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# Missile-Type Molecular Breeding of Medicinal Plant Using Compact Monoclonal Antibody Gene

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## Abstract

In the first step we constructed a recombinant single chain fragment variable (scFv) antibody from hybridoma cell lines expressing anti-solamargine (As) monoclonal antibody (MAb). The As-scFv protein characteristics expressed in both recombinant *Escherichia coli* and hairy root were almost same with those of the original MAb. Up to 220 ng recombinant As-scFv per milligram of soluble protein was produced in transgenic hairy root cultures of *S. khasianum*. The solasodine glycosides concentration was 2.3 fold higher in the transgenic, than in the wild-type hairy root, reflecting from the soluble as-scFv level. The recombinant S. khasianum plant regenerated from recombinant hairy roots contained 2.3 fold concentration of solasodine glycoside, compared to the regenerated plant from wild-type hairy root. It is easily suggested that the scFv antibody expressed in transgenic plant controlled the antigen level. This methodology is the first success for molecular breeding of secondary metabolites without understanding of biosynthetic enzyme, and named as missile-type molecular breeding.

***Keywords:*** *Solanum species; Solasodine glycoside; Monoclonal antibody; Single chain fragment variable antibody; Transgenic plant; Molecular breeding*

## Introduction

Plant breeding contains various field like selection, closing, gene construction, biosynthetic enzyme cloning and so on. In the case of tree breeding including medicinal tree a long-term process that takes more than a decade from the original cross to new cultivar release and its extension is needed, during which much work to manage the seedlings and much thought to select those with excellent characteristics. The excellent characteristics such as productivity, quality, labor saving, marketing and so on are different in different scion crops, rootstocks and environment. The major trends in modern breeding are to develop labor saving cultivars with resistance to biotic and abiotic stress, to create those with high quality and productivity, and to enhance bioactive for health. For these, for example, mutation breeding resulted in the creation of self-compatible cultivars in self-incompatible Japanese and Chinese pairs [1,2] and a disease resistant Japanese pear cultivar from susceptible one [3]. In citrus, monoembryonic cultivar 'Kiyomi' was bred with apomictic satsuma and orange cultivars, and generated high quality tangor cultivars with bioactives such as β-cryptoxanthin [4]. Breeding of rootstock is also developing to broaden the adaptability to circumstances and enhancement of fruit production [5]. Recent advances in genome science in fruit crops will provide new tools hasten the coming breeding programs.

On the other hand, the breeding of medicinal plant is much shorter than the tree breeding as described above. Objectives for medicinal plant breeding are the preparation of clonal cultivars having homogenous gene resulting in homogenous concentration of bioactive components. The authors investigated the clonal propagation of *Aconitum carmichaeli* (Ranunculaceae) [6,7], and *Panax ginseng* [8,9]. We also succeeded to clonally propagate *Pinellia ternate* (Araceae) *via* protocorm-like body and by liquid phase culturing, which theoretically produced 4 × 1023 clonal plants from a single tuber in a year [10]. Furthermore, we confirmed the relation between the virus infection and the component concentration, comparing with the virus free plant obtained by a tip tissue culture of *Rhemannia* species (Scrophulariaceae) [11]. In the second stage of medicinal plant breeding, the authors made evident that the medicinal plants clonally propagated promoted the homogeneity of bioactive component concentration, compared to the parent plant for aconitine-type alkaloid [12] (*Aconitum* spp.), atractylon (*Atractylodes* spp.) [13], and gentiopicroside [14] (*Gentiana* spp.). Their clonal propagation methods further owe the increase of medicinal plant resources even though the resources of medicinal plants have been insufficient worldwide recently.

In our ongoing projects on monoclonal antibody (Mab) against natural products, the authors prepared many MAbs like against ginsenosides, glycyrrhizin [15], saikosaponin [16], sennosides [17], mahihuana compound [18], for skolin [19], berbarine [20], aconitine alkaloid [21], aristrochic acid [22] and so on, and set up their assay systems. We also prepared a MAb against solamargine [23] contained in Solanum species belonging to Solanaceae family containing steroidal alkaloid glycoside like solasonine, solamargine and khasianine (Figure 1). *Solanum* species have been widely used as herb medicines in China and Asian countries. In fact, solasodine glycosides have anti-skin carcinoma properties in humans [24,25], and anti-neo plastic activity in mice [26]. Solamargine and solasonine (Figure 1) inhibited an acetyl-cholinesterase [27]. Solamargine is anti-herpes simplex virus type I [28], and a more powerful anti-fungal agent than ketoconazole [29]. Furthermore, since natural resources of adreno cortical and sex hormones, particularly from *Dioscorea* spp. have been insufficient, steroidal alkaloid glycosides like solamargine and solasonine having solasodine in a molecule as an a glycone (Figure 1) from *Solanum* spp., have become important as starting resources for the production of steroid hormones. It became evident that the above mentioned MAb can be used for the survey of all glycosides containing solasodine as and a glycone, because the MAb have wide cross-reactivity against all solasodine glycosides. This property is a big advantage for the survey of resource of solasodine glycosides [23]. Furthermore, we set up a simple staining system named as eastern blotting [30], and one step immune affinity separation [31].

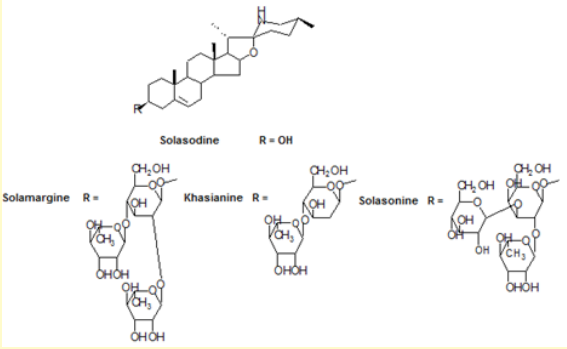



***Figure 1:*** *Structure of solasodine glycosides contained in Solanum species.*

Recently, a single-chain fragment-variable (scFv) antibody has been expressed in plants to protect for viral attack [32,33,34]. Moreover, scFv can be used to study plant growth, and to interfere with the biological activity of antigens [35,36,37]. We there for epostulate that the recombinant scFv can target small antigens like solasodine glycosides, which can then lead to the accumulation of antigen-antibody complexes in plant organs. In order to open a positive molecular breeding for the increase of bioactive component like solasodine glycoside, we constructed a functional anti-solamargine (As)-scFv antibody gene and introduced into a host plant, *S. khasianum*. In

this review, the authors will discuss whether such the transformation of scFv gene into plants can be used as a new molecular breeding methodology, for the increase of secondary metabolite concentration.

## Construction of ScFv and Induction into *Escherichia coli* and *Agro bacterium Rhizogenes*, and Expression of ScFv

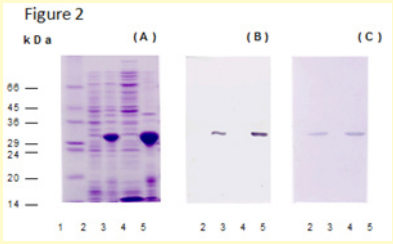
The authors constructed scFv using an *E. coli* expression vector, essentially according to the protocols of the recombinant phage antibody system purification and expression modules [38]. We extracted m RNA from hybridoma cells secreting the As-MAb, SM-BD9 [23], and synthesized first-strand cDNAs primed by random hexamers, using murine reverse transcriptase. PCR was performed using VH and VL primers from the scFv module of the RPAS kit and KOD DNA polymerase. The cDNAs of the VH and VL chain were assembled by seven incubation cycles, using a DNA linker fragment encoding the amino acid sequence (Gly4Ser) 3 supplied with the RPAS kit (Pharmacia). The resulting fragment was ligated into the pCANTAB5E phagemid (Pharmacia), from which high affinity scFv protein was selected. The DNA encoding scFv fused to an E-tag, and an amber stop codon was amplified by PCR from the template pCANTAB5E. The gene was sub cloned into the pET-28 a (+) expression vector to contain His- and T7-tags at the N-terminus, and an E-tag at the C-terminus. We determined the nucleotide and deduced amino-acid sequences of As-scFv and the hyper variable regions or complementarity determining regions (CDRs) were identified. The nucleotide sequence data appear in the GenBank nucleotide sequence data base under the accession number AF332008. The sequence was compared with those compiled in the Kabat antibody sequence database. The VH region of MAb (SM-BD9) belonged to the mouse heavy chain subgroup II (A), whereas the VL region belonged to the mouse kappa light chain V group. Expression of the recombinant scFv protein in *E. coli* was induced by incubation with isopropyl-thio-β-d-galactopyranoside (IPTG). After harvesting induced cells, denaturation was initiated by rapid 100-fold dilution of the denatured protein into refolding buffer, consisting of Tris-HCl, arginine and EDTA. Samples were incubated, dialyzed against Tris-HCl, and then purified by metal chelate affinity chromatography. Bound protein was eluted using Tris-HCl, containing imidazole and NaCl. The binding activity and binding inhibition of purified scFv protein were determined by direct and competitive ELISA. Plant expression vectors were transferred with the PRK 2013 helper plasmid into *Agro bacterium rhizogeness* train 15834 by tri parental mating, and were used to transform *S. khasianum* using standard procedures [39].Transgenic hairy roots were cultured on hormone-free half-strength MS medium containing 60 mg/l kanamycin. Total soluble protein was prepared by homogenizing hairy roots in extraction buffer. Soluble protein was quantified using a Bio-Rad protein assay kit, with bovine serum albumin as standard.

## Determination of scFv Protein by Western Blotting and Maldi Mass Spectrum

The molecular weight of As-scFv protein produced by transgenic hairy roots was the same as that of the protein expressed in *E. coli*, having 31 kD as indicated in Figure 2, whereas wild type hairy roots did not produce As-scFv [38]. Recombinant protein was reacted with T7-tag antibody, followed by peroxidase-labeled anti-mouse IgG, and then stained with 4-chloro-1-naphthol. Lines 1: Protein marker, 2: 200ng scFv from *E. coli*, 3: affinity purified scFv from hairy root clone SR2, 4: affinity-purified scFv from hairy root clone SR19, 5: soluble protein from wild-type hairy root. MALDI mass spectrometry confirmed the exact molecular weight of scFv protein produced by hairy root. A molecular peak [M+H] + appeared at 31,529 *m/z*, in good agreement with the molecular weight of 31, 504 calculated from amino acid sequence [38], as shown in Figure 3. From this result, it becomes clear that the constructed gene functioned normally without any mutation.

### Cross-Reactivity of As-ScFv against Solasodine Glycoside

In order to confirm the equivalence between the As-scFv produced by hairy root and the original As-MAb, we investigated the cross reactivity of As-scFv and MAb (SM-BD9) [23], against related steroidal alkaloid glycosides. Cross-reactivity's of As-scFv fragments against solasodine glycosides having different sugar moieties, such as khasianine (80.7%), solasonine (92.1%), 3-O-β-d-glucopyranosylsolasodine (112.9%), O-α- l -rhamnosyl-(1→2)-3-O-β-d-glucopyranosylsolasodine (98.5%), 3-O-β-d-gala-copyranosylsolasodine (115.4%), O-β-d-glucopyranosyl -(1→3)-3-O-β-d-galactopyranosylsolasodine(118.2%), and isoanquivine (150.4%), were almost same to those for the MAb [31]. The property having wide cross-reactivity against all solasodine glycosides is an advantage, because it is important to accumulate all solasodine glycosides, which are used as the resource of steroidal hormone. Moreover, we analyzed the binding kinetics of Ac-scFv from *E. coli,* hairy root and original MAb, using competitive ELISA, resulting in almost identical [23]. From these results, it is easily suggested that As-scFv can function for the antibody-antigen reaction in a plant.




Expression analysis of scFv in transformed *E. coli* BL21 using a 12.5% SDS-polyacrylamide gel electrophoresis followed by Coomassie brilliant blue staining (A), western blot (B) developed with anti E-tag antibody and peroxidase-labeled goat anti-mouse IgG and western blot (C) immersed in solamargine-HSA and developed with peroxidase-labeled anti-HSA antibody. Lane 1, protein molecular weight marker (kDa); lane 2 and 3, whole cell extract before and after IPTG induction; lane 4, soluble fraction; lane 5, inclusion bodies.

***Figure 2:*** *Western blotting of soluble protein from transgenic hairy root expressing As-scFv.*

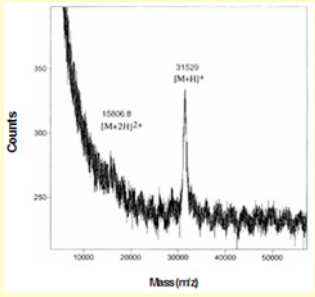
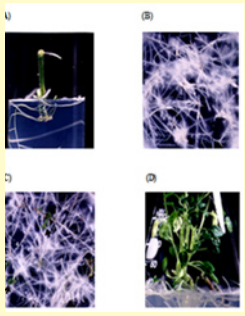



***Figure 3:*** *MALDI-to f-mass spectrum of As-scFv. Molecular peak appeared at 31,529 m/z.*

**Determination of Solasodine Glycoside Concentration in Transgenic Hairy Root and Transgenic Plant by Anti-Solamargine MAb**

The concentration of solasodine glycosides in all clones of As-scFv transgenic hairy roots ranged from 62 to 100 μ g/g dry wt., whereas that of the wild type was 43 to 45 μg/g dry wt. It is 2.3-fold higher than that of the wild type hairy root. Of 37 cloned transgenic hairy roots, 7 produced double the amount of As-scFv. The solasodine glycoside concentration increased in proportion to the soluble scFv level [28]. Therefore, the expression level of as scFv seems to be important for the increase of solasodine glycoside concentration. The solasodine glycoside concentration was two times or more in the transgenic hairy root, indicating that As-scFv protein combines directly to solasodine glycosides, and enhances their biosynthetic pathways in transgenic hairy roots. This evidence suggests that transgenic hairy roots expressing As-scFv produce an antigen-antibody complex that accumulates in plant cells. In order to confirm the above hypothesis, we regenerated plantlets from hairy roots. Figure 4 shows the regeneration step of *S. khasianum* plant from transgenic hairy root, and Figure 5 indicated the photograph of regenerated plant leaf and fruit.



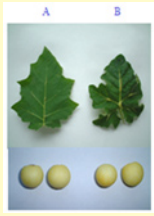
A: Infected shoot segment with *Agrobacterium rhizogenes*

B: Growth of hairy root

C: Appearance of green spot

D: Regenerated *S. khasianum* plant

***Figure 4:*** *The step of generation of plant from hairy root.*



Leaf and fruit of control (A) and transgenic leaf and fruit (B)

***Figure 5:*** *Photograph of regenerated S. khasianum. Leaf and fruit of control (A) and transgenic leaf and fruit (B).*

**Analysis of Solasodine Glycoside by Gastern Blotting in Transgenic *s. Khasianum* Plant**

Since the ELISA survey using As-MAb analyzed the total concentration of solasodine glycosides, we determined the qualitative analysis of solasodine glycosides contained in transgenic *S. khasianum* plant. We indicated the finger printing of solasodine glycoside contained in transgenic *S. khasianum* by Eastern blotting. Since anti-solamargine MAb has a wide cross-reactivity [31], all solasodine glycosides can be stained by Eastern blotting [30] indicating that three major steroidal alkaloid glycosides; khasianine, solamargine and solasonine, can be clearly detected compared to that of finger printing by TLC, although the ratio of individual solasodine glycoside concentration was not clear.

## Conclusion

Recombinant antibodies, such as scFv antibodies, have opened biomedical and biotechnological methodology [40], since they possess the same affinity and monovalent binding specificity compared to their parent antibodies [41]. The application of scFv engineering as a diagnostic tool in plant pathology has been demonstrated [42,43,44]. Other advances in antibody construction owe various means of conferring novel properties upon plants. Resistance to plant viruses can be enhanced using scFv antibody engineering against virus coat protein in plants [32,33,34] as previously described. Although molecular breeding of secondary metabolites has been owned to biosynthetic enzyme, we discovered that the transformation with the scFv fragment improves the yield of a pharmacologically active compound in this review. We, therefore, postulate that the recombinant scFv can target small antigens like solasodine glycosides, which can then lead to the accumulation of antigen-antibody complexes in plant organs. This system also allows there covery of both antigen and scFv protein, which can be used for further analytical studies. Our system established in this review may open upa new plant molecular breeding methodology for pharmacologically active glycosides, such as ginsenosides (*Panax* species), glycyrrhizin(*Glycyrrhiza*

species), saikosaponins (*Bupleurumfalcutum*), sennosides (*Rheum* species) and crocin (*Crocus* sativus), that we prepared MAbs against those compounds [45], without understanding of their biosynthetic pathway like a neutralized antibody. Therefore, we named it a missile type molecular breeding. Recently, we succeeded the production of plumbag in *Plumbag ozeylanica* hairy root, using a scFv against plumbag in as evidence exhibiting 2.2 times higher than those obtained from wild-type plant [46]. From this result we have believed that the scFv gene induction into a host plant can enhance the corresponding biosynthetic enzyme to accumulate the antigen molecule not limited to glycoside like solasodine glycoside as already discussed, but available for non-glycoside compound like a naphthoquinon, plumbag in. Therefore, we have been further investigating the molecular breeding, by using scFv gene in our ongoing study on plant breeding [47].

## Bibliography

1. Mase N., *et al*. "A segmental duplication encompassing S-haplo type triggers pollen-part self-compatibility in Japanese pear (*Pyruspyrifolia*)". *Molecular Breeding* 33 (2014): 117-128.
2. Wu J., *et al*. "Genetic features of the spontaneous self-compatible mutant, 'JinZhui' (*Pyrusbretschneideri* Rehd.)". *PLoS ONE* 8.10 (2013): e76509.
3. Sanada T., *et al*. "Resistant Mutant to Black Spot Disease of Japanese Pear 'Nijisseiki' Induced by Gamma Rays". *Journal of the Japanese Society for Horticultural Science* 57.159 (1988): 159-166.
4. Yano M., *et al*. "Quantitation of carotenoids in raw and processed fruits in Japan". *Food Science and Technology Research* 11.1 (2005): 13-18.
5. Soejima J., *et al*. "New semi- and extremely dwarfing apple rootstocks, 'JM2' and 'JM5' ". *Bulletin NARO Institute of Fruit Tree Science* 16 (2013): 19-36.
6. Hatano K., *et al*. "Somatic embryogenesis and plant regeneration from the anther of Aconitum carmichaeli Debx". *Plant Cell Reports* 6.6 (1987): 446-448.
7. Shoyama Y., *et al*. "Aconitum spp. (Monkshood): Somatic embryogenesis, plant regeneration, and the production of aconitine and other alkaloids". *Biotechnology in Agriculture and Forestry* 15 (1991): 58-72.
8. Shoyama Y., *et al*. "Somatic embryogenesis and clonal multiplication of Panax ginseng". *Planta Medica* 54.2 (1988): 155-156.
9. Shoyama Y., *et al*. "Somatic embryogenesis in ginseng (*Panaxspecies*)". *Biotechnology in Agriculture and Forestry* 31 (1995): 343-356.
10. Shoyama Y., *et al*. "Rapid and simple multiplication of Pinelliaternataby tissue culture". *Planta Medica* 47.2 (1983): l03-105.
11. Matsumoto M., *et al*. "Identification of viruses infected in Rehmannia glutinosa Libosch. varpurpureaMakino and effect of virus infection on root yield and iridoid glycoside contents". *Plant Cell Reports* 7.8 (1989): 636-638.
12. Hatano K., *et al*. "Clonal multiplication of Aconitum carmichaeli by tip tissue culture and alkaloid contents of clonally propagated plant". *Planta Medica* 54.2 (1988): l52-155.
13. Hatano K., *et al.* "Clonal propagation of Atractylodes japonica and A. ovata by tip tissue culture and the atractylon content of clonally propagated plants". *Planta Medica* 56.1 (1990): l31-132.
14. Yamada Y., *et al*. "Clonal micropropagation of Gentiana scabra Bunge var. buergeri Maxim. And examination of the homogeneity concerning gentiopicroside content". *Chemical and Pharmaceutical Bulletin* 39.1 (1991): 204-206.
15. Shan S L., *et al*. "Enzyme-linked immunosorbent assay for glycyrrhizin using anti-glycyrrhizin monoclonal antibody and a new eastern blotting for glucronides of glycyrrhetinic acid". *Analytical Chemistry* 73.24 (2001): 5784-5790.
16. Zhu Shu-hang., *et al*. "Development of an assay system for saikosaponin a using anti saikosaponin a monoclonal antibodies". *Biological and Pharmaceutical Bulletin* 27.1 (2004):66-71.
17. Morinaga O., *et al*. "Production of monoclonal antibody against a major purgative component, sennoside A, its characterization and ELISA". *Analyst* 126.8 (2000): 1372 -1376.

18. Tanaka H., *et al*. "Monoclonal antibody based enzyme immunoassay for marihuan (cannabinoid) compounds". *Journal of Immunoassay* 17.4 (1996): 321-342.
19. Sakata R., *et al*. "Production of monoclonal antibodies and enzyme immunoassy for typical adenylate cyclase activater, forskolin". *Cytotechnology* 16.2 (1994): 101-108.
20. Kim J S., *et al*. "Immuno quantitative analysis for berberine and its related compounds using monoclonal antibody in herbal medicines". *Analyst* 129 (2004): 87-91.
21. Kido K., *et al*. "An enzyme-linked immune sorbent assay for aconitine-type alkaloids using an anti-aconitine monoclonal antibody". *Analytica Chimica Acta* 616.1 (2008): 109-114.
22. Li XW., *et al*. "Development of Eastern blotting technique for the visual detection of aristolochic acids in Aristolochia and Asarum species using monoclonal antibody against aristolochic acids I and II". *Phytochemical Analysis* 24.6 (2013): 645-653.
23. Ishiyama M., *et al*. "Production of monoclonal antibodies and development of an ELISA for solamargine". *Cytotechnology* 18.3 (1995): 153-158.
24. Daunter B and Cham BE. "Solasodine glycosides: *In vitro* preferential cytotoxicity for human cancer cells". *Cancer Letters* 55.3 (1990): 209-220.
25. Cham BE., *et al*. "Topical treatment of malignant and premalignant skin lesions by very low concentrations of a standard mixture (BEC) of solasodine glycosides". *Cancer Letters* 59.3 (1991): 183-192.
26. Cham BE., *et al*. "Anti tumour effects of glycoalkaloids isolated from Solanum sodomaeum". *Planta Medica* 53.1 (1987): 34-36.
27. Roddick JG. "The acetylcholinesterase-inhibitor activity of steroidal glycoalkaloids and their aglycone". *Phytochemistry* 28.10 (1989): 2631-2636.
28. Ikeda T., *et al*. "Anti-herpes virus activity of Solanum steroidal glycosides". *Biological and Pharmaceutical Bulletin* 23.3 (2000): 363-364.
29. Chataing B., *et al*. "Inhibition of Trypanosoma cruzi growth *in vitro* by Solanum alkaloids: a comparison with ketoconazole". *Planta Medica* 64 .1 (1998): 31-36.
30. Tanaka H., *et al*. "A simple determination of steroidal alkaloid glycosides by thin-layer chromatography immunostaining using monoclonal antibody against solamargine". *FEBS Letter* 404.2-3 (1997): 279-282.
31. Putalun W., *et al*. "Rapid separation of solasodine glycosides by an immune affinity column using anti-solamargine monoclonal antibody". *Cytotechnology* 31.1-2 (1999): 153-158.
32. Tavladoraki P., *et al*. "Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack". *Nature* 366.6454 (1993): 469-472.
33. Fiedler U and Conrad U. "High-level production and long-term storage of engineered antibodies in transgenic tobacco seeds". *Biotechnology* (N Y) 13.10 (1995): 1090-1093.
34. Fecker LF., *et al*. "Expression of single-chain antibody fragments (scFv) specific for beet necrotic yellow vein virus coat protein or 25 k Da protein in Escherichia coli and Nicotiana benthamiana". *Plant Molecular Biology* 32.5 (1996): 979-986.
35. Fecker LF., *et al.* "Nicotiana benthamiana plants expressing beet necrotic yellow vein virus (BNYVV) coat protein-specific scFv are partially protected against the establishment of the virus in the early stages of infection and its pathogenic effects in the late stages of infection". *Archives of Virology*142.9 (1997): 1857-1863.
36. Artsaenko O., *et al*. "Expression of a single-chain Fv antibody against abscisic acid creates a wilty phenotype in transgenic tobacco". *Plant Journal* 8.5 (1995): 745-750.
37. Harper K and Ziegler A. "Recombinant antibodies: Applications in plant science and plant pathology". CRC Press, Dundee, UK (1999).
38. Putalun W., *et al*. "antisolasodine glycoside single-chain Fv antibody stimulates biosynthesis of solasodine glycoside in plants". *Plant Cell Reports* 22.5 (2003): 344-349.
39. Pollard JW and Walker JM. "Methods in molecular biology 6: plant cell and tissue culture". Humana, Clifton, N.J., USA (1990).

40. Huston JS., *et al*. "Medicinal applications of single-chain antibodies". *International Reviews of Immunology* 10.2-3 (1993): 195-217.
41. Tang PM., *et al*. "A high affinity dioxin binding protein displayed on M13 is functionally identical to the native protein". *Journal of Biological Chemistry* 270 (1995): 7829-7835.
42. Ziegler A., *et al*. "Cucumber mosaic cucumo virus antibodies from a synthetic phase display library". *Virology* 214.1 (1995): 235-238.
43. Harper K., *et al*. "A scFv-alkaline phosphatase fusion protein which detects potato leaf rollluteo virus in plant extracts by ELISA". *Journal of Virological Methods* 63.1 (1997): 237-242.
44. Hoogenboom HR. "Designing and optimizing library selection strategies for generating high-affinity antibodies". *Trends Biotechnology* 15.2 (1997): 62-70.
45. Tanaka H., *et al*. "Application of monoclonal antibodies against bioactive natural products: Eestern blotting and preparation of knockout extract". *International Journal of Analytical Chemistry* (2012).
46. Sakamoto S., *et al.* "Modulation of plumbagin production in Plumbag ozeylanica using a single-chain variable fragment antibody against plumbagin". *Plant Cell Reports* 31.1 (2012): 103-110.
47. Hiroyuki Tanaka., *et al*. "Formation of monoclonal antibody against a major ginseng component, ginsenoside Rg1 and its characterization". *Cytotechnology* 29.2 (1999): 115-120.

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