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专论与综述

根癌农杆菌介导真菌遗传转化的研究及应用

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摘 要：根癌农杆菌介导转化法(*Agrobacterium tumefaciens*-Mediated Transformation，ATMT)具有转化效率高、遗传稳定、适用范围广等诸多优点，已成为真菌遗传转化研究中的强有力手段，在真菌基因资源开发、真菌性疾病研究和外源蛋白表达研究中发挥巨大作用。本文概述了根癌农杆菌转化法在真菌转化中的研究进展、技术优缺点、转化机制、实验方法和应用现状，着重介绍影响其转化效率的因素并对优化方法进行探讨，展望了该技术在真菌基因资源发掘、基因编辑等方面的应用前景，为今后真菌的遗传转化研究提供参考。

关键词：根癌农杆菌，真菌，遗传转化

Research and application of *Agrobacterium tumefaciens*-mediated fungal genetic transformation

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Abstract: *Agrobacterium tumefaciens*-mediated transformation (ATMT) has many advantages such as high transformation efficiency, genetic stability, and a wide range of applications. It has become a powerful method in the study of fungal genetics. It plays a huge role in fungal disease research and heterologous protein expression research. This article summarizes the research progress, technical advantages and disadvantages, transformation mechanism, experimental methods and application status of ATMT technology in fungi studying. This review also focuses on the influencing factors of ATMT transformation efficiency and discusses the optimization methods. The application prospects of genome editing and other aspects are expected to provide references for future fungal genetic transformation research.

Keywords: *Agrobacterium tumefaciens*, fungi, genetic transformation

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真菌作为人类最早利用的功能微生物之一,已成为重要的酶制剂“微生物细胞工厂”。目前已在真菌中表达了植酸酶[1]、脂肪酶[2-3]、酸性果胶酶[4]、半乳糖苷酶[5]等众多水解酶。

真菌的遗传转化一般有以下几种方法:原生质体转化法[6]、脂质体转化法[7]、电击转化法[8]、限制性酶介导整合法[9]、根癌农杆菌介导转化法(*Agrobacterium tumefaciens*-Mediated Transformation, ATMT)[10]等。原生质体转化法转化周期短,但阳性率偏低且原生质体制备烦琐;脂质体转化法运载能力强,但会对细胞产生毒性且容易污染;电击转化法转化速度快但转化率较低;限制性酶介导整合法需确定内切酶的种类和浓度,不适于推广使用;ATMT 相较于其他转化方法具有显著优越性,首先,该技术实现了对真菌孢子、菌丝体、菌褶、原生质体、子实体等材料的遗传转化,避免了烦琐的原生质体和渗透性敏感细胞的制备,极大简化了转化过程并降低了转化难度;其次,转化效率高,是传统转化方法的 140−1 000 倍[11-14];转化子遗传稳定性高,后代转化子遗传特性仍保持 85%−98%[15-17];转化子 T-DNA 单拷贝插入比例高,为 66%−96%[18-19];此外,成为马拉色菌(*Malassezia* spp.)等特殊真菌遗传转化的有效工具[20-22],为特殊真菌遗传研究奠定基础。

1995 年,Bundock 等[10]、Piers 等[23]利用 ATMT 介导了酿酒酵母(*Saccharomyces cerevisiae*)的遗传转化;1998 年,De Groot 等[13]利用 ATMT 转化了菱镰刀菌(*Fusarium venenatum*)等 9 种真菌;2000 年,对双孢蘑菇(*Agaricus bisporus*)进行 ATMT 转化[24],并逐步应用到金针菇(*Flammulina velutipes*)[25]等 20 余种食用真菌中[26-30];2007 年已经实现了 60 多种真菌的遗传转化[31-34];2019 年,利用 ATMT 在金针菇中成功进行 CRISPR/Cas9 基因编辑系统研究[35-37];到 2020 年,ATMT 已经对 100 余种真菌进行遗传转化[38-39]。部分研究成果见表 1。但由于转化材料、共培养时间与温度、诱导剂浓度等多种因素的限制,至今还无一个普遍适用于大多数真菌的转化条件及指标,此技术在真菌中的应用尚不成熟[87]。本文概述了该技术在真菌领域的研究进程、技术优缺点和应用现状,主要介绍其转化机制和操作步骤,着重分析了影响转化效率的因素并对实际转化操作中的各种问题提出优化建议,此外,总结了近年来 ATMT 成功转化的真菌种类和在病原真菌研究及外源蛋白表达方面的研究成果,旨在为真菌遗传转化研究提供参考。

1 ATMT 转染机制及操作流程

1.1 ATMT 中 T-DNA 转移过程

农杆菌主要有根癌农杆菌(*Agrobacterium tumefaciens*)和发根农杆菌(*Agrobacterium rhizogenes*)2 种,是普遍存在于土壤中的一种革兰氏阴性细菌,在植物酚类物质如乙酰丁香酮(*Acetosyringone*, AS)的吸引下经创口感染进入宿主细胞,致癌(Tumor-Inducing, Ti)质粒毒力基因随即开始表达,将农杆菌的 T-DNA 转移至宿主细胞,完成转基因过程[88]。

农杆菌内 Ti 质粒上 Vir (Virulence)基因区能与 T-DNA 边界上高度保守的约 25 bp 的碱基序列发生特异性结合,使 T-DNA 在转化过程中不受序列特异性的影响,因此可借助分子克隆技术将内源的 T-DNA 替换成外源基因来完成遗传转化,得到表达外源基因的植物或真菌[89]。

农杆菌转化过程主要包括:吸附宿主细胞,Vir 区基因的激活,T-DNA 切割、包装、转移等[90]。在一系列 Vir 毒力蛋白调控下将 T-DNA 左右边界(LB 和 RB,两端长约 25 bp 重复序列)内序列转移到真菌等宿主细胞核 DNA 中[89-90]。*vir* 基因至少包含 6 个不同的毒力基因编码区:*virA*、*virB*、*virC*、*virD*、*virE* 和 *virF*,每个 *vir* 基因座对应一个 Vir 转录单位,其中 *virA* 为组成型表达,*virB*、*virC*、*virD* 和 *virE* 基因仅在被植物细胞激活时表达[91];T-DNA 转移机制如图 1 所示:首先 AS 诱导膜蛋白 VirA 形成复合物,使 VirG 磷酸化,激活 Vir 区转录出核酸内切酶 VirD1 和 VirD2,2 种内切酶分

表 1　ATMT 技术成功转化的真菌种类

Table 1　Fungi successfully transformed by ATMT

真菌种类 Fungal species	农杆菌种类 *Agrobacterium* species	双元载体 Binary vectors	选择标记 Screening markers	表达基因 Expressed genes	转化率 Conversion rate	参考文献 References
Mortierella alpina	AGL-1	pBIG2-ura5s	Hyg B	ura5s gene oPpFADS17	−	[40]
Mortierella alpina	AGL-1, EHA105 C58C1, LBA4404	pBIG2-ura5-ITs	Hyg B	ura5	−	[41]
Macrocybe gigantea	EHA105	Plasmid4	Hyg B	eGFP	−	[42]
Cylindrosporium eleocharidis	−	pEX4	Hyg B	GFP	600–700 transformants/10^7 spores	[11]
Colletotrichun gloeosporioides	−	PSK2251	Hyg B	GFP	−	[43]
Sclerotium rolfsii	LB4404	−	Basta	DsRed, tdTomato, GUSPlus	−	[44]
Aspergillus niger	AGL-1	pCAMBIA	Hyg B	Halophilic, eosinophilic β glucosidase	−	[45]
Flammulina velutipes	EHA105	pCAMBIA0390	Hyg B	FVcas9	−	[37]
Aspergillus niger	AGL-1	pCAMBIA1301	Hyg B	Rhizopus chinensis lipase	60±5 transformants/10^7 spores	[46]
Penicillium digitatum	AGL-1	pPK2	Hyg B, NTC	DsRed, GFP	1 240±165 transformants/10^6 spores	[47]
Morchella importuna	EHA105	p1391-U-GUS	Hyg B	eGFP, β-glucuronidase	−	[30]
Agaricus bisporus	LB4404	pYN6981	Hyg B	eGFP, β-glucuronidase	53.85%	[16]
Fusarium oxysporum	Agr0	pXEN	G418	−	250 transformants/10^4 spores	[32]
Aspergillus oryzae	AGL-1	pEX2	−	Knockout of Pyr G gene, DsRed	1 060 transformants/10^6 spores	[48]
Malassezia furfur, *Malassezia pachydermatis*	AGL-1	pBHg	Hyg B	GFP	*M. furfur*: 0.75%−1.5%, N. *M. pachydermatis*: 0.6%−7.5%	[49]
Flammulina velutipes	LB4404	FpiC	Hyg B	FVCas9	6.84%	[36]
Trichophyton mentagrophytes	EHA105	pDHt	Hyg B	ZafA	−	[50]
Malassezia furfur, *Malassezia sympodialis*	−	pPZP-201BK	NTC, G418	ADE2, LAC2	−	[20]

(待续)

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(续表 1)

Penicillium digitatum	AGL-1	pTFCM	Hyg B	Hygromycin B phosphotransferase	−	[51]
Harpophora oryzae	AGL-1	pKOHo	Hyg B	eGFP	−	[52]
Aspergillus niger	AGL-1	−	Hyg B	Hygromycin B phosphotransferase	35 transformants/10^7 spores	[53]
Trichoderma reesei	AGL-1	pCAMBIA1300-hph-PsCT	Hyg B	Cellulase	13 000 transformants/10^6 spores	[12]
Cladonia metacorallifera	LBA4404	pCAMBIA1300	Hyg B	eGFP	−	[54]
Flammulina velutipes	GV3101	pBHG-BCA1	Hyg B	HMG-box-transcription factorfvhoml	−	[55]
Phytophthora infestans	AGL-1	pBHtl	Hyg B	Hygromycin B phosphotransferase	50−60 transformants/10^6 spores	[56]
Penicillium chrysogenum	LBA1100 AGL-1	p2PEN0014	NTC	Knowlesin acetyltransferase	LBA1100: 104 transformants/10^6 spores AGL-1: 273 transformants/10^6 spores	[57]
Trichoderma harzianum	EHA105	pCAMBIA1301-perg22	Hyg B	Hygromycin B phosphotransferase	Solid phase: 20 transformants/10^7 spores Lquid phase:100 transformants/10^7 spores	[58]
Colletotrichum glloeosporioides Pens	AGL-1	pCAMBIAl300	Tetracycline	eGFP	300−400 transformants/10^6 spores	[59]
Ganoderma lucidum	LBA4404	pCAMBIA1300	Hyg B	Glyceraldehyde-3-phosphate dehydrogenase, eGFP	−	[29]
Lentinus edodes	EHA105	pCAMBIA1301	Hyg B	Hygromycin B phosphotransferase	30%	[27]
Aspergillus aculeatus	C58C1	pBIG2RHPH2	Hyg B	Polyketide synthase	30 transformants/10^4 spores	[60]
Blastocladiella emersonii	EHA105	pBINPLUS	Hyg B	eGFP	−	[61]
Pleurotus ostreatus	AGL-1, GV3101	pCAMBIA1300	Hyg B	Hygromycin B phosphotransferase	75%	[28]
Sporothrix schenckii	LBA4404, EHA105 AGL-1	pBHt1	Hyg B	Hygromycin B phosphotransferase	600 transformants/10^6 spores −	[62]
Aspergillus japonicus	EHA105	pBI-hphII	Hyg B	Hygromycin B phosphotransferase	−	[63]
Curvularia lunata	AGL-1, EHA105 LBA4404	pBHt1	Hyg B	Hygromycin B phosphotransferase	85±4 transformants/10^6 spores	[64]

(待续)

(续表 1)

Cordyceps militaris	AGL-1	pATMT1 pCAMBIA1300	Hyg B	Hygromycin B phosphotransferase	30−600 transformants/10^5 spores	[65]
Mortierella alpina	C58C1	pBIG2RHPH2	Hyg B	Ura5	400 transformants/10^8 spores	[66]
Volvariella volvacea	EHA105	pLin235	Hyg B	Afp	−	[26]
Penicillium digitatum	AGL-1	pTFCM	Hyg B	Hygromycin B phosphotransferase	60 transformants/10^6 spores	[67]
Glomus intraradices		pNHFoxdsRedstuA pNHATPdsRedstA	−	DsRed GFP	pNHF: 8 300 transformants/10^7 spores pNHA: 1 700 transformants/10^7 spores	[68]
Trichoderma reesei	AGL-1	pBIN121	Hyg B	Hygromycin B phosphotransferase	8 500 transformants/10^7 protoplasts	[69]
Metarhizium anisopliae	EHA105	pPZP201BK	Glufosinate ammonium	trp1	22%	[34]
Paecilomyces lilacinus	GV3103	pCAMBIA1302	bar	Chitinase, GFP	−	[70]
Phanerochaete chrysosporium		pCAMBIA	Hyg B	β-glucuronidase, GFP	48%	[71]
Flammulina velutipes	AGL-1	pBG-gHg	Hyg B	Hygromycin B phosphotransferase	16%	[25]
Magnaporthe grisea	AGL-1	pCAMBIA1300	Hyg B	Hygromycin B phosphotransferase	>300 transformants/10^6 spores	[15]
Agaricus bisporus	AGL-1, LBA1126	pBIN19 pGREEN	Hyg B	Hygromycin B phosphotransferase	−	[72]
Trichoderma viride				Chitinase	30−50 transformants/10^5 spores	[73]
Trichoderma spp.	AGL-1	pPK2	Hyg B	Hygromycin B phosphotransferase	190 transformants/10^7 spores	[74]
Colletotrichum gloeosporioides	AGL-1, C58C1	pDHt, pJF1	Hyg B	GFP	−	[75]
Mucor circinelloides	AGL-1	pBHt2	Hyg B	Hygromycin B phosphotransferase	−	[33]
Aspergillus fumigatus	EHA105	pdht-hph pdht-sk	Hyg B	Homologous recombination knockout polyketide synthase	>100 transformants/10^7 spores	[76]
Beauveria bassiana	LBA1126	pAIM3	Hyg B	Hygromycin B phosphotransferase	163±65 transformants/10^6 spores	[77]

(待续)

(续表 1)

Colletotrichum trifolii	C58C1	pBIG2RHPH2	Hyg B	Hygromycin B phosphotransferase	After optimization: 300−500 transformants /10^6 spores Before optimization: 150−300 transformants/10^6 spores	[78]
Rhizopus oryzae	LBA1100	pKS118	Spectinomycin Kanamycin	Pyr4	−	[79]
Aspergillus giganteus	LBA1100	pUR5750	Hyg B	Hygromycin B phosphotransferase	7 900 transformants/10^8 spores	[14]
Phytophthora infestans	LBA1100	pNptII	Neomycin	Neomycin phosphotransferase β-glucuronidase	30 transformants/10^7 spores	[80]
Colletotrichum lagenarium	C58C1	pBIG2RHPH2	Hyg B	Hygromycin B phosphotransferase	150−300 transformants/10^6 spores	[81]
Monascus purpureus	LBA1100, AGL-1	pUR5750, pBGgHg	Hyg B	GFP	−	[17]
Magnaporthe grisea	AGL-1	pBHt1	Hyg B	Hygromycin B phosphotransferase	385 transformants/10^7 spores	[82]
Suillus bovinus	AGL-1	pBGgHg	Hyg B	eGFP	−	[83]
Agaricus bisporus	−	pUR5750	Hyg B	Hygromycin B phosphotransferase	−	[84]
Fusarium circinatum	AGL-1	pPZP201	Hyg B	Hygromycin B phosphotransferase	2−150 transformants/10^5 spores	[85]
Fusarium oxysporum	AGL-1	pCAMBIA1300	Hyg B	Hygromycin B phosphotransferase	300−500 transformants/10^6 spores	[86]
Agaricus bisporus	AGL-1, EHA105	pCAMBIA1300	Hyg B	eGFP	Bacterial fold: 64%, Thallus: 9%	[24]
Aspergillus awamori *Aspergillus niger* *Colletotrichum gloeosporioides* *Fusarium venenatum* *Trichoderma reesei* *Neurospora crassa* *Agaricus bisporus*	LBA1100	pUR5750	Hyg B	Hygromycin B phosphotransferase	9 000 transformants/10^7 spores 5 transformants/10^7 spores 25 transformants/10^7 spores 1 200 transformants/10^7 spores 5 000 transformants/10^7 spores 1 000−9 000 transformants/10^7 spores 300−7 200 transformants/10^7 protoplasts	[13]
Saccharom yces cerevisiae Hamsem	EHA105, A348, At1000, At12506, At11067	pBIN19	Kanamycin	ARSI, TRPJ, GAL3		[23]
Saccharomyces cerevisiae	LBA1100	pBINPLUS	Carboxybenzylpenicillin	URA3	The probability is 1.7×10^{-6}/cell	[19]

注：−：未报道

Note: −: No reference

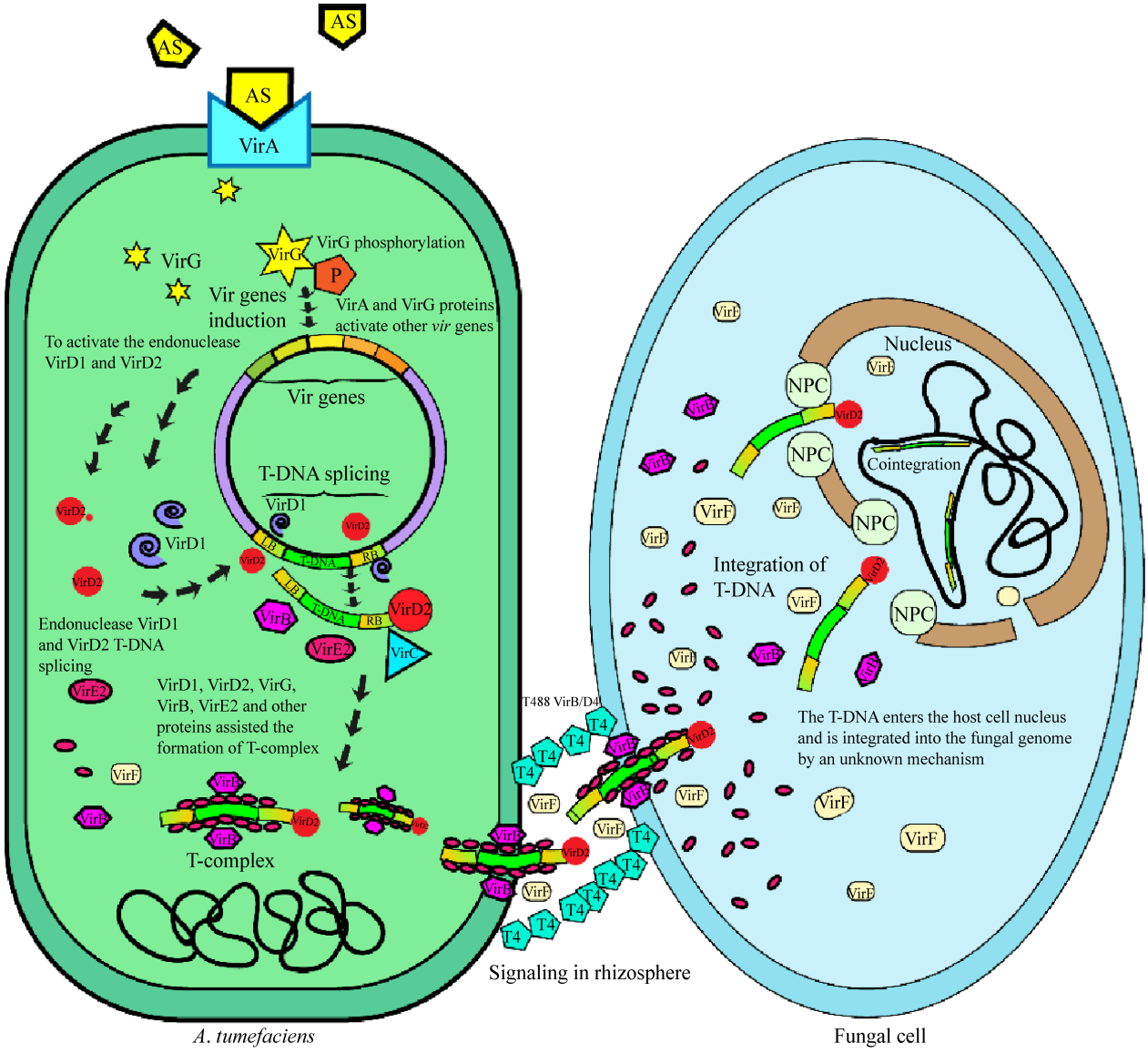

图 1　T-DNA 转移机制

Figure 1　T-DNA transfer mechanism

注：在 AS 诱导下，膜蛋白 VirA 接受刺激，VirA 蛋白结合物磷酸化 *virG* 基因，并转录激活 Vir 区，Vir 区被激活转录出 *virD1*、*virD2*、*virB* 和 *virE* 等蛋白基团，VirD1 和 VirD2 为核酸内切酶，并持续发挥作用，剪切 T-DNA，在 VirD1、VirD2、VirE2、VirB 等蛋白作用下形成 T-DNA 复合体，多种蛋白互作进入宿主细胞核，最终整合到宿主基因组

Note: Under the induction of AS, the membrane protein VirA is stimulated, and the VirA protein conjugate phosphorylates the *virG* gene and transcriptionally activates the Vir region. The Vir region is activated to transcribe the protein groups such as *virD1*, *virD2*, *virB* and *virE*, and VirD1 and VirD2 are nucleic acid Dicer, and continue to work, shear T-DNA, under the action of VirD1, VirD2, VirE2, VirB and other proteins to form a T-DNA complex, multiple proteins interact with the host cell nucleus, and finally integrate into the host genome

别在 LB 和 RB 的第 3 个碱基和第 4 个碱基之间进行切割，并持续发挥作用，T-DNA 的被切割片段从 Ti 质粒中释放出来，产生单链 DNA 分子(T 链)，这些分子在其 5′端共价连接到 VirD2 形成共价复合物(VirD2-T 链)，紧接着与 VirD5、VirE3、VirE2 等蛋白结合形成 T-DNA 复合体，同时，VirD4 与 VirB 形成 T4SS-VirB/D4 通道复合体，进而形成 T4 细胞通道，T 复合体在 VirF 蛋白的辅助下通过

T4 细胞通道进入宿主细胞，在宿主细胞内发生分解，T 链在 VirD2 的牵引下作为线性非置换片段进入宿主细胞核，整合到宿主基因组中，因此能在宿主中稳定存在和遗传[92]。

1.2 ATMT 操作流程

ATMT 操作过程中主要包括农杆菌诱导培养、共培养、转化子筛选 3 个部分(图 2)。农杆菌诱导培养：挑取含有双元载体的根癌农杆菌单菌落，添加 AS 避光诱导培养至指定浓度；黑曲霉(*Aspergillus niger*)与农杆菌诱导共培养：取适量 *A. niger* 孢子悬浮液和诱导过的农杆菌菌液充分混合，均匀涂布于诱导培养基平板上，避光正置培养，AS 添加量、共培养膜基质、培养时间及温度视具体情况而定；转化子筛选：转膜至初筛培养基上进行转化子筛选并杀灭根癌农杆菌，提取转化子基因组并以之为模板进行 PCR 鉴定[46]。

2 ATMT 转化效率的影响因素及优化

根癌农杆菌种类、AS 浓度、双元载体种类、受体材料、共培养时间和温度等众多因素都会对转化效率产生显著影响[39,41,48,60,67,72,76]。需要在保证 T-DNA 转移的同时，兼顾转化材料的生长状况，重点对农杆菌和宿主材料的数量、生长时间及温度进行调控。

2.1 受体材料

适宜的受体材料是转化成功的关键，一般选取幼嫩的组织或细胞，如新鲜或刚萌发的孢子、原生质体、渗透型敏感细胞等，更容易吸纳外源 DNA。

ATMT 适用范围广泛，成功对真菌分生孢子、菌丝体、原生质体、子实体等多种转化材料进行遗传转化[48,54,60]，但不同细胞结构对根癌农杆菌敏感度存在差异，双孢蘑菇(*A. bisporus*)菌褶作为受体材料比孢子获得更高的转化效率[24,84]；金针菇菌丝

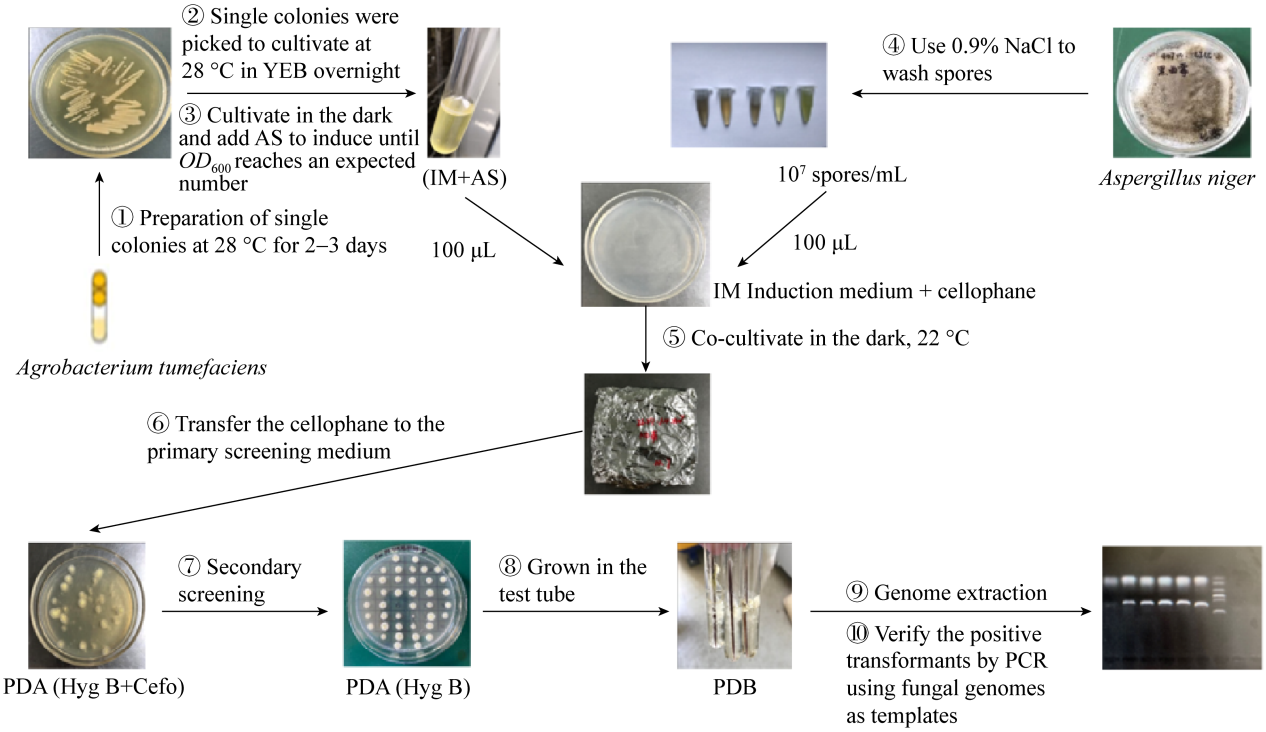

图 2 ATMT 流程图

Figure 2 Flowchart of ATMT

体转化效率是原生质体的 2 倍[93]；而部分真菌只能实现特定材料的 ATMT 转化，毛霉菌和根霉菌的菌丝体、萌发和未萌发孢子、子实体均未能实现 ATMT 转化，仅在原生质体中获得成功[79,92]。此外，受体材料的生长情况也会对转化效率产生极大影响，受体生长过快导致农杆菌侵染过程不完整，受体生长过慢，转化子获得率低且筛选困难。在实验中需对真菌产孢能力、孢子萌发情况和不同细胞结构生长速度等指标进行监测，优先对原生质体、孢子、幼嫩菌丝体进行 ATMT 预实验，确定最佳受体材料，并对受体材料的生长情况进行监控，建议每隔 12 h 进行监测，在根癌农杆菌增殖和受体细胞生长中寻找平衡，不使一方生长过快而影响转化效率，保证 T-DNA 转移过程完整，以获得更高的转化效率。

2.2 根癌农杆菌的种类

根癌农杆菌在转化过程中起到承载、转移和整合目的片段的作用。实现高效侵染必须借助 Ti 质粒上 Vir 区编码的毒力蛋白，因此农杆菌毒力强弱决定了 T-DNA 转移能力的高低。目前可用于真菌 ATMT 转化的农杆菌有：AGL-1[62,75,94-95]、GV3101[55]、LBA4404[62]、LBA1101[80]、C58C1[71,78]、EHA105[42,94-95]、LBA1126[77]等。在实际研究中也发现不同农杆菌菌株转化效率存在差异，甚至少数根癌农杆菌不能转化真菌。Wang 等[41]测试了 4 种农杆菌对高山被孢霉(*Mortierellaoeae alpina*)的转化效率，从高到低依次为 AGL-1、EHA105、C58C1、LBA4404，并且 LBA4404 没有获得转化子。在众多真菌 ATMT 研究中，AGL-1 菌株转化效率普遍偏高。对黑曲霉(*A. niger*)[94]和交织顶孢霉(*Acremonium implicatum*)[95]的转化效率分别是 EHA105 的 12 倍和 600 倍；在申克氏孢子菌丝(*Sporothrix schenckii*)的研究中发现 AGL-1 转化效率是 EHA105 的 5 倍，LBA4404 的 10 倍[62]。但 AGL-1 不能介导斑玉蕈菌(*Hypsizygus maculatus*)的 ATMT 转化[96]。尽管如此，AGL-1 相较于其他菌株适用范围更为广泛。研究过程中建议选择 2–3 种较高毒力菌株，如 AGL-1、EHA105 或 GV3101 进行对比转化，以确定最适宜的根癌农杆菌菌株。

2.3 双元系统、启动子及标记基因

适宜的载体系统是实现转基因操作的桥梁，在转基因过程中起到承载目的片段和赋予宿主新基因功能的作用。Lacroix 等[97]对野生型根癌农杆菌菌株进行了修饰，以适用于广泛多样的宿主为目标，使其能够应用于其他真核物种。在此基础上改造出适用于真菌转化的双元载体系统(二元系统)和共整合系统，除 Ori 序列等载体基本骨架外，均具备完整的 Vir 基因区、T-DNA 及左右边界，以供核酸内切酶特异性识别并切割。双元载体由含有 T-DNA 的多功能克隆质粒和含有 Vir 区的 Ti 衍生质粒构成，均位于根癌农杆菌内，前者负责在大肠杆菌和根癌农杆菌内进行复制和运载 T-DNA 边界内的 DNA 序列(目的基因及标记基因)，后者提供反式毒性区功能，催动 T-DNA 的转移。共整合系统由同时含有转移区和毒力区的 Ti 质粒和大肠穿梭质粒构成，Ti 质粒在根癌农杆菌内，穿梭质粒在大肠杆菌内，通过与大肠穿梭质粒发生同源重组将外源基因整合到 Ti 质粒的 T-DNA 区形成共整合质粒，使之成为 T-DNA 的一部分。

常用的双元载体有 pBI-121[69]、pBNI19[72]、pGreen[72]、pCAMBIA[45,86]系列等，通常以这几种载体为基本骨架通过更换启动子、更换抗性基因、重组进目的基因等方式构建出适用的双元载体。不同载体的转化效率差异明显，在双孢蘑菇(*A. bisporus*)菌的转化中，pCAMBIA 载体的转化效率明显优于 pBNI19 和 pGreen[72]。除常用骨架外，通常利用 *glaA*、*gpdA*、*cbh*、*trpC* 等启动子启动目的基因[40,98]，并以潮霉素磷酸转移酶基因为标记基因，此外标记基因的启动子会对转化效率产生显著影响，在双孢蘑菇(*A. bisporus*)中，选用 *gpdA* 启动子的转化效率是 *trpC* 启动子的 15 倍，CaMV35S 启动子没有转化成功[24]。然而，CaMV35S 启动子成功

驱动了 *rac* 基因在哈茨木霉(*Trichoderma harzianum*)中的表达[99]。在设计表达载体时建议以通用型双元质粒做骨架，如 pCAMBIA 系列[19,27,29,37,94]或 pBI-121[69]系列，此外建议选用 *glaA* 和 *gpdA* 等广谱型强启动子来驱动目的基因或标记基因。

2.4 诱导物浓度

Shaw 等[100]发现在 *virA* 和 *virG* 基因作用下根癌农杆菌对酚类化合物具有趋化性。后续发现，在对绝大部分真菌进行 ATMT 转化时需要 AS 的诱导[62,83]。

在 ATMT 中，AS 主要添加于共培养阶段和农杆菌预培养时期。共培养阶段的 AS 诱导对转化成功必不可少，在指状青霉的 ATMT 转化共培养阶段必须添加 AS 才能成功转化[67]。此外，AS 浓度会对转化效率和插入拷贝数有直接影响，共培养中 AS 浓度通常为 200−300 μmol/L[83,101]。在黑曲霉(*A. niger*)的研究中，AS 浓度为 200 μmol/L 时转化率最高，超过或低于这一浓度，转化效率均下降[102]。众多研究结果与此相近，即转化效率与 AS 浓度之间呈现出一种单峰型的线性关系。在一定浓度范围内，转化效率随着 AS 浓度的升高而增加，超过最适浓度后，转化效率没有明显增加甚至下降[52-53,91-92]，这是因为过少的 AS 无法诱导 *vir* 基因表达，过多则会引起宿主 T-DNA 拷贝数增加，甚至导致根癌农杆菌中毒。目前预培养时期 AS 的添加与否对转化效率的影响评价不一。在转化炭疽病菌(*Colletotrichum trifolii*)[78]和外生菌根真菌(*Hebeloma cylindrosporum*)[103]的过程中是否进行根癌农杆菌 AS 预培养对转化效率没有明显影响。但在须癣毛癣菌(*Trichophyton mentagrophytes*)[50]、稻瘟病菌(*Magnaporthe grisea*)[104]和球状白僵菌(*Beauveria bassiana*)[77]转化中进行 AS 预培养得到了更高的转化效率。建议通过 AS 浓度梯度测试(50、100、200、300、400 μmol/L)确定最适浓度，并进行根癌农杆菌 AS 预培养，预培养 AS 浓度与共培养阶段保持一致。

2.5 共培养温度和时间

共培养温度对转化成功与否至关重要，温度过高，受体生长过快，导致农杆菌侵染不完全就已复苏为完整菌体，也会导致农杆菌生长过快，致毒素积累使 T-DNA 转移受阻，转化率极低或为零。温度过低，农杆菌生长缓慢，Vir 毒力蛋白活力低下，孢子萌发率低，转化子数量少且阳性率不高。T-DNA 转移适宜在低温条件下进行，根癌农杆菌最适生长温度为 28 °C，因此共培养温度在 20–25 °C时转化效率最高。如米曲霉(*A. oryzae*)[48]、巨大口蘑(*Tricholoma giganteum*)[42]、叶斑病菌(*Curvularia lunata*)[64]、指状青霉(*Penicillium digitatum*)[47]、日本曲霉(*A. japonicus*)[63]和须癣毛癣菌(*T. mentagrophytes*)[50]的 ATMT 转化中，最适温度分别为 22、25、25、25、24、20 °C，低于最适温度，转化效率随温度的升高而增加，超过则迅速下降。这与 T-DNA 切割、组装和转移的相关蛋白适宜于低温条件有关，高温易失活，导致转化率降低[105-106]。因此可通过低温共培养显著提高转化率，既能保证毒力蛋白正常发挥功能又能平衡宿主菌与根癌农杆菌的生长。

根癌农杆菌与易感材料接触 36−48 h 内导致细胞改变，完成外源 DNA 转移[107]，因此共培养时间一般为 24−60 h，此外，在一定范围内，转化效率与共培养时间呈正相关，在巨大口蘑(*T. giganteum*)的遗传转化中，共培养时间低于 36 h 时，转化效率随时间延长而增加，36 h 时获得最高转化率，延长时间至 60 h 时转化率逐渐下降，至 84 h 时，转化率极速下降 40%[42]。少数转化的共培养时间需要 72−192 h[11,36,48]。这与宿主菌生长特性有关，以孢子、原生质体等作为受体材料共培养时间较短，以子实体、菌丝体等作为受体材料则转化时间较长[24,84,93]。在实际转化中可通过共培养温度梯度测试(20−28 °C)和时间梯度测试(24、36、40、48、56、64、80 h)获得最佳共培养条件。

2.6 受体材料数量与农杆菌浓度比例

适宜的受体细胞和根癌农杆菌浓度比可以获得更多单拷贝转化子及更高转化效率。受体材料或根癌农杆菌浓度低，导致转化子数量稀少或为零，

假阳性率高。受体或农杆菌过多，真菌生长容易连片，农杆菌生长过快影响受体材料萌发，转化子挑取困难[11-12,51,53,59,76]。

共培养的关键参数不仅包括细菌细胞与受体细胞之间的比例，还涉及侵染期间混合物的密度。只有农杆菌 OD_{600} 值为 0.2−0.3，孢子浓度为 10^6 个/mL 时才能对淡紫拟青霉(*Paecilomyces lilacinus*)进行 ATMT 转化，高出或低于该浓度均未成功[70]。较高浓度的根癌农杆菌和真菌细胞可以提高转化效率，但最高浓度有一定界限，在此浓度范围内，转化率随着农杆菌数的增加而递增，这一现象在尖曲霉(*A. aculeatus*)[60]、灵芝(*Ganoderma lucidum*)和糙皮侧耳(*Pleurotus ostreatus*)[108]的 ATMT 转化中均有体现，此外受体材料与根癌农杆菌的数量比分别保持在 1:10 000、1:1 000、1:100 时转化效率最高。建议控制根癌农杆菌与受体细胞数量比值在 100−10 000 之间进行转化。

2.7 其他因素

影响 ATMT 转化效率的因素还包括共培养膜基质、培养基 pH 值、氧气含量、选择标记和根癌农杆菌杀灭剂等。

目前真菌 ATMT 转化中常使用硝酸纤维素滤膜、尼龙膜、纤维滤纸、玻璃纸、醋酸纤维膜、醋酸硝酸混合膜等作为共培养基质。不同膜基质之间转化率差异较大，黑曲霉(*A. niger*)中不同基质转化效率排序从高到低为：硝酸纤维素滤膜、醋酸硝酸混合膜、醋酸纤维膜、尼龙膜[53]；荸荠秆枯病菌中不同共培养基质转化效率从高到低排序为：硝酸纤维素膜、Hybond N^+膜、玻璃纸、滤纸[11]；烟曲霉中尼龙和纤维素滤膜的转化率最高，硝化纤维素膜效果最差[76]；指状青霉(*P. digitatum*)中滤纸转化效率是可米拉布膜的 2 倍[47]；致病疫霉(*Phytophthora infestans*)中 Hybond N^+杂交膜转化效率是尼龙膜的 2−3 倍[80]。此外以玻璃纸为共培养基质分别成功转化了 *P. infestans*[56]、哈茨木霉(*T. harzianum*)[58]、黑曲霉(*A. niger*)[46]。以上结果表明，不同膜基质会对转化效率产生一定的影响，这与膜材料渗透能力和化学性质相关，膜的化学性质可能影响根癌农杆菌细胞和分生孢子的分布并抑制其相互作用，建议选择硝酸纤维素膜作为共培养基质。

酸性环境更有利于真菌 ATMT 转化。炭疽病菌(*C. gloeosporioides*)[81]和里氏木霉(*T. reesei*)[12]的 ATMT 转化最适 pH 值为 5.3，高于或低于这一数值都会导致转化率降低。在蛹虫草(*C. militaris*)中 pH 值 5.5 时转化效率最高，pH 值稍高或略低于 5.5，转化率都明显下降[65]。综合其他研究结果，建议共培养培养基 pH 为 5.0−5.6 之间[11,78]。

溶氧量、标记基因和农杆菌杀灭剂是 ATMT 转化中很容易忽略的因素。随着诱导过程中溶氧量的增加，转化效率呈上升趋势[53]，这与根癌农杆菌代谢水平增强、细胞活力增加使毒力蛋白分泌量增加有关[77]。适宜地选择标记对转化子的筛选尤为重要，除少数使用营养缺陷型进行筛选外，多采用潮霉素磷酸转移酶(Hygromycin B Phosphotransferase)基因作为标记基因，此外不同菌种对潮霉素的敏感度差异较大，需对宿主菌进行抗生素敏感度测试，找到最低抑制浓度。头孢噻肟是 ATMT 转化过程中常用的根癌农杆菌杀灭剂，对真菌没有抑制作用，通常在筛选培养基中添加 200−300 μmol/L 的头孢噻肟对农杆菌进行杀灭，防止其继续生长覆盖转化子。

3 ATMT 在真菌疾病研究中的应用

病原真菌是指寄生于动植物体表或体内导致动植物病害的真菌。真菌性植物病害达 30 000 余种，占植物病害的 70%−80%，其中炭疽病菌和枯萎病菌占比较大[109-110]。动物性病原真菌通过侵染人体及动物体浅表组织或入侵内部器官引起扩散性深层病害。ATMT 为瓜类炭疽病[81]、荸荠秆枯病[11]，玉米弯孢菌叶斑病[64]、稻瘟病[15,19]、角膜炎[32]、孢子丝菌病[62]等数十种动植物疾病机理研究和耐药基因发掘提供技术支持(表 2)。

表 2 根癌农杆菌介导的真菌性疾病的研究

Table 2 Functional analyses on mycosis by ATMT

真菌疾病 Mycosis	病原真菌 Pathogenic fungus	主要感染物种 Major infectious species	文献来源 Literature sources
Rice blast	*Magnaporthe grisea*	*Oryza sativa* L., *Triticum aestivum* L., *Hordeum vulgare L.*	[15,19]
Fusarium graminearum	*Fusarium graminearum Schw*	*Oryza sativa* L., *Triticum aestivum* L., *Hordeum vulgare* L., *Avena sativa* L.	[13]
Gloeosporium theae-sinensis miyake	*Colletotrichum gloeosporioides*	*Camellia sinensis*	[43]
Cucurbits anthracnose	*Colletotrichum lagenarium*	*Cucurbitaceous plant*	[81]
Stylo anthracnose	*Colletotrichum glloeosporioides* *Colletotrichum damatium*	*Stylosanthesguianensis*	[59]
Anthracnose of alfalfa	*Colletotrichum destructivum* *Colletotrichum trifolii*	*Medicago Sativa Linn*	[78]
Fusarium oxysporum f. sp. conglutinans	*Fusarium oxysporum*	*Brassica oleracea* L., *Brassicaoleracea* L. *var. italica Plenck*	[32]
Apple tree canker	*Valsa mali*	*Malus domestica*	
Stem blight of water chestnut	*Cylindrosporium eleocharidis*	*Eleocharis dulcis*	[11]
Green mold	*Penicillium digitatum*	*Citrus reticulata Blanco*	[51]
Sclerotium rolfsii	*Sclerotium rolfsii*	*Camellia oleifera Abel*, *Vernicia fordii*, *Catalpa bungei*, *Citrus reticulata Blanco*, *Malus domestica*, *Firmiana platanifolia*	[44]
Flax rust	*Melampsora lini*	*Linum usitatissimum* L.	[21]
White rot	*Phanerochaete chrysosporium*	*Vitis vinifera*	[71]
Potato late blight	*Phytophthora infestans*	*Solanum tuberosum*	[56,80]
Pine resin canker	*Fusarium circinatum*	*Pinus*	[85]
Maize curvularia leaf spot	*Curvularia lunata*	*Zea mays* L.	[64]
Fungal keratitis	*Fusarium oxysporum*	*Homo*	[32]
Pityrosporum folliculitis	*Malassezia furfur*	*Homo*	[49]
Sporotrichosis	*Sporothrix schenckii*	*Homo*	[62]

研究者们借助 ATMT 转化绿色荧光蛋白(Green Fluroscent Protein，GFP)等报告基因，通过观察荧光信号进而监测细胞核分裂运动及病原真菌调控蛋白的移动,或构建突变体库进行高通量基因型和表型的筛选，利用 Tail-PCR 进行 T-DNA 标记，筛选出致病位点，达到研究致病真菌侵染途径和定殖机理的目的。Guo 等[19]通过 ATMT 将 eGFP 基因靶向整合到 *Maynaporthe oryzae* 基因 Mosdi-R 位点，证明对 Mosdi1 位点进行靶向整合是稻瘟病遗传互补分析的有效方法;对茶树炭疽病原菌进行 GFP 荧光标记，发现胶孢炭疽菌的分生孢子可以在叶片的气孔和叶片组织内定殖[43]；通过构建以 GFP 为报告基因的荸荠秆枯病病原菌 T-DNA 插入突变体库，获得表型和致病性缺陷突变体，为荸荠秆枯病菌基因功能和病菌与寄主互作研究提供了参考依据。

4 ATMT 介导外源蛋白在真菌中的表达

丝状真菌、酵母菌和食用菌为代表的大部分真菌具有蛋白分泌能力旺盛、安全性高、生长繁殖迅速、发酵工艺简单等优点,它们作为优良的工程菌，被广泛运用到外源蛋白的高效表达。采用 ATMT 技术在海洋黑曲霉(*A. niger*)中定向表达了 6 种纤维素酶组分(AnCel6、AnCel7A、AnCel7B、Aneg1、AnBGL1 和 AnBGL2)，通过酶学性质研究，解释了其分泌的纤维素酶混合物的耐盐性机制[45]。凭此技术在金针菇中稳定表达了乙型肝炎病毒表面抗原(HbsAg)[111],此外还介导了大量异源蛋白和同源蛋白的高效表达，如脂肪酶、β-葡萄糖苷酶、抗原蛋白等(表 3)。

表 3 ATMT 介导外源蛋白的表达

Table 3 Expression of exogenous proteins in fungi mediated by the ATMT

外源基因 Exogenous genes	受体 Hosts	双元载体 Binary vector	参考文献 References
eGFP	*Macrocybe gigantea*	Plasmid4	[42]
GFP	*Cylindrosporium eleocharidis*	pEX4	[11]
GFP	*Colletotrichun gloeosporioides*	PSK2251	[43]
DsRed, TdTomato, GUSPlus	*Sclerotium rolfsii*	−	[44]
Lipase of *Rhizopus chinensis*	*Aspergillus niger*	pCAMBIA1301	[46]
HBsAg Hepatitis B virus surface antigen encoding gene	*Flammulina velutipes*	p0390-AiH-FmHB	[111]
Halophilic, eosinophilic β-glucosidase	*Marine Aspergillus niger*	pCAMBIA	[45]
VgbVitreoscilla hemoglobin	*Lentinus edodes*	pBHg-vgb-gpd	[112]
DsRed, GFP	*Penicillium digitatum*	pPK2	[47]
eGFP, β-glucuronidase	*Morchella esculenta*	p1391-U-GUS	[30]
eGFP, β-glucuronidase	*Agaricus bisporus*	pYN6981	[16]
DsRed	*Aspergillus oryzae*	pEX2	[48]
eGFP	*Harpophora oryzae*	pKOHo	[52]
Cellulase	*Trichoderma reesei*	pCAMBIA1300-hph-PsCT	[12]
P. ostreatus hydrophobin	*Tremella fuciformis*	pGEH-GH	[113]
eGFP	*Cladonia metacorallifera*	pCAMBIA1300	[54]
Enterovirus 71 structural protein P1 and protease3C	*Flammulina velutipes*	p0390-AiH-VLP	[114]
eGFP	*Colletotrichum glloeosporioides Pens*	pCAMBIAl300	[59]
Glyceraldehyde-3-phosphate dehydrogenase, eGFP	*Ganoderma lucidum*	pCAMBIA1300	[29]
eGFP	*Blastocladiella emersonii*	pBINPLUS	[61]
DsRed, GFP	*Glomus intraradices*	pNHFoxdsRedstuA pNHATPdsRedstA	[68]
Spruce budworm antifreeze protein	*Volvariella volvacea*	pLin235	[26]
Chitinase of *Metarhizium anisopliae*, GFP	*Paecilomyces lilacinus*	pCAMBIA1302	[70]
β-glucuronidase, GFP	*Phanerochaete chrysosporium*	pCAMBIA	[71]
chitinase of *Bacillus subtilis*	*Trichoderma viride*	−	[73]
GFP	*Colletotrichum gloeosporioides*	pDHt, pJF1	[75]
Polyketide synthase	*Aspergillus fumigatus*	pdht-hph pdht-sk	[76]
β-glucuronidase	*Phytophthora infestans*	pNptII	[80]
GFP	*Monascus purpureus*	pUR5750, pBGgHg	[17]
eGFP	*Suillus bovinus*	pBGgHg	[83]
eGFP	*Agaricus bisporus*	pCAMBIA1300	[24]

注：−：未报道

Note: −: No reference

5 总结与展望

1995 年 ATMT 首次应用到酿酒酵母中，此后便在真菌遗传研究中广泛运用，由最初的 1–6 种宿主真菌发展到担子菌门、子囊菌门、壶菌门、接合菌门等百余种真菌。本文综述了 ATMT 的研究进程、技术优缺点、转化机制和应用现状，反映了该技术在真菌基因资源开发、病原真菌研究、真菌基因组学等方面的巨大潜力。

然而 ATMT 仍然存在不足：(1) ATMT 在真菌中的应用不完全成熟。农杆菌种类、共培养温度及时间等众多因素都会对转化效率产生影响，不同菌株甚至同一菌株不同受体材料之间转化效率差异较大，限制了该技术的推广使用；(2) ATMT 在构建 T-DNA 突变体库中存在局限性。T-DNA 插入的拷贝数不定，存在单拷贝和多拷贝串联排列现象，整合位点的特异性也尚未明确，此外引入了左右边界两端约 25 bp 的重复序列，对于定点突变和侧翼序列的鉴定产生阻碍；(3) T-DNA 整合机制尚不清楚。T-DNA 进入宿主细胞后在 VirD2 蛋白的牵引下以尚不清楚的方式整合到宿主基因组中；(4) 目前关于 ATMT 的报道较为单一，多为转化条件的优化，对于整合机制的研究较少，缺乏相关基因的功能研究。

尽管如此，ATMT 仍然是真菌遗传研究中强有力的分子手段，它使众多难以进行遗传转化的真菌的研究成为可能，由于具有更高的同源重组频率和 T-DNA 单拷贝插入率，在真菌基因突变体库构建方面具有很好的应用价值。有报道利用 ATMT 在金针菇中成功进行了 CRISPR/Cas 9 基因编辑系统的研究，该技术介导真菌 CRISPR/Cas 9 系统的开发将是热点，将会为真菌领域基因编辑系统的开发与应用提供强有力支持。

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