Development of *Cucumber mosaic virus* as a vector modifiable for different host species to produce therapeutic proteins

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Abstract

We have developed *Cucumber mosaic virus* (CMV) as a plant virus vector especially for production of pharmaceutical proteins. The CMV vector is a vector modifiable for different host plants and does not require further engineering steps. CMV contains three genomic RNA molecules (RNAs 1-3) necessary for infectivity. With this system, instead of creating different vector constructs for each plant we use, we take advantage of the formation of pseudrecombinants between two CMV isolates by simply reassembling a vector construct (RNA 2 base) and an RNA molecule containing the host determinant (mostly RNA 3). In this study, the gene for acidic fibroblast growth factor (aFGF), one of the human cytokines, was cloned under the control of the subgenomic promoter for RNA 4A of the CMV-based vector, C2-H1. Infected *Nicotiana benthamiana* plants produced aFGF at levels up to 5-8% of the total soluble protein. The tobacco-produced aFGF was purified, and its biological activity was confirmed. Using this system, which provides a versatile and viable strategy for production of therapeutic proteins in plants, we also demonstrated a high level of aFGF in *Glycine max* (soybean) and *Arabidopsis thaliana*.

Keywords modifiable vector · *Cucumber mosaic virus* · virus vector · acidic fibroblast growth factor · transient expression

Abbreviations RT-PCR: Reverse transcription-polymerase chain reaction · aFGF: acidic fibroblast growth factor · CMV: *Cucumber mosaic virus* · TMV: *Tobacco mosaic virus*

Introduction

Production of pharmaceutical proteins in plants has generated great interest in recent years for several reasons. For example, we can mass-produce therapeutic proteins in their active forms in edible plants, enabling the oral-delivery of protein drugs (Arazi et al., 2001; Ma et al., 2005). Currently, heterologous genes can be expressed in plants using either transgenic plants or plant viral vectors (Matsumura et al., 2000; Menassa et al, 2000; Gils et al., 2005). The disadvantages of transgenic plants reside in the system itself; the method is time-consuming and an efficient transformation system is lacking for many plants. In addition, we cannot generally produce high levels of heterologous proteins in transgenic plants. Plant RNA virus-based vectors, on the other hand, are rapidly amplified and produce large amounts of recombinant proteins, and in theory we can use any plant that can be infected by the viral vector. In fact, many foreign proteins have been successfully expressed using plant viral vectors (Dirnberger et al. 2001; Pérez Filgueira et al. 2003; Wagner et al. 2004).

Cucumber mosaic virus (CMV), the type member of the genus *Cucumovirus*, belongs to the family *Bromoviridae*. CMV has a very broad host range including more than 1200 species in over 100 families (Edwardson and Christie 1991). It has three single-stranded, genomic RNAs (RNAs 1 to 3). RNAs 1and 2 encode, respectively, the 1a and 2a proteins, which are the components of the viral replicase complex (Nitta et al. 1988). RNA 3 encodes the movement protein (3a) and the coat protein (CP), which is expressed through a subgenomic RNA (RNA 4) (Schwinghamer and Symons 1975). Many studies have mapped CMV host-range determinants to RNA 3 (Carrère et al.

1999; Palukaitis and García-Arenal 2003). Another subgenomic RNA, RNA 4A, is derived from the 3' half of RNA 2 and encodes the 2b protein (Ding et al. 1995), which is a suppressor of RNA silencing (Brigneti et al. 1998). We previously developed a system to generate *in vitro* transcripts from viral cDNA clones (Suzuki et al. 1991). In this study, we successfully used CMV as a viral vector that can systemically infect many plant species. Though a few attempts have been made to develop CMV as a vector to express a foreign gene, engineering RNA 3 (Zhao et al. 2000), the constructs suffered from RNA recombination and difficulty in systemically spreading to produce the target protein throughout the entire plant.

We here utilized the CMV-based vector to express human acidic fibroblast growth factor (aFGF) in plants. aFGF is also known as FGF-1, a nonglycosylated heparin-binding protein, which is expressed in many organs including brain, kidney, retina, smooth muscle cells, bone matrix, osteoblasts, astrocytes and endothelial cells. It stimulates proliferation of a wide range of cells of mesenchymal, neuroectodermal and endothelial origin (Burgess and Maciag 1989). Human aFGF is a 17.5-kDa protein containing 155 amino acid residues. Full-length aFGF can be cleaved after the lysine (position 15) or glycine (position 20) residues but the biological activities of the truncated forms are similar to those of the intact one (Burgess 1991; Landgren et al. 1998). The potential therapeutic use of aFGF is based on its mitogenic and angiogenic activities. For example, aFGF treatment might be used to treat wounds, periodontal disease and cardiovascular disease.

In this article, we describe the CMV-based vector system to produce therapeutic

proteins in different plants. Furthermore, we were able to produce high levels of aFGF in soybean plants as well as in *N. benthamiana* and *Arabidopsis* in order to use plant tissues to produce protein drugs.

Materials and methods

Construction of the CMV-based expression vector

For *in vitro* transcription, we had already obtained full-length cDNA clones of the genomic RNAs 1 to 3 of CMV-Y (pCY1, pCY2 and pCY3, respectively) (Suzuki et al. 1991). Plasmid pCY2 was developed as a viral vector by replacing the 2b gene with a multiple cloning site containing restriction sites (*Stu*I, *Mlu*I and *Spe*I), creating the vector C2-H1. The aFGF gene (Accession; X65778) was PCR-amplified using Human Universal QUICK-Clone cDNA II (Clontech, Palo Alto, CA, USA), sense primer (5'-ATGGCTGAAGGGGAAATCAC-3') and antisense primer (5'-GCTCTAGATTAATCAGAAGAGACTGGCAG-3') with an *Xba*I site. The amplified aFGF gene was then inserted between the *Stu*I and *Spe*I sites of C2-H1 to generate H1-aFGF. The 5' end of the aFGF gene was blunt end-ligated to the *Stu*I site and the 3' end *Xba*I site was ligated to the compatible end of the *Spe*I site.

In vitro transcription and inoculation

The recombinant CMV RNAs were in vitro-synthesized from the plasmids, pCY1, H1-aFGF and pCY3 using the kit, RiboMAX Large Scale RNA Production System-T7

(Promega, Madison, WI, USA). Five plants of *Nicotiana benthamiana* (two-month-old), *Glycine max* (three-week-old) and *Arabidopsis thaliana* (three-week-old) were used for each inoculation. The inoculation method was essentially the same for the three plant species. After the largest leaves were dusted with carborundum, transcribed viral RNAs were rub-inoculated by fingers, followed by immediate washing with water. Two weeks after inoculation, the systemically infected leaves were harvested, and protein levels were analyzed by Western blot. For soybean inoculation, the cDNA clone of RNA 3 of CMV-Sj, a soybean strain of CMV, was used instead of pCY3.

Western blot analysis

Production of aFGF was analyzed by Western blotting. We harvested one leaf from each of the five inoculated plants and extracted protein from the mixed preparations. Leaf samples were ground in liquid N_2, homogenized in three volumes (mass/v) of phosphate-buffered saline containing 0.05% Tween 20 (PBS-T). After centrifugation (15,000 rpm, 10 min) of the samples, the supernatants were used as total soluble protein preparations. Protein levels were estimated using the DC protein assay (Bio-Rad, Hercules, CA, USA). Total soluble protein preparations were then separated on SDS-PAGE in 8 or 14% gels, and the separated proteins were transferred to nylon membrane, Hybond-P (Amersham Biosciences, Piscataway, NJ, USA). The blots were then incubated overnight at room temperature in PBS containing 0.1% Tween 20 and 5% ECL blocking agent (Amersham Biosciences), and incubated at 37°C for 1 h in Can Get Signal solution 1 (TOYOBO, Osaka, Japan) containing the rabbit anti-human aFGF

antibodies (diluted 1: 5000 in blocking buffer; PeproTech, Rocky Hill, NJ, USA). The blots were subsequently incubated at 37°C for 1 h in Can Get Signal solution 2 (TOYOBO) containing the secondary antibody, goat peroxidase-labeled anti-rabbit IgG (diluted 1:16000 in blocking buffer; Sigma-Aldrich, St. Louis, MO, USA), and treated for 5 min at room temperature with ECL plus Western Blotting Detection System (Amersham Biosciences). After 3 times washing, the protein bands were eventually detected by VersaDoc (Bio-Rad) under chemiluminescence mode. The *E. coli*-produced recombinant human aFGF was purchased from Diaclone (Besançon, France).

Reverse transcription-PCR analysis

Total RNA was extracted from the leaf samples using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 5 μg of total RNA using Ready-To-Go RT-PCR Beads (Amersham Biosciences) and the oligo(dT) primer. The aFGF DNA was then PCR-amplified using the aFGF specific primers (the sense primer, 5'-ATGGCTGAAGGGGAAATCACC-3' and the antisense primer, 5'-TTAATCAGAAGAGACTGGCAGGG-3'). The PCR products were analyzed by 1% agarose gel electrophoresis.

Purification of the recombinant aFGF

After filtration through a 0.2-μm filter unit (Sartorius, Hannover, Germany), the total soluble protein obtained was applied to a HiTrap Heparin HP column (1 ml bed volume, Amersham Biosciences). The aFGF was purified by stepwise elution. The column was

first washed with 10 ml of PBS-T and then washed with 10 ml of PBS-T containing 0.6 M NaCl. The column-bound aFGF was eluted with PBS-T containing 1.2 M NaCl. Each eluent was desalted, concentrated and subsequently used for Western blot analysis and a biological activity assay.

Biological activity assay

Bovine aortic endothelial (BAE) cells (Cell Systems, Kirkland, WA, USA) were grown in serum-free medium (CS-C Serum free medium, Cell Systems) with growth factors "RocketFuel" (Cell Systems). Cultures were incubated at 37°C in the humidified air containing 5% CO_2. For the cell-proliferation assay, the medium did not contain growth factors but contained 17.6 units/ml heparin.

The assay was carried out as described previously (Matuo et al. 1991). BAE cells were used between passages 2 and 3. Cells were seeded into a 35-mm collagen-type-coated dish (Asahi Technoglass, Tokyo, Japan) at 5×10^3 cells/dish. The aFGF preparations extracted from the CMV-infected tobacco leaves were added at the indicated concentrations. The cultures were incubated at 37°C, 5% CO_2. The cells were trypsinized and counted at 48 hours and 72 h after aFGF addition, then the average numbers were calculated the stimulation factor over negative control results.

Results

Construction of the CMV-based vector for the expression of a foreign gene

In this study, we have developed a plant viral vector that is easily adjustable to different

host plants depending on the use of target proteins. CMV has three genomic RNA molecules (RNAs 1-3), which are exchangeable between two different isolates; we here engineered RNA 2 to express hetelorogous genes. Many studies have reported that RNA 3 contains the viral host range determinants among the viral RNAs, although RNAs 1 and 2 sometimes contribute to further restriction in the host range. Therefore, by combining viral RNAs of different origins, we can theoretically selectively modify the vector for different host plants. To demonstrate a modifiable vector for plants (MVP) (Fig. 1b), we successfully expressed a human cytokine, acidic FGF (aFGF) in three different hosts (*N. benthamiana*, *Arabidopsis* and soybean) by simply changing one of the viral genomic RNAs (RNA 3). For example, as shown in Fig. 1a, we can create a reassortant virus containing Y2 (Y1Y2S3) that infects soybean by exchanging Y3 and S3. Previously, we could produce infectious transcripts from full-length cDNA clones of CMV-Y RNAs 1, 2 and 3 (pCY1, pCY2 and pCY3, respectively) (Suzuki et al. 1991). We also modified the same system for the soybean strain of CMV, CMV-Sj (Hong et al. 2003; Senda et al. 2004). We first developed pCY2 as a viral vector by replacing the 2b gene (nucleotide positions 2420-2752) with a DNA fragment containing the cloning sites, generating a viral vector, C2-H1 (Fig. 2). The cDNA of aFGF was then obtained by a conventional PCR from the human cDNA library. The aFGF clone was inserted between *Stu*I and *Spe*I sites of the cloning sites in C2-H1 to obtain H1-aFGF (Fig. 2).

Infectivity and stability of the CMV vector containing the aFGF gene in plants

Viral RNAs were *in vitro* transcribed from pCY1, H1-aFGF and pCY3. All the

transcripts were mixed together to inoculate *N. benthamiana*. For soybean, the cDNA clone of CMV-Sj RNA 3 (pCSj3) was used instead of pCY3 because RNA 3 of CMV-Sj determines the systemic infection in soybean (Senda et al. 2004). Mild mosaic symptoms were observed on the upper leaves of both *N. benthamiana* and soybean (Fig. 3a and 3b). The infected plants did not develop any severe symptoms; the symptoms even disappeared as the plant grew, although we could still detect high levels of viral accumulation (data not shown), suggesting that our vector has a significant advantage as a non-pathogenic expression vector. There was no symptom on the infected *Arabidopsis* plants. The stability of H1-aFGF was confirmed by conventional RT-PCR using total RNAs from the upper systemic leaves and the aFGF-specific primers (Fig. 3c). Direct sequencing of the RT-PCR product confirmed the correct sequence of the aFGF gene, demonstrating the stability of the inserted sequence in the progeny viruses.

Production and yield of aFGF in plants

Fourteen days postinoculation, the infected leaves of *N. benthamiana* were harvested and total soluble proteins were extracted, subjected to SDS-PAGE, electroblotted onto nitrocellulose membrane and detected by the aFGF-specific antibodies. We clearly detected the aFGF bands at the expected size of 17.5 kDa (155 amino acids) in the extracts from both the inoculated and upper systemic leaves (Fig. 4a). The purchased *E. coli*-produced aFGF protein (positive control), which is a truncated form, had the molecular weight of 16 kDa. Natural aFGFs exist in a few forms, including the full-length form of 17.5 kDa. The amount of aFGF in infected leaves was estimated

using a dilution series of the protein extracts (Fig. 4b). We found a very high amount of aFGF protein, up to 5-8% of the total soluble protein. The amounts of protein in infected soybean and *Arabidopsis* were also estimated as done for *N. benthamiana*. Western blot analysis showed that aFGF was efficiently produced also in soybean and *Arabidopsis*, suggesting that we could produce an important animal protein in different host plants without the various engineering steps required to prepare another recombinant for a different host (Fig. 5). As demonstrated in Fig. 4b, we estimated the yields to be about 2.5% and 1.5% of the total soluble protein extracted from the inoculated leaves, respectively, suggesting that the protein yield varies depending on the host plant. We detected 2- to 4-fold less target protein in upper tissues of *Arabidopsis* and soybean, compared to the inoculated leaves.

Production of a biologically active human aFGF in *N. benthamiana* by the CMV-based vector

The recombinant aFGF was purified from the infected tissues on a heparin column because aFGF binds to heparin. Total soluble protein extracted from the virus-infected tissues was loaded on the heparin column. Western blot analysis showed that the target protein was mostly in the fraction of 1.2 M NaCl eluent (Fig. 6). The yield of the purified aFGF was colorimetrically estimated. The protein purity and the recovery efficiency were monitored by coomassie staining (Fig. 6a). We then analyzed the biological activity of the purified aFGF. Fig. 7 shows the effects of aFGF on bovine aortic endothelial (BAE) cell proliferation. When the protein extract from the leaves

infected with H1-aFGF was added to the assay cells, cell proliferation was observed (Fig. 7a and 7c) and the effect was dose-dependent (Fig. 7b). We thus confirmed the biological activity of the aFGF protein that was produced in plants using the MVP system.

Discussion

Because ordinary CMV strains including CMV-Y have a broad host range, our CMV-based vector can also infect many host species. However, it is not of course universal. Some plant species such as legume and *Lilium* plants cannot be systemically infected by CMV-Y but are infected by the specific host-adapted CMV isolates (Senda et al. 2004; Yamaguchi et al. 2005). Because the genomic RNAs are readily interchangeable between two CMV isolates to produce pseudorecombinant viruses, we used these particular CMV isolates for the development of "a modifiable vector for different plants (MVP system)." We can create any pseudorecombinant depending on the plants we use by simply changing one of the genomic RNAs that contains the host determinant. To demonstrate this technique, we here enabled our vector to systemically infect soybean by using RNA 3 from soybean strain CMV-Sj (Senda et al. 2004). We have another reason to choose soybean as an alternative host plant for this demonstration. The fact that we have efficiently expressed a foreign gene in soybean shows that there is another way to study the function of soybean genes without using stable transformation, which has been a limiting factor because of the lack of tissue culture methods for efficient plant regeneration.

Harnessing the gene expression system in plants provides many benefits, such as the low cost for cultivation, the low risk of contamination by human pathogens and toxins, the use of eukaryotic posttranslational modification (although it is different from the animal counterpart), the high yields of target protein, the ease of scale-up, the low capital cost and so on. Of the gene expression systems in plants, an expression system based on viral vectors has more promising potential over other stable transformations in terms of the very fast gene-to-protein time in the expression process. Many virus-based vector systems with excellent expression of heterologous genes *in planta* have been developed so far (Arazi et al., 2001; Marillonnet et al., 2004). However, there are not many reports that describe a high level of production of pharmaceutical proteins by virus-based vectors. They often suffer from various limitations, such as low yield or lack of biological activity of the target protein and rapid degradation of the animal protein in the plant (Ma et al., 2005).

Table 1 summarizes some successful reports on production of cytokines in plants. If we compare our system with the data in those successful reports, our results are quite outstanding with regard to yield (5-8% of soluble protein) and use of intact plant. However, the protein yield seems to vary depending on the host plants used. In our experiments, *N. benthamiana* plants produced the highest levels (5-8% of soluble protein) of aFGF in both the inoculated and the systemic leaves. On the other hand, soybean and *Arabidopsis* plants produced aFGF at levels of 2.5% and 1.5% of soluble protein in the inoculated leaves, respectively; the protein yields decreased less than half in the systemic tissues. The virus generally accumulates at a maximum level in

inoculated leaves. Because in *N. benthamiana*, the virus can spread much more efficiently than in many other plants including soybean and *Arabidopsis*, it is conceivable that slower movement of the virus caused lower levels of protein accumulation in upper tissues of soybean and *Arabidopsis*. In addition, it is noteworthy and even surprising that except for interferon, there has been little success in the production of human cytokines by viral vectors in plants. The reason is not clear, but cytokines may be rapidly degraded in plants after synthesis because cytokines are unstable in nature, with very short half-lives both *in vitro* and *in vivo* (Ma et al., 2005). We fortunately overcame this problem through the use of the CMV-based vector system because it may direct the production of very large amounts of foreign proteins.

As far as the yield is concerned, our vector system does not hold the record for best yield. Marillonnet et al. (2004) previously demonstrated the highest yield of heterologous proteins (0.5-1 g/kg of fresh mass) using agroinfiltration and *in planta* recombination of the *Tobacco mosaic virus* (TMV)-based viral modules. In this system, the TMV vector expressing a foreign gene becomes infectious in the areas where *Agrobacterium* is infiltrated and can systemically spread. In comparison with our CMV system, CMV would be easier in preparation for an inoculum but agroinfiltration would be better in infection efficiency. They also succeeded in high-yield production (10% of soluble protein) of active human growth hormone using the same system (Gils et al. 2005). However, in this system, they extracted the expressed protein from the *Agrobacteirum*-infiltrated areas before the tissues died. Considering that the bacteria are still present in the plant tissues, this technique for oral delivery of protein drug may be

difficult to use. Presently, the administration of protein drugs depends on their parenteral injection, which is extremely painful to the patient. Recently, oral administration of protein drugs has been extensively investigated (Charmandari et al. 2002; Lee 2002). For example, oral administration of interferon was found to be efficient in animals and humans (Marcus et al. 1999; Cummins et al. 1999). Moreover, Molecular Targeting Technologies, Inc. (MTTI) (PA, USA) is currently selling an interferon-alpha for oral administration (commercial name, Greenferon), which is produced by *Zucchini yellow mosaic virus* (ZYMV)-AG expressing the interferon gene (Arazi et al. 2001). If we can supply edible plants expressing therapeutic proteins for oral use, therapeutic proteins can be administered through inexpensive vegetables and fruits; very expensive protein drugs can be distributed to developing countries that cannot afford to buy such drugs. In our case, aFGF may be purified from infected *N. benthamiana* for industrial production. Although we cannot eat raw soybean seeds without heat treatment, there is perhaps some potential for oral delivery of aFGF by taking raw sprouts of soybean, which are actually eatable. Using our MVP system, we can select the plant best suited for the use of the target protein.

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Figure legends

Fig. 1 Schematic representation of the MVP (modifiable vector for plants) system. **a** RNAs 1 to 3 are the genomic RNAs of CMV. CMV-Sj is specifically adapted to soybean, and its RNA 3 is the host determinant. CMV-Y can infect many host species but not soybean. The C2-H1 vector is RNA Y2-based; **b** Selection of the host plants for production of pharmaceutical proteins depending on the use of protein. The foreign gene is inserted to C2-H1, followed by selecting RNA1 and RNA3 from respective RNA pools according to their uses.

Fig. 2 Construction of the CMV-based vector and cloning of the aFGF gene. Cloning sites *Stu*I, *Mlu*I and *Spe*I were introduced between nucleotide positions 2420 and 2752 in the CMV-Y RNA 2 clone after deletion of the 2b gene. Gene aFGF was cloned between the *Stu*I and *Mlu*I sites.

Fig. 3 Symptoms on plant inoculated with H1-aFGF and stability of the inserted gene. **a** Very mild symptom with vein clearing on the upper systemic leaves of *N. benthamiana* 5 days post inoculation (dpi); **b** Mild mosaic symptoms on *G. max* plant 8 dpi; **c** Agarose gel electrophoresis after RT-PCR to detect the aFGF gene in *N. benthamiana* leaves infected with H1-aFGF (14 dpi). *Lanes*: *M,* DNA size marker (φ174X/*Hae*III digest); *1,* inoculated leaf; *2,* upper leaf; *3,* aFGF gene amplified from the human cDNA library; *4,* healthy plant; *5,* leaf inoculated with the vector, C2-H1. Red arrow indicates

position of the aFGF gene.

Fig. 4 Expression of aFGF in plants by the viral vector. **a** Western blot of aFGF from *N. benthamiana* plants infected with H1-aFGF. Proteins were separated on SDS-PAGE using a 14% gel. *Lanes*: *P,* aFGF produced by *E. coli* (25 ng, positive control); *1,* inoculated leaf (200 ng protein); *2,* upper leaf (200 ng protein); *3,* healthy plant (200 ng protein); *4,* leaf inoculated with H1 (vector). Numbers on the left indicate the positions of protein size markers; **b** Yield estimation of *in planta*-produced aFGF. Total soluble protein was isolated from the leaves infected with H1-aFGF. The original concentration of the protein extracts was 2.2 mg/ml. The 2-fold dilution series of total soluble proteins were separated by 8% SDS-PAGE, blotted onto PVDF membrane and probed with an anti-aFGF antibody. *Lanes*: *P, E. coli*-produced control aFGF (25 ng); *1,* contains 2.2 μg of total soluble protein (from the original extracts); *2-5,* 2-fold dilution series of total soluble protein. Based on the band intensity, we estimated the yield of aFGF to be 5-8% of the total soluble protein. Because an 8% gel was used, considering the efficiency of electro-transfer of protein to membrane, the size difference between the *E. coli*-produced aFGF and the *in planta*-produced aFGF was not resolved.

Fig. 5 Western blot analysis of aFGF from *G. max* and *A. thaliana*. **a** Western blot analysis of aFGF from *G. max* plants infected with H1-aFGF. *Lanes*: *P*, aFGF produced by *E. coli* (25 ng, positive control); *1,* inoculated leaf (200 ng protein); *2,* upper non-inoculated leaf (200 ng protein); *3,* healthy plant (200 ng protein). Total soluble protein preparations

were separated by 14% SDS-PAGE. The *in planta*-produced aFGFs were observed at 17.5 kDa, which is the full-length aFGF size. *E. coli*-produced aFGF was detected at 16 kDa because it is a truncated form. The concentration of the original protein extracts for lane 1 was about 2 mg/ml. Based on the 2-fold dilution series of control aFGF (right picture), we estimated the yield of aFGF from the soybean tissues to be about 2.5% (about 5 ng in lane 1); **b** Western blot analysis of aFGF from *A. thaliana* plants infected with H1-aFGF. *Lanes*: *P*, aFGF produced by *E. coli* (25 ng, positive control); *1*, inoculated leaf; *2*, stalk of inoculated plant; *3*, healthy plant. The concentration of the original protein extracts for lane 1 was about 0.7 mg/ml. Based on the 2-fold dilution series of control aFGF (right picture), we estimated the yield of aFGF from the *Arabidopsis* tissues to be about 1.5% (about 3 ng in lane 1). Numbers on the left indicate the positions of protein size markers.

Fig. 6 Purification profile of *in planta*-produced aFGF. Two weeks after inoculation, total soluble protein was isolated from the leaves infected with H1-aFGF and loaded on a heparin column. aFGF was then eluted with 1.2 M NaCl in PBS-T. The eluted proteins were analyzed by 14% SDS-PAGE. **a** Coomassie-stained gel containing the purified aFGF. *Lanes*: *M*, protein size markers; *P*, *E. coli*-produced control aFGF (200 ng); *1*, total soluble protein (5 μg); *2*, flow-through; *3*, eluted proteins by PBS-T containing 0.6 M NaCl; *4*, eluted proteins by PBS-T containing 1.2 M NaCl. Red arrow indicates the purified aFGF; **b** Western blot anaysis. The proteins separaed by SDS-PAGE were blotted onto PVDF membrane and probed with an anti-aFGF antibody. *Lanes*: *P*, *E.*

coli-produced aFGF (2.5 ng); *1*, total soluble protein (2 μg); *2*, flow-through; *3*, eluted proteins by PBS-T containing 0.6 M NaCl; *4*, eluted proteins by PBS-T containing 1.2 M NaCl. Numbers on the left indicate the positions of protein size markers.

Fig. 7 Effects of aFGF produced in plants on BAE cell proliferation. **a** *A,* Five ng/ml of recombinant human aFGF expressed by *E. coli*; *B,* five ng/ml of aFGF produced in leaves infected with H1-aFGF; *C,* preparation from H1 (vector)-infected leaves (control); *D,* preparation from healthy tobacco leaves (control); *E,* PBS (control). Statistically significant values compared to the control: **P*<0.05, ***P*<0.01. The bar represents standard deviations; **b** Dose-dependent effects of aFGF produced in plants on cell-proliferation of BAE cell (72-h culture). BAE cells (5×10^3) were seeded onto 35-mm dish; **c** Ten ng/ml of aFGF was added to the BAE cells. For clear observation with light microscopy, BAE cells (5×10^4) were seeded into a 35-mm dish. Photographs were taken 48 and 72 h after adding aFGF to the cell cultures (upper pictures). Controls were treated with PBS (lower pictures).

Table 1 Examples of successful production of human cytokines in plants

Cytokines	Expressionsystem	Plant	Yield	Biological activity	Reference
Virus vector					
IFN[a] α	*Cauliflower mosaic virus*	Turnip plant	2 μg/g of FW[f] (0.03% of TSP[g])	+	De Zoeten et al. 1989
IFN α	*Zucchini yellow mosaic virus*	Squash cucumber plant	3.4×10^4~1.57×10^5 IU/g of leaves (FW)	+	Arazi et al. 2001
IFN α	*Clover yellow vein virus*	Broad bean plant (*Vicia faba*)	Not shown	+	Wang et al. 2003
IFN γ	*Brome mosaic virus*	Tobacco cell suspension	3.2~3.7 μg /g of protoplast (FW) (0.05% of TSP)	Not shown	Mori et al. 2001
Transgenic plant					
IFN α	Transgenic	Potato and Tobacco plants	560 IU/g of potato leaves (FW) 1×10^8 IU/g tobacco leaves (FW)	+	Matsumura et al. 2000
IFN α	Transgenic	Potato plant and cell suspension	923~3,029 U/g of tissue or callus (FW)	+	Sawahel et al. 2002
IL[b]-2	Transgenic	Potato plant	115 U/g of potato tubers (FW)	+	Park and Cheong 2002
IL-2 and -4	Transgenic	Tobacco cell suspension	IL-2: 0.10 μg/ml of medium, 0.8 μg/ml of intracellular IL-4: 0.18 μg/ml of medium, 0.28 μg/ml of intracellular	+	Magnuson et al. 1998
IL-4	Transgenic	Potato and Tobacco plants	0.1% of TSP/tobacco leaves 0.08% of TSP/potato tubers	+	Ma et al., 2005
IL-10	Transgenic	Tobacco plant	55 ng/mg of TSP (0.006% of TSP)	Not shown	Menassa et al. 2000
IL-12	Transgenic	Tobacco cell suspension	175 μg/l of medium	+	Kwon et al. 2003
TNF[c] α	Transgenic	Potato plant	15 μg/g of potato plant tissue (FW) (0.2% of TSP)	+	Ohya et al. 2002
GM-CSF[d]	Transgenic	Tobacco cell suspension	240 μg/l of medium, 150 μg/l of intracellular	+	James et al. 2000
GM-CSF	Transgenic	Tobacco plant	0.03% of TSP	+	Sardana et al. 2002
GM-CSF	Transgenic	Tobacco cell suspension	180 μg/l of medium, 783 μg/l of medium with gelatin	Not shown	Lee JH et al. 2002
GM-CSF	Transgenic	Rice cell suspension	129 mg/l of medium (25% of total medium protein)	+	Shin et al. 2003
EPO[e]	Transgenic	Tobacco cell suspension	0.8 μg/l of cultured cell (0.0026% of TSP)	+	Matsumoto et al. 1995

[a]Interferon

[b]Interleukin

[c]Tumor necrosis factor

[d]Granulocyte-macrophage colony-stimulating factor

[e]Erythropoietin

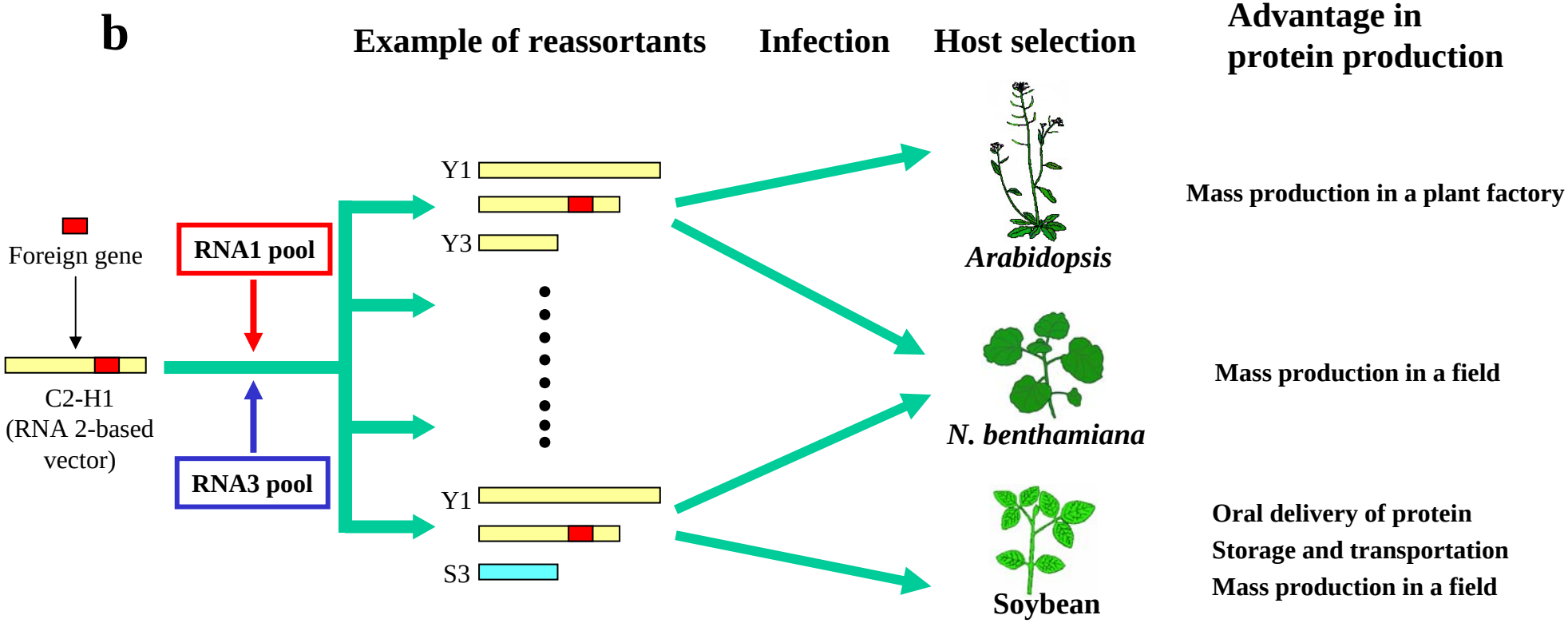
[f]Fresh weight

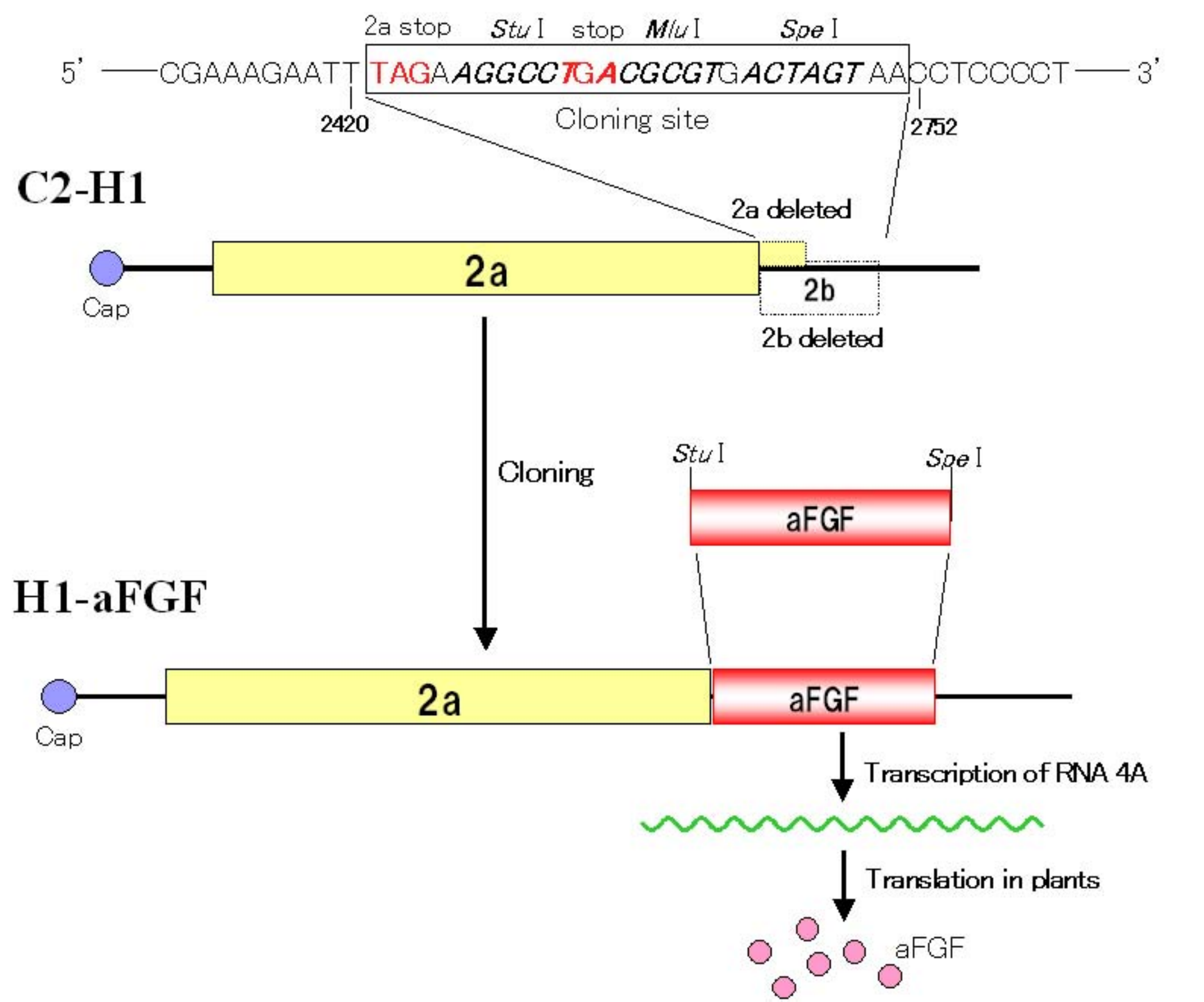
[g]Total soluble protein

a

Virus	RNA	Systemic Infection: *N. benthamiana*	Soybean	*Arabidopsis*
CMV-Y	Y1, Y2, Y3	+	—	+
CMV-Sj	S1, S2, S3	+	+	—
Y1Y2S3	Y1, Y2, S3	+	+	—

b

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stop
Mlu I
Spe I
5'
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2752
C2-H1
2a deleted
2a
2b
Cap
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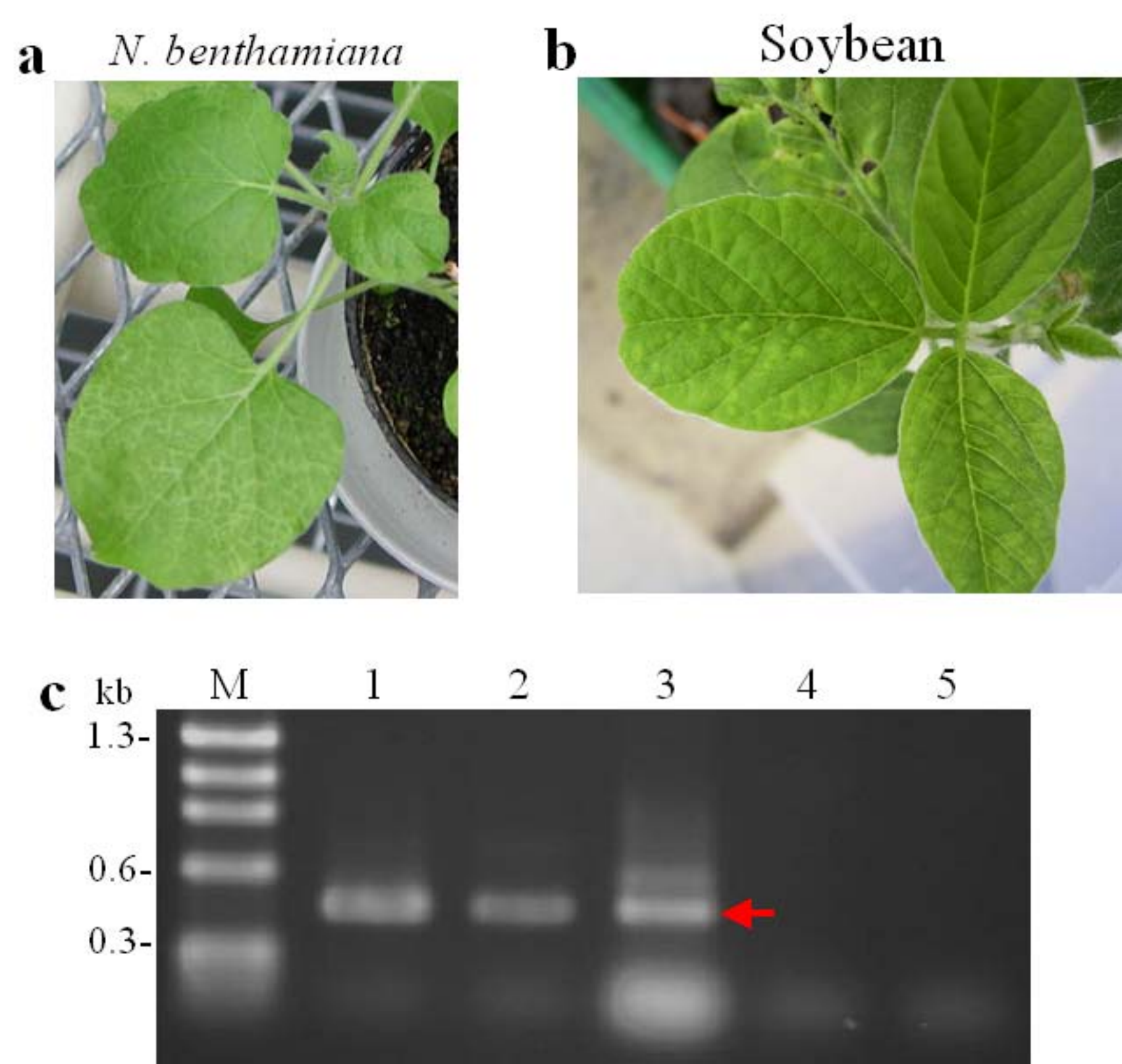
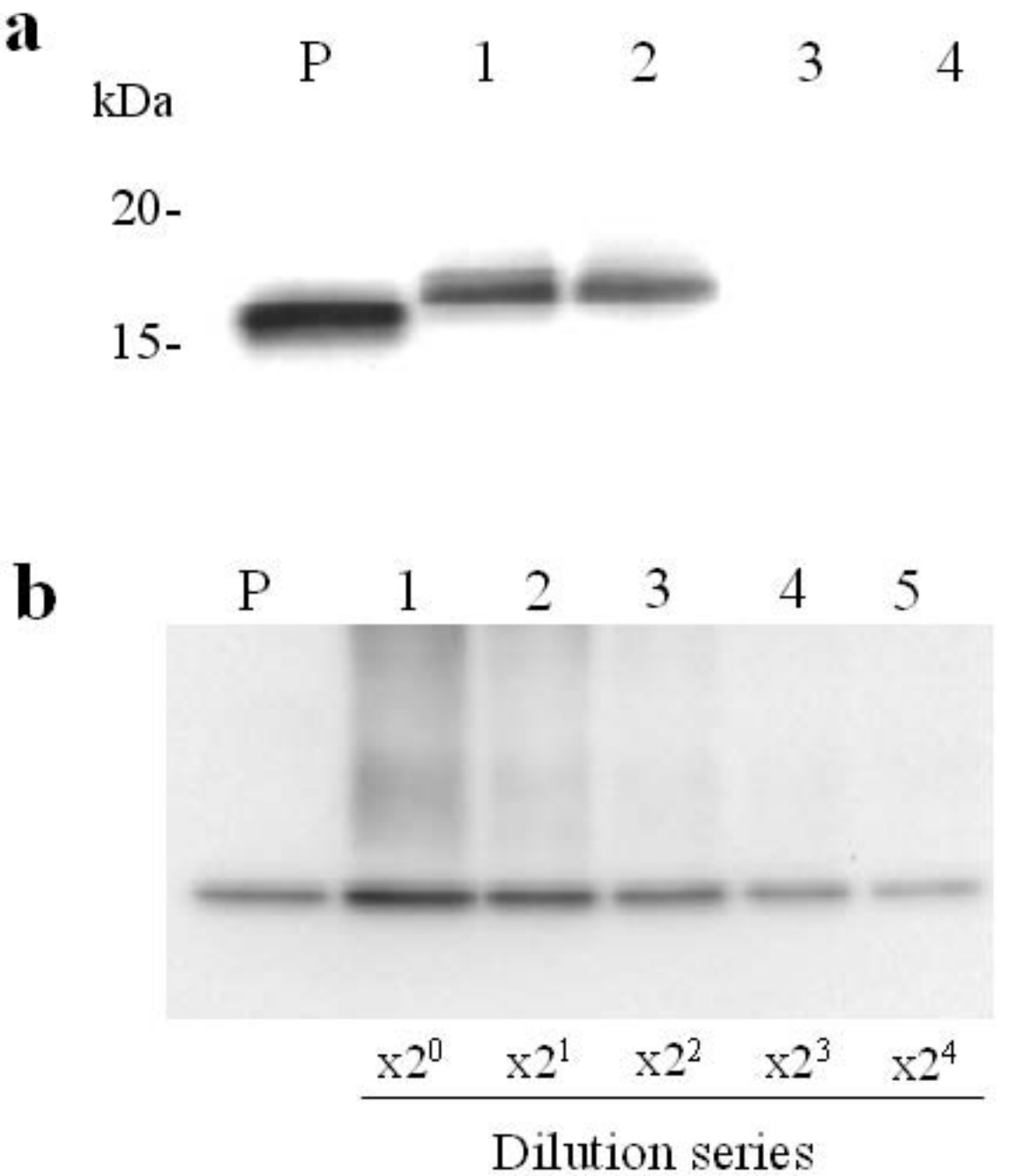
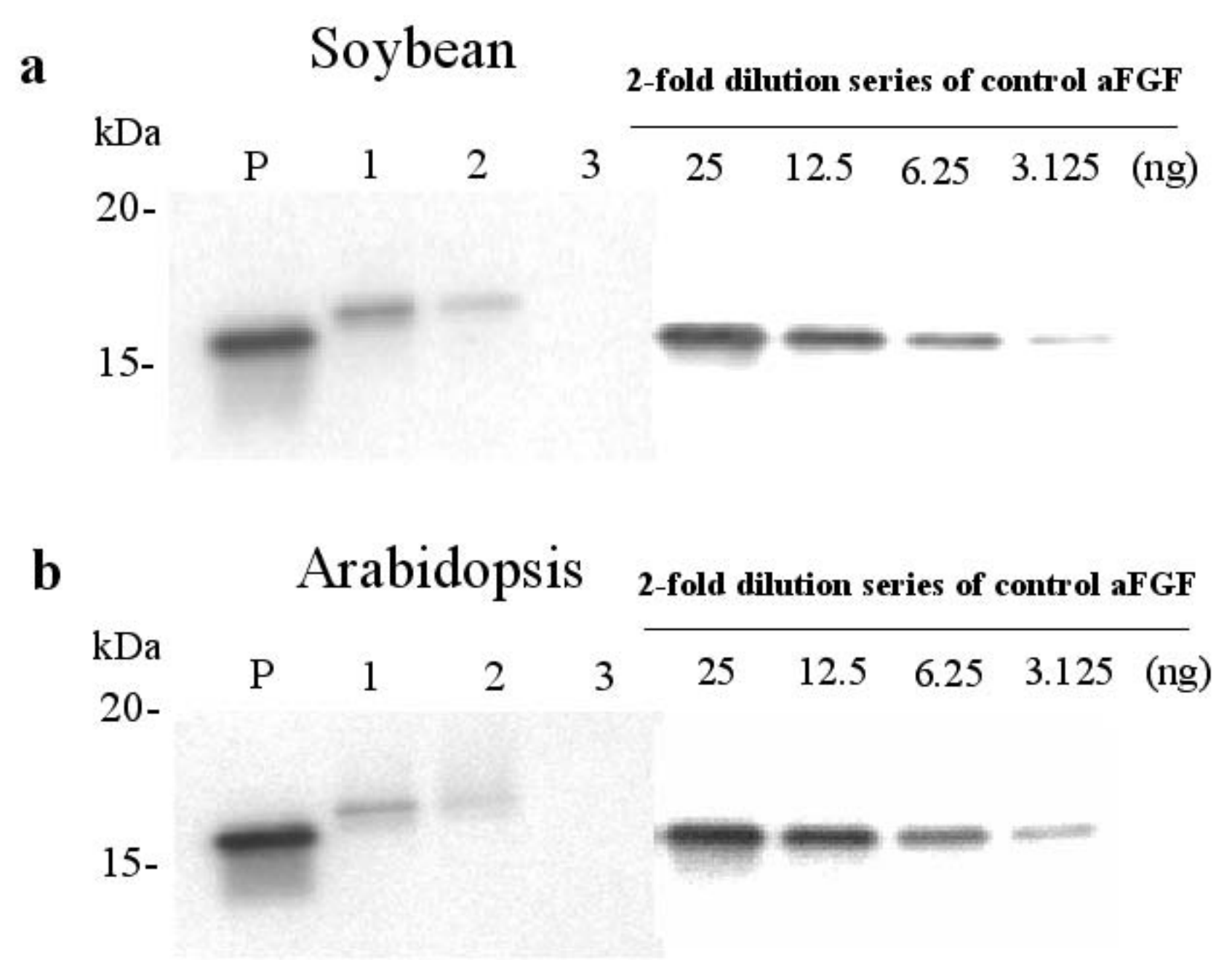
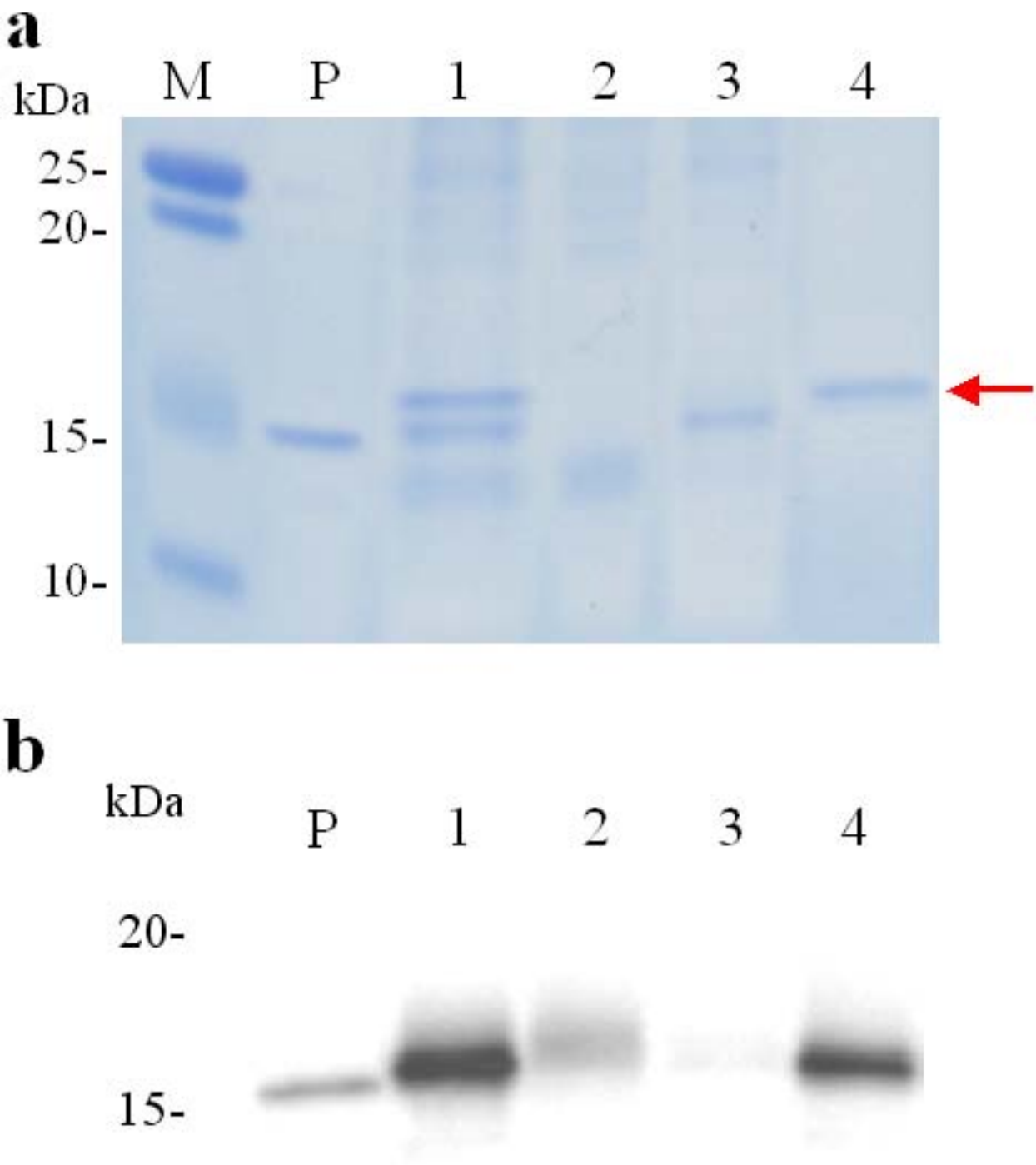
Spe I
aFGF
H1-aFGF
2a
aFGF
Cap
Transcription of RNA 4A
Translation in plants
aFGF


a
N. benthamiana
b
Soybean
c
kb
M
1
2
3
4
5
1.3-
0.6-
0.3-


a
P
1
2
3
4
kDa
20-
15-
b
P
1
2
3
4
5
x2^0
x2^1
x2^2
x2^3
x2^4
Dilution series


a
Soybean
2-fold dilution series of control aFGF
kDa
P
1
2
3
25
12.5
6.25
3.125
(ng)
20-
15-
b
Arabidopsis
2-fold dilution series of control aFGF
kDa
P
1
2
3
25
12.5
6.25
3.125
(ng)
20-
15-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kDa
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b
kDa
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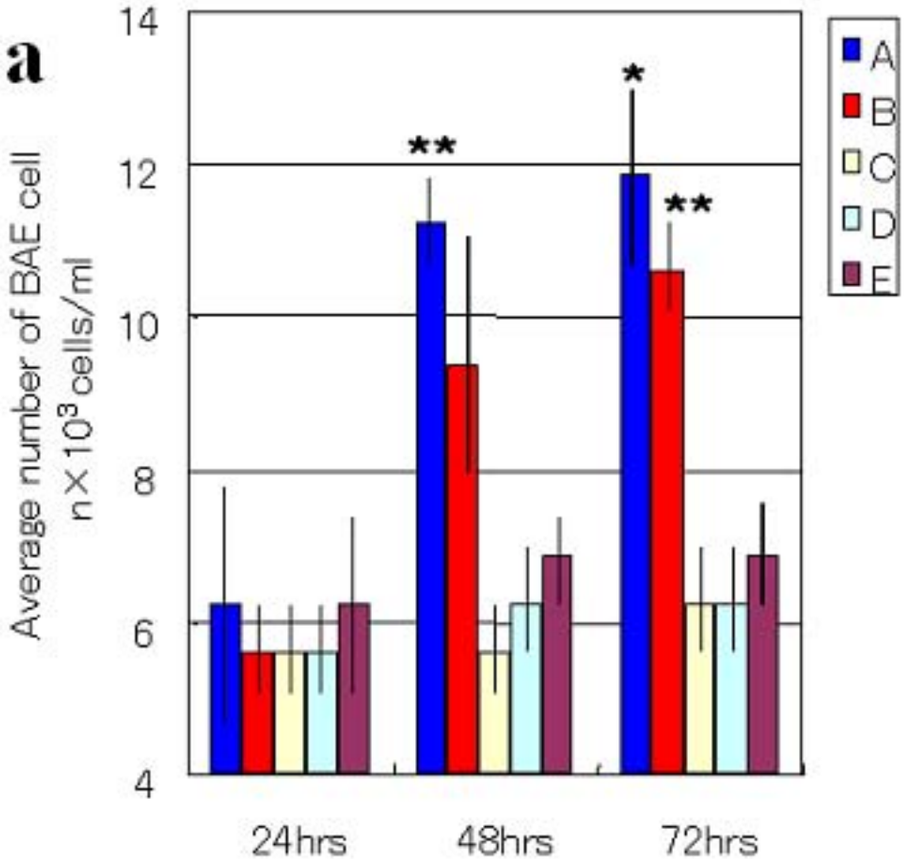
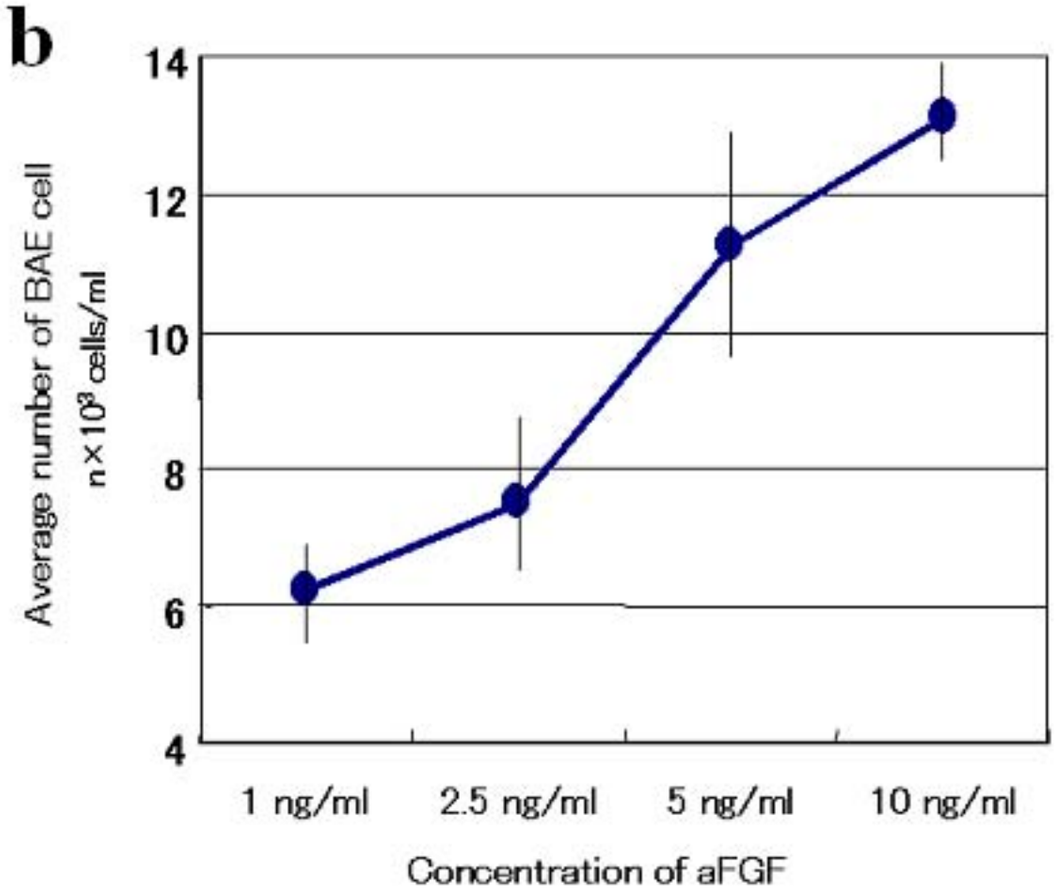
15-


a
Average number of BAE cell
n × 10³ cells/ml
14
12
10
8
6
4
**
*
**
A
B
C
D
E
24hrs
48hrs
72hrs


b
Average number of BAE cell
n × 10³ cells/ml
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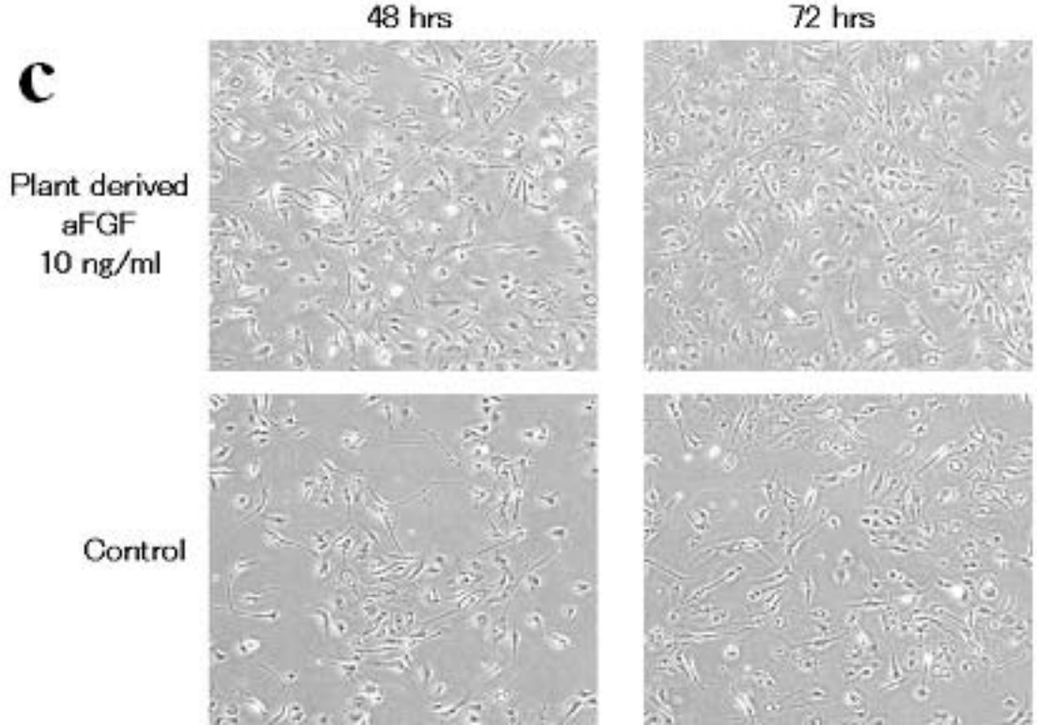
12
10
8
6
4
1 ng/ml
2.5 ng/ml
5 ng/ml
10 ng/ml
Concentration of aFGF


c
48 hrs
72 hrs
Plant derived
aFGF
10 ng/ml
Control