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

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Corresponding author (Hesse Grundlagen der Pflanzenzüchtung, Carl von Linne Weg 10, 50829 Köln, Germany (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). 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 ethylmethylsulfonate (EMS; Maher and Martindale, 1980; Estelle andSomerville, 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; Picket 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 I has homology to amino acid permesases, 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

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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). 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.

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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 cyt 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 Na+/K+/2Cl–-co-transporters. In addition, transient expression of a shark cDNA encoding a Na+/K+/2Cl–-co-transporter in protoplasts also results in auxin-independent and inhibitor-tolerant cell division, underlining functional similarities of AXI 4 and Na+/K+/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 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 transformed with p19 containing the HPT gene were hygromycin resistant, but were unable to grow in the absence of auxin. In contrast, protoplasts transfected
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 EcoRI–XhoI 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. 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 EcoRI (RI), BamHI (B) and KpnI (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 BamHI 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, ClaI; RV, EcoRV; X, XhoI). 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. EcoRI-digested DNA from SR1 and a homozygotic axi 4/1 plant 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], the flatfish (H. Harling et al., 1995; Payne and Forbush, 1994) probe. According to their specificities for the transported ions, 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 Pseudopleuronecotus americanus (winter flounder, flNC; Gamba et al., 1993), a Na⁺/K⁺/2Cl⁻-co-transporter from Squalus acantbias (shark, ShNKC11; Xu et al., 1994) and a K⁺/Cl⁻-co-transporter from rat (KCC1; 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...
A cation–chloride co-transporter in plants

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. flNCC, Na\(^{+}\)/Cl\(^{-}\)-co-transporter from \textit{P. americanus}; shNKCC1, Na\(^{+}\)/K\(^{+}\)/Cl\(^{-}\)-co-transporter from \textit{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\(^{-}\)-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
respectively), and killing curves with metolazone produced to suspect that a portion of AXI 4 and a NKCC in involved in the regulation of cell volume, but might be proteins might have similar functions in plant and vertebrate cells (reviewed in Hoffmann of tobacco protoplasts can be blocked efficiently using controls in inhibitor-free medium, respectively, whereas Overexpression of the genomic plant DNA rescued in undergoing cell division was reduced to 58 and 0% of the individuals.

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 control 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 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 was cloned in pRT plant expression vectors (To¨pfer et al., 1994) 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.

The 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 ClaI 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 protoplast 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 pRT105axi4AC-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–Xhol axi 4 fragment contained in pRT108axi4C-term encodes a large portion of the AXI 4 C-terminus. For animal CCCs, a distinct function of the cytosolic C-terminus 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⁺:2Cl⁻ 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 KKCCs and 16–35% identity to non-vertebrate CCCs. Previously, it has been noted that the greatest apparent structural difference between KCCs and NKCCs 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 NKCCs (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; Rücker 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 cotransporter 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 tobacco 6- to 8-week-old axenic plants following a modified protocol of Negrutiu et al. (1987). Protoplasts were cultured in K⁺ medium (Nagy and Maliga, 1976) in the presence of auxin (5.5 μM 1-NAA, Sigma) and cytokinin (0.9 μM 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 × 10⁴ freshly isolated SR1 mesophyll protoplasts were transfected with 10 μg 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 HindIII fragment of the HPT gene from pPVICEnH4HPT (Hayashi et al., 1992; Walden et al., 1995) and the 1.8 kb EcoRI–HindIII plant DNA fragment from p19En4 (Figure 2B). Probes were purified from agarose gels by electrophoresis (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 at 3 min at 18000 × g in a swinging 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 μg of +NAA samples and 0.5 μg of −NAA samples, respectively) were size-fractionated on 1.1% agarose 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 λglt11D (Pharmacia) using poly(A⁺) RNA purified from 2-, 3- and 5-day-old protoplasts cultivated in media.
GenBank nucleotide sequence database and has the accession number AF021220.

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References


A cation–chloride co-transporter in plants


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Retraction

Site-specific deoxynucleotide substitutions in yeast U6 snRNA block splicing of pre-mRNA in vitro


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We (the authors) wish to report the following correction regarding our studies of deoxynucleotide-substituted U6 snRNAs in yeast spliceosomes. In our original work, we tested 50 site-specific deoxynucleotide substitutions in U6 RNA for their effects on splicing. Of these, only four specific deoxynucleotides blocked splicing and did so reproducibly. Recently, we repeated these experiments using the original stocks of the deoxy-substituted pieces, and we observed that splicing was not blocked or diminished relative to controls. Multiple attempts to reproduce our published results have failed. However, some of the original conditions cannot be replicated. The U6 RNA is synthesized in vitro in these experiments via ligation of four or five synthetic oligonucleotide pieces. Although we still have stocks of the original yeast extract and deoxy-substituted oligonucleotides, the original stocks of the flanking pieces of U6 RNA had been depleted. Hence, we are unable to duplicate the reported experiments exactly. Although we have tested various parameters, including various extracts, preparations of the U6 RNA pieces, and U6 reconstitution conditions, we are unable to find conditions under which the four deoxy substituents in question have any deleterious effect on splicing. In any case, the recent observations of normal splicing for these four substituents mean that they do not block splicing generally. Though we are not now able to reproduce the reported observations for the four deoxy substitutions, they may well have a deleterious effect on splicing under conditions not yet understood. We are left with the revised conclusion that synthetic U6 RNAs substituted with a single deoxynucleotide at any of the 50 positions tested (39–88 in yeast U6) are able to reconstitute splicing activity under standard conditions in vitro.

Retraction

Auxin inducibility and developmental expression of axi 1: a gene directing auxin independent growth in tobacco protoplasts

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The EMBO Journal, 13, 4729–4736, 1994

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

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The EMBO Journal, 16, 5855–5866, 1997

In a recent article in the journal Science volume 283, pages 1987–1989 (1999) it is said that I (Schell) had no plans to publish retractions of the papers in the journals in which they had originally appeared. In fact, I wanted to stress the point that the first responsibility the collaborating colleagues in and outside the Institute and I had felt was to publish our results showing that the previously published data could not be reproduced by another, more objective method. Therefore, the members of the investigating team decided to publish all further data re-evaluating this fraud as a regular scientific paper in The Plant Journal. After peer review and acceptance of the paper, it was agreed with the Editor-in-Chief of The Plant Journal, Professor Diana Bowles, that after publication short correction statements should be sent to individual journals, which could refer to this paper for full details of new experiments confirming the irreproducibility of the protoplast assays in question (Schell et al., 1999). Since the paper has now appeared, we hereby retract officially the results regarding auxin independent division of tobacco protoplast-derived cells in our papers mentioned above.

Reference