 2% PVP-40, 2 mmol L^{-1} DTT, 2% 多聚甲醛(Paraformaldehyde)，0.1% BSA中固定20 min后,移至1% Triton于–20℃下过夜。取出切片解冻,用冷水洗 4 次,每次间隔 30 min。MDH 的另一种反应介质含 50 mmol L^{-1} Tris-HCl 缓冲液(pH 7.0)，0.5 mmol L^{-1} NAD 或 NADP，0.025% BSA，0.03% NBT，1 mmol L^{-1} EDTA，5 mmol L^{-1} $MgCl_2$，8 mmol L^{-1} 苹果酸钠，室温温育 15 min,加 4% 甲醛停止反应,以不加苹果酸钠者作对照。

葡糖-6磷酸脱氢酶 (Glucose-6-phosphate dehydrogenase, G6PDH, EC 1.1.1.49)　定位原理与 MDH 相同。冷冻组织切片 10~12 μm 厚,反应介质含 20% 聚乙烯醇(Polyvinylalcohol, 作为稳定剂)的 0.2 mol L^{-1} Tris-maleats 缓冲液 20 mL (pH 7.2)，NBT 10 mg，NADP 10 mg，G6P (Glucose-6-phosphate, 葡糖-6-磷酸) 60 mg。37℃温育 20~45 min 或室温温育 60~75 min 后水洗,封入甘油,镜检蓝色沉淀的部位。对照不加 G6P[14]。

乙醇酸脱氢酶 (Glycolate dehydrogenase, GDH, EC 1.1.99.14)　采用金属盐法,与 LDH 的定位法相似,酶活性位点呈褐棕色[11]。样品可用组织切片或细胞,用 50 mmol L^{-1} 磷酸钾缓冲液(pH 7.2)洗 2 次后,放入反应介质中于 25℃暗下温育 20 min。反应介质为:50 mmol L^{-1} 磷酸钾缓冲液(pH 7.2)，5 mmol L^{-1} Na-K-tartrate，5 mmol L^{-1} $CuSO_4$，20 mmol L^{-1} K-ferricyanide，20 mmol L^{-1} 乙醇酸钠(Na-glycolate)。

GSH-甲醛脱氢酶 (GSH-formaldehyde dehydrogenase, FALDH，EC 1.2.1.1., 也称 Class III ADH)定位原理也是利用 NBT 还原的显色法。整株幼苗或组织切片放于含 15 mL 反应介质的液闪瓶中，反应介质为:100 mmol L^{-1} 磷酸钠缓冲液(pH 7.5)，9 mmol L^{-1} 丙酮酸钠(Na-pyruvate), 0.1% Triton X-100, 0.6 mmol L^{-1} NAD, 0.02 mg mL^{-1} PMS, 0.2 mg mL^{-1} NBT,用4.8 mmol L^{-1} 甲醛和1 mmol L^{-1} 谷胱甘肽(Glutathione, GSH)反应产生此酶的真正底物羟甲基谷胱甘肽(S-Hydroxymethylglutathine, 0.73 mmol L^{-1})。真空渗入 10 min, 42℃暗下保温 1 h,水洗停止反应,用 70% 乙醇洗几次以限制非专一背景色,酶活性部位显现蓝色。对照只加甲醛或 GSH[15]。

氨基醛脱氢酶 (Aminoaldehyde dehydrogenase, AMADH，EC 1.2.1.19)　利用 NBT 还原法显色。酶染色液含 0.15 mol L^{-1} Tris-HCl 缓冲液(pH 8.5), 1 mmol L^{-1} 3-氨基丙酰醛(3-Aminopropionaldehyde, APAL)，1 mmol L^{-1} NAD, 0.15 mmol L^{-1} PMS, 0.75 mmol L^{-1} NBT，25℃暗下温育 1~3 h。对照不加底物 APAL[16]。

黄嘌呤脱氢酶 (Xanthine dehydrogenase, XDH, EC 1.2.1.37)　通过 NBT 还原形成蓝色不溶物而定位。切片在反应介质中室温暗下温育 3 h,反应介质含 0.55 mL 1.8 mg mL^{-1} 的 NBT, 1.15 mL 次黄嘌呤(Hypoxanthine)溶液(3 mL 10 mmol L^{-1} 次黄嘌呤 + 0.1 mol L^{-1} KOH + 2 mL 0.5 mol L^{-1} Tris-HCl 缓冲液，pH 7.1), 0.05 mL 10 mg mL^{-1} $MgCl_2$, 0.1 mL 4.5 mg mL^{-1} NAD, 2.5 mL 聚乙烯醇 (Polyvinyl alcohol，20 g 在 90℃下溶于 50 mL 的 50 mmol L^{-1} Tris-HCl, pH 7.4),最后加入 0.1 mL 32 mg mL^{-1} 的叠氮化钠(NaN_3)和 0.1 mL 1.0 mg mL^{-1} 的 PMS。对照不加底物或加 XDH 的抑制剂别嘌呤醇(Allopurinol)[17]。

乙醇酸氧化酶 (Glycolate oxidase, GO, EC 1.3.3.1)定位原理是金属盐法显色。反应介质含 8.5 mL 25 mmol L^{-1} 磷酸钠缓冲液(pH 7.2), 0.5 mL 60 mmol L^{-1} $CuSO_4$, 40 mmol L^{-1} Na-K-tartrate (pH 7.2)，1 mL 铁氰化钾，5 mg PMS, 2 mg 黄素单核苷酸(Flavin mononucleotide, FMN)，12.5 mg 乙醇酸钠[18]。

琥珀酸脱氢酶 (Succinate dehydrogenase, SDH, EC 1.3.5.1)　利用 NBT 还原显色法。8 μm 厚的冷冻切片置于 160 mmol L^{-1} 琥珀酸钠(Na-succinate), 0.82 mmol L^{-1} NBT 的 63 mmol L^{-1} 磷酸钾缓冲液(pH 7.6)中，37℃温育 60 min,再用 4% 中

性福尔马林固定 15 min,重蒸馏水洗,甘油-白明胶封片,镜检。对照不加底物[12]。

胺氧化酶 (Amino oxidase, DAO, EC 1.4.3.6;PAO, EC 1.5.3.3) DAB 被 H_2O_2 氧化形成棕色不溶性聚合物或氯萘酚(4-Chloronaphthol)氧化为苯胺红沉淀。多胺氧化酶染色液:1 mmol L^{-1} 亚精胺(Spermidine, SPD)或腐胺(Putrescine, Put)和 1 mg mL^{-1} DAB 的 pH 3.8 缓冲液。光下放置 18 h,酶催化反应产生的 H_2O_2 与 DAB 生成红棕色不溶物[19]。或用 POD 偶联试验,切片在含 60 μg mL^{-1} POD, 0.04% DAB 的 10 mmol L^{-1} 磷酸钠缓冲液(pH 6.5)中预保温 10 min,然后加 3 mmol L^{-1} Spd 保温,水洗 3 min 停止反应。切片封入 25% 甘油,光镜检测,对照不加 Spd[20]。含 Cu 的胺氧化酶染色液为:50 mmol L^{-1} 磷酸缓冲液(pH 5.0), 5 mmol L^{-1} Put, 2.5 mmol L^{-1} 氯萘酚, 5 U mL^{-1} POD,室温温育 30 min,水洗停止反应。酶催化反应产生的 H_2O_2 与氯萘酚被 POD 催化生成蓝色的苯胺红(Magenta)沉淀[21]。

细胞激动素氧化酶/脱氢酶 (Cytokinin oxidase/dehydrogenase, CKX, EC 1.5.99.12) 通过 NBT 还原形成蓝色不溶物。20~40 μm 厚的切片浸泡于含 5 mmol L^{-1} 异戊烯腺嘌呤(Isopentenyladenine, 底物), 0.15 mmol L^{-1} PMS, 0.75 mmol L^{-1} NBT 的 Tris-HCl 缓冲液(pH 8.0)的染色液中,暗下 37℃温育 0.5~24 h, 检查紫色产物形成。为了防止温育过程中试剂产生沉淀,每隔 6 h 用 0.2 mol L^{-1} Tris-HCl 缓冲液(pH 8.0)冲洗切片并转入新鲜的染色液中,光镜观察蓝色产物出现的部位[22–23]。对照的染色液中不加底物。

细胞色素氧化酶 (Cytochrome oxidase, Cyox, EC 1.9.3.1) 以 α-萘酚(α-Naphthol)为底物催化的反应产物与对氨基二甲基苯胺(N-Dimethylaniline hydrochloride)的重氮基结合产生偶联偶氮反应,形成偶氮色素(Azo-dyes)。新鲜切片放入 pH 5.8 的 100 mmol L^{-1} 磷酸缓冲液,室温温育 5~10 min 后,移入 1∶1 (V/V)的 1% α-萘酚和 1% 盐酸对氨基二甲基苯胺液中染色 5 min,生成蓝色靛酚产物。对照样品先煮沸杀灭酶活性再加底物混合液或先用 20 mmol L^{-1} KCN 抑制酶活性后再加同上的反应底物[24]。

多酚氧化酶 (Polyphenol oxidase, PPO, EC 1.10.3.1) 酶催化多酚氧化生成褐色的醌类或经 DAB 或 3-甲基苯并噻唑啉酮腙(3-Methyl-benzotiazolinone hydrazine, MBTH)氧化而显色。切片放入 pH 7.2 的磷酸缓冲液于 2℃~5℃放置 5 min,再移入 1% 邻苯二酚(Catechol)液中, 37℃温育 10 h, 出现茶褐色或棕色络合物[21]。当检测儿茶酚氧化酶 / 酪氨酸酶 / 漆酶 (Catechol oxidase/tyrosinase/laccase) 的组织定位时,染色液含各自所需的底物及 0.05% DAB, 0.1% CAT 的 0.1 mol L^{-1} 磷酸钾(pH 7.0)缓冲液(加 CAT 降解内源 H_2O_2,防止 POD 染色对 PPO 活性定位的干扰),组织片段 / 切片在 37℃暗下温育 30~90 min,观察出现橙色产物的部位。对照不加 DAB[25]。López-Serrano 等[26]报道的 PPO 组织定位反应介质为:5 mmol L^{-1} 酪胺(Tyramine), 2 mmol L^{-1} MBTH 的 50 mmol L^{-1} K-phosphate 缓冲液(pH 6.8)。在有或无 5 mmol L^{-1} 环庚三烯酚酮(Tropolone,一种 PPO 抑制剂)下 25℃温育 15~30 min,观察桃红色产物出现的部位。

过氧化物酶 (Peroxidase, POD, EC 1.11.1.7) 用联苯胺蓝色显示或 H_2O_2 介导酚类氧化显色。POD 组织定位有多种方式:(1) 新鲜切片放入磷酸缓冲液(pH 7.2)中于 25℃浸泡 5 min,移入 1% 钼酸中 5 min,再用 0.1% 联苯胺(加 1 滴 30% H_2O_2)处理 0.5~1.0 min,镜检蓝色络合物生成的部位,对照加 10 mmol L^{-1} NaF 抑制 POD 或先用沸水杀死酶[24]。(2) 直接将切片置于 50 μmol L^{-1} 的四甲基联苯胺(3,5,3,5-tetramethylbenzidine-HCl, TMBZ, 一种 POD 的底物)的 Tris-acetate 缓冲液(pH 5.0)中,加入 0.33 mmol L^{-1} H_2O_2, 25℃温育 15~30 min。对照不加 H_2O_2,观察亮绿产物出现的位点[26]。(3) 染色液包含 50 mmol L^{-1} KH_2PO_4/K_2HPO_4 缓冲液(pH 5.3), 5 mmol L^{-1} H_2O_2, 10 mmol L^{-1} 愈创木酚(Guaiacol)。切片浸泡于染色液中 10 min, POD 活性位点呈红棕色,对照不加 H_2O_2[27]。

1.2 转移酶类

蔗糖合酶 (Sucrose synthase, SUS 或 Susy, EC 2.4.1.13) 在几种工具酶参与下,蔗糖合酶催化蔗糖降解,使 NBT 还原而显色。反应介质含 50 mmol L^{-1} Hepes-NaOH 缓冲液(pH 7.4), 5 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} EDTA, 0.1% BSA, 1 mmol L^{-1} EGTA, 1 mmol L^{-1} NAD, 1 U 葡糖磷酸变位酶(Phosphoglucomutase, PGM), 1 U G6PDH, 20 mmol L^{-1} 葡糖-1,6-二磷酸 (Glucose-1,6-diphosphate, G-1-6P), 1 U 尿苷二磷酸焦磷酸化酶(Uridine-5′-diphosphoglucose

pyrophorylase, UDPase), 0.03% NBT。反应由加入底物终浓度分别为 3.6 mmol L^{-1} 蔗糖、71 μmol L^{-1} UDP、71 mmol L^{-1} 焦磷酸(PPi)而开始。对照不加蔗糖或 PGM, G-1-6P, PPi 或 NAD[28]。Wittich 等[29]以 200 μm 或 1 mm 厚的玉米(*Zea mays*)粒切片为试材,先用 2% 多聚甲醛 + 2% PVP-40，5 mmol L^{-1} DTT 于 4℃固定 1 h,水洗 5 次除去可溶性糖。反应介质含 5 μL 150 mmol L^{-1} NAD，1 U PGM 5 μL，5 μL 3 mmol L^{-1} G-1,6-P，1 U G6PDH 5 μL，5 μL UDPase, 280 μL 0.07% NBT，350 μL 缓冲液(100 mmol L^{-1} Hepes, 10 mmol L^{-1} $MgCl_2$, 2 mmol L^{-1} EDTA, 0.2% BSA, 2 mmol L^{-1} EGTA, pH 7.4)和 50 μL 底物(含 0.75 mol L^{-1} 蔗糖，15 mmol L^{-1} UDP, 15 mmol L^{-1} PPi)。SUS 催化蔗糖降解,引起 NBT 还原产生蓝色不溶产物。

己糖激酶 (Hexokinase, HK, EC 2.7.1.1)**和果糖激酶** (Fructokinase, FK, EC 2.7.1.4)　在相关的工具酶和 ATP 参与下,酶催化底物葡萄糖或果糖转化为 G-6-P, 氧化 NBT 而显色。反应介质含 50 mmol L^{-1} 二羟基氨基-2 羟甲基-1,3-丙二醇(BisTris) 缓冲液 (pH 8.0)、6.25 mmol L^{-1} $MgCl_2$、2.5 mmol L^{-1} ATP、1 mmol L^{-1} NAD、1 U G6PDH、1 U 磷酸葡糖异构酶(Phosphoglucose isomerase, PGI,只在 FK 定位时加入)、12.5 mmol L^{-1} Hepes-NaOH pH 7.4、0.25 mmol L^{-1} EGTA、0.25 mmol L^{-1} EDTA、0.025% BSA、0.03% NBT。酶反应以加 0.5 mmol L^{-1} 葡糖(测 HK 时)或 0.5 mmol L^{-1} 果糖(测 FK 时)开始。 对照不加底物或不加 ATP 或 G6PDH 或 NAD[28]。

腺苷二磷酸焦磷酸化酶 (Adenosine-5′-diphosphoglucose pyrophosphorylase, AGPase, EC 2.7.7.7)　利用相关工具酶，ATP 和 PPi 催化底物 ADPGlc (Adenosine-5′-diphosphoglucose，腺苷二磷酸葡糖)转化为 G-1-P, 氧化 NBT 而显色。反应介质含 75 mmol L^{-1} Hepes-NaOH 缓冲液 (pH 8.0)、0.44 mmol L^{-1} EDTA、5 mmol L^{-1} $MgCl_2$、0.1% BSA、1 mmol L^{-1} NAD、20 mmol L^{-1} G-1,6-P、2 U PGM、6 U G6PDH、2 mmol L^{-1} 3-磷酸甘油酸(3-Phosphoglyceric acid, 3-PGA)、10 mmol L^{-1} NaF、1.4 mmol L^{-1} PPi、0.03% NBT。以加入 2 mmol L^{-1} ADPGlc 开始反应。对照不加 ADPGlc 或 ADPGlc+PPi 或 NAD[28]。

尿苷-5′-二磷酸焦磷酸化酶 (Uridine-5′-diphosphoglucose pyrophosphorylase, UGPase, EC 2.7.7.9)　以尿苷二磷酸葡萄糖(UDPGlc)为底物,催化其转化为 G-1-P, 还原 NBT 而显色。反应介质含 100 mmol L^{-1} Hepes-NaOH 缓冲液 pH 7.5、1 mmol L^{-1} EDTA、2 mg $Mg(AC)_2$、1 mmol L^{-1} NAD、1 U PGM、1 U G6PDH、20 mmol L^{-1} G-1-6-P、0.9 mmol L^{-1} PPi、0.03% NBT。反应由加入 5 mmol L^{-1} UDPGlc 开始。对照不加 UDPGlc+PPi 或 NAD 或 UDPGlc[28]。

1.3 水解酶类

非专一性酯酶 (Non-specific esterase, NE, EC 3.1.1.1)　利用偶联偶氮色素法(Azo-dye method)。样品于 4% 甲醛的磷酸缓冲液(pH 7.2)中固定 1.5 h,移入 7.5% 蔗糖溶液低压渗入 10 min。反应介质含 30 mL 磷酸缓冲液(pH 6.4)，0.5 mL 1% 萘酚-AS-乙酸盐的二甲基酰胺(Naphthol AS-acetatedimethylformamide)溶液，20 g 快蓝 B (Fast blue B 盐，FB)和 4 mL 25% 二甲亚砜(Dimethylsulphoxide, DMSO)。温育 3 h 过程中置换反应介质几次,然后用缓冲液冲洗样品以停止反应,再放于 100℃中 2 s,甘油-白明胶封片,镜检蓝色偶氮染料产物。利用成像分析技术,用 Lucis M 软件(Lim CZ)测定切片上相同面积的染色部位和对照的光密度,计算 NE 的相对活性[30]。

酸性磷酸化酶 (Acid phosphorylase, ACPase, EC 3.1.3.2)　利用金属盐法形成 PbS 沉淀。切片放入 80% 乙醇中，5 min 更换 1 次,共 3 次,接着换成无水乙醇,共 3 次。再移入 0.5%~1% 的火棉胶中 30 s,取出用 80% 乙醇洗 1 次,水洗 3 次,浸水 24 h。取出吸干,加反应试剂(4 mL 0.1 mol L^{-1} 醋酸缓冲液 pH 5.1, 1 mL 0.1 mol L^{-1} 醋酸铅, 0.6 mL 水, 0.4 mL 3.2% 甘油磷酸钠), 37℃温育 75~90 min 后,用水洗 3 次, 2% HAC 作用 1~2 min,再用水洗 3 次。最后加 2% 硫化铵 0.5~1 min,水洗 3 次,镜检或用甘油封藏,观察 PbS 黑色沉淀[21]。Cashikar 等用的固定液包含 3.5% 甲醛，5% 醋酸，50% 乙醇,而反应介质含 52 mmol L^{-1} 醋酸缓冲液(pH 5.0), 4 mmol L^{-1} $Pb(NO_3)_2$, 1 mmol L^{-1} ATP, 温育 1~2 h 后水洗,加入 0.5% $(NH_3)_2S$,水洗封片后,用相差显微镜观察 PbS 黑色沉淀的位点[31]。

酸性转化酶 (Acid invertase, AI，EC 3.2.1.26)　在葡萄糖氧化酶(Glucose oxidase, GOD)参与下, AI 转化蔗糖为葡萄糖并还原 NBT 为蓝色沉淀。

新鲜切片用4%福尔马林(pH 7.0)于4℃固定3 min后，水洗除去内源的糖类(1 h内至少洗10次)，然后移入含0.96 mg mL^{-1} NBT，0.56 mg mL^{-1} PMS，25 U mL^{-1} GOD及100 mg mL^{-1} 蔗糖的0.38 mol L^{-1} 磷酸钠缓冲液(pH 6.0)中，室温下温育，水洗后光镜拍照，观察不溶性蓝色沉淀的部位[32]。对照不加蔗糖。Sergeeva等[28]报道的反应介质略有不同，为38 mmol L^{-1} 磷酸钠缓冲液(pH 6.0), 25 U GOD, 0.024% NBT, 0.014% PMS, 1%蔗糖。对照不加蔗糖或GOD或PMS。

三磷酸腺苷酶(Adenosine triphosphatase, ATPase，EC 3.6.1.35)　采用金属盐法，酶催化反应释出的磷酸用 $Pb(NO_3)_2$ 捕获，转化为PbS沉淀而定位。反应介质含20 mL ATP (1.5 mg mL^{-1})、20 mL 0.2 mmol L^{-1} Tris-HCl缓冲液(pH 7.2)、3 mL $Pb(NO_3)_2$, 5 mL 0.1 mol L^{-1} $MgSO_4$, 2 mL蒸馏水。切片在反应介质中37℃温育1~3 h，水洗，加1% $(NH_4)_2S$ 显色1 min，水洗，观察黑色PbS的部位[24]。

1.4 裂解酶类

碳酸酐酶(Carbonic anhydrase, CA, EC 4.2.1.1) 采用金属盐法。1~5 mm的手切根段和下胚轴段用2%多聚甲醛，2% PVP, 1 mmol L^{-1} DTT在室温下固定30 min, 水洗4℃过夜，移入反应介质中温育30 min, 反应介质含17 mL溶液A (12 mmol L^{-1} $NiSO_4$, 39 mmol L^{-1} KH_2PO_4)和40 mL溶液B (220 mmol L^{-1} $NaHCO_3$), 均于用前制备。温育后样品用0.67 mmol L^{-1} 磷酸缓冲液(pH 5.9)洗10 min，再用0.5% $(NH_4)_2SO_4$ 显色，观察深褐色的部位。对照不加底物 $NaHCO_3$[33]。

1.5 异构酶类

葡萄糖磷酸异构酶(Phosphoglucose isomerase, PGI, EC 5.3.1.9)**和葡萄糖变位酶**(Phosphoglucomutase, PGM, EC 5.4.2.6)　PGI催化F-6-P为G-6-P，PGM催化G-1-P为G-6-P，均偶联NBT还原显色。反应介质含42 mmol L^{-1} Hepes-NaOH缓冲液(pH 7.4)、4.2 mmol L^{-1} $MgCl_2$、0.84 mmol L^{-1} EDTA、0.84 mmol L^{-1} EGTA, 0.084% BSA、1.4 mmol L^{-1} NDA、1 U G6PDH, 0.03% NBT、4.35 mmol L^{-1} F-6-P (PGI测定)或4.35 mmol L^{-1} G-1-P (GPM测定)。对照不加底物F-6-P、G-1-P或 NAD^+，水洗终止反应[28]。Sergeeva等还对拟南芥(*Arabidopsis thaliana*)整株幼苗进行PGM染色，将染色强度转为定量数据，分析PGM在不同器官中的定量分布[34]。

2 酶的细胞化学定位研究方法

通常体外分离不同酶活性的生化分析方法存在酶蛋白的稳定性及其所得组分的纯化程度的不确定性，且并非所有的植物种类都能做细胞分离分析，不可能全部分离纯化出高产量的细胞类型[35–36]。酶的细胞化学活性定位是一种在亚显微结构原位上检测细胞内酶的分布位点和活性丰度的精确方法，其优点在于能同时提供形态学与生化代谢功能的信息。

植物酶细胞化学定位技术主要有电镜细胞化学(Electronmicroscope cytochemistry, EMCC)技术和免疫细胞化学(Immunocytochemistry, ICC)技术两类。EMCC技术使酶的定位研究从显微水平发展到超微结构水平，研究专一性酶在完整细胞内不同组分部位的定位，其原理是使细胞内的目标酶与专一底物产生特异反应，在 Ce^{3+}，Cu^{2+} 和 Pb^{2+} 等金属离子的参与下产生不溶性的电子致密化合物，用电镜观察这些化合物在细胞内的分布位点，结合计算机的自动成像分析软件进行定量分析[37–38]。EMCC技术大多来自组织化学的经典方法，不同的是样品在反应介质温育后尚需要脱水，树脂包埋，超薄切片和二次固定[1–2]。

ICC技术则是利用抗体与抗原特异性结合的原理和特殊的标记技术，提供植物结构中不同组织与细胞间的代谢如何分隔，各目标抗原(酶)在细胞内的分布位点及丰度。此技术结合抗原和抗体的高化学专一性和电镜的高度空间分辨力，具有高度专一性、高灵敏性的特点，是理想的酶细胞定位的方法。自1970年开始应用以来，已被广泛使用[35–36]。

ICC技术因其标记物种类不同和所需检测仪器不同而可区分为免疫电子显微镜技术和免疫荧光抗体技术，抗体可连接到一系列标记，如酶、荧光分子、金颗粒和同位素等。标记抗体并用标记物与其他物质的反应将阳性结果放大后，转换成可见的发光或显色。不同的免疫技术有各自独特的试剂和方法，但基本的过程相似，都包括抗体制备，组织材料处理，免疫染色，对照实验，显微镜观察等。免疫荧光细胞化学技术的建立最早，该技术是用荧光素标记已知的抗体，与切片组织、细胞的抗原(酶)反

应，在荧光显微镜或激光电子共聚焦显微镜下观察呈特异荧光的抗原抗体复合物存在的部位，确定抗原在细胞内的定性和定位。常用的荧光标记物有异硫氰酸荧光素(Flurescein isothiocyanate, FITC, 呈黄绿色荧光)、四甲基异硫氰酸荧光素(Tetramethyl flurescein isothiocyanate, TMRITC, 呈橙红色荧光)和四乙基罗丹明(Tetraethyl rhodamine, RB200, 呈亮橙红色荧光)[3]。

免疫电子显微镜法包括免疫酶标法和免疫金银法等。免疫酶标法的酶标抗体上结合的主要是辣根过氧化物酶(以 DAB 作供氢体)、碱性磷酸酶(以快蓝或快红染色)和葡萄糖氧化酶(以 NBT 作电子供体)。这些酶标抗体与目标酶特异结合产生的抗原抗体复合物可形成有色的产物，从而对目标酶进行精细的亚细胞定位。免疫金(Immunogold)或免疫金银(Immunogold-silver)细胞化学技术则以胶体金颗粒(1~100 mm)标记特异的免疫球蛋白(常用的山羊抗家兔免疫球蛋白 IgG 酶抗体)作为探针，对组织或细胞内的目标酶(抗原)进行定性、定位及定量研究。金颗粒有很高的电子密度，在显微镜下清晰可辨，不必进行呈色反应。若再配合银离子(如乙酸银)显影液的还原反应，可使目标酶与抗体反应部位的免疫金颗粒增大，显示更清晰的棕黑色，显著提高灵敏度。免疫金法还可根据反应部位的金颗粒数量进行酶活性的免疫细胞化学定量。目前，免疫金银细胞化学法已被视为是适用于酶的细胞化学检测的最广泛、最有效和专一的方法。此法的特点是高敏感性，定位准确，方法安全，成本低，实验标本可长期保存[3]。与免疫电子显微镜术相比，免疫荧光法的特异性较高，但敏感度较低，且反应后的标本因荧光衰减而不能长期保存[39]。此两种免疫技术依染色步骤的多少均有直接法(一步法)与间接法(二步法)之分，直接法简便、快速、特异性强，间接法可用一种酶标抗体与多种特异性的一抗配合而检测多种抗原，且其敏感性高，故比直接法更佳，应用更广[3–4]。免疫酶细胞化学定位法的重点在于免疫技术，其他的操作过程包括材料制备，固定脱水，包埋，超薄切片等均相类似，只是定位不同酶时所用的固定剂、缓冲液及其 pH 值、温度、温育时间和包埋材料等有所不同。关于影响免疫组织化学(细胞化学)染色的主要因素，Fung 等已作了详细的评述[40]。

由于细胞化学定位方法的操作过程较复杂，难以一一描述。本文只列举 88 篇有关 46 种酶的细胞化学定位方法的文献。这些酶包括：山梨醇脱氢酶[41]、苹果酸脱氢酶[42]、NADP-苹果酸酶[43–45]、异柠檬酸脱氢酶[46–47]、葡萄糖-6-磷酸脱氢酶[48]、肉桂醇脱氢酶[49–50]、甘油醛-3-磷酸脱氢酶[51–52]、乙醇酸氧化酶[53]、NADH-谷氨酸脱氢酶[54–55]、胺氧化酶[56–57]、硝酸还原酶[58–60]、多酚氧化酶[61–62]、NADH-脱氢酶[63–64]、过氧化物酶和过氧化氢酶[65–67]、抗坏血酸氧化酶和谷胱甘肽还原酶[68–69]、脂氧合酶[70–71]、花色素苷合成酶[72]、NO 合成酶[73–75]、ACC 合成酶[76–78]、酪氨酸酶[79–80]、超氧物歧化酶[81–83]、查尔酮合成酶[84–85]、蔗糖合成酶[86]、丙酮酸磷酸双激酶[87–89]、果胶甲酯酶[90–92]、叶绿素酶[93]、酸性磷酸酶和 ATP 酶[94–96]、果糖-1,6-二磷酸酶[97–98]、淀粉酶[99–102]、纤维素酶[103–104]、溶菌酶[105]、酸性转化酶[106]、黑芥子酶[107–108]、羧肽酶[109–110]、焦磷酸化酶[111–112]、PEP 羧化酶[45,89,113–114]、Rubisco[115–116]、羟氰裂解酶[117–118]、苹果酸合成酶[119–120]、碳酸酐酶[121–122]、苯丙氨酸解氨酶[123–124]、丙二烯氧化物环氧化酶[125–126]和谷氨酰胺合成酶[55,127–128]。

3 结语和研究建议

采用组织化学和细胞化学定位研究技术，可在光镜或电镜水平上观察酶在细胞组织内的空间分布特点和动态，进而结合图像分析技术做定量评估。这种技术对于阐明和定量参与植物重要代谢活动的关键调节酶的特性与功能尤为重要。组织化学定位法易于操作，但精度较差，迄今能用其了解组织与细胞中目标酶分布定位的数量仍相当有限。细胞化学定位法是后期发展起来的技术，其精度和专一性高，能同时提供目标酶的超微结构形态学与生化功能的信息，其中尤以免疫细胞化学定位技术更为理想有效。近年来，随着免疫细胞化学的双重 / 多重染色技术和图像分析技术的发展，人们能在同一细胞 / 切片上同时定量地显示两种以上共存的抗原。而其与分子生物学的结合，更使免疫细胞化学技术达到基因、分子水平的定位，成为生命科学研究前沿的一种重要技术方法。与生命科学其他研究领域相比，植物学方面的相关研究在技术、材料与数量上均相对落后。在我国，免疫细胞化学 / 免疫组织化学技术在 20 世纪 80 年代才逐步普及[3]，而用于植物学研究方面的报道不多。据

此,我们认为要广泛而深入开展植物学中细胞化学酶的定位研究,必需解决如下问题:1、尽量借鉴其他生命科学领域的有效技术方法,应用于植物酶细胞化学定位研究中。2、探索和制备更适用于植物酶类定位研究用的专一性抗体,新的发色剂和荧光性物质。3、探索提高细胞壁的透性,使介质中的底物能在短时间内快速进入细胞并使其产物保留在酶活性原位的方法。4、避免植物细胞的内源荧光性物质,尤其是叶片的具自发荧光性色素对免疫荧光检测的干扰。

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