大豆疫霉菌多聚半乳糖醛酸酶 *pspg*1 基因的克隆及表达分析

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摘 要:大豆疫病严重影响我国及世界各国的农业生产,为探讨多聚半乳糖醛酸酶在大豆疫霉菌致病过程中的作用,采用 PCR 的方法从大豆疫霉菌中克隆了多聚半乳糖醛酸酶 *pspg*1 基因,并利用 RT-PCR 法对其在大豆中的表达进行了分析。结果表明:大豆疫霉菌 *pspg*1 基因开放阅读框长 1236 bp,编码一个长 412 氨基酸的蛋白质。对其进化关系进行分析,发现该基因与其它卵菌的 *pg* 基因亲缘关系最近,形成一个独立的分支。RT-PCR 分析表明:*pspg*1 基因在接种大豆疫霉菌的大豆下胚轴中大量表达,而在健康大豆下胚轴中未检测到。克隆了大豆疫霉菌 *pspg*1 基因,并发现该基因在大豆疫霉菌侵染大豆过程中发挥重要作用。

关键词:大豆疫霉菌;多聚半乳糖醛酸酶;克隆;表达

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Molecular Cloning and Expression Analysis of a Polygalac turonase *pspg*1 from *Phytophthora sojae*

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Abstract: Soybean blight is a worldwide disease, which impacted on agriculture of China and other countries badly, and caused enormous loss on economy. In order to explore the pathogenic role of polygalacturonase from *Phytophthora sojae*, a polygalacturonase gene *pspg*1 was cloned from *P. sojae* by using PCR. And the phylogenetic tree of *pspg*1 was constructed with PAUP software. Then the expression of *pspg*1 in soybean was analyzed by RT-PCR. The results showed that the length of open reading frame of *pspg*1 was 1236 bp, which encoded a protein of 412 amino acid residues. The result of evolutionary analysis indicated that *pspg*1 was gathered with other *pg* genes from other oomycetes, which formed an independent branch. More importantly, the evolutionary analysis further defined the status of oomycete in the evolution of the nature. RT-PCR was performed and the results demonstrated that *pspg*1 expressed in hypocotyl of inoculated soybeans, and the expression was increasing with extending of inoculated time. However, *pspg*1 was not detected in hypocotyl of healthy soybeans. In this paper, a polygalacturonase gene *pspg*1 was cloned, and sequence of *pspg*1 was analyzed. In addition, *pspg*1 was detected in hypocotyl of inoculated soybeans, which testified that *pspg*1 played an important role in pathogenic process of *P. sojae*.

Key words: *Phytophthora sojae*; Polygalacturonase; Cloning; Expression

植物病原卵菌种类多,分布广,引起的病害发生严重,给国内外经济造成巨大损失。卵菌能够侵染多种植物,引起根腐、茎腐等,且病害难于防治。大豆疫病是大田作物中唯一危害严重的疫霉病害,该病由大豆疫霉菌(*Phytophthora sojae*)引起,具有危害面积大、程度重的特点,已被列为大豆毁灭性病害之一[1]。

植物细胞壁是寄主与病菌互作的重要场所,病菌定植于寄主植物表面后须穿透细胞壁的屏障,建立寄生关系,才能表现出致病性,该过程中细胞壁降解酶发挥了极其重要的作用。研究表明,多聚半乳糖醛酸酶(Polygalacturonase, PG)在植物病原菌侵染寄主引起病害过程中起关键作用,是重要的致病因子之一。国内外学者对植物病原真菌多聚半乳糖醛酸酶的基因克隆及表达特性研究资料较多[2-9],但对植物病原卵菌 PG 的研究则较少,主要集中在樟疫霉(*P. cinnamomi*)[10-12]、致病疫霉(*P. infestans*)[13]和寄生疫霉(*P. parasitica*)[14]。

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目前,国内外关于大豆疫霉菌多聚半乳糖醛酸酶的研究报道较少。通过克隆大豆疫 *pspg*1 基因,并对其在寄主体内的表达特性进行分析,探讨该基因在大豆疫霉侵染大豆过程中的致病作用,为建立对大豆疫霉病菌持续有效的基因防治策略提供依据。

1 材料与方法

1.1 大豆疫霉菌总 RNA 的提取

将供试大豆疫霉菌从试管中转移至燕麦琼脂培养基(OMA)平板上,培养 7 d 后过滤菌丝,干燥,将其冻存于 -80℃超低温冰箱。大豆疫霉菌总 RNA 提取采用 Trizol (Invitrogen)一步法提取,方法按照说明书进行。取 2 μg 大豆疫霉菌总 RNA 进行反转录反应。cDNA 合成采用 M-MLV 反转录酶 (Promega)进行,合成的 cDNA 冻存于 -20℃下备用。

1.2 引物设计和 cDNA 的扩增

根据 http://www.jgi.doe.gov/上的大豆疫霉基因组序列及报道的植物病原卵菌的 PG 基因序列设计了 1 对特异性引物,上游引物 pspg1F(5′-TACTTCGAAAAGAGGCGGTA-3′)和下游引物 pspg1R(5′-CTAGCACTTGACGTTGCTGG-3′),用来扩增大豆疫霉菌 cDNA。PCR 反应条件按照以下进行:94℃预变性 4 min,94℃ 1 min,54℃ 30 s,72℃ 1 min,35 个循环,接着进行 10 min 的 72℃延伸。PCR 产物纯化后连接到 pMD18-T 上,转化大肠杆菌 DH5α,挑取阳性克隆送上海英骏生物科技有限公司测序。

1.3 序列分析

应用 DNAMAN 分析软件将所 DNA 序列翻译成蛋白质序列,将推断的氨基酸序列在 Genbank 上进行同源检索。利用 PAUP 软件构建进化树。

1.4 大豆疫霉菌接种和 RNA 提取

将供试携带抗病单基因大豆种子播种于营养钵中,至第 1 片子叶充分展开时,采用下胚轴伤口菌丝接种法接种。接种后 1、3、5 和 7 d 取下胚轴迅速冻存于液氮中,于 -80℃保存备用。接种后的下胚轴总 RNA 的提取方法同 1.1 所述。以健康大豆的下胚轴提取的 RNA 为对照。

1.5 *pspg*1 基因在大豆中的表达

采用 RT-PCR 的方法,利用基因特异性引物 pspg1F 和 pspg1R 来扩增接种大豆疫霉菌后的下胚轴和健康大豆下胚轴中的 *pspg*1 基因。PCR 反应程序如 1.2 所述。设计的一对 β-actin 的特异性引物为 actinN1(5′-ctgggacgacatggagaagatc-3′)和 actinC1(5′-cgctccgtcaggatcttcatc-3′)。取等体积的 pspg1 和 actin 扩增产物进行 1.2% 的琼脂糖凝胶电泳检测。

2 结果与分析

2.1 大豆疫霉菌 *pspg*1 基因的 cDNA 序列分析

取大豆疫霉菌总 RNA,进行 RT-PCR,用引物 pspg1F 和 pspg1R 扩增得到了一个长为 1200 bp 左右的条带,将其插入质粒,测序结果表明序列长 1236 bp,经 BLAST 软件分析,确定为多聚半乳糖醛酸酶基因。该基因开放阅读框长 1236 bp,编码一个 412 个氨基酸残基的蛋白质,如图 1。

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1     ATGAAGCTTTTCTCCGCCATCTTCGCTGTTTCTCTTCTGGTGGCTAGCACCACTATCGCCGACGCTGGAGGCAAC
1      M  K  L  F  S  A  I  F  A  V  S  L  L  V  A  S  T  T  I  A  D  A  G  G  N
76    GCACTGCAGTCTGACAACTGCGATTCAGGTTTCCCCAAGACCAGCGTTGACTCCAGCGGAAAGCAAGGTGACAAC
26     A  L  Q  S  D  N  C  D  S  G  F  P  K  T  S  V  D  S  S  G  K  Q  G  D  N
151   CCTGACCAAAACAACCCAGGACAGAACACCCCCGACCAAAACTCCACGAGTTCTGACGGATCGCAGACGCAAACT
51     P  D  Q  N  N  P  G  Q  N  T  P  D  Q  N  S  T  S  S  D  G  S  Q  T  Q  T
226   CCACCTGTCCTCAAGGGCGACTGCATCCTCACTGGGGACTACATCAACAACACCGACGTGAGGAAGTGCGACAGT
76     P  P  V  L  K  G  D  C  I  L  T  G  D  Y  I  N  N  T  D  V  R  K  C  D  S
301   ATCATCGTCGACTCTCTTCGGGTGCCACCGGGCGTGACGCTGAACTTGACCAAGTTGCAGGACAACGCCAAGGTT
101    I  I  V  D  S  L  R  V  P  P  G  V  T  L  N  L  T  K  L  Q  D  N  A  K  V
376   TCGTTCAAGGGAACCTCCACTTTCGGCACCAAGCTGTGGGATGGCCCGCTCGTTAAGCTGCAAGGAAACAACCTC
126    S  F  K  G  T  S  T  F  G  T  K  L  W  D  G  P  L  V  K  L  Q  G  N  N  L
451   ACCGTCACCGGACCCGGCACGCTCGACGGACAGGGGGCTTACTACTGGCCCCTGGGACAGAAGGTCGACAAGCCC
151    T  V  T  G  P  G  T  L  D  G  Q  G  A  Y  Y  W  P  L  G  Q  K  V  D  K  P
526   GTCTTCTTCAAAATCTACAGGGTCGCCCACTCCACGCTCTCGGGCTTCACCCTTCTCAACGTGCCGTACCGAACC
176    V  F  F  K  I  Y  R  V  A  H  S  T  L  S  G  F  T  L  L  N  V  P  Y  R  T
601   TTCAGCGTCGGAAACTCCAACTACACCACCATCACCGGCCTCACTCTCAACACCACGGCTGGCAATGGCGTCGCC
201    F  S  V  G  N  S  N  Y  T  T  I  T  G  L  T  L  N  T  T  A  G  N  G  V  A
676   CAAAACACGGACGGCTTCGACCTCAGCAAGAATGACCACATCACGATCACCAAGAACCGCATCTTCAACCAGGAC
226    Q  N  T  D  G  F  D  L  S  K  N  D  H  I  T  I  T  K  N  R  I  F  N  Q  D
751   GACTGCCTCGCCATGCAGTCCAGCACCAACACCGTCTTCAGCGACAACTACTGCTGCGGCTCGCACGGCATCTCC
251    D  C  L  A  M  Q  S  S  T  N  T  V  F  S  D  N  Y  C  C  G  S  H  G  I  S
826   ATCGGCTCGCTCGGGGGTCCCGAGCAGAACTCGTCCACCACCGTGGACGGACTCACGGTCACGGGCAACACCATC
276    I  G  S  L  G  G  P  E  Q  N  S  S  T  T  V  D  G  L  T  V  T  G  N  T  I
901   GAGAACAGCACCAACGGCATCCGTATCAAGACCATCATCGGGCTCAAGGGACTCGTCACCAACGCTGTCTACACG
301    E  N  S  T  N  G  I  R  I  K  T  I  I  G  L  K  G  L  V  T  N  A  V  Y  T
976   AACAACAAGCTCATCAACGTCACCAACGCCATCGTCATGCACTCGGACTACAACAAGACCAAGGGCGGCTACGCG
326    N  N  K  L  I  N  V  T  N  A  I  V  M  H  S  D  Y  N  K  T  K  G  G  Y  A
1051  GGCACGCCCACGAGTCTGGTCAAGATCCAGAGCATCAAGATCGACGGGCTCCAGGGCACGGCCGAGAACCTCTAC
351    G  T  P  T  S  L  V  K  I  Q  S  I  K  I  D  G  L  Q  G  T  A  E  N  L  Y
1126  GACATCGTGGCCAACCCGGACGTCGTGTCGGACTGGACGTTCACCAACATCGACGTGACGGCTGCGAGCATCGGC
376    D  I  V  A  N  P  D  V  V  S  D  W  T  F  T  N  I  D  V  T  A  A  S  I  G
1201  AACTGCACGGGTGAGCCCAGCAACGTCAAGTGCTAG
401    N  C  T  G  E  P  S  N  V  K  C  *
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图 1 大豆疫霉菌 *pspg*1 基因核苷酸序列及推断的氨基酸序列

Fig. 1 Nucleotide and deduced amino-acid sequences of *pspg*1 from *Phytophthora sojae*

2.2 大豆疫霉菌 *pspg*1 基因的系统发育分析

由进化树可以看出,大豆疫霉菌 *pspg*1 基因与其它 3 个疫霉种的 *pg* 基因亲缘关系最近,形成一个独立支。来自植物和细菌的 *pg* 基因各自成一分支,而来自其它真菌的 *pg* 基因与卵菌的 *pg* 基因共同组成一个大分支,如图 2。由此可以看出,植物病原卵菌的 *pg* 基因和植物病原真菌的 *pg* 基因起源于一个共同祖先。这种系统发育关系与传统的分类基本一致,也进一步明确了卵菌在自然界的进化地位。

2.3 大豆疫霉菌 *pspg*1 基因在寄主中的表达

以健康大豆下胚轴和接种大豆疫霉菌的大豆下胚轴为材料,以反转录合成的 cDNA 第一链为模板,应用 RT-PCR 方法对大豆疫霉菌 *pspg*1 基因的表达进行了分析。结果表明,在接种病菌的大豆下胚轴

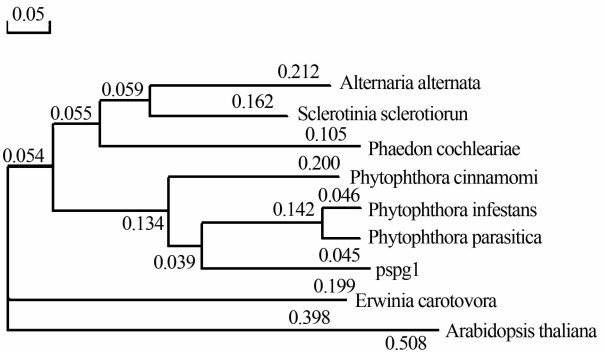

图 2　大豆疫霉菌 *pspg*1 基因与其它物种的进化树分析

Fig. 2　Phylogenetic analysis of *pspg*1 from *Phytophthora sojae* with other species

中检测到了 *pspg*1 基因的表达,且随着接种时间的延长,表达量逐渐升高;而在健康大豆下胚轴中没有检测到 *pspg*1 基因,如图 3。

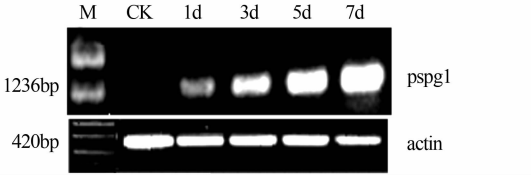

图 3　大豆疫霉菌 *pspg*1 基因在大豆下胚轴中的表达

Fig. 3　Expression of *pspg*1 from *Phytophthora sojae* in soybean hypocotyl

3　讨论

研究发现很多物种的多聚半乳糖醛酸酶是由多基因家族编码的,在黑曲霉、丝核菌、青霉等真菌中克隆了 2 个以上的 *pg* 基因。对樟疫霉 *pg* 基因的研究发现,该基因家族包含 19 个 *pg* 基因,由此推测大豆疫霉菌也有多个 *pg* 基因。本实验仅克隆了其中一个 *pg* 基因,有必要进一步对其他 *pg* 基因的多态性、遗传进化关系及其在侵染寄主过程中的作用进行研究。

多聚半乳糖醛酸酶是一种细胞壁降解酶,能够降解寄主植物的细胞壁,为病原菌顺利侵入寄主建立寄生关系创造条件。研究表明,大多数病原菌的 *pg* 基因在病害的侵染初期大量表达,表达量的高低受到多种因素的影响。Rugang 等研究发现,在腐生生长状态下,来自 *S. sclerotiorum* 的 *sspg*1*d*、*sspg*3、*sspg*5 和 *ssxpg*1 的表达受果胶和半乳糖醛酸的诱导而被葡萄糖所抑制;在寄生生长条件下,*sspg*1*d* 能高效表达,*sspg* 的表达量低于 *sspg*1*d*,但 *sspg*5、*sspg*6 和 *ssxpg*1 却只能微量表达。Li 等[15] 发现 *Colletotrichum gloeosporioide* 的 *scgmpg*2 基因在死体营养和活体营养中均能表达,且在侵染初期的死体营养生长过程表达量较高。通过 RT-PCR 进行检测,发现 *pspg*1 在健康大豆下胚轴中没有表达,而在接种大豆疫霉菌的大豆下胚轴检测到该基因的表达。但试验仅对 *pspg*1 基因在病害侵染初期的表达进行了研究,有必要进一步探讨该基因在侵染后期的表达情况,为更好的防治大豆疫霉奠定理论基础。

克隆了大豆疫霉菌的 *pspg*1 基因,并利用 RT-PCR 对该基因的表达进行了检测,但没有对 *pspg*1 基因的表达产物的致病性进行研究。由于多聚半乳糖醛酸酶是一种细胞壁降解酶,因此可以从 *pg* 基因的表达产物着手,借助电镜手段,进一步探索 PG 在病原菌侵染寄主过程中的致病作用,为从基因和蛋白水平上控制和防治植物病害打下坚实的基础。

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