新世纪微生物学者的一项重要任务——未培养微生物的分离培养

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摘 要 扼要介绍近年来有关分离培养未培养的微生物(Unculturable microorganisms)、扩大生态环境微生物多样性的认识等方面取得的重要进展。首先,是采用非常规的,甚至认为有毒性的电子传递物质,获得了多种新生理型(physiotypes)的纯种微生物。另外,在多种生态域中分离出非同寻常的微生物,扩大了这些生境微生物的多样性的视野,如普遍存在于大洋的浮游细菌 SAR11 类群,其微小菌体呈半月形,在海水中细菌浓度可达(10^5 ~ 10^6)/mL,基因组估计为 1.54 Mb。更值得注意的是从极端高温环境分离的嗜热纳米古细菌属(*Nanoarchaeota*),其基因组仅有 500 kb,是已知原核生物的最小者。应重视的是在分离这些多样性微生物的过程中所发展的新型培养技术,如微滴胶囊化法和扩散小室法都是具有革命性的方法,既有通用性,又无需昂贵设备,对今后这一领域的发展将起重要推动作用。

关键词 未培养的微生物,生理型,大洋微小细菌 SAR11,嗜热纳米古细菌

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著者在最近出版的《微生物工程》[1]首章中讨论了微生物的多样性和微生物资源的开拓,目的在于说明为了持续开发微生物产物,造福人类,人们必须寻找更多类型的微生物。众所周知,现在人们所培养的微生物仅为自然界存在微生物中的少数,约有 1.0%,或更少,究其原因是在自然界存在的微生物不能在常规的分离平板上生长,它们是活的,但是尚未培养的微生物[2,3]。另一方面,生态学和系统发育学家采用分子 DNA 技术,确认在海洋、土壤等环境中存在着大量前所未知的微生物类型。Olsen 等[4]在 1986 年发表应用这一技术探索自然环境中微生物多样性的论文后,引起人们在这领域的重视。在过去的 20 年里,通过核酸技术人们在对微生物多样性的深度和广度的认识取得空前的进展,例如在 1987 年已知可培养的微生物有 26 个类群(phyla),20 年后的今天微生物类群已多达 52 个类群,其中半数是未经培养的,而是经核酸技术获得的。这就扩大了人们对微生物世界多样性的视野[5]。

当然,尽管已取得上述这些进展,并不说明分离培养微生物工作已不再重要。因为利用核酸技术鉴定的类群为数巨大,对这些类群只知其存在,但对它们的形态、生理特性、代谢功能、对环境的影响等等,是难以进行实验研究的,更谈不到获取有用的代谢产物,发展生物技术了。所幸,还有不少学者致力于新的分离培养技术的探索,突破传统的概念,建立新的方法,获得过去未曾想过的新类型,例如利用有毒的电子传递物质的类群,微小的纳米级的细菌等。本文扼要介绍这些新的类型微生物的获得。

1 新生理型(physiotypes)微生物的发现

由于不同电子供体和受体成功地应用,近年不断发现前所未知的生理型微生物,如表 1 所示,采用非传统的生长底物可以促进新型微生物的生长。

表 1 新型生化反应支持的新型微生物的培养

Table 1 Growth-supporting reactions relating to the recent cultivation of novel organisms

Growth-supporting reactions	References
1. $2H_3AsO_3$(亚砷酸) + O_2 → $HAsO_4^{2-}$ + $H_2AsO_4^-$ + $3H^+$	[6]
2. $H_2AsO_3^-$ + NO_3^- → $H_2AsO_4^-$ + NO_2^-	[7]
3. $4HPO_3^{2-}$(亚磷酸) + SO_4^{2-} + H^+ → $4HPO_4^{2-}$ + HS^-	[8]
4. $4HPO_3^{2-}$ + $2CO_2$ + $2H_2O$ → $4HPO_4^{2-}$ + $CH_3CO_2^-$(乙酸) + H^+	[9]
5. $5AHDS_2^-$ + $2NO_3^-$ + $7H^+$ → $5ADS_2^-$ + N_2 + $6H_2O$	[10]
6. C_6H_6(苯) + $6NO_3^-$ + $6H^+$ → $6CO_2$ + $3N_2$ + $6H_2O$	[11]
7. 4 2-Cl-C_6H_4-OH(2-氯苯酚) + CH_3CO_2H(乙酸) + $2H_2O$ → 4 C_6H_5OH + $4Cl^-$ + $4H^+$ + $2CO_2$	[12]
8. $HClO_4$(过氯酸) + CH_3CO_2H(乙酸) → Cl^- + H^+ + $2CO_2$ + $2H_2O$	[13]
9. 4Fe(Ⅱ) + O_2 + $4H^+$ → 4Fe(Ⅲ) + $2H_2O$	[14][15]

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1.1 亚磷酸作为电子供体,硫酸为受体

磷几乎全部以V价的磷酸存在于自然界。最近Schink等[8,9]于厌氧条件下,从海底沉积物中分离出氧化亚磷酸,同时还原硫酸盐的无机化能自养细菌,在无机培养连续传代2~4月的纯培养,经分类研究,认为属产脱硫菌(*Desulfatogenum*)新种,定名为亚磷酸氧化产脱硫菌(*D. phosphitoxidans* sp.nov.)。该菌能在硫酸和亚磷酸存在下,以CO_2为唯一碳源生长,倍增期为3~4天。除亚磷酸外,尚能氧化乙酸、氢、甲酸、富马酸、葡萄糖等。菌体形态为具尖端的直杆菌,(0.6~0.8)×(2~4)μm,具运动功能,革兰氏阳性。其在自然界的生态功能尚不明确,作者推测,远在无氧存在时期地球上存在还原性磷,其在厌氧条件下还原硫酸可能是进化过程的残留反应。

1.2 亚砷酸作为电子供体

与上述亚磷酸氧化的化能自养菌相似的是氧化亚砷酸的化能自养菌(NT-26)的分离。Santini等[6]从澳大利亚金矿中分出,革兰氏阴性,具双鞭毛杆菌,在无机培养基中,以亚砷酸为电子供体,氧为受体,以CO_2为唯一碳源生长。16S rDNA序列分析表明NT-26属于α-变形杆菌的土壤杆菌—根瘤菌分支,可能是一新种。

另外,Oremland等[7]从湖水中分离出兼性亚砷酸化能自养菌(MLHE-1)。细胞可在黑暗中固定$^{14}CO_2$,经PCR试验证明核酮糖-1,5-二磷酸羧化酶/氧化酶的存在。除化能自养生活外,该菌尚能利用乙酸在有氧和硝酸盐存在下无氧生活。16S rDNA序列分析,表明MLHE-1属于α-变形杆菌的外硫红螺菌属(*Ectothiorhodospira*)。

1.3 芳香基卤化物大多是以农药施于土壤,其降解微生物广泛存在于多种环境中。

Sanford等[12]采用2-氯苯酚富集方法从土样和沉积物中分离到5株能于厌氧条件下,利用这类物质的菌种,属革兰氏阴性杆菌,产生红色素,具滑行运动,有孢子结构,能在2,5-二氯苯酚,2-溴苯酚等为电子受体,乙酸为电子供体,厌氧条件下生长。其16S rDNA序列与其他粘菌序列有9.0%的差别,作者认为应列为新属,脱卤厌氧粘菌(*Anaeromyxobacter dehalogenans* gen.nov., sp.nov.)。

类似的情况是在污染过氯酸环境,如火箭推进剂和炸药生产厂附近土壤中,Achenbach等[13]分离到能分解过氯酸的菌种,并分别定名为活力脱氯单胞菌(*Dechloromonas agitata*)和猪脱氯体(*Dechlorosoma suillum*)。

1.4 苯的厌氧降解

苯是石油来源燃料的主要组份,也应用于多种化工制造业,从而构成地下水和地表水的污染。在厌氧条件下,其生物降解极为缓慢,迄今尚未分离出能在厌氧条件下降解苯的微生物。最近,Coates等[11]分离出在这一条件下,以硝酸盐为电子受体能完全氧化,单环芳香化合物的细菌,定名为脱氯单胞菌(*Dechloromonas*, strains RCB和JJ),两株菌均为革兰氏阴性杆菌,兼性厌氧。系统分类上两株菌十分相近,16S rDNA序列相似性达98.1%。

1.5 腐殖质物质作为电子载体

腐殖质物质(humic substances, HS)普遍存在于土壤、海洋和淡水域的沉积物中,前人认为HS在厌氧环境是不易分解的有机物。近年的研究证明HS对厌氧呼吸和发酵细菌起着电子槽(electron sinks)的重要作用,就是在微生物细胞和不溶解性电子受体,如Fe(Ⅲ)氧化物之间充当可溶性电子载体,并重新氧化,进行循环反应,促进有机物降解。Coates等[10]借用HS的结构相似物质,2,6-蒽氢醌二磺酸(AHDS)作为电子供体,硝酸盐为电子受体,分离获得6株能在厌氧下氧化AHDS的新细菌,分别属于α,β,γ和δ变形杆菌。这一工作证明HS的微生物氧化是环境中普遍存在的反应。

1.6 Fe(Ⅱ)的生物氧化

有关Fe(Ⅱ)的化能自养细菌早有报道,如赭色纤发菌(*Leptothrix ochracea*),加氏铁柄细菌(*Gallionella* sp.)等。但这方面的深入研究面临的困难是好氧菌从下列反应$Fe(Ⅱ)+0.25O_2+H^+\rightarrow Fe(Ⅲ)+0.5H_2O$所获得的可供利用的能量很低(△G~29KT/mol的铁),以致化能自养铁细菌的倍增期需几个小时。另外,在中性pH条件,Fe(Ⅱ)的半衰期不过几分钟,要准确测定Fe(Ⅱ)的生物氧化,必须排除非生物氧化的干扰。

Emerson和Moyer[14]在夏威夷群岛附近深海低温喷泉观察到泉水中富含CO_2和Fe(Ⅱ),但H_2S却极少,喷泉口四周由胶态细菌垫层和锈色氧化铁所包围,细菌浓度高达$(1.9\times10^7)\sim(5.3\times10^8)$/mL胶态物。作者在无机培养基,用稀释法获得纯种培养,并观察到在纯种培养中产生的丝状氧化铁喷泉口堆积者相似,指明海底氧化铁沉积是由细菌的氧化作用产生。

Neubauer等[15]在湿地植物根际分离到化能自养铁氧化细菌(Br T株),菌种只能在Fe(Ⅱ)存在下才能生长,在反应器培养倍增期为25 h。对活细胞与叠氮化钠致死细胞进行比较,结果指出BrT氧化活力占总铁氧化量的18%~53%,平均细胞得率约为0.70 g/mol Fe(Ⅱ)氧化。

上述工作,澄清了Fe(Ⅱ)氧化细菌的生化反应和它的生物地质功能。

2 多种生态域微生物多样性的扩大

2.1 广泛分布于大洋的浮游细菌的分离与培养

Giovannoni等[16,17]对广泛存在于美国西海岸Sargasso海域及其他海洋的浮游细菌(SAR11)进行了细致的研究。首先,从美国Oregon州沿海的海水采样,以补充磷酸盐、铵盐和有机碳源的海水为培养基,养料浓度比常规实验室所用低三个数量级。在微孔平板上培养较长时间(23 d),获得的11个分离物,经16S rRNA和16S~23S rDNA基因区间序列分析,找出系统发育关系,明确SAR11是属于α-变形杆菌的一个分支。SAR11菌体较小,呈半月形,长0.37~0.89 μm,直径0.12~0.2 μm,体积0.01 μm^3。基因组为1.54 Mb。作者比较了美国太平洋沿岸和北大西洋52个水样中细菌rRNA基因,结果表明SAR11是最丰富的类群,占有些地域表层总微生物群体的50%,亚透光区(subeuphotic)微生物群体的25%,经过

推算,全球海洋中 SAR11 细胞数目为 2.4×10^{28},其中半数定居于亚透光区。显然,这一类群是地球上最兴旺的生物。

2.2 土壤微生物的多样性

众所周知,土壤有丰富的微生物,水稻田土壤又是影响全球气候变暖的甲烷产生地,所以土壤微生物多样性受到注意。Chin 等[18]对水稻田中主要的厌氧降解纤维素、木聚糖、果胶等物质的微生物进行分离,所用培养基为 Difco 营养汤,浓度是常规的 1/100,结果表明水稻田土样所含细菌细胞总数测定每克干样为 2.4×10^{8}。采用逐步稀释法获得能培养的 9 个分离株,约占总计数的 5%,分属于 6 个群:*Verrucomicrobia*, *Cytophaga-Flavobacterium-Bacteroids*,梭菌簇 XLVa。9 株分离株的 16S rRNA 基因序列均不同于已知序列。

Jansson 等[19]对澳大利亚草原土样经超声匀浆、离心处理提高了活菌计数,采用常规营养液 1/100 浓度的培养基,获得 30 个分离株,其 16S rRNA 基因序列大都属于新的谱系,如 *Actinobacteria*, *Acidobacteria*, *Proteobacteria*,和 *Verrucomicrobia*.

2.3 昆虫消化道螺旋体的重要性

昆虫消化道是微生物的重要生境,特别是螺旋体。Leadbetter 等[20]从白蚁消化道取样,在 H_2 和 CO_2 气相下培养,获得 2 株螺旋体纯培养(ZA S-1,ZA S-2),形态与大小都相似[0.2 μm×(3~7) μm],其 16S rRNA 序列相似程度高达 98%。ZA S-2 生长需要 H_2,用 $^{14}CO_2$ 标记物证明其代谢产物为甲酸与乙酸,如下式所示:$4H_2+2CO_2\rightarrow CH_3CO_2H+2H_2O$,$H_2+CO_2\rightarrow HCO_2H$ 乙酸为主产物(80%)。与 ZA S-2 不同,ZA S-1 可以在 N_2 下生长,同时产生甲酸和乙酸,甲酸多于乙酸。螺旋菌在白蚁消化道产生的挥发酸为宿主提供了碳源和能源。

同一群学者[21]又进一步证明白蚁后消化道和淡水域沉积物中的螺旋体具有同源固氮基因(nif H)和固氮活力。这一活力的确证,使我们认识到螺旋体对白蚁生活的重要性。白蚁所食用的木材含氮极低(0.05%),如无螺旋体固氮的补充,昆虫就难以正常生活。

2.4 极端环境中纳米级古细菌的发现

上述从广大海域分离到的 SAR11 类群就其形态大小,属微小型细胞,其基因组与弯曲杆菌、支原体等相近。但更为微小的细菌是最近 Huber 等[22]所报道的超高温古细菌纳米细菌。作者从冰岛深海热泉口取样,于 90℃在 S、H_2 和 CO_2 存在下,分离出超高温的自养古细菌,——火焰球菌(*Ignicoccus*),并发现火焰球菌表面附有极小球形细胞,平均每个大球上有两个小球。小球细菌可与大球细胞分离,但单独培养不能生长,两者共同培养则取得成功。作者研究了小球菌 rRNA 序列,证明 ss rRNA 基因与火球菌不同,也与已知的古细菌不同,属于一个新的古细菌群。根据细胞形态微小程度属纳米范围,并附生于火球菌细胞,故定名为骑火球纳米古细胞(*Nanoarchaeum equitans*)。

纳米古细菌生理性状知之甚少。细胞不能在火球菌细胞匀浆生长,必须与火球菌细胞接触,才能生长。两者共同培养于 30 L 发酵罐(70~90℃),提高通气($H_2:CO_2$),可使纳米古细菌细胞浓度提高 10 倍,而火球菌并无变化。在发酵后期,80% 的纳米古细菌即行脱落,经高速离心,可得 1.5 g 湿细胞。其基因大小,经测定为 500 kb,是已知原核细胞中最小者。鉴于其高温厌氧生活与微小的基因组,纳米古细菌可能是微生物生命比较更原始的形式,对其基因组和后基因组的深入探求,可为生命起源与进化提供有价值的资料。

3 新颖的分离培养方法

新的分离培养方法的精髓是放弃传统的平板技术所采用的人工合成、高浓度养料的培养基等。这里,扼要介绍细胞微胶囊与扩散小室两种新方法。

3.1 细胞微胶囊法[23]

海水或土壤样品的微生物都须加以浓缩,逐步稀释,再与琼质(海水)混合,并制成多数微滴胶囊(microdroplets, GMDS),其中含有单个细胞的微胶囊,装入灭菌层析柱,柱的入口和出口两端都装有过滤膜(图 1),入口膜(0.1 μm)防止游离活细胞进入,出口膜(8 μm)阻止柱内繁殖的游离细胞逃逸。培养海洋样品微生物样品时层析柱灌注的培养基为灭菌海水,补充少量 K_2HPO_4、NH_4Cl、微量金属与维生素。土壤样品则用土壤抽出液用 0.85% 的 NaCl 液适当稀释。

上述微胶囊中的细胞都是在同一开放式供应的培养液中生长,在很大程度上模拟了自然环境。图 1 的第二阶段(phase Ⅱ)是将第一阶段(phase Ⅰ)获得的微菌落接种到丰富的有机培养基,微胶囊中的细胞群可长出胶囊,经 1 周培养,多数微孔菌浓度可达 10^7/mL,并从中获得有抑制真菌活力的培养。以上结果表明微胶囊技术具有高通量,并可为生理学、代谢产物以及细胞-细胞相互作用提供足够的细胞量。

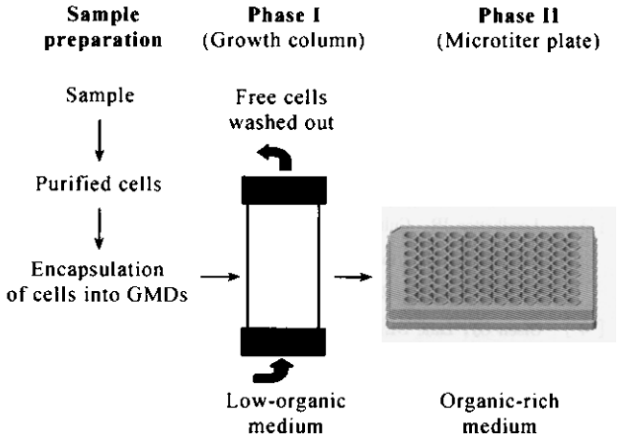

图 1　实验装置的模式图

Fig.1　Model of the experiment setup

Cells captured from environmental samples were encapsulated into GMDs and inoculated in growth columns (phase Ⅰ). GDMs containing microcolonies were detected and separated by flow cytometry into 96-well microtiter plates containing a rich organic medium (phase Ⅱ)

3.2 扩散小室法[24]

Kaberlein 等作者设计扩散小室,用于海洋微生物的分离

培养。海洋沉积物富含好氧异养微生物,浓度可达每克沉积物含10^9细胞,其中绝大部分是未经培养的。沉积物的微生物经过浓缩,适当稀释,接种于海水制成琼脂,注入垫圈,并加盖上膜。多个装置的垫圈可放入养鱼罐中,培养1周后,即有微菌落产生,其数可高达接种细胞的40%,微菌落中多数为混合培养,须再次分离,以获得纯种。作者指出,此法的特色是模拟自然环境,使不同细胞经过互喂,形成菌落。

4 结 语

从上文所述,可以看出过去认为不能培养的微生物也逐步成为纯培养出现,取得这些进展的因素是多方面的。如采用非常规的电子供体与电子受体,低浓度的培养液,如海水、土壤抽出液,以流动方式供应培养液等。此外新颖的培养方法——微胶囊法与扩散小室都模拟了天然环境,使不同微生物细胞之间进行信息交流,发挥细胞之间的互喂作用,促进菌落形成。

再者,通过对大洋海水悬浮细菌的研究,发现了形态大小与基因组均微小的SAR11群,其基因组与支原体相仿。SAR11在大洋中存在细胞数是巨大的,如何利用这一资源是值得考虑的。

更值得重视的是在极端高温下发现的纳米古细菌,其基因组仅为500 kb,是已知原核细胞中的最小者,为研究地球上生命起源提供了优越材料。

致 谢 本文写作过程,得到李金城和张伟同志的大力协助,特致谢忱。

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Feedback-insensitive Anthranilate Synthase Gene as a Novel Selectable Marker for Soybean Transformation

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Abstract Because of the concern about escape of antibiotic- or herbicide-resistant transgenes from transgenic crops, selectable marker genes from plant origin would be an alternative choice for plant transformation. In this study, a feedback-insensitive anthranilate synthase gene (*ASA2*) cloned from a tobacco cell line was tested for *Agrobacterium*-mediated transformation of axis tissue of soybean mature embryo, with a tryptophan analogue 5-methyltryptophan (5-MT) as the selective agent. Southern blot analysis of the T_0 transgenic lines confirmed the integration of the *ASA2* gene into the soybean genome. Northern blot analysis showed the *ASA2* gene was also expressed in the leave tissue, and the free tryptophan content in the leaf tissue of transgenic soybean was about 59% to 123% more than that in the wild type. PCR analysis of the T_1 progeny showed that the transgene was inherited in a Mendelian fashion. All these results indicate that this feedback-insensitive *ASA2* gene can be used as a selectable marker gene for plant transformation. This work also demonstrated that the *ASA2* gene coding for the α-subunits from one plant (tobacco) can interact with the β-subunits of a heterologous plant (soybean) to form an active anthranilate synthase enzyme. The use of this feedback-insensitive gene as a novel selectable marker for plant transformation is also discussed.

Key words Anthranilate synthase, feedback-insensitive, selectable marker, 5-methyltryptophan (5-MT), soybean transformation

For plant transformation, a selectable marker gene is required to distinguish transformed cells from non-transformed ones. Over 50 selective agents/marker gene combinations representing more than 40 unique selectable, lethal or assayable genes have been used for plant transformation[1]. The widely used antibiotic-or herbicide-resistant selectable markers raise the concerns of the transfer of these marker genes to other organisms[2]. Some of these selective agents may be efficient for certain plant species transformation but may not be suitable for other plants. In addition, some of these selectable markers have already been patented and the commercial use of these genes faces proprietary limitations.

Due to the reasons stated above, there is always a need to develop novel and acceptable selectable marker genes for plant transformation. Tryptophan (Trp) is an essential amino acid and is not synthesized by animals and human. It is also a precursor for the Trp-dependent biosynthesis of the auxin IAA[3,4]. Anthranilate synthase (AS) has been shown to be the committing enzyme in the Trp biosynthetic pathway. This enzyme is a tetramer containing two α-subunits and two β-subunits, it converts chorismate into anthranilate, which is the precursor for tryptophan biosynthesis[5]. This enzyme is feedback-inhibited by the end product tryptophan (Fig. 1), which binds to an allosteric site on the AS catalytic α-subunit. Mutant *ASA2* genes isolated from maize and rice have been used as selectable markers for maize[6] and rice[7] transformation, respectively, with 5-MT as the selective agent. Tryptophan analogues such as 5-MT and α-MT are toxic to plant cells and have been used as selective agents to generate *Arabidopsis* mutant lines which contain elevated levels of free auxin[4]. The mechanism of using 5-MT or α-MT for selection is that these Trp analogues inhibit tryptophan biosynthesis and disrupt the functions of proteins in which they have been incorporated[3,4], while mutant or feedback-insensitive AS genes may have a feedback-altered AS form which leads to higher free Trp accumulation.

A feedback-insensitive AS gene (designed as *ASA2*) was isolated from a selected tobacco suspension cultured cell line which was resistant to 5-MT[8]. Transformation of this *ASA2* gene into the hairy roots of a legume plant *Astragalus sinicus* resulted in an increase in free Trp and the hairy roots were shown to be resistant to 5-MT[9], but the selectable marker gene used to obtain the hairy roots was *npt* Ⅱ. Re-engineering of this tobacco *ASA2* gene into the tobacco chloroplast genome also increased free Trp content in transplastomic tobacco, but

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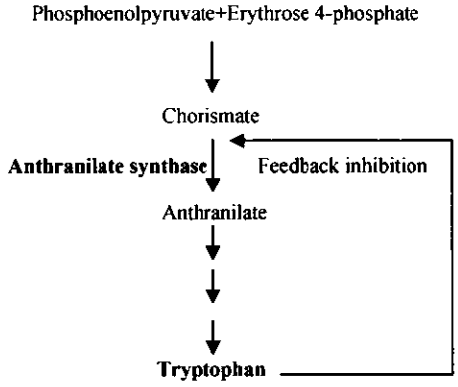

Fig.1 Feedback inhibition of anthranilate synthase by tryptophan

the selectable marker for chloroplast transformation used was *aadA*[10]. To test if this gene can be used as a novel selectable marker for plant transformation, a soybean transformation system based on using the feedback-insensitive *ASA2* gene as a novel selectable marker with 5-MT as the selective agent has been developed. Transgenic soybean plants were generated and confirmed by Southern blot, and the *ASA2* gene expression was confirmed by Northern blot. PCR analyses of the T_1 progeny lines showed that the transgene was inherited in a Mandelian fashion. Advantages as well as limitations of using this selectable marker for plant transformation are discussed.

1 Materials and Methods

1.1 Plasmid construction

A *Bam*H Ⅰ-and *Eco*R Ⅰ-digested fragment of pBIN-ASA2[9] was used to replace the 35S-*bar* fragment of pCAMBIA3301 to produce the binary vector as shown in Fig. 2. Both the intron-containing *gus* gene and the *ASA2* gene were driven by the CaMV 35S promoter. The trascription of the *ASA2* gene was terminated by its own terminator. This construct was first transferred into *E. coli* DH5α competent cells using the protocol as described[11]. The plasmid purified from *E. coli* was then introduced into *A. tumefaciens* strain EHA 105 by the protocol from Chung and Miller[12].

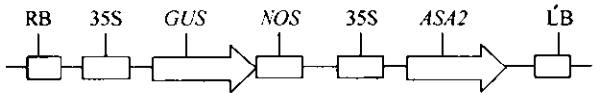

Fig.2 T-DNA region of the binary vector using the 35S-ASA2 selectable marker for soybean transformation.
RB: right border; LB: left border

1.2 Agrobacterium-mediated transformation of soybean embryo axis

Mature soybean (*Glycine max*) seeds (genotype: Dwight) were sterilized in 15% Clorox for 15 min, then thoroughly washed with sterilized distilled water, and the seeds were soaked in sterilized distilled water overnight. The next day, embryo axes were isolated from the seeds by cutting in the middle of the seeds, then removing one of the cotyledons and pushing the embryo axes out from the other cotyledon. Primary leaf tissues were carefully removed, and wounding was made by slicing through the meristem tissue about 1mm deep using a # 11 surgical blade. The explants were then put into *A. tumefaciens* re-suspension medium. After all the explants were prepared, they were either left in the hood for 1 ~ 2 h with shaking, or vacuum infiltrated for 1 ~ 2 min, then left for 1 ~ 2 h. The embryo axes were blotted dry on sterilized filter paper (Whatman # 3), and co-cultivated on sterilized filter paper with 1 ~ 2 mL liquid co-cultivation medium in a 100 × 15 mm Petri dish (1/10 B5 salts + B5 vitamins + 2 mg/L BA + 20 g/L sucrose + 400 mg/L cysteine + 200 μmol/L acetosyringone). Cysteine and acetosyringone were filter-sterilized using a 0.25 μm filter. The plates were placed in the dark at 23℃.

After 3 ~ 5 d of co-cultivation, embryo axes were transferred to selection medium (MS medium with 4 × of micronutrients, designed as 4 MS Ⅱ medium) with 100 mg/L timentin and 100 ~ 200 μmol/L 5-MT (Fluka Chemika, Germany) that had been dissolved in hot water, filter- sterilized and added to the medium after autoclaving. The cultures were kept in the dark at 23℃ for 2 ~ 3 d, and transferred to 24 h light at 28℃ for 4 ~ 5 d. The explants with visible shoot-buds were transferred to shoot elongation medium (WPM salts, B5 vitamins, 2% sucrose, 0.4 mg/L IAA, 0.4 mg/L GA_3, 50 μmol/L 5-MT, 100 mg/L timentin). IAA, GA_3 and 5-MT were all filter-sterilized and added to the medium after autoclaving. The explants were transferred every 2 weeks until the shoots were 1 ~ 2 cm long. The elongated shoots were then rooted in MS rooting medium without 5-MT.

1.3 GUS expression in embryo axes

Ten days after selection, some of the embryo axes were incubated in GUS assay buffer (0.5 mol/L KH_2PO_4, pH 7.0, 1 μg/mL X-Gluc) at 37℃ overnight. The tissues were treated with 95% EtOH and photographed under a dissecting microscope.

1.4 HPLC analysis of free tryptophan content

Fully developed soybean leaf tissues from the greenhouse were collected and immediately frozen in liquid nitrogen. The samples were powdered in liquid nitrogen and about 100 mg was placed in a screw cap tube with 500 μL of 0.1mol/L HCl (pH 7.0). One bead was added into the tube and the tissues were homogenized in a disrupter machine for 45 seconds at setting 4. Following centrifugation at 4℃ for 10 min, the supernatant was transferred to a Millipore Ultra-FREE centrifugal filter unit (Millipore, Bedford, MA). The filtrate was centrifuged at 2400 RCF for 15 min and 100 μL was placed in an HPLC sample tube for Trp analysis by methods similar to that of Berardino *et al.*[13] and Cho *et al.*[9], using a 250 mm × 4.6 mm Adsorbosil C18 column (Alltech Associates, USA) and an 85% isocratic buffer system.

1.5 Southern blot analysis

Partially expanded trifoliate leaf tissues were collected from the T_0 transgenic plants in the greenhouse and were immediately frozen in

liquid nitrogen. The tissues were ground into fine powder in liquid nitrogen. Genomic DNA was isolated using a nucleon PhytoPure plant DNA extraction kit (# RPN8511, Amersham Life Science, England) following the manufacturer's protocol. About 10 μg DNA from each sample was digested with *Bam*H Ⅰ and the protocols described[14] were used for the hybridization. A 2.2 kb *Bam*H Ⅰ/*Eco*R Ⅰ ASA2 cDNA fragment isolated from the plasmid DNA was purified with a plasmid kit (Qiagen, Valencia, CA) and radio-labeled as a probe.

1.6 PCR analysis of T_1 progeny

Genomic DNA was isolated from T_1 transgenic and wild type soybean leaves using the method stated above. The PCR primers are 5′-GCTGGTAGATATCACAGTC-3′ and 5′-TATAATACTCTCCGGATGAAACTG-3′, which used for amplification of a 1.3 kb fragment of the *ASA2* cDNA. The DNA samples were denatured at 94℃ for 5 min, followed by 30 cycles of 94℃, 45 s; 55℃, 45 s; 72℃, 90 s, and extension at 72℃ for 10 min. The PCR products were separated on a 0.8% agarose gel.

1.7 Northern blot analysis

Total RNA was isolated from young leaf tissue of transformed soybean lines by a RNeasy Plant Mini Kit (Qiagen, USA). Purified RNA samples were mixed with 5 × RNA loading buffer, incubated for 5 min at 65℃, and loaded onto a 1.2% formaldehyde agarose gel. After electrophoresis, the RNAs were blotted onto Hybond$^+$ membrane (Amersham, England), fixed onto the membrane by baking at 80℃ for 2 h and then pre-hybridized at 65℃ for 1 h and hybridized with the radio-labeled *ASA2* cDNA probe that was prepared as described for the Southern blot analysis. The overnight-hybridized membrane was washed in high stringency buffer for 15 min at room temperature, washed at low stringency buffer until the background disappeared. The membrane was then exposed to X-ray film at −80℃.

1.8 Enzymatic assay of anthranilate synthase

About 2.5 g leaf tissue from the T_1 PCR positive and wild type control plants in the greenhouse were collected and placed on ice. The tissue was first chopped with a razor blade and then ground in ASA1 extraction buffer (2 mL/g tissue) as described by Bernasconi *et al*[15]. After removal of cellular debris by centrifugation at 35 000 × g for 10 min at 4℃, the supernatant was desalted by an Econo-Pac 10DG column (Bio-Rad, Hercules, USA) using ASA1 assay buffer as described by Bernasconi *et al*[15], but without NH_4Cl in the buffer. 100 μL of enzyme solution was added to a mixture containing 200 μL of ASA1 assay buffer, 50 μL of 100 mmol/L glutamine, 50 μL of tryptophan at concentrations of 1, 5, 10, 20, 50, 100, 500 or 1000 μmol/L and 100 μL of 5 mmol/L chorismate. The mixture was incubated in a 30℃ water bath for 30 min and the reaction was stopped by adding 100 μL 1 mol/L HCl. After adding 1 mL ethyl acetate and inverted several times to mix, the fluorescence of the supernatant was measured using a F-2000 spectrophotometer (Hitachi, Tokyo, Japan).

2 Results and Discussion

2.1 Test of 5-MT concentration for soybean transformation

To test the appropriate 5-MT concentration to inhibit shoot regeneration of soybean embryo axis, the explants were prepared as described in 1.2 and placed on medium with 50, 100, 200 or 400 μmol/L 5-MT. 5-MT can effectively suppress shoot organogensis at the concentration of 100 μmol/L. This concentration was chosen for the following transformation process.

2.2 Development of soybean transformation system

Embryo axes isolated from mature seeds of soybean genotype Dwight were used for transformation (Fig. 3 A). After co-cultivation with *A. tumefaciens* for 3 d, the axes were transferred into selection medium containing 100 μmol/L 5-MT and incubated in the dark at 23℃ for 4 ~ 5 d. Under the dark conditions, shoot regeneration was inhibited and the selective agent was translocated into the apical meristem so that the non- transformed cell growth was inhibited while the transformed cells began to grow. This dark treatment is crucial since if the tissues were transferred directly into the light at 28℃, the non-transformed cells which are in the majority would grow rapidly and a few transformed cells present would have little chance of growing into whole plants. After 5 d in the dark, all of the explants were white in color and no shoots had regenerated (Fig. 3 B). When transferred to the light, the axes became green and shoot buds began to emerge from the tip (Fig. 3 C). At this stage, the embryo axes were tested GUS positive as shown in Fig. 3 D. After transfer of the shoot buds to shoot elongation medium with 100 μmol/L 5-MT, some of the shoots elongated (Fig. 3 E) and these shoots were then transferred to rooting medium. However, some of the shoots did not form roots in the rooting medium but continued to form callus at the base of the shoots(Fig. 3 F). Even if the shoots formed roots, the regenerated plants looked unhealthy and grew very slowly in the greenhouse, but these plants did eventually grow and set seeds. The rooting inhibition and poor plant growth may be due to the fact that 5-MT greatly inhibited root formation as reported by others[4].

2.3 Confirmation of primary transformed events

Since this *ASA2* selectable marker gene encodes the feedback insensitive α-subunit of anthranilate synthase, transgenic soybean plants expressing this gene should have higher free Trp than the wild type plants. Fully expanded soybean leaves were used for the analysis of free Trp by HPLC. As shown in Fig. 4, the four putative transgenic lines tested had 59% to 123% more free Trp than the wild type. However, the Trp increase is not as high as reported for *Astragalus sinicus* hairy roots[9] or tobacco chloroplast transformation[10]. This may be due to the different expression level of this gene in different tissues.

To further confirm that the *ASA2* gene was integrated into the soybean genome, Southern blot analysis was carried out on four of the putative transgenic lines which had higher free Trp tested by HPLC.

As shown in Fig. 5, all four lines were tested positive for the *ASA2* gene, and had 1 ~ 2 copies of the transgene. Northern blot analysis using leaf total RNA from two of the Southern positive lines also showed the *ASA2* gene was expressed (Fig. 6). A summary of the *ASA2* transgenic lines derived from these transformation experiments is shown in Table 1.

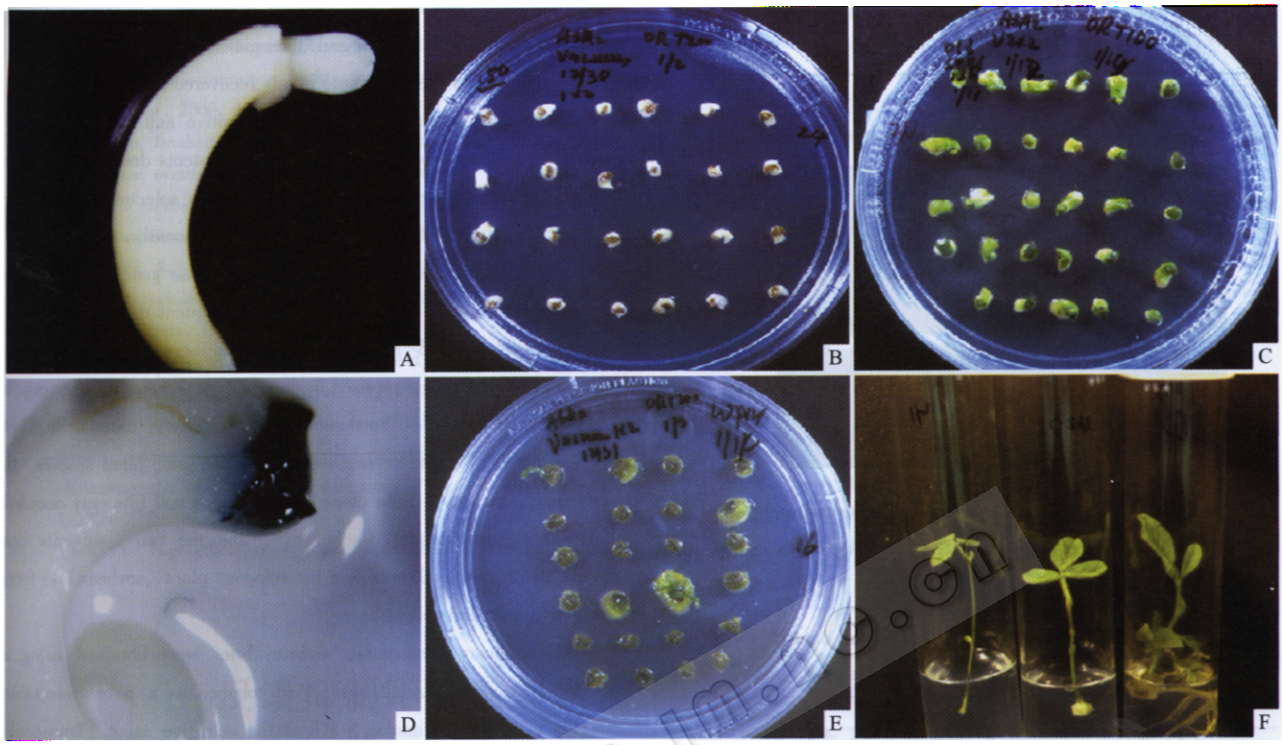

Fig.3 Transformation of soybean embryo axes with the *ASA2* selectable marker.
A: embryo axis used for transformation. B: selection in 4MS Ⅱ medium with 200 μmol/L 5-MT in the dark for 5 d. C: selection with 100 μmol/L 5-MT under light for 14 d. D: GUS assay of the embryo axis after 14 d selection. E: elongated shoots (arrow) with 50 μmol/L 5-MT after 21 d in shoot elongation medium. F: rooting of elongated shoots. The diameter of the Petri dish was 10 cm and the test tube was 25 mm.

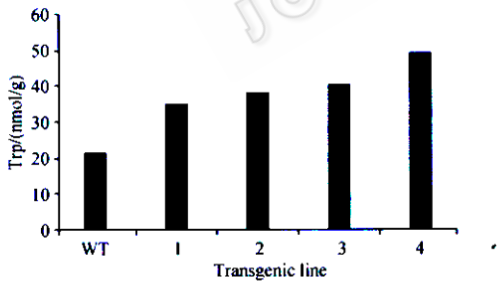

Fig.4 HPLC analysis of free Trp in T_0 transgenic soybean plant leaves
Trp content of the wild type (WT) and 4 putative transgenic lines

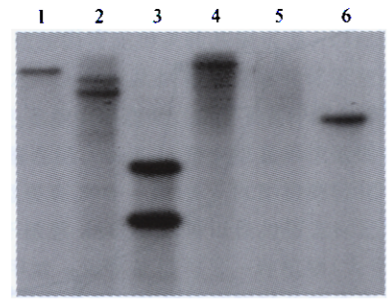

Fig.5 Southern blot analysis of transgenic *ASA2* soybean lines
The genomic DNA was digested with *Bam*H Ⅰ. The probe used was the 2.2 kb *ASA2* cDNA fragment. 1 ~ 4: independent transgenic soybean lines. 5: wild type soybean as the negative control; 6: plasmid control

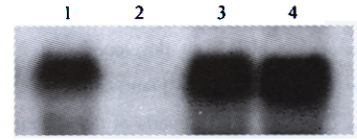

Fig.6 Northern blot analysis of transgenic soybean lines
The probe used was the 2.2-kb *ASA2* cDNA. 1: Transgenic *ASA2* Arabidopsis; 2: Soybean wild type ; 3 & 4: Two independent transgenic soybean lines.

2.4 Progeny analysis of *ASA2* transgenic lines

T_1 progeny of the three transgenic lines that were Southern blot positive were further analyzed by PCR to confirm the integration and segregation of the transgene. As shown in Fig. 7, PCR analysis of the progeny from one line showed segregation of the ASA2 gene in T_1 progeny. A summary of the PCR results at the progeny of three T_0 is shown in Table 2. Enzymatic assay for the AS activity of T_1 progeny of one transgenic line showed that the enzyme activity of transgenic line is less feedback sensitive than that from the wild type plant (Fig. 8).

Table 1 Summary of soybean embryo axes transformation using 5-MT for selection

Total embryo axes	Plants recovered	Southern blot
120	1	+
150	2	+
200	5	+

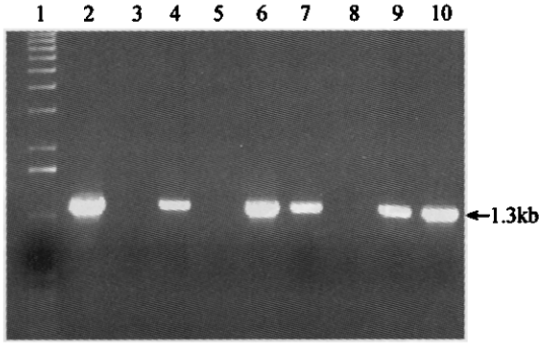

Fig.7 PCR analysis of T_1 plants from one transgenic line

1: 1 kb DNA ladder; 2: Plasmid DNA; 3: Wild type soybean; 4 ~ 10: Seven T_1 plants from one transgenic line.

Table 2 Summary of the segregation data of three ASA2 transgenic lines

T_0 line #	Total T_1 plants tested	PCR positive
1	7	5
2	7	5
3	7	5

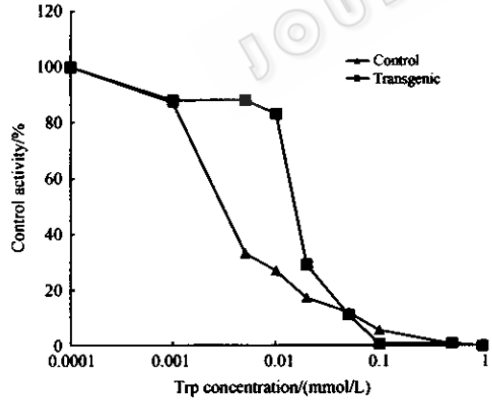

Fig.8 AS enzyme assay of T_1 progeny of transgenic line

Due mainly to consumer concerns, strategies have been developed to eliminate the marker gene after selection[16,17]. Alternatively, the use of marker genes that do not rely on antibiotic or herbicide resistance has also been developed[18]. Finding new and useful selectable marker genes for plant transformation is always a great challenge. There are a few reports of using amino acids or their analogues for plant transformation selection. A tryptophan decarboxylase gene(*tdc gene*) from *Catharanthus roseus* was used for tobacco transformation with 4-MT as the selective agent, this enzyme can convert the toxic 4-MT into non-toxic 4-methyltryptamine[19]. Since many plants have endogenous TDC activity, this gene has not been widely used as a selectable marker. A mutant *lys* C gene from *E. coli* encoding a lysine-threonine feedback insensitive form of aspartate kinase has also been used as a selectable marker for barley transformation, with lysine and threonine as selective agents[20]. However, most of the transgenic plants recovered were albino.

As far as the use of feedback-insensitive anthranilate synthase genes for selectable markers, there are two patents describing the use of mutant genes encoding the AS α-subunit as selectable marker for maize[6] or rice[7] transformation. More recently, a feedback-insensitive point mutation of anthranilate synthase gene from rice was engineered into rice, and the calli and subsequent regenerated plants showed resistance to 5- MT and had a 36-fold increase in free Trp, but the selectable marker gene used in that study was *hpt* with hygromycin as a selective agent[21]. Furthermore, in both cases, the mutant genes were isolated from and re-engineered into the same plant species. In this study, the *ASA2* gene cloned from tobacco cell line only encoded for anthranilate synthase α-subunit[8], and the two α-subunits can interact with the β-subunits of a heterologous plant (soybean) to form an active anthranilate synthase.

In summary, transgenic soybean lines were obtained using a feedback-insensitive *ASA2* gene from tobacco as a novel selectable marker with the Trp analog 5-MT as the selective agent. Transgenic shoots were recovered in only 6 ~ 8 weeks. Since this feedback-insensitive *ASA2* gene was isolated from an unselected, but 5-MT tobacco cell line, and was not a mutation from another plant species (tobacco)[8], it can be used as a novel selectable marker gene for soybean transformation with no concerns about the environmental issues such as those associated with using antibiotic or herbicide resistance genes. A possible disadvantage of using this selection system is that the transgenic shoots were difficult to root and the growth of the primary transgenic events was inhibited at the beginning. This problem may be solved by the addition of Trp into the rooting medium[21].

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