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Over-expression of plant 14-3-3 proteins in tobacco: enhancement of the plasmalemma K^+ conductance of mesophyll cells

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Abstract Two cDNA clones encoding 14-3-3 homologous proteins were isolated from *Vicia faba*. Deduced amino acid sequences share different degrees of homology with other plant 14-3-3 proteins. Both clones, under the control of the CaMV 35S promoter, were transformed into tobacco plants. Immunoblotting showed three different forms of ca. 31, 34, and 37 kDa, indicating a covalent modification of the expressed 14-3-3 proteins. These forms were mainly present in the microsomal fraction. Patch-clamp studies of mesophyll protoplasts of the transformants revealed a strongly enhanced K^+ conductance compared to the wild type. This indicates the involvement of 14-3-3 proteins in ion channel regulation, presumably by modulating kinase activities or binding the channel.

Key words: 14-3-3 Protein; *Vicia faba* cotyledon; Tobacco transformation; Covalent modification; Ion channel activation; Signal transduction

1. Introduction

The 14-3-3 proteins (14-3-3s) constitute a family of highly homologous, ubiquitous proteins that were first discovered as abundant proteins in the brain (reviewed in [1,2]). Since then, they have been found in a wide range of eukaryotic organisms including yeast and plants (reviewed in [3]). In mammalian cells seven different isoforms have been identified (reviewed in [1]). 14-3-3s have been found to be involved in a number of different biological activities. The first reported function of 14-3-3s was the activation of neuronal tyrosine and tryptophan hydroxylases dependent on their prior phosphorylation [4,5]. 14-3-3s also bind and regulate protein kinase C (PKC). Both inhibition and activation of PKC have been reported [6,7]. More recently, 14-3-3s have been found to bind a number of proteins involved in signal transduction (reviewed in [8]). These activities include binding and activation of the Raf-1 kinase (e.g. [9,10]).

Studies of 14-3-3s in plants comprised the cloning of several isoforms in spinach and *Oenothera* [11], pea [12], maize [13], *Arabidopsis* [14,15], and other plant species. Phylogenetic analyses indicated an early and clear separation of the plant and animal lineages [3,16]. Therefore, some functions of 14-3-3s might be limited to either plants or animals. At least one finding on 14-3-3 function in plants supports this conclusion: 14-3-3s have been found to be involved in the binding of the fungal phytotoxin fusicoccin at the plasma membrane (e.g. [17]). Furthermore, 14-3-3 homologues in *Arabidopsis* were detected as a component of the protein–DNA complex occurring at the G-box region of certain plant promoters [18].

In the present paper we report on the characterization of two plant 14-3-3 clones isolated from cotyledons of *Vicia faba*. When these clones were over-expressed in transformed tobacco plants, three different specific products were observed on immunoblots. Using the patch-clamp technique we observed a strong activation of K^+ channels in the plasmalemma of mesophyll protoplasts of the transformed plants. This finding indicates the participation of 14-3-3s in K^+ channel regulation, a function which has not yet been reported for 14-3-3s.

2. Materials and methods

2.1. Isolation and characterization of cDNA clones

Two degenerate oligonucleotides (GTNGCNTAYAARAAYGT and RTTRTCNCKNARNARYTGCATDAT) were deduced from the highly conserved peptide sequences VAYKNV and IMQLLRDN, respectively. These oligonucleotides were used in RT-PCR (Perkin-Elmer *rTth*-Kit) with total RNA isolated from developing cotyledons of *Vicia faba*. The PCR products were used to screen a *V. faba* cotyledon cDNA library kindly provided by H. Bäumlein (Gatersleben). Hybridizing clones were subjected to DNA sequence analysis using the A.L.F-DNA-Sequencer (Pharmacia). For subcloning, inserts were amplified by PCR using oligonucleotides adding appropriate restriction sites to the ends. These fragments were inserted into pBluescribe (Stratagene) to verify the DNA sequence before using them for further experiments.

2.2. Plant transformation and analysis of expression

Fragments comprising the coding region of Vf1433a and -b were placed under the control of the CaMV 35S promoter and stably transformed into tobacco plants (*Nicotiana tabacum* Samsun NN) by using *Agrobacterium tumefaciens* as described previously [19]. Callus tissue was induced on MS medium (Sigma) containing 2 mg l^{-1} 2,4-dichlorophenoxy-acetic acid (2,4-D).

Proteins were extracted from plant material in SDS sample buffer (50 mM Tris-HCl, 2% SDS, 50 mM DTT, 10% glycerol, 0.1% bromophenol blue, pH 6.8) at ca. 250 mg (fresh weight) ml^{-1}, separated by SDS-PAGE, and transferred to nitrocellulose membranes (Amersham) by electroblotting [20]. Protein levels in plant extracts were estimated by using the Bio-Rad Protein Assay Kit with BSA (Serva) as standard. For this, extracts were prepared from leaves ground under liquid nitrogen with 50 mM Tris-HCl, pH 7.5, 1% Triton X-100.

For differential centrifugation, fresh leaves from plants grown in the greenhouse were ground in an ice-cold mortar in 2 volumes of 50 mM HEPES-KOH, pH 7.5, 2 mM EDTA, 0.5 M sucrose, and 1.6 mg ml^{-1}

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This work has been dedicated to K. Müntz in honor of his 65th birthday.

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'Complete' anti-protease mixture (Boehringer Mannheim). The homogenate was squeezed through nylon tissue and the filtrate was centrifuged at 4°C consecutively for 5 min at 1000×*g* (pellet P1), for 10 min at 4000×*g* (P2), for 30 min at 10000×*g* (P3), and for 1 h at 150000×*g* (P4) to yield fractions mainly containing (according to standard centrifugation protocols) nuclei and plastids (P1), plastids (P2), mitochondria (P3), and microsomes/membranes (P4). Pellets recovered from 3.5 g leaf tissue were resuspended in 400 μl of SDS sample buffer and used for immunoblot analysis. The supernatant (10 ml) after the 150000×*g* centrifugation was mixed with an equal volume of SDS sample buffer and used as the soluble fraction.

2.3. Isolation of recombinant Vf1433a and immunological procedures

Recombinant Vf1433a was isolated from *E. coli* strain DH5α harboring the pQE60 plasmid (Qiagen) with the Vf1433a encoding insert. For the production of antigen, total bacterial lysate was separated by SDS-PAGE. The band corresponding to recombinant Vf1433a was excised from the gel and used after homogenization for the generation of antibodies in rabbits and as marker on immunoblots. Rabbits were immunised according to a standard protocol by three injections with intervals of 4 weeks. Crude serum was purified by ammonium sulfate precipitation (50%) and dialysis against 10 mM phosphate buffer, pH 7.2, 150 mM NaCl (PBS). Blotted membranes were treated with a 1:500 dilution of this antibody preparation for 4 h. After washing they were incubated for 30 min with secondary antibodies coupled to alkaline phosphatase (Sigma, Deisenhofen, Germany) (dilution 1:10000), and stained with the chromogenic substrates 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium chloride (Boehringer Mannheim).

2.4. Patch-clamp measurements

Protoplasts were isolated from leaves of tobacco plants grown in vitro (glass vessels ca. 13 cm high) by incubation for overnight in a solution containing mannitol (500 mOsm), 2 mg ml^{-1} cellulase (Onozuka R10), 960 μg ml^{-1} Driselase, and 320 μg ml^{-1} Macerozym R10 (all from Serva), pH 5.8. The bath solution contained 30 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM K_2H- and KH_2PO_4 buffer, 10 mM 2-[*N*-morpholino]-ethanesulfonic acid/1,3-bis[tris(hydroxymethyl) methylamino]-propane [MES/BTP, Sigma], pH 5.8, adjusted to 500 mOsm with mannitol) and was continuously exchanged during electrical recordings using a peristaltic pump. The membrane potential and currents across the entire surface of the plasmalemma were measured in the whole-cell configuration using standard patch-clamp techniques [21] at room temperature (ca. 22°C) by using polished patch pipettes made from thin-walled Kimax-51 glass capillaries (Kimble, Toledo, OH). The pipette solution contained 180 mM KCl, 6.7 mM EGTA, 2 mM $MgCl_2$, 3.5 mM $CaCl_2$, 4 mM MgATP (Sigma), 10 mM MES/BTP, pH 7.2, adjusted to 550 mOsm with mannitol. The relationship between whole-cell currents and membrane potentials was determined from steady-state currents induced by 1.8-s voltage pulses. The membrane potential, E_m, was held at −50 mV (V_H), pulsed to the indicated potentials and then returned to V_H. This pulse protocol was repeated in increments of 20 mV at intervals of 5 s. The membrane potential at zero net current, the reversal potential (E_{rev}), was determined from tail currents using a double-pulse protocol [22]. By means of E_{rev} the membrane conductance was identified as K^+ conductance which could be ascribed to K^+ channel activity by the application of the K^+ channel blockers $BaCl_2$ (4 mM) and CsCl (5 mM). Patch-clamp recordings were performed and low-pass-filtered with an Axopatch-1D amplifier (Axon Instruments, Foster City, CA). The software package pCLAMP 5.5 (Axon) was used for the generation of sequences of test voltage potentials, data recording and data storage.

3. Results and discussion

3.1. Isolation and sequence analysis of three clones encoding plant 14-3-3 proteins

Degenerate primers corresponding to the conserved peptide sequences VAYKNV occurring close to the N-terminus and IMQLLRDN located close to the C-terminus were used in RT-PCR reactions with total RNA isolated from immature cotyledons of *V. faba*. DNA sequence analysis of some cloned PCR products revealed that 14-3-3-specific DNA fragments were obtained (data not shown). Products from PCR reactions were directly used for screening of a *V. faba* cotyledon cDNA library. A saturation screen yielded dozens of signals. Analysis of some clones yielded one full-length clone encoding a 14-3-3 homologue. This clone (Vf1433a) was used to re-screen other clones selected from the primary screen under more stringent conditions. DNA sequencing of clones not hybridizing under these conditions yielded a second, different *V. faba* 14-3-3 full-length clone (Vf1433b). Further extensive sequencing of cDNA clones revealed a considerable micro-heterogeneity (data not shown) indicating the existence of a 14-3-3 multigene family. Sequencing of PCR fragments obtained with RNA from roots of *V. faba* yielded another (incomplete) 14-3-3 sequence (Vf1433c) differing from the two cDNA clones.

A comparison of the deduced amino acid sequences of the three *V. faba* 14-3-3 clones showed that they contain all the functional domains described so far for animal and plant isoforms (Fig. 1). This indicates that the isoforms may have similar or overlapping functions and might be able to substitute each other in the different functions. This is supported by the finding that one single plant isoform was shown to activate animal PKC, tryptophan hydroxylase, exoenzyme S, as well as to be phosphorylated and to bind calcium, all properties known from diverse animal isoforms [23,24]. In addition, at least 4 of the 6 known 14-3-3 isoforms from *Arabidopsis* were found to complement the lethal disruption of the two yeast genes encoding 14-3-3s [25].

The degrees of homology of the three *V. faba* 14-3-3 sequences to other plant 14-3-3 sequences are given in Table 1. These data show that Vf1433a shares a very high degree of homology (around 90%) with almost all of the plant 14-3-3 sequences investigated in this comparison. The only exception is a 14-3-3 from tobacco [26] with only 78% homology. The homology of the incomplete Vf1433c sequence to the investigated group is only slightly lower (ca. 85%). It shares the same low homology with the tobacco 14-3-3 as Vf1433a. Since it shares a relatively high homology to RCI2, it might correspond to this cold-inducible 14-3-3 from *Arabidopsis* [15]. The degree of homology of Vf1433b to the other plant 14-3-3 sequences is clearly lower (ca. 79–80%). But, contrary to

Table 1
Homologies of the deduced peptide sequences of the three *V. faba* 14-3-3 clones shared with other plant 14-3-3s

		Vf1433a	Vf1433b	Vf1433c
*Ara*GF14w	[14]	93.8	80.3	85.5
*Ara*GF14y	[14]	90.6	81.2	86.0
*Ara*GF14c	[14]	91.9	79.7	86.5
*Ara*GF14f	[14]	90.1	77.7	82.5
*Ara*GF14n	[14]	90.3	78.1	84.0
*Ara*RCI2	[15]	86.5	78.5	91.0
Oenothera	[11]	93.9	78.9	85.0
Hordeum	[35]	88.9	79.7	86.5
Pisum	[12]	96.0	80.0	87.0
Nicotiana1	[a]	91.8	79.5	82.0
Nicotiana2	[26]	77.7	86.5	77.0
Vf1433a		–	80.5	86.0
Vf1433b		80.5	–	80.0
Vf1433c		86.0	80.0	–

Values were determined by using the PALIGN program of the PCGENE sequence analysis package (Intelligentics) and are given in % (identity plus similarity).

[a]W.H. Shen and C. Gigot, unpublished, EMBL accession number Y11212.

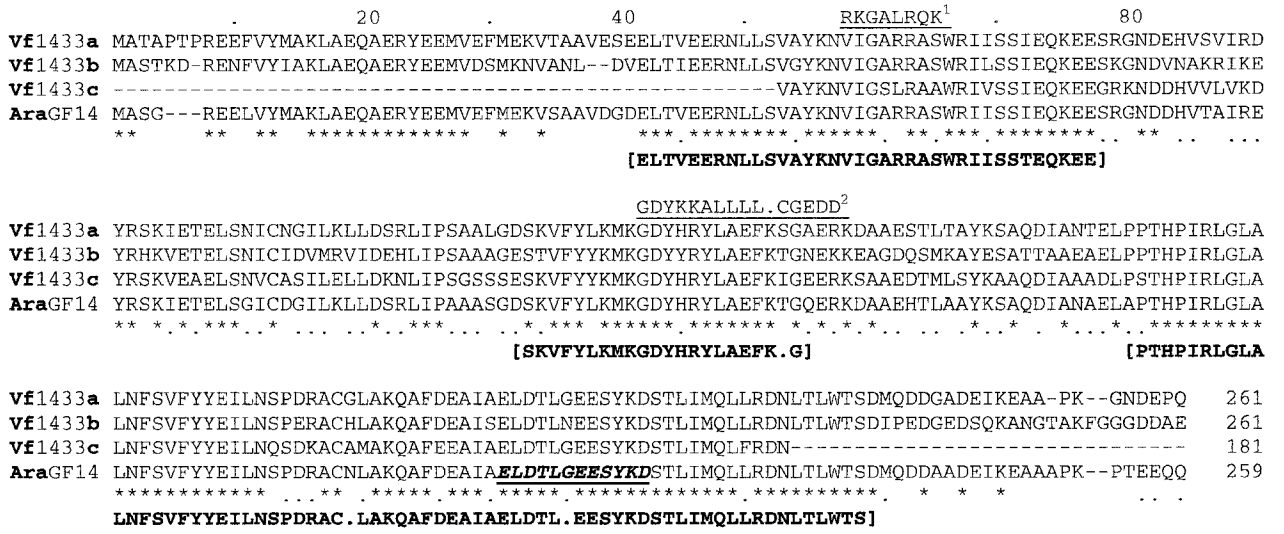

Fig. 1. Alignment of the deduced amino acid sequences of Vf1433a, Vf1433b, Vf1433c, and GF14ω from *Arabidopsis* [14] by using the CLUSTAL program from the PCGENE sequence analysis package (Intelligenetics). In addition, the consensus sequences of the conserved domains A, B, and C [16] are shown in brackets. The putative Ca^{2+} binding domain [24] is underlined in the GF14w sequence. The two short peptide sequences in the upper line indicate regions of homology of 14-3-3 to the pseudosubstrate sequence of PKC (peptide 1) [36] and to the annexin C-terminal region known to bind PKC (peptide 2) [1,37].

Vf1433a and -c, it shares relatively high homology (87%) to the above mentioned tobacco 14-3-3 and might therefore correspond to this specific NaCl-inducible protein [26].

3.2. *Over-expression of V. faba 14-3-3s in transformed tobacco plants*

The two full-length cDNA clones Vf1433a and -b were placed under the control of the CaMV 35S promoter and transformed into tobacco plants. The transformed tobacco plants grew normally and did not show an altered phenotype. When total SDS extracts from leaves of transformed plants were analyzed by immunoblotting, three specific bands of ca. 31, 34, and 37 kDa were observed on the immunoblots (Fig. 2a). This banding pattern was identical between Vf1433a and -b. The 31 kDa product corresponded to the size expected from the deduced amino acid sequence and co-migrated with recombinant Vf1433a protein obtained from expression in *E. coli*. This result indicates that 14-3-3s can be modified in plant cells by a yet unknown covalent attachment resulting in a considerably slower migration in SDS gels. Post-translational modifications such as phosphorylation [24,27] and N-acetylation [28] have been reported for 14-3-3s. However, both the data of these works and our own preliminary results from labelling experiments with ^{32}P (data not shown) demonstrated that these modifications did not markedly affect the migration in SDS gels. Covalent binding of arachidonic acid to human 14-3-3 has been shown in a work demonstrating phospholipase A_2 activity of 14-3-3 [29]. However, work of a different group did not confirm this phospholipase A_2 activity [30]. In addition, the gel migration of the observed acyl-enzyme (14-3-3) intermediate [29] was not different from unmodified 14-3-3, although a low amount of an additional unidentified product migrating at 40 kDa was observed.

When leaf extracts from untransformed tobacco plants were applied at levels comparable to that used for the detection of the 14-3-3 over-expression products no background was observed (see lane Lwt, Fig. 2a). However, when large amounts of total leaf extract from untransformed tobacco were applied, a weak banding pattern similar to that found in the transformants was observed (see lane Lwt2, Fig. 2b). Since the occurrence of 14-3-3s in tobacco homologous to the *V. faba* 14-3-3s used in the present study has been reported (see Table 1) cross-reactivity of the antibodies to different 14-3-3 isoforms of tobacco can not be excluded. On the other hand, this result could indicate that the modifications observed with the over-expressed *V. faba* 14-3-3s are not only due to the over-expression of the heterologous proteins in tobacco, but that 14-3-3s are normally modified in leaves by a yet unknown covalent attachment.

The expression level of the transformed 14-3-3s was similar in different individual transformants (data not shown). Plants t1433/32 and t1433/35 with a relatively high expression level of Vf1433a and -b, respectively, were chosen for further experiments. The protein level was estimated from the band intensities on the immunoblots shown in Fig. 2. The samples applied in lanes La and Lb correspond to ca. 25 μg protein. By comparison to the band in lane R (30 ng of recombinant Vf1433a), we estimated the total amount of the three 14-3-3 products in lanes La and Lb to ca. 100 ng. From this, the expression level of the transformed 14-3-3s in plants t1433/32 and t1433/35 was estimated to ca. 0.3–0.5% of total leaf protein.

Cell fractionation by differential centrifugation (Fig. 2b) revealed that all three forms were predominantly present in the microsomal fraction (150 000 × *g* pellet, P4). The soluble fraction (lane S) contained only the 31 kDa product and a low amount of this form could also be detected in P1 and P2. This indicates that the 31 kDa form may be present in different compartments of the cell such as nuclei, mitochondria, and cytosol. This is in accordance to the many different functions and locations reported for 14-3-3s (see Section 1) including the binding to the G-box transcription complex in the nucleus [18], the action as mitochondrial import stimulation factor [31], and the binding and regulation of cytosolic nitrate reductase [32]. The two additional upper bands were not present in callus tissue from the transformants (Fig. 2a). Contrary to

leaves, this tissue did not contain green chloroplasts. However, neither of the larger products was found in the leaf chloroplast fraction (pellets P1 and P2, Fig. 2b).

3.3. Patch-clamp analysis reveals enhanced plasmalemma K^+ conductance by 14-3-3 over-expression

Similar to other ion channels, the activity of several K^+ channels in the plasmalemma of plant cells is regulated by their phosphorylation/dephosphorylation. This has been also proved for tobacco mesophyll cell protoplasts [33]. Since 14-3-3s are known to modulate the activity of kinases, they may participate in ion channel regulation. Consequently, we compared the K^+ conductance of mesophyll protoplasts of tobacco wild-type and 14-3-3-over-expressing plants by using the patch-clamp technique. The determination of the reversal potential and the application of the K^+ channel blockers, Ba^{2+} and Cs^+, indicated that the obtained I-V curves reflect the K^+ channel activity (data not shown).

The systematical analysis of as many as 45 protoplasts from wild-type plants showed a considerable variability of the specific membrane conductance. Despite of this variance, which is typical for tobacco mesophyll protoplasts, the outward currents could be always induced to much higher levels than the inward currents (see Fig. 3). The comparison of maximum outward current density (at +90 mV) of wild-type and 14-3-3-transformed protoplasts indicates that in transformed protoplasts the K^+-outward rectifier is more activated than in the control. Considering only measurements which strictly fulfilled certain criteria (serial resistance <20 MΩ, resting potential <-20 mV, seal resistance >1 GΩ, membrane capacitance ca. 0.9 μF cm^{-2}) the current densities observed in protoplasts from the transformed plants, t1433/32 and t1433/35 (expressing Vf1433a and -b, respectively), were significantly enhanced (72.4% and 70.0%, respectively) over those of the wild type. At +90 mV the current densities in the two transformants reached 60.5 ± 11.2 pA pF^{-1} ($n=26$) and 59.7 ± 12.3 pA pF^{-1} ($n=27$), respectively, versus 35.1 ± 9.8 pA pF^{-1} ($n=22$) in the wild type (see also Fig. 3). The tobacco transformant, K11-12, expressing a myc-tagged rab-homologous protein from field bean (J. Thielmann and G. Saalbach, unpublished), constructed by means of the same vector as the 14-3-3-over-expressing plants, displayed similar current densities as the wild type. This control experiment showed that the transformation itself did not cause enhanced plasmalemma conductance. Though the inward currents are relatively small in tobacco mesophyll protoplasts they may also be enhanced in the transformed plants (see Fig. 3), however the data did not yield statistical significance.

Taken together, these results clearly show that the over-expression of 14-3-3s causes an enhanced K^+ conductance of the plasmalemma in tobacco mesophyll cells. This elevated conductance could be generated by different mechanisms. In view of the known regulatory role of 14-3-3s, an activation of K^+ channels e.g. via the activation of protein kinases as reported for animal cells (see [2,8]) is more likely than the direct modulation of channel conductance or of the open probability. However, a direct interaction of the 14-3-3s with the phosphorylated K^+ channels cannot be excluded, since 14-3-3s have been found to bind phosphoserine residues [34]. As 14-3-3-over-expressing plants display a normal phenotype, it seems too early to speculate about the physiological consequences of this new finding.

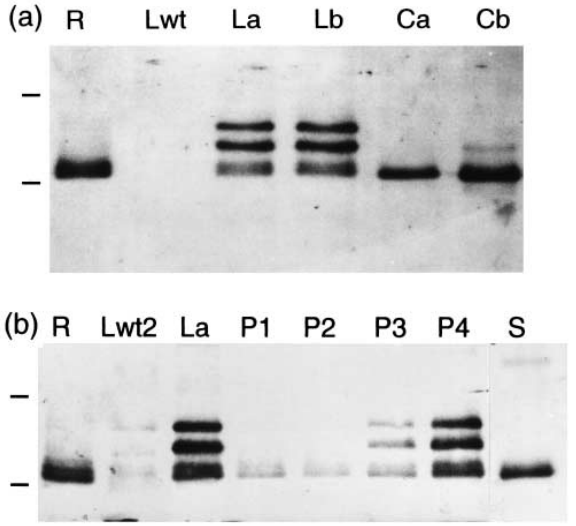

Fig. 2. Immunoblots demonstrating the expression and intracellular localization of Vf1433a and -b under the control of the CaMV 35S promoter in transformed tobacco plants. a: Immunoblot showing the expression of Vf1433a and -b in leaves and callus tissue of the transformed tobacco plants t1433/32 and t1433/35, respectively. R: recombinant Vf1433a (30 ng) purified from *E. coli*; other lanes: total SDS extracts from 1 mg (fresh weight) of leaves of untransformed tobacco plants (Lwt), of transformed tobacco plants expressing Vf1433a (La) and -b (Lb), and from callus tissue of transformed tobacco plants expressing Vf1433a (Ca) and -b (Cb). Marks at the left indicate the location of molecular mass markers of 30 and 40 kDa. b: Immunoblot showing the distribution in fractions of differential centrifugation of the three 14-3-3 specific products observed in leaves of transformed plants. P1, P2, P3, P4: pellets after 5 min at $1000\times$ *g* (nuclei, plastids), 10 min at $4000\times g$ (plastids), 30 min at $10\,000\times g$ (mitochondria), and 1 h at $150\,000\times g$ (microsomes, membranes), respectively; S: soluble proteins (supernatant after 1 h at $150\,000\times g$). All three 14-3-3 forms are found predominantly in P4. Total leaf extracts from Vf1433a-expressing plants (La) and from untransformed tobacco plants (Lwt2) as well as recombinant Vf1433a (R) were applied for comparison at the left. In lane Lwt2 a 3-fold amount was applied compared to lane Lwt in part (a) of this figure (see also text).

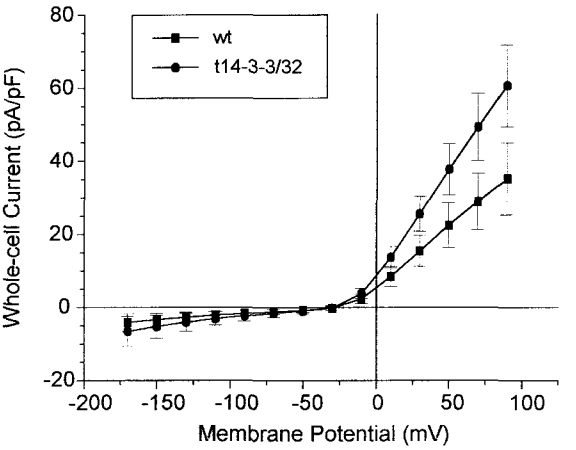

Fig. 3. Averaged steady-state I-V curves of the whole-cell K^+ currents of mesophyll protoplasts from *Nicotiana tabacum*. Comparison between wild-type (wt, $n=22$) and Vf1433a-over-expressing plants t1433/32 ($n=26$). Bars represent the standard deviation. Similar results were obtained with the Vf1433b-over-expressing plants t1433/35 (see text).

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