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ORIGINAL ARTICLE

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Prevention of stomatal closure by immunomodulation of endogenous abscisic acid and its reversion by abscisic acid treatment: physiological behaviour and morphological features of tobacco stomata

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Abstract Transgenic tobacco (*Nicotiana tabacum* L.) plants ubiquitously accumulating a single-chain variable-fragment (scFv) antibody against abscisic acid (ABA) to high concentrations in the endoplasmic reticulum (RA plants) show a wilty phenotype. High stomatal conductance and loss of CO_2 and light dependence of stomatal conductance are typical features of these plants. ABA was applied to these plants either via the petioles or by daily spraying over several weeks in order to normalise the phenotype. During the long-term experiments, scFv protein concentrations, total and (calculated) free ABA contents, and stomatal conductance and its dependence on CO_2 concentration and light intensity were monitored. The wilty phenotype of transgenic plants could not be normalised by short-term treatment with ABA via the petioles. Only a daily long-term treatment during plant development normalised the physiological behaviour completely. Scanning electron microscopy of stomata showed morphological changes in RA plants compared with wild-type plants that, for structural reasons, prevented regular stomatal movements. After long-term treatment with ABA this defect could be completely eliminated. Guard-cell-specific expression of the anti-ABA scFv did not cause any changes in physiological behaviour compared to the wild type. In addition, mesophyll-specific expression starting in leaves that were already fully differentiated resulted in normal phenotypes, too. We conclude that changes in distribution and availability of ABA in the cells of developing leaves of RA plants cause the development of structural features in stomata that prevent normal function.

Keywords Abscisic acid · Guard cell · Immunomodulation · *Nicotiana* (stomata) · Single-chain antibody · Stomatal conductance

Abbreviations ABA: abscisic acid · AGPase: ADP-glucose pyrophosphorylase · cytFBPase: cytosolic fructose-1,6-bisphosphatase · ELISA: enzyme-linked immunosorbent assay · PPFD: photosynthetically active photon flux density · scFv: single-chain variable fragment · TSP: total soluble protein · WT: wild type

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Introduction

The phytohormone abscisic acid (ABA) has several important physiological functions in plants, such as the maintenance of seed dormancy (Koornneef 1986) and desiccation tolerance (Kermode and Bewley 1987). ABA was shown to trigger regulation of stomatal closure under water-deficiency conditions. Most of the ABA-

deficient and ABA-insensitive mutants of different plant species show an increased tendency to wilt under normal humidity (for reviews, see Giraudat et al. 1994; Leung and Giraudat 1998).

Interestingly, the ABA amount per unit leaf area does not change before stomatal closure. Redistribution of ABA between different cell compartments was proposed as a model for the water-stress-dependent ABA function (Hartung and Slovik 1991). This redistribution of ABA was explained by water-stress-induced changes in pH in different plant cell compartments (Hartung et al. 1988; Hartung and Radin 1989).

Several papers provide evidence for the existence of ABA-binding proteins at the outer part of the plasmalemma or even in intracellular compartments, including the microsomal fraction (Schwartz et al. 1994; Allan and Trewavas 1994; Pedron et al. 1998). So far, ABA receptors or binding proteins have not been identified.

Many ABA-responsive genes have been identified in several species (for reviews, see Chandler and Robertson 1994; Shinozaki and Yamaguchi-Shinozaki 1996). Specific genes are expressed under stress conditions and by ABA induction in non-stressed tissue, too (Singh et al. 1987; Gomez et al. 1988; Mundy and Chua 1988). Nevertheless, the investigation of gene expression in ABA-biosynthetic mutants showed that the inducibility of a given gene by exogenous ABA under non-stress conditions does not necessarily imply the regulation of this gene by endogenous ABA upon stress (Gilmour and Thomashow 1991; Nordin et al. 1991; Imai et al. 1995). Inhibitors of carotenoid biosynthesis such as fluridone or norflurazon have been used to decrease ABA concentrations in plants to about 15%, resulting in phenotypes typical of ABA deficiency, and improved germination and vivipary (Fong et al. 1983; Oishi and Bewley 1990; Black 1991).

A new approach based on complexing endogenous ABA intracellularly with specific recombinant antibodies expressed in transgenic plants has been developed (Artsaenko et al. 1995; Phillips et al. 1997). An important prerequisite for these experiments was the construction of a recombinant antibody fragment (scFv: single-chain variable fragment) from a hybridoma cell line producing antibodies with high specificity and high affinity to free ABA (Mertens et al. 1983) and the characterisation of this scFv (Artsaenko et al. 1994; Phillips et al. 1997). The antibody does not cross-react with (-)-*cis*, *trans*-ABA, (±)-*trans*, *trans*-ABA, (±)-*cis*, *trans*-ABA methylester or other main ABA precursors and metabolites (Mertens 1985). The accumulation of this specific scFv to high concentrations in the endoplasmic reticulum (ER) of cells of transgenic plants (designated RA plants) driven by the ubiquitous CaMV 35S promoter led to a wilty phenotype. Stomatal conductance was increased even at high CO_2 concentrations. Symptoms of ABA deficiency were generated in the transgenic plants although they have even higher levels of ABA than wild-type (WT) plants (Artsaenko et al. 1995; Artsaenko et al. 1999). As a control for these experiments, transgenic tobacco plants were produced that accumulate in the ER an scFv antibody binding to the hapten oxazolone (Fiedler et al. 1999). These plants show normal growth and physiological behaviour comparable to WT plants.

The anti-ABA scFv was also ubiquitously expressed in transgenic potato plants, in which the subcellular distribution of ABA was influenced by the specific antibody. Leaf stomatal conductance was increased due to larger stomata. A detailed analysis of changes in plant metabolism showed that leaves of young transgenic plants developed in ABA deficiency and leaves of older plants in ABA excess (Strauss et al. 2001).

Furthermore, we asked if specific expression of anti-ABA scFv exclusively in guard cells or in mesophyll cells could also cause physiological changes.

Tobacco plants were transformed with a construct using the guard-cell-specific truncated version of the ADP-glucose pyrophosphorylase (AGPase) promoter from potato (construct 4 in Fig. 6 of Müller-Röber et al. 1994). In addition we constructed a mesophyll-cell-specific anti-ABA scFv expression cassette using the cytosolic fructose-1,6-bisphosphatase (cytFBPase) promoter also from potato (Ebneth 1996). The resulting transgenic tobacco plants were compared with RA plants and anti-oxazolone scFv transgenic plants.

We found that short-term application of ABA to excised leaves via the petioles cannot normalise the transgenic phenotype. Long-term treatment over several weeks by spraying, however, leads to a normalised phenotype showing lower stomatal conductance and dependence on CO_2 concentrations and light intensity comparable to control plants. The morphology of stomata has been changed in immunomodulated RA plants and we show normalisation by long-term treatment, too. The dependence of normal development of guard cells on compartment-specific ABA concentrations will be discussed.

Materials and methods

Plasmid construction and plant transformation

DNA manipulations were performed according to standard protocols (Sambrook et al. 1989). The CaMV 35S promoter constructs with the anti-ABA scFv (RA) and anti-oxazolone scFv (designated UF9 in this report) have been described previously (Artsaenko et al. 1995).

The guard-cell-specific construct was produced by cloning the *Sal*I/*Xba*I fragment from pAHCRaABA (Artsaenko 1996) containing the antibody-encoding sequence into an *Sal*I/*Xba*I-restricted pBin19 derivative containing the full-length AGPase promoter, and then recloning the *Hin*dIII fragment containing the truncated promoter into the *Hin*dIII site of pBin19 (Bevan 1984). This construct was designated SP.

The mesophyll-cell-specific construct was produced by cloning the same *Sal*I/*Xba*I fragment from pAHCRaABA into pBin19 containing the cytFBPase promoter (Ebneth 1996). This construct was designated ME. The SP and ME constructs were transferred into *Agrobacterium tumefaciens* C58C1 (pGV2260; Deblaere et al. 1985) by electroporation and used for leaf-disc transformation of tobacco, *Nicotiana tabacum* L. cv. Samsun NN (Horsch et al.

1985). Kanamycin-resistant plants were grown to maturity in the greenhouse.

Plant material and ABA treatment

Offspring of RA, ME, SP and UF9 plants were selected by germination on quarter-strength agar-solidified MS medium (Murashige and Skoog 1962) without sucrose or vitamins but containing 75 mg/l kanamycin, transferred to soil and grown in a phytochamber at 18 °C with 95% humidity and a 16-h daylength. The photosynthetic photon flux density (PPFD) of the light was 245 μmol m^{-2} s^{-1}. For histochemical analyses, RA lines expressing the scFv at less than 1% of total soluble protein (TSP; lines 41 and 51; Artsaenko 1996) were selected.

For the ABA-treatment experiment, in vitro-generated anti-ABA scFv transgenic tobacco plants (line RA 27), WT tobacco plants (*N. tabacum* cv. Samsun NN; SNN) and anti-oxazolone scFv transgenic plants (line UF9/38) as controls were grown in a phytochamber under defined conditions (18 °C, 95% humidity) for 9 weeks. Starting in week 3, plants were sprayed daily with either 50 μM (±)-*cis*, *trans*-ABA (Sigma) or water for 6 weeks. Line RA 27 was selected for ABA treatment because of its high stability of antibody expression. The use of transgenic in vitro plants ensures relatively constant expression levels.

Immunohistochemistry

Leaf tissue was fixed in 4% paraformaldehyde, 0.1% Triton X-100, phosphate-buffered saline (PBS; pH 7.3) for 2 h, dehydrated in ethanol and embedded in polyethylene glycol (van Lammeren et al. 1985). For immunolabelling we used the ELF-cytological-labelling-kit2 (Molecular Probes Europe BV, Leiden, The Netherlands), which utilises 2-(5′-chloro-2′-phosphoryloxyphenyl)-6-chloro-4-[^{3}H]quinazolinone as phosphatase substrate. This compound produces a bright yellow-green-fluorescing precipitate (maximum excitation at 365 nm, maximum emission at 500–580 nm) with standard alkaline phosphatase-mediated immunohistochemical techniques. In detail, 2-μm sections were pre-incubated in aldehyde blocking buffer (PBS, 50 mM glycine) for 15 min and in protein blocking buffer (PBS, 1% BSA) for 30 min. Then the sections were incubated with monoclonal 9E10 mouse anti-c-myc antibodies (hybridoma supernatant, 1:20,000) for 12 h at 4 °C, with rabbit anti-mouse IgG1 (DAKO Diagnostica, Hamburg, Germany) for 30 min at room temperature followed by biotinylated goat anti-rabbit antibody (DAKO Diagnostica) for 30 min at room temperature. All antibodies were diluted in protein-blocking buffer. The next incubation steps were carried out according to the instructions for the ELF-cytological-labelling-kit.

Fluorescence was visualized with a Zeiss Axioplan Epi-fluorescence microscope using the filter set G 365/FT 395/ LP 420.

Scanning electron microscopy

Leaf samples (three to four discs per leaf, one leaf from each of five plants per line) were fixed in a mixture of ethanol/formalin/glacial acetic acid (90:5:5, by vol.) and the water was removed in an ethanol series. After critical-point drying, samples were sputtered with gold and investigated in a Zeiss DSM 962.

Estimation of scFv accumulation in leaves

Total soluble proteins were extracted from leaf discs of transgenic RA, ME and UF plants and control plants by homogenisation and boiling in SDS sample buffer (Sambrook et al. 1989).

The amount of scFv protein in the extracts was estimated by Western blot analysis according to Conrad et al. (1998).

Detection of scFv protein in SP plants was impossible in whole-leaf extracts, so material enriched for epidermal fragments, by the blender method as described by Raschke and Hedrich (1989), was used. Pieces of laminar tissue were cut from between the midrib and secondary veins of fully expanded leaves and chopped in ice-cold water in a blender (Homogenisator 4; Edmund Bühler, Tübingen, Germany) for 30 s at 30,000 rpm. The epidermal fragments were collected by passing through a 200-μm mesh, washed, and the homogenisation step was repeated. After sieving and washing again the material consisted of epidermal fragments contaminated by small pieces of vascular tissue. This was ground with sand in liquid nitrogen for several minutes and lysis of the guard cells checked by microscopic examination. The powder was mixed with 2× SDS sample buffer [72 mM Tris-HCl (pH 6.8), 5% β-mercaptoethanol, 2% SDS, 0.2% bromophenol blue, 10% glycerol] and boiled for 5 min. The extracts were cleared by centrifugation and used for Western blot analysis. RA and UF9 lines with expression of scFv below 1% TSP were selected for direct physiological comparison with SP and ME plants.

Estimation of ABA content and calculation of free ABA

Extracts from ME, RA, SP, UF and WT plants were made from leaf discs, taken shortly after gas-exchange measurements from leaves next to those used for the transpiration measurement.

During the ABA-treatment experiment, leaf discs from 6- and 9-week-old plants (ABA- and water-treated) were harvested before spraying. The leaf material was frozen in liquid nitrogen, powdered and homogenised in 1 ml of 80% acetone. The extraction was performed at 4 °C for 24 h by shaking in the dark. After centrifugation the supernatant was used for ABA measurement by competitive enzyme-linked immunosorbent assay (ELISA), as described by Artsaenko et al. (1995).

The resulting values for total ABA in ng g^{-1} fresh weight were converted into nM considering the molar mass of ABA and assuming that 1 g fresh weight corresponds to 1 ml leaf volume.

The dissociation constant K_d (1.5×10^{-9} M) of the anti-ABA scFv antibody isolated from tobacco leaves for free ABA was evaluated by competition ELISA of affinity-purified scFv protein (Artsaenko et al. 1995).

Calculation of free ABA content was done based on the equation according to Neri et al. (1996).

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

The theoretically free ABA content results from subtracting the concentration of scFv×ABA complex from the total amount of ABA. For these calculations the density of the leaves was assumed to be the same as water.

Gas-exchange measurements

Leaf segments were enclosed in a standard MK-022/A leaf chamber connected to a GK-022 climate unit (Heinz Walz, Effeltrich, Germany). Different CO_2 concentrations were obtained by mixing CO_2 and CO_2-free gas at varying ratios as described elsewhere (Peisker et al. 1998). The water vapour concentration of the air entering the leaf chamber was held constant with a cold trap (KF 18/2; Walz). The CO_2 and water vapour concentrations of gases entering and leaving the leaf chamber were measured with two BINOS 1000 gas analysers (Rosemount, Hanau, Germany) operated in the absolute mode with nearly identical calibration curves. For compensation of remaining small differences in the sensitivity (span) of gas analysers, one of them was adjusted to the other one in each case before steady-state gas-exchange rates were measured. Gas flow rates through the leaf chamber and the reference channel were regulated by mass-flow controllers (1259 C; MKS Instruments). Leaf temperature was determined with a thermocouple and held constant at 25.0 ± 0.2 °C throughout the experiments. Illumination was provided by a light unit (LA-4; Walz) equipped with a 75-W quartz filament tube and a cold light reflector. The PPFD at the leaf surface was varied by neutral density filters and measured with an internal sensor calibrated against the quantum sensor of a LI 189

quantum/radiometer/photometer (LI-COR, Lincoln, Neb., USA). Transpiration rate and stomatal conductance were calculated according to von Caemmerer and Farquhar (1981).

Steady-state values of transpiration rate and stomatal conductance were determined with attached leaves first at a constant PPFD of 370 μmol m^{-2} s^{-1} and various levels of ambient CO_2 concentration between 600 and 25 μmol mol^{-1}. Thereafter, measurements were made at an ambient CO_2 concentration of about 330 μmol mol^{-1} and various PPFDs between 650 and 25 μmol m^{-2} s^{-1}. The mentioned relatively low level of the highest PPFD was chosen because in the anti-ABA scFv transgenic plants excessive losses of water by transpiration were to be avoided. When the effect of feeding ABA was studied, excised leaves were provided with water through the petioles and exposed to 330 μmol mol^{-1} CO_2 and a PPFD of 370 μmol m^{-2} s^{-1} until the steady state was attained. After replacing the water with 50 μM ABA solution the time course of stomatal conductance was followed.

Results

Design of constructs, plant transformation and selection of transgenic plants

The expression cassettes used in this investigation are illustrated in Fig. 1. *Nicotiana tabacum* plants were transformed by *Agrobacterium tumefaciens*, and plants expressing the anti-ABA scFv genes were selected for further work by Western blot analysis. Plants transformed with the mesophyll-cell-specific construct and the guard-cell-specific construct were designated ME and SP, respectively. RA plants and plants that produce anti-oxazolone scFv antibodies, designated in this report UF9 plants, have been described elsewhere (Artsaenko et al. 1995). For the present study, RA lines expressing relatively low levels of scFv (RA 41/16 and RA 51/7) were chosen for comparison with ME and SP plants. The high-producer lines RA 27 and UF9 were used for ABA-treatment experiments.

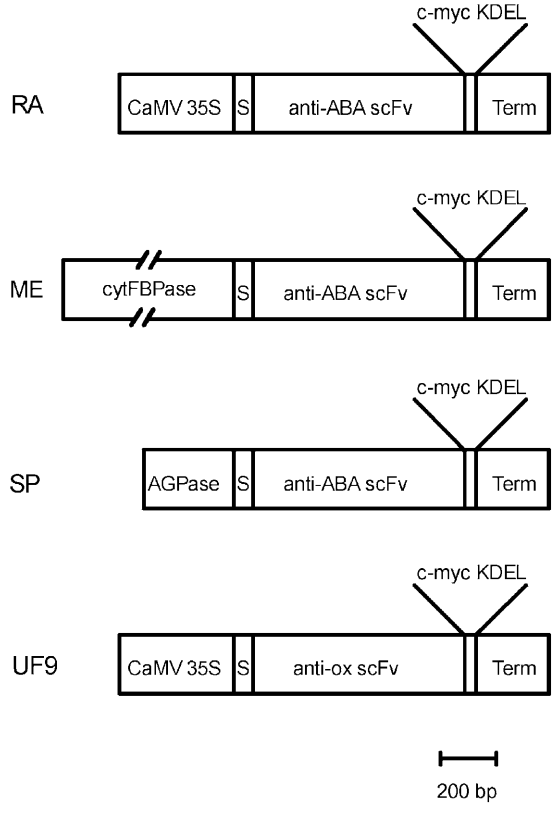

Fig. 1 Expression cassettes designed for ubiquitous or cell-type-specific accumulation of scFv antibodies. Transcriptional control sequences: *CaMV 35S* CaMV 35S promoter, *cytFBPase* cytFBPase promoter, *AGPase* AGPase promoter, *Term* transcriptional terminator. Coding sequences: *S* legumin signal peptide, *scFv*, anti-ABA single-chain Fv, *c-myc KDEL* c-myc epitope tag with KDEL endoplasmic retention signal. The 1.7-kb cytFBPase promoter is not drawn to scale

Immunohistochemical localisation of anti-ABA scFv antibodies

Immunohistochemical localisation of the scFv accumulation using the 9E10 anti-c-myc antibody (Evan et al. 1985) showed that expression was as expected for the promoters used (Fig. 2). ME plants accumulated scFv protein exclusively in mesophyll cells in older leaves while scFv protein was undetectable in the young leaves used for immunohistochemical localisation. A similar expression pattern has already been described for transformants expressing the *uidA* reporter gene under the control of the cytFBPase promoter (Ebneth 1996) and was also found by Western blot analysis of the ME plants (data not shown). In SP plants, scFv was detected in guard cells as soon as they developed. In RA plants, scFv protein was detected in all leaf cell types. The intensity of signals resulting from scFv accumulation in the guard cells of low-expressing RA plants, selected for comparison with SP plants (see *Materials and methods*), was comparable to the intensity of signals in the guard cells of SP plants (Fig. 2).

Phenotypes of transgenic plants

The SP and ME plants were indistinguishable from WT control plants regenerated from leaf discs and grown under similar conditions in the greenhouse. To compare the phenotypes caused by the different expression patterns, plants were grown from seeds of the transformants in a phytochamber under controlled conditions with 95% humidity. High humidity was chosen because RA plants wilt so much under conditions with lower humidity that they are severely morphologically abnormal (Artsaenko 1996).

Initial experiments showed that only stomatal conductances of RA plants did not respond to changes in CO_2 concentration and irradiance while ME, SP and UF9 plants were similar to WT plants (data not shown). We further investigated this by measuring stomatal conductance in the light and then in the dark after switching off the lamp. Reduction in stomatal conductance was monitored and the results compared. Figure 3a shows typical

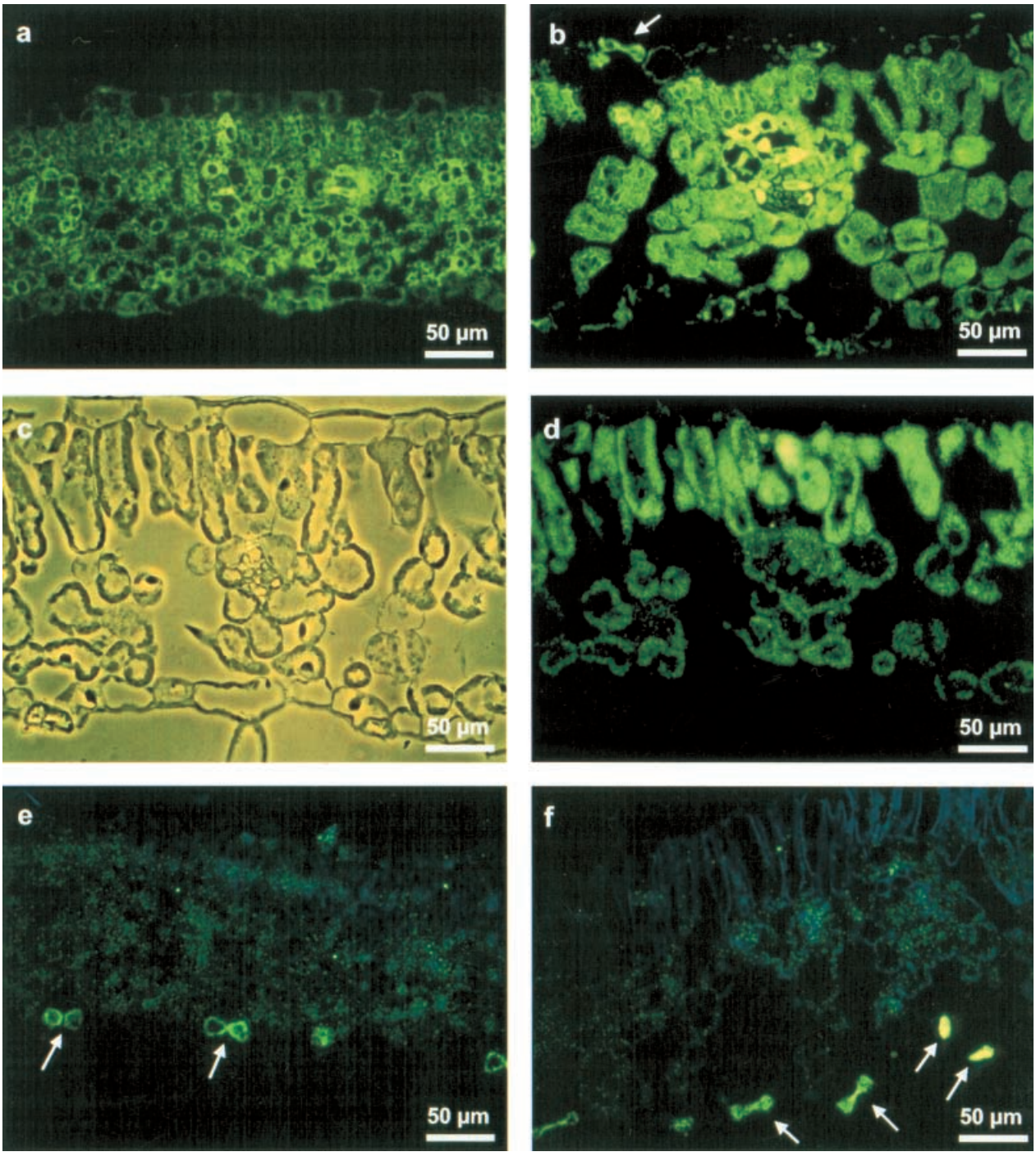

Fig. 2a–f Immunohistochemical localization of anti-ABA scFv antibodies in leaves of tobacco (*Nicotiana tabacum*). ScFv protein in young (**a, e**) or mature (**b, d, f**) leaves from RA (**a, b**), ME (**d**), and SP (**e, f**) plants was detected with the 9E10 anti-c-myc antibody as described in experimental procedures; scFv protein was not detected in young leaves from ME plants (not shown). Expression was ubiquitous in RA plants, restricted to mesophyll cells in ME plants and restricted to guard cells in SP plants. Signals in guard cells were similar in intensity to those in RA and SP plants (*arrowed* in **b**, **e** and **f**). Integrity of the leaf sections was seen by differential interference contrast microscopy (shown for the ME section: **c** corresponds to **d**). The blue colour visible is caused by autofluorescence at 365 nm (**e, f**)

results with respect to the rate of closure. Plants investigated were grown under identical conditions at the same time in the phytochamber (see *Materials and methods*). RA plants did not close their stomata in response to darkening while ME, SP and UF9 plants closed their stomata at rates similar to those of WT plants.

Comparison of the initial stomatal conductances under light conditions (before turning off the lamp) from these experiments shows that among the transgenic plants tested only RA plants had substantially higher stomatal conductances than WT plants (Fig. 3b). In addition, ABA

levels were measured, as these were previously found to be higher in RA plants than in WT plants (Artsaenko et al. 1995). Figure 3c shows that only RA plants were substantially different from WT plants in this respect.

We found no evidence for any phenotypic effects caused by the guard-cell-specific accumulation of anti-ABA scFv antibodies (SP plants) in spite of the fact that RA plants, which accumulate anti-ABA scFv throughout the plant, have unresponsive guard cells.

Normalisation of immunomodulated RA plants by long-term spraying with ABA

We tested if the wilty phenotype could be normalised by long-term spraying with ABA solution during growth and development of the anti-ABA transgenic plants. Wild-type plants and anti-oxazolone transgenic plants were included in this experiment. ScFv contents and ABA concentrations were monitored, and stomatal conductance and its dependence on CO_2 concentration and light intensity were measured at the fifth week of the ABA-treatment time. The results of the experiment presented here were confirmed by five similar repeated experiments (data not shown).

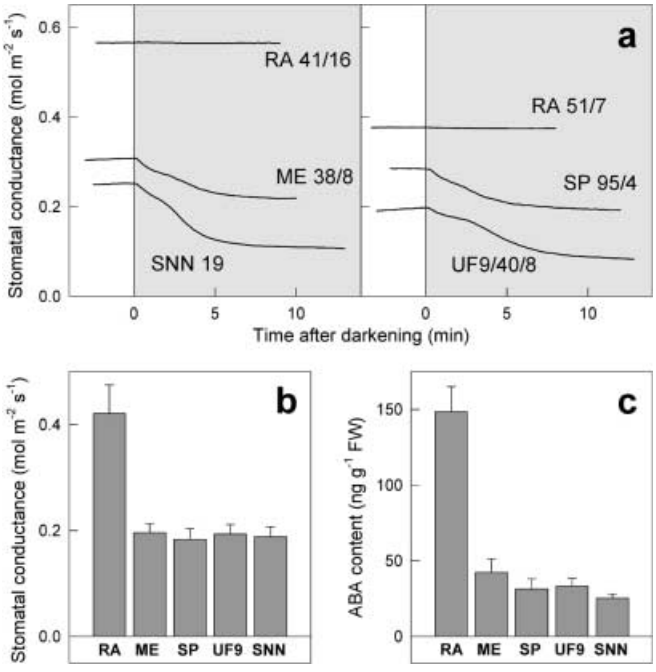

Fig. 3 **a** Typical examples for the time course of changes in stomatal conductance after darkening leaves from RA, ME, SP, UF9 and WT (*SNN*) tobacco plants. Measurements were performed at a PPFD of 705 ± 11 µmol m^{-2} s^{-1}, ambient CO_2 concentration of 135 ± 7 µmol mol^{-1}, and leaf temperature of 25.0 ± 0.1 °C. Water vapour pressure deficit varied between 1.0 and 1.5 kPa. **b** Steady-state mean values and standard errors of stomatal conductance in leaves from various types of transgenic (RA, number of plants $n = 10$; ME, $n = 12$; SP, $n = 14$; UF9, $n = 10$) and WT (*SNN*, $n = 6$) tobacco plants. **c** Leaf ABA levels in leaves from transgenic (RA, number of plants $n = 9$; ME, $n = 12$; SP, $n = 14$; UF9, $n = 10$) and WT (*SNN*, $n = 5$) tobacco plants

Morphology of guard cells

SEM pictures of the leaf surface of the WT and transgenic (RA) tobacco leaves are shown in Fig. 4. The overall size of the stomata was the same in WT and RA leaves. Unlike the WT, stomata of the RA 27 leaves showed an abnormal morphology. Three different leaves from five RA 27 plants were investigated. In a 1-cm^2 area of each leaf, asymmetrical stomata with one guard

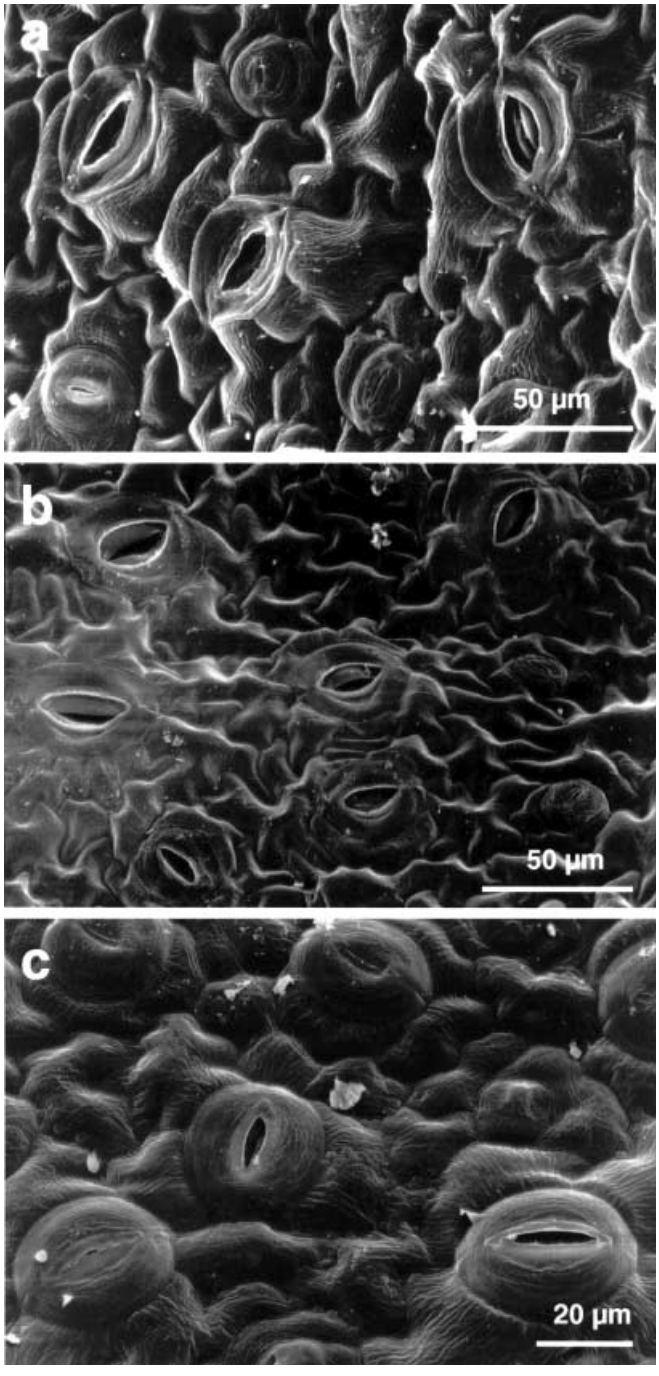

Fig. 4 SEM micrographs of the leaf surfaces of transgenic anti-ABA scFv tobacco plants (RA 27, **a**), transgenic plants (RA 27) that had been sprayed with ABA for six weeks (**b**) and WT plants (**c**)

cell substantially smaller than the other were detected. On an area of 0.128 mm^2, 19 fully developed guard cells were clearly visible, and 14 stomata showed this abnormal morphology. Very often the central opening between guard cells was triangular. Such stomata seemed to be locked open; closure was likely not possible. In controls (three leaves of SNN plants treated with water), no such malformed guard cells could be detected, as evaluated in a 1-cm^2 area for each leaf. When RA 27 plants (Fig. 4b) were treated over their whole growth period with ABA, leaves developed normal, functional stomata.

Additionally, it was tested if ABA treatment can restore WT morphology in differentiated RA guard cells. In vitro RA 27 plants were transferred to soil and grown for 2 weeks without treatment. When the first adult leaves had developed, the plants were sprayed daily with 50 μM ABA over a period of 2 weeks. Leaves already present before ABA treatment showed the abnormal guard cell morphology mentioned above. A remodeling by ABA was not observed. Only guard cells of leaves newly developed during ABA-spraying generally showed a normal WT morphology.

Short-term treatment of transgenic RA and WT tobacco leaves with ABA via petioles

Excised leaves of WT and transgenic RA 27 plants were fed with 50 μM ABA solution via the petioles. In WT plants, this treatment induced stomatal closure, which started between 10 and 20 min after application. This was found in both WT plants derived from in vitro culture (Fig. 5a) as well as in WT plants grown from seeds (data not shown). Transgenic plants (line UF9/38, grown from seeds) accumulating an scFv antibody against oxazolone to high concentrations in the leaves (Fiedler et al. 1997) were used as additional controls to exclude unspecific effects of such proteins on stomatal closure. Plants of line UF9/38 behaved similarly to WT plants and closed their stomata around 10 min after application of ABA (Fig. 5a). In contrast, application of ABA to anti-ABA scFv transgenic plants via the petioles did not bring about stomatal closure (Fig. 5a).

These results show that the application of ABA via the petioles cannot complement the specific phenotype of anti-ABA transgenic plants. Furthermore, the long-term-treated plants were also subjected to short-term treatment with ABA via the petioles at week 5 (Fig. 5b).

Total ABA content during the long-term treatment with ABA

Water-treated anti-ABA transgenic plants showed total ABA contents (free ABA and ABA attached to the antibody) in the range 541–1165 nM at week 6 and 2512–2928 nM at week 9 (Table 1). The ABA level was 10- (week 6) to 16-fold (week 9) higher than in leaves of

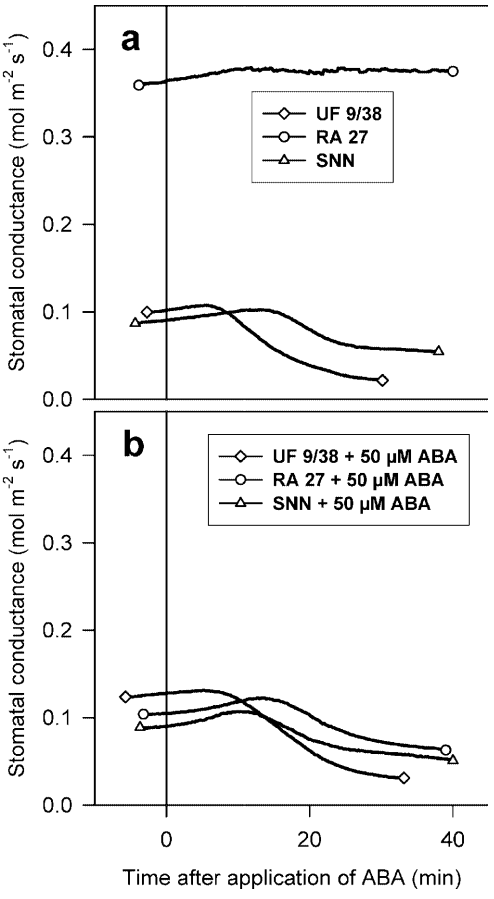

Fig. 5a, b Changes in stomatal conductance after application of 50 μM ABA solution via the petioles of detached leaves of tobacco. WT plants (*SNN*) and transgenic plants that expressed anti-ABA scFv antibodies (in vitro clone RA 27) were obtained from in vitro culture, whereas plants with antibodies against oxazolone (line UF9/38) were grown from seeds. Plants were sprayed daily during cultivation either with water (**a**) or with 50 μM ABA solution (**b**). Data from one plant of each line are shown. Identical results were obtained in several parallel experiments

WT tobacco plants. This effect has been reported in an earlier study (Artsaenko et al. 1995).

ABA-treated transgenic RA plants contained total ABA amounts in the range 2478–3152 nM at week 6 and 2096–4336 nM at week 9. Compared with WT leaves sprayed daily with ABA, the ABA level was similar at week 6 and 6-fold lower at week 9.

In transgenic plants sprayed with ABA the ABA content increased considerably during development. In contrast, in WT plants the content of total ABA increased during the first 3 weeks of treatment and decreased after 6 weeks of treatment.

Effects of ABA treatment on the CO_2 and light dependence of stomatal conductance

RA plants accumulating an scFv antibody to high concentrations in the ER show much higher values of stomatal conductance than WT plants (Artsaenko et al.

Table 1 Effects of various ABA concentrations and treatment times on the ABA content, CO_2 dependence and stomatal conductance of 12 individual anti-ABA scFv transgenic and WT tobacco (*Nicotiana tabacum*) plants (see also Fig. 6)

Plant No	Line	ABA treatment (μM)	scFv (% TSP)	Total ABA content (nM)		Theoretically free ABA (nM)		CO_2 dependence	Stomatal conductance (mol m^{-2} s^{-1})
		Weeks 3–9		Week 6	Week 9	Week 6	Week 9		
1	RA 27	0	4.8	1165	2928	0.1	0.2	–	0.312
2	RA 27	0	4.5	677	2838	0.1	0.3	–	0.392
3	RA 27	0	5.1	541	2512	0	0.2	–	0.350
4	SNN	0	0	91	68	91	69	+	0.033
5	SNN	0	0	102	144	102	144	+	0.032
6	SNN	0	0	83	288	83	298	+	0.030
7	RA 27	50	2.7	2478	4336	0.4	0.9	+	0.080
8	RA 27	50	2.4	3102	3095	0.5	0.5	+	0.273
9	RA 27	50	4.7	2282	3859	0.2	0.3	+	0.043
10	RA 27	50	4.7	3152	2096	0.3	0.2	+	0.041
11	SNN	50	0	2758	511	2758	511	+	0.034
12	SNN	50	0	3178	636	3178	636	+	0.034

1995). This physiological behaviour was not changed by long-term treatment with water (Fig. 6). As in the earlier study, the known effect of CO_2 on stomatal conductance was observed in the WT but not in the transgenic plants (Fig. 6). The measurements shown in Fig. 6 were performed for all individual plants ($n=7$ for RA 27, $n=5$ for SNN) included in the treatment experiment with similar results (Table 1). In addition, the expression of the anti-ABA scFv antibody also gave rise to a suppression of the light dependence of stomatal conductance (Fig. 6). Maximal stomatal aperture was probably observed in RA plants; therefore, they could not respond to an additional stimulus. However, daily treatment of leaves with ABA solution during the whole period of cultivation resulted in a complete recovery of the CO_2 dependence as well as of the light dependence of stomatal conductance in the transgenic plants.

Daily spraying of leaves with ABA solution during the whole cultivation period did not influence the behaviour of WT plants to which ABA had been applied via petioles and also did not change the pattern obtained in anti-oxazolone transgenic plants. However, in the anti-ABA transgenic leaves sprayed with ABA solution, stomatal closure could be induced by application of ABA solution via the petioles (Fig. 5b). Spraying with ABA during plant development could normalise the specific phenotype of immunomodulated plants.

Calculated free ABA

Anti-ABA immunomodulated plants, water-treated as well as ABA-treated, contain levels of theoretically free ABA, not bound by the antibody, in the range 0–0.9 nM (Table 1). In spite of the long-term treatment with applied ABA, almost the whole amount of leaf ABA is bound by the antibody. Theoretically, the scFv protein accumulated in the ER could bind even higher amounts of ABA, resulting from long-term treatment, nearly completely. Interestingly, normalisation was observed even in such cases (Table 1, plants 7–10).

Discussion

Transgenic tobacco RA plants accumulated anti-ABA scFv antibodies to high concentrations in the ER. Expression was driven by the ubiquitous CaMV 35S promoter, resulting in high expression in all cell types throughout the leaves. The recombinant antibodies were retained in the ER due to the C-terminal ER retention signal KDEL (Artsaenko et al. 1995). They show high-affinity binding to free ABA, as has been shown by the determination of the affinity constant by competitive ELISA (Artsaenko et al. 1995; Phillips et al. 1997).

All transgenic tobacco lines expressing different levels of anti-ABA scFv from 0.5 to 6.8% TSP in the ER show a wilty phenotype (Artsaenko et al. 1995, 1999).

The in vitro clone RA 27 was selected because of the stable expression of the transgene in all individual plants included in the experiment (Table 1). Total ABA content was measured in all individual plants, too. Therefore, free ABA was calculated for each plant in the experiment and could be related to the individual physiological behaviour. This wilty phenotype has been characterised by physiological measurements. Typical features are generally high stomatal conductance (Artsaenko et al. 1995, 1999; Table 1), loss of CO_2 dependence of stomatal width (Artsaenko et al. 1995; Fig. 6a) and loss of light dependence of stomatal closure (Fig. 6b), including the absence of any response to darkening (Fig. 3a). These observations indicate that the transgenic plants were unable to modify the aperture of their stomata.

Scanning electron microscopy showed that, compared with SNN plants, there were morphological changes to stomata of RA plants that could prevent regular closure (Fig. 4a, c). The plants with malformed

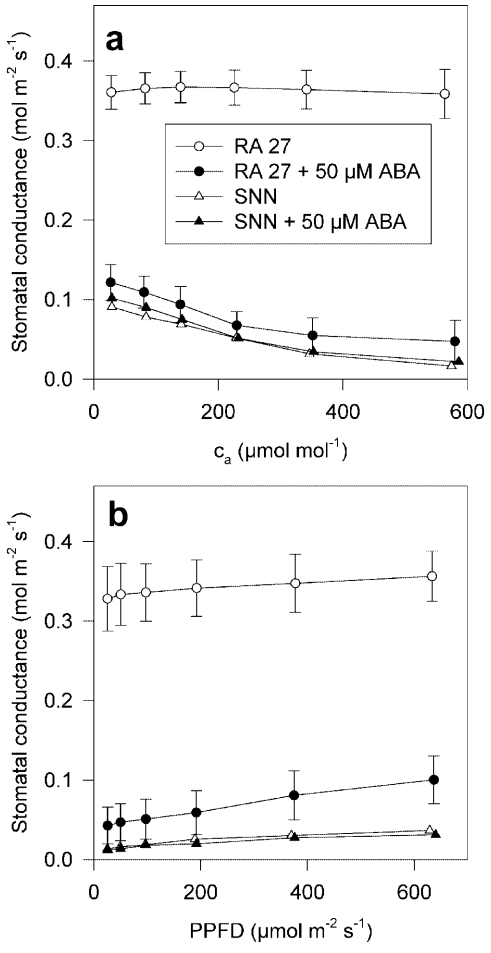

Fig. 6 The influence of ambient CO_2 concentration c_a (**a**) and photon flux density (**b**) on steady-state levels of stomatal conductance in attached leaves of tobacco. WT plants (*SNN*) or transgenic plants that expressed an anti-ABA scFv antibody (in vitro clone RA 27) were sprayed daily during cultivation in a phytochamber (95% humidity, temperature 18 °C, 16-h daylength, PPFD = 245 µmol m^{-2} s^{-1}) either with water or with 50 µM ABA solution. The gas exchange of one leaf from three different plants of each line (RA 27, RA 27 + ABA, SNN, SNN + ABA) was studied. Mean values ± SD are presented for the leaves of transgenic plants (RA 27 and RA 27 + ABA). The SD values of SNN plants are not given because they were smaller than those of transgenic plants

guard cells had been cultivated as in vitro clones and under 95% humidity in a phytochamber (see *Material and methods*). Water stress during the cultivation could, therefore, be ruled out.

During the growth and development of stomata in developing and growing leaves of RA plants, intracellular ABA distribution should be changed compared with WT plants. High concentrations of anti-ABA scFv antibody in the ER can provide an ABA-sink in this compartment because the protonated form of ABA can easily permeate biomembranes (Hartung and Slovik 1991). The accumulation of ABA bound by antibodies located in the ER could cause a deficiency of the phytohormone in other cellular compartments, provided ABA synthesis and metabolism remains unaffected. In addition, the biological activity of ABA could be inactivated by antibody binding itself, as has been suggested by comparison of the structure-function relations in the ABA molecule (Walton 1983) and the epitope specificity of the parental antibody of the scFv (Walker-Simmons et al. 1991; Artsaenko et al. 1999). Although the physical structure and the subcellular distribution of hypothetical ABA receptors are not known, the cytoplasmic perception of ABA molecules has been discussed (Allan and Trewavas 1994). In RA plants, normal development of functioning stomata seemed to be prevented by one of the possible mechanisms mentioned above.

The RA-specific morphological features of stomata could be completely restored by long-term treatment with ABA (Fig. 4b). Calculations of free ABA concentrations indicate that, theoretically, even in long-term treated plants, nearly all available ABA in the leaves could be bound by the antibody in the ER (Table 1).

Despite these theoretical considerations based on the calculations of bound and free ABA, the spraying treatment enables the normal development of guard cells. We conclude that ABA synthesis and/or ABA metabolism may also be affected in the transgenic plants. In WT plants the content of ABA increased during the first 3 weeks of treatment and decreased after 6 weeks. This could be explained by the induction of ABA-degrading enzymes by high ABA amounts. In the transgenic plants the ABA is bound by the antibody and protected from degradation by accumulation in the ER, where the scFv is retained.

Thus, filling up the level of free ABA in the leaf compartments may be retarded or even prevented. Spraying seems to be much more effective at saturating the compartments with free ABA. It should also be mentioned that the first step of oxidative ABA degradation, the hydroxylation of the 8′-methyl group, is a P450 cytochrome-dependent process (Cutler and Krochko 1999). This enzyme system is located in the membranes of cytosolic vesicles. The incorporation of high amounts of ABA antibody into the vesicle may affect the ABA-hydroxylation activity.

Further evidence that stomata of RA plants have lost their ability to modify their aperture comes from the physiological experiments. ABA was introduced into excised leaves via the petioles to overcome the ABA binding in the ER caused by the antibody. No closure of stomata in the RA plants could be induced in these experiments (Fig. 5). Daily long-term treatment of transgenic RA plants during the growth period (6–8 weeks), however, normalised the physiological behaviour completely. Even the inducibility of stomatal closure by ABA treatment via petioles was restored (Fig. 5a, b; Fig. 6). The expression of the recombinant antibody exclusively in guard cells by a specific promoter resulted in physiologically normal transgenic plants (Fig. 3).

Furthermore, the cell-specific expression of the anti-ABA antibody in mesophyll cells driven by the cytFB-Pase promoter also resulted in transgenic tobacco plants showing normal stomatal behaviour (Fig. 3). This

promoter is mainly active in source leaves (Ebneth 1996). We could not detect any scFv expression by immunohistochemical analysis of young leaves (data not shown), as shown for the CaMV 35S promoter or the guard-cell-specific promoter (Fig. 2). This shows that only expression in all cells early in leaf development could provide sufficient recombinant antibody to substantially reduce the level of free ABA in compartments of developing stomata.

Altogether these results show that ABA is necessary not only for the movement of functional stomata, but also for their development. At present, little is known about the role of ABA in guard cell differentiation and development. In long-term fluridone-treated *Vicia faba* plants the appearance of defective guard cells probably lacking a functional closing mechanism, and undeveloped stomata could be observed (Popova and Riddle 1996). A further hint comes from the investigation of the heterophyllous semi-aquatic plant *Potamogeton nodosus*. Application of 1 μM ABA for 4 h induced entirely submerged plants to produce leaves that developed guard cells that would normally only have been formed at the water surface. Leaves already present prior to the ABA treatment did not response to ABA (Gee and Anderson 1998). Conversion of differentiated RA guard cells with abnormal morphology to WT (e.g. by cell wall remodeling) by ABA could not be observed. Only guard cells newly developed during the long-term ABA treatment exhibited WT morphology. Altogether our results show that ABA seems to act in guard cell differentiation and/or development.

Changes in distribution of ABA in the cells cause structural features during the development of stomata that prevent normal function. Stomatal differentiation is a specific process that causes the development of specialised cells with unique morphological and functional properties. The recombinant antibody is expressed in all cells, but only the specific stomatal development is influenced. Further experiments will show the biochemical and molecular nature of these structural and physiological alterations.

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