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ORIGINAL PAPER

Production of recombinant single chain antibodies (scFv) in vegetatively reproductive *Kalanchoe pinnata* by in planta transformation

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Abstract We developed an asexual reproductive plant, *Kalanchoe pinnata*, as a new bioreactor for plant-based molecular farming using a newly developed transformation method. Leaf crenate margins were pin-pricked to infect the plant with the *Agrobacterium* strain LBA4404 and vacuum infiltration was also applied to introduce the target gene into the plants. Subsequently, the young mother leaf produced new clones at the leaf crenate margins without the need for time- and labor-consuming tissue culture procedures. The average transformation rates were approximately 77 and 84% for pin-prickling and vacuum-infiltration methods, respectively. To functionally characterize an introduced target protein, a nucleic acid hydrolyzing recombinant 3D8 scFv was selected and the plant based 3D8 scFv proteins were purified and analyzed. Based on abzyme analysis, the purified protein expressed with this system had catalytic activity and exhibited all of properties of the protein produced in an *E. coli* system. This result suggested that vegetatively reproductive *K. pinnata* can be a novel and potent bioreactor for bio-pharmaceutical proteins.

Keywords In planta transformation · *Kalanchoe pinnata* · Molecular farming · scFv · Vegetative reproduction

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Introduction

Recently, transgenic plants have become one of several novel hosts that can be used for the production of recombinant bio-pharmaceuticals such as cytokines, hormones, monoclonal antibodies, enzymes, and vaccines (Miele 1997). Plant system has several advantages over other eukaryotes used for the production of recombinant proteins, namely: (1) the production cost of proteins from plant is less expensive than those from transgenic animals, fermentation, bioreactors, and microbial or animal cell culture-based systems; (2) plant systems can be scaled up quickly; (3) plants do not have any known human or mammalian pathogens; (4) plant cells are able to carry out all of the correct folding, glycosylation, and other post-translational activities necessary for the accurate production of foreign proteins; and (5) plant cells can direct proteins to environments to reduce degradation and therefore increase stability (Horn et al. 2004). Following the first report of successful production of a mouse monoclonal antibody in a plant (Hiatt et al. 1989), many other therapeutic proteins have been expressed in transgenic plants. In general, four approaches have been considered for developing molecular farming system in crop species (Fischer et al. 2004): nuclear transformation, plastid (chloroplast) transformation, virus-mediated transient transformation,

and stable transformation of hydroponically-grown plant species.

Genetic engineering techniques, at their core, involve transferring the gene(s) of one species to another species. Thus, the essence of genetic manipulation in a plant is based on the fact that the new genetic traits can be added into a plant species genome that is totally unrelated to the donor plant species (Job 2002). In recent years, various transformation techniques for plants have been developed, and in most current transformation protocols, a tissue culture and regeneration stage is necessary to recover transgenic plants (Walden and Wingender 1995). The incorporation of genes into the nuclear genome of plants can be carried out by using different techniques, namely, protoplast transformation, biolistics or micro-projectile bombardment, and *Agrobacterium*-mediated transformation. The *Agrobacterium* system is the most useful approach due to its relatively easy protocol with minimal equipment cost requirements and the fact that transgenic plants produced with this method often contain single copy insertions (Lin et al. 1995; Hansen and Wright 1999). *Agrobacterium*-mediated transformation was initially applied only to dicotyledonous plant species; however, it has recently been employed for the transformation of monocotyledonous plant species as well (Hellens et al. 2000; He et al. 2003). Development of in planta transformation as a simple transformation method is of considerable interest, as it does not require a plant tissue culture process (Kojima et al. 2004). In this system, meristematic cells and fertilized egg cells become the primary targets for DNA delivery, and following delivery, multiple shoots are induced from the transformed tissues to regenerate a whole transgenic plant (Supartana et al. 2005).

In the genus *Bryophyllum, Kalanchoe sp.,* along with its hybrids, are generally known to have the ability to produce plantlets (bulbils or gemmae) on their leaves by adventitious (asexual) propagation (Fig. 1). Such epiphyllous budding is a unique morphogenetic phenomenon that is limited to only a few plant species. In *Kalanchoe*, foliar budding is an integral part of the leaf ontogeny, whereby the species has adopted two alternative developmental strategies. Specifically, monophasic type species exhibit a continuous bud differentiation process from the initiation of the primordial stages all the way to plantlet formation. In biphasic type species, such as *K. pinnata*, the first phase extends from initiation to the in situ development of morphologically mature but physiologically dormant buds, and the second phase leads to plantlet formation that is normally triggered in response to leaf detachment (Sawhney and Sawhney 2002).

In this paper, we describe a new in planta *Agrobacterium*-mediated transformation system for *K. pinnata* by using nucleic acid hydrolyzing recombinant antibody, 3D8 scFv, which we isolated and characterized (Kim et al. 2006) and discuss the possibility of its use as a novel bioreactor system for the production of recombinant antibodies.

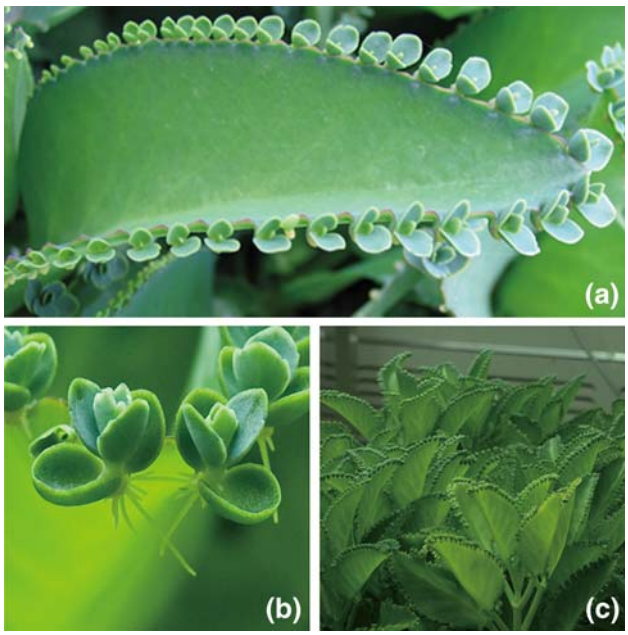

Fig. 1 *K. pinnata* leaf plantlet development. **a**, **b** A mature leaf of the *K. pinnata* plant. Plantlets develop on the leaf crenate margins of the mother leaf. **c** Plantlet formation on a leaf

Materials and methods

Plant materials and growth conditions

Kalanchoe pinnata were grown at 38–40°C, 65–70% relative humidity with 8/16 h dark/light photoperiods under 3000-lux standard light.

Vector construction

For reporter gene transformation, we used the pCAMBIA1303 plant expression vector (Center for Application of Molecular Biology to International Agriculture, Austria). pCAMBIA1303 contains two reporter genes [*β-glucuronidase* (*GUS*) and g*reen fluorescence protein* (*GFP*)]. For transformation with 3D8 scFv, we used a pBI121 to express 3D8 scFv in transgenic *K. pinnata.* Two tags of His(6) and Protein-A at *C*-terminal were introduced into the pBI121 expression vectors to facilitate enrichment and purification of the 3D8 scFv protein using nickel-nitrilotriacetic acid (Ni-NTA) resin or Sepharose IgG resin, respectively (Fig. 4a, b).

Agrobacterium culture and transformation

Each plasmid was introduced into *Agrobacterium* LBA4404 by freeze-thaw method (Hofgen and Willmitzer

1988). *Agrobacterium* LBA4404 harboring each vector was cultured until the absorbance at 600 nm reached 0.7–0.8. Cultured *Agrobacterium* was harvested by centrifugation at 3,000 rpm for 15 min. The cells were then re-suspended with transformation buffer [100 μM of 3′5′-dimethoxy-4′-hydroxyacetophenone (acetosyringone) and 200 μl/l of Silwet L-77(Lehle Seeds Co., USA) in 1/2 Murashige and Skoog (MS) media (Murashige and Skoog 1962)] (Bechtold and Pelletier 1998).

K. pinnata transformation

Pin-prickling transformation method

K. pinnata was grown under the controlled conditions and mature leaves (~20 cm in size) with no plantlets were used for transformation. Leaf crenate margins were assumed to be a potential site for plantlet development. To allow *Agrobacterium* to deliver the target genes into plant cells, epidermal tissues of leaf margins were scraped with carborundum or alternatively, leaf notches were pricked with a tungsten pin (<0.2 mm in diameter). Wounded leaves were co-cultured with *Agrobacterium* at 28°C in 1500 lux for about 10 days until new plantlets formed on the infected leaf notches (Fig. 2).

Vacuum-infiltration method

K. pinnata with four leaves were pre-incubated with high humidity (over 75%) in the dark for 20–30 h at 25°C. The pretreated plants were then submerged in *Agrobacterium* transformation buffer described previously under the vacuum pressure of 300–500 mmHg/cm for 30 min. After removing the vacuum pressure rapidly, plantlets were air-dried for 10–20 min on 3MM paper. The infected plantlets were then incubated in the dark at 25°C for 24 h. The plants were subsequently incubated at 28°C for 7 days to allow for recovery from physical stress. Finally, putative transgenic plants were screened on MS media with kanamycin (100 mg/l) for pBI121 vector or hygromycin (30 mg/l) for pCAMBIA1303 vector (Fig. 2).

GUS staining and GFP fluorescence detection

GUS staining was basically carried out by the standard protocol (Jefferson et al. 1987). Sample tissues were fixed in iced 90% acetone for 20 min. The fixed tissues were then incubated overnight in a GUS staining solution [500 mM $NaPO_4$, pH 7.0, 2 mM $K_3(Fe[CN_6])$, 2 mM $K_3(Fe[CN_6])\cdot 3H_2O$, 0.1% (v/v) Triton X-100, 10 mM EDTA, and 200 mM 5-bromo-4-chloro-3-indoly-β-D-glucuronide (X-GlucA)]. After staining, the samples were treated with 70% (v/v) ethanol for 1 h, followed by 100% (v/v) ethanol at room temperature overnight to remove chlorophylls. Protoplasts were isolated enzymatically (Smith et al. 1984). Mesophyll tissue pre-incubation for 15 min in MM solution (400 mM of mannitol and 25 mM of 2-(*N*-morpholino)ethanesulfonic acid, pH 5.7). The tissue was transferred into the cell wall lysis enzyme mixture buffer for 45 min at 37°C. Finally, the tissue was transferred to the experimental solution (400 mM of mannitol, 5 mM of $MgSO_4$, 0.2% (w/v) polyvinylpyrrolidone-40, 1 mM of dithiothreitol, 25 mM of MES, pH 5.7). Protoplasts were washed in the experimental solution by mild centrifugation (Hafke et al. 2001). GFP fluorescence of transgenic *K. pinnata* was detected under UV light (380 nm) by Confocal Laser Scanning Microscope (MRC-1024, Bio-Rad, UK). The selected transgenic plants were then transferred to individual pots and subjected to re-screening for GFP fluorescence from R_1 (the first reproduction from the first transformation, T_0) to R_3.

Genomic polymerase chain reaction (PCR)

Genomic DNA was extracted by using lysis buffer [0.05 M Tris–HCl, pH 7.6, 0.1 M NaCl, 0.05 M EDTA, pH 8.0, 0.5% sodium dodecyl sulfate (SDS), 0.01 M β-mercaptoethanol] (Edwards et al. 1991). For the genomic PCR analysis, purified genomic DNA was digested by *Bam*H I and *Hin*dIII (Takara-bio, Japan) for 45 min in a 37°C water bath and 3 h in a 37°C incubator. A total of 3 μg of digested genomic DNA was used for PCR reaction with gene-specific primers (*GUS* specific primer forward: 5-CTGATA GCGCGTGACAAAAA-3 and backward: 5-GGCACAG CACATCAAAGAGA-3 and *GFP* specific primer forward: 5-TCAAGGAGGACGGAACATC-3 and backward: 5-AA AGGGCAGATTGTGTGGAC-3). PCR was performed using TaKaRa Taq™ DNA polymerase (Takara-bio, Japan) with the following conditions: the 1st denaturation at 95°C for 10 min, the 2nd denaturation at 94°C, annealing at 56°C and extension at 72°C for 30 s each for 32 cycles, and a final extension at 72°C for 10 min. Amplified PCR products were separated on a 1% agarose gel.

Reverse transcriptase polymerase reaction (RT-PCR)

Total RNA was extracted from transgenic and wild type plants using Trizol® reagent (Invitrogen, USA) (Gehrig et al. 2000). Samples were treated with DNase prior to RT-PCR with the specific primer sets. For PCR reactions, 3 μg of synthesized cDNA was used with gene-specific primers (3D8 scFv specific primer forward: 5-CAGGGCCTTG AGTGGATTGGA-3 and backward: 5-GGTCCCCGA TCCGAACGTATA-3). PCR was performed using TaKaRa Taq™ DNA polymerase (Takara-bio, Japan) with the following conditions; 95°C for 10 min, 30 cycles of 94°C

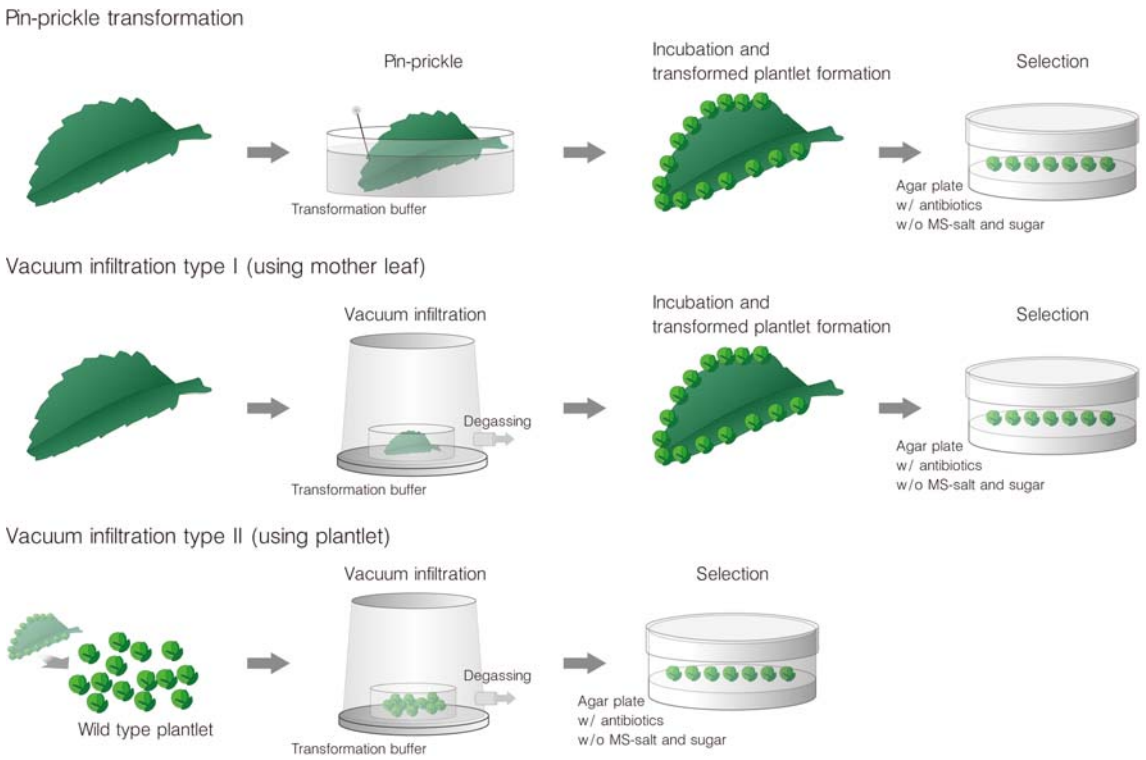

Fig. 2 Illustration of the transformation methods for *K. pinnata*. Two principal technical approaches, pin-prickling and vacuum infiltration, were performed to deliver *Agrobacterium* into *K. pinnata*. For the pin-prickling method, the mature leaf without plantlet development was soaked in transformation buffer mixture containing *Agrobacterium* and prickling of the crenate margins was performed using a fine micro-needle 5–10 times. Vacuum-infiltration methods were divided into two types. In the first type, a mature leaf without plantlet formation was soaked in transformation buffer with *Agrobacterium* and was subjected to vacuum pressure (500 mmHg/cm) for 30 min. In the second type, the same procedure was performed using plantlets

for 30 s, 56°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 10 min. Amplified PCR products were analyzed on a 1% agarose gel.

Purification of 3D8 scFv protein

Total plant proteins were extracted from leaves of 3D8 scFv transgenic plants such as *K. pinnata*, *Nicotiana tabacum* (prepared by CelTech laboratory, Sungkyunkwan Univ., Korea), *Lycopersicon esculentum*, *and Brassica rapa* (kindly provided by Dr. DS Kim, Rural Development Administration, Korea). Leaves were homogenized in liquid nitrogen and re-suspended in cold protein extraction buffer [100 mM Tris–HCl, pH 8.0, 10 mM EDTA, 5% SDS, 20% sucrose, 5% β-mercaptoethanol, and 0.1% protease inhibitor cocktail (Sigma-Aldrich, USA)]. Samples were then centrifuged for 45 min at 4°C, and the collected supernatant was filtrated with a 0.25 μm filter. Protein concentrations were measured according to the Bradford method by using the Sigma reagent with bovine serum albumin (BSA) (Sigma-Aldrich, USA) as a standard. For purification of 3D8 scFv, a 1/5 volume (v/v) of IgG Sepharose^{TM} 6 Fast Flow gel matrix (Amersham Bioscience, USA) that was pre-equilibrated to pH 7.2 with PBS (1.2 g NaH_2PO_4, 7.591 g NaCl, made up to 1000 ml, pH 7.2) was added into filtrated supernatants. Binding of the Protein-A tag to an IgG resin was conducted at 4°C in a cold chamber for 60 min by inversion. The IgG Sepharose^{TM} resin was then packed in a column tube and the 1st wash was performed with 200 ml of PBS-T (PBS with 0.05% Tween-20, pH 7.4) followed by the 2nd wash with two volumes of 5 mM ammonium acetate (pH 5.0). Resin bound proteins were then eluted with 0.1 M acetic acid (pH 3.4) and 1 M Tris–HCl (pH 9.5) was added for neutralization and concentration. Buffer exchange to TBS (150 mM NaCl, 10 mM Tris–HCl, pH 7.5) was performed using a Vivaspin 20^{TM} with a 10,000 MWCO (Viva science, Germany) (Desai et al. 2002).

Enzyme-linked immunosorbent assay (ELISA)

The amounts of 3D8 scFv produced in various plant leaves were quantified by ELISA (Engvall and Perlmann 1971). Briefly, the samples were diluted to 20 μg/ml with coating buffer (1.59 g sodium carbonate (anhydrous), 2.93 g sodium bicarbonate, and 0.2 g sodium azide dissolved in

1000 ml distilled water) and bound to a 96-well polyvinyl chloride micro-titer plate at 4°C overnight in a humidity box followed by two washes with PBS. Background was blocked with 5% (w/v) BSA in PBS at 4°C overnight in a humidity box. Next, the samples were washed with PBS three times and incubated with anti-3D8 scFv polyclonal antibody at a 1:1000 dilution with buffer (1% BSA (w/v) in PBS) for 2 h at room temperature in a humidity box. The wells were then washed three times with PBS-T and incubated with a 1:5000 dilution of anti-rabbit IgG-conjugated HRP for 2 h at room temperature. The wells were washed with PBS-T four times and reacted with a PNP solution (0.1 g of magnesium chloride hexahydrate, 0.2 g of sodium azide, 97 ml of diethanolamine, pH 9.8 with hydrochloric acid in a total volume of 1000 ml) until the reaction changed to a yellow color (about 1 h) at room temperature. To terminate the reaction, 50 µl of 0.75 M NaOH was added (Kingan 1989). Purified proteins from *E. coli* were used to generate a standard curve to calculate the amount of 3D8 scFv protein.

Abzyme assay

The catalytic activity of the purified 3D8 scFv protein was determined by a modified abzyme assay using dsDNA (Kim et al. 2006). Briefly, dilutions of standard protein purified from *E. coli*, negative control protein (BSA), and purified *K. pinnata* 3D8 scFv were added to micro-tubes containing nucleic acid substrates (dsDNA) in 2X abzyme buffer [20 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA]. Samples were taken every 10 min during an incubation that lasted 2 h at 37°C. To terminate the reaction, 5 mM EDTA was added to micro-tubes. For detection of the degradation pattern of nucleic acid, samples were analyzed on a 1.5% agarose gel.

Western blot analysis

A total of 30 µg of protein from each plant was diluted with TBS and boiled for 5 min with 2X protein loading buffer (2.4 g of urea, 20 µl of ampholyte solution, pH 3.5–10, 100 µl of ampholyte solution, pH 4–6, 500 µl of 20% (w/v) Triton X-100, 50 µl of β-mercaptoethanol, and 200 µl of 1% (w/v) bromophenol blue in total volume of 5 ml). Next, the samples were electrophoresed on a 12% SDS polyacrylamide gel. The separated proteins were then transferred to a nitrocellulose membrane for immunoblotting. 3D8 scFv from *E. coli* was used as a positive control. The primary anti-3D8 scFv polyclonal antibody was used at a 1:1000 dilution, and the secondary antibody (goat anti-rabbit IgG-HRP conjugate) was used at a 1:5000 dilution. Detection was performed using an ECL Western blotting system (Amersham Bioscience, USA) (Tazaki et al. 1995).

Results

Molecular analysis of transgenic *K. pinnata*

To validate the successful production of transgenic *K. pinnata*, genomic PCR and RT-PCR techniques were employed. First, DNA and total RNA were extracted from transgenic plants, respectively, and the presence of a *GUS* gene in the transgenic plants was confirmed with PCR using *GUS*-specific primers. The *GUS* gene was successfully amplified from the genomic PCR. In vacuum infiltration conditions at 300 and 400 mmHg/cm, plantlets were produced from transformed mother plant leaves; however, RNA transcripts were not detected from their tissues (Fig. 3a). Conversely, under the same vacuum conditions, transformed young small *K. pinnata* plants produced plantlets in which the PCR band had the expected size. The optimal vacuum pressure was fixed to 500 mmHg/cm plantlet transformation.

In transgenic plants generated with the pin-prickling method, the *GUS* gene was also amplified from total RNA. To confirm the transgene transmittance through asexual propagation, *GUS* gene amplification was checked by genomic PCR and RT-PCR with R_1 and R_2 plants (Fig. 3b, c).

Histochemical analysis by GUS staining and analysis of GFP expression

In planta transformed *K. pinnata* was subjected to asexual reproduction to produce the R_1 generation by plantlet formation on the crenate margins of transgenic mother leaves. All transgenic plants exhibited normal growth and development. To monitor any possible *Agrobacterium* contamination during transformation, GFP expression in transgenic *K. pinnata* leaves was initially analyzed by confocal microscopy; the results showed the green fluorescence on the reverse side of leaves. Both R_1 and R_2 plants had green fluorescence at the tissue level (Fig. 3d), while R_2 and R_3 leaf-derived protoplasts were analyzed for GFP expression at the cellular level. The results showed that there was GFP expression in cytosol (Fig. 3d). Together, these findings strongly indicate that GFP was inserted and actively expressed in individual cells of transgenic *K. pinnata*.

Other analysis for monitoring the transgenic plants was carried by GUS staining. Leaf tissues from T_0 *K. pinnata*, which *GUS* gene was delivered into, exhibited a blue color upon GUS staining. In the same manner, the transformed

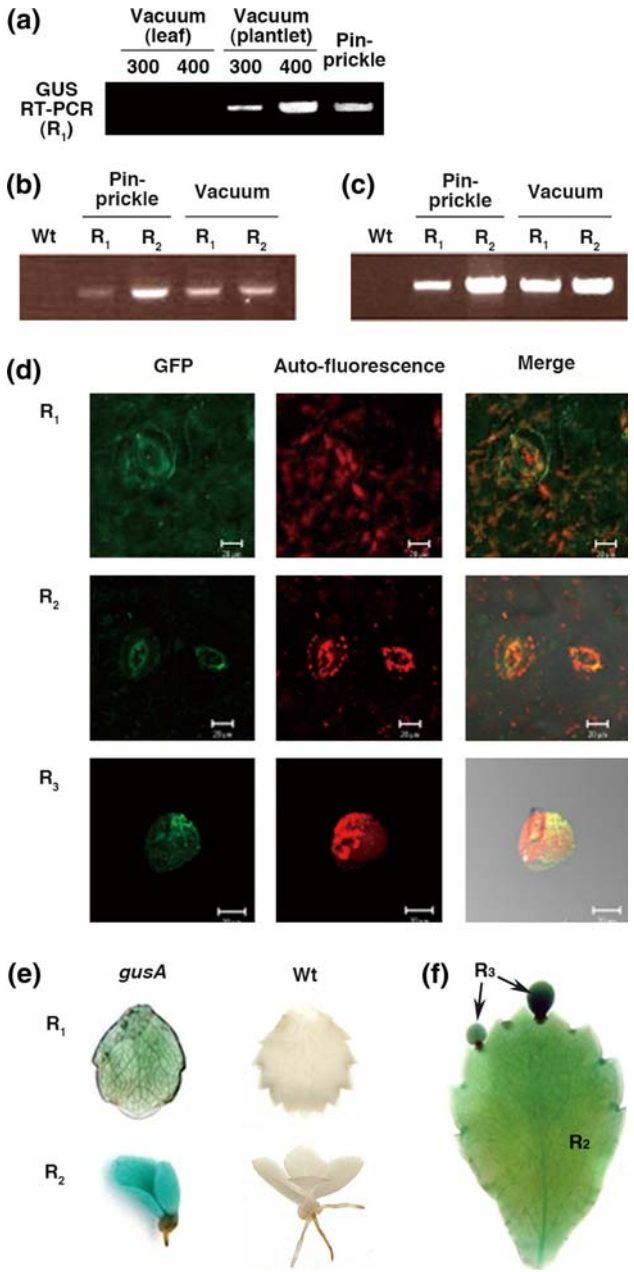

Fig. 3 Transgene analysis of transgenic *K. pinnata*. **a** RT-PCR analysis of GUS in transgenic *K. pinnata* R_1 with various vacuum pressure conditions (300 and 400 mmHg/cm) and plant materials (mature mother leaves and young plantlet). The pin-prickling transformated *K. pinnata* (*lane 5*) as contrast. **b** Genomic PCR analysis results of *GUS* in transgenic *K. pinnata* by pin-prickling and vacuum-infiltration transformation. *GUS* gene amplification in the R_1 and R_2 plants and RT-PCR results (**c**). **d** Confocal microscopy analysis of GFP transgenic *K. pinnata*. **e** GUS staining of *GUS* transgenic *K. pinnata*. **f** GUS staining of *GUS* transgenic *K. pinnata* during vegetative regeneration of R_2 to R_3

tissues stained blue around leaf veins in R_1 plantlets. Likewise, the R_2 regenerated from R_1 stained totally blue in all leaf (Fig. 3e). A transgenic R_2 leaf with plantlets on its notches also stained blue (Fig. 3f). In most case, newly produced R_1 regenerated from T_0 or R_2 from R_1 at the leaf margin of T_0 or R_1 leaf, respectively, stained darker blue than T_0 or R_1 leaf.

Transformation efficiency

The efficiencies of the two transformation methods (pin-prickling and vacuum-infiltration) were estimated to be 77 and 84%, respectively, based on T_0 transformants per an initial plant identified with kanamycin selection (100 mg/l) and GUS staining. The frequency of transmission of transgenes from R_1 to R_2 was more than 97% in both transformation methods. Interestingly, the pin-prickling transformation system showed about 2% higher transgene transmission to subsequent generations than the vacuum-infiltration system (Table 1).

Transformation of *K. pinnata* with the 3D8 scFv gene

To transform *K. pinnata*, the plant expression vector pBI121 harboring 3D8 scFv was delivered into the *K. pinnata* genome (Fig. 4a, b). The vacuum-infiltration method was used for delivery and a large number of plantlets was propagated and subjected to random selection. Next, putative transgenic plants were re-selected by kanamycin resistance and RT-PCR analysis was conducted to assess of 3D8 scFv expression. The *phosphoenolpyruvate carboxylase* (*PEPC*) gene was used as a positive control for RT-PCR. As shown in Fig. 4c, expression of 3D8 scFv was detected as a single band ($\sim$600 bp in size) by RT-PCR in R_1 to R_3 plants which were transformed by vacuum-infiltration methods (V-01-III-32 and V-01-V-12) and pin-prickling method (P-96-II-32 and P-96-iV-13). Genomic PCR also showed a single band in two transformation methods (Fig. 4c). RT-PCR confirmed expression of the 3D8 scFv gene from each transgenic lines and generations, albeit with different expression levels amongst the selected lines. Finally, V-01-V-12 line from 3D8 scFv-expressing plants on lane number 4 in the R_2 generation was selected for protein analysis (Fig. 4c).

Purification of 3D8 scFv::Protein-A from transgenic *K. pinnata*

The 3D8 scFv protein was purified from transgenic *K. pinnata* and verified as a band approximately 38-kD in size by using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western analysis by using antiserum specific to 3D8 scFv (Fig. 5a). To measure the purification efficiency, anti-3D8 scFv polyclonal antibody was examined by western blot and ELISA analysis at each step of purification. From crude extracts (WT-T) through to final elutes purified on an IgG Sepharose™

Table 1 Efficiency and inheritance of in planta transformation of *K. pinnata* determined by antibiotic resistance

(a) Transformation efficiency

Method	Material	Execution number (*n*)	Regenerated buds (R_1/T_0)	Km^+ R_1 number (Km^+ R_1/T_0)	Km^+ R_1 efficiency (%)
Pin-prickling	Crenate margin	72	486 (6.76 ± 0.35)	409 (5.69 ± 0.72)	84.2
Vacuum	Young mother leaf	137	1887 (13.78 ± 0.41)	1468 (10.72 ± 1.02)	77.8
Vacuum	Budded plantlet	350 (50 × 7 set)	267 (survival number)	219 (Km^+ number)	82.0

(b) Frequency of transgene inheritance

Method	Material	Km^+ R_1 number	Regeneration buds (R_2) (R_2/R_1)	Km^+ R_2 number (Km^+ R_2/R_1)	Km^+ R_2 efficiency (%)
Pin-prickling	Crenate margin	54	1059 (19.6 ± 4.2)	1045 (19.3 ± 2.54)	98.7
Vacuum	Young mother leaf	72	665 (9.2 ± 3.2)	644 (8.9 ± 0.84)	96.8
Vacuum	Budded plantlet	120 (30 × 4 set)	449 (3.7 ± 0.72)	443 (3.6 ± 0.28)	98.6

Efficiencies are expressed as the percentage of antibiotic resistance shoots (plantlets) relative to the total number of regenerated shoots (plantlets)

Efficiency mean = (Km^+ R_1/total R_1) × 100

Efficiency mean = (Km^+ R_2/total R_2) × 100

resin column (Tx-P), a clear target size band (about MW 38 kD) became concentrated and purified. As shown in Fig. 5a, 3D8 scFv expression was confirmed by the presence of band (38 kD) on western blots of transgenic *K. pinnata*. But in some cases, 3D8 scFv protein which was expressed in *E. coli* and plants was detected by two bands in sizes of 29 and 38 kD (Fig. 5a). 3D8 scFv::Protein-A (Fig. 5a, *lane 1*) produced in an *E. coli* also had two bands and identical to the transgenic plants. These two bands were 3D8 scFv proteins and both functionally active shown by nuclease hydrolyzing catalytic activity as confirmed by zymography (Fig. 5b). We need to figure out why 3D8 scFv protein was shift down from 38 kD to 29 kD in size on the SDS-PAGE analysis.

Enzyme activity test

3D8 scFv is a recombinant antibody and was found to have nucleic acid hydrolyzing catalytic activity (Kim et al. 2006). For abzyme analysis, Protein-A tagged 3D8 scFv protein was incubated with dsDNA (pUC18 plasmid DNA) upto 120 min at 37°C and then the reactants were analyzed on 1.5% agarose gel (Fig. 5c). 3D8 scFv proteins expressed in *E. coli* and *K. pinnata* started to show the DNase activity from 10 min after incubation. *E. coli* based 3D8 scFv showed a little bit more active DNase activity than *K. pinnata* based 3D8 scFv. However plant based 3D8 scFv hydrolyzed dsDNA band to small pieces of DNA (shown by smeared band pattern) at 120 min after incubation and completely digested at 4 h after incubation (Fig. 5d). Taken together, these results suggest that plant-derived 3D8 scFv retains its functional activity.

Production yield of 3D8 scFv protein in transgenic *K. pinnata*

K. pinnata based 3D8 scFv production efficiency was compared to three other plant species, *N. tabacum*, *L. esculentum*, and *B. rapa*. The contents of total soluble protein were measured from four plant species harboring 3D8 scFv gene by a Bradford assay. *N. tabacum* had the highest value (>8 mg/g), while the lowest value was from *K. pinnata* (<1 mg/g) (Fig. 6a, b). Production levels of 3D8 scFv out of total soluble proteins were quantitatively analyzed by an ELISA. The 3D8 scFv production from *K. pinnata* was measured as 0.126 mg per 1.0 mg of total soluble protein, indicating that 3D8 scFv comprised about 12% of total soluble protein with a 0.95 error rate, while production levels of the other plants range from 2% to 4% of total soluble protein. Indeed, the 3D8 scFv production yield per total soluble protein of *K. pinnata* was six times greater than that of *B. rapa* (Fig. 6). This quantitative difference of 3D8 scFv protein expression yield per total soluble protein shown by ELISA was confirmed by western analysis (Fig. 6c).

Discussion

Agrobacterium-mediated plant transformation requires the selection of putative transformants to three generations for homozygote fixation, because the transgene can be removed from chromosomes by genetic recombination during sexual reproduction (Birch 1997). Moreover, conventional transformation methods require in vitro culture

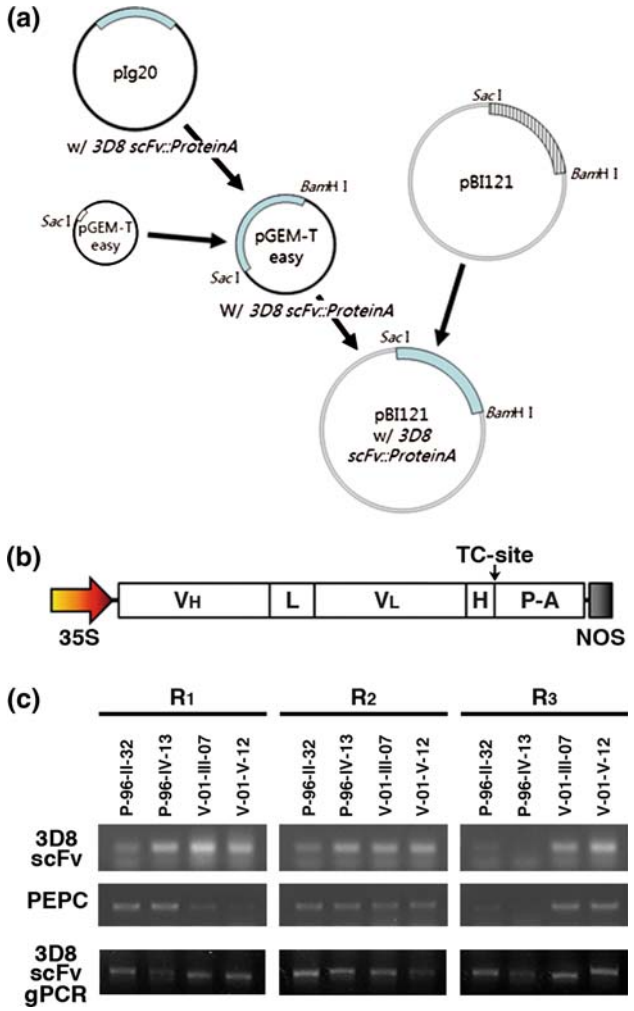

Fig. 4 Plant transformation vector for the expression of the 3D8 single chain antibody (3D8 scFv) in *K. pinnata* and transgene conformation with RT-PCR and genomic PCR. **a** Process of 3D8 scFv expression vector construction. **b** The recombinant T-DNA region consisting of the Cauliflower Mosaic Virus 35S (35S) promoter and nopaline synthase terminator (NOS). 3D8 scFv was fused to a His(6) tag (H) and protein A tag (P-A). The TC-site has a thrombin cleavage recognition site. **c** RT-PCR analysis of 3D8 scFv mRNA expression and genomic PCR analysis in transgenic *K. pinnata*. Total RNA was extracted from R_1, R_2, and R_3 fully expanded leaves. The expected 3D8 scFv RT-PCR product was amplified from all plants. The middle section is a phosphoenolpyruvate carboxylase (PEPC) amplicon used as an RT-PCR positive control. The bottom section is genomic PCR results of transgenic *K. pinnata*. The single band indicates amplification from targeted 3D8 scFv gene. Transgenic line named by pin-prickling (P) or vacuum-infiltration (V) method and the year of transformation and serial number T_0 and serial number R_1

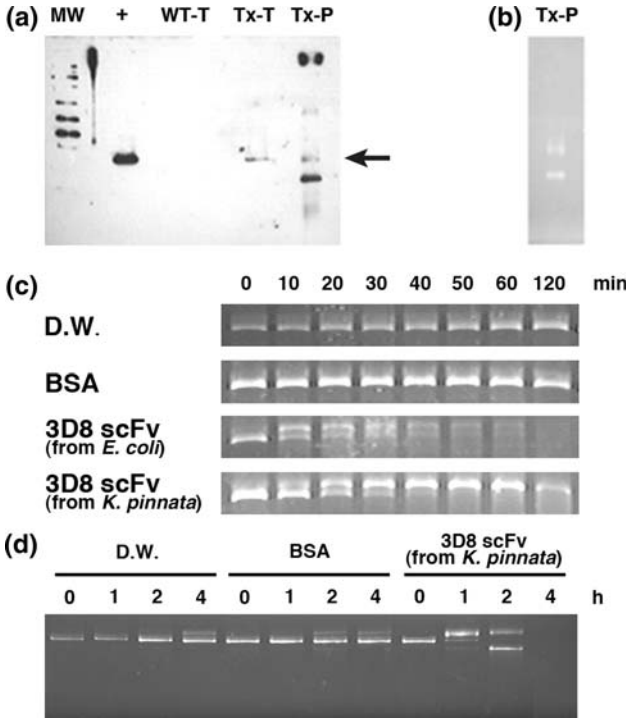

Fig. 5 DNA dehydration of purified 3D8 scFv from *K. pinnata*. **a** Western blot of 3D8 scFv proteins purified from wild type and transgenic *K. pinnata* leaves with anti-3D8 scFv polyclonal antibody. The *arrow* indicates the expected 3D8 scFv MW. *E. coli* purified positive control (+), total soluble protein from wild type *K. pinnata* (WT-T), total soluble protein from transgenic *K. pinnata* (Tx-T), and purified soluble protein from transgenic *K. pinnata* (Tx-P) **b** Zymography analysis of the purified 3D8 scFv protein. The two separated bands of purified protein have nuclease activity. **c**, **d** In vitro abzyme analysis of scFv from *K. pinnata* and *E. coli*. 3D8 scFv expressed from *E. coli* was used as a positive control for abzyme analysis. Distilled water and BSA were used as negative controls

and regeneration (Aida and Shibata 1996), which are the main factors responsible for low transformation efficiency due to gene silencing and tissue culture-induced mutations (Garces et al. 2007). Here, our study provides an alternative method for establishing reliable and stable transformants. The newly introduced genetic trait can be maintained through vegetative propagation of the transgenic *K. pinnata* plant. Moreover, the formation of multiple plantlets on the transgenic plant leaves provides an excellent opportunity for mass production of transgenic plants without the need for tissue culture. Based on the results of GUS staining and GFP fluorescence (Fig. 3) and antibiotics selection from R_1 to R_2 generation (Table 1), it can be inferred that the regenerated plantlets from transformed mother plant had identical genetic traits to the mother plant.

In this study, two in planta transformation approaches (pin-prickling and vacuum-infiltration) were developed to transform asexually reproducible plant, *K. pinnata*. This study began with the assumption that if the meristem is located in the crenate margin of a *K. pinnata* leaf and a whole plantlet can be regenerated from this site, any foreign gene inserted into the meristem area will be present in the entire developed plantlet. The pin-prickling method produced a low frequency (2.4-fold lower) of plantlet

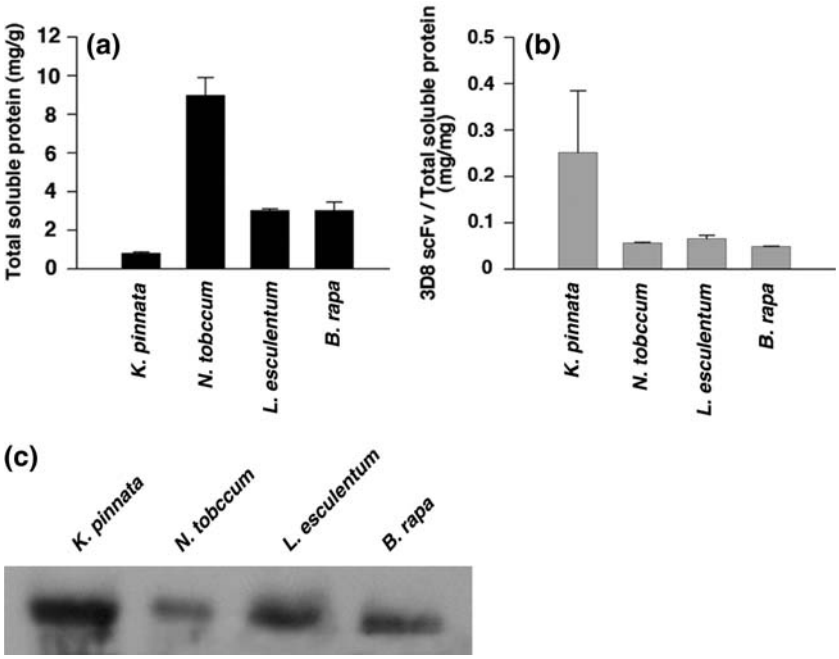

Fig. 6 3D8 recombinant antibody production by *K. pinnata, N. tabacum, L. esculentum*, and *B. rapa*. **a** Total soluble protein was extracted from the various plants with non-denaturing extraction buffer and quantitatively measured by the Bradford method. **b** *K. pinnata* had high 3D8 scFv content with respect to the total soluble protein. Results are representative of three independent experiments. **c** Western blot analysis with respect to the same volume of total soluble protein from various plants

formation on leaf notches compared to vacuum-infiltration method, possibly due to wounding by pin stabbing, even though the budding plantlet could nevertheless be transformed (Table 1). In contrast to the pin-prickling method, the vacuum-infiltration method produced a higher number of developed plantlets than the pin-prickling method; however, fewer transformants were produced when young mother leaf was used for transformation (Table 1). However, when budded plantlets were used for vacuum infiltration in transformation buffer, the transformation efficiency (TE) was 82.0%. The TE of pin-prickling transformation was 1.08 times higher than the vacuum-infiltration method with young mother leaf and 1.02 times higher than vacuum-infiltration method with budded plantlet.

To test foreign protein production from transgenic *K. pinnata* plants, the recombinant 3D8 scFv catalytic antibody was introduced into *K. pinnata*. Plant-based production of monoclonal antibody and scFv has already been reported by several groups (Larrick et al. 2001; Peeters et al. 2001; Semenyuk et al. 2007). There was notable variability in the expression levels of 3D8 scFv in individual transgenic lines from the same mother *K. pinnata* plant (Fig. 4). It is thought that such variable expression was due to position effects. In our study, 3D8 scFv was expressed as a His(6) and Protein-A tagged fusion protein in transgenic plants. The addition of the Protein-A tag to 3D8 scFv simplified purification of the recombinant protein from the crude plant extracts and thus allowed rapid biochemical and functional characterization of the recombinant protein. An abzyme assay demonstrated that the plant-derived 3D8 scFv fusion protein showed the catalytic activity closely comparable with a standard protein produced in *E. coli* (Fig. 5c), suggesting that the Protein-A tag did not interfere with the biological activity of plant 3D8 scFv. The expected size of 3D8 scFv is 29 kD and the molecular weight of the Protein-A tag is 9 kD. So 38-kD protein in Fig. 5a came out the sum of 3D8 scFv and Protein-A. However, in this study the 29-kD protein was more band intensity than 38-kD protein (Fig. 5a) and this may be due to the spontaneous cleavage of the Protein-A tag from the fusion protein of 3D8 scFv and Protein A because 29-kD protein has a catalytic activity like 38-kD scFv protein shown in zymography analysis (Fig. 5b).

The production yields for the foreign protein were compared amongst transgenic *K. pinnata*, *E. coil*, and other three plant systems. In the *E. coli* system, the yield of 3D8 scFv ranged from 2.1 mg to 7.9 mg per liter, with an average of 5.1 mg in our previous works (data not shown). For *K. pinnata*, the production yield was measured as 0.1–0.04 mg/g (purified protein/initiate biomass). Therefore, 100 g of *K. pinnata* was estimated to be sufficient to produce the same volume of protein from *E. coli*. Here, the *K. pinnata* system is CAM species with large biomass so that *K. pinnata* system ultimately assumed to be a stable bioreactor system for large-scale production of foreign proteins. When the yields were compared between *K. pinnata* and other three plant systems, the highest value of total soluble protein contents per fresh tissue weight was assigned to the tobacco plant, while the lowest value belonged to that of *K. pinnata* (Fig. 6). However the yield efficiency of target foreign protein expression per total soluble protein of each plant system was 3–4 times higher in *K. pinnata* than other plant system which we

investigated in this study. These results suggest that the production yield of foreign protein in *K. pinnata,* a succulent CAM plant species, was approximately 12% of total soluble protein, despite the large quantity of water retained by the plant.

In general, plant bioreactor systems require non-toxicity, easy cultivation, safety, and stable protein production yield (Twyman et al. 2003; Maliga and Graham 2004). In addition to the requirements for plant bioreactor, approximately 3–5-folds higher foreign protein production yield per total soluble protein of *K. pinnata* compared to three other systems in this study gives another advantage for further bioreactor development of *K. pinnata.*

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