Notice

DESCRIPTION CN115161340A

Highly efficient genetic transformation methods for duckweed and their applications in utilizing duckweed to express cytokines method

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高效浮萍遗传转化方法及应用与利用浮萍表达细胞因子的 方法

[0001]

Technical Field

技术领域

[n0001]

This invention belongs to the fields of genetic engineering and biotechnology, and relates to an efficient method for the genetic transformation of duckweed and a method for expressing cytokines using duckweed.

本发明属于基因工程和生物技术领域，涉及一种高效浮萍遗传转化方法及应用与利用浮萍表达细胞因子的方法。

[0003]

Background Technology

背景技术

[n0002]

Biologics are typically expressed in bacteria, fungi, mammals, insect cells, and plants.

生物制剂通常在细菌、真菌、哺乳动物、昆虫细胞以及植物中表达。

In comparison, plant bioreactors have unique advantages, such as simple cultivation conditions, low transportation costs, high safety, and the advantages of protein post-translational processes.

相比较而言，植物生物反应器具有独特的优势，如培养条件简单、运输成本低、安全性高、具有蛋白的翻译后优势等。

Especially in resource-scarce environments for large-scale vaccine production, such as COVID-19 vaccines, plant bioreactors offer a cheaper and more practical strategy.

特别是在资源匮乏的环境中去大规模生产疫苗，如新冠疫苗，植物生物反应器提供了一个更便宜且能实际操作的策略。

Novel genetically engineered subunit vaccines often face problems such as poor immunogenicity, and need to be combined with vaccine adjuvants to improve the immune effect. Immune adjuvants can enhance the body's immune response to antigens, resulting in stronger immune protection and longer-lasting immune memory, effectively improving vaccine efficacy. Currently, most commercial adjuvants are aluminum adjuvants and oil emulsion adjuvants, whose use may cause adverse local reactions such as inflammation and fever, which is detrimental to animal welfare. In addition, aluminum adjuvants are sensitive to freezing conditions, making them difficult to store and transport. Recent research has

provided new insights into innate immune receptors and signaling pathways, offering opportunities for the rational design of novel adjuvants. Several new adjuvants have been approved, such as alum, monophospholipid A (MPL), ASO4 (containing both alum and MPL), and ASO3 (a squalene-based adjuvant).

新型的基因工程亚单位疫苗通常面临着免疫原性差等问题，需要结合疫苗佐剂来提高免疫效果。 免疫佐剂可增强机体对抗原的免疫应答，产生更强的免疫保护和更久的免疫记忆，有效提升疫苗效果。 目前，大多数商业佐剂都是铝佐剂和油乳佐剂，它们的使用可能会导致炎症、发热等不良的局部反应，不利于动物福利。 此外，铝佐剂对冷冻条件敏感，因此很难进行存储和运输。 最近的研究对先天免疫受体和信号通路的新认识为合理设计新型佐剂提供了机会，一些新的佐剂获得了许可，例如，明矾、单磷酰脂质A(MPL)、AS04(同时含有明矾和MPL)和AS03(一种基于角鲨烯的佐剂)。

[n0003]

The combined use of cytokines and chemokines with DNA vaccines, or the expression of cytokines alone and their combination with traditional vaccines, can enhance the immune efficacy of vaccines.

细胞因子和趋化因子与DNA疫苗联合使用，或者将细胞因子单独表达后与传统疫苗联合使用，可提高疫苗的免疫效果。

Using plant expression systems to produce cytokines has advantages such as low cost, simple culture conditions, and the ability to perform protein post-modification in eukaryotes. Currently, most cytokines expressed in plants use tobacco as a chassis plant, but tobacco is generally not considered safe, which limits its clinical application. Therefore, there is an urgent need for safe and efficient chassis plants to meet market demands.

利用植物表达体系生产细胞因子具有成本低、培养条件简单以及具有真核生物的蛋白后修饰等优点。 目前，在植物中表达的大多数细胞因子都以烟草作为底盘植物，但烟草通常不被认为是安全的，这就限制了其在临床上的应用。 因此，亟需安全和高效的底盘植物去满足市场需要。

[n0004]

Duckweed is the fastest growing and smallest flowering plant in the world, comprising 5 genera and 36 species. It is characterized by asexual reproduction, not competing with food crops for land, small genome size, simple cultivation conditions, and low cultivation cost.

浮萍是世界上生长最快、形态最小的开花植物，包括5个属和36个种，具有无性繁殖、“不与粮争地”、基因组小、培养条件简单及培养成本低等特点。

The commercial application of genetically modified plants is limited because they can easily escape into the environment, while the closed culture mode of duckweed can avoid the pollution of the environment by genetically modified plants. Therefore, duckweed is an ideal

model carrier for producing exogenous proteins, studying growth and development mechanisms, and gene editing. Currently, duckweed has been used as a bioreactor to successfully express some exogenous proteins, including vaccines, antibodies, enzymes, and interferons.

转基因植株因容易逃逸到环境中而限制了其商业化应用，而浮萍的封闭培养模式可避免转基因对环境的污染。 因此，浮萍是一种用于生产外源蛋白、研究生长发育机理以及基因编辑的理想模式载体。目前，利用浮萍作为生物反应器已成功表达了包括疫苗、抗体、酶、干扰素等在内的一些外源蛋白。

[n0005]

Although duckweed can be used as a bioreactor to produce some exogenous proteins, it still suffers from problems such as low conversion efficiency, long screening and regeneration time, and few transgenic positive plants.

虽然浮萍作为生物反应器可用于生产一些外源蛋白，但是整体上还存在着转化效率不高、筛选及再生时间长、获得转基因阳性植物少等问题。

The induction of duckweed callus tissue determines the development of its molecular biology. There are 36 species of duckweed, but only 9 species have established genetic transformation systems. Callus induction depends on genotype selection. When the same

callus induction scheme is used for different strains of the same species of duckweed, there will be large differences in induction rates between strains, and some strains may even fail to induce callus. Liu et al. reported the induction of callus from 100 Lemna aequinoctialis lines using the same induction method. The results showed that only 7 lines could be induced to produce callus [Y.Liu,Y.Wang,S.Xu,X.Tang,J.Zhao,C.Yu,G.He,H.Xu,S.Wang,Y.Tang,C.Fu,Y.Ma,G. Zhou,Efficient genetic transformation and CRISPR/Cas9-mediated genomeediting in Lemna aequinoctialis]. Plant biotechnology journal, (2019).]. Yang Guili et al. reported on callus induction in three different Lemn. minor lines of Lemna minor. Only one line could be induced to produce callus. Based on this, a genetic transformation system was constructed. Although the callus induction rate and transient transfection efficiency were very high, reaching 93% and 80% respectively, the regeneration stage and stable transformation rates were low, only 30% and 4% respectively. [G.L.Yang,Y.Fang,Y.L.Xu,L.Tan,Q.Li,Y.Liu,F.Lai,Y.L.Jin,A. P.Du,K.Z.He, X.R.Ma,H.Zhao,Frond transformation system mediated by Agrobacterium tumefaciens for Lemnaminor.] Plant Molecular Biology 98,319-331(2018).]. Therefore, callus induction is the foundation of duckweed genetic transformation system, and it is essential to establish a more efficient and rapid duckweed expression system for screening and regeneration to produce valuable exogenous proteins. Furthermore, there are currently no reports of using duckweed as a substrate plant to produce cytokines and as a vaccine adjuvant, nor on its immune effects.

浮萍愈伤组织的诱导决定着其分子生物学的发展，浮萍有36个种，但目前只有9个种建立了遗传转化体系。 愈伤组织诱导依赖基因型的选择，同种不同株系的浮萍采用相同的愈伤组织诱导方案，会出现株系之间的诱导率差异大，甚至部分株系无法诱导出愈伤组织的问题。 刘宇等报道了利用同一种诱导方法对100 个浮萍株系进行愈伤组织的诱导，结果表明仅有7个株系能被诱导出愈伤组织[Y.Liu, Y.Wang, S.Xu,X.Tang,J.Zhao,C.Yu,G.He,H.Xu,S.Wang,Y.Tang,C.Fu,Y.Ma,G.Zhou,Efficient genetic transformation and CRISPR/Cas9-mediated genomeediting in Lemna aequinoctialis. Plant biotechnology journal,(2019).]。 杨贵利等报道了对绿萍种的三个不同Lemn.minor株系进行愈伤组织诱导，其中仅有一个株系能被诱导出愈伤组织，在此基础上进行遗传转化体系的构建，虽然愈伤组织诱导率和瞬时转染效率很高分别达到93%和80%，但再生阶段和稳定转化率低，分别仅为30%和4%[G.L.Yang,Y.Fang,Y.L.Xu,L.Tan,Q.Li,Y.Liu,F.Lai,Y.L.Jin,A. P.Du,K.Z.He,X.R.Ma,H.Zhao, Frond transformation system mediated by Agrobacterium tumefaciens for Lemnaminor. Plant Molecular Biology 98,319-331(2018).]。 因此，愈伤组织的诱导是浮萍遗传转化体系的基础，建立更高效快速筛选和再生的浮萍表达体系去生产有价值的外源蛋白是十分必要的。 此外，目前尚未见以浮萍作为底盘植物来生产细胞因子并作为疫苗佐剂及其免疫效果的报道。

[0008]

Summary of the Invention

发明内容

[n0006]

To address the problems of low transformation rate, long regeneration time, and few positive transgenic lines obtained by using duckweed callus as genetic transformation material in existing technologies, as well as the lack of existing technologies that use duckweed as a substrate plant to produce cytokines, this invention provides an efficient duckweed genetic transformation method and its application in the production of exogenous proteins, thereby shortening the screening and regeneration culture time and improving the genetic transformation efficiency. This invention also provides a method for expressing cytokines using duckweed, providing a new approach for cytokine production.

针对现有技术以浮萍愈伤组织作为遗传转化材料存在着转化率低、再生时间长、获得阳性转基因株系少等问题，以及现有技术尚无以浮萍作为底盘植物来生产细胞因子的不足，本发明提供了一种高效浮萍遗传转化方法以及该方法在生产外源蛋白中的应用，以缩短筛选培养和再生培养的时间、提高遗传转化效率，本发明还提供了一种利用浮萍表达细胞因子的方法，为细胞因子的生产提供新方法。

[n0007]

To achieve the above-mentioned objectives, the technical solution adopted by the present invention is as follows:

为实现上述发明目的，本发明采用的技术方案如下：

[n0008]

An efficient method for genetic transformation of duckweed includes the following steps:

一种高效浮萍遗传转化方法，包括以下步骤：

[n0009]

(1) Induction and culture of duckweed callus

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(1)浮萍愈伤组织的诱导培养

[n0010]

Duckweed thallus was inoculated into callus induction medium for induction culture to obtain callus. The induction culture conditions were as follows: photoperiod of (16-14)h:(8-10)h, light intensity of 80-180μmol/mNER1/s, and MS basal medium supplemented with 5-50μM 2,4-dichlorophenoxyacetic acid, 0.45-5μM thidiazuron, 10-30g/L sucrose, and 3-5g/L gellan gum. The pH of the callus induction medium was 5.5-5.7.

将浮萍叶状体接种于愈伤组织诱导培养基中进行诱导培养，得到愈伤组织，诱导培养条件：光周期为(16～14)h:(8～10)h，光照强度为80～180μmol/m²/s，愈伤组织诱导培养基为添加

5～50μM 2,4-二氯苯氧乙酸、0.45～5μM噻苯隆、10～30g/L蔗糖、3～5g/L结冷胶的 MS基础培养基，愈伤组织诱导培养基的pH值为5.5～5.7；

[n0011]

(2) Subculture of duckweed callus

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(2)浮萍愈伤组织的继代培养

[n0012]

Callus tissue was transferred to subculture medium for subculture. Subculture conditions: photoperiod of (16-14)h: (8-10)h, light intensity of 80-180μmol/m²/s, and MS basal medium supplemented with 1-1.5μM 2,4-dichlorophenoxyacetic acid, 0.2-0.25μM 6-benzylaminopurine, 25-30g/L sucrose, and 0.3-0.4g/L gellan gum. The pH of the subculture medium was 5.5-5.7.

将愈伤组织转接至继代培养基中进行继代培养，继代培养条件：光周期为(16～14)h: (8～10)h，光照强度为80～180μmol/m²/s，继代培养基为添加1～1.5μM 2,4-二氯苯氧乙酸、 0.2～0.25μM 6-苄氨基腺嘌呤、25～30g/L蔗糖、0.3～0.4g/L结冷胶的MS基础培养基，继代培养基的pH值为5.5～5.7；

[n0013]

(3) Infecting callus tissue with Agrobacterium containing the target gene.

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(3)利用含目的基因的农杆菌侵染愈伤组织

[n0014]

A plant expression vector containing the target gene was constructed, and the constructed plant expression vector containing the target gene was transformed into Agrobacterium. The Agrobacterium transformed with the target gene was used to infect callus tissue. The infected callus tissue was then inoculated into a co-culture medium and cultured in the dark for 3–4 days. The co-culture medium was MS basal medium supplemented with 1–1.5 μM 2,4-dichlorophenoxyacetic acid, 0.2–0.25 μM 6-benzylaminopurine, 80–120 μM acetylsylgenone, 25–30 g/L sucrose, and 0.3–0.4 g/L gellan gum. The pH of the co-culture medium was 5.5–5.7.

构建含目的基因的植物表达载体，将构建的含目的基因的植物表达载体转入农杆菌，用转入了目的基因的农杆菌侵染愈伤组织，将侵染后的愈伤组织接种至共培养培养基中，在黑暗条件下培养3～4天，共培养培养基为添加1～1.5μM 2,4-二氯苯氧乙酸、0.2～0.25μM 6-苄氨基腺嘌呤、80～120μM乙酰丁香酮、25～30g/L蔗糖、0.3～0.4g/L结冷胶的MS基础培养基，共培养培养基的pH值为5.5～5.7；

[n0015]

(4) Screening and culture of callus tissue infected with Agrobacterium containing the target gene

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(4)含目的基因的农杆菌侵染后的愈伤组织的筛选培养

[n0016]

The co-cultured callus tissue was transferred to a selection medium for selection culture. The selection culture conditions were as follows: photoperiod of (16-14)h:(8-10)h, light intensity of 20-60μmol/m²/s, and MS basal medium supplemented with 1-1.5μM 2,4-dichlorophenoxyacetic acid, 0.2-0.25μM 6-benzylaminopurine, 80-120mg/L selection agent G418, 200-250mg/L cephalosporin, 25-30g/L sucrose, and 0.3-0.4g/L gellan gum. The pH of the selection medium was 5.5-5.7.

将共培养后的愈伤组织转接至筛选培养基上进行筛选培养，筛选培养条件：光周期为 (16～14)h:(8～10)h，光照强度为20～60μmol/m²/s，筛选培养基为添加1～1.5μM 2,4-二氯苯氧乙酸、0.2～0.25μM 6-苄氨基腺嘌呤、80～120mg/L的筛选剂G418、200～250mg/L头孢、25～30g/L蔗糖、0.3～0.4g/L结冷胶的MS基础培养基，筛选培养基的pH值为5.5～5.7；

[n0017]

(5) Regeneration of callus containing the target gene

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(5)含目的基因的愈伤组织的再生

[n0018]

The selected callus tissue containing the target gene was transferred to regeneration medium for regeneration culture to obtain transgenic duckweed containing the target gene (complete transgenic duckweed line). The regeneration culture conditions were as follows: 24-hour photoperiod under full light, light intensity of 80-180 μmol/mNER4/s, regeneration culture time of 28-32 days, and the regeneration medium was 1/2SH medium supplemented with 4-6 g/L sucrose, 80-120 mg/L screening agent G418, 200-250 mg/L cephalosporin, and 0.3-0.4 g/L gellan gum. The pH of the regeneration medium was 5.5-5.7.

将筛选得到的含目的基因的愈伤组织转接至再生培养基中进行再生培养，得到含目的基因的转基因浮萍(完整的转基因浮萍株系)；再生培养的条件：光周期为24h全光照，光照强度为80～180μmol/m²/s，再生培养时间为28～32天，再生培养基为添加4～6g/L蔗糖、80～120 mg/L筛选剂G418、200～250mg/L头孢、0.3～0.4g/L结冷胶的1/2SH培养基，再生培养基的 pH值为5.5～5.7。

[n0019]

In the above-mentioned efficient duckweed genetic transformation method, the method for obtaining duckweed thallus in step (1) is as follows:

上述高效浮萍遗传转化方法的技术方案中，步骤(1)所采用的浮萍叶状体的获取方法如下：

[n0020]

Duckweed thallus specimens preserved in the seed resource bank were selected and inoculated into a pre-culture medium for pre-culture. The pre-culture conditions were: temperature 25±1°C, photoperiod of (16～14)h:(8～10)h, light intensity of 80～180μmol /m²/s, and the pre-culture medium was SH liquid medium supplemented with 10～15g/L sucrose. The pH of the pre-culture medium was 5.6～5.7.

挑取保存于种子资源库的浮萍叶状体，接种于预培养基中进行预培养，预培养条件：温度25±1°C，光周期为(16～14)h:(8～10)h，光照强度为80～180μmol/m²/s，预培养基为添加10～15g/L蔗糖的SH液体培养基，预培养基的pH值为5.6～5.7。

During pre-culture, the thallus is cultured until it reaches the amount required for subsequent steps. The specific amount can be determined based on factors such as the duckweed strain and the state of the duckweed thallus. After the pre-culture is completed, duckweed thallus in good growth condition is selected for step (1).

预培养时，预培养至叶状体达到后续步骤所需要的量即可，具体可根据浮萍株系、浮萍叶状体的状态等因素进行确定；预培养结束后，选取生长状态良好的浮萍叶状体用于步骤(1)。

[n0021]

In step (2) of the above-mentioned efficient duckweed genetic transformation method, it is preferable to change the subculture medium once a month during subculture.

上述高效浮萍遗传转化方法的技术方案的步骤(2)在继代培养时，优选每隔一个月更换一次继代培养基。

[n0022]

In step (3) of the above-mentioned efficient duckweed genetic transformation method, after the plant expression vector containing the target gene is transformed into Agrobacterium, single colonies that have been successfully transformed are selected, inoculated into LB liquid medium, and cultured at 28±1°C with shaking until OD₆₀₀ is 0.6-0.8. Then, the bacterial cells are collected and resuspended in MS basal medium supplemented with 6-6.5 mM mannitol and 95-100μM acetylsyl syringone until OD₆₀₀ is 1-1.2 to obtain a resuspended bacterial solution. The resuspended bacterial solution is used to infect callus tissue at 28±1°C for 10-15 min to obtain the infected callus tissue.

上述高效浮萍遗传转化方法的技术方案的步骤(3)中，含目的基因的植物表达载体转入农杆菌之后，挑选转化成功的单菌落，接种于LB液体培养基中，在28±1°C振荡培养至OD₆₀₀为0.6～0.8，然后收集菌体，用添加了6～6.5mM甘露醇、95～100μM乙酰丁香酮的MS基础培养基重新悬浮菌体至OD₆₀₀为1～1.2，得到重悬菌液；用重悬菌液在28±1°C侵染愈伤组织10～15min，即得到侵染后的愈伤组织。

The preferred Agrobacterium species used is Agrobacterium GV3101.

所采用的农杆菌优选为农杆菌GV3101。

[n0023]

In step (4) of the above-mentioned efficient duckweed genetic transformation method, the screening culture time is preferably 28 to 32 days, and the screening culture medium does not need to be changed during the screening culture process.

上述高效浮萍遗传转化方法的技术方案的步骤(4)中，筛选培养的时间优选为28～32 天，筛选培养过程中不用更换筛选培养基。

[n0024]

In step (5) of the above-mentioned efficient duckweed genetic transformation method, the regeneration culture medium does not need to be replaced during the regeneration culture process.

上述高效浮萍遗传转化方法的技术方案的步骤(5)中，再生培养过程中不用更换再生培养基。

[n0025]

In the above-mentioned efficient duckweed genetic transformation method, it is preferable to carry out each step at a temperature of 25±1°C.

上述高效浮萍遗传转化方法的技术方案中，优选将各步骤均在25±1°C的温度条件下进行培养。

[n0026]

In the above-mentioned efficient duckweed genetic transformation method, the duckweed thallus is preferably derived from duckweed, for example, duckweed thallus can be derived from duckweed M0157.

上述高效浮萍遗传转化方法的技术方案中，所述浮萍叶状体优选来源于绿萍，例如，浮萍叶状体可来源于绿萍M0157。

[n0027]

In the above-mentioned efficient duckweed genetic transformation method, the photoperiod is (16-14)h:(8-10)h, which means that the light time is 16-14 hours and the corresponding darkness time is 8-10 hours.

上述高效浮萍遗传转化方法的技术方案中，光周期为(16～14)h:(8～10)h是指一天之中，光照时间为16～14小时，相应地，黑暗时间为8～10小时。

[n0028]

This invention also provides the application of the above method in the production of exogenous proteins.

本发明还提供了上述方法在生产外源蛋白中的应用。

Furthermore, in application, the transgenic duckweed containing the target gene obtained using the above-mentioned efficient duckweed genetic transformation method is expanded and cultured, and the target gene expresses the target protein in the transgenic duckweed, thus obtaining transgenic duckweed containing the target exogenous protein.

进一步地，在应用时，将利用上述高效浮萍遗传转化方法得到的含目的基因的转基因浮萍进行扩培，目的基因在转基因浮萍中表达目的蛋白，得到含目的外源蛋白的转基因浮萍。

[n0029]

Furthermore, in the above applications, the culture medium used for the propagation of transgenic duckweed containing the target gene is SH medium supplemented with 5-10 g/L sucrose, 80-120 mg/L screening agent G418, and 200 mg/L cephalosporin, and the pH value of the culture medium is 5.5-5.7.

更进一步地，上述应用中，对含目的基因的转基因浮萍进行扩培所采用的培养基为添加 5～10g/L蔗糖、80～120mg/L筛选剂G418、200mg/L头孢的SH培养基，所述培养基的pH 值为5.5～5.7。

[n0030]

This invention also provides a method for expressing cytokines using duckweed. The method first uses the above-mentioned efficient duckweed genetic transformation method to obtain transgenic duckweed containing the target gene. When obtaining transgenic duckweed containing the target gene, the target gene used is a gene encoding cytokines. Then, the transgenic duckweed containing the target gene is expanded and cultured, thus realizing the expression of cytokines using duckweed.

本发明还提供了一种利用浮萍表达细胞因子的方法，该方法先利用上述高效浮萍遗传转化方法获得含目的基因的转基因浮萍，在获得含目的基因的转基因浮萍时，所采用的目的基因为编码细胞因子的基因，然后对获得的含目的基因的转基因浮萍进行扩培，即实现了利用浮萍表达细胞因子。

[n0031]

Furthermore, in the above-mentioned method for expressing cytokines using duckweed, when expanding the transgenic duckweed containing the target gene, the culture medium used is SH medium supplemented with 5-10 g/L sucrose, 80-120 mg/L screening agent G418, and 200 mg/L cephalosporin, and the pH value of the culture medium is 5.5-5.7.

进一步地，上述利用浮萍表达细胞因子的方法的技术方案中，在对含目的基因的转基因浮萍进行扩培时，所采用的培养基为添加5～10g/L蔗糖、80～120mg/L筛选剂G418、200mg/L 头孢的SH培养基，所述培养基的pH值为5.5～5.7。

[n0032]

Furthermore, in the above-mentioned technical solution of the method for expressing cytokines using duckweed, the gene encoding the cytokine includes an interleukin gene. For example, a feasible interleukin gene is the chicken interleukin gene IL-17B. Of course, the genes encoding cytokines are not limited to the chicken interleukin gene IL-17B.

进一步地，上述利用浮萍表达细胞因子的方法的技术方案中，所述编码细胞因子的基因包括白介素基因，例如，一种可行的白介素基因为鸡白介素基因IL-17B，当然，编码细胞因子的基因并不仅仅包括鸡白介素基因IL-17B。

[n0033]

This invention demonstrates through animal experiments that combining transgenic duckweed containing IL-17B protein obtained using the method described in this invention for expressing cytokines from duckweed with chicken bronchitis vaccine H120, and feeding transgenic duckweed containing IL-17B protein while injecting H120 vaccine, can increase mucosal antibodies, enhance the ability to defend against viruses, and inhibit the proliferation of viruses in tissues.

本发明通过动物实验证实，将利用本发明所述利用浮萍表达细胞因子的方法得到的含 IL-17B蛋白的转基因浮萍与鸡支气管炎疫苗H120组合使用，在注射疫苗H120的同时饲喂含IL-17B蛋白的转基因浮萍，可以增加黏膜抗体，增强对病毒防御能力，能够抑制病毒在组织中的增殖。

The above experimental results demonstrate that the cytokine (IL-17B protein) expressed by the method of the present invention has vaccine adjuvant activity, and the method of expressing cytokines described in the present invention does not cause problems of cytokine inactivation or low activity.

以上实验结果说明，通过本发明的方法表达的细胞因子(IL-17B蛋白)是具有疫苗佐剂的活性的，本发明所述表达细胞因子的方法并不会造成细胞因子失活或活性低的问题。

The transgenic duckweed containing IL-17B protein obtained by the method described in this invention can be used as an adjuvant for mucosal vaccines. Alternatively, the IL-17B protein in the transgenic duckweed containing IL-17B protein can be isolated and purified for use as an adjuvant for mucosal vaccines.

采用本发明所述方法获得的含IL-17B蛋白的转基因浮萍可以作为黏膜疫苗佐剂使用，当然，也可以将含IL-17B蛋白的转基因浮萍中的IL-17B蛋白分离纯化出来作为黏膜疫苗佐剂使用。

[n0034]

Compared with the prior art, the technical solution of the present invention has the following beneficial technical effects:

与现有技术相比，本发明的技术方案产生了以下有益的技术效果：

[n0035]

1.

1.

This invention provides an efficient method for the genetic transformation of duckweed. The method has a high callus induction rate, and the resulting callus is dark green and embryogenic, with high infection efficiency. When screening and culturing the callus, the method can achieve the screening of transformed and untransformed callus within one month. When regenerating the screened callus, it only takes one month to regenerate all the callus into complete and morphologically normal plants.

本发明提供了一种高效浮萍遗传转化方法，该方法的愈伤组织诱导率高，得到的愈伤组织呈深绿色且为胚性愈伤组织，侵染效率高；在对愈伤组织进行筛选培养时，能够在一个月实现转化与未转化的愈伤组织的筛选；在对筛选出的愈伤组织进行再生培养时，仅需一个月即可将所有的愈伤组织再生为完整的、形态正常的植株。

Compared with existing technologies, this invention has the advantages of higher screening and regeneration efficiency.

相对于现有技术，本发明具有筛选和再生效率更高的特点。

[n0036]

2.

2.

Based on the efficient duckweed genetic transformation method described in this invention, this invention also provides the application of this method in the production of exogenous proteins, providing a certain methodological basis for the production of exogenous proteins in duckweed bioreactors.

以本发明所述高效浮萍遗传转化方法为基础，本发明还提供了该方法在生产外源蛋白中的应用，为浮萍生物生物反应器生产外源蛋白提供了一定的方法基础。

[n0037]

3.

3.

Based on the efficient duckweed genetic transformation method described in this invention, this invention also provides a method for expressing cytokines using duckweed, filling the gap in the existing technology for producing cytokines using duckweed as a substrate plant. This

solves the safety issues and clinical limitations of using tobacco as a substrate plant to express cytokines in the existing technology, and can better meet the needs of practical applications.

以本发明所述高效浮萍遗传转化方法为基础，本发明还提供了利用浮萍表达细胞因子的方法，填补了现有技术尚无以浮萍作为底盘植物来生产细胞因子的空白，解决了现有技术以烟草作为底盘植物来表达细胞因子存在的安全性问题和临床使用受限的问题，可以更好地满足实际应用需求。

[n0038]

4.

4.

This invention demonstrates through animal experiments that combining transgenic duckweed containing IL-17B protein obtained using the method described in this invention for expressing cytokines from duckweed with chicken bronchitis vaccine H120, and feeding the transgenic duckweed containing IL-17B protein while injecting the H120 vaccine, can increase mucosal antibodies, enhance the ability to defend against viruses, and inhibit the proliferation of viruses in tissues.

本发明通过动物实验证实，将利用本发明所述利用浮萍表达细胞因子的方法得到的含 IL-17B蛋白的转基因浮萍与鸡支气管炎疫苗H120组合使用，在注射疫苗H120的同时饲喂含 IL-17B蛋白的转基因浮萍，可以增加黏膜抗体，增强对病毒防御能力，能够抑制病毒在组织中的增殖。

This indicates that the cytokine (IL-17B protein) expressed by the method of the present invention has vaccine adjuvant activity, and the method of expressing cytokines described in the present invention does not cause problems of cytokine inactivation or low activity.

说明通过本发明的方法表达的细胞因子(IL-17B蛋白)是具有疫苗佐剂的活性的，本发明所述表达细胞因子的方法并不会造成细胞因子失活或活性低的问题。

This invention provides a more convenient, lower-cost, and safer method for the production of cytokines.

本发明为细胞因子的生产提供了更方便、更低成本和更安全的方法。

[0042]

Attached Figure Description

附图说明

[n0039]

Figure 1 shows the callus induction rate of experimental groups T-1 to T-6 in Example 1.

图1是实施例1中的T-1～T-6实验组的愈伤组织诱导率。

[n0040]

Figure 2 shows photographs of the T-3 experimental group of Example 1 and the callus tissue obtained by induction culture in Comparative Example 1.

图2是实施例1的T-3实验组和对比例1中诱导培养得到的愈伤组织的照片。

[n0041]

Figure 3 is a photograph of the callus tissue obtained by screening and culturing under different concentrations of G418 in Example 2.

图3是实施例2在不同浓度的G418的条件下进行筛选培养得到的愈伤组织的照片。

[n0042]

Figure 4 is a schematic diagram of the construction of vectors pCambia2301:GUS and pCambia2301:17B.

图4是载体pCambia2301:GUS和载体pCambia2301:17B的构建示意图。

[n0043]

Figure 5 shows the screening and culture results of duckweed callus containing reporter genes and target genes, respectively.

图5的(A)(B)两图分别是含报告基因和含目的基因的浮萍愈伤组织的筛选培养结果。

[n0044]

Figure 6 shows the regeneration culture results of duckweed callus containing reporter gene and target gene, respectively.

图6的(A)(B)两图分别是含报告基因和含目的基因的浮萍愈伤组织的再生培养结果。

[n0045]

Figure 7 shows the DNA level identification results of transgenic IL-17B and the reporter gene gus duckweed.

图7是转基因IL-17B和报告基因gus浮萍的DNA水平鉴定结果。

[n0046]

Figure 8 shows the protein level identification results of transgenic IL-17B and the reporter gene gus (Lysimachia foetida).

图8是转基因IL-17B和报告基因gus浮萍的蛋白水平鉴定结果。

[n0047]

Figure 9 shows the antibody titer determination results in serum from Example 9 and Comparative Example 3.

图9实施例9和对比例3中的血清中抗体效价测定结果。

[n0048]

Figure 10 shows the results of mucosal antibody assays in the trachea and intestines of Example 10 and Comparative Example 4.

图10实施例10和对比例4气管和肠道的黏膜抗体测定结果。

[n0049]

Figure 11 shows the viral load determination results in tissues of Example 11 and Comparative Example 5.

图11实施例11和对比例5组织中病毒载量测定结果。

[0054]

Detailed Implementation

具体实施方式

[n0050]

The following examples further illustrate the efficient duckweed genetic transformation method and its application in expressing cytokines using duckweed provided by the present invention.

下面通过实施例对本发明提供的高效浮萍遗传转化方法及应用与利用浮萍表达细胞因子的方法作进一步说明。

It should be noted that the following embodiments are only used to further illustrate the present invention and should not be construed as limiting the scope of protection of the present invention. Those skilled in the art can make some non-essential improvements and adjustments to the present invention based on the above-described invention content, and these improvements and adjustments still fall within the scope of protection of the invention.

有必要指出，以下实施例只用于对本发明作进一步说明，不能理解为对本发明保护范围的限制，所属领域技术人员根据上述发明内容，对本发明做出一些非本质的改进和调整进行具体实施，仍属于发明保护的范围。

[n0051]

Unless otherwise specified, the experimental methods used in the following examples and comparative examples are conventional methods in this technical field; the reagents and instruments used are commercially available unless otherwise specified; the duckweed used is Lemnagibba M0157, which is preserved in the germplasm resource bank of the Chengdu Institute of Biology, Chinese Academy of Sciences; the SH, MS, B5 and LB media were purchased from Beijing Coolabor Technology Co., Ltd.; the 2,4-dichlorophenoxyacetic acid (2,4-D), thidiazuron (chemical name N-phenyl-N-1,2,3-thiadiazole-5-urea, TDZ), 6-

benzylaminopurine (6-BA), 2-isopentene adenine (2-IP), and 1-naphthaleneacetic acid (NAA) were purchased from Beijing Solarbio Science & Technology Co., Ltd.; the His antibody was purchased from Beijing TransGen Biotech Co., Ltd.

下述各实施例和对比例中，所使用的试验方法，如无特殊说明，均为本技术领域的常规方法；所使用的试剂、仪器等，如无特殊说明，均可从商业途径获得；所采用的浮萍为绿萍 M0157，属于Lemnagibba，保存于中国科学院成都生物研究所种质资源库；所述SH、MS、B5和LB培养基均购于北京酷来博有限公司；所述2,4-二氯苯氧乙酸(2,4-D)、噻苯隆(化学名称为N-苯基-N-1,2,3-噻二唑-5-脲，TDZ)、6-苄氨基腺嘌呤(6-BA)、2-异戊烯腺嘌呤(2-IP)、 1-萘乙酸(NAA)均购于北京索莱宝科技有限公司；His抗体购置于北京全式金生物技术股份有限公司。

Agrobacterium GV3101 can be purchased from Beijing Tianenze Gene Technology Co., Ltd.

农杆菌GV3101可以从北京天恩泽基因科技有限公司购买得到。

[n0052]

Example 1: Comparison of induction efficiency of duckweed callus under different induction culture conditions

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实施例1：不同诱导培养条件对浮萍愈伤组织的诱导效率比较

[n0053]

In this embodiment, the effect of different induction culture conditions on the induction efficiency of duckweed callus was investigated. The steps are as follows:

本实施例中，考察不同的诱导培养条件对浮萍愈伤组织诱导效率的影响，步骤如下：

[n0054]

(1) Pre-culture of duckweed thallus

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(1)浮萍叶状体的预培养

[n0055]

Duckweed thallus specimens preserved in the seed resource bank were selected and inoculated into a pre-culture medium for pre-culture. The culture conditions were: temperature 25±1℃, photoperiod 16h:8h, light intensity 100μmol/m²/s, and the pre-culture medium was SH liquid medium with 10g/L sucrose added. The pH of the pre-culture medium was 5.6.

挑取保存于种子资源库的浮萍叶状体，接种于预培养基中进行预培养，培养条件：温度 25±1℃，光周期为16h:8h，光照强度为100μmol/m²/s，预培养基为添加10g/L蔗糖的SH 液体培养基，预培养基的pH值为5.6。

[n0056]

(3) Induction and culture of duckweed callus

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(3)浮萍愈伤组织的诱导培养

[n0057]

The duckweed thallus precultured in step (1) until it reached a certain number and was in good growth condition was inoculated into various callus induction culture media and induced under different conditions to obtain duckweed callus tissue.

将步骤(1)预培养至浮萍叶状体达到一定数量且生长状态良好的浮萍叶状体分别接种于多种愈伤组织诱导培养基中，在不同的条件下进行诱导培养，得到浮萍愈伤组织。

This step was carried out simultaneously in 7 experimental groups (T-1 to T-7). The callus induction culture medium and induction culture conditions used in each group are shown in Table 1.

该步骤一共分7个实验组(T-1～T-7实验组)同时进行，各组采用的愈伤组织诱导培养基和诱导培养条件如表1所示。

[n0058]

Table 1. Callus induction and culture conditions

表1愈伤组织诱导培养条件

[n0060]

The callus induction rate was calculated for each group of duckweed according to the formula: callus induction rate = leaf of induced callus / total leaf. The results are shown in Figure 1.

按照公式：愈伤组织诱导率＝诱导出的愈伤组织的叶片/总叶片，计算各组浮萍的愈伤组织诱导率，结果如图1所示。

As shown in Figure 1, the callus induction rate of duckweed varied significantly under different induction culture conditions, indicating that duckweed callus is dependent on certain concentrations of hormones and certain induction culture media.

由图1可知，在不同诱导培养条件下，浮萍的愈伤组织诱导率差异显著，说明浮萍愈伤组织对某些浓度的激素和某些诱导培养基具有依赖性。

Among them, the induction rate of duckweed callus in the T-3 experimental group was as high as 83%, and the photograph of the duckweed callus obtained by induction culture is shown in the left figure of Figure 2.

其中，T-3 实验组的浮萍愈伤组织的诱导率高达83%，诱导培养得到的浮萍愈伤组织的照片如图2的左图所示。

[n0061]

Comparative Example 1: Induction and culture of duckweed callus using the method described in CN 110616182 A

对比例1：运用CN 110616182 A的方法诱导培养浮萍愈伤组织

[n0062]

The method of Example 1 in CN 110616182 A was used to induce and culture duckweed callus tissue, specifically Y3 medium.

采用CN 110616182 A中实施例1方法诱导培养浮萍愈伤组织，具体采用的培养基为Y3 培养基。

The photograph of the duckweed callus tissue obtained by induced culture is shown in the right image of Figure 2.

诱导培养得到的浮萍愈伤组织的照片如图2的右图所示。

[n0063]

As shown in Figure 2, the duckweed callus obtained by induction culture using Y3 medium according to the method of Example 1 in CN 110616182 A is yellow in color, smaller in size and looser in structure; while the duckweed callus obtained by induction culture under the T-3 group according to the method of Example 1 of this invention is green and the tissue is harder.

由图2可知，采用CN 110616182 A中实施例1的方法利用Y3培养基诱导培养得到的浮萍愈伤组织整体呈现黄色、形态更小且疏松；而采用本发明实施例1的方法由T-3组的诱导培养条件得到的浮萍愈伤组织呈绿色、组织更坚硬。

The two have distinct differences in form.

二者的形态具有明显差异。

Further comparisons were made of the composition and content of the induction culture medium used by the two methods, as well as the differences in hormone concentration, as shown in Table 2.

进一步比较二者所采用的诱导培养基的成分与含量以及激素浓度的差异，如表2所示。

[n0064]

Table 2 Comparison of the composition and content of induction culture medium and hormone concentration

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表2诱导培养基的成分与含量以及激素浓度比较

[n0066]

As shown in Table 2, the induction medium used in the T-3 experimental group of the present invention has significant differences in composition compared with the induction medium used in CN 110616182 A. For example, there are significant differences in the types and amounts of macroelements, as well as significant differences in the amounts of microelements and carbon sources, and differences in the content of organic components.

由表2可知，本发明的T-3实验组所用诱导培养基与CN 110616182 A所用诱导培养基的成分有明显差别，例如，大量元素的种类和用量有明显差异，微量元素和碳源的用量也有较大的差异，有机成分的含量也存在差异。

Meanwhile, there were also significant differences in hormone levels among the induction culture cultures.

同时，诱导培养基种的激素水平也存在显著的差异。

It is precisely these differences that lead to significant differences in the morphology of duckweed callus tissues obtained from the two induced cultures.

正是这些差异的存在，使得二者诱导培养获得的浮萍愈伤组织的形态出现了明显差异。

[n0067]

Example 2: Screening and culture of duckweed callus

实施例2：浮萍愈伤组织的筛选培养

[n0068]

(1) The duckweed callus tissue obtained by the T-3 experimental group in Example 1 was transferred to the subculture medium for subculture. The subculture conditions were: temperature 25±1°C, photoperiod 16h:8h, light intensity 100μmol/m²/s. The subculture medium was changed once a month during the subculture period. The subculture medium was MS basal medium supplemented with 1μM 2,4-dichlorophenoxyacetic acid, 0.2 μM 6-benzylaminopurine, 30g/L sucrose, and 0.35g/L gellan gum. The pH of the subculture medium was 5.6.

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(1)将实施例1的T-3实验组诱导培养得到的浮萍愈伤组织转接至继代培养基中进行继代培养，继代培养条件：温度25±1°C，光周期为16h:8h，光照强度为100μmol/m²/s，继代培养期间，一个月更换一次继代培养基；继代培养基为添加1μM 2,4-二氯苯氧乙酸、0.2μM 6-苄氨基腺嘌呤、30g/L蔗糖、0.35g/L结冷胶的MS基础培养基，继代培养基的pH值为5.6。

[n0069]

(2) The duckweed callus obtained by subculture was inoculated onto the screening medium for screening culture. The screening culture conditions were: temperature 25±1°C, photoperiod 16h:8h, and light intensity 100μmol/m²/s.

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(2)将继代培养获得的浮萍愈伤组织接种至筛选培养基上进行筛选培养，筛选培养条件：温度25±1°C，光周期为16h:8h，光照强度为100μmol/m²/s。

[n0070]

In this step, a total of 6 experimental groups were set up. The screening medium used in each experimental group contained different concentrations of screening agent G418. The screening medium of each experimental group was obtained by adding 0 mg/L, 10 mg/L, 20 mg/L, 50 mg/L, 80 mg/L and 100 mg/L of G418 to the subculture medium described in step (1).

该步骤中，共设置6个实验组，各实验组采用的筛选培养基中含有不同浓度的筛选剂 G418，各实验组的筛选培养基是在步骤(1)所述继代培养基的基础上添加0mg/L、10mg/L、 20mg/L、50mg/L、80mg/L、100mg/L的G418得到的。

[n0071]

The screening results are shown in Figure 3. When the concentration of G418 is 20 mg/L, the growth of callus tissue is slow and the color turns yellow, but there is no whitening.

筛选结果如图3所示，当G418的浓度为20mg/L时，愈伤组织的生长较缓慢，颜色变黄，但没有出现变白的情况。

When the concentration of G418 reaches 50 mg/L, the callus tissue gradually turns white and the growth rate slows down further.

当G418的浓度达到50mg/L时，愈伤组织逐渐变白，生长速度进一步变缓。

When the concentration of G418 is 100 mg/L, the growth of callus tissue is inhibited and begins to whiten.

当G418的浓度为100mg/L时，愈伤组织的生长受阻且开始白化。

Therefore, in the subsequent transgenic screening experiment, the G418 concentration of 100 mg/L was selected for screening. This ensured that most of the non-transgenic callus tissue was whitened, while also allowing for the screening of transgenic callus tissue.

因此，在后续转基因筛选实验选取G418浓度为100mg/L的条件进行筛选，既能保证未转基因的愈伤组织大部分白化，又能筛选到转基因的愈伤组织。

[n0072]

Example 3: Rapid regeneration of duckweed callus

实施例3：浮萍愈伤组织的快速再生

[n0073]

The duckweed callus tissue obtained in Example 2 under the condition of G418 concentration of 100 mg/L was transferred to the regeneration medium for regeneration culture. The regeneration culture conditions were: temperature 25±1°C, photoperiod of 24h full light, light intensity of 100 μmol/m²/s. The regeneration medium did not need to be changed during the regeneration period. The regeneration medium was 1/2SH medium with 5 g/L sucrose and 0.35 g/L gellan gum added, and the pH value of the regeneration medium was 5.6.

将实施例2在G418浓度为100mg/L的条件下筛选得到的浮萍愈伤组织转接至再生培养基中进行再生培养，再生培养条件：温度25±1°C，光周期为24h全光照，光照强度为100μmol/m²/s，再生期间不用更换再生培养基，再生培养基为添加5g/L蔗糖、0.35g/L结冷胶的1/2SH培养基，再生培养基的pH值为5.6。

[n0074]

After 15 days of regeneration culture, the selected duckweed callus tissue clearly showed that leaves and roots differentiated from the callus tissue. After another 15 days of culture, all the callus tissue regenerated into complete plants, and the regenerated leaves were all in normal shape, with a regeneration rate as high as 94%.

筛选出的浮萍愈伤组织在再生培养15天时，能明显看见叶片和根从愈伤组织中分化出来，继续培养15天，所有的愈伤组织都再生出了完整的植株，并且再生出的叶片形状都正常，再生率高达94%。

[n0075]

Comparative Example 2: Regeneration using the method described in CN 110616182 A

对比例2：运用CN 110616182 A的方法进行再生

[n0076]

The regeneration culture was carried out using the method of Example 6 in CN 110616182 A. The results showed that it took 3 months to regenerate a complete plant, and the regeneration rate was only 30%.

采用CN 110616182 A中的实施例6的方法进行再生培养，结果发现，采用该方法需要3 个月的时间才能再生得到完整的植株，并且再生率仅为30%。

Further comparison is made of the differences in the regeneration culture medium used in Example 3 of this application and Example 6 of CN 110616182A during the regeneration culture.

进一步比较本申请实施例3和 CN 110616182A的实施例6在进行再生培养时采用的再生培养基的差异。

The basal medium used in the regeneration culture of this invention is 1/2SH medium, while CN 110616182 A uses B5 medium as the basal medium in the regeneration culture, as shown in Table 3.

本发明在再生培养时所用的基础培养基为1/2SH培养基，CN 110616182 A在再生培养是所采用的基础培养基为 B5培养基，具体如表3所示。

[n0077]

Table 3 Comparison of components and contents of regeneration culture medium

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表3再生培养基的成分与含量比较

[n0080]

As shown in Table 3, there are significant differences in the macro-elements, micro-elements, iron salts, organic components and carbon sources in the regeneration culture medium used in Example 3 of this application and Example 6 of CN 110616182 A during the regeneration culture.

由表3可知，本申请的实施例3和CN 110616182 A的实施例6在进行再生培养时采用的再生培养基中，大量元素、微量元素、铁盐、有机成分和碳源上都具有较为明显的差异。

These differences result in a significant difference in regeneration efficiency between the two. This application can shorten the regeneration time in CN110616182 A from 3 months to 1 month, effectively improving the regeneration efficiency.

这些差异的存在使得二者再生时，在再生效率上出现了明显的差异，本申请可将CN110616182 A中的再生时间由3个月缩短至1个月，有效提高了再生效率。

[n0081]

Example 4: Transferring a target gene or reporter gene into duckweed callus tissue.

实施例4：将含有目的基因或者报告基因转入到浮萍愈伤组织中

[n0082]

The pCambia2301:GUS vector containing the reporter gene was used as the reference group for the duckweed genetic transformation system. The genetic transformation efficiency of the system was measured by the GUS staining results of callus tissue and transgenic leaves.

将含有报告基因的pCambia2301:GUS载体作为浮萍遗传转化体系的参照组，通过愈伤组织和转基因叶片GUS染色结果来衡量该体系的遗传转化效率。

In addition, the gus gene in pCambia2301:GUS was replaced with the cytokine chicken interleukin IL-17B to construct the vector pCambia2301:17B.

此外，将pCambia2301:GUS 中的gus基因替换为细胞因子—鸡白介素IL-17B，构建载体pCambia2301:17B。

Figure 4 shows a schematic diagram of the construction of vectors pCambia2301:GUS and pCambia2301:17B.

载体pCambia2301:GUS和载体pCambia2301:17B的构建示意图见图4。

[n0083]

Vectors pCambia2301:GUS and pCambia2301:17B were transformed into Agrobacterium GV3101. After colony PCR verification, the cells were inoculated into LB medium and cultured at 28±1°C and 150 rpm until the bacterial OD_NER12 was about 0.6. The cells were collected and resuspended in MS medium (containing 6 mM mannitol, 100 μM acetylsyl syringone, pH=5.6) until the OD_NER13 reached 1.2. The cells were then incubated at 28±1°C for 1 h, and

green duckweed callus was added and allowed to stand for 10 min. After removing the bacterial solution, the callus was placed on co-culture medium and incubated in the dark for 3 days.

将载体pCambia2301:GUS和载体pCambia2301:17B转入到农杆菌GV3101中，菌落PCR 验证正确后接种于LB培养基中，在28±1°C、转速150rpm培养至菌体OD₆₀₀为0.6左右，收集菌体并用MS培养基(含有6mM甘露醇、100μM乙酰丁香酮，pH＝5.6)重悬菌体至 OD₆₀₀达到1.2，在28±1°C放置1h，加入绿色状态的浮萍愈伤组织静止10min，去除菌液后将愈伤组织放入共培养培养基上黑暗放置3天。

The co-culture medium was MS basal medium supplemented with 1 μM 2,4-dichlorophenoxyacetic acid, 0.2 μM 6-benzylaminopurine, 100 μM acetylsalicylic acid, 30 g/L sucrose and 0.35 g/L gellan gum, and the pH of the co-culture medium was 5.6.

所述共培养的培养基为添加1μM 2,4-二氯苯氧乙酸、0.2μM 6-苄氨基腺嘌呤、100μM乙酰丁香酮、30g/L蔗糖和0.35g/L结冷胶的 MS基础培养基，共培养培养基的pH值为5.6。

[n0084]

Example 5: Screening and culture of duckweed callus containing the target gene or reporter gene

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实施例5：含目的基因或者报告基因的浮萍愈伤组织的筛选培养

[n0085]

The duckweed callus tissue infected for 3 days in Example 4 was transferred to screening medium for screening culture. The screening culture conditions were: temperature 25±1°C, photoperiod of 16h:8h, light intensity of 40μmol/m²/s, and screening culture time of 1 month. The screening medium was MS basal medium supplemented with 1μM 2,4-dichlorophenoxyacetic acid, 0.2μM 6-benzylaminopurine, 30g/L sucrose, 100mg/L LG418, 200mg/L cephalosporin, and 0.35g/L gellan gum. The pH of the screening medium was 5.6.

将实施例4中侵染3天的浮萍愈伤组织转接至筛选培养基中进行筛选培养，筛选培养条件：温度25±1°C，光周期为16h:8h，光照强度为40μmol/m²/s，筛选培养时间为1个月；筛选培养基为添加1μM 2,4-二氯苯氧乙酸、0.2μM 6-苄氨基腺嘌呤、30g/L蔗糖、100mg/LG418、200mg/L头孢、0.35g/L结冷胶的MS基础培养基，筛选培养基的pH值为5.6。

[n0086]

The screening and culture results are shown in Figure 5, where Figures (A) and (B) show the screening and culture results of duckweed callus containing the reporter gene and the target gene, respectively.

筛选培养结果如图5所示，其中的(A)(B)两图分别是含报告基因和含目的基因的浮萍愈伤组织的筛选培养结果。

As shown in Figure 5, this embodiment can achieve a good screening effect within one month. The unconverted callus tissue eventually turns white under the condition of G418 concentration of 100 mg/L, while the successfully converted callus tissue grows vigorously and remains green under the condition of G418 concentration of 100 mg/L.

由图5可知，本实施例在一个月即可达到很好的筛选效果，未转化的愈伤组织在G418浓度为100mg/L的条件下最终变白，而转化成功的愈伤组织在G418 浓度为100mg/L的条件下生长旺盛且颜色依然为绿色。

[n0087]

Example 6: Rapid regeneration of callus containing the target gene or reporter gene

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实施例6：含目的基因或者报告基因的愈伤组织的快速再生

[n0088]

The morphologically normal callus tissue obtained from screening and culturing for one month in Example 5 was transferred to regeneration medium for regeneration culture. The

regeneration culture conditions were: temperature 25±1°C, photoperiod of 24h full light, light intensity of 100μmol/m²/s, and regeneration culture time of 1 month. The regeneration medium was 1/2SH medium supplemented with 5g/L sucrose, 100mg/L G418, 200mg/L cephalosporin, and 0.35g/L gellan gum. The pH value of the regeneration culture was 5.6.

将实施例5中筛选培养一个月得到的形态正常的愈伤组织转入再生培养基中进行再生培养，再生培养条件：温度25±1°C，光周期为24h全光照，光照强度为100μmol/m²/s，再生培养时间为1个月；再生培养基为添加5g/L蔗糖、100mg/L G418、200mg/L头孢、0.35g/L结冷胶的1/2SH培养基，再生培养的pH值为5.6。

[n0089]

The regeneration culture results are shown in Figure 6, where Figures (A) and (B) show the regeneration culture results of duckweed callus containing the reporter gene and the target gene, respectively.

再生培养结果如图6所示，其中的(A)(B)两图分别是含报告基因和含目的基因的浮萍愈伤组织的再生培养结果。

As shown in Figure 6, after one month of regeneration culture, all callus tissues were able to regenerate into complete plants, and nearly 100% of them were tolerant to 100 mg/L of G418.

由图6可知，再生培养一个月，所有的愈伤组织都能再生出完整的植株，且接近100%都耐受100mg/L的G418。

[n0090]

Example 7: DNA level identification of regenerated transgenic plants

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实施例7：再生转基因植株的DNA水平鉴定

[n0091]

The transgenic plants obtained from the regeneration culture in Example 6 were expanded in liquid culture medium under the following conditions: temperature 25±1°C, photoperiod of 24h full light, light intensity of 100μmol/m²/s; the liquid culture medium was SH medium supplemented with 10g/L sucrose, 100mg/L screening agent G418, and 200mg/L cephalosporin, with a pH of 5.6.

将实施例6再生培养得到的转基因植株在液体培养基中进行扩培，扩培条件：温度25±1°C，光周期为24h全光照，光照强度为100μmol/m²/s；液体培养基为添加10g/L蔗糖、100mg/L筛选剂G418、200mg/L头孢的SH培养基，该培养基的pH值为5.6。

A portion of the transgenic plants were selected for DNA-level verification. The specific procedures are as follows:

从中挑取部分转基因植株进行DNA水平验证，具体操作如下：

[n0092]

After grinding the transgenic plants with liquid nitrogen, they were placed in 1.5 mL EP tubes, and 200 μL of DNA extraction buffer was added. The tubes were then placed in a constant temperature metal bath at 70 °C for 30 min. After centrifugation at 13500 × g at 4 °C for 5 min, the supernatant was discarded. 200 μL of isoamyl alcohol was added to the precipitate, and the mixture was incubated at -20 °C for 10 min. After centrifugation at 13500 × g at 4 °C for 5 min, the supernatant was discarded. The precipitate was resuspended in 1 mL of 75% ethanol, and after centrifugation at 13500 × g at 4 °C for 1 min, the supernatant was discarded. The mixture was washed again with 75% ethanol, and the ethanol was allowed to evaporate at room temperature for 10 min. Finally, 100 μL of water was added to dissolve the DNA.

液氮研磨转基因植株后放于1.5mL EP管中，加入200μL的DNA提取液buffer，将其放入恒温金属浴，条件为70°C，30min；在13500×g、4°C离心5min，去上清，沉淀加入 200μL的异戊醇，混匀后在-

20°C放置10min，在13500×g、4°C离心5min，弃上清，加入 1mL的75%的乙醇重悬沉淀，在13500×g、4°C离心1min后弃上清，再重复加入75%乙醇洗涤，室温放置10min挥发乙醇，最后加入100μL水溶解DNA。

The extracted DNA was detected using GUS-specific primers and 17B-specific primers, and then analyzed by agarose gel electrophoresis.

将提取的DNA采用gus 的特异性引物和17B的特性性引物进行检测，并用琼脂凝胶电泳进行检测。

The results are shown in Figure 7. As can be seen from Figure 7, both the gus gene and 17B were successfully transferred into the genome of duckweed.

结果如图7所示，由图7可知，gus基因和17B都成功转入到浮萍的基因组上。

[n0093]

Example 8: Verification of protein levels in regenerated transgenic plants

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实施例8：再生转基因植株的蛋白水平验证

[n0094]

Protein levels of the transgenic duckweed containing the reporter gene gus, obtained after expansion in liquid culture medium in Example 7, were identified. The specific operation was performed according to the gus staining kit. A certain amount of transgenic duckweed leaves were placed in a 5 mL EP tube, and the prepared substrate containing x-gal was added. After mixing, the mixture was placed in the dark at room temperature for 24 h.

对实施例7在液体培养基中扩培后得到的含报告基因gus的转基因浮萍进行蛋白水平鉴定，具体操作按gus染色试剂盒进行，取一定量的转基因浮萍叶片在5mL EP管中，加入配置好的含x-gal的底物，混匀后黑暗常温放置24h。

Discard the staining solution and add anhydrous ethanol to elute until all the duckweed chlorophyll is washed away.

弃掉染色液加入无水乙醇进行洗脱，直到将所有浮萍叶绿素洗脱干净为止。

Finally, the gus protein was observed under a microscope, and the results are shown in Figure 8.

最后进行gus蛋白的显微镜观察，结果如图8所示。

[n0095]

Weigh 1g of fresh weight of transgenic duckweed containing IL-17B protein, grind it in liquid nitrogen, homogenize it with 2 times the volume of PBS buffer, place it on ice for 2h, centrifuge at 13500×g, 4°C for 20min, collect the supernatant and determine its soluble protein content.

称取含IL-17B蛋白的转基因浮萍1g鲜重加入液氮研磨，并用2倍体积的PBS缓冲液匀浆，冰上放置2h，在13500×g、4°C离心20min，收集上清液并测定其可溶性蛋白含量。

Soluble proteins from transgenic plants were isolated using a 10% SDS-PAGE gel, and the isolated proteins were transferred to a PVDF membrane on ice at 95V for 50 min.

用10%SDS-PAGE胶分离转基因植株中的可溶性蛋白，并在冰上将分离的蛋白转移至PVDF膜，转膜条件为95V，50min。

After the transfer is complete, add 5% skim milk and seal for 2 hours.

转膜结束后加入5%脱脂牛奶封闭2h。

After the blocking process, add His-monoclonal antibody diluted 3000 times and incubate overnight.

封闭结束后加入稀释3000 倍的His-单克隆抗体过夜孵育。

After eluting the excess primary antibody with elution buffer, add secondary antibody diluted 10,000 times and incubate for 1 hour. Elute the secondary antibody with elution buffer again, and finally add chemiluminescence staining solution to obtain the results in a chemiluminescence imaging system. The results are shown in Figure 8.

用洗脱液洗脱多余的一抗后加入稀释10000倍的二抗孵育1h，加入洗脱液洗脱二抗，最后加入化学荧光染色液在化学发光成像仪中获取结果，结果如图8所示。

[n0096]

Example 9: Effect of transgenic plants containing the IL-17B gene combined with chicken infectious bronchitis virus vaccine on antibodies in chicken serum.

实施例9：含IL-17B基因的转基因植株结合鸡传染性支气管炎病毒疫苗对鸡血清中抗体的影响

[n0097]

The transgenic duckweed containing IL-17B protein obtained after expansion in liquid culture medium in Example 7 was vacuum freeze-dried and pulverized.

将实施例7在液体培养基中扩培后得到的含IL-17B蛋白的转基因浮萍真空冷冻干燥并粉粹。

Seven-day-old pathogen-free chickens were vaccinated with chicken bronchitis vaccine H120, and each chicken was fed transgenic duckweed powder containing 3 μg of IL-17B protein resuspended in PBS.

对7日龄的无病原菌的鸡接种鸡支气管炎疫苗H120，同时给每只鸡饲喂含IL-17B蛋白3 μg的PBS重悬的转基因浮萍粉末。

Chicks aged 21 days were given a booster vaccine H120 under the same vaccination conditions as the initial vaccination and fed the same amount of transgenic duckweed powder resuspended in PBS.

对21日龄的小鸡按首免相同的接种条件接种加强疫苗 H120，并饲喂相同量的PBS重悬的转基因浮萍粉末。

It is designated as group IL-17B-H120.

记作IL-17B-H120组。

[n0098]

Blood was collected from under the wings of chickens at 0, 7, 14, 21 and 35 days after the first vaccination. The serum was separated and stored at -80°C.

首免后0d，7d，14d，21d，35d进行鸡翅下采血，分离血清，放置于-80°C。

IBV-specific antibodies in serum were detected using an ELISA kit, with the secondary antibody being IgG. The results are shown in Figure 9.

利用ELISA试剂盒检测血清中的IBV特异性抗体，其中二抗为IgG，结果如图9。

As shown in Figure 9, the antibody titer of the IL-17B-H120 group reached over 1200 21 days after the first immunization.

由图9可知，在首免后21d，IL-17B-H120组的抗体效价达到了1200以上。

[n0099]

Comparative Example 3: Effects of wild-type duckweed and PBS buffer combined with chicken infectious bronchitis virus vaccine on antibodies in chicken serum.

对比例3：浮萍野生型植株和PBS缓冲液结合鸡传染性支气管炎病毒疫苗对鸡血清中抗体的影响

[n0100]

In this embodiment, the following three experimental groups were conducted:

本实施例中，共进行如下3个实验组：

[n0101]

The plants of wild-type duckweed M0157 were vacuum freeze-dried and pulverized.

将浮萍野生型M0157的植株真空冷冻干燥并粉粹。

Seven-day-old pathogen-free chickens were vaccinated with chicken bronchitis vaccine H120, and each chicken was fed duckweed powder resuspended in PBS with wild-type duckweed M0157, at the same amount as in Example 9.

对7日龄的无病原菌的鸡接种鸡支气管炎疫苗H120，同时给每只鸡饲喂浮萍野生型M0157的PBS重悬的浮萍粉末，饲喂量与实施例9相同。

Chickens aged 21 days were vaccinated with booster vaccine H120 under the same vaccination conditions as the initial vaccination and fed the same amount of duckweed powder resuspended in PBS for wild-type duckweed M0157.

对21日龄的鸡按首免相同的接种条件接种加强疫苗H120，并饲喂相同量的浮萍野生型M0157的PBS重悬的浮萍粉末。

It is designated as group M0157-H120.

记作M0157-H120组。

[n0102]

The plants of wild-type duckweed M0157 were vacuum freeze-dried and pulverized.

将浮萍野生型M0157的植株真空冷冻干燥并粉粹。

Seven-day-old pathogen-free chickens were vaccinated with chicken bronchitis vaccine H120, and each chicken was fed PBS buffer at the same amount as in Example 9.

对7日龄的无病原菌的鸡接种鸡支气管炎疫苗H120，同时给每只鸡饲喂PBS缓冲液，饲喂量与实施例9相同。

Chickens aged 21 days were vaccinated with booster vaccine H120 under the same vaccination conditions as the first immunization and fed the same amount of PBS buffer.

对21日龄的鸡按首免相同的接种条件接种加强疫苗H120，并饲喂相同量的PBS缓冲液。

This is designated as the PBS-H120 group.

记作PBS-H120组。

[n0103]

PBS buffer was fed directly to 7-day-old pathogen-free chickens at the same amount as in

Example 9.

直接对7日龄的无病原菌的鸡饲喂PBS缓冲液，饲喂量与实施例9相同。

Chickens aged 21 days were fed the same amount of PBS buffer.

对21日龄的鸡饲喂相同量的PBS缓冲液。

This is referred to as the PBS group.

记作PBS组。

[n0104]

Blood was collected from under the wings of chickens at 0, 7, 14, 21 and 28 days after the first vaccination. The serum was separated and stored at -80°C.

首免后0d，7d，14d，21d，28d进行鸡翅下采血，分离血清，放置于-80°C。

IBV-specific antibodies in serum were detected using an ELISA kit, with IgG as the secondary antibody. The results are shown in Figure 9.

利用ELISA试剂盒检测血清中的IBV特异性抗体，其中二抗为IgG，结果如图9所示。

[n0105]

As shown in Figure 9, in the IL-17B-H120 group, which was fed transgenic duckweed containing IL-17B protein while being injected with vaccine H120, the titer of IBV antibody in chicken serum was significantly higher than that in the MO157-H120 group and the PBS-H120 group.

由图9可知，在注射疫苗H120的同时饲喂含IL-17B蛋白的转基因浮萍的IL-17B-H120 组中，鸡血清中的IBV抗体的效价显著高于M0157-H120组和PBS-H120组。

Furthermore, no IBV-specific antibodies were detected in the serum of chickens in the unvaccinated PBS group, indicating that the chickens were not infected with IBV virus during the immunization process.

此外，未接种疫苗的PBS组的鸡血清中，未检测到IBV特异性抗体，表明鸡在免疫过程期间未感染IBV 病毒。

[n0106]

Example 10: Effect of transgenic plants containing the IL-17B gene combined with chicken infectious bronchitis virus vaccine on mucosal antibodies in chicken trachea and intestine.

实施例10：含IL-17B基因的转基因植株结合鸡传染性支气管炎病毒疫苗对鸡气管和肠道中黏膜抗体的影响

[n0107]

Chickens 28 days after the first vaccination in Example 9 were culled, and 8 cm of each of the trachea and intestines were taken and rinsed three times with PBS buffer. The mixtures were then centrifuged at 13500×g and 4°C for 5 min, and the supernatant was collected.

将实施例9中首免后28d的鸡扑杀，分别取气管和肠道各8cm用PBS缓冲液灌洗三次，在13500×g、4℃离心5min，取上清。

The secondary antibody in Example 9 was replaced with sIgA antibody, and the mucosal antibodies in the trachea and intestine were measured in the same way. The results are shown in Figure 10.

将实施例9中的二抗替换为sIgA抗体按同样的方法测定气管和肠道中黏膜抗体，结果如图10所示。

[n0108]

Comparative Example 4: Effects of wild-type duckweed and PBS buffer on mucosal antibodies in the trachea and intestines of chickens immunized against infectious bronchitis virus vaccine.

对比例4：浮萍野生型植株和PBS缓冲液对鸡传染性支气管炎病毒疫苗的免疫气管和肠道中黏膜抗体的影响

[n0109]

Chickens in Comparative Example 3 were culled 28 days after their first immunization. Eight cm sections of their trachea and intestines were taken and rinsed three times with PBS buffer. The trachea and intestines were then centrifuged at 13500×g and 4°C for 5 min, and the supernatant was collected.

将对比例3中首免后28d的鸡扑杀，分别取气管和肠道8cm用PBS缓冲液灌洗三次后，在13500×g、4°C离心5min，取上清。

The secondary antibody in Example 9 was replaced with sIgA antibody, and the mucosal antibodies in the trachea and intestine were measured in the same way. The results are shown in Figure 10.

将实施例9中的二抗替换为sIgA抗体按同样的方法测定气管和肠道中黏膜抗体，结果如图10所示。

[n0110]

As shown in Figure 10, in the IL-17B-H120 group, which was fed transgenic duckweed containing IL-17B protein while being injected with vaccine H120, the level of IBV mucosal antibody sIgA in the chicken trachea and intestine was significantly higher than that in the MO157-H120 group and the PBS-H120 group.

由图10可知，在注射疫苗H120的同时饲喂含IL-17B蛋白实的转基因浮萍的IL-17B-H120 组中，鸡气管和肠道中的IBV黏膜抗体sIgA的水平显著高于M0157-H120组和PBS-H120组。

As shown in Figures 9 and 10, the combination of the IL-17B protein expressed by the transgenic duckweed in this invention with the chicken infectious bronchitis vaccine H120 can increase mucosal antibodies and enhance the ability to defend against viruses.

结合图9和图10可知，本发明中转基因浮萍表达的IL-17B蛋白与鸡传染性支气管疫苗H120 组合使用可以增加黏膜抗体，增强对病毒防御能力。

[n0111]

Example 11: Effect of transgenic plants containing the IL-17B gene combined with chicken infectious bronchitis virus vaccine on viral load in chicken trachea, kidney and lungs.

实施例11：含IL-17B基因的转基因植株结合鸡传染性支气管炎病毒疫苗对鸡气管、肾和肺病毒载量的影响

[n0112]

Chickens that were first immunized in Example 9 were challenged with the virus 28 days later at a dose of 2 MOI IBV SC-MY-19 (GenBank Accession no.).

对实施例9中首免后28d后的鸡进行攻毒，攻毒剂量为2MOI IBV SC-MY-19(GenBank Accession no. MT563407.1), ten days after challenge, all chickens in the group were dissected, and the viral load in the trachea, kidneys and lungs of the chickens was analyzed.

MT563407.1)，攻毒后十天解剖该组所有的鸡，并对鸡的气管、肾和肺中病毒载量分析。

The specific steps are as follows: a standard curve is established by RT-PCR using a plasmid containing a partial fragment of the virus. The viral load in different tissues is calculated based on the standard curve, and the results are shown in Figure 11.

具体步骤为，以含有该病毒部分片段的质粒进行RT-PCR建立标准曲线，不同组织病毒载量根据标准曲线进行计算，结果如图11所示。

[n0113]

Comparative Example 5: Effects of wild-type duckweed plants and PBS buffer combined with chicken infectious bronchitis virus vaccine on viral load in chicken trachea, kidneys, and lungs.

对比例5：浮萍野生型植株和PBS缓冲液结合鸡传染性支气管炎病毒疫苗对鸡气管、肾和肺病毒载量的影响

[n0114]

In Comparative Example 3, chickens in the M0157-H120, PBS-H120, and PBS groups were challenged with 2 MOI IBV SC-MY-19 (GenBank Accession no.) 28 days after their initial immunization.

在对比例3中M0157-H120组、PBS-H120组和PBS组首免后28d后，对各组的鸡进行攻毒，攻毒剂量为2MOI IBV SC-MY-19(GenBank Accession no.
MT563407.1), ten days after challenge, all chickens in each group were dissected, and the viral load in the trachea, kidneys and lungs of the chickens was analyzed.

MT563407.1)，攻毒后十天解剖各组所有的鸡，并对鸡的气管、肾和肺中病毒载量分析。
The specific steps are as follows: a standard curve is established by RT-PCR using a plasmid containing a partial fragment of the virus; the viral load in different tissues is calculated based on the standard curve; and the results are shown in Figure 11.

具体步骤为，以含有该病毒部分片段的质粒进行RT-PCR建立标准曲线，不同组织病毒载量根据标准曲线进行计算，结果如图11所示。

[n0115]

As shown in Figure 11, in the IL-17B-H120 group, which was fed transgenic duckweed containing IL-17B protein while being injected with vaccine H120, the viral load in the trachea, kidneys, and lungs of chickens was significantly lower than that in the MO157-H120 group, PBS-H120 group, and PBS group.

由图11可知，在注射疫苗H120的同时饲喂含IL-17B蛋白实的转基因浮萍的IL-17B-H120 组中，鸡的气管、肾和肺的病毒载量显著低于M0157-H120组、PBS-H120组和PBS组。

This indicates that the combination of the IL-17B protein expressed by the transgenic duckweed in this invention with the chicken infectious bronchitis vaccine H120 can more effectively inhibit the proliferation of the virus in tissues.

表明本发明中转基因浮萍表达的IL-17B蛋白与鸡传染性支气管疫苗H120组合使用能更有效地抑制病毒在组织中的增殖。