果 树 学 报 2018,35(5):602-609
Journal of Fruit Science

DOI:10.13925/j.cnki.gsxb.20170386

杧果炭疽病菌谷氨酸转运蛋白基因*Cgglt1*功能分析

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摘 要:【目的】探明谷氨酸转运蛋白基因*Cgglt1*与杧果炭疽病菌(*Colletotrichum gloeosporioides*)致病力的关系,以便为该病害发生机制的研究提供理论依据。**【方法】**利用In-Fusion® HD Cloning Kit技术构建*Cgglt1*敲除载体,转化杧果炭疽病菌原生质体,对获得的敲除突变体进行表型分析。**【结果】**获得敲除载体pCgglt1GH1和敲除突变体△*Cgglt1*,表型测定显示,与野生型相比,突变体△*Cgglt1*菌落颜色变浅、生长速率略下降、产孢量显著下降、孢子萌发加快但不能形成附着胞,对pH的适应性有所改变,对杧果叶片的致病力显著下降。**【结论】***Cgglt1*基因调控*C. gloeosporioide*菌落生长、产孢量、孢子萌发率、附着胞的形成、对pH的敏感性及对杧果叶片的致病力。

关键词:杧果;胶孢炭疽菌;谷氨酸转运蛋白基因*Cgglt1*;敲除;表型

中图分类号:S667.7 **文献标志码:**A **文章编号:**1009-9980(2018)05-0602-08

Functional analysis of the glutamate transporter gene *Cgglt1* from *Colletotrichum gloeosporioides* on the mango

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Abstract:【Objective】*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc is the main causal agent of anthracnose in mangos (*Mangifera indica*), which is very prevalent in the field and postharvest storage stage, and has great economic impact on the mango industry worldwide. Mango anthracnose is one of the main diseases in all mango planting countries, usually causing mango leaf spot, flower wilt, fruit soft rot, and directly decreases its fruit yield and commercial value. China is one of the main growing areas of mango; in 2014, the cultivated areas reached 17 320 hm^2, and the total yield reached 143.77 million ton, which was ranked eighth in the world. A large amount of fungicides are used to control mango anthracnose every year, but it still causes significant economic losses. So it is necessary to develop more effective control measures. Studies of the infection mechanism of *C. gloeosporioides* on mango can help to improve our knowledge and develop some new control targets. It has been reported that *C. gloeosporioides* could alkalinize the host tissue during disease development. The pH modulation is not a host-mediated effect, but rather it is pathogen dependent. Host tissue alkalinization results from active secretion of ammonia which is induced by relatively low pH values. The *glt1* is a nitrogen metabolism related gene, which regulates the synthesis and metabolism of ammonia. In our previous work, a glutamate transporter gene, named *Cgglt1*, was cloned from *C. gloeosporioides* isolated from diseased mango leaves. In order to identify the role of the glutamate transporter gene *Cgglt1* in the infection process,

收稿日期:2017-11-01 接受日期:2018-03-29

基金项目:国家自然科学基金(31460455);海南省重大科技专项(ZDKJ2017003);海南省高等学校教育教学改革研究项目(Hnjg2016ZD-2)

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particularly, to reveal the relationship between virulence and the gene, the *Cgglt1* is disrupted and the phenotypes of the mutant are analyzed in the present study.【Methods】Resulting 5’ and 3’ *Cgglt1* fragments and *gfp-hygB* gene are inserted into pUC19 via T-cloning site using an In-Fusion ®HD Cloning Kit (Clontech, Mountain View, CA, USA), and then transferred into *Escherichia coli* DH5α. So a knockout vector is constructed by replacing the *Cgglt1* coding region with the *hygB* gene and *sGFP* gene from pGH33. The positive clones are sequenced and the *Cgglt1* gene replacement vector with the right sequence is stored and named pCgglt1GH1, in which the *gfp-hygB* gene is flanked by *Cgglt1* fragments. The protoplasts of *C. gloeosporioides* are prepared using the lysozyme degrading method. The knockout fragment GLTGH amplified from pCgglt1GH1is transformed into *C. gloeosporioides* protoplasts using the PEG4000-mediated method. Hygromycin-resistant transformants are screened and purified on hygromycin-containing SR plates and confirmed by applying PCR methods. Finally, by performing a series of comparative phenotype analysis, including culture morphology, mycelial growth, conidia production, germination and appressorium formation, suitable pH value ranges and pathogenicity on mango leaves, are performed between the obtained knockout mutant △*Cgglt1* and its wild-type.【Results】The *Cgglt1* gene knockout vector pCgglt1GH1was successfully constructed. The mutant △*Cgglt1* was obtained and confirmed by transforming the knockout fragment glt1GH into the *C. gloeosporioides* protoplasts and PCR detection. Phenotypic analysis showed that the colony of mutant △*Cgglt1* became light in color and was always white, and grew slower in terms of the radial growth rate than with the blackish-brown wild-type. The mutant △*Cgglt1* failed to produce conidia on the PDA, even after a prolonged incubation of 12 days, and only produced 10^3 spores per mL in PD broth or seldom generated spores when inoculated on detached wounded leaves with mycelial plugs. The conidial germination ratio of mutant △*Cgglt1* significantly accelerated in the presence of free water but could not form the appressoria on moist glass slides. The wild-type produced a large number of conidia by macroscopic observation in PD broth and on detached wounded leaves. The ends of the wild-type germ tubes swelled and formed melanized appressoria after incubation for 4 h. This indicated that the mutants lose their ability to form infection structure appressorium. Mycelial adaptability of the mutant to ambient pH changes in terms of radial growth rates and the most suitable pH value ranges changs from 7-9 for wild types to 6-8 for the mutants, indicating that mycelia growth prefers relative low pH. When slightly wounded leaves were inoculated with wild-type mycelial plugs, the lesions were visible 2 days after inoculation, and became typical anthracnose spots with an average lesion diameter around 21.7 mm (on bronze-colored tender leaves) and 21.0 mm (on light green leaves) 5 days after inoculation. However, △*Cgglt1* caused smaller necrotic spots than the wild-type on slightly wounded leaves, with necrotic spot size of 18.8 mm (on bronze-colored tender leaves) and 18.7 mm (on light green leaves) 5 days after inoculation. When unwounded leaves were inoculated with wild-type mycelial plugs, the lesions were visible 2 days after inoculation, then enlarged to form typical anthracnose necrotic spots of 19.7 mm (on bronze-colored tender leaves) and 17.9 mm (on light green leaves) 5 days after inoculation. In contrast, no lesions emerged on unwounded leaves inoculated with △*Cgglt1* mycelial plugs or control agar plugs 5 days after inoculation. This suggests that the knockout of the *Cgglt1* gene results in the loss of the ability to directly penetrate into the mango leaf epidermal surface as well as reduce its expansion efficiency in leaf tissue.【Conclusion】The glutamate transporter gene *Cgglt1* of *C. gloeosporioides* on mangos was involved in colony pigmentation and mycelial growth, conidia production, conidia germination rate, appressorial formation, adaptability to relative alkalic pH, and virulence to mango leaves. The glutamate transporter gene *glt* has been cloned in several other pathogenic fungi, but its function was only

identified in *C. gloeosporioides* on avocados. The knockout of the *glt1* gene resulted in only significant reductions in appressorium formation and pathogenicity on avocados. This indicates that the functions of *glt1* genes are significantly different in *C. gloeosporioides* from different hosts.

Key words: Mango; *Colletotrichum gloeosporioides*; Glutamate transporter gene *Cgglt1*; Gene knockout; Phenotype

胶孢炭疽菌[*Colletotrichum gloeosporioides*（Penz.）Penz. & Sacc.]是杧果炭疽病的主要病原菌，侵染寄主后会造成落叶、果实腐烂等症状，直接影响其产量和商品价值，损失率超过40%[1]。炭疽菌在侵染寄主的过程中，会形成一系列专门的侵染结构，如芽管、附着胞、侵染钉、初生菌丝等[2]。芽管顶端膨大形成黑色素化的附着胞并产生侵染钉直接侵入寄主是炭疽菌的主要侵染方式[3-4]。*C. gloeosporioides* 是一种碱化病原真菌，在侵染油梨、番茄等果实时会产生氨，促使受侵染的寄主组织pH值逐渐升高，增强*C. gloeosporioides*其他毒力因子的表达和分泌，加速腐烂症状的出现[5]。氨的合成和代谢过程中涉及到与氮代谢相关的一系列基因，如谷氨酸脱氢酶基因*GDH2*（NAD^+-specific glutamate dehydrogenase）、氨转运蛋白基因*AMET*（ammonia transporter）、氨透性酶基因*MEPB*（ammonia permeases）、谷氨酸转运蛋白基因*GLT*（glutamate transporter）和谷氨酰氨合成酶基因*GS1*（glutamine synthetase）等，在果实组织碱化前的早期阶段（从pH=4.0到pH=4.5），*GDH2*和*AMET*的表达有助于氨的产生和分泌，果实组织pH从6上升到7的过程中，*MEPB*、*GLT*和*GS1*表达被激活上调，菌丝中氨的浓度增加[6-7]。进一步研究表明，*GLT*对碱化病原真菌致病力的影响主要有2个方面，一是参与附着胞的形成，二是通过参与氨的产生来调控果实pH。笔者实验室前期从杧果炭疽病菌（*C. gloeosporioides*）中克隆获得1个谷氨酸转运蛋白基因*Cgglt1*，全长为3 114 bp，其中包括2 162 bp ATG-TGA区域、482 bp的5′端上游序列、464 bp的3′端下游序列，且含有GLT典型序列的3个保守结构域（钠结合位点，钾结合位点和谷氨酸羧酸盐结合位点）。为了明确*Cgglt1*在*C. gloeosporioides*-杧果病害系统中的作用，笔者构建了其敲除载体，分析了敲除突变体的表型，以便更好地了解*C. gloeosporioides*与杧果之间的互作机制，为寻找新的防治靶标打下基础。

1 材料和方法

1.1 供试培养基

Czapek培养基（查彼培养基）、PDA培养基和PD培养液配制参照方中达《植病研究方法》[8]，SR固体培养基配制参照翟李刚[9]的方法。

1.2 供试病原菌和载体

杧果炭疽病菌胶孢炭疽菌[*C. gloeosporioides*（Penz.）Penz.& Sacc.]A2菌株。含绿色荧光蛋白基因（*gfp*）和潮霉素抗性基因（*hygB*）的载体pGH33。

1.3 引物

本研究中所有引物信息见表1。

表1 本试验所用引物

Table 1 Primes used in this study

引物名称 Prime name	序列 Sequence (5’−3’)	退火温度 Annealing temperature/℃	长度 Length/bp	用途 Application
glt1GH-F1 glt1GH-R1	TCGTTATTTACACGGATCGCCTCA CCCTGCCTCTTTCTCGGTCTC	58.0	2 588	扩增敲除片段 For amplifying the knockout fragment
GLTG-F2 GLTG-R2	CATCACCCTTGAGCGTTTC CGTTGTGGCTGTTGTAGTTGT	56.5	1 065	检测上游片段至*gfp*基因 For detecting the upstream fragment of *Cgglt* and the *gfp* gene
HGLT-F2 HGLT-R2	ATCACGCCATGTAGTGTATTGA CTGGATTGTCGCATGTATCTT	54.5	1 250	检测*hygB*基因至下游片段 For detecting the *hygB* gene and the downstream fragment of *Cgglt*

1.4 *Cgglt1*敲除载体构建

参照《分子克隆实验指南》[10]，分别扩增*Cgglt1*基因上下游片段及*gfp*::*hygB*基因片段并回收。按照In-Fusion HD® Cloning Kit说明书操作，将各片段

按顺序连接后插入载体pUC19 T-Cloning位点，转入*Escherichia coli* DH5α，筛选出阳性克隆寄往北京六合华大基因科技股份有限公司测序，保存序列正确的质粒，命名为pCgglt1GH1。

1.5 *Cgglt1*基因敲除及验证

以构建好的敲除载体pCgglt1GH1为模板，用引物glt1GH-F1/R1扩增敲除片段，参照韦运谢[11]的方法转化*C. gloeosporioides*原生质体后，于含潮霉素的SR平板上筛选，并用glt1GH-F1/R1对获得的转化子进行PCR初步验证，再用特异性引物GLTG-F2/R2、HGLT-F2/R2进行PCR交叉验证，以验证敲除位置是否正确和是否被彻底敲除。

1.6 表型测定

1.6.1 菌落形态及生长速率测定　参照翟李刚[9]的方法，分别接种突变体和野生型菌饼于PDA上，拍照并采取十字交叉法测量菌落直径。每处理3次重复。

1.6.2 产孢量、孢子萌发率及附着胞形成率测定　参照韦运谢[11]的方法，将突变体和野生型菌饼分别接种在PDA、PD及‘台农杧’（*Mangifera indica* ‘Tainong mango’）叶片上培养，每天测定其产孢量。

参照翟李刚[9]的方法，接种菌饼于PD培养液，8 d后过滤制成孢子悬浮液并调整孢子浓度，悬滴法观测孢子萌发率；涂抹于无菌载玻片上保湿培养，观测附着胞形成率（OLYMPUS BX51正立显微镜）。每处理3次重复。

1.6.3 pH敏感性测定　参照张春霞等[12]的方法，以灭菌的0.1 mol·L^{-1} HCl和0.1 mol·L^{-1} NaOH调节无菌Czapek培养基pH至3~11，接种培养6 d后测量菌落直径。共9个处理，每个处理3次重复。

1.6.4 致病力测定　参照Cai等[13]的方法，以刺伤和未刺伤2种方式，分别将菌饼接种于‘台农杧’古铜期和淡绿期叶片。共6个处理，每处理重复10枚叶片。对照接种无菌琼脂饼。

2 结果与分析

2.1 敲除突变体△*Cgglt1*的获得

用引物glt1GH-F1/R1扩增一段大小2 588 bp的片段，转化原生质体并纯化，用引物glt1GH-F1/R1检测，从突变体△*Cgglt1*和野生型A2中分别扩增出了约2 588 bp和2 253 bp的单一亮带，说明*Cgglt1*基因中间1 801 bp的序列被2 136 bp的*gfp*::*hyg B*替代，即转化子较野生型多了约335 bp序列。再用引物GLTG-F2/R2和HGLT-F2/R2分别进行扩增，从突变体中分别扩增出约1 000 bp以及1 300 bp的单一亮带，而在野生型A2中并未扩增出任何条带，说明敲除位置是正确的。用GLTG-F2和HGLT-R2引物扩增，在野生型A2中扩增出约2 800 bp的条带，在突变体中只扩增出约3 200 bp的单一亮带（没有2 800 bp的条带），说明*Cgglt1*被彻底敲除。

2.2 表型测定

2.2.1 菌落形态及生长速率测定　野生型A2菌落初期为白色，随着培养时间的延长，菌落背面颜色变为黑褐色，而突变体△*Cgglt1*菌落始终为白色，且气生菌丝不及野生型旺盛（图1）。可见*Cgglt1*的敲除抑制了菌落色素沉积和气生菌丝生长。

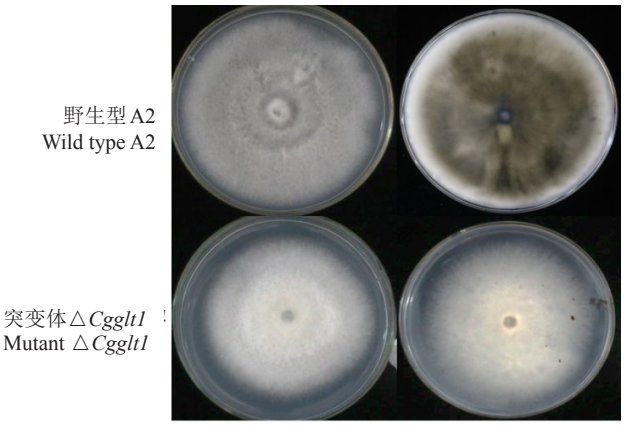

图1　野生型A2和突变体△*Cgglt1*菌落形态比较

Fig. 1 Comparison of colony morphology between the wild-type A2 and its mutant △*Cgglt1*

与野生型A2相比，突变体△*Cgglt1*菌落生长速率有所下降（图2），且在α=0.05水平上差异显著，说

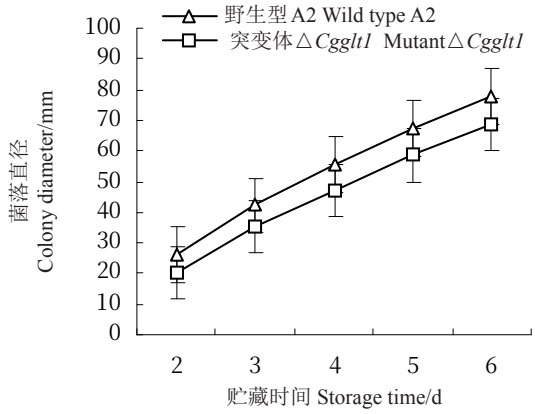

图2　野生型A2和突变体△*Cgglt1*菌丝生长速率比较

Fig. 2 Mycelium growth rates of the wild-type A2 and mutant △*Cgglt1*

明*Cgglt1*基因敲除显著降低了杧果炭疽病菌的营养生长速率。

2.2.2 突变体△*Cgglt1*产孢量、萌发率及附着胞形成的观测 显微检查产孢量发现，突变体△*Cgglt1*在PDA上培养12 d仍未产生分生孢子，而野生型培养6~7 d即有少量分生孢子产生。在PD培养液中，△*Cgglt1*培养7 d时偶见分生孢子，10 d时分生孢子产量约为每mL 10^3个；而野生型在培养5 d后即可产生大量分生孢子，在瓶壁上有肉眼可见的橘黄色分生孢子堆。在刺伤的叶片上诱导产孢，4 d后接种野生型的病部可见分生孢子堆，而突变体△*Cgglt1*接种7 d后，制片镜检可观察到个别分生孢子。可见*Cgglt1*的敲除严重降低了杧果炭疽病菌的产孢量。

由图3和图4可以看出，突变体△*Cgglt1*分生孢子在自由水中培育2 h即开始萌发，6 h时萌发率已接近饱和，但未见附着胞的形成；野生型A2培育2 h开始萌发，12 h才基本达到饱和，附着胞从4 h开始形成，到12 h时形成率为75%左右，可见*Cgglt1*敲除促进了杧果炭疽病菌分生孢子萌发，但却完全抑制了附着胞的形成。

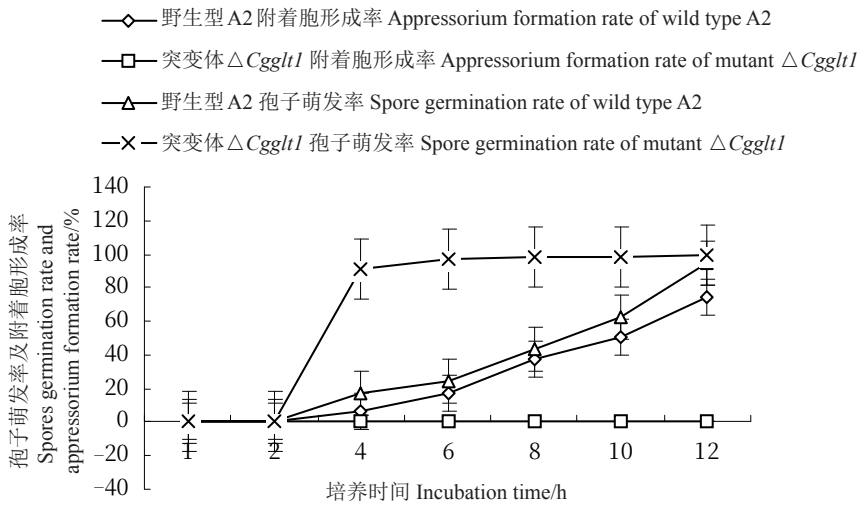

图3 野生型A2与突变体△*Cgglt1*孢子萌发率和附着胞形成率

Fig. 3 Germination and appressorium formations of the wild-type A2 and mutant △*Cgglt1*

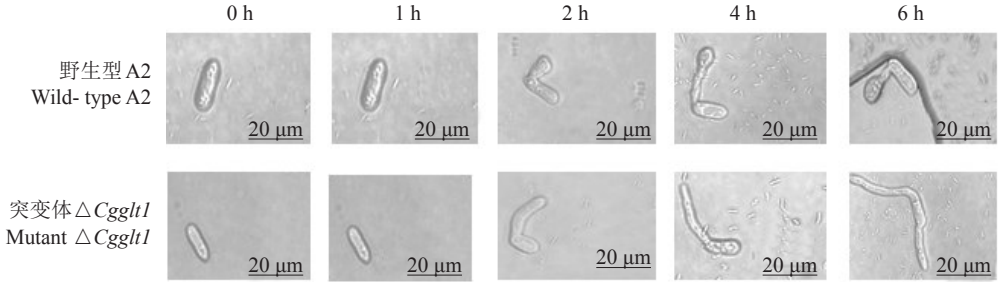

图4 野生型A2和突变体△*Cgglt1*分生孢子萌发及附着胞观察

Fig. 4 Observation of conidia germination and appressorium of the wild-type A2 and mutant △*Cgglt1*

2.2.3 pH敏感性测定 由表2可知，在pH 3~11范围内，野生型和突变体均能生长。野生型A2的最适生长pH范围为7~9，pH为8时生长最快；突变体△*Cgglt1*的最适pH为6~8，pH为7时生长最快。说明相对野生型，*Cgglt1*敲除突变体更加适应偏酸至中性环境。

2.2.4 致病力测定 (1)刺伤接种叶片。从图5看出，用菌饼刺伤接种‘台农杧’古铜期叶片，野生菌株A2 2 d后在接种处即可观察到褐色坏死斑，3 d后病斑平均直径为5.6 mm，4 d后坏死斑明显扩展，直径为11.4 mm，5 d后病斑扩展到主脉，在病斑上可见橘红色分生孢子堆及稀疏的菌丝，病斑直径为21.7 mm。用突变体△*Cgglt1*接种3 d和4 d后，病斑平均直径分别为4.7 mm和10.8 mm，略小于野生型，5 d后病斑直径为18.8 mm，明显小于野生型，并在$\alpha=0.05$水平上达到显著差异。同样，用菌饼接种刺伤淡绿期叶片后出现类似的结果，用野生菌株A2接种3、4、5 d后，病斑直径分别为4.0、11.3和21.0 mm；而

表2　野生型A2和突变体△*Cgglt1*菌丝对pH的敏感性测定

Table 2　The sensitivity test of pH for vegetative growth of the wild-type A2 and mutant △*Cgglt1*

菌株 Strains	不同pH下菌落直径The colony diameter at different pH/mm								
	3	4	5	6	7	8	9	10	11
野生型A2 Wild- type A2	28.33 fD	51.17 eC	60.27 deBC	64.33 cdBC	73.33 abA	75.00 aA	68.17 bcAB	62.83 cdBC	60.50 deBC
突变体△*Cgglt1* Mutant △*Cgglt1*	38.33 eDE	56.00 bcdABCD	60.67 bcABC	68.33 abAB	77.03 aA	64.00 abcAB	49.00 cdeBCD	41.33 deCDE	22.00 fE

注：不同小写字母表示差异显著（$p < 0.05$），不同大写字母表示差异极显著（$p < 0.01$）。

Note: Different small letters indicate significant difference at $p < 0.05$, different capital letters indicate extremely significant difference at $p < 0.01$.

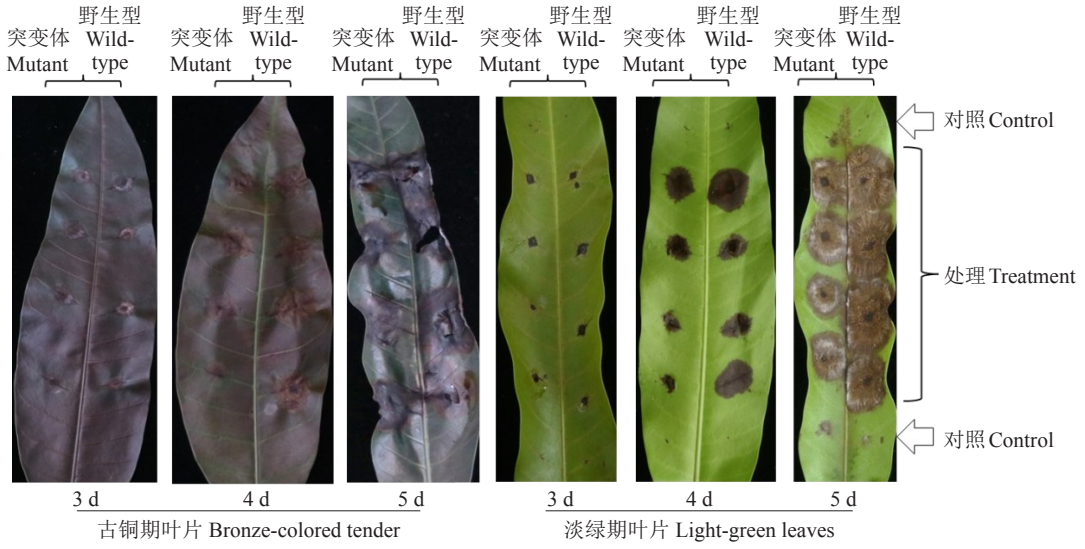

图5　野生型A2和突变体△*Cgglt1*对刺伤古铜期和淡绿期叶片的致病力

Fig. 5　Test of the virulence of the wild-type A2 and its mutant △*Cgglt1* on wounded bronze-colored tender leaves and light-green leaves

用突变体△*Cgglt1*接种3、4、5 d后，病斑直径分别为3.8、10.0和18.7 mm。说明*Cgglt1*基因的敲除降低了杧果炭疽病菌伤口接种的侵染力，即伤口侵入后的定殖扩展能力。

（2）接种未刺伤叶片。由图6可知，用菌饼接种未刺伤的‘台农杧’古铜期叶片，野生菌株A2 2 d后

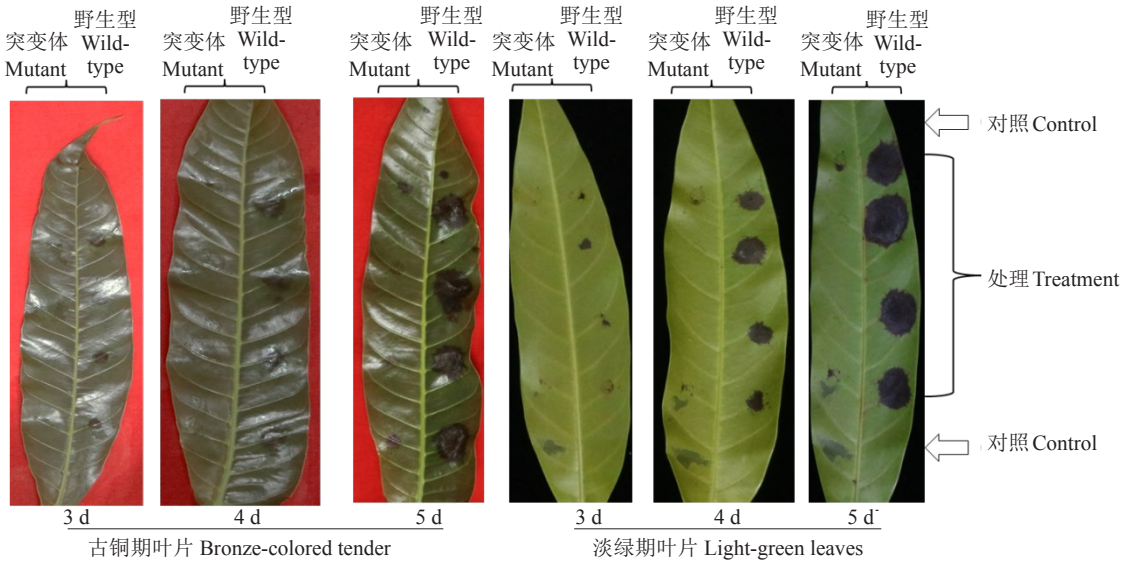

图6　野生型A2和突变体△*Cgglt1*对未刺伤古铜期和淡绿期叶片的致病力

Fig. 6　Test of the virulence of the wild-type A2 and its mutant △*Cgglt1* on unwounded bronze-colored tender leaves and light-green leaves

在接种处出现褐色坏死斑点，3 d后病斑平均直径为5.05 mm，4 d后坏死斑明显扩展，直径为10.4 mm，5 d后病斑上可见到少量稀疏的菌丝，病斑直径为19.7 mm。接种淡绿期叶片后3、4、5 d后，病斑直径分别为3.0、9.2和17.9 mm；而用突变体△*Cgglt1*接种5 d后仍未观察到任何症状。说明*Cgglt1*突变体在寄主无伤口的情况下基本丧失了侵染能力。

3 讨 论

谷氨酸转运蛋白基因*GLT*广泛存在于人、老鼠、家蚕、真菌及线虫中，但仅在医学领域研究取得了一定的进展，并发现它与人的癫痫、神经系统疾病等有关。在真菌中，仅在几种病原菌中被克隆，且仅在*C. gloeosporioides*-油梨病害系统中*GLT*功能得到鉴定，该基因被敲除后，*C. gloeosporioides*突变体附着胞形成率显著降低，对油梨的致病力明显下降[7]。本研究结果表明，来源于杧果*C. gloeosporioides*菌株的*Cgglt1*被敲除后，突变体△*Cgglt1*菌落生长减缓、黑色素沉积下降，分生孢子产量、萌发率均下降，且不能形成附着胞，对pH的适应性有所改变，对刺伤的杧果叶片致病力下降，对完整的杧果叶片基本失去致病力。可见，同样都是*C. gloeosporioides*的*GLT*基因，在不同的病害系统中，其功能却有明显的差异。

病原真菌与寄主组织开始接触，即处在寄主组织动态pH环境之中，其对寄主组织的侵染与定殖受到寄主组织pH环境的影响，通过氨的积累碱化寄主的组织是炭疽菌（*Colletotrichum* spp.）成功定殖的关键[6-7, 14]。*C. gloeosporioides*的谷氨酸转运蛋白基因*GLT*是氨合成途径上的重要基因之一，它将环境中谷氨酸运输到病原真菌中，使得谷氨酸一方面在ATP供能的情况下可以与NH_4结合形成谷氨酰胺，另一方面在NAD^+作为辅酶的谷氨酸脱氢酶GDH_2作用下分解，释放出的NH_4在AMET和MEPB作用下，被运输到环境中，动态调控着菌丝体内与外界环境中的NH_4浓度，从而改变着寄主的pH环境，使其更有利于病原菌侵染及定殖[7]。而氨的积累可以作为信号促进萌发的分生孢子形成附着胞，同时碱化环境pH，诱导果胶裂解酶等毒力因子的表达和分泌[9]。本研究结果表明，*Cgglt1*基因参与调控杧果炭疽病菌*C. gloeosporioides*的菌落生长、分生孢子产生及萌发和附着胞形成，且影响对杧果叶片的侵染力和侵入后的扩展能力。综合分析，笔者推测*Cgglt1*对胶孢炭疽菌上述生物学性状的影响，可能与它通过调控氨的合成代谢进而影响其内外环境pH有关。此外，本研究还发现，突变体△*Cgglt1*菌落正反面颜色明显变浅，显示*Cgglt1*可能参与了杧果炭疽病菌的黑色素合成，具体机制仍需进一步研究证实。

4 结 论

借助In-Fusion® HD Cloning Kit技术构建了*Cgglt1*敲除载体，并通过转化原生质体获得敲除突变体，表型测定结果表明，*Cgglt1*参与调控杧果炭疽病菌*C. gloeosporioides*的菌落生长、产孢量、孢子萌发率、附着胞形成以及对杧果叶片的致病力，该基因有作为防治靶标的潜力。

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