A plant cation–chloride co-transporter promoting auxin-independent tobacco protoplast division

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Although auxin plays a central role in plant development, little is known about the signal transduction pathways triggered by auxin regulating cell elongation, division and differentiation. We describe the molecular analysis of the mutant tobacco line *axi 4/1*, which was regenerated from an auxin-independent callus created by activation T-DNA tagging. Transcriptional enhancer-mediated deregulated expression of the tagged plant gene *axi 4* uncouples division of *axi 4/1* protoplasts from external auxin stimuli, whereas in untransformed protoplasts expression of *axi 4* and cell division are auxin dependent. *axi 4* encodes a 109 kDa protein with significant homology to a family of electroneutral cation–chloride co-transporters (CCCs). We show that overexpression of AXI 4 or another member of the CCC family, the Na⁺/K⁺/2Cl[−]-co-transporter from shark, triggers auxin-independent growth of tobacco protoplasts. We suggest that Na⁺/K⁺/2Cl[−]-co-transporters may play a role in signalling cell division and that this function is highly conserved between AXI 4 and the shark Na⁺/K⁺/2Cl[−]-co-transporter. We also demonstrate that a C-terminal fragment of AXI 4 is sufficient to promote auxin-independent cell division, showing that the C-termini of CCCs are functional subunits triggering cell division. This may allow a molecular dissection of this process not only in plants but also in animal cells.

Keywords: auxin/bumetanide/cell division/ion co-transporter/T-DNA tagging

Introduction

The phytohormone auxin (indole 3-acetic acid, IAA) is an indispensable regulator of plant growth and development. In combination with other plant growth regulators, it exerts pleiotropic effects *in planta*, including regulation of growth rate, initiation of lateral roots, control of shoot formation and vascular differentiation. In addition, auxin mediates the plant's tropic responses to light and gravity (Davies, 1995). Many of these effects can be attributed to auxin-regulated cell elongation and division. With this in mind, it may be no surprise that auxin is an important component of media used in plant tissue culture. Its presence is normally required to promote protoplast division *in vitro*, and ratios of auxin to cytokinins define shoot

or root organogenesis from undifferentiated callus (Skoog and Miller, 1957; Krikorian, 1995).

The molecular basis of auxin action is an area of intense study. Biochemical and electrophysiological evidence indicates that auxin can bind a variety of plant proteins (reviewed in Jones, 1994) of which one, ABP1 from maize (Hesse *et al.*, 1989), has the characteristics of being a receptor mediating auxin-induced membrane hyperpolarization (Barbier-Brygoo, 1995; Napier and Venis, 1995). Hyperpolarization is thought to reflect the stimulation of proton extrusion from the cytosol via the H⁺-ATPase and acidification of the apoplast (Hager *et al.*, 1991). It has been proposed that this leads to a loosening of non-covalent bonds in the cell wall, allowing cell expansion, an assumption made in the context of the 'acid-growth' theory of auxin action (Rayle and Cleland, 1970, 1992). A consequence of the electrochemical gradient established in this manner is the energization of solute uptake into the cytosol. This is a prerequisite for osmotically driven processes mediating elongation and tropic movements of plant tissues (Michelet and Boutry, 1995). Moreover, as in other eukaryotes, cell size is an essential determinant for cell division in plants (Francis and Halford, 1995). However, identification of volume-regulating signalling pathways in plants remains a major challenge, and how auxin acts with regard to regulating cell volume and division is essentially unknown.

Genetic approaches to dissect the molecular basis of auxin action rely on the creation of mutants modified in their response to auxin (Walden and Lubenow, 1996). For example, *Arabidopsis* populations mutated by ethylmethyl-sulfonate (EMS; Maher and Martindale, 1980; Estelle and Somerville, 1987) or by T-DNA tagging (Feldmann *et al.*, 1994) have been screened by the ability of germinating seedlings to grow on normally toxic levels of auxin. The mutants obtained display an array of phenotypic effects indicative of changes in auxin action or response, including: altered growth, root length, apical dominance, changes in flowering and reduced gravitropism (Lincoln *et al.*, 1990; Pickett *et al.*, 1990; Wilson *et al.*, 1990; Hobbie and Estelle, 1995; Leyser *et al.*, 1996). To date, two *Arabidopsis* genes with a possible role in auxin action have been cloned. *axr 1* encodes a peptide with homology to the ubiquitin-activating enzyme E1, and thus is thought to play a role in protein turnover (Leyser *et al.*, 1993), and the protein encoded by *aux 1* has homology to amino acid permeases, suggesting a role in auxin uptake (Bennett *et al.*, 1996).

We have taken another approach to isolating genes involved in auxin signal transduction. Tobacco protoplasts, under defined culture conditions, have an absolute requirement for exogenously applied auxins and cytokinins for cell division and callus growth (Murashige and Skoog, 1962; Nagata and Takebe, 1970). We have used activation

T-DNA tagging to create tobacco mutants able to form callus in the absence of auxin (Hayashi *et al.*, 1992; Walden *et al.*, 1994a). Activation tagging involves the use of a T-DNA containing multiple transcriptional enhancers so that, following insertion of the T-DNA into the plant genome, expression of flanking plant genes comes under the influence of the enhancers and is deregulated, thus creating dominant mutations, which are independent of externally supplied auxin. The T-DNA tag contains sequences of a bacterial plasmid so that it and flanking plant DNA sequences can be recovered with relative ease by plasmid rescue in bacteria. In this way, we have cloned *axi 1*, a gene encoding a potential transcriptional activator (Hayashi *et al.*, 1992; Walden *et al.*, 1994b; R.Walden, H.Lubenow, M.J.Soto, I.Czaja, C.Schommer and J.Schell, in preparation), *caxi 7*, which encodes a protein with similarity to pathogenesis-related proteins (M.J.Soto, J.Brandle, H.Schaller, I.Czaja, J.Schell and R.Walden, in preparation), and *cyi 1* encoding a small peptide growth factor (Miklashevichs *et al.*, 1997).

Here we describe the characterization of *axi 4/1*, a mutant derived from a T-DNA-tagged auxin-independent tobacco cell line. The protein deduced from the tagged plant gene *axi 4* displays significant sequence and structural homology to a family of electroneutral cation-chloride co-transporters (CCCs). CCC sequences previously have been cloned from a prokaryote, lower eukaryotes and vertebrates including humans (Gillen *et al.*, 1996; Hebert *et al.*, 1996), but this is the first report of a putative CCC from plants. Overexpression of *axi 4* in tobacco leads to auxin-independent cell division and increased resistance of protoplasts towards a specific inhibitor of one class of CCCs, namely $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ -co-transporters. In addition, transient expression of a shark cDNA encoding a $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ -co-transporter in protoplasts also results in auxin-independent and inhibitor-tolerant cell division, underlining functional similarities of AXI 4 and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ -co-transporters. In vertebrate cells, this class of CCCs plays a key role in cell volume regulation and has also been implicated in cell proliferation, suggesting that, by analogy, AXI 4 might be an important factor in a signalling cascade from auxin to cell enlargement and division. In addition, we demonstrate that the C-terminal region of AXI 4 is required and sufficient to promote auxin-independent cell division, suggesting that this region of CCCs is most likely to play a regulatory role in their function.

Results

axi 4/1 protoplasts display auxin-independent cell division in vitro

The tobacco line *axi 4/1* (auxin-independent) was generated by activation T-DNA tagging of SR1 mesophyll protoplasts followed by selection for auxin-independent cell division and callus growth (Hayashi *et al.*, 1992; Walden *et al.*, 1994a, 1995). Plants regenerated from auxin-independent calli were selfed in order to obtain homozygotic lines with respect to the T-DNA insertion. Homozygotic *axi 4/1* plants display no obvious phenotypic differences as compared with untransformed SR1 tobacco. Nevertheless, whereas division of untransformed SR1 protoplasts has an absolute requirement for auxin (Nagata and Takebe, 1970), *axi 4/1* protoplasts divide and form

Fig. 1. Auxin-independent division of *axi 4/1* protoplasts. Protoplasts were isolated from 6-week-old untransformed tobacco (SR1) and *axi 4/1* plants, respectively, and cultured in medium with or without auxin. All media contained cytokinin (0.9 μM kinetin). After embedding, microcalli were cultured for 6 weeks.

microcalli in the absence of exogenously supplied auxin (Figure 1). Thus, activation T-DNA tagging uncouples protoplast division in *axi 4/1* from the effects of auxin, suggesting that the tagged plant gene might code for a protein involved in the auxin response.

Southern analysis of *axi 4/1* genomic DNA digested with either *EcoRI*, *KpnI* or *BamHI* was performed using hybridization probes derived from the hygromycin phosphotransferase (HPT) gene and the enhancer sequences of the T-DNA-tagging vector pPCVICEn4HPT (Walden *et al.*, 1995). These analyses revealed that two T-DNA copies are inserted into the plant genome as a dimer, apparently linked at their right borders (Figure 2A).

Cloning and functional analysis of plant DNA tagged in *axi 4/1*

To rescue the T-DNA together with flanking plant DNA, *axi 4/1* genomic DNA was digested with *BamHI*, followed by self-ligation and transformation into *Escherichia coli*. The *E.coli* origin of replication and the ampicillin resistance gene present within the T-DNA tag allowed recovery of the resultant plasmid p19 in *E.coli*. Plasmid p19 contains a partial T-DNA tag (*oriC*, ampicillin resistance gene, and the HPT marker gene at the left border) plus ~4.7 kb of plant DNA, flanking the left T-DNA border (Figure 2B). The presence of the T-DNA within p19 was confirmed by Southern blots (data not shown). The strategy used in plasmid rescue did not allow the recovery of the enhancer tetramer from the T-DNA tag. In order to restore linkage of the multiple enhancers with the rescued plant DNA, the enhancer tetramer subsequently was religated into the unique *BamHI* restriction site of p19, resulting in p19En4 (Figure 2B).

To test whether the rescued plant DNA was able to confer auxin-independent cell division, we transformed tobacco SR1 mesophyll protoplasts with p19 and p19En4 by polyethylene glycol (PEG)-mediated DNA uptake. Transformants were selected in media containing hygromycin in the presence or absence of auxin (Figure 2C). Protoplasts transfected with p19 containing the HPT gene were hygromycin resistant, but were unable to grow in the absence of auxin. In contrast, protoplasts transfected

Fig. 2. Organization of the T-DNA in *axi 4/1* and functional analysis of rescued plant DNA. (A) Map of the T-DNA and flanking plant DNA sequences in *axi 4/1* derived from Southern blot analysis of genomic *axi 4/1* DNA digested with *Eco*RI (RI), *Bam*HI (B) and *Kpn*I (K), respectively, and hybridized successively with a 35S enhancer and HPT probe (see Materials and methods; data not shown). Open boxes indicate plant DNA, hatched boxes functional elements of the T-DNA [HPT, hygromycin phosphotransferase gene; oriC, *E. coli* origin of replication and amp, ampicillin resistance gene, both derived from pC19H (Marsh *et al.*, 1984)] and arrows 35S enhancer sequences (–427 to –90 bp) cloned as a tetramer (En4; Fritze, 1992). LB and RB indicate left and right border sequences in the T-DNA. (B) Re-isolation of a partial T-DNA together with flanking plant DNA. Plant DNA rescued following *Bam*HI digestion of *axi 4/1* genomic DNA before (plasmid p19) and after recloning of the enhancer tetramer (plasmid p19En4). Sites for relevant restriction enzymes (see deletion analysis of p19En4, Materials and methods) are as shown (C, *Cl*aI; RV, *Eco*RV; X, *Xho*I). The black box indicates the region of plant DNA required for auxin-independent protoplast division defined by deletion analysis of p19En4 (data not shown). (C) Functional analysis of rescued *axi 4/1* plant DNA. Freshly isolated SR1 protoplasts were transfected with a water control, p19 and p19En4, respectively, by PEG-mediated DNA uptake, and cultured in media with different auxin (NAA) and hygromycin (Hyg) combinations as indicated. The photograph was taken 7 weeks after embedding. (D) Southern blot analysis of SR1 and *axi 4/1* genomic DNA. *Eco*RI-digested DNA from SR1 and a homozygotic *axi 4/1* plant were hybridized with the functional genomic *Eco*RI–*Xho*I plant DNA fragment from p19En4.

with p19En4 were not only hygromycin resistant, but also displayed auxin independence. Thus, we can conclude that indeed the gene tagged in *axi 4/1* producing auxin-independent growth has been isolated and that enhancer-mediated overexpression is an absolute requirement to produce the selected phenotype.

Using deletion derivatives of p19En4 in DNA uptake

experiments with SR1 protoplasts, the region of the rescued plant DNA required for auxin-independent cell division was mapped to a 1.8 kb *Eco*RI–*Xho*I fragment of p19En4 (Figure 2B). The plant gene tagged in *axi 4/1* was called *axi 4*.

Southern blot analysis of *axi 4/1* and SR1 genomic DNA was carried out to confirm that *axi 4* is linked to

Fig. 3. Northern blot analysis of SR1 and *axi 4/1* protoplasts. Protoplasts were cultured for 2 days in the presence or absence of auxin as indicated. Equal amounts of poly(A)⁺ RNA were hybridized with the functional genomic *EcoRI*–*XhoI* plant DNA fragment from p19En4.

the T-DNA insertion in *axi 4/1* and to determine the number of *axi 4*-related sequences in the tobacco genome. To do this, SR1 and *axi 4/1* genomic DNA were digested with *EcoRI*, that cleaves neither in the T-DNA nor in the functional genomic *axi 4* fragment (Figure 2A and B), and the membrane was probed with the 1.8 kb *EcoRI*–*XhoI* genomic *axi 4* sequence from p19En4 (Figure 2B). The result indicates that the hybridizing 8 kb fragment in SR1 is shifted to ~20 kb in the tagged mutant. This shift of 12 kb corresponds to the insertion of two T-DNA copies into the *axi 4/1* genome and is therefore consistent with the linkage of *axi 4* and the T-DNA tag in *axi 4/1*. In addition, it appears that in tobacco, *axi 4* is a member of a small gene family comprising probably two or three members (Figure 2D).

***axi 4* is overexpressed in *axi 4/1* protoplasts**

axi 4/1 protoplasts divide *in vitro* in auxin-free medium, whereas SR1 protoplasts have an absolute requirement for auxin. In addition, the rescued genomic *axi 4* sequence confers auxin-independent cell division in transient expression assays, but only when physically linked to the transcriptional enhancers. To investigate the accumulation of the *axi 4* transcript in SR1 and *axi 4/1*, protoplasts were isolated from SR1 and *axi 4/1* plants and cultured for 2 days in the presence or absence of auxin under normal culture conditions. At this timepoint, SR1 protoplasts supplemented with auxin and *axi 4/1* protoplasts in media with or without auxin enter cell division. The proportion of dividing protoplasts was similar in all three samples, except that SR1 protoplasts in auxin-free medium did not divide (data not shown). Poly(A)⁺ RNA was extracted and subjected to Northern analysis using the genomic *axi 4* *EcoRI*–*XhoI* fragment (Figure 2B) as a hybridization probe. *axi 4* transcripts of 3.4 kb are clearly detectable in SR1 and *axi 4/1* protoplasts cultured in medium containing auxin (Figure 3). In the tagged mutant, the presence of the enhancers within the T-DNA tag apparently promote overexpression of *axi 4* in comparison with SR1. In protoplasts cultured in the absence of auxin, *axi 4* transcripts are only detectable in *axi 4/1* protoplasts. Therefore, *axi 4* expression in SR1 protoplasts is auxin dependent,

but is uncoupled from external auxin stimuli in the tagged mutant.

***axi 4* codes for a 109 kDa protein with homology to electroneutral ion co-transporters**

Northern analysis revealed that the *axi 4* transcript is ~3.4 kb (Figure 3), whereas the functional genomic *axi 4* sequence was mapped to a 1.8 kb fragment (Figure 2B). This suggests that a functional, but partial, *axi 4* sequence was rescued in p19. To clone a full-length *axi 4* cDNA, we constructed a cDNA library from SR1 mesophyll protoplasts cultured for 2, 3 and 5 days, respectively, in medium containing auxin. At each timepoint, *axi 4* expression was verified by Northern blot analysis (data not shown). Screening the library with the genomic *axi 4* *EcoRI*–*XhoI* fragment as a hybridization probe resulted in the recovery of a 3.44 kb *axi 4* cDNA 1. Sequencing *axi 4* cDNA 1 revealed an open reading frame starting with the first ATG at nucleotide 268 encoding a 109 kDa protein of 990 amino acids. Database searches with the predicted AXI 4 protein sequence showed significant homology over its complete length to members of a family of electroneutral cation–chloride co-transporters (CCCs).

According to their specificities for the transported ions, CCCs can be grouped into three classes: (i) Na⁺/Cl[−]-co-transporters (NCCs) cloned from flounder, rat, mouse and human (Gamba *et al.*, 1993, 1994; Kunchaparty *et al.*, 1996; Simon *et al.*, 1996a); (ii) Na⁺/K⁺/2Cl[−]-co-transporters (NKCCs) cloned from shark, rat, rabbit, mouse and human (Delpire *et al.*, 1994; Gamba *et al.*, 1994; Payne and Forbush, 1994; Xu *et al.*, 1994; Igarashi *et al.*, 1995; Payne *et al.*, 1995; Simon *et al.*, 1996b); and (iii) K⁺/Cl[−]-co-transporters (KCCs) cloned from human, rabbit and rat (Gillen *et al.*, 1996; Payne *et al.*, 1996).

Other sequences encoding members of the CCC family were derived from the amphibian *Necturus maculosus* (Soybel *et al.*, 1995), from the insect *Manduca sexta* (Reagan, 1995), the nematode *Caenorhabditis elegans* (Wilson *et al.*, 1994), the yeast *Saccharomyces cerevisiae* [E. Dubois, M. El Bakkoury, N. Glansdorff, F. Messenguy, A. Pierard, B. Scherens and F. Vierendeels (1994) Unpublished, EMBL accession No. Z36104] and from the cyanobacterium *Synechococcus* (Cantrell and Bryant, 1987).

CCCs are proposed to be integral proteins of the plasma membrane, with 12 predicted transmembrane helices and hydrophilic N- and C-termini, which probably reside within the cytoplasm (Xu *et al.*, 1994; Payne *et al.*, 1995; D'Andrea *et al.*, 1996). Hydropathy analysis of AXI 4 also revealed the existence of 12 potential membrane-spanning domains flanked by large, mainly hydrophilic, N- and C-termini. An alignment of the AXI 4 protein sequence with a Na⁺/Cl[−]-co-transporter from *Pseudopleuronectes americanus* (winter flounder, flNCC; Gamba *et al.*, 1993), a Na⁺/K⁺/2Cl[−]-co-transporter from *Squalus acanthias* (shark, shNKCC1; Xu *et al.*, 1994) and a K⁺/Cl[−]-co-transporter from rat (rtKCC1; Gillen *et al.*, 1996) reveals that the highest sequence identity between AXI 4 and animal co-transporters is found within the putative transmembrane domains and the predicted intracellular loops (Figure 4). Most of the predicted extracellular loops and the C-termini show less sequence identity and are more variable in length, and the N-termini show only minor conservation. These regions of homology are similar

f1NCC	MEPAFPASSAGVQSQSGPEPGAGQ	EPPPATPLRPVASQSRFQVDLVE	GGGGGDKQKQTAAQPAAAAKDKR	GDGGAAPSPASPAAPAAEPAAAE	47
shNKC1					
rtKCC1					
ntAXI4			MHSQNKAEIDGEDIADDI	NQFPTGVGRKYSEVVAHDVNDASVV	
f1NCC	MELPGDGVHLASYGP	RAPQLAVNGKAPAGGLDGSYYQCY	RD.....GSSVASRGSADPT	GYDQVADPHYDFY	
shNKC1	EAQRGRVNFVDPASDEPLSS.QQ	QPPPPSSASSAHGHQPPSESMNGY	PQNGDTMMSEGLHSSGTGAHHYYD	THNTNYLRTFGHNTIDAVPRIDHY	
rtKCC1	PVDGPRRDYDNL..EGLSWVDYG.	..ERAEREDSDGQGNHRENSPFLS.		PLDASRGNDY	
ntAXI4	EMTSIHPGSSSFPHLEKKVKGV	QPNMASEEESASANNHNGP			93
f1NCC	ANTEVWGRRRR.VRPSLYQLSVDPE	QDDFKPPMYEETAGERMGGDSSEE	EEEEHKEPPPEPP..RFQVQGVMI	RCMLNIWGVILYLRLPWITAGIG	
shNKC1	RHTVAQLGEKL.IRPSLAEL....	HDELDKEPFEDGY...VNGEESPA	EEAVSKHVADNKGVVKFGWIKGVLV	RCMLNIWGVMLFIRLSWVGHAGIG	
rtKCC1	DRNLALFEELDIDRPKVSLLGLV	SYTNLTQGAKEHEE	AESGEGGRRRAAKAPSMGTIMGVYL	PCLQNIQFVILFLRLTWVGTAGLV	
ntAXI4	QRESKL..ELFGFDSLNLGLKSM	TGDTQAPSSPRDG	EDVTITFEQPKPTADKSGTMGVFM	PCLQNIQGIYIYIRFSWVGMAGIG	180
f1NCC	LTWVILLSSFTIGITGLSTSAIAT	NGKVGGGTYFLISRLSGPELGGSI	GLIFAFANAVAVAMHTVGFATVTD	LMRENGVVMVD.....	
shNKC1	LALLVIGTATVTTITGLSTSAITT	NGFVRGGGAYYLLSRLSGPEFGAI	GLIFAFANAVAVAMHTVGFATVTD	LLVEHNALMID.....	
rtKCC1	QALLIVLCCTCTLTATSMIAIT	NGVVPAGGSYFMLSRLSGPEFGAV	GLCFYLTGTFFAAMYIIGAIELIT	YIAPPAATFYPSPGTH.....DM	
ntAXI4	ESLLLVVFCGSCFTLTTVSLSAIAS	NGAMKGGPYLLIGRALGPEVGVSI	GLCFFLGNAGIAGAMYIIGAVETFLN	AV.PSAGIFRETITRVNGTDIAEPI	279
f1NCC	...PINDIRIVGVITVTCLLGISMA	GMWESKAQVLLFVIMVSFVNIYV	GTIIPA.....		
shNKC1	...EMSDIRIGSVTIVLFGISVA	GMWEAKAQIVLLGILLAIIVNFTV	GTIIPA.....		
rtKCC1	SSATLNNMRYVGTIFLTMTLVFV	GKRYVNFASLFLACVLIISILSIYV	GGIKSAFDPVPFVCMGNRTLSRD	QFDICAKTVVVDNETVATRLWTFFC	
ntAXI4	TSPSLHDLDIYIGIVSILLCFVVF	GKMINRVAAPFLVPLFSLLCFV	GIFSAHRDPAV.....		341
f1NCC	SPQKQAKGF	FSYKAEIFANFVPGWRGKEG...		SFGMFSTFFPSTATGILAGANIGD	
shNKC1	N.DKRAKGF	FNYRGEIFSENFVDFRDEG...		FFSVFAIFPPAATGILAGANIGD	
rtKCC1	HSPNLTADESCDYPFLNTEIPGI	PGAAAGVLQENFSLAELKEVEVEK	HGLPSTDTLGLKESLSLVVADIAT	SETVLVGIFFPSTVTGIMAGSNRSGD	
ntAXI4	GI	TGLNLESFKNWSSS...QMTNN	AGIPDPN.....GKIYW	DENALVGLFFPAVTGIMAGSNRSGS	401
f1NCC	LKDPTVAIPRGTMAIFWTTISYLI	ISATIGACVVRDASGELNDTLYSS	SSENCGLACQYRWDFSECIKNNCT	XHGIMNYYQSMLSVSAFAPLISAGI	
shNKC1	LADQLAIPKGLLAILITTVIYAG	AAVSQSGCIVREATGNLTDAL.IPG	TVTNTCTNACKLGNFNSVCCATNK.C	SYGLNMDQVMSLVSGFGLPLIAGI	
rtKCC1	LRDAQKSIPIVGTILAIVTSLVYFS	SVILFGACI.....	EGVVLRDYK	GDGVSRLNVVGTAWPSPWIVVGS	
ntAXI4	LKDPTQSIPIVGTILAIVTSLVYFS	SVILFGACI.....	VSTRDL	LDTRLISAIVAMPPLAIVVGI	462
f1NCC	FGATLSSALACLVAPKVFQCLCKD	QLYPLIGFFGKGKNAEPLRAILL	TYVIAVCFVLAELNTIAPILISNFF	LCSYALINFSCFHASVTNSPGWRPS	
shNKC1	FSATLSSALASLVAPKVFQCLCKD	NIPYGLHVFVSVGKKNPELRGVLV	TYFIFGLFILAEINVTIAPILISNFF	LASYALINFVSVFHASLAKSPGWRPA	
rtKCC1	FFSTCGAGLQSLTAPRLQAIAKD	NIPYGLHVFVSVGKKNPELRGVLV	TALIAELGILIASLDMVAPILSMFF	LMCYLFVNACAVQLLRTFPNWRPR	
ntAXI4	LLSTLGAALQSLTAPRLQAIAKD	DILPVLNYFKVADG.H.EPHVATLF	TAFICIGCVVIGNLDLVSPSTMFY	LVCYAGVNLSSFLDLLDAPSWRPR	560
f1NCC	FRYYSKWLSSLAGVCCVIMFILTIN	WAALIAFGVVFLLGYTLKKPAVN	WGSSVQASSYSMALNQCVGLNQVED	HVKNYRQCCLVL.....TGPPC	
shNKC1	FRYNNMWISLGLLCCGVFMFINN	WAALLTNVIVLALYIVVTYKKPDVN	WGSSTQALTYLNLQHAIRLTGVED	VGLGMRNPNTLVFGFKDQRLIATG	
rtKCC1	FKYVHWALSFLGMSLCLALMFVSSW	YYALVAMVYAGMIYKYIEVQGAKE	WGDGIRGLSLSAARYALLRLEEGEP	HTKKNRPPQLLV.....LKLDELHIV	
ntAXI4	WKRHHWGLSLVGLLCIIVIMFLISW	AFTIVSLALASLYYVVSIRKAGAD	WGDGFSKAYQLALRSRLSGANQV	HPKNWYPIPLVFCRPWGLPENVP	660
f1NCC	CRPALVDLVGLCTKRL.SLMCGHV	VTAGP.....SPVSEHVTWLNQ	RKVSFYRGVVAADLRSGVNMLLQ	AGLGRKPNVLLMGFKDKWGC.DSP	
shNKC1	SRPALLHLVHAFSTNV.GLVVCGHV	HTGPRRLQALKEISTDQAKYQRLV	NKMKAFYAPYAEIDLREGTQFLLOA	VGLGMRNPNTLVFGFKDQRLIATG	
rtKCC1	KYPRLLTFASQL.KAGKGLTVGSV	IQGSFLESYGAAEQTIKNMMEI	EKVKGFCQVVAASKVREGLAHLIQS	CGLGMRHNSVVLGWPGYWRQSEDP	
ntAXI4	HPKLADPANCCKMGRMSIFVSI	IDGDYHERAEDAKACEQLSTYIEY	KQCEGVAEIVVAPNMSEGRFVIGT	MGLGNLKPNIIMRYPEIWRRENLT	759
f1NCC	QAAHHYIGILHDAFLHYGVCVLRV	KEGLDAS.....HP	PQCHVNPGF.....	DGGPESINTVCA	
shNKC1	KDVENYINAIHDAFDYQYGVVIRL	KEGFNISHIQAQELCTSQEKSAPH	KDITVNLHSDADSKPSSKSVSET	NSPAVCQDQKDEEDGKASTQPLLK	
rtKCC1	RAWTFIDTTRCTTAHLALVLPKN	IAFYPSNH.....			
ntAXI4	DIPATFVGIINDCIVANKAVIVKG	LDEWPNEY.....			792
f1NCC	PACVQTSVTSVSMDDPDPQSSVFQ	KKQGGKTTIDYWLSDGGLTLLPY	LLTRRKRWAGCKVRVFGGDTDKKE	EQ.KEEVLALIKKFRGLGHDVEVLP	
shNKC1	KEVDPSVPLNMTDQKLLQASSQFQ	KKQGGKTTIDYWLSDGGLTLLPY	LLTRKKKWKDCKIRVFIGGKINRID	HD.RRTMATLLSKFRIDFSDITVLG	
rtKCC1		ERYLEGHIDVWIVHDGGMMLLFP	LLRQHKVWKKCRMRIFTVAQMDNS	IQMKKDLAIFLYHLRLA.AEVVVE	
ntAXI4		QRQY.GTIDLYWIVRDGGLMLLSQ	LLLTCKSFEGCKIQVFCIAEEDSDA	EGLKADVKKFLYDLRMQ.AEIVTIS	865
f1NCC			DIHQPPQPGNVDFEDS.....VN	RFRLETNPKQSDSDSGPQQQQQEEPW	
shNKC1			DMNTKSKDNITAFEM.....IE	PFRLHEDDKEQAS..EKMKEEPW	
rtKCC1	MHNSDISAYTYERTLMMEQRSQMLR	QMRLTKTERDRQAOLVKDRHSALRL	ESLYSDEDESVTGADKIQMTWTRD	KYMAEPWDPKSHAPDNFRELVIKIP	
ntAXI4	M.....	KSWEAQ	GEQQESVEAFSAAQQ..RVA	SYLEEMKEQAR.RDGTPLADGKPV	914
f1NCC	MITEQDLERNRAKSLRQIRLNEVLQ	VHSREAAALIVITMPVRRGVCPSL	FLAWLDVLSRDLRPPVLLVRGNQEN	VLTIFYCQ	
shNKC1	RITNLELEIYRMKTYRQIRLNEVLQ	ENSGTANLIVMSLPVARKGAVSSAL	YMAWIELSKDL.FPILLVRGNQES	VLTIFYS	
rtKCC1	...DQSNVRRMHTA...VKLNEVIV	TRSHDARLVLLNMPGPKNSEGDN	YMEFLEVLTGL.ERVLLVRGGGRS	VITIYS	
ntAXI4	FVEEQVEKFLYTT...LKLNLMIQ	KYSRMAAVVLVSLPPPPFN.HPASF	YMEYMDLLVENV.PRLLVIRGYHND	VVTLFT 990	

Fig. 4. Alignment of AXI 4 peptide sequence with electroneutral ion co-transporters. AXI 4 residues matching with at least one representative of the CCC family are coloured red, identical residues among animal co-transporters not matching with AXI 4 are coloured blue. Boxes indicate predicted transmembrane domains. f1NCC, Na⁺/Cl⁻-co-transporter from *P.americanus*; shNKC1, Na⁺/K⁺/2Cl⁻-co-transporter from *S.acanthias*; rtKCC1, K⁺/Cl⁻-co-transporter from rat; ntAXI4, tobacco AXI 4. Numbers refer to amino acid residues of AXI 4.

to those derived from sequence and structural comparisons of animal co-transporters (Palfrey and Cossins, 1994; Gillen *et al.*, 1996; Payne *et al.*, 1996). Thus, regions that are well conserved among animal co-transporters are also homologous between AXI 4 and members of the CCC family. Individual sequence alignments of AXI 4 with members of the CCC family revealed significantly higher sequence identity of AXI 4 to KCCs (36–38%) compared with NCCs and NKCCs (27–30%). Nevertheless, defined domains and conclusive structural features conferring ion selectivity to individual co-transporters have yet to be identified. Thus, sequence and structural comparisons of AXI 4 with animal co-transporters alone do not allow us

to conclude whether *axi 4* encodes a plant homologue of one of the known co-transporters, or a new member of the CCC family with different ion specificities.

Functional comparison of AXI 4 and vertebrate CCCs

In addition to their different ion selectivities, CCCs can be grouped according to their affinities for specific inhibitors. Na⁺/Cl⁻-co-transport is generally found to be sensitive to thiazide diuretics like metolazone, but not affected by 'loop' diuretics of the sulfamoylbenzoic acid class, such as furosemide and bumetanide. In contrast, ion transport via both NKCCs and KCCs can be inhibited

Fig. 5. Cell division of SR1 and *axi 4/1* protoplasts at different bumetanide concentrations. Freshly isolated SR1 (open bars) and *axi 4/1* protoplasts (filled bars) were cultured in media containing increasing bumetanide concentrations in the presence of auxin and cytokinin. The proportion of dividing protoplasts was estimated microscopically after 5 days. Bars represent the means of three individual experiments.

selectively by 'loop' diuretics, but is not affected by thiazides (O'Grady *et al.*, 1987; Palfrey and O'Donnell, 1992; Haas, 1994). Bumetanide is a more potent inhibitor of NKCCs than furosemide, and the order of potency is reversed for inhibition of KCCs (Gillen *et al.*, 1996, and references therein). Thiazide and 'loop' diuretics have been used intensively for the characterization of specific ion transport *in situ* and via cloned co-transporters (e.g. Gamba *et al.*, 1993; Xu *et al.*, 1994; Gillen *et al.*, 1996).

CCCs catalyse electroneutral ion transport across the plasma membrane and play a key role in cell volume regulation in vertebrate cells (reviewed in Hoffmann and Simonsen, 1989; Hoffmann and Dunham, 1995). Application of furosemide or bumetanide not only disrupts cell volume control, but also resulted in a significant reduction of mitogen-induced DNA synthesis preventing progression of the cell cycle in cultured cell lines (Panet and Atlan, 1991; Panet *et al.*, 1994; Bussolati *et al.*, 1996). It was proposed that (N)KCCs might not simply be involved in the regulation of cell volume, but might be a component of mitogen-induced signalling pathways regulating cell proliferation (Palfrey and O'Donnell, 1992; McManus and Churchwell, 1994).

To analyse the sensitivities of SR1 and *axi 4/1* protoplasts towards specific inhibitors of CCCs, protoplasts were cultured in media containing auxin and cytokinin plus different concentrations of either metolazone, bumetanide or furosemide. The sensitivities towards the inhibitors were measured as the proportion of dividing cells after 5 days in culture. At this timepoint, ~50% of SR1 and *axi 4/1* protoplasts undergo cell division in inhibitor-free control medium. While all tested inhibitors reduced the proportion of both dividing SR1 and *axi 4/1* protoplasts in a concentration-dependent manner (data not shown), division of SR1 protoplasts was significantly more sensitive towards bumetanide as compared with *axi 4/1* protoplasts (Figure 5). In media containing either 100 or 300 μM bumetanide, the proportion of SR1 protoplasts undergoing cell division was reduced to 58 and 0% of the controls in inhibitor-free medium, respectively, whereas division of *axi 4/1* protoplasts was only affected at bumetanide concentrations >300 μM. Parallel experiments with furosemide revealed only a slightly increased resistance of *axi 4/1* compared with SR1 protoplasts (80 and 60% cell division of the controls in 100 μM furosemide, respectively), and killing curves with metolazone produced no difference in sensitivity between SR1 and *axi 4/1*

Fig. 6. Expression of *axi 4* and a $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ -co-transporter in transfected SR1 protoplasts. *axi 4* cDNA 1 in pRT106 and a cDNA encoding a $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ -co-transporter from *S. acanthias* (shNKCC1) in pRT105 were transiently expressed in SR1 protoplasts. pRT106 was included as a control. Protoplasts were cultured in medium with auxin (NAA) and media lacking auxin or containing 300 μM bumetanide (Bum). All media contained cytokinin. Microcalli were embedded in agarose and photographed 7 weeks later.

protoplasts (68% cell division compared with the controls in 100 μM metolazone, data not shown). Thus, division of tobacco protoplasts can be blocked efficiently using specific inhibitors of animal CCCs. Overexpression of *axi 4* in *axi 4/1* as compared with SR1 protoplasts (Figure 3) correlates with a weakly enhanced resistance towards furosemide and a strongly enhanced bumetanide resistance of *axi 4/1* as compared with SR1 protoplasts. These inhibitor sensitivities of AXI 4 resemble qualitatively the sensitivities of animal NKCCs, suggesting that both proteins might have similar functions in plant and animal cells.

To compare the function of AXI 4 and a NKCC in tobacco protoplasts, *axi 4* cDNA 1 and the cDNA encoding the shark co-transporter (shNKCC1; Xu *et al.*, 1994) were cloned in pRT plant expression vectors (Töpfer *et al.*, 1993) under the control of the 35S RNA promoter. SR1 protoplasts were transfected with the resulting constructs pRTaxi 4 and pRTshNKCC 1, respectively, and cultured in media with or without auxin as well as medium containing auxin plus 300 μM bumetanide. As observed with *axi 4*-overexpressing *axi 4/1* protoplasts (Figure 5), transient expression of both *axi 4* and shNKCC1 in transfected SR1 protoplasts also confers auxin independence and increased bumetanide tolerance (Figure 6). Thus, functional similarities of AXI 4 appear to be conserved with an animal NKCC in tobacco protoplasts.

The AXI 4 C-terminus is sufficient to confer auxin-independent protoplast division *in vitro*

Overexpression of the genomic plant DNA rescued in plasmid p19 is sufficient to promote auxin-independent protoplast division. However, *axi 4* transcripts were found to be 3.4 kb, corresponding to the size of the *axi 4* cDNA 1, whereas the functional part of the genomic *axi 4* sequence rescued with p19 was only 1.8 kb. This led us to suspect that a portion of *axi 4* would be sufficient to produce auxin-independent growth. To define this

Fig. 7. Functional analysis of *axi 4* deletions. **(A)** Schematic representation of the genomic plant DNA rescued with p19, *axi 4* cDNA 1 and two deletion derivatives thereof, pRT108axi4C-term and pRT105axi4ΔC-term (see Materials and methods). Relevant vector sequences are represented by open, *axi 4* sequence by filled boxes (35S, CaMV 35S RNA promoter; poly A, CaMV polyadenylation signal). The non-coding genomic plant DNA is shown as thick lines. LB and HPT represent T-DNA sequences of plasmid p19 as described in Figure 2. Vertical black bars indicate *axi 4* segments encoding putative transmembrane domains, and numbers refer to amino acid residues of the deduced AXI 4 protein. The positions of some restriction sites are indicated using the same abbreviations as in Figure 2 (N, *NotI*). Stippled vertical lines embrace the region of complete sequence identity between the genomic *axi 4* sequence and the *axi 4* cDNA 1. **(B)** Transient expression of the *axi 4* cDNA 1 constructs and a pRT106 vector control in SR1 protoplasts. Media compositions were as described in Figure 6. The photograph was taken 5 weeks after embedding in agarose.

functional part of AXI 4, the genomic plant DNA rescued with p19 was fully sequenced (data not shown). Sequence comparison revealed complete identity of a 1279 bp genomic *axi 4* sequence close to the left border of the T-DNA in p19 to the 3' end of the *axi 4* cDNA 1 (Figure 7A), indicating that cDNA 1 corresponds to the *axi 4* gene tagged in *axi 4/1*. The 2.36 kb 5' part of the rescued genomic sequence shows no homology to the *axi 4* cDNA 1, suggesting that this genomic sequence might comprise an intron. This is supported by the finding of a putative intron–exon border formed by the sequence TGCAGCA, which is in agreement with the proposed plant intron–exon consensus TGYAGGT (Simpson and Filipowicz, 1996; nucleotides of the exon underlined). Thus, the T-DNA tag inserted ~1 kb downstream of the *axi 4* gene in *axi 4/1*, and apparently only one exon of *axi 4* encoding the amino acid residues 633–990 of the AXI 4 C-terminus was rescued in p19.

Deletion of the genomic *axi 4* sequence downstream of the *XhoI* site in p19 (AXI 4 residues 940–990) resulted in a functional *axi 4* fragment (Figure 2B). In contrast, extending the deletion of the *axi 4* sequence to the *Clal* site in p19En4 (residues 798–990) abolishes function of the rescued genomic DNA (Figure 2B). This suggests that deregulated expression of a defined fraction of the genomic *axi 4* exon encoding the amino acid residues 633–939 of AXI 4 is sufficient to promote auxin-independent proto-

plast division *in vitro*. In p19En4, the multiple enhancers could activate transcription, possibly from a cryptic site in the plasmid, with translation being initiated from the first in-frame ATG in the *axi 4* exon (AXI 4 residue 671). To confirm the biological activity of the AXI 4 C-terminus, the 833 bp *HindIII*–*XhoI* fragment of the *axi 4* cDNA 1, which is almost equivalent to the functional part of the genomic *axi 4* exon (residues 633–939) and encodes the AXI 4 residues 663–939, was cloned as a fusion to the translation start site of the plant expression vector pRT108 (Töpfer *et al.*, 1993), resulting in pRT108axi4C-term (Figure 7A). In parallel, a C-terminal deletion of AXI 4 encoding the residues 1–750 was cloned into pRT105 (Töpfer *et al.*, 1993), giving pRT105axi4ΔC-term (Figure 7A). Both constructs were transfected into SR1 protoplasts followed by selection for either auxin-independent or bumetanide-resistant cell division. The result confirms that overexpression of an *axi 4* fragment, encoding residues 663–939 of the AXI 4 C-terminus, is sufficient to confer auxin independence (Figure 7B). However, overexpression of the AXI 4 C-terminus does not result in bumetanide resistance. In contrast, pRT105axi4ΔC-term produces bumetanide resistance but not auxin independence.

These deletion analyses of the *axi 4* cDNA 1 show that auxin independence and bumetanide tolerance clearly can be allocated to distinct domains of AXI 4, which can be physically separated from each other without affecting the

specific function. The 2.5 kb 5' part of *axi 4* contained in pRT105*axi4*ΔC-term encodes the N-terminus and the putative transmembrane domains of AXI 4. The transmembrane domains and the connecting loops of animal CCCs form the catalytic domain involved in ion translocation and the proposed bumetanide-binding site (Haas, 1994). Overexpression of these AXI 4 domains confers bumetanide resistance, but not auxin independence. The 833 bp *HindIII*–*XhoI* *axi 4* fragment contained in pRT108*axi4*C-term encodes a large portion of the AXI 4 C-terminus. For animal CCCs, a distinct function of the cytosolic C-termini has yet to be described, although it has been proposed that the C-termini might be regulatory elements of CCCs (Palfrey and Cossins, 1994). Our analyses indicate that the AXI 4 C-terminus alone has biological activity in our growth assays and promotes auxin-independent cell division.

Discussion

AXI 4 is the first description of an electroneutral CCC in plants. To date, the most studied CCCs are those from vertebrates where two isoforms of both the furosemide-sensitive K^+/Cl^- -co-transporters (KCC1 and KCC2) and the bumetanide-sensitive $Na^+/K^+/2Cl^-$ -co-transporters (NKCC1 and NKCC2), along with the metolazone-sensitive Na^+/Cl^- -co-transporter (NCC) have been cloned. In secretory and absorptive epithelia, CCCs promote vectorial salt transport (Kaplan *et al.*, 1996). In non-epithelial cells, they play a key role in cell volume regulation (Hoffmann and Simonsen, 1989). Based on physiological studies (Hoffmann and Simonsen, 1989, and references therein) and structural analyses (Gamba *et al.*, 1993; Xu *et al.*, 1994; Gillen *et al.*, 1996), it has been proposed that CCCs are integral proteins of the plasma membrane, a notion that has been confirmed by immunolocalization of human NKCC (D'Andrea *et al.*, 1996). Our inhibitor studies with untransformed tobacco protoplasts clearly demonstrate that metolazone, furosemide and bumetanide reduce cell division in a concentration-dependent manner. On the other hand, *axi 4* overexpression increases the tolerance of protoplasts to bumetanide (Figure 5). This, coupled with a similar result obtained by the overexpression of the shark NKCC1 cDNA in protoplasts (Figure 6), leads us to suspect that AXI 4 is a bumetanide-sensitive CCC. In vertebrates, bumetanide-sensitive CCCs generally promote the coupled uptake of 1 Na^+ :1 K^+ :2 Cl^- into the cell, fuelled by ATP hydrolysis by the Na^+/K^+ pump which creates an inwardly directed electrochemical Na^+ gradient across the plasma membrane. This represents the driving force for Na^+ -coupled co-transport, accumulating Cl^- and K^+ within the cell and allowing cell expansion. To our knowledge, only one plant ion transporter, HKT1 from wheat, with a proposed high affinity K^+ uptake mechanism has been described which mediates both Na^+ and K^+ influx when expressed in yeast or oocytes (Rubio *et al.*, 1995). However, no data on the function of HKT1 in *planta* are available, and Na^+ coupling as a means of energizing solute transport has been proposed to have a limited or no physiological relevance in terrestrial plants (Maathius *et al.*, 1996) which use H^+ -coupled solute transport energized by the H^+ -ATPase (Michelet and Boutry, 1995).

The central hydrophobic domain containing the putative transmembrane segments and the proposed intracellular loops is the region displaying the highest sequence identity among all CCCs, including AXI 4 (Palfrey and Cossins, 1994; Gillen *et al.*, 1996; and Figure 4). This is consistent with the proposed importance of the transmembrane segments in ion translocation. The C-termini of CCCs are also well conserved, whereas the N-termini show only very little conservation even between CCCs with the same ion specificity (e.g. Delpire *et al.*, 1995). However, based on their amino acid sequence, all CCCs from non-vertebrates cannot be related unambiguously to one functional CCC class and form separate branches on the CCC phylogenetic tree (Gillen *et al.*, 1996; Kaplan *et al.*, 1996). The same applies to AXI 4, which displays 36–38% sequence identity to KCCs, 27–30% identity to NKCCs and NCCs and 16–35% identity to non-vertebrate CCCs. Previously, it has been noted that the greatest apparent structural difference between KCCs and N(K)CCs is in the one extracellular loop which contains several potential glycosylation sites and that this might be used to discriminate between KCCs and Na^+ -dependent N(K)CCs (Gillen *et al.*, 1996; Payne *et al.*, 1996). Though AXI 4 contains extracellular loops similar to those seen in KCCs, NKCCs and NCCs, no potential glycosylation sites could be identified in any of the predicted extracellular AXI 4 loops. Thus, sequence and structural data do not allow us to draw conclusions on the ion specificity of AXI 4. Routinely, bumetanide sensitivity correlates with $Na^+/K^+/2Cl^-$ -co-transport in most vertebrate systems. However, the final functional characterization of AXI 4 requires the demonstration that the transport of any of the involved ions requires the presence of all other co-ions and is inhibited when any of these co-ions is removed (Palfrey and O'Donnell, 1992; Haas, 1994). Currently, we are analysing the ion specificity of AXI 4 by tracer studies using tobacco protoplasts and heterologous expression systems.

In tobacco, AXI 4 is encoded by a small gene family of possibly two or three members. The *axi 4* cDNA 1 corresponds to a 3.4 kb signal in Northern analysis (Figure 3). *axi 4* expression is induced by auxin in protoplasts from untransformed plants and it is expressed in the absence of auxin in cells of the tagged line. Transcripts of *axi 4* accumulate in all tested tissues of SR1 plants (flower, stem, leaf and root; data not shown). Similarly, in vertebrates, NKCC1 and KCC1 are also expressed ubiquitously, and it is suggested that they represent the 'house-keeping' isoforms regulating cell volume (Delpire *et al.*, 1995; Gillen *et al.*, 1996).

Possibly the most intriguing question raised by this work is how does *axi 4* overexpression result in auxin-independent protoplast growth? Though the auxin signal transduction pathway in plants is little understood, it is well established that auxin application to cells or tissues results in changes of ion transport (Blatt and Thiel, 1993; Barbier-Brygoo, 1995). Ion uptake by plant cells has been shown to be driven by the electrochemical gradient generated by the H^+ -ATPase, which is activated by auxin (Lohse and Hedrich, 1992; Rück *et al.*, 1993). In addition, auxin regulates the activity of anion and K^+ channels in guard cells (Marten *et al.*, 1991; Blatt and Thiel, 1994). Auxin-induced growth of oat coleoptile protoplasts and

maize coleoptile segments depends on the presence of potassium in the culture media, and the auxin-stimulated potassium uptake has been suggested to be a rate-limiting factor for osmoregulation and cell swelling (Keller and Van Volkenburgh, 1996; Claussen *et al.*, 1997). In the context of these observations, AXI 4 may prove to contribute to ion uptake and osmoregulation in plant cells. Clearly, further investigations are required to analyse whether deregulated expression of *axi 4* results in changes of ion fluxes. Therefore, we currently are involved in measuring bumetanide-sensitive K^+ (Rb^+) uptake by SR1 and *axi 4/1* mesophyll protoplasts cultured in the presence or absence of auxin. We can be relatively certain that the AXI 4 effect is a result of deregulated expression as it is clear that auxin independence of transfected protoplasts relies on the plasmid construct containing multiple transcriptional enhancers (Figure 2C). On the face of it, one might propose that deregulated expression of an ion co-transporter might result in changes in cell volume which in turn trigger cell division, irrespective of auxin presence. This is an attractive proposition given that NKCCs are thought to act in regulating increases in cell volume during the cell cycle (Bussolati *et al.*, 1996). Indeed, it has been proposed that NKCCs might not only be important for achieving a critical cell size prior to cell division, but may also have a more direct physiological role in mitogen-induced signalling pathways triggering cell proliferation (reviewed in Palfrey and O'Donnell, 1992; McManus and Churchwell, 1994). However, this notion ignores our observation that overexpression of the AXI 4 C-terminus alone, lacking any membrane-spanning region, is sufficient to trigger auxin-independent protoplast growth. Thus, in this case, auxin independence might not be linked to changes in ion transport *per se*.

Currently, little is known concerning the functional domains of CCCs. As mentioned previously, the transmembrane domains and the connecting loops are thought to be important for ion translocation and possibly ion specificity (Haas, 1994). Attention has also focused on the N- and C-termini of CCCs, which contain several phosphorylation sites, because it is known that phosphorylation plays a role in controlling CCC activity for regulating cell volume (Cossins, 1991; Haas, 1994; Hoffmann and Dunham, 1995). We wonder, however, whether conservation of sequences of the C-terminus of CCCs suggests an important role for this part of the protein. What this role may be can currently only be the subject of speculation. It has been proposed previously that the C-terminus of NKCCs might be a regulatory domain and attachment site for other proteins (Palfrey and Cossins, 1994). Thus, we currently are interested in using the carboxy portion of AXI 4 in the yeast two-hybrid system to isolate proteins that might interact with it.

At present, our working hypothesis is that the co-transporter AXI 4 does in fact play an important role in an auxin-triggered mitogenic signalling cascade in plants. This may be linked to AXI 4-mediated ion transport and changes in cell volume, which might exert a signalling function. However, transient overexpression of not only the full-length AXI 4 but also of the AXI 4 C-terminus alone disrupts the normal auxin signal transduction cascade leading to cell division. This could result from interaction of the AXI 4 C-terminus with other proteins of the auxin

signalling pathway and lead to the observed gain-of-function effect. Alternatively, the C-terminus could function as a regulatory domain of the co-transporter, which even when not physically linked to the catalytic domain can regulate the ion transport properties of AXI 4. Interestingly, a similar mode of regulation has been proposed for Shaker voltage-gated K^+ channels (reviewed in Jan and Jan, 1994). Ion transport studies of protoplasts overexpressing only the AXI 4 C-terminus might allow further analysis of the mechanisms involved.

Focusing more on CCC function in general, we note that deletions of AXI 4 can be screened simply by assaying for protoplast division, and this finding may allow the further analysis of the structural–functional relationships of CCCs not only from plants but also from animals.

Materials and methods

Plant material and tissue culture conditions

Nicotiana tabacum Petit Havanna SR1 (Maliga *et al.*, 1975) was used in all experiments. The tissue culture conditions have been described previously (Walden *et al.*, 1994a, 1995). Briefly, mesophyll protoplasts were isolated from 6- to 8-week-old axenic plants following a modified protocol of Negrutiu *et al.* (1987). Protoplasts were cultured in K_3 medium (Nagy and Maliga, 1976) in the presence of auxin (5.5 μ M 1-NAA, Sigma) and cytokinin (0.9 μ M kinetin, Sigma) or cytokinin alone. Selective agents were added as indicated [15 mg/l hygromycin B, Boehringer Mannheim; variable concentrations of bumetanide (Sigma) from a 100 mM stock solution in absolute ethanol]. The proportion of dividing protoplasts was determined microscopically 5 days after protoplast isolation by counting at least 50 protoplasts in duplicate samples using a Neubauer cell counting chamber. The error was judged at 5% or less between samples.

For transient gene expression assays, 3.3×10^5 freshly isolated SR1 mesophyll protoplasts were transfected with 10 μ g of plasmid DNA, obtained from two successive CsCl bandings (Sambrook *et al.*, 1989), by PEG-mediated DNA uptake (Negrutiu *et al.*, 1987; Walden *et al.*, 1994a). To obtain microcalli, protoplasts were embedded in low melting agarose 7 days after isolation, and cultured under selective conditions as indicated.

Southern and Northern blot analysis

Southern blot analysis was performed using plant genomic DNA isolated from leaf tissue (Dellaporta *et al.*, 1983) digested with the indicated restriction enzymes as recommended by the supplier (New England Biolabs) and fractionated on 0.8% agarose gels followed by transfer to nylon membranes (Hybond-N, Amersham). Hybridization probes were derived from the cauliflower mosaic virus (CaMV) 35S RNA promoter enhancer sequence (–90 to –427 bp), the 2.1 kb *Hind*III fragment of the HPT gene from pPVICen4HPT (Hayashi *et al.*, 1992; Walden *et al.*, 1995) and the 1.8 kb *Eco*RI–*Xho*I plant DNA fragment from p19En4 (Figure 2B). Probes were purified from agarose gels by electroelution (Sambrook *et al.*, 1989) and radiolabelled by random priming using the Prime-It II kit (Stratagene).

Northern blots were performed using poly(A)⁺ RNA isolated from 2-day-old protoplasts. Protoplast cultures were transferred to 12 ml plastic centrifuge tubes (Nunc), mixed with 1 vol. of W5 buffer (Walden *et al.*, 1995) and centrifuged for 3 min at 1800 r.p.m. in a swingout bucket rotor centrifuge (Hettich) at room temperature. After aspiration of the supernatant, the protoplast pellet was frozen in liquid nitrogen. Poly(A)⁺ RNA was isolated directly from frozen protoplast pellets using oligo(dT)–dynabeads as recommended by the supplier (Dynal). Equal amounts of poly(A)⁺ RNA (1.0 μ g of +NAA samples and 0.5 μ g of –NAA samples, respectively) were size-fractionated on 1.1% formaldehyde gels and transferred to nylon membranes (Sambrook *et al.*, 1989).

Southern and Northern hybridizations, washing at high stringency and complete removal of probes from membranes were carried out as described (Sambrook *et al.*, 1989). Southern and Northern blot membranes were autoradiographed for 6 and 17 days, respectively.

Construction and screening of a cDNA library from tobacco mesophyll protoplasts

A cDNA library was constructed in λ gt11D (Pharmacia) using poly(A)⁺ RNA purified from 2-, 3- and 5-day-old protoplasts cultivated in media

containing hormone concentrations for promoting optimal frequencies of division (5.5 μM 1-NAA, 0.9 μM kinetin). Poly(A)⁺ RNA (0.7 μg) from each sample was pooled and subjected to cDNA synthesis using the Gibco/BRL cDNA synthesis kit. For the addition of *NotI* oligo(dT) primers, the synthesis of DNA strands and the ligation of *EcoRI* adaptors to the 5' ends of the cDNAs, the manufacturer's protocols were followed. After digestion with *NotI*, the resulting cDNAs with 5' *EcoRI* and 3' *NotI* overhangs were ligated into $\lambda\text{gt}11\text{D}$ (Pharmacia). Packaging was performed using Stratagene Gigapack II packaging extracts and the library was amplified using *E.coli* Y1090 (Pharmacia) as recommended by the supplier. About 3×10^5 plaques were screened using the 1.8 kb *EcoRI*–*XhoI* genomic *axi 4* fragment from p19En4 (Figure 2B) as a hybridization probe under standard high stringency conditions (Sambrook *et al.*, 1989). DNA from single plaques producing strong hybridization signals was isolated, and *axi 4* cDNAs were subcloned into pBluescript II⁺ phagemids (Stratagene).

Recombinant DNA techniques and cloning for functional assays

Basic recombinant DNA methods were as described (Sambrook *et al.*, 1989). The protocol for plasmid rescue of T-DNA and flanking plant DNA sequences from tagged mutants was reported elsewhere (Walden *et al.*, 1995), and resulted in the recovery of plasmid p19, which contains a partial T-DNA (*oriC*, ampicillin resistance gene and the HPT marker gene at the left T-DNA border) plus some 4.7 kb of flanking plant DNA. Plasmid p19En4 resulted from recloning the enhancer tetramer obtained as a 1.3 kb *BamHI*–*BglIII* fragment from pICEn4 (Fritze, 1992) into the unique *BamHI* restriction site of p19.

Deletion analysis of p19En4 (Figure 2B) was carried out by digestion of the plasmid with either *XhoI* or *ClaI*, to remove the 1.4 kb *XhoI* or the 1.85 kb *ClaI* plant DNA fragment respectively, followed by self-ligation of the plasmids. A 2.7 kb fragment representing plant DNA was removed by digestion of p19 with *EcoRV* followed by self-ligation and recloning of the enhancers as described above. To delete the 1.45 kb *BamHI*–*EcoRI* fragment most distant from the left border of the T-DNA, p19 was digested with *BamHI* and *EcoRI* and the plant DNA fragment was replaced by an En4 fragment with compatible overhangs. This enhancer fragment resulted from subcloning the *BamHI*–*BglIII* En4 fragment from pICEn4 into pBluescript, followed by digestion with *BamHI* and *EcoRI*. The ability of the obtained deletion derivatives of p19En4 to promote auxin-independent protoplast division was tested by transient expression in SR1 protoplasts.

A full-length *axi 4* cDNA 1 was removed from $\lambda\text{gt}11\text{D}$ by digestion with *SfiI* and mung bean nuclease treatment (Kowalski *et al.*, 1976), followed by digestion with *NotI*. The cDNA was electroeluted from an agarose gel and ligated into an *EcoRV*–*NotI*-digested pBluescript II vector. For functional assays, *axi 4* cDNA 1 was subcloned as an *Apal*–*NotI* fragment into the plant expression vector pRT106 between the CaMV 35S RNA promoter and poly(A) addition sequences (Töpfer *et al.*, 1993). The shNKCC1 cDNA (Xu *et al.*, 1994) was subcloned as an *XhoI*–*NotI* fragment from pBluescript into pRT105 (Töpfer *et al.*, 1993).

Plasmid pRT108*axi4C*-term was constructed by digestion of the full-length *axi 4* cDNA 1 in pRT106 with *HindIII*–*XhoI* and subcloning of the resulting 833 bp fragment into pBluescript. This construct was digested with *XhoI* and the 5' overhangs were removed by mung bean nuclease treatment. After *BamHI* digestion, the resulting 857 bp fragment was electroeluted from an agarose gel and ligated as a translational fusion into a *BamHI*–*SmaI*-digested plant expression vector pRT108 (Töpfer *et al.*, 1993). The derived protein starts with a vector-encoded methionine followed by 23 amino acids encoded by vector sequences (lower case letters) and 277 amino acid residues of the AXI 4 C-terminus (upper case letters, residues 663–939 of AXI 4): mgelhrgggrsrtspsglqf-diKLAD....KYS.

Construction of plasmid pRT105*axi4AC*-term was performed by digestion of the full-length *axi 4* cDNA 1 in pBluescript with *ClaI*–*KpnI* and subcloning the resultant 2.5 kb *axi 4* fragment into pRT105 digested with the same enzymes. The protein derived from this construct starts with the *axi 4* endogenous methionine and contains the AXI 4 amino acid residues 1–750.

Sequencing and computer programs

DNA sequencing was performed using overlapping exonuclease III-generated deletion clones (Henikoff, 1987) by the dideoxy method (Sanger *et al.*, 1977) using an A.L.F. DNA sequencer (Pharmacia). The sequence was analysed with the GCG VAX package (Devereux *et al.*, 1984). The *axi 4* sequence has been submitted to the DDBJ/EMBL/

GenBank nucleotide sequence database and has the accession number AF021220.

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