

联合固氮40年: 从发现到应用

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摘要 生物固氮作为将大气氮气转化为植物可利用铵的关键生物学过程, 目前仅由原核生物实现, 对减少化学氮肥依赖、保障粮食安全和改善生态环境至关重要; 尽管根瘤-豆科共生体系效率最高, 但水稻、小麦、玉米等主粮作物无法建立此类共生关系, 而联合固氮菌可定殖禾本科及果蔬作物根表、维管束或内生组织, 通过固氮、分泌植物激素、合成抗菌物质及增强宿主抗逆性形成多维促生机制. 中国农业大学生物学院微生物系四十年来在联合固氮领域构建了覆盖“资源发掘-机制解析-合成生物学改造-田间应用”的全链条技术体系: 分离获得2000多株固氮菌, 建立国内外最大的种质资源库并命名15个固氮新种; 解析固氮类芽孢杆菌中丙氨酸脱氢酶介导的氮代谢-固氮协同通路, 突破高铵抑制固氮的瓶颈; 在合成生物学领域实现突破——酵母体系(15个固氮基因共表达)中成功组装大小正确的NifDK四聚体及活性的NifH蛋白, 水稻体系(13个固氮基因共表达)中实现NifDK四聚体组装并创制抗降解NifH突变体; 开发的固氮菌剂在减氮15%条件下显著提升小麦/玉米产量7.64%~23.20%. 这些系统性成果为谷类作物生物固氮技术开发奠定理论和技术双重基础, 标志着我国在农业微生物领域已构建起具有自主知识产权的完整创新链.

关键词 联合固氮, 资源挖掘, 调控机制, 异源表达, 菌剂应用

生物固氮是农业生态系统氮素循环的核心环节. 联合固氮作为其重要分支, 核心特征在于固氮微生物与宿主植物形成非根瘤型松散共生关系, 定殖于根表、根皮层内或植株地上部分. 自1975年概念提出以来, 联合固氮研究在全球范围内受到广泛关注. 早期研究证实甘蔗、水稻、玉米等禾本科作物及牧草根系具显著固氮活性, 初步揭示了其在减少化学氮肥依赖的应用潜力. 自20世纪80年代起, 中国农业大学李季伦、陈三凤等学者组建核心研究团队, 围绕联合固氮开展系统性研究. 从固氮菌种资源发掘、固氮调控机制解析到合成生物学改造及抗病促生应用, 构建了覆盖全链条的技术体系, 为我国农业绿色转型储备了关键菌种资源与技术基础. 本文旨在系统梳理中国农业大学生物

学院微生物系建系40年来在该领域取得的研究进展.

1 联合固氮菌种资源发掘与多样性研究

禾本科作物的生物固氮潜力最早于1961年由Doberiner证实, 其在甘蔗根际成功分离出具有固氮能力的拜叶林克氏菌属(*Beijerinckia*)^[1]. 1975年, 具有里程碑意义的研究发现了与禾本科植物共生的关键固氮微生物——固氮螺菌属(*Azospirillum*), 并由此正式提出了“根际联合固氮”概念^[2]. 后续研究进一步揭示, 甘蔗、水稻、玉米、牧草等重要禾本科作物均存在显著的固氮现象, 极大地推动了该领域的研究热潮. 自联合固氮概念确立以来, 在各类植物根际不断发现新的相关菌株, 联合固氮菌的多样性不断扩展. 目前已鉴定包括醋

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酸固氮菌(*Acetobacter*)、草螺菌(*Herbaspirillum*)、固氮弧菌(*Azoarcus*)、产碱菌(*Alcaligenes*)、肠杆菌(*Enterobacter*)、克雷伯氏菌(*Klebsiella*)、假单胞菌(*Pseudomonas*)、伯克氏菌(*Burkholderia*)、不动杆菌(*Acinetobacter*)、欧文氏菌(*Erwinia*)、黄杆菌(*Flavobacterium*)、类芽孢杆菌(*Paenibacillus*)等在内的诸多属的代表菌株^[3]。相较于根瘤菌与豆科植物形成的紧密共生体系(根瘤结构),联合固氮菌与宿主植物的关系更为松散,它们主要定殖于植物根表或根际土壤,部分可侵入植物根皮层组织或维管系统,依赖根系分泌物生存繁殖,与根系关系密切但并不形成特化的共生结构。这些固氮菌广泛分布于各种植物根际,在禾本科作物(如玉米、水稻、甘蔗、小麦)中固氮潜力尤为突出。例如,在墨西哥玉米品种的气生根黏液中富集伯克氏菌、假单胞菌和固氮螺菌,其生物固氮贡献可达植株总氮吸收量的80%以上^[4]。研究表明,联合固氮可满足甘蔗年需氮量的60%^[5]。

为深入探索这一新兴固氮资源,李季伦团队自1979年起开展了研究。历经40年持续努力,团队累计从

全国30余省份的植物根际、土壤及特殊生境(如盐碱地、干旱区、高海拔地区)分离获得联合固氮菌株2000多株。采样区域重点覆盖华北(北京、河北、山东)、华中(湖北、河南)、西北(甘肃、内蒙古)、西南(云南、四川)及东北(黑龙江)等地(图1(a)),分离的菌株涵盖*Azospirillum*、*Paenibacillus*、*Fontibacillus*、*Klebsiella*、*Burkholderia*、*Cohnella*、*Arthrobacter*、*Acinetobacter*、*Ochrobactrum*等属。菌株来源组成分析表明85%以上分离自主粮作物(玉米、小麦、水稻)根际,其余则源自各类瓜果蔬菜及野生植物根际。

李季伦团队于1984年成功分离并报道了两株高活性联合固氮菌——玉62(玉米根系)和高63(高粱根系),经鉴定均为*Azospirillum brasilense*(巴西固氮螺菌)。其中,玉62菌株的固氮酶活性(乙炔还原法测定)比国际参考菌株Sp81高60%,展现了显著的固氮潜力^[6]。该菌株具有独特的跨作物系统性定殖能力,能在玉米、小麦、水稻及烟草的根表及根内组织及茎、叶细胞间隙稳定定殖^[7~9];同时通过分泌吲哚乙酸(indoleacetic acid, IAA)等促生物质^[10],显著提升宿主作物生物量。这一

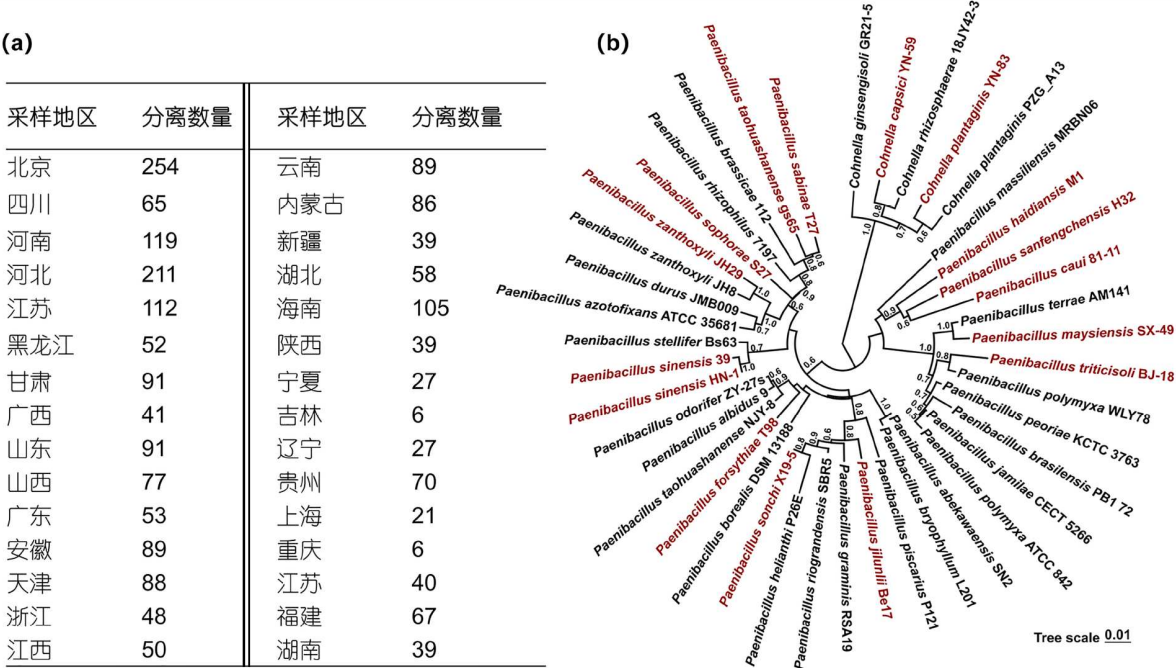


图1 联合固氮菌资源分布与系统发育分析。(a) 分离菌株的地理分布及数量统计;(b) 基于16S rRNA基因构建的固氮类芽孢杆菌、柯恩氏菌的系统发育树。整合含保守固氮基因簇的菌株(含国际已发表种及团队通过基因组分析鉴定种),其中红色标注为本团队发表的15个固氮新种(13个*Paenibacillus*属及2个*Cohnella*属)

Figure 1 Distribution and phylogenetic analysis of associative diazotrophs. (a) Geographic distribution and quantitative statistics of isolated strains. (b) Phylogenetic tree based on 16S rRNA gene sequences of nitrogen-fixing *Paenibacillus* and *Cohnella* strains, incorporating isolates with conserved *nif* gene clusters (including internationally published species and those identified through genomic analysis), with red labels indicating the 15 novel nitrogen-fixing species published by our team (13 in *Paenibacillus* and 2 in *Cohnella*)

发现不仅揭示了巴西固氮螺菌在植物体内的跨组织迁移特性及其与宿主互作机制，更从生态学与细胞形态学层面为菌株-植物协同进化研究提供了关键实证，为联合固氮菌剂的田间应用开发奠定了理论和应用双重基础。

陈三凤团队自2001年起聚焦于固氮类芽孢杆菌(*Paenibacillus*)的研究。早期分类曾将此类菌归入芽孢杆菌属(*Bacillus*)。1993年, Ash等人^[11]提出另立类芽孢杆菌属。截至2025年6月1日(<http://www.bacterio.net/paenibacillus.html>), 该属已包含415个种和8个亚种, 其模式种为*Paenibacillus polymyxa* ATCC 842^T。尽管类芽孢杆菌属成员广泛分布于根际土壤、水体及食品等环境中, 但绝大多数菌株不具固氮能力。迄今全球已发表的固氮类芽孢杆菌新种仅25个, 其中陈三凤团队发表和命名了13个新种, 包括: *P. sabinae* (高山柏根际)、*P. zanthoxyli* (花椒根际)、*P. forsythia* (连翘根际)、*P. sonchi* (苦苣菜根际)、*P. sophorae* (槐树根际)、*P. jilunlii* (秋海棠根际)、*P. taohuashanense* (锦鸡儿根际)、*P. triticisoli* (小麦根际)、*P. maysiensis* (玉米根际)、*P. caui* (桃树根际)、*P. sinensis* (水稻、乔木根

际)、*P. haidiansis* (玉簪根际)、*P. sanfengchensis* (水稻根际)^[12-23], 这些新种在系统发育树中广泛分布于各进化分支(图1(b)), 覆盖灌木、禾本科作物及果蔬等多样化生态位, 充分体现了其广谱环境适应性。团队还建立了拥有1000余株固氮类芽孢杆菌的资源库, 是国内外该领域资源最多的实验室。固氮类芽孢杆菌产芽孢, 抗逆性强, 耐贮藏, 被认为是制备微生物肥料的优良候选菌株。这些菌株在生物活性方面呈现丰富的多样性, 包括固氮酶活性、生长素合成、溶磷、产铁载体、耐铵性、耐盐性、抑菌活性及促生长作用^[24-29](表1)。例如代表性菌株*P. triticisoli* BJ-18和*P. polymyxa* WLY78 (竹子根际)经GFP标记证实能在小麦、玉米、黄瓜等作物的根表、根内、茎及叶组织中稳定定殖^[30,31], 有效拮抗病原真菌并促进多种作物生长^[26](图S1)。比较基因组分析表明其固氮作用通常依赖于一个保守而紧凑的固氮基因簇(nitrogen fixation (*nif*) cluster)(约10 kb), 包含9个*nif*基因(*nifBHDKENXhesAnifV*); 部分菌株的*nif*簇中还含有*orfl*, 位于*nifENX*基因下游; 有些菌还有多拷贝的*nifB*、*nifHDK*基因, 4~6拷贝的nitrogenase-like基因(*nifH-like*, *nifD-like*, *nifK-like*基因), 个别菌还存在铁

表 1 部分联合固氮菌株的生理生化特性^{a)}

Table 1 Physiological and biochemical properties of representative associative diazotrophic strains

菌株	固氮酶活性 (nmol C ₂ H ₄ /(mg protein/h))	IAA产量 (μg/mL)	溶磷能力(mg/L)	耐铵固氮 (mmol/L)	抑菌活性
<i>A. brasilense</i> Yu62 (玉62)	938.9	>50	—	ND	—
<i>P. polymyxa</i> WLY78	3385±214.8	46.6±2.8	溶解有机磷85.5±3.1, 无机磷120±3.8	—	强(产Fusaricidin、嗜铁素), 对灰葡萄孢菌、禾谷丝核菌、尖镰孢菌、核盘菌等有抑制作用
<i>P. sabinae</i> T27	7218±392.8	—	—	300	弱
<i>P. triticisoli</i> BJ-18	992.8±95.0	24.9±1.2	溶解有机磷2.4±0.3, 无机磷3.6±0.2	ND	强(Surfactin、产嗜铁素等), 对灰葡萄孢菌、禾谷丝核菌、尖镰孢菌、核盘菌等有抑制作用
<i>Paenibacillus</i> sp. 1-43	908.2±27.6	49.9±3.6	溶解有机磷100±5.6, 无机磷140±6.8	ND	强(产Fusaricidin等), 对尖孢镰刀菌、禾谷丝核菌、核盘菌、灰葡萄孢有抑制作用
<i>P. maysiensis</i> SX-49	1124.6±11.9	26.3±4.2	溶解有机磷4±0.3, 无机磷5±0.7	ND	强, 对尖孢镰刀菌、禾谷丝核菌、核盘菌、灰葡萄孢有抑制作用
<i>Paenibacillus</i> sp. AH-4	7660.3±603.6	—	ND	300	弱
<i>Paenibacillus</i> sp. BJ-4	3083.9±239.5	>4	ND	ND	强, 对禾谷丝核菌、禾谷镰刀菌、小麦赤霉病菌、腐皮镰刀菌、棉刺盘孢菌和长柄链格孢等有抑制作用
<i>Paenibacillus</i> sp. TD94	1555.8±110.0	30.1±4.7	溶解有机磷90±8.9, 无机磷150±9.7	ND	强(产Tyrocidine等), 对灰葡萄孢、禾谷丝核菌、尖镰孢菌、核盘菌等有抑制作用
<i>Paenibacillus peoriae</i> A22	2289±169.2	—	—	ND	强, 对卷枝毛霉、黄萎病菌、褐丝梗孢菌、串珠镰孢菌、立枯病菌、亚洲镰孢菌等都有抑制作用

a) 数据来自参考文献 [6,10,24-29]。—, 未检测; ND (not detected), 未检测到

铁固氮酶(*anf*)或钼铁固氮酶(*vnf*)相关基因^[29,32-34]。除固氮基因外,部分菌株基因组中还包含溶磷、IAA合成、抗菌物质合成等相关基因(图S1)^[28]。此外,团队还发表了两个柯恩氏菌属(*Cohnella*)的两个新种: *Cohnella capsici* (辣椒根际)和*Cohnella plantaginis* (车前草根际)^[35,36]。这些丰富的菌种和基因资源,为固氮合成生物学研究及联合固氮菌肥的开发奠定了坚实的理论基础。

2 联合固氮菌固氮酶催化与固氮调控的分子机制

李季伦、陈三凤团队在固氮酶催化机制和固氮表达调控网络调控机制领域开展了系统性研究。研究对象涵盖多个重要模式菌株,包括巴西固氮螺菌(*Azospirillum brasilense*)、深红红螺菌(*Rhodospirillum rubrum*)、棕色固氮菌(*Azotobacter vinelandii*)和产酸克氏杆菌(*Klebsiella oxytoca*)以及固氮类芽孢杆菌(*Paenibacillus polymyxa*、*Paenibacillus sabinae*)。

2.1 固氮酶的催化机制

生物固氮的核心催化元件是一类金属酶蛋白复合物-固氮酶。根据活性中心金属元素分为钼铁(MoFe)、铁铁(FeFe)和钒铁(VFe)三种类型,其中钼铁固氮酶存在于所有固氮微生物中且研究最为深入。它由两种蛋白组分构成: Fe蛋白是一个由*nifH*基因编码的 γ_2 二聚体,包含[4Fe-4S]簇和Mg-ATP结合位点,负责电子传递; MoFe蛋白是一个 $\alpha_2\beta_2$ 型四聚体(α 和 β 亚基分别由*nifD*和*nifK*基因编码),含有FeMo-co (FeMo-cofactor)和P-cluster两个关键金属簇——FeMo-co[7Fe-9S-Mo-C-homocitrate]位于 α 亚基内,是活性中心,直接负责 N_2 结合与还原,而P-cluster[8Fe-7S]位于 α/β 亚基界面,作为电子传递中间体,负责接收来自Fe蛋白[4Fe-4S]簇的电子并将电子传递给FeMo-co。两者单独存在时不具备催化活性,只有两者结合形成复合体后,通过Mg-ATP驱动电子从Fe蛋白传递给MoFe蛋白的P-cluster,最终到达FeMo-co,才能完成固氮反应^[37,38]。尽管近几十年生物固氮领域取得了突破性进展,但对固氮酶催化核心机制的深入理解仍存在挑战,特别是在以下几个方面尚缺乏充分的实验证据: 催化过程中的详细电子/质子传递路径,底物(N_2)进入及产物(NH_3/H_2)离开FeMo-co活性中心的分子通道等,这源于其结构的极端复杂性、高度氧敏感性及严苛实验要求。深入解析这些机制,不

仅有助于阐明基本生化原理,也将为降低工业合成氨成本以及探索作物固氮策略提供重要的科学参考。

李季伦团队长期深耕固氮酶催化机制研究。早期即建立厌氧提取与纯化系统,为后续多种来源固氮酶的催化机制研究奠定了技术基础。20世纪80年代初,与美国Burris教授合作,证明了固氮酶催化的氢氘(hydrogen-deuterium molecule, HD)形成反应是普遍共性,且该反应是绝对依赖于 N_2 的,有力反驳了当时认为HD形成不依赖 N_2 的观点^[39]。基于对固氮酶还原氮气和质子关系的研究,结合氢爆发现象,提出了固氮酶催化质子还原的“双位点放氢模型”^[40]。为验证模型,分别以*K. oxytoca*和*A. vinelandii*为对象,证实第一条可能的电子/质子传递通路,即P-cluster $\rightarrow\alpha$ -Cys62 $\rightarrow\alpha$ -Gly61 $\rightarrow\alpha$ -Gln191 $\rightarrow\alpha$ -His195 $\rightarrow$ S2B (FeMoco)^[41,42]。通过细胞学和酶学的多项生化、物化性质分析,解析特定氨基酸在还原底物中的关键作用,获得第二条电子通路: P-cluster $\rightarrow\alpha$ -Cys61 $\rightarrow\alpha$ -Ala62 $\rightarrow\alpha$ -Tyr63 $\rightarrow\alpha$ -Ala64 $\rightarrow$ homocitrate (O1) $\rightarrow$ Mo^[43,44]。为理解底物还原分子机制提供关键实验证据,完善了模型。Siegbahn的量子化学模拟进一步佐证该模型,表明高柠檬酸在活性状态下构象变化形成局部“空穴”储存质子;质子化的高柠檬酸烷氧基接受电子驱动 H_2 释放^[45,46]。双位点模型统一解释了HD形成、 H_2 释放与 N_2 还原的偶联机制,但 N_2 进入FeMo-co的分子通道及电子传递时序细节仍需结构生物学验证,固氮酶氧敏感性及金属簇动态变化为原位观测带来挑战。

2.2 固氮基因的表达调控机理

环境中铵(NH_4^+)和氧(O_2)的浓度是调控生物固氮效率的关键因子: 高浓度铵和氧会强烈抑制*nif*基因的转录,进而阻碍固氮作用。但不同种类固氮菌的具体调控机制存在差异。环境铵浓度的变化直接影响特定转录调控因子与*nif*基因启动子的结合状态,从而调控其转录水平。此外,*nif*基因通常与氮代谢相关基因共同构成一个复杂的调控网络,协同决定固氮效率。谷氨酰胺合成酶(glutamine synthetase, GS),由*glnA*基因编码,是广泛存在于生物体内的氮同化核心酶。GS催化生成的产物——谷氨酰胺(glutamine, Gln)——作为细胞内氮水平的关键信号分子,在固氮及氮代谢的整体调控中扮演中心角色。

在巴西固氮螺菌中,团队解析其遗传特性,成功克隆并鉴定了*ntrBC*、*draTG*、*glnB*、*glnZ*、*nifA*等关键

调节基因,其基因组中缺乏典型的抑制子基因*nifL*,并首次阐明其PII蛋白与转录激活蛋白NifA之间的相互作用受到铵浓度的调控^[47-59]。深红螺菌中,固氮酶兼具固氮与光合产氢的双重关键功能,其表达受NifA的驱动,其活性则受DraT/DraG翻译后修饰系统的精细调节。环境铵浓度升高会双重抑制产氢过程:既通过抑制NifA活性阻碍固氮酶转录合成,又通过激活DraT促使已存在的固氮酶发生翻译后失活^[60,61]。以巴西固氮螺菌和深红螺菌为代表的革兰氏阴性菌调控机制具有共性(缺少抑制子NifL)。*nif*启动子均属于 σ^{54} 型,转录严格根据铵和氧浓度受转录激活蛋白NifA的调控。而*nifA*的转录又受一般氮代谢调控(nitrogen regulation, Ntr)系统的调节。Ntr系统由四种蛋白组成:*glnD*编码的尿苷酰转移酶(GlnD或称UTase)、*glnB*编码的GlnB(也称为P II 蛋白)以及双组分调节系统NtrB/NtrC。低铵时,GS则以非腺苷化状态存在,此时GS保持活性;GlnD催化GlnB尿苷酰化形成GlnB-UMP, GlnB-UMP通过NtrB催化NtrC磷酸化(NtrC-P), NtrC-P具有DNA结合特性,能激活*nifA*转录;高铵时, GlnE使GS腺苷化,被修饰的GS失去活性; GlnD催化GlnB脱尿苷酰化,非尿苷化的GlnB不能通过NtrB使NtrC发生磷酸化,非磷酸化的NtrC不能激活*nifA*及其他氮代谢基因的转录,则抑制固氮基因的转录。此外, *nifHDK*编码的固氮酶被翻译形成后,其酶活性还受DraT/DraG系统的调控。高铵下, DraT催化使铁蛋白可逆性失活;低铵下, DraG使铁蛋白恢复活性, PII、GlnB和GlnZ及GlnK参与调控此过程^[62-65](图2(a))。

尽管固氮类芽孢杆菌的认知与应用历史较长,其遗传转化难度曾长期制约固氮调控机理的研究进展。陈三凤团队的系统研究填补了这一空白。比较基因组和进化分析表明固氮类芽孢杆菌分为两个类群^[29]。以*P. polymyxa*为代表的类群中,9个固氮基因(*nifBHD-KENXhesAnifV*)紧密连锁成簇,受 σ^{70} 型启动子(含-35/-10区保守区及GlnR-binding site I和II)调控,仅在无铵或低铵条件下固氮。固氮基因表达受氮代谢全局调控因子GlnR和GS的调控^[65-69]。低铵时, GlnR与GlnR-binding site I 结合,激活*nif*基因转录;高铵时, GS催化合成Gln, Gln反馈抑制GS自身活性,并与GS形成反馈抑制型复合物(feedback inhibited glutamine synthetase, FBI-GS),该复合物促进GlnR与启动子区的GlnR-binding site II 结合,干扰RNA聚合酶与启动子的结合,从而抑制*nif*基因表达。此项工作首次阐明了GlnR在固氮类芽孢

杆菌中正负调控固氮基因表达的独特机制。以*P. sabiniae*为代表的固氮类芽孢杆菌,不仅在低铵(0~4 mmol/L)条件下固氮,更在高铵(30~300 mmol/L)环境中维持固氮酶活性。纯化的固氮酶在体外无论提取自低铵或高铵培养条件均具活性^[70],此为首次发现在如此高铵(30~300 mmol/L NH_4^+)条件下检测到固氮酶活性的直接证据。深入机制研究表明,高铵特异性诱导*ald*基因编码的丙氨酸脱氢酶(ADH)高表达:转录组分析显示*ald*基因在>30 mmol/L铵浓度下表达显著上调,酶活测定证实ADH催化活性提升,酶动力学分析进一步揭示ADH对底物铵离子的亲和性较低($K_m=39.78$ mmol/L, pH 7),表明其仅在超过此阈值的高铵环境中高效激活。基于此,研究揭示了高铵固氮的协同信号通路:高铵诱导ADH催化铵和丙酮酸合成大量丙氨酸;丙氨酸有效抑制GS活性,导致胞内Gln浓度降低。低浓度Gln无法与GS相互作用形成FBI-GS,因此在高铵环境下, GlnR仅能结合GlnR-binding site I 从而持续激活固氮基因表达,模拟了低铵信号通路。在中等铵浓度(4~30 mmol/L)范围,ADH作用不足以大幅降低Gln,经典的FBI-GS依赖抑制机制为主导(GlnR结合GlnR-binding site II 抑制表达);而在高铵(>30 mmol/L)条件下,ADH介导的信号通路被激活^[71](图2(b))。这些原创性发现首次系统阐明了固氮类芽孢杆菌的固氮调控网络,特别是揭示了克服“高铵抑制固氮”这一核心挑战的全新分子机制,为开发具有高铵耐受性的工程化固氮体系奠定了坚实的理论基础并指明了极具前景的优化策略。

为避免固氮酶遭受氧损伤,大多数固氮菌进化出各种生理策略:在厌氧环境中生长、通过呼吸链加速消耗氧、形成物理性氧气扩散屏障、或实现时空上的分隔。同时,固氮菌对氧气高度敏感,严格依据外部氧浓度变化调控*nif*基因的转录。团队解析了FixLJ/FixK信号通路在巴西固氮螺菌氧调控中的作用^[72]。FixL作为含血红素的氧传感蛋白,感知环境氧浓度:在微好氧条件下,FixL激活同组分的响应调节蛋白FixJ的转录激活功能;在好氧条件下,则抑制FixJ活性。被活化的FixJ直接调控下游FixK(一种CRP/FNR家族转录因子)的表达。FixK结合特定DNA序列,最终激活*nifA*的转录,从而开启下游*nif*基因表达。在固氮类芽孢杆菌*P. polymyxa*中,生物膜的形成能够创造低氧微环境以保护固氮作用:在不形成生物膜时,5%氧气即可强烈抑制固氮酶活性;而形成生物膜后,即使暴露于21%氧气下,仍可维持约50%的固氮酶活性^[73]。*P. polymyxa*拥有4个FNR家族蛋

到好氧固氮菌,并赋予其在高氧环境下维持固氮功能的能力^[32].联合固氮菌应对氧胁迫的策略具有共性:均通过生理屏障(如生物膜)或调控蛋白(如FixLJ、FNR)创造低氧微环境.特性上,革兰氏阴性菌(如*Azospirillum*)依赖FixLJ-FixK信号链,而革兰氏阳性菌(如*P. polymyxa*)利用FNR蛋白间接调控.这些发现为设计抗氧固氮体系提供了关键分子基础.

3 固氮酶系统的异源表达

固氮酶及其辅因子(FeMo-co)的合成是一个多基因协同的高度复杂过程.在固氮生物中,这些基因通常聚集成簇形成固氮基因簇(*nif* cluster)(图3(a)).不同固氮菌中*nif*基因簇在基因组成、数量及排列方式上存在显著差异,这可能反映了其对特定生态环境和宿主生理需求适应性演化的结果^[75].在革兰氏阴性固氮菌中,*nif*基因成一簇或几簇排列,基因数目相对较多(通常20个以上),例如*K. oxytoca*的约24 kb基因簇内包含20个*nif*基因(*nifJHDKTYENXUSVWZMFLABQ*),组成7个转录单元^[76],而*A. vinelandii*的*nif*基因(*nifHDKTYEN-*

XUSVZMFLABQ)、辅助因子组装相关基因(*nafY*、*iscA*)及其他未知功能基因(*orf*)则分布相对分散,形成两个主要的*nif*基因簇^[77]; *A. brasilense* *nif*相关基因分布更为分散,一个主要*nif*簇含有*nifHDKYEUSW*和*fixABCX*,而*nifA*、*nifB*分别散落在基因组其他位置^[78].系统基因组学比较揭示,几乎所有固氮生物都保守包含一个由6个基因(*nifHDKENB*)组成的最小功能单元^[79].基于*K. oxytoca*和*A. vinelandii*研究表明,完整的固氮酶的合成通常需要约16个*nif*基因(*nifHDKYTEN-XUSVZWMBQ*): *nifH*、*nifD*和*nifK*编码结构蛋白; *nifU*、*nifS* [Fe-S]簇组装)、*nifE*、*nifN* (FeMo-co骨架合成)、*nifX*、*nifB*、*nifQ* (FeMo-co合成前体加工)、*nifV* (高柠檬酸合成)、*nifY* (FeMo-co插入与MoFe蛋白成熟)基因参与FeMo-co的合成; *nifF*和*nifJ*基因负责电子传递, *nifZ*参与金属簇的合成, *nifM*则促进铁蛋白的正确折叠^[80].相较之下,革兰氏阳性固氮类芽孢杆菌的*nif*基因簇通常结构紧凑且基因数量较少,仅由9个关键基因(*nifBHDKENXhesAnifV*)构成.该基因簇被认为是进化过程中通过水平基因转移获得,与弗兰克氏菌(*Frankia*)

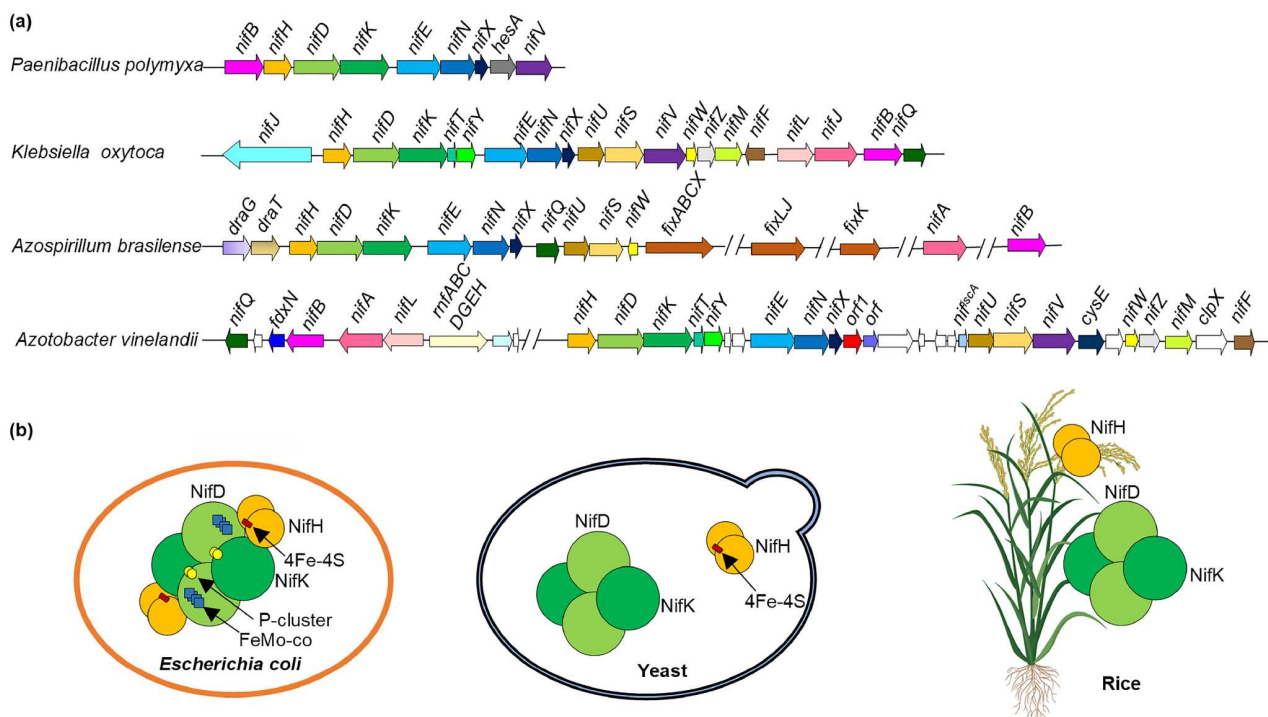


图3 联合固氮菌的固氮基因簇及其异源表达。(a)不同联合固氮菌中固氮基因簇的组成;(b)固氮酶关键组分NifH与NifDK在异源宿主(大肠杆菌、酵母、水稻)的合成进展.水稻元素源于BioGDP.com

Figure 3 Organization and heterologous expression of *nif* gene clusters. (a) Comparative organization of *nif* gene clusters in diverse associative diazotrophs. (b) Advances in heterologous assembly of core nitrogenase components (NifH and NifDK) in hosts (*Escherichia coli*, *Saccharomyces cerevisiae*, *Oryza sativa*). Rice elements sourced from BioGDP.com

的*nif*簇同源性最高,暗示它们可能源自共同的祖先^[29]。这种精简的遗传结构使其成为极具吸引力的合成生物学目标载体。

实现禾本科作物自主固氮的终极目标,需要克服一个根本挑战:生物固氮目前仅为原核生物(细菌和古菌)所固有的能力。为此,研究者们正致力于在大肠杆菌(*Escherichia coli*)、酵母(*Saccharomyces cerevisiae*)和烟草(*Nicotiana tabacum*)等模式生物系统中进行固氮酶系统异源表达的探索性研究^[81]。该领域的重要尝试始于1972年:利用接合转移方法,将*K. oxytoca*的20个固氮基因成功导入了*E. coli*中。然而,所得工程菌仅表现出较弱的固氮活性,且其表达与活性仍受到宿主环境中的铵及氧气的严格调控^[82]。后续研究推动了多种来源*nif*基因簇在大肠杆菌中的异源表达。最近一项突破性进展是构建了一种仅包含NifH和NifE蛋白的双组分截短固氮酶系统,首次在大肠杆菌中实现了N₂还原^[83]。在真核生物中的探索则面临更复杂的挑战。早期将*K. oxytoca*的*nif* cluster转化酵母,结果发现NifH能组装成同源二聚体,但NifD和NifK无法有效装配形成四聚体^[84,85]。由于固氮酶及其组装因子对氧的高度敏感性,线粒体因其内部低氧环境被视为理想的异源表达场所。近年来该方向取得了显著进展:在*S. cerevisiae*线粒体中共表达*A. vinelandii*的*nifH*和*nifM*基因可以合成有活性的铁蛋白,共表达*nifBHDKUSMEN*基因可以形成NifDK四聚体,共表达*nifU*、*nifS*、*fdxN*以及嗜热甲烷杆菌(*Methanocaldococcus infernus*)或嗜热自养甲烷杆菌(*Methanothermobacter thermautotrophicus*)的*nifB*基因实现了功能性NifB蛋白的合成^[86-89]。尽管在酵母中已取得了一定成果,在植物中实现功能性固氮酶的表达仍面临诸多障碍。光合作用产氧与固氮酶活性所需的厌氧环境存在根本冲突、固氮酶组装路径高度复杂、植物生长周期长且遗传操作相对困难等。在烟草中,尝试将*K. oxytoca*的16个核心*nif*基因(*nifBDEFHJK-MNQSUVXYZ*)定向表达在线粒体内。初步发现NifD亚基发生降解,但通过特定位点氨基酸替换成功解决了该问题,最终检测到正确组装大小的NifDK四聚体^[90,91]。水稻中,在T₀代(首代转化)植株的线粒体中共表达*Hydrogenobacter thermophilus*的*nifH*和*A. vinelandii*的*nifM*,合成了有活性的铁蛋白;共转化*A. vinelandii*的*fdxN*和*M. infernus*或*M. thermautotrophicus*的*nifB*基因,合成了有活性的NifB蛋白^[92,93]。

陈三凤团队在固氮酶系统的异源表达研究中建立

了系统性技术路线,实现了从原核生物到真核生物(酵母、植物)的渐进式突破(图3(b)):2013年,将来源于*P. polymyxa*仅含9个基因(*nifBHDKENVXhesA*)的最小固氮基因簇导入了*E. coli*中,使后者获得了固氮能力,而且在重组固氮*E. coli*中,*nif*基因的表达不受铵调控^[66,94],这改变了以前认为固氮至少需要16个基因的认识。进一步将*K. oxytoca*的*nifUS*和*nifFJ*基因导入该重组菌株,使其固氮酶活性由原来的10%提高到50.1%^[95]。2019年,将来自*P. polymyxa*的11个基因(*nifBHDKENVXhesA*和*groESgroEL*)和来自*K. oxytoca*的4个基因(*nifUSFJ*)导入酿酒酵母中,成功在酵母细胞内组装成有活性的Fe蛋白和大小正确的NifDK四聚体^[96]。2023年,采用2A肽自切割系统,将9个Nif蛋白(NifB、NifH、NifD、NifK、NifE、NifN、NifX、HesA和NifV)融合表达为两个多聚蛋白,在酵母细胞内共表达后,成功检测到具有活性的铁蛋白^[88],验证了这种多基因共表达策略的有效性^[97]。植物方面,在转基因棉花T₃代纯合株系中,稳定实现了*P. polymyxa*的NifB、NifH、NifD和NifK蛋白的共表达^[98]。通过多基因组装、单转化、共转化及杂交等技术,将来自*P. polymyxa*和*K. oxytoca*的13个固氮基因(*nifBHDKENXhesAnifV*、*nifSU*、*groESEL*,总长约40 kb)整合导入水稻1号染色体两个特定位点,获得转基因株系L8-17。重测序证实该基因簇在F₄-F₆代稳定遗传,Western blot检测显示NifDK四聚体正确组装(与*P. polymyxa*天然四聚体一致)。然而,该工程水稻株系未检测到体内外固氮酶活性,核心原因可能是水稻细胞质蛋白酶切割NifH蛋白导致其功能丧失,进而阻碍FeMo-co合成受阻——完整NifH是激活NifDK的必要条件。对此,团队已筛选获得抗降解的NifH突变体(T17C、T17V及S18A)^[99],并为所有固氮基因添加线粒体引导肽(MTP)以利用线粒体低氧高还原力环境。目前,携带NifH-T17V突变体与MTP融合固氮基因的工程水稻已进入纯合株系选育阶段,将通过体外酶活检测及¹⁵N₂同位素示踪验证固氮活性。此项研究在禾本科模式作物(水稻)中成功构建并稳定遗传了复杂固氮酶蛋白的核心结构模块,为实现作物“自主固氮”目标迈出了突破性一步。

当前,在禾本科作物实现功能性固氮酶面临三大核心障碍:光合作用产氧与固氮酶氧敏感性的根本矛盾、多基因协同表达及金属簇组装的复杂性以及植物蛋白酶对关键固氮蛋白的降解问题。针对植物细胞器(线粒体与叶绿体)可提供微厌氧环境及高还原力的特

性,团队正采用引导肽靶向策略(线粒体MTP/叶绿体转运肽),将固氮酶合成基因定向导入细胞器,旨在优化靶向效率并提升辅因子组装效率.同时,通过共享*nif*基因和其突变体库筛选可溶蛋白,并基于靶向效率的数据优化载体设计,以提升表达蛋白的稳定性与功能.此外,受天然存在的固氮内共生体——贝氏布拉藻(*Braarudosphaera bigelowii*)硝基体(nitroplast)模型的启发,团队也在积极探索体外重建和构建人工固氮细胞器的可能性.

4 联合固氮菌的抗病促生机制及应用

20世纪70年代,Döbereiner团队发现巴西固氮螺菌,这启动了联合固氮菌的应用研究.作为典型代表,该菌在田间接种试验中统计平均增产5%~30%^[100].然而,田间实践表明,单纯依赖其固氮作用提供的氮素贡献相对有限.这主要是由于高浓度铵态氮对固氮酶活性的强烈抑制作用.实现应用突破的关键在于解决氮肥施用与固氮活性的矛盾.相较于可通过减少氮肥投入以规避铵抑制、充分发挥根瘤菌固氮效能的豆科植物,与之联合固氮的高需氮禾本科作物(如水稻、小麦、玉米)通常需高氮肥投入.因此,选育具有“耐铵固氮”特性的菌株至关重要,在保证作物高产所需施氮的条件下,使固氮酶活性免受铵抑制干扰而持续发挥作用,从而实现显著节肥目标,这对农业绿色可持续发展具有重大意义.随着研究的深入,不仅通过基因编辑等技术致力于改造优化菌株,更重要的是系统揭示了联合固氮菌超越单纯固氮作用的多维促生机制.

固氮内生细菌因其在植物体内低氧环境生长而被认为具有较高固氮效率,对植物的生长、发育及健康起着重要作用.巴西固氮螺菌玉62作为兼性内生菌可进入根、茎、叶组织^[7-9],通过随机诱变,成功筛选到抗氨(耐铵)突变株.田间试验表明,无论野生型还是突变株,其作为追肥施用的促生效果均优于种肥^[6].值得注意的是,在肥沃土壤中增产幅度较小,暗示促生作用可能更多归因于其分泌的植物生长调节物质(而非主要依赖固氮供氮).分子机制研究表明,玉62菌株的促生效应是多维协同作用的结果:其通过核心调控因子NifA与PII蛋白的铵敏感性互作实现精准固氮调控,为耐铵工程菌构建奠定基础;高效趋化与根际定植则依赖*flbD*基因编码转录激活因子调控极生鞭毛(液体游动)和侧生鞭毛(固体表面爬行)实现灵活趋化运动,*cheQ*和*org35*基因编码的杂合蛋白协同调控趋化性,胞

外多糖(extracellular polysaccharide, EPS)合成、孢囊发育驱动菌体向植物根系分泌物定向迁移,增强根表吸附并形成抗干燥休眠体维持根际存活;此外,*fhuE*基因编码铁载体受体高效摄取环境铁元素,兼具抑制病原菌生长和为植物提供铁营养的功能;通过色氨酸依赖途径(*trpE*、*atrC*等基因)合成IAA,直接刺激植物根系发育^[7,101-107].因此,巴西固氮螺菌通过整合高效趋化定殖、精准固氮调控、铁载体介导的抗病与铁营养供给以及IAA驱动根系生长等多重机制,综合提升了植物养分吸收效率与抗逆性,实现了显著的联合促生效果.基于上述机制认知构建的耐铵固氮基因工程菌株,田间应用证明可显著减少玉米氮肥施用量达20%,大幅提升经济效益与环境效益,其核心技术现已实现产业化,有效推动科研成果转化.

固氮类芽孢杆菌可侵入植物体内并在根、茎、叶中稳定定殖,属于典型的内生固氮菌^[30,31].固氮菌的重要指标是它们是否可以向宿主植物提供固定的氮素.利用¹⁵N同位素稀释法证实,该类菌通过生物固氮可为非豆科宿主植物提供其所需氮素的12.9%~36.4%^[108,109].其固氮贡献受氮水平调控(低氮条件下贡献率显著高于高氮条件),且存在植物种属依赖性.相较于能分泌大量IAA的巴西固氮螺菌玉62菌株,部分固氮类芽孢杆菌(如*P. tritici* BJ-18)仅分泌少量IAA,暗示其IAA并非促生主导因子.该类菌的主要促生机制在于显著增强植物氮素吸收与同化能力:接种显著提高了小麦、玉米、黄瓜根部和茎部的NH₄⁺、NO₃⁻及总氮含量;同时上调硝酸盐转运蛋白(nitrate transporter, NRT)基因和GS基因的转录水平,增强GS活性及硝酸还原酶(nitrate reductase, NR)活性,最终导致植物干物质积累量提升20.3%~103.6%,且增幅在根部普遍高于茎部.然而,上述对氮吸收代谢的积极效应及最终的增产效果均会被高氮土壤环境显著抑制^[109].

接种固氮类芽孢杆菌能显著重塑宿主根际土壤及植物内生微生境(根内、茎内)的微生物群落结构与多样性,并改善土壤理化性质.以*P. tritici*为例,其接种可提高根际土壤pH、有机质含量、氮/磷含量及土壤固氮酶活性^[110-112].根际土的细菌种群相对丰度显著高于根内和地上部分,说明只有少数细菌进入植物体内,但接种如BJ-18仍能显著改变小麦根际土壤、根内和茎内细菌群落结构:促进有益菌属(如*Bacillus*、*Pseudomonas*、*Klebsiella*等)的富集,使*Paenibacillus*属成员成为根际土壤和根内优势固氮菌群,并显著降低植物

病原真菌的相对丰度,尤其是引起叶斑病的*Alternaria* (链格孢属)及引起茎基腐病的*Fusarium/Gibberella* (镰刀菌/赤霉菌属)。在玉米上的研究也观察到类似效应:接种显著提高了其根际土壤有益菌(如*Pseudomonas*、*Salinimicrobium* (盐微菌属)及*Bacillus*)和固氮相关菌属(*Klebsiella*、*Azotobacter*、*Azospirillum*、*Azoarous*、*Bradyrhizobium*)相对丰度,抑制致病镰刀菌属(*Fusarium*),同时使*Paenibacillus*在根际、根内及茎内成为优势属。跨地点(河北吴桥 vs. 北京上庄)和作物(小麦 vs. 玉米)的研究均证实接种可富集有益微生物,抑制土传病原真菌(特别是镰刀菌*Fusarium*),并影响原生物群落的组成——后者可能是驱动土壤微生物组成和功能的关键调控者,共同实现多重效益:增强植物对土传病害的抗性,富集的有益菌群通过其促生功能(如溶磷、分泌植物激素)协同提升植物养分获取与健康,提升农业生态系统功能与稳定性。此外,代表性菌株*P. polymyxa*不仅能固氮,还分泌以杀镰孢菌素(Fusaricidins)为主的脂肽类抗生素,有效抑制多种植物病原菌(如黄瓜枯萎病菌、番茄灰霉病菌、小麦纹枯病菌、小麦赤霉病菌等)。其核心抑菌功能依赖于*fus*基因簇,野生型菌株对黄瓜枯萎病的防治效果是其*fusA*突变株的6.4倍,该抗生素通过几乎完全阻断孢子萌发和菌丝生长并诱导孢子破裂来杀伤病原菌,其合成受Kin4833-Spo0A-AbrB信号级联调控(磷酸化Spo0A间接正调控*fus*而AbrB直接负调控),且在芽孢形成期活性最强。除直接抑菌外,Fusaricidin还能诱导植物根部水杨酸(salicylic acid, SA)积累并显著上调叶片病程相关(pathogenesis-related, PR)基因的转录,激活系统获得抗性(systemic acquired resistance, SAR),并通过抑制其他根际芽孢杆菌的生物膜形成能力增强自身定殖^[113~115]。固氮类芽孢杆菌还通过高效合成铁载体发挥多重作用:包括为植物供铁、与病原菌竞争铁资源、支持菌体自身固氮酶活性及IAA合成。从*P. tritici*中首次分离到细菌来源的fusarinines型嗜铁素(含新型化合物deshydroxylferri-triacetylusigen和paenibacillic acid A)及吡啶酸类促生物质^[116],而*P. polymyxa*中bacillibactin型铁载体合成基因簇(*entAdhbCEBF*)受铁响应因子Fur与过氧化氢胁迫因子PerR协同调控,其缺失会导致铁吸收障碍、生长延缓及固氮酶活性下降^[117]。

固氮类芽孢杆菌菌剂在大田应用中显著提升玉米和小麦的产量与农艺性状。为实现生物物质高产,其发酵培养基已优化^[118]。田间试验表明,接种特定菌株(如

BJ-18、1-33、1-43、1-49)可使小麦增产5.1%~26.9%,其中BJ-18效果最为突出(26.9%)^[119];接种*P. polymyxa*对小麦赤霉病的防治效果达64.8%,并显著提升亩产24.1%^[25]。连续3年的玉米随机区组试验中显示,单菌剂(BJ-18)增产率为5.04%~16.05%,复合菌剂(BJ-18与芽孢杆菌L-56)增产效果更显著,达11.74%~18.50%^[119]。河北吴桥4年定位试验证明,在减氮15%条件下施用菌剂可使小麦产量实现稳定增产(增幅7.64%~11.6%)^[110]。山西省韩村基地小麦-玉米轮作区的大田试验进一步验证了其效果:两种固氮菌剂显著提高小麦拔节期/孕穗期株高、鲜重及最终产量(最高6211.24 kg/hm²,增产16.23%);减氮15%条件下,菌剂处理产量(6056.27 kg/hm²)显著高于减氮对照(增产23.20%)和正常施肥对照(增产13.84%)。此外,两种固氮菌剂都能显著促进玉米株高、叶片生长及鲜重,提升穗部性状(鲜/干重、穗长宽、百粒重);产量分别达8566.27与8602.70 kg/hm²,较减氮对照增产18.58%和19.09%,较正常施肥对照增产9.30%和9.78%^[120]。这些广泛的田间数据充分证明,固氮类芽孢杆菌菌剂,尤其是复合菌剂形式,配合减氮施肥策略,能够稳定且显著地提升作物产量与关键农艺性状,为该技术的大规模推广应用提供了坚实支撑。目前,相关固氮类芽孢杆菌技术已成功转化。

综上所述,联合固氮菌通过生物固氮直接供给氮素、合成抗菌物质抑制病原体、调控根际及植物内生微生物群落结构促进有益菌群,以及增强宿主植物对氮素的吸收与同化能力等多重机制的协同作用(图4),有效提升作物的抗病性与产量。其核心优势——在减少氮肥施用的条件下仍能实现显著的“减氮增效”效果,为农业生产的绿色可持续发展提供了关键技术支撑。

四十年间,以中国农业大学生物学院微生物系李季伦教授、陈三凤教授领衔的研究团队始终深耕联合固氮领域研究。在固氮资源库建设、调控机制解析、固氮合成生物学及应用转化方面取得了系统性与开创性成果:构建了重要的固氮菌和固氮基因资源库,为全球研究提供基础支撑;率先阐明革兰氏阳性固氮菌的调控机制,填补该领域研究空白;在固氮合成生物学领域取得国际领先突破;贯通固氮菌“基础研究-调控机制-应用开发”全链条。这些成果显著提升了我国联合固氮研究的国际地位,并为发展可持续农业与绿色生物制造奠定了坚实科学基础。立足过去四十年的深厚积淀,团队将继续聚焦核心方向:发掘新型固氮资源、

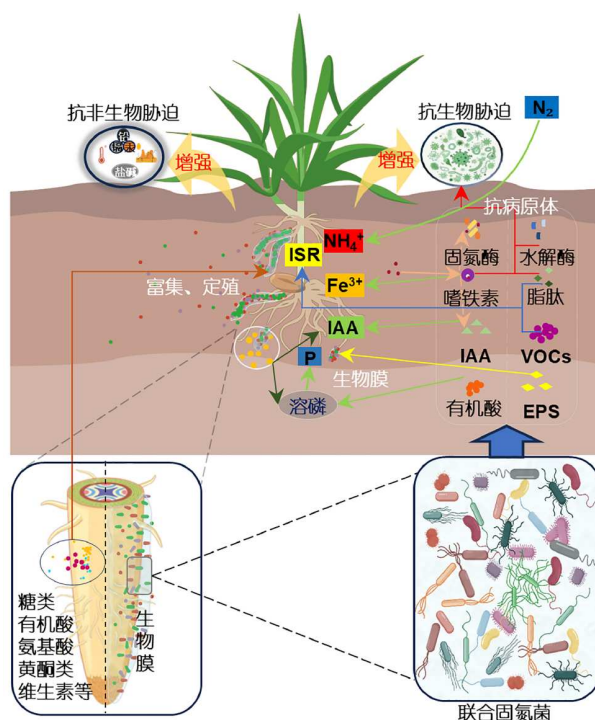


图 4 联合固氮菌的抗病促生机制。植物根系分泌物(如糖类、有机酸、氨基酸)驱动联合固氮菌(如固氮螺菌、假单胞菌、克雷伯氏菌、固氮类芽孢杆菌)在根际富集定殖。促生机制包括: 固氮酶催化 N_2 还原为 NH_4^+ 为植物提供氮素; 分泌嗜铁素螯合铁促进固氮酶/IAA合成并向宿主供铁; 分泌有机酸溶解磷酸盐提高磷有效性。抗病机制涉及: 释放挥发性有机物(volatile organic compound, VOCs)及脂肽诱导植物系统抗性(inducing systemic resistance, ISR); 利用脂肽及水解酶直接抑制病原体, 合成胞外多糖形成保护性生物膜。此外, 固氮菌定殖提升有益菌相对丰度及功能(如溶磷、产IAA), 并增强植物抗生物/非生物胁迫能力。根尖元素取自MedPeer.cn, 其他制图元素源于BioGDP.com

Figure 4 Cooperative mechanisms of growth promotion and disease suppression by associative diazotrophs in cereal crops. Root exudates (e.g., sugars, organic acids, amino acids) drive rhizosphere colonization of associative diazotrophs (e.g., *Azospirillum*, *Pseudomonas*, *Klebsiella*, *Paenibacillus*). Cooperative mechanisms enhance crop growth through nitrogenase-mediated N_2 reduction to NH_4^+ , siderophore secretion for iron chelation (facilitating iron-siderophore complex uptake to boost nitrogenase/IAA synthesis and host iron nutrition), and phosphate solubilization via organic acid secretion. Disease suppression involves direct pathogen inhibition by lipopeptides (e.g., fusaricidin) and cell wall hydrolases, induction of systemic resistance (ISR) through SA signaling pathways activated by volatile organic compounds (VOCs) and lipopeptides, and protective biofilm formation via EPS matrices. Furthermore, diazotroph colonization restructures the rhizosphere microbiome by enriching indigenous beneficial microbiota (e.g., phosphate-solubilizing and IAA-producing bacteria), thereby synergistically enhancing broad-spectrum resilience to biotic stresses and abiotic stresses. Root element sourced from MedPeer.cn; other graphical elements from sourced BioGDP.com

构建高效合成群落; 深入解析固氮菌-植物互作信号通路, 提升固氮菌田间定殖效率与适应性; 开发高效真核表达系统, 力争突破非豆科植物固氮的关键瓶颈。团队

的核心目标是加速高效固氮生物肥料应用进程, 大幅降低农业生产对化学氮肥的依赖, 服务国家粮食安全与生态安全战略。

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图S1 固氮类芽孢杆菌的抗病促生特性

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Summary for “联合固氮40年: 从发现到应用”

40 Years of associative nitrogen fixation: from discovery to applications

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Biological nitrogen fixation (BNF), exclusively performed by prokaryotes, catalyzes the reduction of atmospheric dinitrogen to plant-available ammonium—a cornerstone process for sustainable agriculture by reducing synthetic fertilizer dependence, enhancing global food security, and mitigating environmental degradation. While rhizobia-legume symbiosis represents the most efficient BNF system, staple cereal crops (e.g., rice, wheat, maize) lack such symbiotic capabilities. Alternatively, associative diazotrophs colonize the rhizoplane, vascular tissues, and endophytic compartments of cereals and horticultural crops, deploying multidimensional plant-beneficial mechanisms: through nitrogen fixation, phytohormone secretion, antimicrobial compound synthesis, and enhanced host stress tolerance. To harness this potential, the Department of Microbiology at China Agricultural University has established a comprehensive “resource-to-application” research framework over four decades, integrating strain discovery, mechanistic dissection, synthetic biology engineering, and field deployment.

Systematic exploration has yielded transformative outcomes: isolation of >2,000 diazotrophic strains established the world’s largest germplasm repository, with 15 novel nitrogen-fixing species formally described. Mechanistic studies revealed an alanine dehydrogenase (ADH)-mediated coordination pathway in *Paenibacillus* that overcomes ammonium inhibition—a persistent bottleneck in non-legume BNF—whereby high ammonium induces ADH to deplete glutamine, mimicking low-nitrogen signaling to activate *nif* genes. In synthetic biology, co-expression of 15 *nif* genes in *Saccharomyces cerevisiae* achieved assembly of functional NifH and correctly structured NifDK tetramers (~240 kD), while chromosomal integration of 13 *nif* genes in rice (*Oryza sativa*) enabled stable NifDK assembly and protease-resistant NifH mutants (T17C, T17V and S18A). Field applications of developed inoculants significantly increased crop yields under 15% nitrogen reduction—wheat and maize by 7.64%–23.2%—through synergistic nitrogen provision, iron chelation via siderophores, and systemic resistance induction.

These advances establish a dual theoretical-technical foundation for engineering biological nitrogen fixation in cereals. The constructed innovation chain encompasses foundational resources (globally unique strain library), pioneering mechanisms (ADH-mediated nitrogen-metabolism coordination), transformative technologies (heterologous nitrogenase assembly in eukaryotes), and scalable field solutions (proprietary biofertilizers). This end-to-end progress positions China at the forefront of agricultural microbiology, with a fully integrated intellectual property portfolio spanning microbial resources, genetic toolkits, and sustainable farming practices—significantly accelerating the transition toward green agriculture and fortified food systems.

associative nitrogen fixation, resource exploration, regulatory mechanism, heterologous expression, inoculant application

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