

# Immunomodulation of gibberellin biosynthesis using an anti-precursor gibberellin antibody confers gibberellin-deficient phenotypes

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**Abstract** Immunomodulation is a means to modulate an organism's function by antibody production to capture either endogenous or exogenous antigens. We have recently succeeded in obtaining gibberellin (GA)-deficient phenotypes in *Arabidopsis thaliana* by using anti-bioactive GA antibodies. In this study, a single-chain antibody (scFv) against GA<sub>24</sub>, a precursor GA, was utilized to repress the biosynthesis of bioactive gibberellins. Stable accumulation of the scFv in endoplasmic reticulum (ER) was achieved by being produced as a fusion with GFP as well as KDEL ER-retention signal. The transgenic plants showed GFP fluorescence in the reticulate cortical ER network in epidermal cells. The GFP-scFv fusion produced in plants maintained its binding activity. The transgenic plants showed GA-deficient phenotypes, including reduced rosette leaf development, delayed flower induction and reduced stem

elongation of the main culm, especially in the early stage of inflorescence growth. Contrarily, stem elongation of the main culm at a later stage, or that of lateral shoots was much less affected by scFv production. These phenotypes were different from anti-bioactive GA scFv-producing lines, whose stem elongation was continuously repressed throughout the inflorescence development. The GA-deficient phenotypes were recovered by treatment with GA<sub>24</sub> and bioactive GA<sub>4</sub>, the latter being more effective. The transgenic lines contained conspicuously higher endogenous GA<sub>24</sub> and clearly less GA<sub>4</sub> than wild-type plants. The expression of *GA 20-oxidase* and *GA 3-oxidase* genes, which are feedback-regulated by GA signaling, were up-regulated in those plants. These results demonstrate that the scFv trapped GA<sub>24</sub> in ER and inhibited metabolism of GA<sub>24</sub> to bioactive GA<sub>4</sub>.

**Keywords** Antibody · Dwarf · Endoplasmic reticulum · Gibberellin · Immunomodulation · ScFv

## Abbreviations

GA	Gibberellin
scFv	Single chain variable fragment
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
LD	Long day

## Introduction

The methodology to modulate plant functions by antibody production in planta is called 'immunomodulation'. Since Owen et al. (1992) showed that plants producing anti-phytochrome antibody have decreased responsiveness to light, immunomodulatory studies in plants have seen a steady

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increase in the number of antibodies produced, including those against phytohormones to repress phenomena regulated by phytohormones such as abscisic acid (Artsaenko et al. 1995; Phillips et al. 1997; Strauss et al. 2001; Wigger et al. 2002) and jasmonic acid (ten Hoopen et al. 2007), metabolic enzymes to modulate pigment metabolism (De Jaeger et al. 1999) and starch synthesis (Jobling et al. 2003), plant viral coat proteins to confer virus resistance (Tavladoraki et al. 1993; Voss et al. 1995; Fecker et al. 1997; Zimmermann et al. 1998), herbicides to confer herbicide resistance (Eto et al. 2003; Horsman et al. 2007), and against small heat shock proteins that prevent the normal heat stress response (Miroshnichenko et al. 2005).

Recently, we succeeded in obtaining GA-deficient phenotypes in *Arabidopsis* such as reduced leaf size, delayed bolting, reduced inflorescence length and decreased germination, by producing a single-chain variable fragment (scFv), a recombinant form of antibody, recognizing bioactive GAs (Suzuki et al. 2008). When the scFv was localized in endoplasmic reticulum (ER), it was most stably accumulated and gave clear GA-deficient phenotypes. The transgenic plants responded to exogenous GA<sub>4</sub> and contained 15–40 times higher endogenous GA<sub>4</sub> than wild-type plants. They also showed changes in expression levels of enzyme genes involved in the regulation of GA<sub>4</sub> level similarly to the GA-deficient mutants. These results clearly demonstrated that the level of free functional GA<sub>4</sub> was decreased by being trapped with scFv in ER to the extent that GA-deficient phenotypes were created. A dramatic increase in the total sum of GA<sub>4</sub> (free plus scFv-bound) was attributable to the enhanced GA biosynthesis caused by repressed GA action, and a reduction in GA<sub>4</sub> catabolism due to protection by scFv binding in ER.

While the above study was a good example of immunomodulation where GA function was inhibited by the antibody to prevent bioactive GAs from interacting with the GA receptors, there is another possible mode of immunomodulation for GA function, which is inhibition of GA biosynthesis by using antibodies against biosynthetic precursors of bioactive GAs. We previously reported the preparation of dwarf tobacco plants producing an antibody, which was raised against BSA-conjugated GA<sub>24</sub> (Shimada et al. 1999). The antibody was highly reactive with GA<sub>24</sub> and GA<sub>19</sub>, which are aldehyde-type GAs located two steps upstream of the bioactive GA<sub>4</sub> and GA<sub>1</sub>, respectively. While the antibody showed no cross-reactivity with GA<sub>20</sub>, GA<sub>1</sub> and GA<sub>4</sub>, all having  $\gamma$ -lactone on the A ring of the GA skeleton, GA<sub>17</sub> and GA<sub>13</sub>, having a C-20 carboxyl group, nor with GA<sub>53</sub> having a C-20 methyl group, it did show reactivity to GA<sub>15</sub>, which has a  $\delta$ -lactone on the A ring. The antibody was also not reactive with GA<sub>24</sub> methyl ester. Based on these results, it was argued that the antibody critically recognized either C-19  $\rightarrow$  C-20 lactol or  $\delta$ -lactone on

the A ring, and the dissociated form of C-7 carboxyl group on the B ring (Shimada et al. 1999). The observed dwarf phenotype for transgenic tobacco was considered to be caused by capture of biosynthetic precursor GAs by the scFv, because GA<sub>1</sub>, the major bioactive GA in tobacco, could not be detected from the transgenic tobacco plants whereas wild-type plants contained a detectable level of GA<sub>1</sub>. However, the results were preliminary in the sense that statistical significance was not evaluated for the dwarf phenotype. Afterwards, we tried to produce the same scFv in *Arabidopsis*, so as to demonstrate the significant effect of the antibody to modulate plant growth, and also to show that the effect is universal among different plant species. However, the antibody was not stably accumulated in *Arabidopsis* and conferred no phenotypes to the transgenic plants. In this study, we again tried to produce the same scFv as a fusion with green fluorescence protein (GFP) in ER along the lines of the previous study where GFP-fused anti-bioactive GA scFv was stably accumulated in *Arabidopsis* ER. Previous immunomodulatory studies targeting small molecules supported the idea that the effective inhibition of the target function can be obtained by producing antibodies in the ER (Artsaenko et al. 1995; Eto et al. 2003; Suzuki et al. 2008). This can be interpreted as antibody being generally more stable in ER than in any other compartment tested, and that targets of antibodies with low molecular weight and relatively high hydrophobicity can pass through membrane systems and reach antibodies in ER. Because antigens are sequestered in ER by antibodies and cannot bind their receptors in other compartments, this mode of immunomodulation is called ‘mis-targeting’ of antigens (Conrad and Manteuffel 2001). Since the anti-GA<sub>24</sub> scFv was stably accumulated in ER but not in cytosol in tobacco (Shimada et al. 1999), we followed the mis-targeting system in this study. Consequently, the anti-precursor GA scFv was stably accumulated and gave clear phenotypes in *Arabidopsis*. Interestingly, the transgenic plants producing anti-GA<sub>4</sub> scFv and anti-GA<sub>24</sub> scFv showed different severity depending on the growth stages, although both of them showed GA-deficient phenotypes as a whole.

## Materials and methods

### Construction of the plant expression vector

The anti-GA<sub>24</sub> scFv gene was PCR-amplified with a template plasmid pHIL24B (Shimada et al. 1999), with a primer set for integrating *Bsp*1407I and *Not*I sites (fw, 5'-gggtgaca[*Bsp*1407I]tggaggtgaagctggtggagtc-3'; rv, 5'-tgcgcccgc[*Not*I]tacagttggtgcagcatcagccc-3'). The scFv fragment was replaced with a scFv gene encoding anti-GA<sub>4</sub>

scFv using *Bsp*1407I and *Not*I sites in pBI-ER-D, a binary vector constructed previously (Suzuki et al. 2008).

#### Plant transformation

The binary vector was transferred into *Agrobacterium tumefaciens* strain LBA4404 (pAL4404), which was used to transform *Arabidopsis* plants (*Arabidopsis thaliana* L. Heynh., Brassicaceae, accession Columbia, from Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH, USA) as previously described (Bechtold et al. 1993). Transgenic plants (T1) were selected on Murashige and Skoog (MS) agar medium containing 100 µg/ml of kanamycin. Homozygous lines were established from the independent T1 lines whose next generation showed a 3 to 1 segregation for kanamycin resistance and sensitivity, and which were used for all subsequent analyses.

#### Plant growth conditions

Surface-sterilized seeds were sown on sterilized MS media containing 3% (w/v) sucrose and 0.8% (w/v) agar (Wako, Osaka, Japan). The seeds were moved to a growth chamber at 23°C with continuous fluorescent light (35 µmol m<sup>-2</sup> s<sup>-2</sup>) after chilling at 4°C for 3 days.

#### Microscopic analysis of GFP fluorescence from GFP-scFv proteins

The subcellular localization of GFP-scFv in transgenic plants was analyzed by observing GFP fluorescence under a confocal laser scanning microscope (IX81/FV500, Olympus, Tokyo, Japan). Excitation at 488 nm was provided by Ar ion laser, and fluorescence emission was collected at 500–530 nm. Images were acquired with Olympus Fluoview software. A fluorescence microscope (BX60, Olympus) was also used for GFP observation with either a U-MWIB/GFP or a U-MNIBA filter (Olympus).

#### Radioimmunoassay and Western blotting

Rosette leaves (200 mg) was frozen in liquid nitrogen and ground to a fine powder, and protein was extracted from the powder with 1 ml of 50 mM Tris-HCl (pH 8.0) containing 200 mM NaCl, 5 mM EDTA and 0.1% Tween 20 and one tablet of proteinase inhibitor mixture (Complete Mini, Roche, Mannheim, Germany) for 10 mL of the buffer. The binding activity of scFv from the transgenic plants to GA<sub>24</sub> was detected by radioimmunoassay. The extract (100 µL) was mixed with <sup>3</sup>H-labeled GA<sub>24</sub> (10,000 dpm) in PBS containing 180 µL rabbit normal serum, and incubated at 4°C overnight. For competitive radioimmunoassay, an

excess of non-labeled GA<sub>24</sub> was added to the mixture to give a 0.17 µM final concentration. To measure the unspecific binding, the protein extract was eliminated from the incubation reaction.

The presence of scFv protein was detected by Western blotting. Twenty micro liters of the protein extract was applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were electrotransferred onto a nitrocellulose membrane, and a c-myc tag fused to scFv was detected with the anti-c-myc antibody (9E10 mouse ascites 1,000 times diluted with PBS containing 2% skimmed milk) (Evan et al. 1985) and HRP-conjugated anti-mouse antibody (5,000 times diluted with PBS; GE Healthcare, Uppsala, Sweden). The HRP activity was detected by an ECL Plus Western blotting detection system (GE Healthcare).

#### Quantification of endogenous gibberellins and real-time RT-PCR analysis

The aerial part of wild-type plants (4.0 g), A24-2 (3.9 g) and A24-3 (3.5 g) was spiked with 50 ng of deuterium-labeled GA<sub>4</sub> and GA<sub>24</sub> (purchased from Dr. Mander, Australian National University), and homogenized in methanol. The extract was filtered and concentrated *in vacuo*. The concentrate was acidified with hydrogen chloride to pH 3 and extracted with ethyl acetate, which was then extracted with 0.5 M potassium phosphate buffer (pH 8.5). The aqueous layer was again extracted with ethyl acetate after being adjusted to pH 3. The obtained ethyl acetate acidic fraction was dried over sodium sulfate and concentrated to dryness. The concentrate was dissolved in a small volume of 50 mM potassium phosphate buffer (pH 8.0) and loaded onto a column packed with insoluble polyvinylpyrrolidone (1 g, Tokyo Kasei, Tokyo, Japan), prepared and equilibrated with the same phosphate buffer, and the GA fraction was obtained as the first 10-mL elution with the same buffer. The resulting GA fraction was acidified to pH 3.0 with hydrogen chloride and loaded onto an OASIS HLB cartridge (500 mg, Waters, Milford, MA, USA). After washing with 6 mL of 20% methanol containing 1% acetic acid, the GAs were recovered by elution with 12 mL of 60% methanol containing 1% acetic acid. The eluate was concentrated to dryness, loaded onto Sep-Pak Silica (500 mg, Waters) equilibrated with chloroform-ethyl acetate (1:1, v/v) containing 1% acetic acid, and eluted with 10 mL of the same solvent. The purified GA fraction was analyzed with an API3000 LC-MS/MS device (Applied Biosystems, Foster City, CA, USA). The sample was separated in Senshu Pak Pegasil ODS (2.0 mm i.d. × 150 mm, Senshu Scientific, Tokyo, Japan). Elution was performed as follows, with solvents A [0.1% (v/v) acetic acid in water] and B [0.1% (v/v) acetic acid in acetonitrile]: 0–2 min, isocratic with 10% B; 2–20 min, linear gradient from 10% B to

100% B; 20–24 min, isocratic elution with 100% B. The flow rate was 0.2 mL min<sup>-1</sup>. MS-MS analysis was carried out in the negative-ion mode with a turbo-ion-spray inlet system. GA<sub>4</sub>, GA<sub>24</sub> and their internal standards, d<sub>2</sub>-GA<sub>4</sub> and d<sub>2</sub>-GA<sub>24</sub>, were detected by the combination of the primary and the secondary ions at m/z 331/257 for GA<sub>4</sub>, m/z 345/257 for GA<sub>24</sub>, m/z 333/259 for d<sub>2</sub>-GA<sub>4</sub>, and m/z 347/259 for d<sub>2</sub>-GA<sub>24</sub>, respectively, in the multiple-reaction monitoring mode.

Real-time RT-PCR analysis exactly followed the method in our previous report (Suzuki et al. 2008).

## Results

### Preparation of transgenic *Arabidopsis* producing GFP-scFv fusion proteins

ScFv is composed of variable domains of an H chain (V<sub>H</sub>) and an L chain (V<sub>L</sub>) of an immunoglobulin molecule connected with a flexible linker peptide. The scFv gene used in this study had been prepared from a hybridoma cell line producing a monoclonal antibody against aldehyde-type GAs, GA<sub>24</sub> and GA<sub>19</sub> (Shimada et al. 1999). Here we designate the scFv anti-GA<sub>24</sub> scFv because the major GA biosynthetic pathway in *Arabidopsis* is the early non-3, 13-hydroxylation pathway (Talon et al. 1990; Magome et al. 2004), on which GA<sub>4</sub> and GA<sub>24</sub> are a bioactive GA and its biosynthetic precursor, respectively, i.e., GA<sub>24</sub> is converted to GA<sub>9</sub>, the immediate precursor of bioactive GA<sub>4</sub>. Although the antibody is also reactive with GA<sub>15</sub>, which is the precursor of GA<sub>24</sub>, it is the minor target of the antibody in *Arabidopsis* (Magome et al. 2004). GFP was fused to the N-terminus of the anti-GA<sub>24</sub> scFv, and a c-myc epitope tag and KDEL ER-retention signal to the C-terminus. A cauliflower mosaic virus 35S RNA promoter was used for constitutive expression. More than ten independent transgenic plants were obtained as kanamycin-resistant plants.

### Confocal imaging of GFP fluorescence, Western blotting and radioimmunoassay of scFvs produced in plants

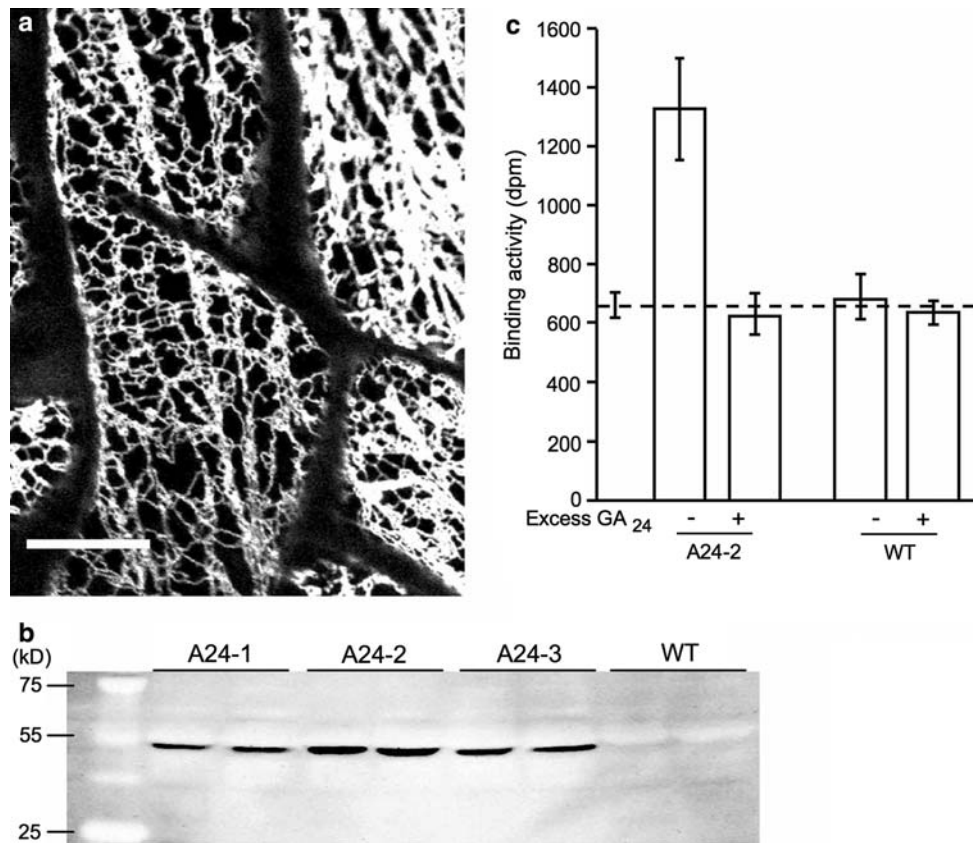
In the initial screening of transgenic plants, some lines here designated as A24-1, 2 and 3 showed clear dwarf phenotypes presumably due to GFP-scFv production (details will be shown later), and showed strong GFP fluorescence. Using these lines, sub-cellular localization of GFP-scFv was examined by confocal laser scanning microscopy. The reticulate shape of fluorescence was observed in the epidermal cells of rosette leaves (Fig. 1a). This reticulate structure is a well-known cortical ER network (Staehelin 1997; Suzuki et al. 2008), indicating that GFP-scFv was successfully targeted to the ER compartment. GFP-scFvs in the

extracts from A24-1, 2 and 3 were clearly detected by western blotting at the expected size on SDS-PAGE (Fig. 1b), which were quite different from our previous experiment where scFv could hardly be detected by the same detection method when we tried to produce scFv without fusing with GFP (data not shown). The protein extract from the transgenic line A24-2, which showed a higher GFP-scFv level than the other two lines, was subjected to radioimmunoassay to confirm that the GFP-scFv maintains its binding activity with GA<sub>24</sub>. After incubation of the protein extract with <sup>3</sup>H-labeled GA<sub>24</sub>, radioactivity was detected in the ammonium sulfate-precipitated protein fraction containing GFP-scFv, and the recovery of <sup>3</sup>H-GA<sub>24</sub> in the protein fraction was decreased by an excess non-labeled GA<sub>24</sub> as a competitor of <sup>3</sup>H-GA<sub>24</sub> to the level of the unspecific binding (Fig. 1c). As the extract from wild-type plants showed no significant binding, the binding activity observed in the A24-2 plants could be attributed to scFv produced in the plants. These results indicate that functional scFv was successfully produced in *Arabidopsis* as fusion with GFP in the ER compartment.

### Phenotypes of transgenic plants

The effects of scFv production on conferring GA-deficient phenotypes to plants were examined in detail using the transgenic lines with a high accumulation of GFP-scFv (A24-1, 2 and 3). Figure 2a shows the typical appearance of the A24-2 line and a wild-type plant at 58 days after germination, the point in time when both lines had already stopped elongation. The plant height of A24-2 was less than one-half of the wild-type plant. The dwarf phenotype was statistically significant for all three lines, A24-1, 2 and 3, and the severity of the dwarf phenotype was approximately correlated with the accumulation level of GFP-scFv protein (Figs. 1b, 2b). Analysis of the temporal fluctuation of the A24-2 plant height showed that stem elongation was extremely slow during the first several days after bolting, whereas the elongation rate at a later stage was almost comparable to that of wild-type plants (Fig. 2c).

Another GA-deficient phenotype was observed as a delay in bolting which was accompanied with increased rosette leaf number, a typical late flowering phenotype. Bolting of A24-2 was 6.3 days later than in wild-type plants and had about four more leaves (Table 1). Table 1 also shows the day at first flower opening and the inflorescence length at that time. The delay of the first flower opening in A24-2 (3.8 days) was not as big as that of bolting (6.3 days), because flowers in the A24-2 line started to open soon after bolting on a shorter inflorescence than wild-type plants (Table 1; Fig. 2d). It is apparent that the antibody is more effective in repressing flower induction than flower development.



**Fig. 1** Production of GFP-scFv protein in transgenic plants. **a** The green fluorescence from GFP-scFv protein was observed with a confocal laser scanning microscope, showing subcellular localization of the protein in the reticulate cortical ER network in epidermal cells. *Bar* 20  $\mu$ m. **b** The production of GFP-scFv was detected by western blotting. The protein extract from rosette leaves (two individual plants from the same transgenic line) corresponding to approximately 4 mg fr. wt. was loaded into each lane and detected with anti-c myc-tag anti-

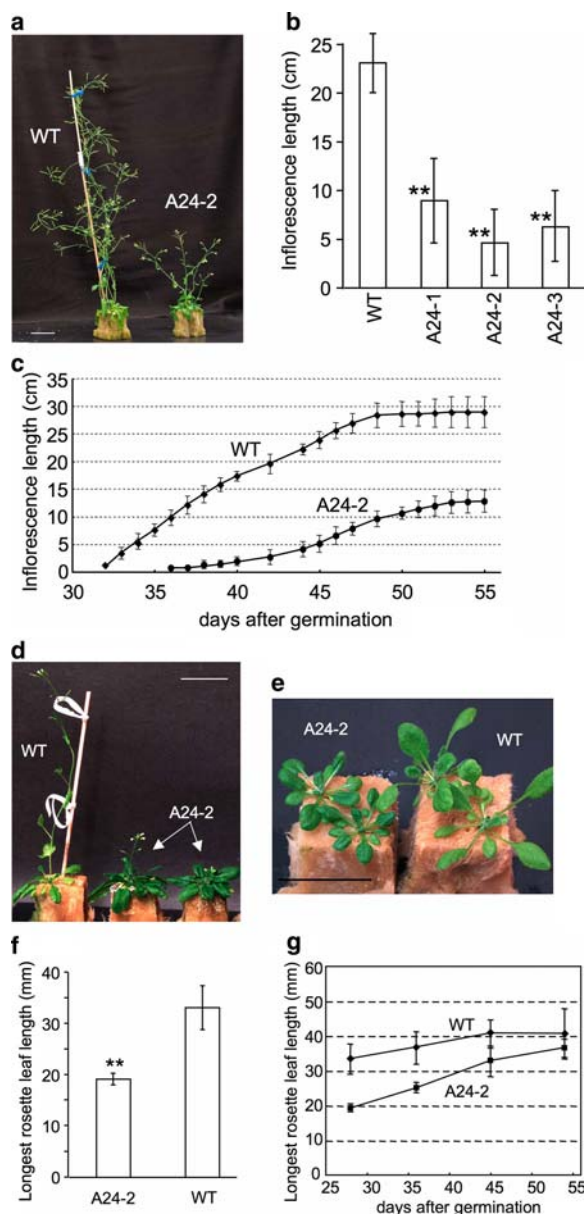
body. *WT* wild-type plant. **c** The binding activity of scFv in plant extract of A24-2 line toward GA<sub>24</sub> was detected by radioimmunoassay using <sup>3</sup>H-labeled GA<sub>24</sub> as a tracer. The level of unspecific binding shown as a dashed line was determined by eliminating the plant extract from the reaction mixture. The binding above the level of unspecific binding was shown to be competitively replaced by excess GA<sub>24</sub>. The assay was also performed with wild-type plant extract to show that the binding activity in the transgenic plant was derived from the scFv

Extension of rosette leaves of the transgenic line was also repressed by antibody production. The leaves had shorter petioles and their overall size was clearly smaller than wild-type plants (Fig. 2e, f). Their leaves also had a darker green color (Fig. 2e) and were less flexible, which was reminiscent of a GA-deficient mutant such as *gal-3* (Sun and Kamiya 1994). The time course of rosette leaf expansion indicated that the leaves of A24-2 continued to extend for a longer period than wild-type plants (Fig. 2g).

Verification of capture of GA<sub>24</sub> by GFP-scFv causing repressed GA<sub>4</sub> production

To verify that the GA-deficient phenotypes can be attributed to the repressed biosynthesis of GA<sub>4</sub> caused by capture of the precursor GA<sub>24</sub> by the produced scFv, first the ability of the plants to respond to GA<sub>4</sub> was confirmed. When an excess amount of GA<sub>4</sub> was applied to the plants by spraying GA<sub>4</sub> solution, the GA-deficient phenotypes, including reduced rosette size, delay of bolting and reduced stem

elongation were clearly rescued (Fig. 3a, b), indicating that the A24-2 plants retained their responsiveness to bioactive GA. In this recovery assay using an excess amount of GA, the effects of treating with GA<sub>4</sub> and GA<sub>24</sub> were not significantly different (data not shown). However, when a small amount of GA solution was applied to the tip of a rosette leaf, the effect of GA<sub>4</sub> on the recovery of stem elongation was much bigger than that of GA<sub>24</sub> (Fig. 3c). There were two possible explanations for this difference. One was that GA<sub>24</sub> is less active than GA<sub>4</sub> regardless of the scFv production because the conversion ratio of GA<sub>24</sub> to bioactive GA<sub>4</sub> is not 100%, and/or transport efficiency of GA<sub>24</sub> is lower than that of GA<sub>4</sub>. The other possibility was that the exogenous GA<sub>24</sub> was captured by scFv in the treated rosette leaf similarly to the endogenous GA<sub>24</sub>, and only part of the applied GA<sub>24</sub> was converted to GA<sub>4</sub>. To examine the former possibility, *gal-3*, a GA biosynthetic mutant, was treated with GAs in an identical manner. When applied to the tip of a rosette leaf, GA<sub>24</sub> was even more effective than GA<sub>4</sub> in recovering stem elongation of *gal-3* (Fig. 3d). This



**Fig. 2** GA-deficient phenotypes of the transgenic plants producing anti-GA<sub>24</sub> scFv. **a** Typical appearance of plants producing anti-GA<sub>24</sub> scFv after transition to reproductive growth. A transgenic plant line (A24-2) and a wild-type plant (WT) 58 days after germination are shown. Bar, 3 cm. **b** Comparison of the inflorescence length of each transgenic line at 45 days after germination. Each data represents the mean ± SD of 10 plants. \*\* indicates significant difference compared to wild-type plants at  $P < 0.01$  by Welch's *t*-test. **c** Temporal fluctuation of plant height of wild-type plants and A24-2 plants. Each point represents the mean ± SD of 10 plants. **d** The A24-2 plants which started flowering are shown beside a WT plant at the same age (42 days after germination). Bar 3 cm. **e** Typical appearance of a A24-2 plant and a WT plant during vegetative growth (28 days after germination). Bar 3 cm. **f** Comparison of the longest rosette leaf length between A24-2 and WT plants. Each data represents the mean ± SD from 30 to 40 plants at 28 days after germination. \*\* indicates significant difference compared with WT at  $P < 0.01$  by Welch's *t*-test. **g** Temporal fluctuation of the longest rosette leaf length of A24-2 and WT. Each point represents the mean ± SD of 10 plants

**Table 1** Phenotype of A24-2 line for bolting and flower opening

	WT	A24-2	Difference
Bolting time (DAG) <sup>a</sup>	32.7 ± 1.8	39.0 ± 2.7	6.3
Rosette leaf number	11.1 ± 0.5	15.0 ± 1.5	3.9
Flower opening time (DAG) <sup>b</sup>	37.3 ± 1.8	41.1 ± 2.5	3.8
Inflorescence length (cm)	7.4 ± 1.2	1.3 ± 0.7	-6.2

Each data represents the mean ± SD of 10 plants. DAG days after germination

<sup>a</sup> The time when elongated inflorescence stem was observed was marked as the bolting time

<sup>b</sup> The time when the first flower opened was marked as the flower opening time

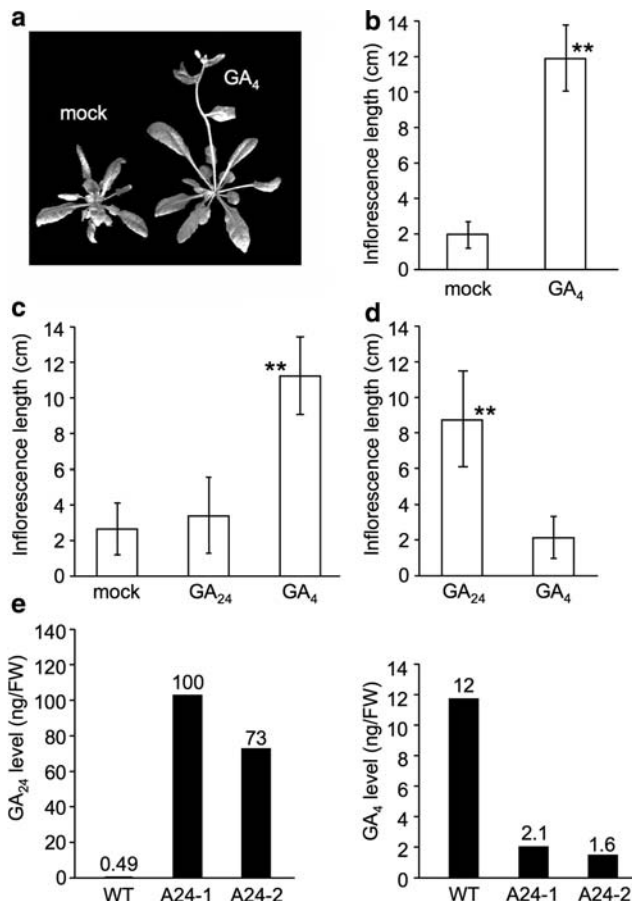
result is consistent with the observation that bioactive GA<sub>1</sub> was less effective than its precursors on shoot elongation of morning glory when they were treated onto the cotyledons (Yang et al. 1995). The precursor GA<sub>20</sub> has also been shown to be more effectively transported from lower leaves to upper apices than bioactive GA<sub>1</sub> in pea (Proebsting et al. 1992). The present result is highly suggestive that the lower effectiveness of GA<sub>24</sub> to recover stem elongation of A24-2 plants was due to the accumulated scFv, which captured the applied GA<sub>24</sub> and inhibited conversion to GA<sub>4</sub>.

If capture of GA<sub>24</sub> by scFvs in the ER is effective enough to repress its conversion to bioactive GA<sub>4</sub> and cause GA-deficient phenotypes, the transgenic plants are expected to have a higher level of GA<sub>24</sub> and a lower level of GA<sub>4</sub> than wild-type plants. The LC-MS/MS analysis showed that the endogenous level of GA<sub>24</sub> in A24-2 and A24-3 was conspicuously higher than in wild-type plants, while the level of GA<sub>4</sub> was less than 20% of that in wild-type plants (Fig. 3e). These results strongly suggest that the GA-deficient phenotypes were caused by decreased biosynthesis of bioactive GA<sub>4</sub> due to repression of GA<sub>24</sub> metabolism as a result of its capture by the antibody.

To obtain a supportive data to show that the transgenic plants are in the GA deficient state, transcript levels of GA biosynthetic genes, *AtGA20ox1* and *AtGA3ox1*, which are feedback-regulated by activated GA signaling, were analyzed. Quantitative real-time RT-PCR analysis showed that expression of *AtGA3ox1* and *AtGA20ox1* are significantly up-regulated in the A24-2 line (Fig. 4). This result also indicated that the conspicuous accumulation of GA<sub>24</sub> in A24-2 plants was due not only to repressed metabolism of GA<sub>24</sub> by binding of scFv but also to its enhanced biosynthesis.

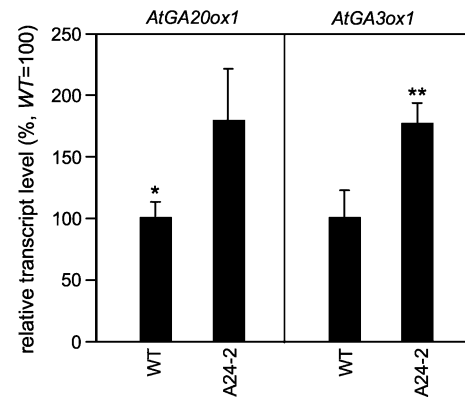
Differences of phenotypes between plants producing anti-GA<sub>4</sub> scFv and anti-GA<sub>24</sub> scFv

Although, the overall phenotypes caused by anti-GA<sub>4</sub> scFv production and anti-GA<sub>24</sub> scFv were similar in the sense that both antibodies gave GA-deficient phenotypes for



**Fig. 3** Verification for capture of GA<sub>24</sub> by GFP-scFv causing repressed GA<sub>4</sub> production. **a** Typical appearance of A24-2 plants treated with GA<sub>4</sub>. The A24-2 plants at 35 days after germination were sprayed with 100 μM GA<sub>4</sub> or mock-treated. The plants were recorded 5 days after the treatment. **b** Inflorescence lengths of A24-2 lines sprayed with GA<sub>4</sub> or mock solution. Inflorescence length was measured 4 days after the treatment as in **a**. \*\* indicates significant difference compared with mock-treated plants at  $P < 0.01$  by Welch's *t*-test. **c** Inflorescence lengths of A24-2 lines whose rosette leaves were treated with GA<sub>4</sub>, GA<sub>24</sub> or mock solution. Ten microliters of 100 μM GA<sub>4</sub> or GA<sub>24</sub> solution was applied to the tip of the longest rosette leaf of each A24-2 plant at 40 days after germination which had not started bolting. The inflorescence lengths were measured 5 days after the treatment. \*\* indicates significant difference compared with both mock-treated and GA<sub>24</sub>-treated plants at  $P < 0.01$  by Welch's *t*-test. The GA<sub>24</sub>-treatment showed no significant difference with mock-treatment. **d** Comparison of GA<sub>24</sub> and GA<sub>4</sub> treatment on recovery of the GA-deficient phenotypes of *gal-3*. Half a microliter of 100 μM GA<sub>4</sub> or GA<sub>24</sub> solution was applied to *gal-3* as in **c**. Note that mock-treated plants produced no elongated inflorescence. \*\* indicates significant difference compared with GA<sub>4</sub>-treated plants at  $P < 0.01$  by Welch's *t*-test. **e** Endogenous levels of GA<sub>24</sub> and GA<sub>4</sub> in wild-type plants (WT), A24-1 and A24-2 lines. Aerial parts of each plant line were extracted, purified and analyzed by LC-MS/MS with deuterium-labeled GA<sub>24</sub> and GA<sub>4</sub> as internal standards. The numerals are GA levels (ng/FW)

rosette leaf size, flower induction and stem elongation, there were apparent differences between them in the extent of effects depending on the growth stages. During the early stage of vegetative growth, rosette leaf development was

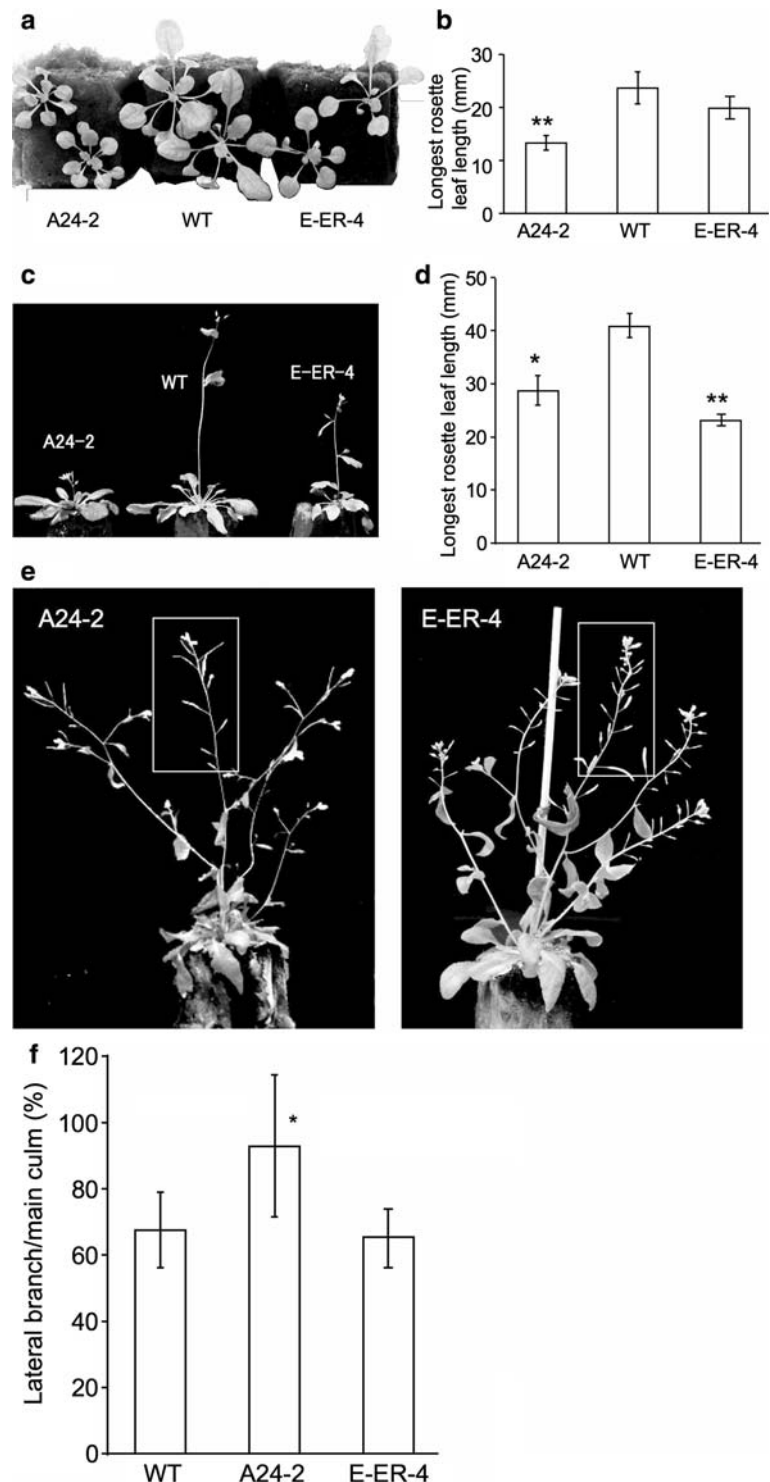


**Fig. 4** The effect of GFP-scFv production on gene expression of GA biosynthetic enzyme genes. The transcript level of *AtGA3ox1* and *AtGA20ox1* was obtained by real-time RT-PCR, and the level in A24-2 was shown as a relative value against that in wild-type plants (WT = 100%). *AtActin2* was used as the internal control in the analysis. Each data column represents the mean + SD of 3 quantification results using cDNAs independently prepared from 3 plants. \*\* and \* indicate significant differences compared with WT at  $P < 0.01$  and  $P < 0.05$ , respectively, by Student's *t*-test

more strongly repressed by anti-GA<sub>24</sub> scFv than by anti-GA<sub>4</sub> scFv. Figure 5a shows the typical appearance of A24-2 and E-ER-4 plants, one of the transgenic *Arabidopsis* lines producing anti-GA<sub>4</sub> scFv (Suzuki et al. 2008) 30 days after germination when no line, including wild-type plants, had started to bolt. At this early growth stage, A24-2 plants have smaller rosette leaves than E-ER-4 (Fig. 5b) and their green color was slightly darker (Fig. 2e). The effect of anti-GA<sub>24</sub> scFv was also bigger than anti-GA<sub>4</sub> scFv on delaying flower induction; the bolting time of A24-2 was more than 6 days later than wild-type plants (Table 1) whereas that of E-ER-4 was only about two days later (Suzuki et al. 2008). Figure 5c shows the time when A24-2 plant started to bolt. At this time, a wild-type plant and E-ER-4 plants had already started their elongation of the inflorescence stem. Note that the rosette leaf size of A24-2 is even bigger than E-ER-4 (Fig. 5c, d), because rosette leaves of A24-2 continued to grow for longer period than wild-type plants and E-ER-4 (cf. Fig. 2g), although it is not clear if the longer expansion growth of rosette leaves is associated with the delayed bolting.

As described before, the inflorescence stem elongation of A24-2 line was extremely slow during the first several days after bolting, whereas the elongation rate at a later stage was recovered almost to the level of wild-type plants (Fig. 2c). In contrast, the elongation rate of E-ER-4 line was constantly about half of that of wild-type plants (Suzuki et al. 2008). Consequently, the internode length between fruits, which is an index of stem elongation at a later stage of inflorescence growth after flowering, of E-ER-4 was shorter than that of A24-2 (Fig. 5e). The effect of anti-GA<sub>24</sub> scFv on repressing elongation of lateral branches

**Fig. 5** Comparison of phenotypes between transgenic lines producing anti-GA<sub>4</sub> scFv (E-ER-4) and those producing anti-GA<sub>24</sub> scFv (A24-2). **a** Typical appearance of A24-2, E-ER-4 and wild-type plants (WT) 20 days after germination. **b** Longest rosette leaf lengths of A24-2, E-ER-4 and WT 20 days after germination. \*\* indicates significant difference compared with WT at  $P < 0.01$  by Welch's *t*-test. The longest rosette size for A24-2 was significantly smaller than that for E-ER-4 at  $P < 0.01$ . **c** Typical appearance of A24-2, E-ER-4 and WT 42 days after germination. **d** Longest rosette leaf lengths of A24-2, E-ER-4 and WT 42 days after germination. \*\* and \* indicate significant differences compared with WT at  $P < 0.01$  and  $P < 0.05$ , respectively by Welch's *t*-test. The longest rosette size for E-ER-4 was significantly smaller than that for A24-2 at  $P < 0.01$ . **e** Typical appearance of A24-2 and E-ER-4 58 days after germination. **f** The value of lateral branch length divided by main culm length as an index for the differential effect of antibody production on repressed growth of main culm and that of lateral branch. The length of main culm and that of longest lateral branch was measured 58 days after germination, and their ratio is shown. \* indicates significant difference compared with WT at  $P < 0.05$  by Welch's *t*-test. The value for E-ER-4 was not significantly different from that of WT



was also smaller than anti-GA<sub>4</sub> scFv. Therefore, the value of lateral branch length divided by main culm length for A24-2 was larger than the value for wild-type plants, whereas that of E-ER-4 was not significantly different from that of wild-type plants (Fig. 5f).

As a whole, anti-GA<sub>24</sub> scFv was more repressive than anti-GA<sub>4</sub> scFv on growth during the early stages including

rosette leaf development, flower induction and inflorescence stem elongation just after bolting, and less repressive on later inflorescence growth, whereas anti-GA<sub>4</sub> scFv showed a constant mild effect on repressing plant growth. These tendencies for the extent of antibody effects were reproducibly observed for other transgenic lines (data not shown).



## Discussion

In our previous study, we showed that immunomodulation could be applied to modulate GA-regulated plant phenomena by using anti-bioactive GA<sub>4</sub> antibody. The antibody in that study was supposed to prevent bioactive GAs from binding with the GA receptor. In this study, we evaluated the possibility to immunomodulate biosynthesis of GA using anti-precursor GA antibody. To this end, we prepared *Arabidopsis* plants producing anti-GA<sub>24</sub> scFv fused to GFP along the lines of the previous study where GFP-fused anti-GA<sub>4</sub> scFv was stably accumulated in *Arabidopsis* ER. Consequently, the GFP-fused anti-GA<sub>24</sub> scFv was also successfully accumulated to a level high enough to obtain GA-deficient phenotypes. This result further supported the usefulness of ‘mis-targeting’ of antigens to block their functions, when antibodies are more stable in a specific compartment such as ER and antigens can reach antibodies. It is likely that GA<sub>24</sub> synthesized in cytosol was diffusible across ER membrane system to the lumen of ER, and was captured by the scFv, resulting in shortage of biosynthetic precursor of bioactive GA<sub>4</sub>. Since the same scFv was not stably accumulated when it was produced without being fused with GFP, it seems that GFP worked to stabilize the fusion protein in ER. Scheller et al. (2006) showed that elastin-like polypeptides fused to the C-terminus of scFv increased the accumulation level of scFv in ER to a large extent. Although KDEL ER-retention signal was shown to stabilize scFvs in the cytosol where scFvs harbouring intramolecular disulphide bonds are generally unstable because of its reducing condition (Schouten et al. 1997), there is only limited information how to stabilize scFvs in other compartments. Because fusing scFvs with other proteins or peptides is simpler than modifying scFv molecules by mutagenesis, it is quite advantageous to establish the fusing component which can be generally applied to stabilize scFvs, not only for the immunomodulatory study but also for obtaining antibodies for pharmaceutical use.

The rescue of phenotypes of A24-lines by GA<sub>4</sub> showed that the transgenic plants could retain their responsiveness to GA. The fact that GA<sub>4</sub> was more effective than GA<sub>24</sub> in rescuing the phenotypes suggests that the scFvs inhibit the conversion of GA<sub>24</sub> to GA<sub>4</sub>. The conspicuous elevation of endogenous GA<sub>24</sub> level and the clear reduction of GA<sub>4</sub> level in the anti-GA<sub>24</sub> scFv-producing plants further supported the hypothesis that the GA-deficient phenotypes were caused by scFvs preventing conversion of GA<sub>24</sub> to bioactive GA<sub>4</sub>. GA<sub>15</sub>, another possible target of the scFv located in the major early non-3, 13-hydroxylation pathway, was not detected by LC-MS/MS analysis even from the transgenic lines (data not shown). The level of GA<sub>15</sub> might be too low to be captured by the scFv. The GA-deficient state was further supported by the expression levels of

GA 20-oxidase and GA 3-oxidase genes. Their higher levels in A24-2 than in wild-type plants suggest that the feedback repression of those genes was relieved by the reduction of GA signaling. Therefore, the increased level of GA<sub>24</sub> is attributable both to the enhanced biosynthesis of GA<sub>24</sub>, and to the protection of GA<sub>24</sub> by the scFv from metabolizing enzymes. It seems that the protecting effect of scFv is enhanced by the subcellular compartment of the scFv accumulation, i.e., the captured GA<sub>24</sub> is separated in ER not only from GA receptors but also from the metabolizing enzymes such as GA 20-oxidases or possibly GA 2-oxidase, which are located in the cytosol. This conspicuous accumulation of target antigen was also observed for GA<sub>4</sub> (Suzuki et al. 2008), abscisic acid (Phillips et al. 1997; Strauss et al. 2001) and jasmonic acid (ten Hoopen et al. 2007) in transgenic plants producing scFvs against respective antigens, suggesting that the content of rare endogenous compounds can be elevated by producing antibodies recognizing those compounds.

Although both the present immunomodulatory study using anti-precursor GA antibody and the previous study using anti-bioactive GA antibody gave GA-deficient phenotypes by repressed GA biosynthesis and repressed action of bioactive GA, respectively, their phenotypes were apparently different in terms of the extent of phenotypes depending on the growth stages. In summary, the early stage of development including rosette leaf extension, flower induction and early inflorescence growth was more effectively repressed by anti-GA<sub>24</sub> scFv than by anti-GA<sub>4</sub> scFv, whereas the later inflorescence growth was less effectively repressed by anti-GA<sub>24</sub> scFv than by anti-GA<sub>4</sub> scFv. Although the extent of phenotype was dependent on the production level of scFv, the tendency of immunomodulated growth was consistent among different transgenic lines, both those producing anti-GA<sub>24</sub> scFv and those producing anti-GA<sub>4</sub> scFv. The fact that the shoot apical meristem (SAM) before bolting is adjacent to rosette leaves, and the results that indicate that the phenotypes on the SAM, i.e., flower induction and early stem elongation, and on rosette leaves were more severe in A24-lines suggest a possibility that flower induction and early stem elongation depend on GAs biosynthesized in rosette leaves. This is supported by the result that GAs applied to rosette leaves can rescue the GA-deficient phenotypes, late flower induction and reduced stem elongation, of *gal-3* mutant (Fig. 3d). It is well known that GAs are involved in flower induction of long-day (LD) plants. In some plant species, the LD condition was shown to promote GA biosynthesis (Talon and Zeevaart 1990; Talon et al. 1991), but this response is not restricted to any specified tissue. In spinach, GA levels increase in the petiole, blade and also in the shoot tip in response to LD. Spinach GA 20-oxidase, which catalyzes the conversion of GA<sub>24</sub> or GA<sub>19</sub> to GA<sub>9</sub> or GA<sub>20</sub>,

the respective immediate precursors of bioactive  $GA_4$  or  $GA_1$ , was shown to be involved in this regulation as a key enzyme; LD increases not only the transcript level but also its protein level (Wu et al. 1996; Lee and Zeevaart 2002). In *Arabidopsis*, although the GA levels in shoot tips have not been measured, GA contents in rosette leaves were shown to increase in response to LD (Xu et al. 1997). Since the transcript level of *GA 20-oxidase* increases in the shoot tips of *Arabidopsis* in response to LD (Xu et al. 1997), it is likely that the biosynthesis of GA in the elongation zone is at least partly responsible for the early stem elongation. It is probable that a precursor GA such as  $GA_{24}$  is translocated from rosette leaves to the SAM and is used for the biosynthesis of  $GA_4$  for flower induction and early stem elongation, in which the LD-induced *GA 20-oxidase* is involved. On the other hand, the mode of GA action for flowering in *Arabidopsis* grown under short-day condition has been studied in more detail (Eriksson et al. 2006). The authors first showed that  $GA_4$  is the active gibberellin in the regulation of flowering based on the observation that  $GA_4$  level in shoot apices increased dramatically just before flower initiation, and that  $GA_4$  showed a promoting effect on flowering when applied to shoot apices while other biologically active GAs,  $GA_1$  and  $GA_5$ , were inactive. Thereafter they suggested that the  $GA_4$  involved in flowering is transported from outside sources based on the fact that the expression levels of *GA 20-oxidase* and *GA 3-oxidase* genes remained unchanged during the period when  $GA_4$  levels in shoot apices increased. They further confirmed the transportation of exogenously applied  $GA_4$  on rosette leaves to shoot apices. All these studies as well as our results suggest the above hypothesis that the GAs synthesized in rosette leaves may be translocated to the SAM and participate in both flower induction and promotion of early stem elongation.

It is difficult to explain why anti- $GA_{24}$  scFv and anti- $GA_4$  scFv gave different extents of severity depending on the growth stages. It is apparent that merely the level of scFv production or their affinity to antigen cannot account for this difference. Many factors must be taken into account. First, the endogenous  $GA_{24}$  and  $GA_4$  are regulated to be at different levels and this regulation is also temporally and spatially specific. This means that the ease with which  $GA_{24}$  and  $GA_4$  are captured is different and this difference depends on the kind of tissue and its age. On the other hand, the accumulation level of scFv is also inconsistent despite the fact that gene expression was driven by a CaMV 35S RNA promoter, which is supposed to be constitutively active. Judging from the fluorescence strength, the level of scFv is higher in older tissues such as old rosette leaves and lower in young tissues such as young leaves or shoot tips. Moreover, the competitor of anti- $GA_{24}$  scFv at capturing targets is different from that of anti- $GA_4$  scFv, i.e., *GA 20-oxidase* for anti- $GA_{24}$  scFv and *GA receptor* for

anti- $GA_4$  scFv. Thus, both the relative affinities and the relative amounts of the antibodies and their competitors must be important factors to determine the phenotype severity. The relative locations of the proteins, ER versus cytosol in A24 lines, and ER versus nucleus in A4 lines, will also influence the outcome. In addition to the combination of these factors, translocation of GAs is another factor that makes it difficult to anticipate the effects of scFv production. As was shown in Fig. 3d, GAs in rosette leaves could migrate to the SAM, promote flower induction, and stem elongation. Given that the GAs in rosette leaves are involved in the GA-regulated events in the SAM, the strong phenotypes in A24-lines mean that anti- $GA_{24}$  scFv captured  $GA_{24}$  and inhibited translocation of  $GA_{24}$  to the SAM, or inhibited the conversion of  $GA_{24}$  to  $GA_9$ , and then to  $GA_4$  in rosette leaves and cut off the supply of  $GA_9$  or  $GA_4$  to the SAM. Since A4-lines showed only mild phenotypes on flower induction and early stem elongation compared to A24-lines,  $GA_{24}$  or  $GA_9$  in rosette leaves of A4-lines were freely migrated to the SAM and converted to  $GA_4$  in the SAM, which could not be captured by anti- $GA_4$  scFv efficiently enough to give strong phenotypes because the scFv level is low in the SAM. On the other hand, the stronger effect of anti- $GA_4$  scFv than anti- $GA_{24}$  scFv on late inflorescence growth might be attributable to a lower endogenous level of  $GA_4$  than  $GA_{24}$  in the inflorescence stem. The above-mentioned factors relating to the competitors of scFvs should also be integrated into this discussion. These speculations must be validated by quantitative estimate of scFvs, GA receptors, *GA 20-oxidases*, and GAs in specific parts of plants related to phenotypes of interest and also by a demonstration of the translocation of endogenous GAs. In spite of these ambiguities, the differential phenotypes by anti-bioactive GAs scFv and anti-precursor GAs scFv expand the capability of immunomodulation.

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