

• 农业生物技术 •

# 抗 FLAG 标签抗体在植物中的高效表达

孔志成<sup>1,2</sup>, 熊潇然<sup>1,2</sup>, 吴川<sup>3</sup>, 潘炜松<sup>1,2\*</sup>

1 湖南农业大学生物科学与技术学院, 湖南 长沙 410128

2 湖南省细胞力学与功能分析工程技术研究中心, 湖南 长沙 410128

3 湖南诺合新生物科技有限公司, 湖南 长沙 410000

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**摘要:** 植物生物反应器是一种新兴的重组蛋白表达系统, 是分子农业的核心内容之一。本研究在本氏烟草(*Nicotiana benthamiana*)中表达了抗八肽(DYKDDDDK, FLAG)标签抗体, 并对其进行了纯化与鉴定。通过多次免疫小鼠获得高效价抗 FLAG 抗体并测出其编码序列, 然后亚克隆至植物 DNA 病毒表达载体, 最后通过农杆菌介导转染烟草叶片。经 Western blotting 检测了转染后 2–9 d 抗体的表达情况: 3 d 后 FLAG 抗体开始在烟草叶片中表达, 5 d 后表达量达到峰值, 每千克鲜叶估计可表达 66 mg FLAG 抗体。抗体经过分离纯化后浓缩为 1 mg/mL, 按 1:10 000 稀释仍可识别 1 ng/mL 的抗原, 表明植物生产的 FLAG 抗体具有高亲和力。植物生物反应器可用于生产高亲和力抗体, 并具有简易、成本低和生产周期短等特点, 具有很高的应用价值。

**关键词:** 重组蛋白; 植物生物反应器; 蛋白纯化; 分子农业

## High-level expression of anti FLAG tag antibody in plants

KONG Zhicheng<sup>1,2</sup>, XIONG Xiaoran<sup>1,2</sup>, WU Chuan<sup>3</sup>, PAN Weisong<sup>1,2\*</sup>

1 College of Bioscience and Biotechnology, Hunan Agricultural University, Changsha 410128, Hunan, China

2 Hunan Engineering Research Center for Cell Mechanics and Functional Analysis, Changsha 410128, Hunan, China

3 Hunan Novomore Biotechnology Co., Ltd., Changsha 410000, Hunan, China

**Abstract:** Plant bioreactor is a new production platform for expression of recombinant protein, which is one of the cores of molecular farming. In this study, the anti DYKDDDDK (FLAG)

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\*Corresponding author. E-mail: joux19@163.com

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antibody was recombinantly expressed in tobacco (*Nicotiana benthamiana*) and purified. FLAG antibody with high affinity was obtained after immunizing mice for several times and its sequence was determined. Based on this, virus vectors expressing heavy chain (HC) and light chain (LC) inoculated into *Nicotiana benthamiana* leaves by using *Agrobacterium*-mediated delivery. Accumulation of the HC and LC was analyzed by SDS/PAGE followed by Western blotting probed with specific antibodies from 2 to 9 days postinfiltration (dpi). Accumulation of the FLAG antibody displayed at 3 dpi, and reached a maximum at 5 dpi. It was estimated that 66 mg of antibody per kilogram of fresh leaves could be obtained. After separation and purification, the antibody was concentrated to 1 mg/mL. The 1:10 000 diluted antibody can probe with 1 ng/mL FLAG fused antigen well, indicating the high affinity of the FLAG antibody produced in plants. In conclusion, the plant bioreactor is able to produce high affinity FLAG antibodies, with the characteristics of simplicity, low cost and highly added value, which contains enormous potential for the rapid and abundant biosynthesis of antibodies.

**Keywords:** recombinant protein; plant bioreactor; protein purification; molecular farming

目前,重组蛋白的生产主要是以原核生物、酵母、昆虫细胞和哺乳动物细胞等作为反应器进行的,然而这些传统的表达体系具有明显的不足之处。首先,这些表达体系需要无菌环境或较复杂的培养基,因此生产成本较高;其次,原核生物由于自身结构不完善,没有内质网等内膜系统,在合成蛋白时不能进行适当的折叠或加工,因此只能用于无需翻译后修饰的简单蛋白;而哺乳动物细胞容易携带动物病毒,具有安全隐患<sup>[1]</sup>。基于这些问题,植物生物反应器是生产特定重组蛋白的良好替代方案<sup>[2]</sup>,其生产过程不需要无菌环境,高等植物作为真核生物能够合成具有生物活性的蛋白,并且没有感染动物性病原体的风险。此外,植物生物反应器还具有易规模化、成本低和操作简便等诸多优势<sup>[3]</sup>,这些优势决定了在低成本下植物反应器应用于重组蛋白生产特别是生物制药的高度可拓展性<sup>[4]</sup>,其成本效益特别适合发展中国家<sup>[5]</sup>,是开展分子农业的核心内容之一<sup>[6-7]</sup>。

FLAG (DYKDDDDK)是由 8 个氨基酸 (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys)组成的短

肽<sup>[8]</sup>,是用于蛋白质免疫吸附纯化的经典标签,具有高亲和力、高泛用性的优点<sup>[9]</sup>。但是,目前商品化抗 FLAG 标签蛋白的单克隆抗体种类不多,且多需从国外进口,价格十分昂贵。因此,开发一种低成本、简易的 FLAG 抗体生产方法,在融合蛋白质的免疫吸附分离纯化中具有广阔的应用前景,而植物生物反应器恰恰具有这些特点。

烟草作为模式植物,具有生长周期短、生长环境要求低、叶片生物量大(10 t/hm<sup>2</sup>)和易于筛选等优点<sup>[10]</sup>,是表达重组蛋白的最佳系统之一<sup>[11]</sup>。因此,本研究以烟草作为反应器应用于 FLAG 抗体的表达,监测表达周期,测定其表达量,提取产物进行纯化并检测其亲和力,旨在基于植物反应器建立安全、快速生产高亲和力重组蛋白的方法,促进分子农业的发展。

## 1 材料与amp;方法

### 1.1 材料

#### 1.1.1 植物材料

本氏烟草(*Nicotiana benthamiana*),种子保存于本实验室。

### 1.1.2 菌株和质粒

大肠杆菌(*Escherichia coli*) DH5 $\alpha$  菌株和发根农杆菌(*Agrobacterium rhizogenes*) C58C1 菌株保存于本实验室。

pMDV 质粒是本实验室基于鹰嘴豆褪绿性矮缩病毒(chickpea chlorotic dwarf virus, CpCDV) 构建的双元载体。

### 1.1.3 主要试剂

质粒提取试剂盒、琼脂糖电泳回收试剂盒购自天根生化科技(北京)公司, 蛋白质分子量标准购自翌圣生物科技(上海)股份有限公司, 考马斯亮蓝购自北京索莱宝科技公司, 显影剂(Western Lightning)购自珀金埃尔默公司。商业抗 FLAG 单抗(Cat.No.F1804)购自 Merck 公司, 二抗(ProteinFind Goat Anti-Mouse IgG HRP conjugate)购自北京全式金生物技术有限公司, 序列合成和测序由北京擎科生物科技有限公司完成。

## 1.2 方法

### 1.2.1 载体构建

利用 FLAG 抗原[八氨基酸短肽 DYKDDDDK-偶联 KLH (keyhole limpet hemocyanin)], 多次免

疫 BALB/c 小鼠, 制备大量杂交瘤细胞株, 筛选分泌抗体阳性细胞并克隆, 提取其序列并测序。具体操作参考前人的方法<sup>[12]</sup>。实验方案遵守相关实验动物福利规定, 经湖南农业大学生物医药研究伦理委员会审查(获批伦审科第 119 号文件)。

经分析, 重链的互补决定区, CDR1: T-Y-T-I-H; CDR2: Y-I-N-P-S-S-G-Y-A-A-Y-N-Q-F-K-D; CDR3: E-K-F-Y-G-Y-D-Y。轻链的互补决定区: CDR1: R-S-S-Q-S-I-V-H-R-N-G-N-T-Y-L-E; CDR2: K-V-S-N-R-F-S; CDR3: F-Q-G-S-H-V-P-Y-T。据此合成其重链和轻链的 DNA 序列, 亚克隆到 pMDV 载体上(图 1), 并转化农杆菌。

### 1.2.2 烟草转染

将携带目的片段的农杆菌在固体培养基上活化, 挑取单菌落接种至 5 mL 液体培养基, 28 °C、220 r/min 过夜。次日接种于 100 mL 液体培养基, 培养至对数生长期。在室温、4 000 r/min 下离心 25 min, 去上清后用吗啉乙磺酸(morpholinoethanesulphonic acid, MES)缓冲液

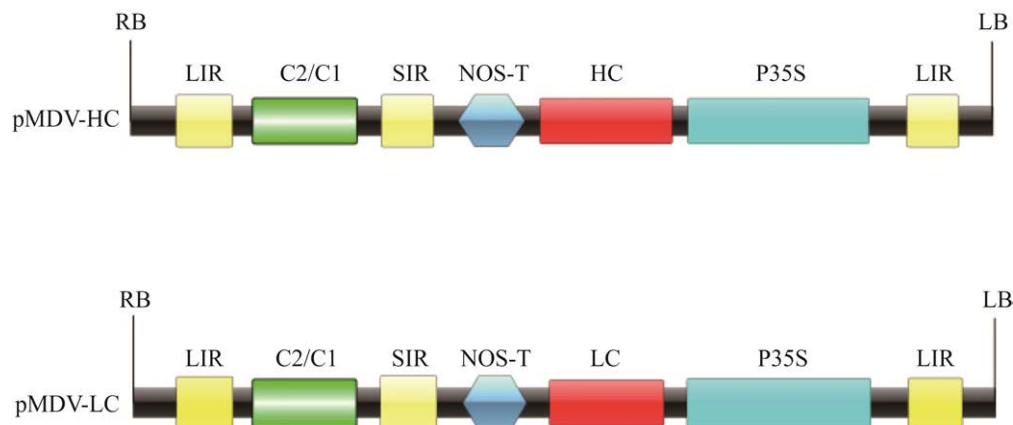


图 1 载体的 T-DNA 区域示意图

Figure 1 T-DNA region of vector. RB and LB: Left and right borders of the *Agrobacterium*; LIR: Long intergenic region of CpCDV genome; SIR: Short intergenic region of CpCDV genome; C2/C1: C1 and C2 gene of CpCDV for replication initiation protein (Rep) and RepA; NOS-T: Nopaline synthase terminator; HC and LC: Heavy and light chains of the antibody; P35S: Cauliflower mosaic virus (CaMV) 35S promoter.

(pH 5.6)将农杆菌重悬浮,加入 10 mmol/L  $MgCl_2$ 、36 mg/L 乙酰丁香酮。放入培养箱(28 °C、220 r/min)孵育 1 h。孵育后稀释至  $OD_{600}$  约为 0.1,分装至 1 L 塑料烧杯中。将生长 5–7 周的烟草倒置,使叶片浸入渗入液并置于真空箱,抽真空至 -0.1 MPa,保持 10 min。侵染结束后将烟草置于 25 °C、16 h 光照/8 h 黑暗循环的温室培养。

### 1.2.3 抗体提取与纯化

分别剪取 2–9 dpi (days post-infiltration)的叶片,在液氮中冷冻,充分研磨后加入适量 PBS。4 °C、12 000 r/min 离心 15 min,留上清,重复 1 次。

取样品过 0.22  $\mu m$  滤膜,上 Protein G 亲和层析柱。先用 5 倍柱体积的去离子水洗涤,去除空气和 20%乙醇;用 PBS 缓冲液平衡柱子,将样品以 0.5 mL/min 的速度流穿柱子,0.1 mol/L 甘氨酸洗脱,收集洗脱样品然后用 PBS 缓冲液透析,超滤浓缩。

### 1.2.4 SDS-PAGE 与 Western blotting 分析

将植物蛋白提取物或纯化后的 FLAG 抗体样品与电泳缓冲液混合,并以商业抗 FLAG 单抗(F1804, Merck)作为阳性对照,100 °C下加热

5 min 使蛋白变性,在聚丙烯酰胺凝胶电泳中分离,用考马斯亮蓝染色或准备转膜。

将分离的抗体转移到聚偏二氟乙烯(polyvinylidene fluoride, PVDF)膜上,用可逆染色剂丽春红验证各泳道上样量的一致性。洗脱膜上的丽春红,用含 5%脱脂牛奶的 TBST 缓冲液封闭,加入二抗孵育。缓冲液清洗 3 次后,加显影剂显影。

### 1.2.5 FLAG 抗体与抗原的结合效率

抗原滴定的蛋白印迹分析:在 SDS-PAGE 上分离重组带 FLAG 标签的红色荧光蛋白(FLAG-DsRed),并用纯化浓缩后的 FLAG 抗体样品检测。抗原(FLAG-DsRed)量梯度为 0.5–10 ng,样品(1 mg/mL)按 1:10 000 稀释。

## 2 结果与分析

### 2.1 FLAG 抗体在烟叶中的瞬时表达

通过对转染的烟草叶片逐天观察发现:转染 3 d 后叶片开始出现斑状,表明 FLAG 抗体开始表达;5–6 d 后发病的表型最为明显;7 d 后发病部位颜色变深,局部组织开始萎蔫直至 8 d 后开始坏死,如图 2 所示。

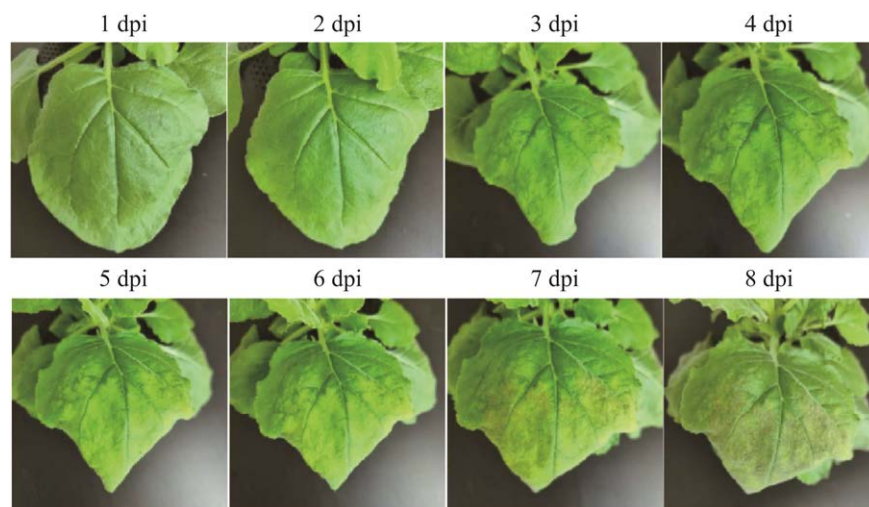


图 2 转染后不同天数的叶片表型

Figure 2 Phenotype of leaves upon transfection for different days.

鉴于叶片表型的变化,对转染后 2–9 d 的叶片中提取的蛋白进行 Western blotting 分析,并通过丽春红对 PVDF 膜染色验证了各泳道上样量的一致性,结果如图 3 所示。转染后第 3 天,抗体开始表达,在 25 kDa 和 50 kDa 处分别出现条带,与抗体的重链和轻链的理论分子量相符。第 5 天时,条带最为明显,表明表达量达到峰值。分析结果与叶片表型基本一致,最佳收获时间为第 5 天。

对 5 dpi 的烟草叶片提取的 FLAG 抗体,进行蛋白免疫印迹分析,结果如图 4 所示。pMDV-HC/LC 孔为取 0.15 g 叶子用 500  $\mu$ L 裂解液提取,再上样相应体积的样品,在理论分子量处有明显的条带。按条带灰度与商业抗体条带比较,每千克鲜叶的抗体表达量为 66 mg。

## 2.2 烟叶生产的 FLAG 抗体的纯化

从 100 株 5 dpi 的烟草(共 1 kg 鲜叶)中提取蛋白,通过 protein G 亲和层析柱纯化后,得到

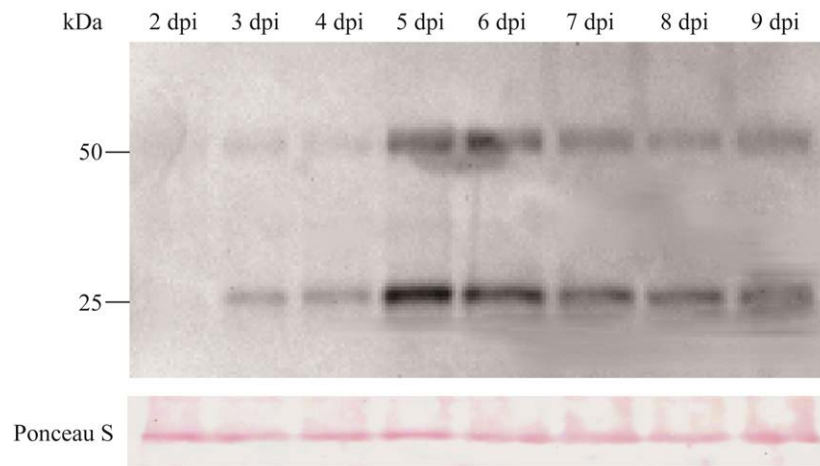


图 3 FLAG 抗体在转染后不同天数的表达情况

Figure 3 Expression of FLAG antibody upon transfection for different days. Ponceau S staining shows equal protein loading across samples.

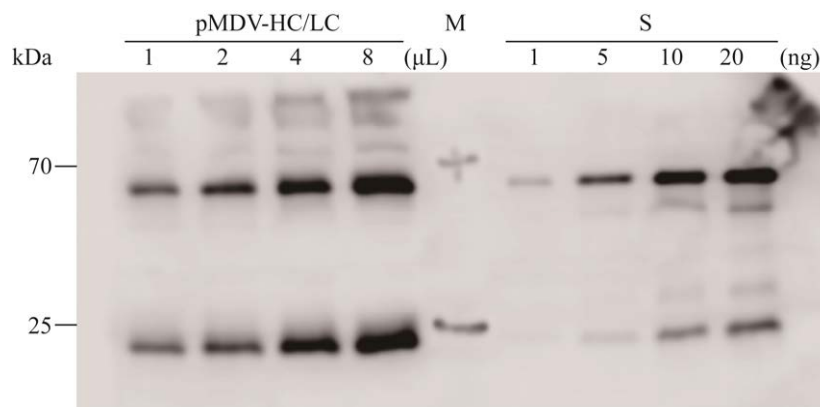


图 4 FLAG 抗体在转染后第 5 天的表达量

Figure 4 Quantitative expression of FLAG antibody on the 5th day upon transfection. M: Molecular weight standard of protein; S: Commercial anti-FLAG antibody.

20 mg FLAG 抗体。SDS-PAGE 分析结果如图 5 所示：只在 FLAG 抗体的重链和轻链的理论分子量处检测到条带，表明经亲和层析纯化后的 FLAG 抗体具有较高的纯度。

### 2.3 烟叶生产的 FLAG 抗体与抗原结合的特性

通过 Western blotting 蛋白印迹分析抗体的亲和力：加抗原 FLAG DsRed，梯度稀释至 0.5–10 ng，与浓度为 0.1  $\mu\text{g/mL}$  FLAG 抗体孵育。如图 6 所示，在抗原稀释至 1 ng 时仍有明显的反应条带，说明通过烟草生产的 FLAG 抗体与抗原的结合效率较高，具有较高的亲和力。

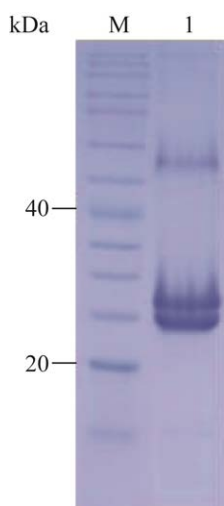


图 5 FLAG 抗体的纯化

Figure 5 Purification of FLAG antibody. M: Molecular weight standard of protein; 1: FLAG antibody.

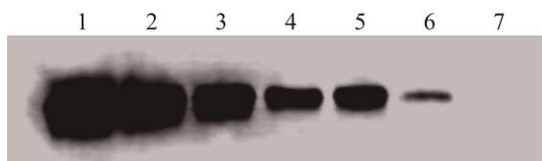


图 6 FLAG 抗体与抗原的结合效率

Figure 6 Binding efficiency of FLAG antibody and antigen. Concentration of antigen. 1: 10 ng; 2: 5 ng; 3: 4 ng; 4: 3 ng; 5: 2 ng; 6: 1 ng; 7: 0.5 ng.

## 3 讨论与结论

植物生物反应器以低成本、简易和安全性等特殊优势实现了对传统方法的完美替代并快速发展<sup>[13-14]</sup>。自美国首次批准一种植物细胞衍生酶(高木糖醇)用于人类疾病治疗取得重大突破以来<sup>[15]</sup>，植物生物反应器正成为分子农业的有力工具<sup>[16]</sup>，越来越多植物生产的重组蛋白投入临床试验，如用苔藓产  $\alpha$ -半乳糖苷酶已用于法布瑞士症的 I 期试验<sup>[17]</sup>；烟草产中和艾滋病病毒人类单克隆抗体 P2G12 被批准人类 I 期试验<sup>[18]</sup>；由胡萝卜细胞生产的用于治疗戈谢病的重组葡萄糖脑苷脂酶(recombinant plant-derived glucocerebrosidase, prGCD)已经投入市场<sup>[19]</sup>；2022 年 2 月，第一个植物源性新冠病毒疫苗产品 Covifenz<sup>[20]</sup>被加拿大卫生部批准上市。

由于起步较早，经过多年的优化，传统的基于动物细胞、酵母和原核生物的重组蛋白表达量可达到 1–10 g/L (kg)<sup>[21-22]</sup>。植物表达体系起步较晚，虽对于荧光蛋白的表达量达到 5 g/kg 鲜叶<sup>[23-24]</sup>，但对于抗体等活性肽的表达量较低<sup>[25]</sup>，而在优化后可达到 500 mg/kg 以上<sup>[3,26]</sup>。植物反应器可以是任意植物个体、器官、组织甚至细胞，其中器官作为反应器无需无菌环境且能快速累积重组蛋白，叶片是烟草生物量最大的器官，以烟叶生产 FLAG 抗体已达到中等水平的 66 mg/kg，且有较大的优化空间。同时烟草作为模式植物具有易培养、技术成熟等特点，且非粮食/饲料作物，是最佳的植物表达载体<sup>[27]</sup>，其生产过程仅需低成本的水、肥和光照。特别是相较于传统表达体系，无需特殊、无菌环境；生长周期较短，表达周期仅为一周，而动物细胞表达周期长达 15–30 d，且存在较长的病毒清理及验证期(3–6 个月)。因此，植物表达体系适合规模化生产重组蛋白。

FLAG 标签在蛋白检测和分离纯化中广泛应用,已经有 4 种抗 FLAG 标签的单克隆抗体,即钙依赖的 M1 和钙不依赖的 M2、M5 和 L5 上市。这 4 种 FLAG 抗体在检测极低表达水平的 FLAG 融合蛋白时不够敏感, Ikeda 等<sup>[8]</sup>对此建立了新的杂交瘤细胞系以制备高亲和力 FLAG 抗体——2H8, 该抗体足以检测氨基末端 FLAG 标记的 G 蛋白偶联受体(G protein-coupled receptors, GPCRs)和粗制剂中的可溶性蛋白,并能够从细胞裂解液中免疫沉淀 FLAG 标记的 GPCRs。本文通过免疫小鼠制备杂交瘤细胞,筛选后亦获得了高亲和力 FLAG 抗体,并于测序后建立了植物表达体系进行生产。相比较 M2 的最低稀释度为 20 ng/mL,可识别 2 ng 抗原,植物源 FLAG 抗体稀释至 100 ng/mL 时可识别 1 ng 抗原(图 6),表明其亲和力已达到商业抗体水平。

本研究的表达体系为农杆菌介导的瞬时表达体系,烟草叶片经过真空渗入后,目的序列立刻被农杆菌导入细胞内并在短时间内(数小时至数天)就能进行表达,因此具有快捷省时的特点。瞬时表达不受基因位置的影响,无需整合到基因组<sup>[28]</sup>,因而表达效率更高,可达稳定转化表达量的数倍<sup>[29]</sup>。此外,瞬时转染只转染体细胞,相对于稳定遗传转染生殖细胞,目的序列不会传递到下一代,因此环境安全性高<sup>[30]</sup>,是转基因舆论环境下绿色生产的有力技术。

瞬时转染往往具有侵染效率低的缺点,侵染时植物体往往只有部分细胞被导入目的序列,对此,本研究构建了基于 CpCDV 病毒的载体,有效地提高了转染效率。由于病毒的高效复制、表达的特性,基于病毒的载体可以在瞬时基因表达后获得高水平的有价值的蛋白<sup>[26,31-35]</sup>,因此经常被纳入农杆菌中,以增加在转染过程中被侵染的植物细胞的数量<sup>[36]</sup>。此外,出于植

物的内在防御反应,外源基因往往会出现转录后基因沉默的现象<sup>[37]</sup>。对此,本研究构建了 p19(基因沉默抑制剂)载体,降低烟草细胞中基因沉默蛋白的表达水平,从而抑制转录后基因沉默<sup>[38]</sup>。

通过瞬时转染的植株,其重组蛋白的表达水平与叶片的位置相关:植株上端的叶片表达水平最高<sup>[39-40]</sup>,特别是从顶端开始的第一叶到第六叶<sup>[41]</sup>。本研究转染的烟叶中观察到类似的结果:顶部叶片的发病表型较明显,特别是发育较快的 3 片大叶。这种现象表明蛋白的表达量与叶片生长发育的典型特征有关:上叶为新生的幼叶,其细胞平均分化水平较低,蛋白表达水平较高;而在成熟叶片中,蛋白质代谢始终处于低水平,在衰老叶片中,蛋白质几乎没有表达且已有的蛋白会降解<sup>[42]</sup>。此外,生长较好并开始开花的植物比未开花的发育较差的植物表现出较低的表达水平,因此转染的最佳时间在植物开花前<sup>[40]</sup>。导致外源蛋白产量低的一个关键因素是合成后和/或分泌后的不稳定性和降解,如蛋白水解和不可逆的表面吸附,会发生外源蛋白的损失<sup>[43]</sup>。因此,需要使用蛋白酶抑制剂<sup>[44-45]</sup>或基于基因编辑技术敲除蛋白酶基因<sup>[46]</sup>以提高蛋白产量。

随着外源蛋白在病毒的扩散作用下高速表达与积累,植物组织细胞的正常生理代谢被打乱因而受到严重胁迫,其结果导致组织坏死,影响蛋白产量<sup>[47]</sup>,正如 8 dpi 时烟叶开始出现黑坏死的现象(图 2)。Diomas 等<sup>[48-49]</sup>构建豆黄矮病毒(bean yellow dwarf virus, BeYDV)表达载体时,通过改变 5'端非翻译区核苷酸序列,降低其复制蛋白 Rep/RepA 表达量,使烟草叶片细胞死亡率明显降低,从而提高重组蛋白的产量。在重组蛋白的提取过程中,应合理安排收获时间,在组织坏死前及时提取蛋白。

动物细胞合成的蛋白活性受糖基化修饰调

节<sup>[50]</sup>，植物细胞的蛋白合成途径与动物细胞类似，但是高尔基体的 N-聚糖加工途径不同，因此植物合成的蛋白会有其独特的糖基化修饰<sup>[51-53]</sup>。具有植物特异性 N-聚糖的重组蛋白与受体的结合力降低，表明植物特异性糖基化修饰也具有调节蛋白亲和力的作用<sup>[54]</sup>。提取时，部分蛋白经过了糖基化修饰，出现同一批蛋白的分子量不一致的现象(图 5)，并可能导致修饰与未修饰的蛋白分子亲和力不一致，从而影响整体质量。为了防止植物特异性复合物 N-聚糖修饰的附着，重组蛋白的合成途径应被设计为停留在内质网中<sup>[55]</sup>或使用糖删除技术敲除相关基因<sup>[56]</sup>，但这可能会加重植物组织坏死<sup>[47]</sup>。此外，也可以将植物糖基化修饰基因敲除后，转入哺乳动物糖基化修饰基因，促进植物表达蛋白修饰的“人源化”<sup>[57]</sup>。

基于植物反应器的 FLAG 抗体生产方法，通过构建病毒载体和基因沉默抑制剂的使用，表达量达到 66 mg 抗体/kg 鲜叶，最佳生产周期为 5 d。抗体经纯化后与抗原有非常好的结合效果，表明其具有极高的亲和力。表达量仍有较大提升空间，后续可通过密码子优化、细胞器特异性启动子的加入和蛋白酶抑制剂的加入等进一步提高 FLAG 抗体等重组蛋白的产量；并可通过敲除植物糖基化基因或糖基“人源化”进一步提高重组蛋白的可靠性。基于植物反应器的分子农业具有低成本、高效率、简易、安全和易规模化的优势，具有极大的应用价值。

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