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Seed-specific immunomodulation of abscisic acid activity induces a developmental switch

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A single-chain Fv antibody (scFv) gene, which has previously been used to immunomodulate abscisic acid (ABA) activity in transgenic tobacco to create a 'wilty' phenotype, was put under control of the seed-specific USP promoter from *Vicia faba* and used to transform tobacco. Transformants were phenotypically similar to wild-type plants apart from their seeds. Anti-ABA scFv embryo development differed markedly from wild-type embryo development. Seeds which accumulated similar levels of a scFv that binds to oxazolone, a hapten absent from plants, developed like wild-type embryos. Anti-ABA scFv embryos developed green cotyledons containing chloroplasts and accumulated photosynthetic pigments but produced less seed storage protein and oil bodies. Anti-ABA scFv seeds germinated precociously if removed from seed capsules during development but were incapable of germination after drying. Total ABA levels were higher than in wild-type seeds but calculated free ABA levels were near-zero until 21 days after pollination. We show for the first time seed-specific immunomodulation and the resulting switch from the seed maturation programme to a germination programme. We conclude that the immunomodulation of hormones can alter the development programme of target organs, allowing the study of the directly blocked endogenous molecules and manipulation of the system concerned.

Keywords: abscisic acid/embryogenesis/immunomodulation/seed development/single-chain Fv antibody

Introduction

In recent years engineered antibodies have been used to perturb physiological processes within transformed mammalian cells by binding to specific endogenous or viral antigens (Richardson and Marasco, 1995). Single-chain Fv antibodies (scFv), which consist of the variable domains of the immunoglobulin heavy and light chains joined by a flexible linker peptide and are encoded by a single gene, have been advantageous for this approach. ScFvs which accumulate in the endoplasmic reticulum (ER) of mammalian cells have been able to bind protein antigens, preventing their further transport to the plasma-membrane and thereby blocking their action (Marasco *et al*., 1993, Beerli *et al*., 1994, Richardson *et al*., 1995). ScFvs accumulated in the cytoplasm of *Xenopus* oocytes have blocked activity of the $p21^{ras}$ proto-oncogene (Biocca *et al*., 1993).

Antibodies have also been expressed in plants (Hiatt *et al*., 1989; for review, see Conrad and Fiedler, 1994). A cytoplasmic scFv has been used to immunomodulate the activity of the phytochrome A protein in transgenic tobacco (Owen *et al*., 1992). Cytoplasmic expression of a scFv against artichoke mottled crinkle virus has been shown to delay infection in transgenic *Nicotiana benthamiana* plants (Tavladoraki *et al*., 1993). Recently this approach has been shown to also be applicable to non-protein endogenous regulatory antigens with the example of abscisic acid (Artsaenko *et al*., 1995).

Abscisic acid (ABA) is an important phytohormone responsible for the regulation of stomatal movements and stress responses as well as seed development processes. Until recently, studies have made use of ABA biosynthesis and response mutants as well as of the application, to plant tissues, of ABA and inhibitors of ABA biosynthesis (for review, see Giraudat *et al*., 1994). In a new approach, Artsaenko *et al*. (1994) constructed and characterized an scFv derived from the 15-I-C5 monoclonal antibody (Mertens *et al*., 1983) which has high affinity and specificity for ABA. Targeting of the anti-ABA scFv to the lumen of the ER in transgenic tobacco plants allowed accumulation in leaves to high levels (up to 4.8% of total soluble protein). Specific binding of the scFv to ABA *in vivo* caused a number of physiological effects including an increase in transpiration rates that resulted in a 'wilty' phenotype and loss of CO_2 dependence of stomatal closure. Seed development in these plants was not obviously affected by the scFv expression, although levels reached up to 0.75% of extracted seed protein. Also, the expression controlled by a legumin B4 seed-specific promoter from *Vicia faba* (Bäumlein *et al*., 1991a) failed to produce any apparent developmental effects (Artsaenko, 1996).

In the study reported here we used the USP promoter from *Vicia faba* (Bassüner *et al*., 1988; Bäumlein *et al*., 1991b) to control seed-specific expression of the anti-ABA scFv gene. The rationale behind this strategy was that this promoter is active several days earlier during seed development than the legumin promoter (Fiedler *et al*., 1993). This was expected to allow accumulation of the scFv antibodies before the rise in ABA during seed development, which has been described for tobacco by Imai *et al*. (1995). It enabled for the first time the seed-specific immunomodulation of ABA activity and study of plants which have normal vegetative growth and

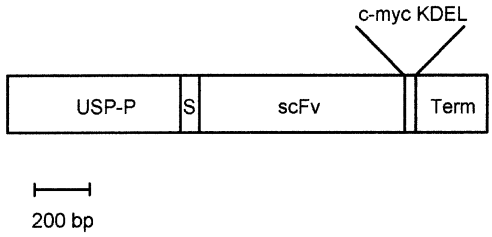


Fig. 1. Expression cassette designed for seed-specific immunomodulation. Transcriptional control sequences: USP-P, USP promoter; Term, CaMV 35S terminator (Töpfer *et al*., 1987). Coding sequences: S, legumin B4 signal peptide; scFv, anti-ABA single-chain Fv; c-myc KDEL, c-*myc* epitope tag with KDEL endoplasmic retention signal.

development patterns while the seeds display a phenotype due to reduction of ABA activity. Specificity of these effects was shown by comparison with seeds accumulating an unrelated scFv antibody that binds to the hapten oxazolone which is not found in plants (Fiedler and Conrad, 1995). This comparison allows us to conclude that the effects are specifically caused by the ABA-binding activity of the anti-ABA scFv antibody and are not secondary effects caused by high-level accumulation of a foreign protein in the lumen of the ER of plant cells.

Results

High-level seed-specific accumulation of anti-ABA scFv antibodies in transgenic tobacco

The USP promoter was used to regulate transcription of the scFv gene (Figure 1). Sequences encoding an N-terminal legumin B4 signal peptide and C-terminal KDEL ER retention signal were included to target antibodies to the lumen of the ER, while the c-*myc* tag was to allow detection of scFv protein with the 9E10 monoclonal antibody (Evan *et al*., 1985).

A series of plants were transformed and grown to maturity under greenhouse conditions. Western blot analysis of seed extracts showed that accumulation of the scFv was very high, up to 4% of extracted protein in mature seeds with more than one-quarter of the seeds tested having more than 2% (Figure 2). Expression was as expected for the USP promoter (Bäumlein *et al*., 1991b) seed-specific with no scFv protein detected in leaf or root extracts (Figure 3A). The scFv antibodies accumulated early during seed development, beginning around 10 days after pollination (DAP) (Figure 3B). Plants expressing similar levels of anti-oxazolone scFv in seeds were also produced (U.Fiedler, J.Phillips, O.Artsaenko and U.Conrad, manuscript in preparation).

Phenotypic effects caused by the seed-specific immunomodulation of ABA activity on seed development

The transgenic plants which accumulated anti-ABA scFv protein only in their seeds were not, apart from their seeds, phenotypically different from wild-type plants. Just before the seed coats had become brown (~17 DAP), embryos within seeds which expressed high-levels of the anti-ABA scFv developed green cotyledons (Figure 4A). The specificity of this effect is shown by the normal appearance of embryos from mature seeds expressing the anti-oxazolone scFv (Figure 4B). Anti-ABA scFv embryos

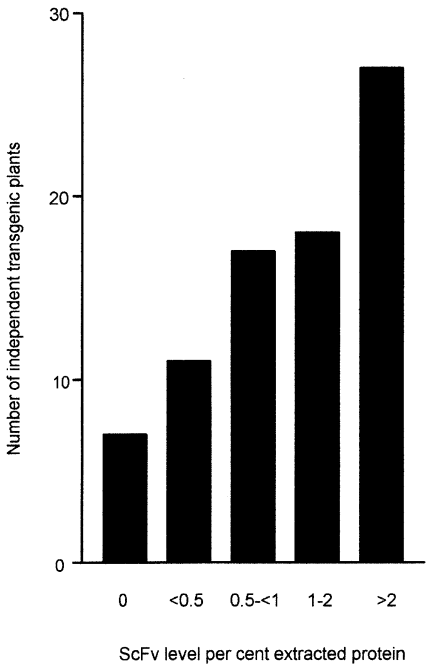


Fig. 2. ScFv levels in protein extracts of mature seeds from kanamycin-resistant plants estimated by comparison with affinity-purified scFv protein in Western blot analysis.

also underwent a phase of rapid growth around the time the cotyledons became green (Figure 4A). This was very noticeable when isolating embryos from seeds harvested 17 DAP as they tended to spring out as soon as the seed coat and endosperm were cut open. If such embryos were placed on MS medium they grew rapidly into small plantlets whereas wild-type control embryos showed no further development. When allowed to develop further until desiccation, embryos of high-expressing seeds dried out and shrivelled, leaving a large air space inside the seed. These seeds were not capable of germinating.

HPLC analysis of acetone extracts revealed that anti-ABA scFv seeds synthesize and accumulate other photosynthetic pigments in addition to chlorophyll a and b which give the cotyledons their green colour (Figure 5). Neither chlorophyll nor carotenoids were found in extracts from wild-type seeds (Figure 5A, lower panel). Extracts from anti-oxazolone seeds also contained no detectable photosynthetic pigments (not shown).

As ABA can stimulate seed storage protein accumulation in *in vitro*-cultured *Vicia faba* cotyledons (Barratt, 1986), the protein patterns from seed extracts were investigated by SDS–PAGE. No differences were initially found between extracts from whole seeds of anti-ABA scFv and wild-type plants when equal quantities of protein were compared from seeds at different stages of development (data not shown). However, when proteins extracted from isolated embryos were compared, dramatic changes were seen (Figure 6A). The most abundant proteins found in wild-type embryos—marked a to g—are much reduced or completely absent from anti-ABA scFv embryos. The specificity of this effect is demonstrated by the similar protein patterns of the wild-type and anti-oxazolone scFv embryos. The protein patterns from whole seed extracts show that most of the tobacco seed storage protein is found in the endosperm. Seed storage protein accumulation

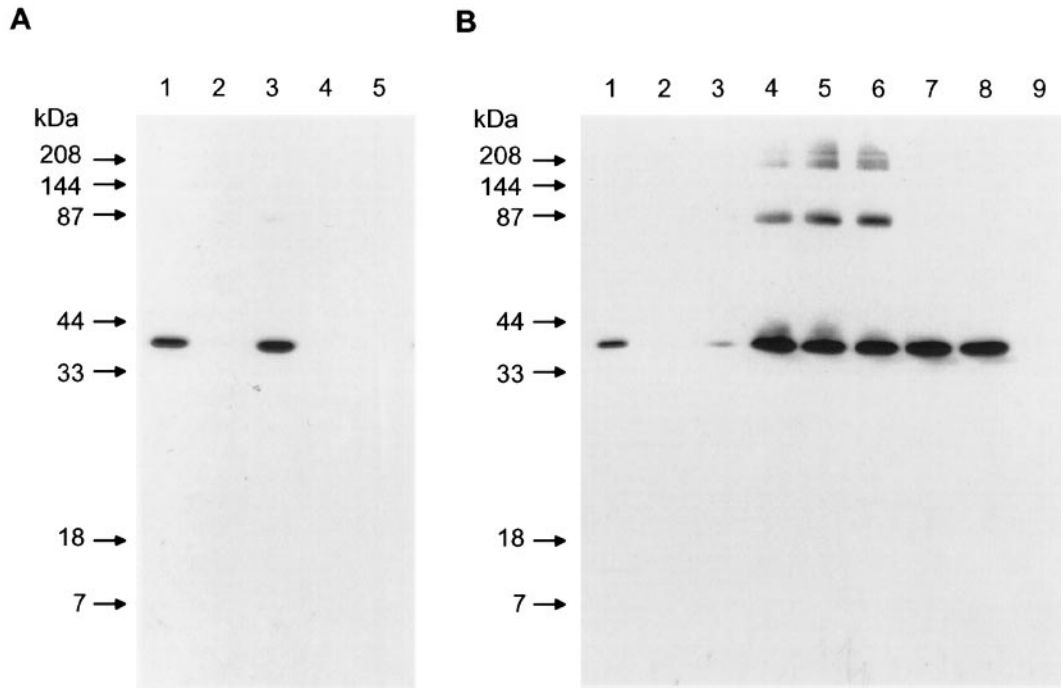


Fig. 3. (**A**) Accumulation of scFv is seed-specific. As positive control, 200 ng affinity-purified scFv protein (lane 1), 10 μg protein extracted from wild-type mature seeds (lane 2) and from mature seeds (lane 3), leaf (lane 4) and roots (lane 5) of an anti-ABA scFv transgenic plant. (**B**) Detection of scFv protein during seed development. As positive control, 100 ng affinity-purified anti-ABA scFv protein was used (lane 1), 10 μg protein extracted from anti-ABA scFv seeds harvested 7, 10, 14, 17, 21, 24 and 28 DAP (lanes 2, 3, 4, 5, 6, 7 and 8, respectively) and mature wild-type seeds (lane 9). The bands corresponding to a protein of ~75 kDa are probably a dimeric form of the scFv protein which has also been observed in bacterial expression systems (Artsaenko, 1996), while the bands corresponding to proteins >144 kDa are due to crossreactions with endogenous seed proteins and have also been observed in wild-type seed protein extracts (data not shown).

in the endosperm of anti-ABA scFv seeds is apparently relatively unaffected by the antibody. Western blot analysis of isolated embryos and endosperm showed that >90% of the scFv-antibody is located in the embryo, providing a possible explanation for this phenomenon (data not shown). N-terminal sequencing of the proteins e, f and g showed that these are β-chains of 12S globulins (Figure 6B). It is suggested therefore, that proteins a, b, c and d are the large acidic α-chains corresponding to these basic β-chains (Sano and Kawashima, 1983; Müntz *et al*., 1993). This is supported by the similarity of the protein pattern in Figure 6A and the pattern obtained by Western blot analysis using anti-tobacco 12S globulin serum (Figure 4; Fiedler and Conrad, 1995).

Artsaenko *et al*. (1995) found that ABA levels in leaves which accumulated scFv-ABA antibodies were 2- to 10-fold higher than levels in wild-type control leaves. Therefore, ABA levels in seeds were measured throughout development. ScFv levels were also measured, by Western blot analysis, in the same material. The expected amount of free, unbound ABA in the anti-ABA scFv seeds was calculated using the formula described by Neri *et al*. (1996) and the dissociation constant for the anti-ABA scFv ($K_d = 1.5\times10^{-9}$ M) estimated from affinity-purified anti-ABA scFv produced in plants by competition ELISA as described by Artsaenko *et al*. (1995).

In wild-type seeds, ABA levels started to rise rapidly 10 DAP, peaking at 17 DAP and rapidly decreasing from 21 DAP onwards (Figure 7). Total ABA levels in anti-ABA scFv transgenic seeds were higher than in wild-type seeds as soon as the scFv antibody was detected, at 10 DAP, they then rose more rapidly than in wild-type seeds and continued to rise throughout development. In mature anti-ABA scFv seeds ABA levels were 30- to 60-fold higher than in mature wild-type seeds. In contrast, free ABA levels remained low in anti-ABA scFv seeds until 21 DAP, only increasing at the end of seed development as scFv levels diminished.

The finding that anti-ABA scFv seeds produce chlorophyll a and b as well as other photosynthetic pigments (see above) suggested that these were likely to have fully developed chloroplasts. This was investigated at the subcellular level by electron microscopy. Comparison of the anti-ABA scFv embryos with anti-oxazolone scFv embryos showed not only the presence of chloroplasts but also a lack of protein bodies and oil bodies in cotyledon cells (Figure 8). This result is in agreement with the finding that seed storage proteins are absent or much reduced in anti-ABA scFv embryos.

Although the cotyledons of anti-ABA scFv embryos synthesized chloroplasts and photosynthetic pigments during their development—processes which are normally part of the germination programme—the seeds were not viviparous. However, a proportion of the anti-ABA scFv seeds underwent a form of precocious germination when removed from capsules and incubated on filter paper soaked with distilled water (Figure 9). Out of 163 anti-ABA scFv seeds, 41 germinated within 3 days, whereas out of 480 wild-type control seeds none germinated within 8 days when seeds 17 DAP were incubated under identical conditions. These rapidly germinating seeds all germinated by the emergence of the cotyledons—in contrast to the wild-type germination process where the radicle is the first to emerge (Figure 9; Avery, 1933). Interestingly, although this primary emergence of cotyledons was never observed in wild-type seeds,

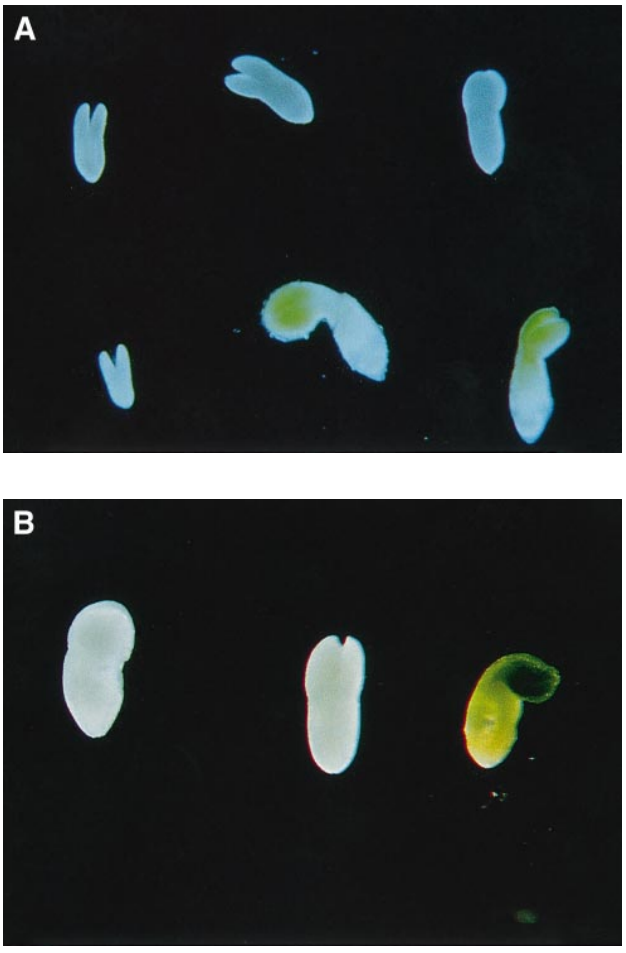
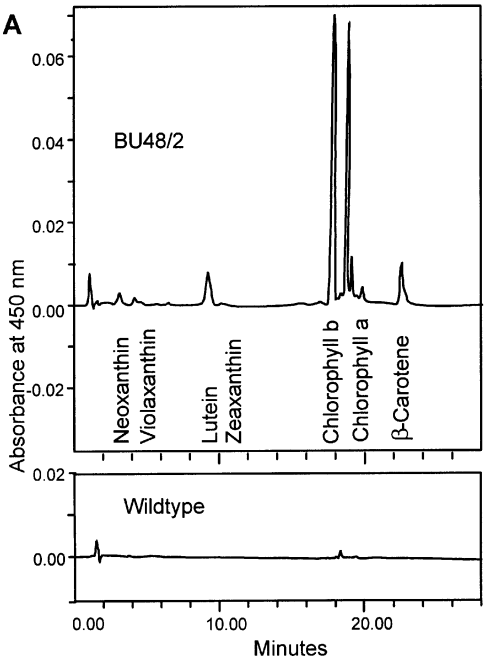


Fig. 4. (**A**) Embryos isolated from developing seeds of wild-type (upper row) and anti-ABA scFv seeds (lower row) 14, 17 and 21 DAP (left to right). (**B**) Embryos isolated from mature seeds from wild-type, anti-oxazolone scFv and anti-ABA scFv seeds (left to right).



B

Pigment	Content (µg/g FW)
Neoxanthin	0.45
Violaxanthin	0.42
Lutein	1.43
Zeaxanthin	0.09
b-Carotene	0.85
Chlorophyll b	4.00
Chlorophyll a	12.10

Fig. 5. (**A**) Photosynthetic pigments were detected, by HPLC, in acetone extracts from anti-ABA scFv mature seeds (BU48/2, upper panel) but not in extracts from wild-type seeds (lower panel). Analysis of the absorption spectra confirmed the identity of the chlorophylls and carotenoids. (**B**) Integration of the peaks allowed quantification of the pigments. FW, fresh weight.

it has in rare cases been seen in anti-oxazolone scFv seed germination, and so we cannot rule out that this is an effect caused by factors other than the blocking of ABA activity.

Discussion

We have shown for the first time that embryo development can be affected by seed-specific expression of an scFv which binds to an endogenous regulatory molecule. This has allowed the observation of changes in development caused by blocking ABA activity only in seeds, the vegetative parts of the plant being unaffected. Thus, the effects are due to processes within the seed and not to a lack of vigour in the parent plant due to a wilty phenotype.

Transcriptional activity directed by the USP promoter, together with targeting of the scFv to the lumen of the ER, caused sufficient accumulation of the scFv to block ABA activity at a crucial time during seed development (between 10 and 21 DAP). Although ABA-immunomodulated seeds did not actually germinate while on the plant to display a viviparous phenotype, processes usually associated with germination did occur, indicating a switch in the development programme.

The induction of synthesis of chloroplasts and photosynthetic pigments in cotyledons of developing immature seeds without viviparous germination in a species in which this does not normally occur has not previously been shown. The most similar situation reported was for the *Arabidopsis thaliana aba*, *abi3* double mutant seeds which have mutations in both ABA biosynthesis and ABA response genes and a resultant extreme phenotype (Koorneef *et al*., 1989). *Arabidopsis* embryos usually become green during development, losing this coloration towards maturity. The *aba*, *abi3* mutant seeds retained this green colour throughout development but also often showed viviparous germination inside the siliques. Development was however, normal if the maternal plant had a normal ABA biosynthesis phenotype (*Aba*, *aba* genotype) or if the plant was treated with ABA or an ABA analogue. The maintenance of the green seed colour until maturity was also found in the strong *abi3-3* allele mutants (Nambara *et al*., 1992).

Xu and Bewley (1995) applied the ABA biosynthesis inhibitor fluoridone to developing alfalfa pods and found that normally green pods and seeds became white. This may have been due to the inhibition of carotenoid synthesis and consequent photobleaching, masking possible effects of inhibition of ABA action, and thus highlighting a

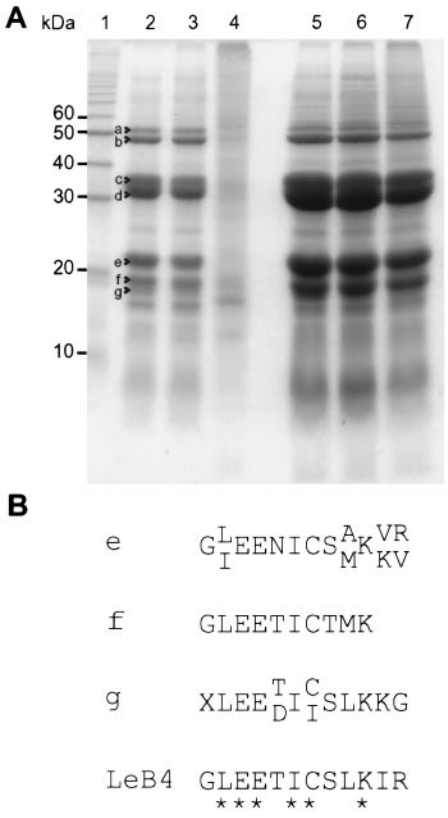


Fig. 6. (**A**) Comparison of the protein patterns in extracts from wild-type (lanes 2 and 5), anti-oxazolone scFv (lanes 3 and 6) and anti-ABA scFv (lanes 4 and 7) seeds. Proteins were extracted from 20 isolated embryos (lanes 2–4) and 20 whole seeds (lanes 5–7). (**B**) The proteins e, f and g were N-terminally sequenced and found to be 12S globulin β-chains. For comparison, the N-terminal sequence of the legumin B4 β-chain of *Vicia faba* is shown (LeB4). Asterisks mark amino acids common to all four sequences.

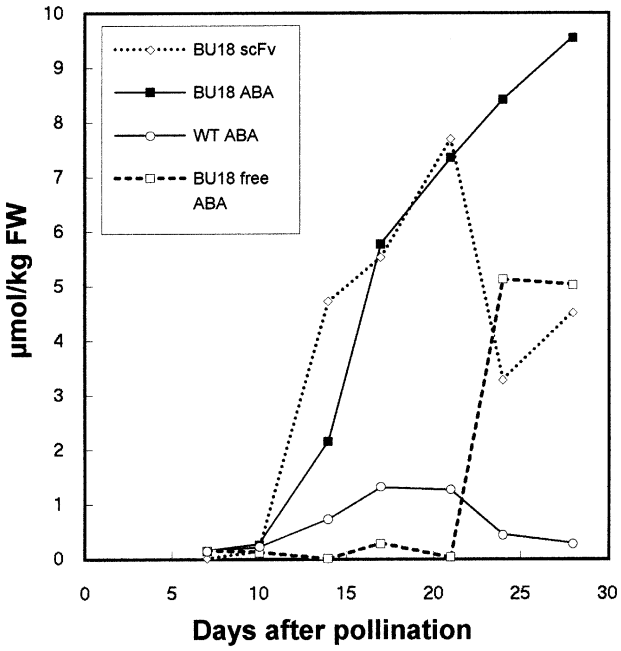


Fig. 7. ABA levels in developing wild-type (WT ABA) and anti-ABA scFv seeds (BU18 ABA) compared with scFv levels in anti-ABA scFv seeds and free ABA calculated as described in Materials and methods. Data points are mean values for seeds from three plants of each type. The anti-ABA plants were offspring of primary transformants.

disadvantage of chemical treatment strategies which block ABA synthesis very early in its pathway. It is this effect that causes several viviparous maize mutations, which are blocked in carotenoid biosynthesis, to be lethal as the seedlings are albino (Rock and Quatrano, 1995).

We found that accumulation of the 12S globulin proteins—the most abundant proteins in mature tobacco seeds (Sano and Kawashima, 1983)—was dramatically reduced in embryos and to a lesser degree in endosperm by immunomodulation of ABA activity. The less dramatic reduction in the endosperm was perhaps due to an insufficient level of anti-ABA scFv protein in this tissue or due to a gradient of ABA concentration caused by transport from the mother plant. The observation that most ABA biosynthesis mutants in *Arabidopsis* and maize accumulate normal seed storage protein levels (Giraudat *et al*., 1994) suggests that there are redundant biosynthetic pathways for ABA in seeds and only low levels are needed to induce correct seed development. This underlines the power of the immunomodulation approach where the ABA molecule itself is directly blocked by specific scFv binding. This could be tested by transformation of *Arabidopsis* and maize with suitable seed-specific anti-ABA scFv constructs. The same argument applies for the onset of desiccation tolerance.

In the *Arabidopsis abi3-3* (Nambara *et al*., 1992) and *fus3* (Bäumlein *et al*., 1994) mutants, which have a similar green seed phenotype to our ABA-immunomodulated seeds, lower levels of seed storage protein accumulation and desiccation intolerance have been reported.

The extremely high levels of ABA measured in the anti-ABA scFv transgenic seeds compared with the wild-type seeds reflect the situation found in leaves which accumulated the anti-ABA scFv (Artsaenko *et al*., 1995). Although the total ABA levels are extremely high in comparison with those in wild-type seeds, the anti-ABA scFv antibody is present at sufficiently high levels and early enough during development to prevent the accumulation of free ABA. Figure 7 clearly shows that free ABA levels are nearly zero or much lower than in wild-type seeds up to 21 DAP. By this time, the embryos have already undergone a switch in development and have green cotyledons (Figure 4A) and have failed to accumulate storage proteins (data not shown). Interestingly, the free ABA which begins to accumulate from 21 DAP to levels above those in wild-type seeds, cannot alter embryo development at this stage to allow normal seed maturation to take place.

The increased total ABA content in anti-ABA scFv seeds could be due to increased synthesis or decreased catabolism or conjugation of ABA within the seeds, or increased net transport from maternal tissues, or a combination of these. The scFv antibody probably sequesters ABA in the ER, providing a sink in this cell compartment which could inhibit a feedback mechanism regulating ABA synthesis. Alternatively, a combination of the phenotype of the embryos with green cotyledons and their dehydration could induce increased ABA synthesis. Enzymes which usually catabolize ABA may not be induced or may—at least until 21 DAP—be prevented physically from processing ABA due to antibody binding.

The observation that oil bodies appear to be absent in

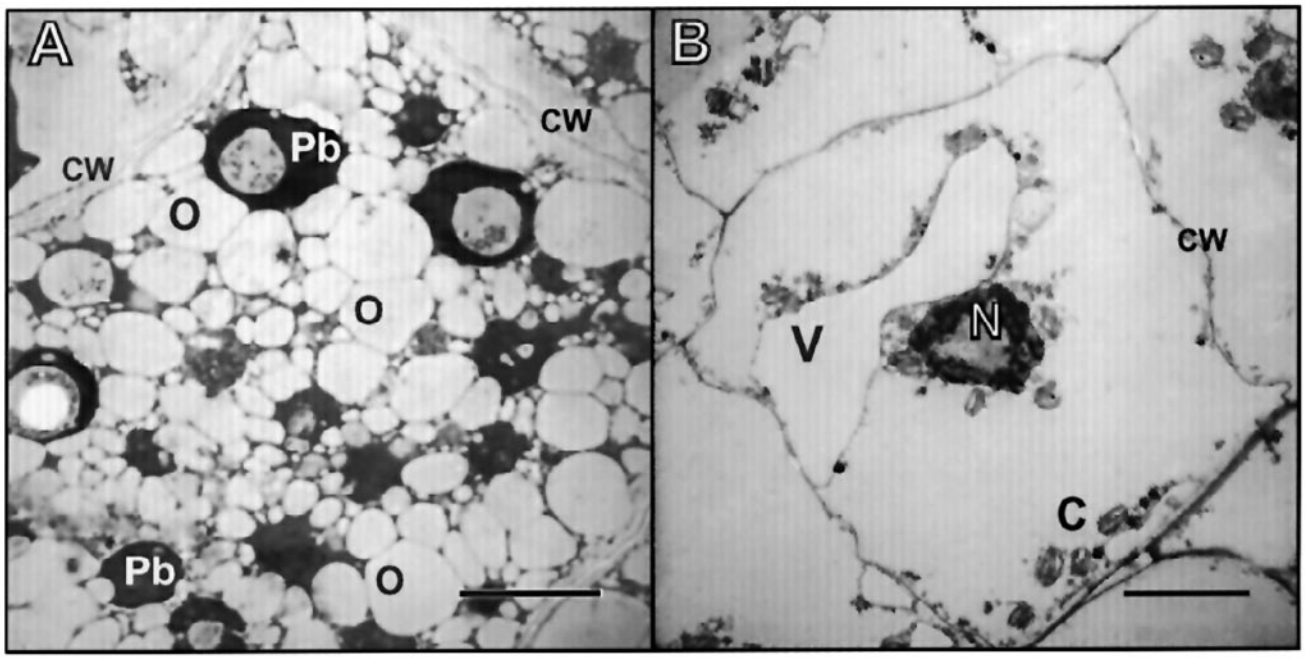


Fig. 8. Electron micrographs of cotyledon cells from embryos isolated from (**A**) anti-oxazolone scFv and (**B**) anti-ABA scFv mature seeds. Anti-oxazolone scFv cells are full of oil (o) and protein (Pb) bodies. In contrast, anti-ABA scFv cells lack these organelles but contain chloroplasts (c). Cw, cell wall; V, vacuole; N, nucleus. Bars represent 2.5 μm and 5.0 μm in (A) and (B), respectively.

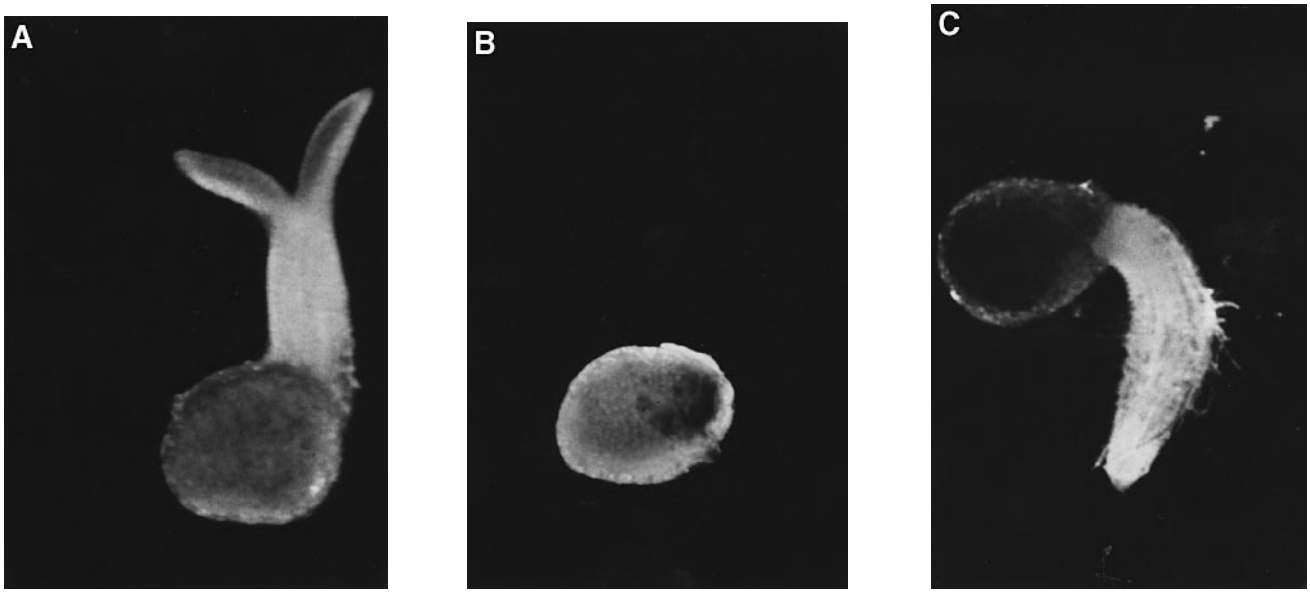


Fig. 9. Precocious germination of anti-ABA seed (**A**) removed from its seed capsule 17 DAP and incubated for 2 days on filter paper soaked with water. Similarly treated wild-type seeds did not germinate in this time (**B**). A wild-type seed germinating by emergence of its radicle, 14 days after incubation on wet filter paper (**C**), is shown in contrast to the anti-ABA scFv seed germinating by emergence of the cotyledons.

anti-ABA scFv embryos is reminiscent of the reduction in lipid content in *fus3* mutant *Arabidopsis* seeds when compared with wild-type (Bäumlein *et al*., 1994). The lack of oil and protein bodies and presence of chloroplasts in anti-ABA scFv embryo cotyledon cells is clear from Figure 8. In Figure 8B some of the cytoplasm might have been damaged. This may have been because the method of preparation used was optimized for embryos of mature wild-type seeds and is perhaps less than optimal for the dried anti-ABA scFv embryos.

Under the conditions in which the plants were grown, the anti-ABA scFv seeds did not germinate while still in capsules on the plant. Possibly the endosperm and seed coat physically prevent further growth of the embryo, as suggested by the *in vitro* growth of isolated embryos 17 DAP to form plantlets. Alternatively, water relations could be unfavourable for germination *in planta*.

To summarize, the anti-ABA scFv was able seed-specifically to repress ABA activity and allowed the study of the resultant physiological changes. The usual maturation programme was blocked and a vegetative programme was initiated. Detailed investigation of plants expressing lower levels of anti-ABA scFv will address the question whether this reprogramming is a strict process

or if some seeds have embryos which accumulate seed storage proteins and chloroplasts. The tobacco plants described here represent a model system for the further elucidation of ABA regulation of seed development processes.

The immunomodulation approach is particularly suited to blocking activity of small endogenous regulatory molecules which are present and biologically active at low concentrations in cells, such as hormones. It is expected that the ever-increasing array of promoters, to regulate specific spatial and temporal expression patterns, combined with improvements in the isolation of suitable scFv genes from phage display libraries, encoding high-specificity, high-affinity antibodies, will allow the immunomodulation of a wide range of targets for the study and manipulation of both animal and plant systems over the next few years.

Materials and methods

Construction of plant transformation vectors

DNA manipulations were performed according to standard protocols (Sambrook *et al*., 1989). A *Nco*I–*Sca*I fragment containing the USP promoter (Bäumlein *et al*., 1991b) was ligated to the *Nco*I–*Sca*I fragment containing the anti-ABA scFv coding region from pRTRA7/3 (Artsaenko *et al*., 1995). The seed-specific anti-ABA scFv gene was then cloned as a *Hin*dIII fragment into the *Hin*dIII site of pBin19 (Bevan, 1984). The anti-oxazolone scFv construct was cloned in a similar procedure (U.Fiedler, J.Phillips, O.Artsaenko and U.Conrad, manuscript in preparation).

Plant transformation

The plant transformation vectors were transferred to *Agrobacterium tumefaciens* C58C1 (pGV2260; Deblaere *et al*., 1985) by electroporation and used for leaf disc transformation of tobacco, *Nicotiana tabacum* cv. Samsun NN (Horsch *et al*., 1985). Kanamycin-resistant plants were grown to maturity in the greenhouse and pollinated by hand, and seeds were harvested at different times after pollination. To obtain offspring, 17 DAP seed capsules were surface-sterilized and seeds transferred aseptically to MS medium (Murashige and Skoog, 1962) to allow precocious germination.

Estimation of scFv expression in seeds

Proteins were extracted from mature seeds in 50 mM Tris–HCl, pH 7.7, 200 mM NaCl, 5 mM EDTA, 0.1% (v/v) Tween-20 and protein concentration was estimated by the method described by Bradford (1976). The amount of scFv protein in the extracts was estimated by Western blot analysis as described previously (Fiedler and Conrad, 1995).

Quantification of pigment content

The HPLC analysis of acetone extracts was described by Kruse *et al*. (1995).

Seed storage protein analysis

Embryos were isolated from mature seeds by hand with the aid of fine forceps and a binocular microscope. Twenty embryos were pooled and ground in 30 µl SDS sample buffer (36 mM Tris–HCl, pH 6.8, 2.5% β-mercaptoethanol, 1% SDS, 0.1% bromophenol blue, 5% glycerol) using an electric drill with a small plastic pestle. The homogenates were boiled for 5 min and cleared by centrifugation. Proteins were separated by SDS–PAGE and stained with Coomassie blue (Laemmli, 1970).

N-terminal protein sequencing

Proteins were extracted from 20 isolated wild-type embryos, separated by SDS–PAGE, and electrotransferred to PVDF membrane. The membrane was washed three times with water, stained for 2 min with 40% methanol, 0.025% Coomassie brilliant blue R250, destained with 50% methanol and washed with water four times. After drying, protein bands were cut out and used for N-terminal protein sequencing with a gas-phase sequencer LF 3400 (Beckman instruments). Phenylthiohydantoin amino acids (Pth-Xaa) were analysed by HPLC. Cysteine was detected as Pth-Cys S-propionamide that is formed during PAGE by reaction with unpolymerized acrylamide remaining in the gel (Brune, 1992).

Estimation of abscisic acid content, scFv content and calculation of free ABA

Throughout seed development, seeds from a single capsule were ground in liquid nitrogen and extracted overnight at 4°C in 10 ml 80% acetone. The extracts were cleared by centrifugation and ABA contents estimated by competitive ELISA as described by Artsaenko *et al*. (1995).

The pellets were stored at –20°C until extracts were made in 20 ml/g SDS sample buffer by incubation in a boiling water bath for 15 min. Estimation of anti-ABA scFv was by Western blot analysis as described previously (Fiedler and Conrad, 1995).

Estimation of the dissociation constant, K_d, of the anti-ABA scFv antibody for free ABA was by competition ELISA of affinity-purified scFv protein as described (Artsaenko *et al*., 1995).

Free ABA levels were calculated by solving the equation

$$K_d = \frac{[\text{scFv}][\text{ABA}]}{[\text{scFv}\cdot\text{ABA complex}]}$$

as described by Neri *et al*. (1996), for the different time points and subtracting the concentration of scFv·ABA complex from the total amount of ABA. For these calculations the density of the seeds was assumed to be the same as water and so mol/kg is equivalent to M.

Electron microscopy

Electron microscopy was performed by the method described by Fiedler (1996).

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