Anti-herbicide single-chain antibody expression confers herbicide tolerance in transgenic plants

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Abstract An anti-chlorpropham single-chain variable-fragment (scFv) gene was introduced into *Arabidopsis* in a manner to express the antibody fragment in each of four different subcellular compartments. The accumulation of scFv in transgenic plants was detected by targeting the fragment in the endoplasmic reticulum or apoplastic space, or by expressing the fragment as a glycosylphosphatidylinositol-anchored protein, while no accumulation could be detected by targeting the fragment in the cytosol. Transgenic plants accumulating the scFv gene at a high level in the endoplasmic reticulum had enhanced tolerance to chlorpropham in comparison with the non-transformants.

Key words: Chlorpropham; Single-chain antibody; Herbicide tolerance; Glycosylphosphatidylinositol anchor; *Arabidopsis thaliana*

1. Introduction

Many reports in recent years have described the expression of antibodies or antibody fragments in plants [1]. They have proven the effectiveness of this technique for modulating phytohormone activity [2–4], in blocking plant-pathogen infection [5], and in producing vaccines [6]. Recombinant antibodies such as Fab and single-chain variable fragments (scFv) as well as original antibodies have been used in this technique. ScFv is the minimum recombinant antibody with antigen-binding activity, in which the variable regions of the heavy and light chains are connected by a flexible linker peptide [7,8]. Since scFv is encoded by a single structural gene, it can be more easily produced in plants than the complete antibody.

The development of herbicide-tolerant plants has been achieved in different ways to increase the weed control in crops. Most of them have so far been transformed with genes isolated from microorganisms or plants. However, alternative methods are needed to meet the requirements for other herbicides. The effectiveness of anti-herbicide antibody expression has been suggested by the exogenous application of an anti-diuron antibody to isolated thylakoids or green algae that were protected from photosynthetic inhibition by the herbicide [9].

We have previously succeeded in producing scFv against chlorpropham (isopropyl-*N*-(3-chlorophenyl) carbamate) in *Escherichia coli* [10]. Chlorpropham, a pre-emergence herbicide, can be used for the control of weeds and as a sprout suppressant on potatoes [11]. We report here the preparation of transgenic plants expressing anti-chlorpropham scFv as a new approach to developing herbicide-tolerant plants. The expression of scFv and protection from chlorpropham-dependent inhibition were optimized by its accumulation in different cellular compartments, using five plant expression vector constructs. First, a plant expression vector harboring the scFv gene was designed to retain scFv in the lumen of the endoplasmic reticulum (ER), where stable and high-level expression of antibodies has been shown [2]. Second, the scFv expression was targeted in the cytosol (CY), where chlorpropham action is supposed to take place. Third, scFv was expressed in a secreted form by targeting scFv via ER to the apoplastic space (AP). The expression of scFv as a glycosylphosphatidylinositol (GPI)-anchored protein was also examined. GPI is a type of lipid membrane anchor containing a conserved glycan linkage covalently attached to the C-terminus of a protein, with phosphatidylinositol, which is embedded in the outer layer of the plasma membrane, attaching the protein to the plasma membrane [12]. Takos et al. have recently reported that GPI-addition signals derived from yeast and a plant functioned to link a GPI anchor to a reporter protein in plant cells [13]. In this present study, we expressed scFv as a GPI-anchored protein by using GPI-addition signals isolated from *Arabidopsis thaliana AtAGP5* or *Saccharomyces cerevisiae GAS1*.

We finally tested the tolerance of transgenic plants to chlorpropham. The relationship between the tolerance and the expression level or subcellular localization of scFv is also discussed.

2. Materials and methods

2.1. Construction of the plant expression vectors

Five vectors incorporating the anti-chlorpropham scFv gene were constructed for plant transformation (Fig. 1). The scFv gene was amplified by polymerase chain reaction (PCR) to create restriction enzyme sites and a KDEL sequence, and was replaced with the GUS reporter gene of binary vector pBI121 [14], which contains a kanamycin-resistance gene, CaMV 35S promoter and nopaline synthase terminator, between the left and right borders of the T-DNA, giving the plant expression vector pBISCY. To construct pBISER, the PCR product was fused to the 5′ end of the legumine B4 signal sequence (LeB4) in pRTRA15 [2], and the resulting fusion gene was cloned into pBI121. pBISAP was constructed by fusing LeB4 to the PCR product without the KDEL sequence in the same way as used for pBISER construction. pBISGA and pBISGS were respectively

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constructed by fusing the GPI-addition signals isolated from *Arabidopsis AtAGP5* or *S. cerevisiae GAS1* [14] to the 3′ end resulting from the fusion of scFv and LeB4, and by inserting the resulting fusion genes in pBI121.

2.2. Plant transformation

pBISER, pBISCY, pBISAP, pBISGA and pBISGS were introduced into *Agrobacterium tumefaciens* strain GV3010 (pMP90), which was used to transform *Arabidopsis* plants (*A. thaliana* ecotype Columbia) as previously described [15]. Transgenic plants were selected on Murashige–Skoog (MS) agar medium containing 100 μg/ml of kanamycin. The independent T1 transformants are identified by number (#) as AER#, ACY#, AAP#, AGA# and AGS# from respective constructs pBISER, pBISCY, pBISAP, pBISGA and pBISGS. Each T2 plant is identified by another number like AER#-#. The homozygous T3 lines were selected by segregation for their kanamycin resistance.

2.3. ELISA and Western blotting

Whole plants were frozen in liquid nitrogen and ground to a fine powder, and proteins were extracted from the powder with phosphate-buffered saline (PBS).

The expression and binding activity of scFv in the transgenic plants toward chlorpropham were detected by enzyme-linked immunosorbent assay (ELISA) as previously described [10]. Indirect ELISA (i-ELISA) was performed by successively incubating the following solutions: 100 μl of chlorpropham hapten conjugated with rabbit serum albumin (2 μg/ml in 50 mM $NaHCO_3$, pH 9.6) at 4°C for 16 h as the plate-coating antigen, 200 μl of 2% skim milk in PBS for blocking at 25°C for 2 h, 100 μl of the protein extract containing 300 μg of bovine serum albumin-equivalent protein at 25°C for 1 h, 100 μl of the anti-c-myc antibody (9E10 mouse ascites 1000 times diluted with PBS) [16], 100 μl of the horseradish peroxidase (HRP)-conjugated anti-mouse antibody (5000 times diluted with PBS; Amersham Biosciences, USA) as a secondary antibody, and 100 μl of a substrate solution (100 mM sodium acetate, pH 6) containing 3,3′,5,5′-tetramethylbenzidine (100 μg/ml) and hydrogen peroxide (0.6%). The enzyme reaction was stopped by adding 50 μl of 1 M H_2SO_4. The peroxidase activity was evaluated by calculating the difference in absorbance (A) at 630 nm and 450 nm ($A_{450}-A_{630}$). An excess of chlorpropham (10 μg/ml) was mixed with the soluble protein extract before the reaction with the coated antigen for competitive indirect ELISA (ci-ELISA).

The expression of scFv was also detected by Western blotting. A sample of the amount indicated was applied to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were electrotransferred on to a nitrocellulose membrane, and a c-myc tag fused to scFv was detected with the 9E10 antibody and HRP-conjugated anti-mouse antibody as for ELISA. The HRP activity was detected by an ECL Plus Western blotting detection system (Amersham Biosciences).

2.4. Cell fractionation

A microsomal fraction was prepared from whole plants, all procedures being conducted at 4°C or on ice. Plants were homogenized in liquid nitrogen, and an extraction buffer (100 mM Tris–HCl at pH 7.8, 10 mM KCl, 12% w/v sucrose, 1 mM EDTA and 2 mM $MgCl_2$) was added at 1 ml/g fresh weight (fw). The homogenate was centrifuged at 12 600×g for 10 min, and the resulting supernatant was centrifuged at 120 000×g for 100 min. The microsomal fraction was prepared by resuspending the resulting pellet in PBS. The soluble proteins in the remaining supernatant were precipitated with cold acetone, and the pellet was resuspended in PBS to give the soluble protein fraction.

The microsomal fraction was used for Triton X-114 phase partitioning as previously described with minor modifications [17]. The microsomal fraction was equilibrated with a lysis buffer (150 mM NaCl, 2% Triton X-114 and 10 mM Tris–HCl at pH 7.4), incubated at 32°C for 12 min and then centrifuged (3000×g, 32°C, 3 min). The detergent phase was resuspended in cold buffer A (150 mM NaCl, 0.06% Triton X-114 and 10 mM Tris–HCl at pH 7.4) and incubated at 0°C for 10 min. Triton X-114 partitioning was repeated. The detergent phase was then resuspended in buffer A, incubated at 0°C for 10 min, and centrifuged (18 000×g, 0°C, 10 min). The resulting supernatant was again partitioned with Triton X-114. Proteins in the final detergent phase and in the aqueous phase from the partitioning steps were precipitated with cold acetone, resuspended in PBS and applied to a Western blotting analysis. When the detergent phase was used for phosphatidylinositol-specific phospholipase C (PI-PLC) treatment, the pellet from the detergent phase was resuspended in an incubation buffer containing *Bacillus thuringiensis* PI-PLC (Glyko, Novato, CA, USA) at 2 U/ml, 0.1% w/v sodium deoxycholate and 100 mM HEPES (pH 7.2), and then incubated at 37°C for 3 h. Triton X-114 phase partitioning was repeated as just described.

2.5. Chlorpropham tolerance test

Seeds of transgenic plants and wild-type plants were surface-sterilized, before being planted on MS agar medium containing chlorpropham at the indicated concentrations. The chlorpropham tolerance was evaluated by measuring the length of the longest leaf in each plant 10 days after germination.

3. Results

3.1. Transformation of Arabidopsis with the plant expression vectors

Plant expression vectors pBISER, pBISCY, pBISAP, pBISGA and pBISGS were constructed for the expression of scFv in ER, CY and AP, and as a GPI-anchored protein by respectively using the GPI-addition signals from *Arabidopsis AGP5* or *S. cerevisiae GAS1* (Fig. 1). These constructs were introduced into *Arabidopsis* by *Agrobacterium*-mediated transformation, respectively generating the AER, ACY, AAP, AGA and AGS transgenic lines. More than 10 initial independent transformants (T1) for each construct were selected by their kanamycin resistance.

3.2. Expression of scFv in the transgenic plants

A protein extract was prepared from the progeny (T2) of 10 independent T1 lines for each construct, and the binding ac-

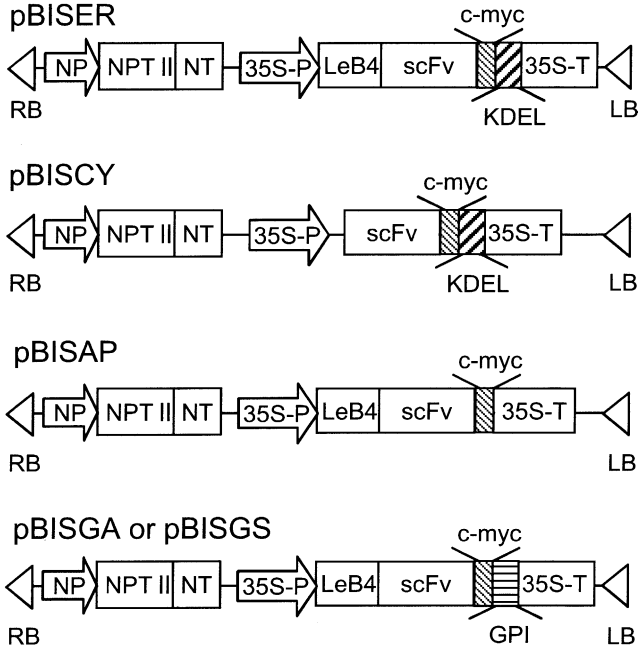

Fig. 1. Construction of the plant expression vectors. The GPI-addition signals were isolated from *Arabidopsis* or *S. cerevisiae*, giving two constructs for the expression of scFv as a GPI-anchored protein. NP, nopaline synthase promoter; NPTII, neomycin phosphotransferase gene; NT, nopaline synthase terminator; 35S-P, CaMV 35S promoter; LeB4, legumine B4 signal peptide; c-myc, c-myc tag; KDEL, ER retention signal; GPI, GPI-addition signal; 35S-T, CaMV 35S terminator.

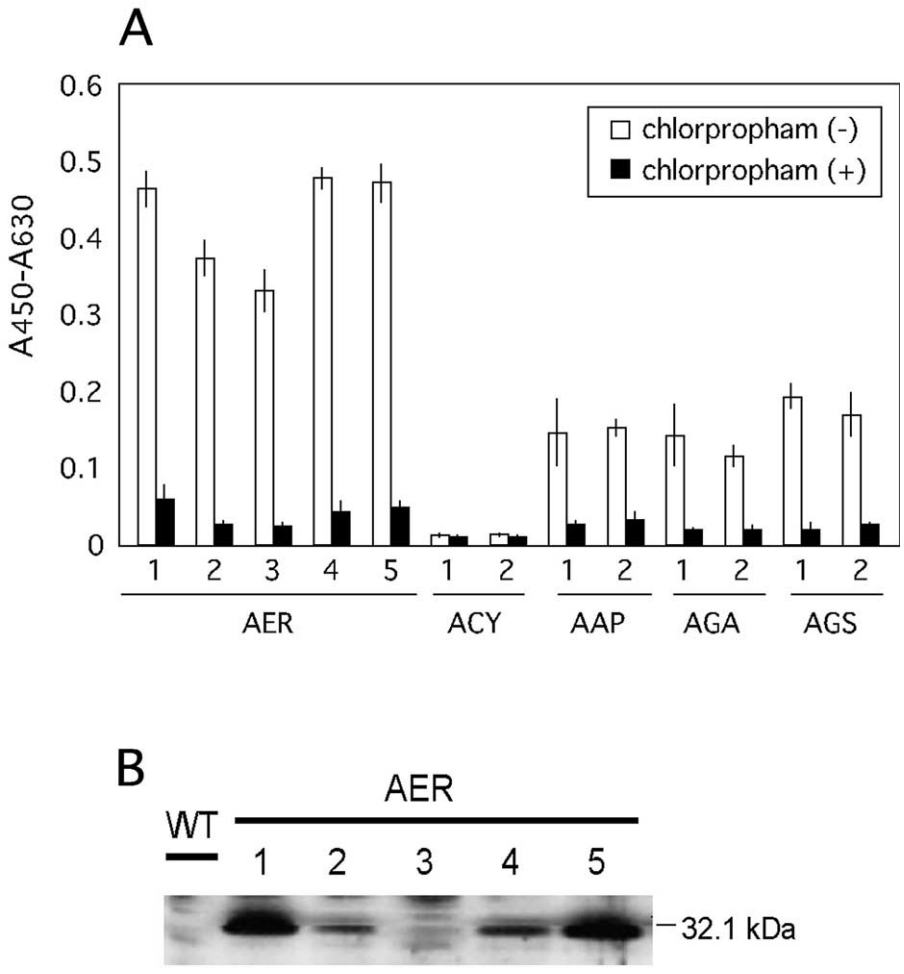

Fig. 2. Expression of scFv in representative transgenic plants. The protein extract was used for these analyses. A: The expression and binding activity of scFv toward chlorpropham were detected by i- and ci-ELISA. Chlorpropham was not added for i-ELISA [chlorpropham(−)], and an excess of chlorpropham (10 μg/ml) was added for ci-ELISA as a competitor [chlorpropham(+)]. B: The expression of scFv was also confirmed by Western blotting. 30 μg protein was loaded for each lane. Numbers indicate the T2 progeny from independent T1 transformant lines.

tivity of scFv in each extract was examined by i-ELISA. Activity toward chlorpropham was detected in the extract of all the AER, AAP, AGA and AGS plants, while the extract of any ACY line showed no binding activity. It seems that the KDEL sequence, which has been reported to stabilize scFv in cytosol [18], did not work in our case. The reversibility of the binding was confirmed by ci-ELISA for some of the lines (Fig. 2A). The binding of the coating antigen with scFv was clearly replaced by excess chlorpropham as a competitor. The production of scFv in the AER, AAP, AGA and AGS plants was also confirmed by Western blotting (Fig. 3A), as was the expression level of scFv in the extract of each AER line (Fig. 2B). In both analyses, the highest scFv expression level was obtained in the AER plants.

3.3. Subcellular localization of scFv

The microsomal fraction and the soluble protein fraction were prepared by cell fractionation to confirm the subcellular localization of scFv. Western blotting (Fig. 3A) detected scFv mainly in the microsomal fraction from the AER plants, and in the microsomal fraction and soluble protein fraction from the AAP plants. scFv was detected in the microsomal fractions from the AGS and AGA plants.

In order to investigate whether scFv in the microsomal fractions of AGA and AGS was embedded in the plasma membrane or anchored to the plasma membrane by a GPI anchor, the microsomal fraction was used for a further analysis. After Triton X-114 phase partitioning had been performed, scFv from AGA and AGS was detected in the detergent phase to which GPI-anchored proteins and other hydrophobic membrane proteins are presumed to have been partitioned, while scFv from AER and AAP could not be detected in the detergent phase (Fig. 3B). Treatment with PI-PLC, which specifically cleaves phosphatidylinositol of a GPI-anchored protein, and further Triton X-114 phase partitioning shifted scFv in the detergent phase from AGA and AGS to the aqueous phase, while mock-treated scFv stayed in the detergent phase (Fig. 3C). This indicates that scFv in AGA and AGS was expressed as a GPI-anchored protein.

3.4. Chlorpropham tolerance of the transgenic plants

Seeds of the transgenic plants and of the wild-type plant were planted on MS medium containing 1 μg/ml of chlorpropham to examine the chlorpropham tolerance. The chlorpropham concentration was determined by cultivating wild-type plants at various concentrations (shown in Fig. 4E). The

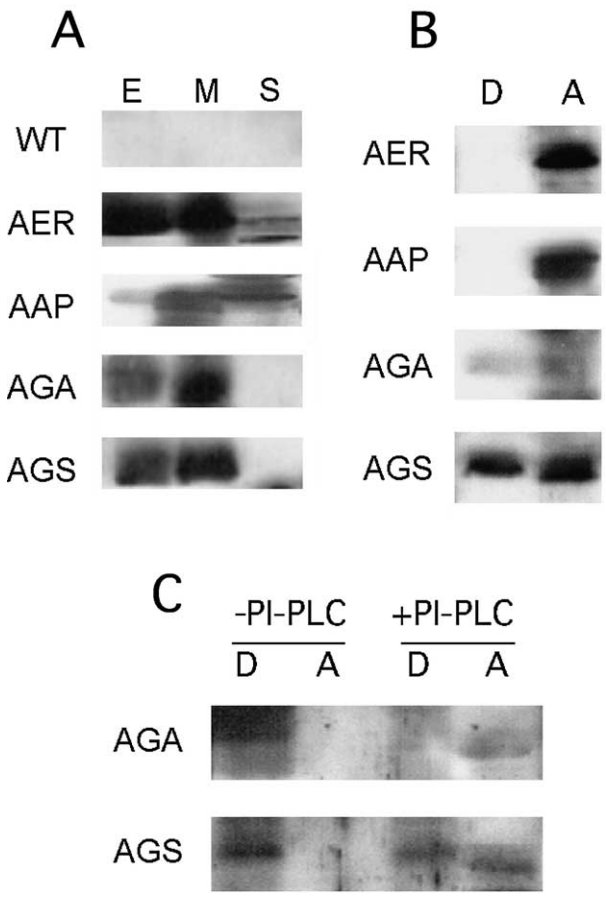

Fig. 3. Subcellular localization of scFv. The scFv localization was detected by Western blotting after each fractionation. A: The microsomal fraction was prepared by ultracentrifugation of the cell homogenate. E, protein extract from 10 mg fw; M, microsomal fraction from 100 mg fw; S, soluble protein fraction from 100 mg fw. B: The GPI-anchored protein and other hydrophobic proteins were extracted in the detergent phase by Triton X-114 phase partitioning. D, detergent phase; A, aqueous phase equivalent to 100 mg fw. C: The detergent phase was incubated in the absence (−PI-PLC) or presence (+PI-PLC) of PI-PLC, and Triton X-114 phase partitioning was repeated. D, detergent phase; A, aqueous phase equivalent to 100 mg fw.

wild-type plants showed obvious sensitivity to chlorpropham 10 days after germination: the leaves had curled and the development was strongly inhibited. Of five AER T2 plants examined (the progeny of T1 lines AER1, 2, 3, 4 and 5), many plants in all the lines grew bigger than the wild-type under the same condition as shown in Fig. 4A, while all plants of the AAP, AGA and AGS lines were equally sensitive to chlorpropham as the wild-type (data not shown). The tolerance was evaluated as the length of the longest leaf in each plant (Fig. 4B). The values for the AER lines showed a significant difference from those of the wild-type plants when cultivated with chlorpropham. Thus, the AER lines showed obvious tolerance to chlorpropham in comparison with the wild-type plants. Although the AER plants were not fully tolerant to chlorpropham, their leaves did not curl and they grew to maturity, while the wild-type plants did not survive (Fig. 4C). To provide a detailed analysis of the phenotype, homozygous T3 lines were established from each independent T2 (AER1–3 and AER4–5) by testing their kanamycin resistance. They showed a more homogeneous distribution of the longest leaf length than the heterozygous lines (Fig. 4D). The dose–response characteristics to chlorpropham were compared between the wild-type and one of the homozygous lines, AER4–5 (Fig. 4E). The results show that the reagent was toxic at a concentration higher than 0.32 μg/ml for both the wild-type and AER4–5 plants, but the latter had better growth in the concentration range of 0.32–1.0 μg/ml. Within this range, AER4–5 showed comparable growth to the wild-type plants at about a two-fold higher concentration of chlorpropham.

4. Discussion

Transgenic plants expressing anti-chlorpropham scFv in different cellular compartments (ER, CY and AP) and as a GPI-anchored protein were prepared. Functional scFv expression in the transgenic plants was achieved by targeting scFv into ER and AP; targeting into CY did not accumulate scFv at a detectable level, this being a similar pattern to that reported elsewhere [4,19]. We also succeeded for the first time in expressing scFv in a GPI-anchored form. The results of cell fractionation provide strong evidence to prove that scFv was localized to ER in the AER plants, to AP in the AAP plants, and to the plasma membrane in the AGA and AGS plants.

scFv in the AER plants was designed to be transported into the lumen of ER under the direction of the LeB4 signal peptide and retained in ER by the KDEL sequence; the highest level of scFv accumulation would have been due to the environment of ER that is favorable for the folding of scFvs [20]. The AER lines showed enhanced tolerance to chlorpropham. This tolerance was to some extent correlated with the expression level and binding activity of scFv; the expression level and binding activity of scFv were relatively low in the AER3 plants which showed less tolerance than the other AER lines. It can be deduced that, in the cells of AER plants, chlorpropham passed through the ER membrane, and was captured by scFv that had accumulated in ER, resulting in a decreased intracellular chlorpropham concentration.

We successfully expressed scFv with antigen-binding activity as a GPI-anchored protein by using GPI-addition signals. Takos et al. have reported that the yeast GPI-addition signal from *GAS1* and the putative plant GPI-addition signal from *LeAGP-1* were capable of directing the addition of a GPI anchor to a reporter protein [14]. In this present study, the GPI-addition signals isolated from *A. thaliana AGP5* and *S. cerevisiae GAS1* were used for the expression of scFv as a GPI-anchored protein. The results of Triton X-114 phase partitioning and PI-PLC treatment reveal that the GPI-addition signal from *A. thaliana AGP5* as well as that from *S. cerevisiae GAS1* functioned to link scFv to the plasma membrane by the GPI anchor.

The AAP, AGA and AGS plants had no enhanced tolerance to chlorpropham, indicating that scFv expressed in AP or on the cell surface was not able to successfully prevent chlorpropham from entering the cells. Thus, an enhanced tolerance to chlorpropham was only obtained when the scFv expression was targeted to ER. Considering the correlation between the scFv level and the tolerance to chlorpropham among different AER lines (Figs. 2B and 4B) and among the transgenic plants with different scFv compartments, the expression level of scFv seems to have been the key factor for

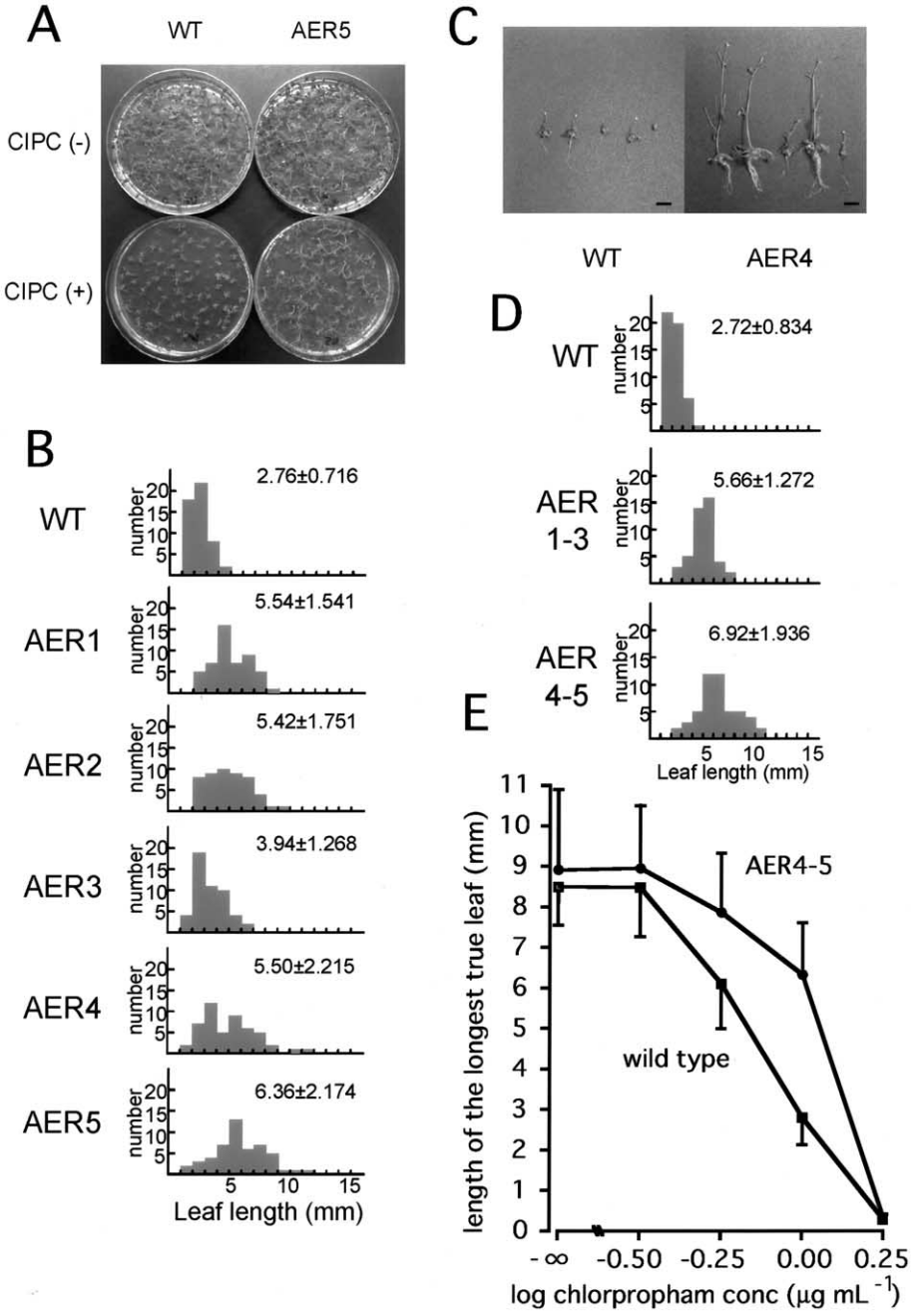

Fig. 4. Chlorpropham tolerance of the transgenic plants. A: 10-day-old plants of the wild-type and T2 heterozygous (progeny of AER5) lines cultivated on MS medium containing no or 1 μg/ml of chlorpropham. B: Distribution of the longest leaf length in each plant of the wild-type and T2 progeny from the T1 AER lines (#1–5) cultivated on MS medium containing 1 μg/ml of chlorpropham. Each value represents the mean of the leaf length ± S.D. C: 24-day-old plants of the wild-type and T2 progeny of the AER4 line cultivated on MS medium containing 1 μg/ml of chlorpropham. Bars = 1 cm. D: Distribution of the longest leaf length in each plant of the wild-type and T3 homozygous lines cultivated on MS medium containing 1 μg/ml of chlorpropham. Each value represents the mean of the leaf length ± S.D. E: Comparison of the dose–response curves between the wild-type plants and T3 homozygous progeny from AER4–5 against different concentrations of chlorpropham.

the tolerance. It is also possible, however, that the environment of each cell compartment affected the functionality of scFv; binding activity and/or accessibility of scFv to chlorpropham may vary due to the different environments.

There are some potential applications from the results of this study. Since the agent neutralizing the action of the herbicide with this approach was an antibody fragment, the method can be applied to many other herbicides for a diverse range of antibodies, and the tolerance can be improved by engineering the antigen-binding activity. The antibody-producing plants could be utilized for phytoremediation by their ability to capture herbicides in the environment [21].

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References

[1] De Jaeger, G., De Wilde, C., Eeckhout, D., Fiers, E. and Depicker, A. (2000) Plant Mol. Biol. 43, 419–428.
[2] Artsaenko, O., Peisker, M., Nieden, U.Z., Fielder, U., Weiler, E.W., Müntz, K. and Conrad, U. (1995) Plant J. 8, 745–750.
[3] Phillips, J., Artsaenko, O., Fielder, U., Horstmann, C., Mock, H.-P., Müntz, K. and Conrad, U. (1997) EMBO J. 16, 4489–4496.
[4] Shimada, N., Suzuki, Y., Nakajima, M., Conrad, U., Murofushi, N. and Yamaguchi, Y. (1999) Biosci. Biotechnol. Biochem. 63, 779–783.
[5] Tavladoraki, P., Benvenuto, E., Trinca, S., Martins, D.D., Cattaneo, S. and Galeffi, P. (1993) Nature 366, 469–472.
[6] Daniell, H., Streatfield, S.J. and Wycoff, K. (2001) Trends Plant Sci. 5, 219–226.
[7] Huston, J.S., Mudgett-Hunter, M., Tai, M.-S., McCartney, J., Warren, F., Haber, E. and Oppermann, H. (1991) Methods Enzymol. 203, 46–88.
[8] Johnson, S. and Bird, R.E. (1991) Methods Enzymol. 203, 88–98.
[9] Werner, D., Behrsing, O., Scharte, G., Woller, J., Steup, M. and Micheel, B. (2002) FEBS Lett. 523, 95–98.
[10] Eto, J., Suzuki, Y., Ohkawa, H. and Yamaguchi, I. (2002) J. Pesticide Sci. 27, 384–387.
[11] Tomlin, C. (1995) The Pesticide Manual, 10th edn., British Crop Protection Council, The Royal Society of Chemistry, Cambridge.
[12] Englund, P.T. (1993) Annu. Rev. Biochem. 62, 121–138.
[13] Takos, A.M., Dry, I.B. and Soole, K.L. (2000) Plant J. 21, 43–52.
[14] Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) EMBO J. 6, 3901–3907.
[15] Bechtold, N. and Pelletier, G. (1998) Methods Mol. Biol. 82, 259–266.
[16] Evan, G.I., Lewis, G.K., Ramsay, G. and Bishop, J.M. (1985) Mol. Cell. Biol. 5, 3610–3616.
[17] Ko, Y.-G. and Thompson Jr., G.A. (1995) Anal. Biochem. 224, 166–172.
[18] Schouten, A., Roosien, J., Van Engelen, F.A., De Jong, G.A.M., Borst-Vrenssen, A.W.M., Zilverentant, J.F., Bosch, D., Stiekema, W.J., Gommers, F.J., Schots, A. and Bakker, J. (1996) Plant Mol. Biol. 30, 781–793.
[19] Ramírez, N., Ayala, M., Lorenzo, D., Palenzuela, D., Herrera, L., Doreste, V., Pérez, M., Gavilondo, J.V. and Oramas, P. (2002) Transgenic Res. 11, 61–64.
[20] Conrad, U. and Fielder, U. (1998) Plant Mol. Biol. 38, 101–109.
[21] Harris, B. (1999) Trends Biotechnol. 17, 290–296.