CrossMark

RESEARCH

High-Yield Expression of M2e Peptide of Avian Influenza Virus H5N1 in Transgenic Duckweed Plants

Aleksey Firsov · Irina Tarasenko · Tatiana Mitiouchkina ·
Natalya Ismailova · Lyubov Shaloiko ·
Alexander Vainstein · Sergey Dolgov

Abstract Avian influenza is a major viral disease in poultry. Antigenic variation of this virus hinders vaccine development. However, the extracellular domain of the virus-encoded M2 protein (peptide M2e) is nearly invariant in all influenza A strains, enabling the development of a broad-range vaccine against them. Antigen expression in transgenic plants is becoming a popular alternative to classical expression methods. Here we expressed M2e from avian influenza virus A/chicken/Kurgan/5/2005(H5N1) in nuclear-transformed duckweed plants for further development of avian influenza vaccine. The N-terminal fragment of M2, including M2e, was selected for expression. The M2e DNA sequence fused in-frame to the 5′ end of β-glucuronidase was cloned into pBI121 under the control of CaMV 35S promoter. The resulting plasmid was successfully used for duckweed transformation, and western analysis with anti-β-glucuronidase and anti-M2e antibodies confirmed accumulation of the target protein (M130) in 17 independent transgenic lines. Quantitative ELISA of crude protein extracts from these lines showed M130–β-glucuronidase accumulation ranging from 0.09–0.97 mg/g FW (0.12–1.96 % of total soluble protein), equivalent to yields of up to 40 μg M2e/g plant FW. This relatively high yield holds promise for the development of a duckweed-based expression system to produce an edible vaccine against avian influenza.

A. Firsov (✉) · I. Tarasenko · T. Mitiouchkina · N. Ismailova ·
L. Shaloiko · S. Dolgov
Branch of the Shemyakin-Ovchinnikov Institute of Bioorganic
Chemistry of the RAS, Prospekt Nauki, 6, Pushchino,
Moscow region, Russian Federation 142290
e-mail: aleksey_firsov@mail.ru

I. Tarasenko
e-mail: starassenko@rambler.ru

T. Mitiouchkina
e-mail: mitiouchkina@rambler.ru

N. Ismailova
e-mail: natashita-bios@yandex.ru

L. Shaloiko
e-mail: shaloiko@yandex.ru

S. Dolgov
e-mail: dolgov@bibch.ru

A. Vainstein
Robert H. Smith Faculty of Agriculture, Food and
Environment, The Hebrew University of Jerusalem, POB 12,
76100 Rehovot, Israel
e-mail: alexander.vainstein@mail.huji.ac.il

Introduction

Avian influenza is one of the most dangerous diseases to domestic poultry. Mass vaccination of domestic and wild birds is the best method for preventing its spread. This requires a large quantity of vaccines that are inexpensive and convenient for mass immunization. Such vaccines might be plant-produced edible vaccines. The development of plant-based vaccines for veterinary medicine is a rapidly developing field in applied plant molecular biology. Despite the obvious progress in this field [1–3], few examples of successful commercialization of plant-based vaccines are known [4–6].

An important limitation to the commercialization of plant-based vaccines is fear of accidental release of genetically modified plants into the environment during the

Springer

cultivation of transgenic plant producers in the field or greenhouse. Considerable effort has been focused on the development of contained cultivation systems, such as axenic culture of suspension cells or hairy roots. The contained plant systems can be cultivated in bioreactors, preventing the accidental release of genetically modified plant material to the environment [7, 8].

Lemna minor (duckweed) is excellent for use in these contained systems, as it can be effectively and inexpensively cultivated in bioreactors of various types [9, 10]. An additional benefit of duckweed cultivation in bioreactors is secretion of the target product into the culture medium, resulting in simpler and less costly downstream processing and product purification. To date, however, there are only a few examples of successful expression of commercially important proteins in duckweed plants: the industrial enzyme endoglucanase E1 from *Acidothermus cellulolyticus* [11], the monoclonal antibodies anti-CD30, anti-CD20, and anti-interferon α2b [12, Biolex Therapeutics website], and the protective antigen of swine epidemic diarrhea [13]. Moreover, Biolex is currently carrying out clinical trials with recombinant interferon α2b and plasmin expressed in duckweed [10].

Today, the prevailing view among experts is that a "universal" influenza vaccine, effective against many of the circulating strains, can be developed on the basis of the 24-amino acid (aa) extracellular domain of the viral M2 protein (peptide M2e) [14, 15]. M2 is a small (97 aa) protein that functions as a tetrameric ion channel. It is involved in uncoating viral particles in the endosome and subsequently releasing viral RNA into the cytoplasm of host cells. Proper functioning of the M2 protein is critical to viral replication, as well as to blocking the ion channel that prevents infection of a host cell with the virus [16]. The peptide M2e is highly conserved across influenza A subtypes [17] and therefore, an M2e-based vaccine is expected to protect against new influenza virus variants. Conventional influenza vaccines are currently based on variable surface proteins of the influenza virus- hemagglutinin and neuraminidase [18, 19]. However, mutations at their antigenic sites are constantly changing the antigenic properties of these proteins, requiring the continuous development of new vaccines. The production of and trials with transgenic plants are a lengthy process, during which hemagglutinin- and neuraminidase-based vaccines may lose their relevance. Since the M2e sequence has remained almost unchanged for ca. 100 years [20], it is highly suitable for the development of plant-based edible vaccines that will not lose their effectiveness.

There are a number of reports on M2e expression in plants [21–24]. In all of those studies, the M2e peptide was expressed in transient systems using different virus-based vectors. Recombinant M2e reacted with specific antibodies [21] and was used for immunization [22, 23]. Plant-produced M2e peptide as part of a virus-like particle provided complete [22] or partial [23] protection of mice against influenza virus challenge. Nevertheless, we believe that a universal edible vaccine against avian influenza based on nuclear-transformed plants can be a more robust product, which does not require expensive industrial-scale facilities for transient expression.

The aim of this work was to explore the feasibility of M2e expression in nuclear-transformed duckweed (*L. minor* L.) plants. Duckweed is a small aquatic monocotyledonous plant. A rapid growth rate (36 h doubling time) in liquid media, high protein content (up to 45 % of dry weight), and almost exclusively vegetative propagation make this plant attractive for edible vaccine research in a well-controlled format, specifically in different types of photoreactors.

The usual approach to expressing peptides in plants is fusion of the target peptide to a carrier protein. It has been repeatedly shown that use of a carrier protein significantly increases the accumulation of target peptides in plants [25]. We selected β-glucuronidase as the carrier protein, as it is highly stable, accumulates in the cytoplasm in large quantities and has been successfully used to study the development of plant-based vaccines [2, 26, 27].

Here, we report the successful agrobacterial transformation of duckweed and the expression of peptide M2e as part of the fusion protein M2e–β-glucuronidase in transgenic plants. The fusion protein was accumulated to high levels, comparable to those from transient virus-based expression systems, and sufficient for further studies of its immunogenicity.

Materials and Methods

Construction of the Plant Transformation Vector

The 30-aa long N-terminal fragment of the M2 protein of avian influenza virus A/chicken/Kurgan/5/2005(H5N1) (GenBank DQ449633.1) containing 24 aa of peptide M2e, termed M130, was selected for expression in transgenic duckweed plants.

The DNA sequence corresponding to target peptide M130 was optimized for expression in duckweed. A table of codon usage in *Lemna gibba* was used (http://www.kazusa.or.jp/codon/), and optimization was performed using DNA2.0 Gene Designer software. For cloning, restriction sites *Xba*I and *BamH*I were added to the nucleotide sequence of M130, and the synthesized fragment was digested and cloned into the corresponding sites of plasmid pBI121 in the translational fusion upstream of the β-glucuronidase gene. After sequencing, the resulting

plasmid (pBIM130) was transferred into *Agrobacterium tumefaciens* CBE21 and used for transformation of duckweed.

Agrobacterial Transformation of Duckweed

In vitro culture of a local Russian isolate of duckweed from the Oka river was used in our experiments. The sterile duckweed plants were grown in liquid hormone-free MS (LHFM) medium [28] supplemented with 2.0 % (w/v) sucrose.

The calluses were used for agrobacterial transformation. For callus induction, the fronds were separated and placed in Petri dishes on NPM medium (macro and micro salts and vitamins according to Murashige and Skoog [28], 3.0 % sucrose, 0.4 % w/v agar, 0.15 % gelrite) containing 1.0 mg/l thidiazuron. The calluses that developed on the fronds were excised and transferred to NPM medium with 2.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4D) for subsequent growth and proliferation.

The callus pieces (approximately 3–4 mm in diameter) obtained from the NPM medium were used for transformation. *A. tumefaciens* cells were grown overnight in liquid LB medium at 28 °C and 140 rpm. The bacterial cells were washed twice in LHFM medium by centrifugation for 5 min (4000 g), and the bacterial pellet was resuspended in LHFM to an OD_{600} of 0.2.

The calluses [2.0–3.0 g fresh weight (FW)] were transferred to vessels containing 20 ml of the agrobacterial suspension and incubated with *A. tumefaciens* for 30 min. The calluses were then transferred to NPM medium containing 2.0 mg/l 2, 4D and cultivated for 2 days, then transferred to the same medium containing 500 mg/l cefotaxime for callus growth and elimination of *A. tumefaciens*. After 30 days, the calluses were transferred to NPM medium containing 2.0 mg/l 2, 4D, 500 mg/l cefotaxime, and 35 mg/l kanamycin. The kanamycin-resistant calluses reaching 2–3 mm in diameter were detached from nontransgenic dying callus and cultivated in the same medium.

When the calluses reached a size of about 5–6 mm, they were placed on regeneration NPM medium with 2.0 mg/l benzylaminopurine, 0.1 mg/l IAA, 200 mg/l cefotaxime, and 35 mg/l kanamycin. Calluses were cultivated on this medium until kanamycin-resistant fronds appeared, after about 2 to 3 months. Then, kanamycin-resistant fronds were transferred to LHFM medium containing 200 mg/l cefotaxime and 10 mg/l kanamycin for further selection and proliferation.

The calluses at all stages of the transformation procedure were subcultured on fresh medium every 2 weeks. Duckweed growth, callus induction and cultivation, and regeneration of the transgenic plants in all experiments were carried out at 25 ± 1 °C under a 16/8 h photoperiod and illumination intensity of 1.5 W/m^2.

GUS-Expression Assays

The activity of β-glucuronidase in duckweed was analyzed using the histochemical method described by Jefferson et al. [26].

PCR Analysis

For PCR analysis, the genomic DNA of duckweed was isolated from GUS-positive and nontransformed control plants using the method of Dellaporta et al. [29]. PCR analysis of putatively transgenic plants was performed using primers M130f (forward, 5′-catctagaatgtccctcctcactgaag-3′) and uidAlow (reverse, 5′-gaatcctttgccacgcaagtccgcatctt-3′), amplifying a 1024-bp fragment comprising the sequence of M130 and part of the β-glucuronidase gene. Transgenic plants were also tested for the absence of agrobacterial contamination using primers virC1 (forward, 5′-gcactatctacctaccgctacgtcatc-3′) and virC2 (reverse, 5′-gttgtcgatcgggactgtaaatgtg-3′) that amplify the virC gene of *A. tumefaciens*.

Southern Blot Analysis

Duckweed genomic DNA (50 μg) was digested overnight at 37 °C with 100 U *Eco*RI which cut the T-DNA of pBIM130 at a single position (between the left border and nos terminator of the β-glucuronidase gene). The DNA of duckweed plants transformed with pBI121 and digested with *Eco*RI and *Hind*III was used as a positive control. After agarose gel (0.8 %) electrophoresis, the digestion products were transferred and immobilized onto Hybond+ membrane (Amersham, USA) following the manufacturer's instructions. The DNA probe was constructed by PCR using plasmid pBIM130 as the template, and primers M130f and uidAlow. Probe DNA (1024 bp) was labeled with alkaline phosphatase using the AlkPhos Direct Labeling Kit (Amersham Bioscience, USA). Prehybridization, hybridization (overnight at 60 °C) with alkaline phosphatase-labeled probe, and subsequent washings of the membrane were carried out according to the AlkPhos Direct Labeling Kit protocol. Detection was performed using CDP-Star detection reagent following the manufacturer's directions (Amersham Bioscience).

Western Blot Analysis

To prepare total soluble protein, duckweed plants (0.5 g) were ground in liquid nitrogen. The ground material was resuspended in four volumes of extraction buffer containing

50 mM Tris–HCl, pH 8.0, 10 mM EDTA, pH 8.0, 10 % (v/v) glycerol, 1 % (w/v) SDS, 30 mM 2-mercaptoethanol, 4 µg/ml aprotinin, and 4 µg/ml leupeptin. Total proteins were extracted for 20 min at 4 °C, then centrifuged for 10 min at 16,000 g, 4 °C and the supernatant was taken for further analysis. Protein concentration was measured by DC protein assay (BioRad, USA).

Total proteins (25 µg) from each transgenic line were separated by 12 % SDS-PAGE and transferred onto an NC membrane (Amersham). Rabbit anti-β-glucuronidase (diluted 1:2000, Sigma, USA) and anti-M2e (1:1000, Abcam, UK) polyclonal antibodies served as the primary antibodies. Anti-rabbit IgG conjugated to alkaline phosphatase was used as the secondary antibody (1:4000, Pierce, USA). Blots were treated with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) for visualization.

ELISA Quantification of M130–β-Glucuronidase Accumulation

Total soluble protein (TSP) was extracted as described above in extraction buffer without SDS. The protein samples were serially diluted in phosphate buffer saline (PBS) (0.25, 0.5, 1.0, and 2.0 µg TSP per well) and placed into a 96-well microtiter plate, using β-glucuronidase from E. coli (Sigma) as the reference standard. The plates were incubated for 2 h at room temperature, washed three times with washing buffer for 5 min each [PBS plus 0.05 % (v/v) Tween-20] and blocked with blocking buffer (PBS containing 2 % w/v bovine serum albumin and 0.05 % Tween-20) for 1 h at 37 °C. The plates were then incubated with rabbit anti-β-glucuronidase (diluted 1:1000, Sigma) polyclonal antibody overnight at 4 °C. After washing, the plates were incubated with anti-rabbit IgG conjugated to alkaline phosphatase (1:3000, Pierce) for 1 h at 37 °C. The plates were washed and developed by the addition of 100 µl per well of TMB Peroxidase EIA substrate (BioRad) for 30 min at room temperature. The plate was read at 405 nm and the amount of plant-expressed M130–β-glucuronidase was estimated based on reference β-glucuronidase standards.

To determine the expression level of M130–β-glucuronidase in the transgenic lines, three samples of duckweed per line were analyzed, and their average expression level calculated. All measurements were performed in duplicate.

Results

Construction of the Plant Transformation Vector

A comparison of codon usage in L. minor and avian influenza virus showed their essential differences. Assuming that codon usage in the related species L. minor and L. gibba does not vary too greatly, we used the L. gibba codon usage table for optimization of the M130 nucleotide sequence. A codon-optimized nucleotide sequence encoding M130 peptide was synthesized and cloned as a translational fusion with the 5′-end of the β-glucuronidase gene into plasmid pBI121 downstream of the 35S CaMV promoter (Fig. 1). The resulting plasmid pBIM130 was used for the production of M130-β-glucuronidase in the duckweed cell cytoplasm, following the duckweed's Agrobacterium-mediated transformation.

Agrobacterial Transformation of Duckweed

Kanamycin-resistant calluses were obtained after 5–6 weeks of cultivation on NPM medium with cefotaxime and kanamycin. The kanamycin-resistant calluses grew vigorously, reaching 2- to 3-mm diameter in 10–15 days. They were then detached from the initial callus and transferred to fresh medium for further growth. When the calluses reached 5- to 6-mm diameter, they were transferred to NPM regeneration medium for regeneration and transformant selection. The first regenerated fronds appeared after 8–10 weeks of cultivation on this medium from growing sectors of the calluses (Fig. 2a). Each frond was transferred to a separate culture tube with LHFM medium containing kanamycin and cefotaxime for further growth and proliferation. Approximately 40 of the fronds grew and proliferated on the selection medium.

The kanamycin-resistant duckweed plants did not differ morphologically from the nontransformed ones. The development and growth rate of these plants in liquid culture did not differ from the corresponding characteristics of the nontransformed control plants. In total, 33 independent kanamycin-resistant duckweed lines were obtained.

GUS-Expression Assays

After 2 months of growth on LHFM medium with kanamycin and cefotaxime, the kanamycin-resistant plants were assayed for GUS activity. All 33 tested lines showed staining, at intensities ranging from almost indistinguishable pale blue to dark blue—almost black (Fig. 2b–e). At the same time, the nontransformed duckweed plants were not stained by X-Gluc (Fig. 2f).

In most transgenic lines, staining was uniform throughout the fronds. However, six of the lines demonstrated nonuniform spotty blue staining that indicated the possible chimeric nature of these plants or unstable inheritance of the fusion gene through cell division. These lines, as well as weakly stained ones, were excluded from further study; 20 duckweed lines with uniform blue staining were chosen for further analyses.
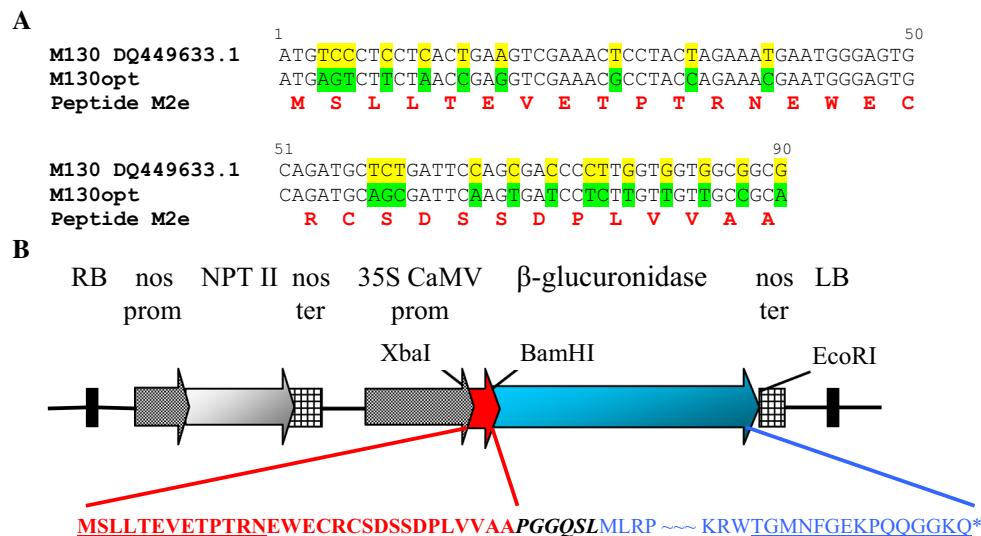
A

```
                    1                                                  50
M130 DQ449633.1   ATGTCCCTCCTCACTGAAGTCGAAACTCCTACTAGAAATGAATGGGAGTG
M130opt           ATGAGTCTTCTAACCGAGGTCGAAACGCCTACCCAGAAACGAATGGGAGTG
Peptide M2e        M  S  L  L  T  E  V  E  T  P  T  R  N  E  W  E  C

                    51                                      90
M130 DQ449633.1   CAGATGCTCTGATTCCAGCGACCCCTTGGTGGTGGCGGCG
M130opt           CAGATGCAGCGATTCAAGTGATCCTCTTGTTGTTGCCGCA
Peptide M2e        R  C  S  D  S  S  D  P  L  V  V  A  A
```

B

RB nos prom NPT II nos ter 35S CaMV prom β-glucuronidase nos ter LB

XbaI BamHI EcoRI

MSLLTEVETPTRNEWECRCSDSSDPLVVAA*PGGQSL*MLRP ~~~ KRW<u>TGMNFGEKPQQGGKQ</u>*

Fig. 1 Schematic depiction of the expression cassette of plasmid pBIM130. **a** Nucleotide sequence of the DNA fragment encoding the peptide M130. *M130 DQ449633.1* original sequence, *M130opt* sequence optimized for expression in *L. minor*. The differences in nucleotide composition of these sequences are indicated by *colors*. **b** Expression cassette obtained after cloning the M130-encoding sequence into plasmid pBI121. The amino acid sequences of M130 peptide and β-glucuronidase are also shown (in *red* and *blue*, respectively) (Color figure online)

Fig. 2 **a** Frond regeneration from kanamycin-resistant callus after 10 weeks of growth on NPM regeneration medium. **b–f** X-Gluc staining of nontransformed control and kanamycin-resistant duckweed plants. **b** and **c** Transgenic *lines 16* and *54*, respectively, with high GUS expression. **d** and **e** Transgenic *lines 19* and *34*, respectively, with moderate GUS expression. **f** Nontransformed duckweed plants. **g** Transgenic duckweed plants (*line 54*) growing on LHFM medium with kanamycin

line 16   line 54   line 19   line 34
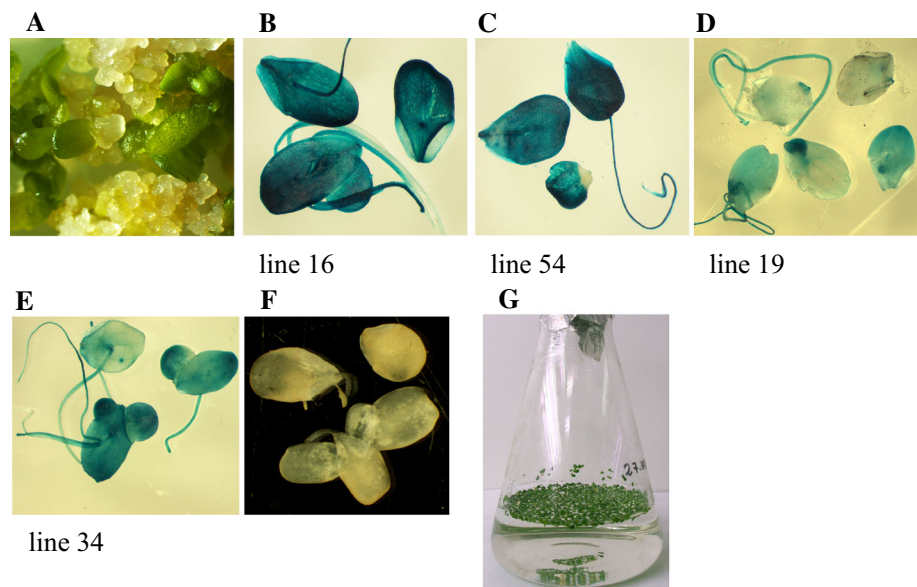
PCR and Southern Blot Analysis of Duckweed Plants

An M130 fragment of the expected size was amplified from the DNA of all analyzed putatively transgenic duckweed lines (Fig. 3a). As revealed by PCR using virC1 and virC2 primers, all lines were also free of agrobacterial contamination (data not shown). To further confirm the transgenic origin of these lines, Southern blot analysis was performed. Genomic DNA was digested with *Eco*RI that cut once within the T-DNA. Results confirmed integration of the M130-β-glucuronidase gene into duckweed genomic

DNA (Fig. 3b). Based on the hybridization profile, there were one (line 72) or two (line 54) insertions of the transgene in the studied lines, which is typical for *Agrobacterium*-mediated transformation. The DNA from nontransformed plants failed to hybridize to the probe.

Western Blot Analysis of Transgenic Duckweed Plants

Western blot analysis using antibody to β-glucuronidase revealed the presence of a 72-kDa band corresponding to the fusion protein M130–β-glucuronidase in 17 transgenic

A

1000bp →

K− K+ M 13 14 16 18 34 51 54 58 72

B

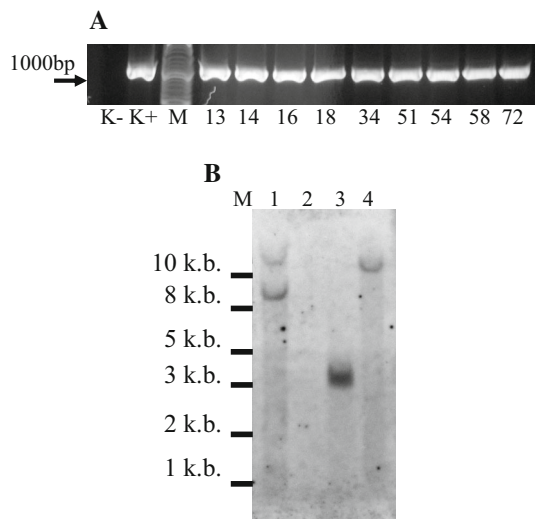M 1 2 3 4

10 k.b.
8 k.b.
5 k.b.
3 k.b.
2 k.b.
1 k.b.

Fig. 3 **a** PCR analysis of duckweed plants from the specified transgenic lines. *K−* nontransformed plant, *K+* DNA of plasmid pBIM130. The expected length of the amplified fragment was 1024 bp. *Numbers* denote independent transgenic lines. **b** Southern blot analysis of transgenic duckweed lines. *1* transgenic duckweed line 54, *2* nontransformed plant, *3* duckweed plants transformed with pBI121, *4* transgenic duckweed line 72, *M* molecular size marker
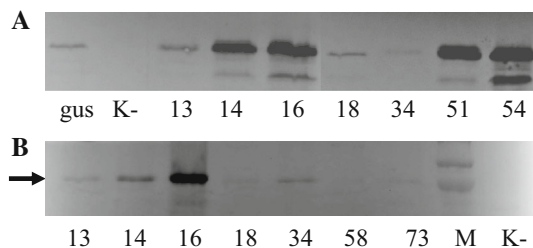
A

gus K− 13 14 16 18 34 51 54

B

→

13 14 16 18 34 58 73 M K−

Fig. 4 Western blot analysis of M130-β-glucuronidase protein expression in transgenic duckweed lines. Western blot analysis using **a** anti-β-glucuronidase antibody and **b** anti-M2e antibody. *K−* nontransformed duckweed plants, *gus* β-glucuronidase from *E. coli* (25 ng), *M* molecular size marker. *Numbers* denote transgenic lines; *arrow* indicates M130–β-glucuronidase fusion protein

lines out of 20 studied (Fig. 4a). Immunoreactive bands of similar weight were not observed in the protein samples from nontransformed control plants.

In some lines (for example, 18 and 34), the target protein was detected at low levels; the immunoreactive bands were weak, although all transgenic lines were similarly stained in the GUS-expression assays. Transgenic duckweed lines that demonstrated high levels of M130–β-glucuronidase expression were further analyzed using antibodies to M2e peptide (Fig. 4b). The highest level of M2e peptide was detected in lines 14 and 16; accumulation of M2e was lower in lines 13, 18, and 34. In the lines expressing β-glucuronidase at relatively low levels, the target peptide was only weakly detected (lines 58 and 73). Overall, Western

blot analysis showed a good correlation between the expressions of β-glucuronidase and M2e peptide.

Quantification of M130–β-Glucuronidase Expression in Transgenic Duckweed

Quantification of the target fusion protein in transgenic duckweed plants using anti-β-glucuronidase antibody showed accumulation of 0.09 and 0.97 mg of M130–β-glucuronidase per g duckweed FW (Fig. 5), corresponding to 0.12–1.96 % of TSP. The highest levels of M130-β-glucuronidase accumulation were observed in lines 16 and 54 (0.82 and 0.97 mg/g FW; 1.89 and 1.96 % of TSP, respectively), and the lowest in lines 19, 49, and 93 (0.16, 0.17 and 0.09 mg/g FW; 0.16, 0.16 and 0.12 % of TSP, respectively).

Taking into account that the target peptide M130 (molecular weight 3.5 vs 68.4 kDa for β-glucuronidase) was expressed as a fusion protein, the maximal accumulation of peptide M130 alone in highly expressing lines 16 and 54 reached more than 40 µg/g FW. The overall results of our study are presented in Table 1.

In our experiments, we observed a wide, almost tenfold variation in target protein accumulation among transgenic lines. Variations in recombinant protein expression in independently derived transgenic lines are common [30, 31]. They are often related to differences in the number of transgene copies or in the position within the genome into which the foreign DNA has integrated. Both of these factors apply to the transgenic duckweed lines that we obtained.

It should be noted that the duckweed expression system has been rather stable for most of the transgenic lines. For example, in the last 3 years, the content of target protein in transgenic lines 13, 17, 51, and 54 changed from 1.72, 0.45, 1.67, and 2.19 % of TSP, respectively, in 2011 (not shown) to 1.80, 0.65, 1.71, and 1.96 % of TSP, respectively, in 2013 (Fig. 5). During this same period, the contents of the target protein in line 50 decreased from 1.9 to 0.64 % of TSP, perhaps due to chromosomal instability during cultivation.

Discussion

The protocol for *Agrobacterium*-mediated transformation of duckweed was originally developed by Yamamoto et al. [32], and then fine-tuned for the physiological characteristics of different geographical isolates of the plant [33, 34]. The original protocol [32] consisted of organogenic callus induction, its transformation via *Agrobacterium*, and subsequent regeneration and selection of transformants in the presence of the selective antibiotic. This scheme proved effective in our case. The organogenic callus induction, *Agrobacterium* transformation, and regeneration of
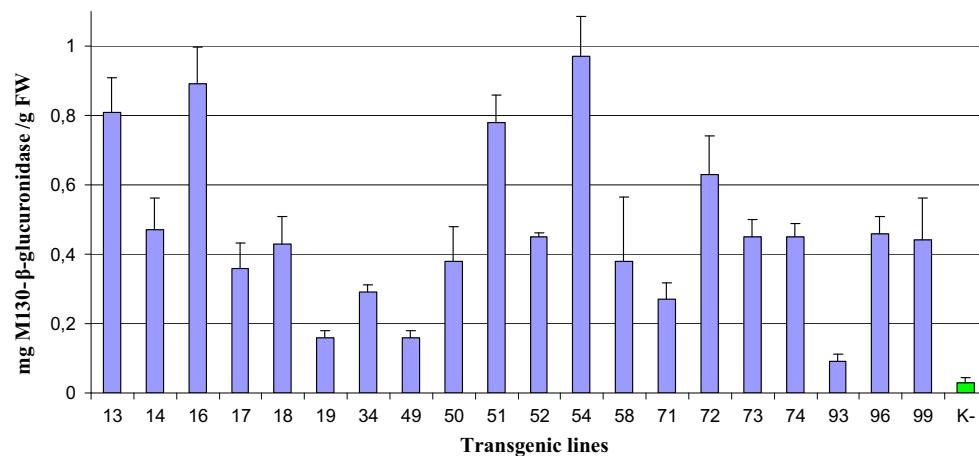
Fig. 5 Quantification of M130–β-glucuronidase fusion protein in transgenic duckweed plants (results from 2013). *K−* nontransformed plants. *Numbers* denote transgenic lines. *Error bars* indicate ± SD

Table 1 Summarized results of duckweed transformation and expression of M130-β-glucuronidase protein in transgenic lines		
Kanamycin-resistant duckweed lines		33
GUS-positive duckweed lines		33
GUS-positive duckweed lines chosen for further analyses		20
GUS-positive duckweed lines tested by PCR		20
PCR-positive duckweed lines		20
PCR-positive duckweed lines tested by Western blot analysis		20
PCR-positive duckweed lines with target protein accumulation estimated by ELISA		20
Duckweed lines showing target protein accumulation		20
PCR- and Western blot-positive duckweed lines tested by Southern blot		6
Southern blot-positive duckweed lines		6

transgenic plants occurred at a reasonably high frequency. In total, we obtained 20 different lines of duckweed with confirmed transgenic status. Transgenic duckweed plants did not differ morphologically from their nontransformed counterparts. Expression of the fusion protein had no effect on growth rate in liquid culture or on TSP content in the plants.

A high level of peptide accumulation is fairly common when the peptide is expressed in a fusion with β-glucuronidase. For example, a high level of vaccine peptide expression was observed in *Arabidopsis* plants transformed with a translational fusion of canine parvovirus 2L21 peptide with β-glucuronidase- the fusion protein 2L21-β-glucuronidase was present at 0.15–3.3 % of TSP in the different expressing lines [35]. This corresponds to an average of 75 µg 2L21-β-glucuronidase/g FW. The highly immunogenic epitope of structural protein VP1 from foot and mouth disease virus fused to the β-glucuronidase gene (VP–βGUS) was expressed in transgenic alfalfa plants at 0.5–1.0 mg/g FW [36]. In those studies, as in ours, the fusion protein accumulated in the cytoplasm. It seems that the high expression of the target fusion protein is determined by the high stability of β-glucuronidase in the cytoplasm (half-life in living mesophyll protoplasts of ∼50 h [26]), allowing it to accumulate in large quantities.

To date, the M2e peptide has only been successfully expressed in plants using virus-based transient expression systems. Meshcheryakova et al. [23] used a transient expression system based on cowpea mosaic virus; the yield of recombinant virus particles expressed in cowpea plants amounted to 15–33 µg/g FW. Tyulkina et al. [24] used hybrid viral vectors constructed on the basis of the potato virus X (PVX) genome and the *Alternanthera* mosaic virus (AltMV) coat protein (CP) gene. The accumulation of chimeric capsid proteins CPAltMV-M2e in *Nicotiana benthamiana* leaves reached over 1 mg/g of green material (in some experiments, up to 3 mg/g). In another study, expression of the hybrid protein M2e-HBc consisting of peptide M2e fused to hepatitis B core antigen (HBc) reached 1–2 % of TSP [37]. In that study, the viral vector was based on PVX, and expression was conducted in *N. benthamiana* plants. Thus, the accumulation level of M2e peptide in nuclear-transformed duckweed plants in our experiments corresponds quite well with the results obtained using of the virus-based transient systems. This is especially attractive for the production of a "universal"

influenza vaccine, which is required in substantial quantities on a regular basis. We believe that if plant producers providing a high level of a particular target protein are available, expression systems based on stably transformed plants have an obvious advantage over transient ones. Because there is need to maintain facilities for transient expression, the cost of recombinant protein production via stably transformed plants is reduced. When these proteins are required regularly and in relatively large quantities (for example, insulin and other hormones, diagnostic and therapeutic antibodies to widespread diseases, human serum albumin, other blood proteins, etc.), this is especially important. Duckweed, as mentioned above, is particularly convenient for growth in contained cultivation systems; moreover, the efficiency of expression systems based on nuclear-transformed duckweed plants is generally comparable to transient- or yeast-based expression.

In summary, our study clearly demonstrates the feasibility of M2e peptide expression in nuclear-transformed duckweed plants with no noticeable impact on the plants' morphology or growth rate. The accumulation of target M2e peptide in the best transgenic duckweed lines reached 40 μg/g FW, corresponding to levels obtained in transient virus-based systems, and opening the way to developing an edible plant-produced vaccine against avian influenza virus.

References

1. Chen, Q. (2011). Expression and manufacture of pharmaceutical proteins in genetically engineered horticultural plants. In B. Mou & R. Scorza (Eds.), *Transgenic horticultural crops: Challenges and opportunities-essays by experts* (pp. 83–124). Boca Raton, FL: Taylor & Francis.
2. Guan, Z. H., Guo, B., Huo, Y.-L., Guan, Z.-P., Dai, J.-K., & Wei, Y.-H. (2013). Recent advances and safety issues of transgenic plant-derived vaccines. *Applied Microbiology and Biotechnology, 97*(7), 2817–2840.
3. Phan, H. T., Floss, D. M., & Conrad, U. (2013). Veterinary vaccines from transgenic plants: Highlights of two decades of research and a promising example. *Current Pharmaceutical Design, 19*(31), 5601–5611.
4. Drake, P. M. W., & Thangaraj, H. (2010). Molecular farming, patents and access to medicines. *Expert Review of Vaccines, 9*(8), 811–819.
5. Fischer, R., Schillberg, S., Hellwig, S., Twyman, R. M., & Drossard, J. (2012). GMP issues for recombinant plant-derived pharmaceutical proteins. *Biotechnology Advances, 30*(2), 434–439.
6. Yusibov, V., Streatfield, S. J., & Kushnir, N. (2011). Clinical development of plant-produced recombinant pharmaceuticals. *Human Vaccines, 7*(3), 1–9.
7. Kirk, D. D., McIntosh, K., Walmsley, A. M., & Peterson, R. K. D. (2005). Risk analysis for plant-made vaccines. *Transgenic Research, 14*, 449–462.
8. Franconi, R., Demurtas, O. C., & Massa, S. (2010). Plant-derived vaccines and other therapeutics produced in contained systems. *Expert Review of Vaccines, 9*(8), 877–892.
9. Gasdaska, J. R., Spencer, D., & Dickey, L. (2003). Advantages of therapeutic protein production in the aquatic plant *Lemna. Bioprocessing Journal, 2*, 50–56.
10. Everett, K. M., Dickey, L., Parsons, J., Loranger, R., & Wingate, V. (2012). Development of a plant-made pharmaceutical production platform. *BioProcess International, 10*(1), 16–25.
11. Sun, Y., Cheng, J. J., Himmel, M. E., Skory, C. D., Adney, W. S., Thomas, S. R., et al. (2007). Expression and characterization of *Acidothermus cellulolyticus* E1 endoglucanase in transgenic duckweed *Lemna minor* 8627. *Bioresource Technology, 98*, 2866–2872.
12. Cox, K. M., Sterling, J. D., & Regan, J. T. (2006). Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemna minor. Nature Biotechnology, 24*(12), 1591–1597.
13. Ko, S.-M., Sun, H.-J., Oh, M. J., Song, I.-J., Kim, M.-J., Sin, H.-S., et al. (2011). Expression of the protective antigen for PEDV in transgenic duckweed *Lemna minor. Horticulture Environment Biotechnology, 52*(5), 511–515.
14. Subbarao, K., & Matsuoka, Y. (2013). The prospects and challenges of universal vaccines for influenza. *Trends in Microbiology, 21*(7), 350–358.
15. Pica, N., & Palese, P. (2013). Toward a universal influenza virus vaccine: Prospects and challenges. *Annual Review of Medicine, 64*, 189–202.
16. Knipe, D. M., Samuel, C. E., & Palese, P. (2001). Virus-host cell interactions, Chapter 7. In D. M. Knipe, P. M. Howley, et al. (Eds.), *Fields virology* (4th ed., pp. 133–170). Philadelphia, PA: Lippincott, Williams and Wilkins.
17. Fiers, W., De Filette, M., Birkett, A., Neirynck, S., & Min Jou, W. (2004). A "universal" human influenza A vaccine. *Virus Research, 103*, 173–176.
18. Reperant, L. A., Rimmelzwaan, G. F., & Osterhaus, A. D. (2014). Advances in influenza vaccination. *F1000Prime Reports, 6*, 47.
19. Marcelin, G., Sandbulte, M. R., & Webb, R. J. (2012). Contribution of antibody production against neuraminidase to the protection afforded by influenza vaccines. *Reviews in Medical Virology, 22*(4), 267–279.
20. Tumpey, T. M., Basler, C. F., Aguilar, P. V., Zeng, H., Solórzano, A., Swayne, D. E., et al. (2005). Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science, 310*(5745), 77–80.
21. Nemchinov, L. G., & Natilla, A. (2007). Transient expression of the ectodomain of matrix protein 2 (M2e) of avian influenza A virus in plants. *Protein Expression and Purification, 56*, 153–159.
22. Denis, J., Acosta-Ramirez, E., Zhao, Y., Hamelin, M.-E., Koukavica, I., Baz, M., et al. (2008). Development of a universal influenza A vaccine based on the M2e peptide fused to the papaya mosaic virus (PapMV) vaccine platform. *Vaccine, 26*, 3395–3403.
23. Meshcheryakova, Y. A., Eldarov, M. A., Migunov, A. I., Stepanova, L. A., Repko, I. A., Kiselev, C. I., et al. (2009). Cowpea mosaic virus chimeric particles bearing the ectodomain of matrix protein 2 (M2E) of the influenza A virus: Production and characterization. *Molecular Biology, 43*(4), 685–694.
24. Tyulkina, L. G., Skurat, E. V., Frolova, O. Y., Komarova, T. V., Karger, E. M., & Atabekov, I. G. (2011). New viral vector for

superproduction of epitopes of vaccine proteins in plants. *Acta Naturae, 3*(11), 73–82.

25. Streatfield, S. J. (2007). Approaches to achieve high-level heterologous protein production in plants. *Plant Biotechnology Journal, 5*, 2–15.

26. Jefferson, R. A., Kavanagh', T. A., & Bevan, M. W. (1987). GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal, 6*(13), 3901–3907.

27. Floss, D. M., Falkenburg, D., & Conrad, U. (2007). Production of vaccines and therapeutic antibodies for veterinary applications in transgenic plants: An overview. *Transgenic Research, 16*, 315–332.

28. Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiologia Plantarum, 15*(3), 473–497.

29. Dellaporta, S. L., Wood, J., & Hicks, J. B. (1983). A plant DNA minipreparation version II. *Plant Molecular Biology Reporter, 1*, 19–21.

30. Richter, L. J., Thanavala, Y., Arntzen, C. J., & Mason, H. S. (2000). Production of hepatitis B surface antigen in transgenic plants for oral immunization. *Nature Biotechnology, 18*, 1167–1171.

31. Sharma, A. K., & Sharma, M. K. (2009). Plants as bioreactors: Recent developments and emerging opportunities. *Biotechnology Advances, 27*, 811–832.

32. Yamamoto, Y. T., Rajbhandari, N., Lin, X., Bergmann, B. A., Nishimura, Y., & Stomp, A.-M. (2001). Genetic transformation of duckweed *Lemna gibba* and *Lemna minor*. *In Vitro Cellular & Developmental Biology-Plant, 37*, 349–353.

33. Li, J., Jain, M., Vunsh, R., Vishnevetsky, J., Hanania, U., Flaishman, M., et al. (2004). Callus induction and regeneration in *Spirodela* and *Lemna*. *Plant Cell Reports, 22*, 457–464.

34. Chhabra, G., Chaudhary, D., Sainger, M., & Jaiwal, P. K. (2011). Genetic transformation of Indian isolate of *Lemna minor* mediated by *Agrobacterium tumefaciens* and recovery of transgenic plants. *Physiology and Molecular Biology of Plants, 17*(2), 129–136.

35. Gil, F., Brun, A., Wigdorovitz, A., Catalá, R., Martínez-Torrecuadrada, J. L., Casal, I., et al. (2001). High-yield expression of a viral peptide vaccine in transgenic plants. *FEBS Letters, 488*, 13–17.

36. Dus Santos, M. J., Wigdorovitz, A., Trono, K., Ríos, R. D., Franzone, P. M., Gil, F., et al. (2002). A novel methodology to develop a foot and mouth disease virus (FMDV) peptide-based vaccine in transgenic plants. *Vaccine, 20*, 1141–1147.

37. Ravin, N. V., Kotlyarov, R. Y., Mardanova, E. S., Kuprianov, V. V., Migunov, A. I., Stepanova, L. A., et al. (2012). Plant-produced recombinant influenza vaccine based on virus-like HBc particles carrying an extracellular domain of M2 protein. *Biochemistry (Moscow), 77*(1), 33–40.