Antisense expression of a gene encoding a calcium-binding protein in transgenic tobacco leads to altered morphology and enhanced chlorophyll§

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Entamoeba histolytica contains a novel calcium-binding protein like calmodulin, which was discovered earlier, and we have reported the presence of its homologue(s) and a dependent protein kinase in plants. To understand the functions of these in plants, a cDNA encoding a calcium-binding protein isolated from *Entamoeba histolytica* (EhCaBP) was cloned into vector pBI121 in antisense orientation and transgenic tobacco plants were raised. These plants showed variation in several phenotypic characters, of which two distinct features, more greenness and leaf thickness, were inherited in subsequent generations. The increase in the level of total chlorophyll in different plants ranged from 60% to 70%. There was no major change in chloroplast structure and in the protein level of D1, D2, LHCP and RuBP carboxylase. These morphological changes were not seen in antisense calmodulin transgenic tobacco plants, nor was the calmodulin level altered in EhCaBP antisense plants.

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1. Introduction

Calcium is an important second messenger which is involved in signal transduction in many developmental and physiological processes in plants (Trewavas and Malho 1997; McAnish and Hetherington 1998). The wide variety of responses triggered by calcium suggests the presence of multiple, yet specific, downstream components. Calcium represents a prime candidate for such crosstalk nodes as it has been shown to serve as a second messenger for both abscisic acid (ABA) and stress responses.

It is intriguing to know how calcium functions in transmitting different signals to trigger stimulus-specific responses in a plant cell. If calcium signalling in the cell represents a molecular relay, the components immediately following calcium changes would be "sensor" molecules

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Abbreviations used: ABA, abscisic acid; CaM, calmodulin; CBl, calcineurin B-like proteins; CDPK, calcium-dependent protein kinase; CIPK, CBL interacting protein kinases; dZR, dihydroxyzeatin riboside; EF, elongation factor motif; EhCaBP, *Entamoeba histolytica* calcium-binding protein; GUS, beta-glucuronidase; IgG, immunoglobulin G; iPA, isopentenyl adenosine; LHCP, light harvesting chlorophyll binding project; LSU, large subunit; PCR, polymerase chain reaction; PKS11, protein kinase 11; PS, photosystem; RuBP, ribulose-1,5-bisphosphate carboxylase/oxygenase; SOS2, salt overly sensitive 2; TLC, thin layer chromatography.

that decode the changes in calcium concentration and other parameters that constitute "calcium signatures" (Sanders *et al* 1999; Rudd and Franklin-Tong 2001). A number of calcium-binding proteins have been identified that may fulfil the role of calcium sensors in plant cells. This is reflected in the diversity of calcium-binding proteins and calcium-stimulated kinases that have been identified in plants (Sopory and Munshi 1998; Pandey *et al* 2000; Reddy 2001; Luan *et al* 2002; Pandey *et al* 2002). These include calmodulin (CaM) and CaM-related proteins (Zielinski 1998; Snedden and Fromm 2001; Luan *et al* 2002), calcium-dependent protein kinases (CDPKs) (Harmon *et al* 2000; Sanders *et al* 2002), and calcineurin B-like proteins (CBLs) (Luan *et al* 2002; Batistic and Kudla 2004; Kolukisaoglu *et al* 2004) In each case, the proteins are encoded by large multigene families suggesting possible redundancy and/or diversity in their functions. For example, CBL1 plays a role in multiple abiotic stress response pathways (Albrecht *et al* 2003; Cheong *et al* 2003) whereas CBL9 is more specifically involved in mediating ABA signalling (Pandey *et al* 2004) despite the fact that both the genes are highly inducible under ABA and abiotic stress conditions. CBL-type calcium sensors are small elongation factor motif (EF)-hand-containing proteins that interact and regulate the function of a group of protein kinases called CBL interacting protein kinases (CIPKs) (reviewed in Luan *et al* 2002; Batistic and Kudla 2004; Kolukisaoglu *et al* 2004). Recent studies have indicated that several CIPK genes such as *CIPK1* (D'Angelo *et al* 2006), *CIPK3* (Kim *et al* 2003), *PKS11* or *CIPK8* (Gong *et al* 2002a), *PKS3* or *CIPK15* (Guo *et al* 2002), *PKS18* or *CIPK20* (Gong *et al* 2002b) *CIPK24/SOS2* (Guo *et al* 2004) function in stress and ABA responses. Other members of the CIPK family such as *CIPK9* (Pandey *et al* 2006) and *CIPK23* (Cheong *et al,* unpublished data; Li *et al* 2006; Xu *et al* 2006) have been implicated in regulating low potassium nutrition. Moreover, CBL and CIPK components have been identified in several other higher plants (Kim *et al* 2003; Kolukisaoglu *et al* 2004; Hwang *et al* 2005; Mahajan *et al* 2006). A large number of Ca^{2+}-binding proteins and kinases of yet unknown functions have been identified (Mayer *et al* 1998; Reddy 2001). It will be a challenge to decipher the function(s) of these molecules. In a few cases, functional analysis of calcium-binding proteins has been undertaken using the transgenic approach. The transformation of tobacco with barley calmodulin (*CaM*) cDNA in sense and antisense orientations resulted in T_1 seedlings that showed a change in internode length and lack of apical dominance (Zielinski *et al* 1990). Similarly, transgenic potato harbouring potato *CaM* cDNA in the antisense orientation exhibited strong apical dominance and elongated tubers (Poovaiah *et al* 1996). Over-expression of *CaM* also confers tolerance to environmental stress (Harding *et al* 1997) and helps in acquiring systemic resistance (Heo *et al* 1999). Transgenic plants have also been raised with genes encoding Ca^{2+}-binding protein from other sources. The over-expression of yeast calcineurin in tobacco conferred tolerance to salt stress (Pardo *et al* 1998). It was found that the gene mutated in a salt-sensitive mutant (*SOS-3*) in *Arabidopsis* codes for a calcium-binding protein showing homology to calcineurin and neural calcium sensor (Liu and Zhu 1997), and its associated protein is a kinase (Halfter *et al* 2000; Zhu 2002).

A novel calcium-binding protein (EhCaBP) showing structural, but not functional, homology to CaM, was reported from the protozoan parasite *Entamoeba histolytica* (Prasad *et al* 1992). This protein has characteristic EF-hand motifs and is able to stimulate specific kinase activity (Yadava *et al* 1997). We have evidence for the presence of homologue(s) of this protein in plants, and a novel kinase that is stimulated by EhCaBP was purified and characterized from *Brassica juncea* (Pandey 1999; Deswal *et al* 2000). Moreover, over-expression of *EhCaBP* in tobacco plants leads to enhanced growth and tolerance to salt stress (Pandey *et al* 2002). In this paper, we show that *EhCaBP* cDNA antisense transgenic plants differ phenotypically from wild-type plants. Furthermore, the phenotypic changes were not observed in plants transformed with a *CaM* cDNA antisense construct.

2. Materials and methods

2.1 *Construction of plant transformation vectors*

The *EhCaBP* gene was excised from pET3cCaBP as a BamHI-NdeI fragment, end-filled using Klenow and ligated into the pBI121 vector (Clontech) digested with SmaI and finally ligated to generate vector pBI121-antisense *EhCaBP* (figure 1a). The *Arabidopsis thaliana* CaM isoform 3 cDNA (*AtCaM3* cDNA) was excised from the pBSAtCaM3 plasmid as an EcoRI fragment, end-filled as above and ligated into the pBI221 vector (Clontech) digested with SmaI, to generate intermediate vector pBI221-antisense (figure 4a). A 3.7 kb cassette containing CaMV 35S promoter-driven *AtCaM3* and *uidA* genes in antisense or sense (data not given) orientation was digested with EcoRI and PstI enzymes and ligated into the plant transformation vector pPZP111 (Hajdukiewicz *et al* 1994) at the same sites. Cloning and orientations of the genes in sense and antisense were confirmed by polymerase chain reaction (PCR) amplification using *uidA* and *EhCaBP/AtCaM3* gene-specific primer combinations, by restriction digestion and by partially sequencing the cloned junctions. Each construct was transferred into *Agrobacterium tumefaciens* strain LBA4404 and used for the transformation of *Nicotiana tabacum* cv. Xanthi by the leaf disc method (Horsch *et al* 1985). Preliminary screening for the transgenic nature of the regenerated plants by kanamycin selection (200 mg/l) was carried out by beta-glucuronidase (GUS) histochemical assay (Jefferson 1987) and by PCR

methods using *EhCaBP* or *AtCaM3* gene-specific primers in combination with *uidA* gene-specific primers.

2.2 *DNA, RNA and protein blot analysis*

Southern, northern and western blot analyses were carried out essentially as described (Sambrook *et al* 1989). For Southern and northern blots, the blots were pre-hybridized for 6–8 hours at 65°C to achieve high stringency for transgenic plants. After hybridization, the blots were washed twice with 1 X SSC, 0.1% SDS; 0.5 X SSC, 0.1% SDS and 0.2 X SSC, 0.1% SDS for 15 min at 65°C. For western blotting, incubation with the primary antibodies was done for 2–3 h at room temperature and, after washing, the blots were incubated with secondary antibodies conjugated to alkaline phosphatase or horseradish peroxidase and developed by BCIP/NBT substrate or ECL (Amersham Bioscience, NJ, USA).

2.3 *Transmission electron microscopy (TEM)*

Immersion fixation of leaf tissues for ultrastructural studies was carried out in Karnovsky fixative (Karnovsky 1967). Embedding of the tissue was done in LR white resin and the specimens were polymerized at 60°C for 2 days. Ultra-thin sections, ranging from 60 to 90 nm, were cut by an ultra-microtome and lifted onto nickel grids (4 grids for each sample). Viewing was done using a Phillips transmission electron microscope. A large number of sections from 3 different plants were analysed and representative pictures were taken.

2.4 *Chlorophyll estimation*

Estimation of chlorophyll in leaves from transgenic plants and in leaf discs used in senescence assay was done according to the procedure of Arnon (1949). The leaf discs were homogenized in 1 ml of 80% acetone and centrifuged at 3000X *g* for 2–3 min. The supernatant was collected and the absorbance was recorded at 710 nm, 663 nm, 646 nm and 470 nm. The amount of pigment was calculated and expressed as chlorophyll content per gram fresh weight of leaf discs.

2.5 *Estimation of polyamines*

The procedure followed was described by Bajaj and Rajam (1996). Leaf tissue (100 mg) was homogenized in 500 μl of 10% perchloric acid (ice cold). This was kept at 4°C for 1 h. Again, 500 μl of 10% perchloric acid was added, and the tissue ground thoroughly. This mixture was centrifuged at 12,000 *g* for 20 min at 4°C. The pellet contained the bound fractions of polyamines whereas the supernatant contained the free and conjugated fractions. To measure the free fractions of polyamines, 100 μl of a saturated solution of Na_2CO_3 was added to 100 μl of the supernatant. After complete mixing, 200 μl of dansyl chloride (5 mg/ml in acetone) was added, mixed and incubated for 15–18 h in the dark at 26°C. Excess dansyl chloride was quenched by adding proline solution (100 μl) and mixed by vortexing. Thereafter, 200 μl of benzene was added to this, vortexed and incubated for 10 min. Finally, 50 μl of the upper organic phase was taken and spotted onto pre-activated TLC plates on pre-marked spots. The TLC plate was placed in a tank containing cyclohexane:ethyl acetate (200:160) and allowed to run for 30 min to 1 h. After dragging the plate, it was visualized under UV light, photographed and bands of putriscine, spermine, spermidine were located and scraped off from the TLC plate. The scraped bands were suspended in ethyl acetate and fluorescence was measured at an excitation of 350 nm and emission at 495 nm.

2.6 *Measurement of cytokinin*

For extraction of cytokinin, the procedure of Hansen *et al* (1984) was followed. The extract was obtained by grinding the tissue in ice-cold ethanol and incubated for 24 h at –20°C. The residue was re-extracted twice with 5 volumes of ethanol. Further purification of the plant extract was done by butanol partitioning and Sep-Pak C18 column chromatography. This removes lipids and pigments. The partially purified fractions were coated onto a solid phase (ELISA plate) and allowed to bind with primary antibodies (anti-iPA, tZR, dZR) and then with a secondary antibody–enzyme conjugate (anti-rabbit immunoglobulin G [IgG] conjugated to alkaline phosphatase) and after washing, the colour was developed and quantitated using an ELISA reader.

3. Results

3.1 *Altered morphology of EhCaBP-antisense transgenic plants*

Transgenic tobacco (*Nicotiana tabacum* cv Xanthi) plants were raised by introducing *EhCaBP* cDNA in antisense orientation under the control of a strong constitutive promoter, *cauliflower mosaic virus 35S* (*CaMV 35S*) (figure 1a) via *Agrobacterium*-mediated transformation. A total of 100 kanamycin-resistant plants from two independent transformation experiments were selected and screened by PCR and GUS assay and finally confirmed by Southern and northern hybridizations (figures 1d and 1e). In Southern hybridization, a band of the expected size (at approximately 1.1 kb) besides a common band of ~600 bp was detected in all transgenic lines (2A, 6A, 8A, 9A, 10A) but not in the wild-type; it corresponded to the *EhCaBP*-pBI121 Hind III fragment (*see* +C in figure 1d). The

Table 1. Growth and developmental features of *EhCaBP* antisense tobacco plants

Characteristic features	Antisense	Sense	Wild-type
Plant height (cm)	92 ± 4.0	94 ± 3.0	95 ± 3.0
Leaves/plant	31 ± 3.9	26 ± 3.8	26 ± 3.1
[a]Plants showing delayed flowering(%)	70–80	None	None
No. of seeds producing siliques/plant	9 ± 0.68	65 ± 3.6	70 ± 3.0
[a]Pollen viability (%)	92 ± 4.0	94 ± 3.0	95 ± 3.0
[a]Pollen germination (%)	35–40	78– 82	85–90
[a]Seed germination (%)	70–75	80–85	90–95

Wild-type and *EhCaBP*-sense plants were taken as control for comparison of phenotype. A total of 50 plants were analysed for antisense and sense transgenics.
[a] Variation obtained in different transgenic plants.

intensity of the hybridization signal was variable, indicating multiple copies.

A 2.27 kb transcript of variable intensity was observed by northern hybridization in different transgenic plants (figure 1e). This transcript is a fusion product of *EhCaBP* (0.405 kb) and *uidA* (1.87 kb). This size was expected since the antisense construct contains a single transcriptional start, stop (Nos-T) and polyadenylation sequence.

The transgenic plants showed altered morphology and an increase in chlorophyll levels. The leaves of transgenic plants were rounder, smaller, thicker and hairy, and gave the plant a compact look due to shortening of the internode length. These characters were observed in a large population of plants and were retained even in T_1 generation plants raised on MS-basal medium (figure 1b). Plants transferred to pots retained the increased greenness and thickness of the leaves, which were inherited in the next generation (figure 1c). The various changes observed in antisense *EhCaBP* transgenic plants are listed in table 1. The plants were partially sterile and flowering was delayed in 80% of the population. There was a 19% increase in the number of leaves per plant without the plant height being affected. The number of flowers per plant was reduced and a decrease in the number and viability of pollen was seen. Seed production and the number of siliques per plant were also reduced. The altered features were observed in 60–70% of the population and in a repeat transformation experiment.

3.2 *Enhanced levels of chlorophyll and increase in thickness of leaves*

The chlorophyll level was estimated in individual antisense transgenic plants from leaves at different stages. The increase in chlorophyll in *EhCaBP* antisense plants was in the range of 50–65% in comparison with the wild-type plants (figure 2a). The transgenic plants raised with sense *EhCaBP* construct did not show any increase in chlorophyll levels. The thylakoids isolated from antisense plants also exhibited a higher rate of PSII and PSI activity (Tripathy *et al* unpublished). In addition, the leaves also showed an increase in thickness compared with sense transgenic and wild-type plants (figure 2b). Electron microscopic analysis showed that the chloroplast structure was not altered (figures 2c and d) although large starch granules were seen inside the chloroplast of antisense transgenic plants. We measured the levels of a few cytokinins such as iPA and dZR (table 2). In general, there was an increase in cytokinin level in most of the antisense transgenics. The increase in levels of cytokinin was, however, variable in different plants. This could be due to the transgene number or an unknown parameter. Of the various free polyamines tested, putricine level was also higher in antisense transgenic plants (table 2).

The mechanism that led to an increase in chlorophyll is not yet clear. It was, however, found that the chlorophyll degradation kinetics was similar for both wild-type and antisense plants (figure 3a). Also, the levels of D1, D2, LHCP1 and RuBP carboxylase (large subunit [LSU]) proteins did not show any significant changes in both transgenic and wild-type plants (figure 3b). This suggests that a decrease in chlorophyll degradation and/or increase in associated proteins are not responsible for the retention of higher chlorophyll levels in *EhCaBP* antisense transgenic plants.

3.3 *EhCaBP effects may not be due to calmodulin*

Western blot analysis using anti-CaM antibodies did not reveal any change in the level of CaM protein in the *EhCaBP* antisense plants (figure 3b), which suggested that CaM may not be responsible for the altered characteristics observed. To further confirm this, tobacco plants with CaM (*AtCaM3*) in antisense orientation were raised (figure 4a). These transgenic plants were screened by PCR, GUS assay and finally by high-stringency Southern and northern hybridizations. Southern hybridization showed fragments of 310 bp corresponding to partial *AtCaM3* cDNA and 1.0 to 1.4 kb bands in different antisense transgenic plants, as expected from the design of the *AtCaM3* antisense construct (figure 4d). Northern hybridization showed two transcripts, a fusion transcript of 2.6 kb formed by ~800 bp (*AtCaM3*) and 1.87 (*uidA*), and another one at ~800 bp corresponding to *AtCaM3* (figure 4e). The fusion transcript was due to a common transcription start, stop (Nos-T) site and polyadenylation sequences. In contrast to the *EhCaBP* antisense transgenic plants, the *AtCaM3* antisense transgenic plants did not show any morphological variations and were similar to the wild-type plants (figures 4b, c).

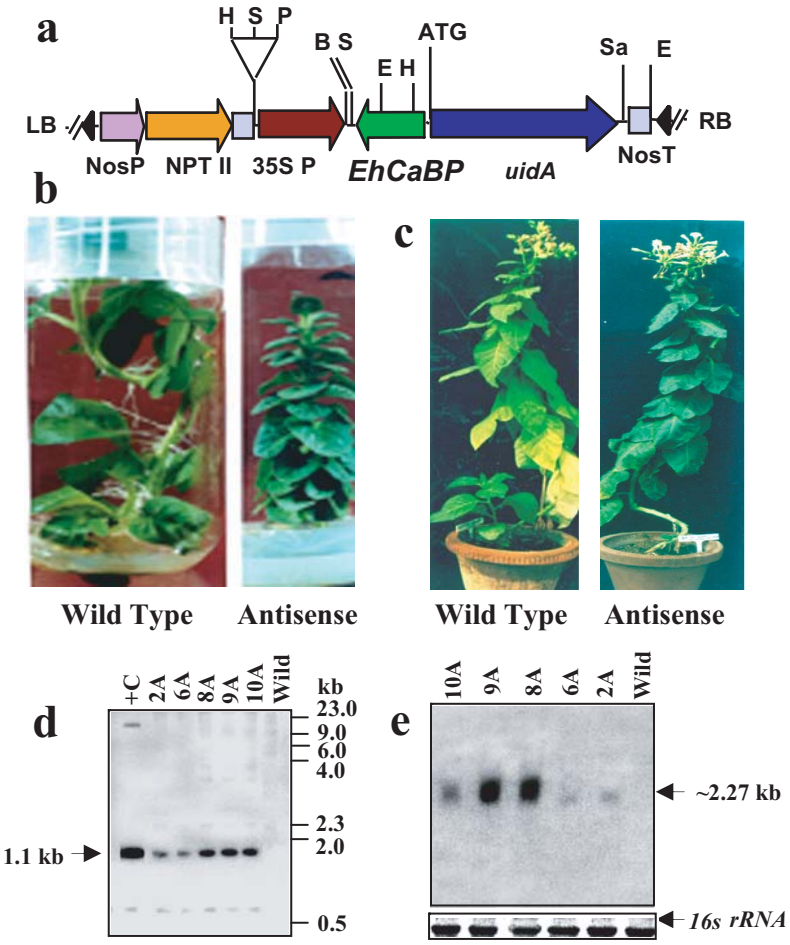

Figure 1. Confirmation and morphology of *EhCaBP* antisense transgenic tobacco plants: **(a)** Schematic representation of *EhCaBP* antisense construct (pBI121-antisense). The open reading frame (ORF) of *EhCaBP* was inserted into vector pBI121 under CaMV 35S promoter in antisense orientation. Morphology of the transgenic plants was transformed with *EhCaBP* antisense construct and wild-type plants grown *in vitro* **(b)** and in the glass house **(c)**. Southern hybridization of genomic DNA from GUS-positive putative transgenic plants digested with HindIII and probed with radiolabelled EhCaBP cDNA. +C; Positive control (pBI121-antisense plasmid digested with HindIII that gives a 1.1 kb signal); Antisense **(a)** and wild-type (WT) plants **(d)**. Northern blot analysis using radiolabelled *EhCaBP* cDNA to show the transcription of *EhCaBP*. To show equal loading of RNA, the same blot was re-probed with *16S rRNA* **(e)**.

4. Discussion

A novel calcium-binding protein from *Entamoeba histolytica* (EhCaBP) was reported to have structural but not functional homology to CaM (Prasad *et al* 1992). We have evidence that homologues(s) of EhCaBP may be present in plants and a protein kinase that was stimulated in the presence of EhCaBP and not by calmodulin was purified from a plant, *B. juncea* (Deswal *et al* 2000). Similar EhCaBP homologue(s) and kinase activities have been identified in *Pisum sativum, Nicotiana tabacum* and *Zea mays* (Pandey *et al* 2001; Chandok *et al* unpublished). In order to identify the role of EhCaBP-like proteins, if present in plants, we made an antisense construct of *EhCaBP* in the pBI121 vector and raised transgenic tobacco plants. The transgenic nature of the plants was confirmed by Southern and northern analyses. More than 100 transgenic plants were analysed, which showed a number of variations in morphological characters. However, only two characters—increase in greenness and leaf thickness—were stably inherited. In one of the plants tested, the photosynthetic efficiency was also increased (Tripathy *et al* unpublished). While there was an overall increase in chlorophyll level and leaf thickness in all transgenic plants expressing antisense transcript, a quantitative correlation between the two parameters was not seen. Plants that showed low expression such as 2A and 6A showed a similar increase in chlorophyll level and leaf thickness as in high transcript-expressing plants such

Table 2. Altered levels of cytokinins and polyamines in antisense transgenic plants*.

Plant no.	Cytokinins (picomol/0.1 ml)		Polyamines (nmol/g)		
	iPA	dZR	Put.	Spmd.	Spm.
8A	4.8 ± 0.04	2.5 ± 0.02	1350 ± 34	325 ± 16	100 ± 3
9A	8.3 ± 0.20	11.0 ± 0.2	1525 ± 17	250 ± 9	95 ± 5
10A	3.8 ± 0.05	7.0 ± 0.08	1250 ± 23	300 ± 10	98 ± 2
WT1	0.3 ± 0.003	1.0 ± 0.007	850 ±18	700 ± 7	150 ± 4
WT2	0.28 ± 0.01	1.1 ± 0.003	700 ± 20	650 ± 12	140 ± 3

Values are means of three replicates ± SE and representative of at least three independent experiments.
*A few cytokinins, viz. isopentenyl adenosine (iPA), dihydroxyzeatin riboside (dZR) and free polyamines Put. (putricine), Spmd. (spermidine), Spm. (spermine) were extracted from T_1-generation transgenic and wild-type tobacco leaves, measured and expressed as picomol/0.1 ml and nmol/g of tissue, respectively.

as 8A, 9A and 10A. This could be due to the fact that a minimal level of transcript may be enough to bring about the antisense effect and the observed changes.

One question that may arise is whether the observed changes in chlorophyll level and leaf thickness are in fact due to an antisense effect of the *EhCaBP* gene. We observed these features in 60–70% of the population and also in two independent transformation experiments. Therefore, we feel that these effects could not be due only to the site of integration of transferred (T)-DNA into the plant genome. The 30–40% of the population of *EhCaBP* antisense plants that showed wild-type features was Southern positive but the *EhCaBP* transcript was not detectable in these plants. Thus, there seems to be a positive qualitative correlation between the observed phenotype and *EhCaBP* transcript level in the transgenic plants. It was also seen that none of the sense *EhCaBP* transgenic plants showed any of the features observed in the antisense transgenic plants, again suggesting that there is an overall correlation between the antisense transcript and an increase in chlorophyll level and leaf thickness. At present, the mechanism leading to these changes is not clear. However, when measured, we found that the rate of chlorophyll degradation was similar in both wild-type and antisense plants. Also, the protein level of D1, D2, LHCPs and RuBP carboxylase (LSU) did not show any significant changes in the antisense transgenics compared with the wild-type plants. This suggests that a decrease in chlorophyll degradation and/or increase in associated proteins are not responsible for the greater retention of chlorophyll in the antisense plants. It is possible that the chlorophyll biosynthetic pathway may have been affected in these plants. It has been reported that hormones affect the morphology and physiology of a plant (Li *et al* 1992; Kusubas *et al* 1999; Fridborg *et al* 1999). We also measured the levels of cytokinins (isopentenyl adenosine, dihydrozeatin) and free polyamines in some of antisense plants. The cytokinins

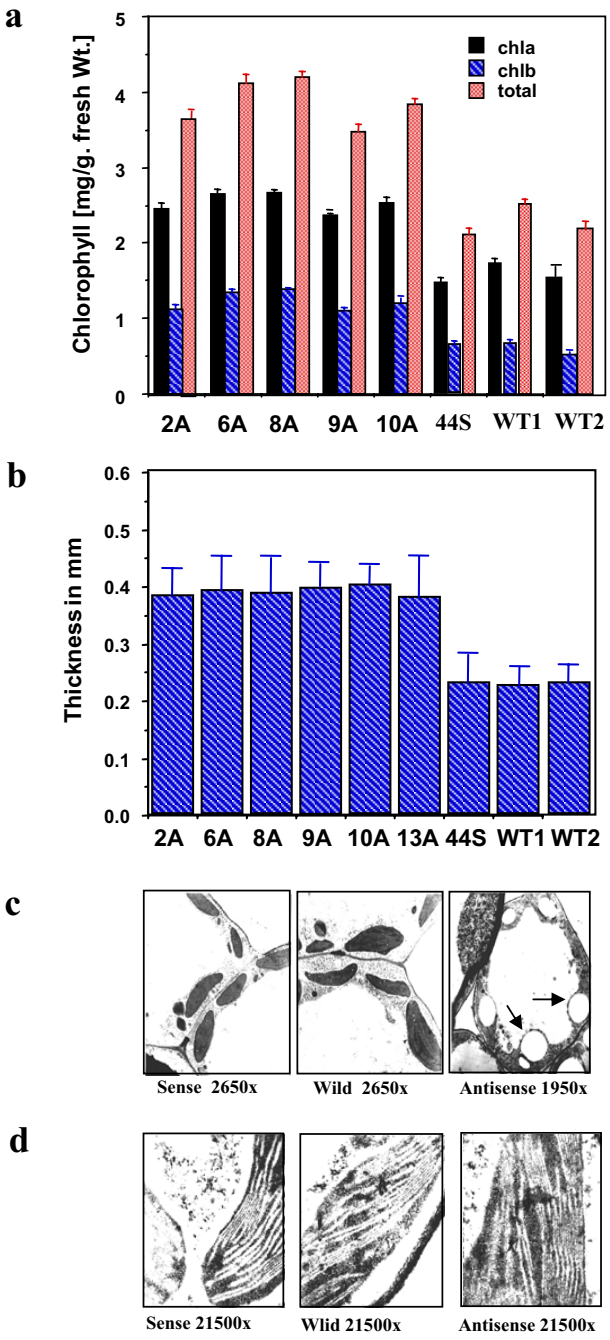

Figure 2. Chlorophyll levels and leaf thickness and structure of chloroplast in antisense transgenic tobacco plants. The chlorophyll level **(a)** and leaf thickness **(b)** in different *EhCaBP* antisense transgenic plants. Structure of chloroplast at low **(c)** and high **(d)** magnification in transmission electron microscopy. The arrowheads show starch granules inside a chloroplast.

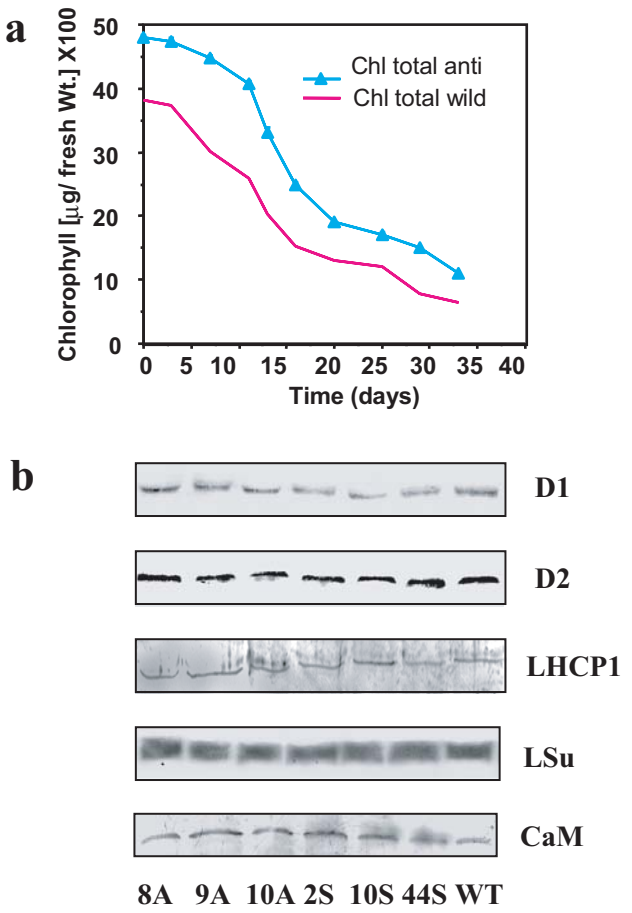

Figure 3. The rate of degradation of chlorophyll and western blot analysis to record changes in the level of certain proteins involved in photosynthetic apparatus and calmodulin. **(a)** Similar aged leaves of *EhCaBP* antisense transgenic and wild-type plants grown in a glass house were briefly washed and leaf discs of 1 cm^3 floated on water at 25 ± 2°C under a regime of 16 h light (100 $\mu E/m^{-2}/s^{-1}$) and 8 h darkness. Two leaf discs were frozen at regular intervals of 2 days and their chlorophyll content estimated. **(b)** Immunoblot analysis of *EhCaBP* transgenic (sense and antisense) and wild-type plants. Protein (30 μg) from the leaves was electrophoresed on 12% SDS-PAGE, transferred onto nitrocellulose and probed with polyclonal antibodies against D1 (1:5000), D2 (1:2000), LHCP1 (1:1000), and LSU (5000), CaM (1:2000) proteins involved in photosynthetic machinery and calmodulin. A, antisense; S, sense; WT, wild type.

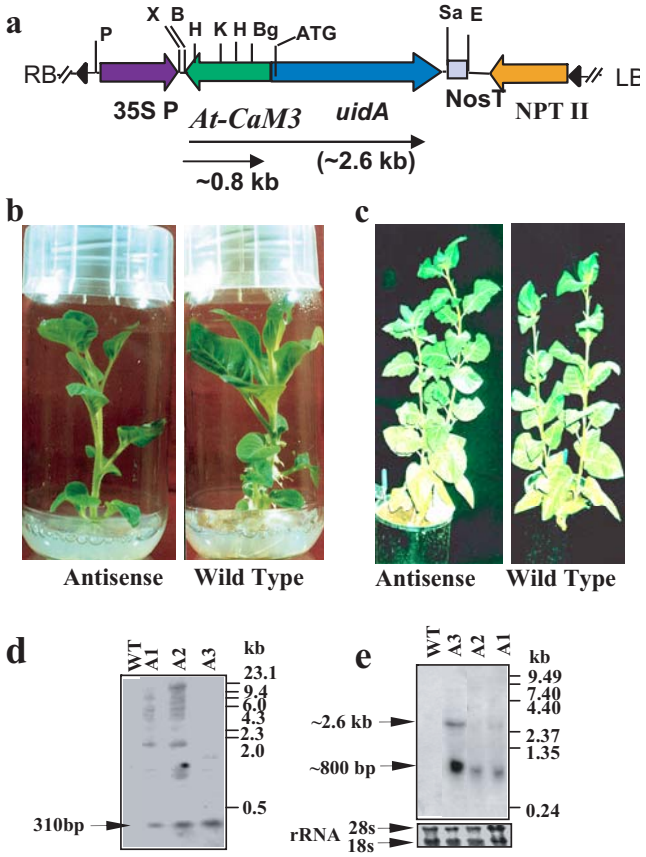

Figure 4. Confirmation and morphology of *CaM* antisense transgenic tobacco plants. Schematic representation of *AtCaM3* antisense construct. The ORF of *AtCaM3* was inserted into vector pPZP111 under CaMV 35S promoter in antisense orientation **(a)**. Morphology of plants transformed with *AtCaM3* in antisense construct (pZP111-antisense) and *in vitro* grown wild-type plants **(b)** and those in a glass house **(c)**. Southern hybridization of genomic DNA from GUS-positive transgenic plants digested with HindIII and probed with radiolabelled *AtCaM3* cDNA **(d)**. A, antisense; WT, wild type. Northern blot analysis using radiolabelled *AtCaM3* cDNA to show the transcription of *AtCaM3* **(e)**. A methylene blue stained blot is shown for equal loading of RNA.

and polyamines are well known for their ability to markedly delay or reverse leaf yellowing in various species of plants (Thimann 1985; Noodén 1988). The increase in cytokinins varied among the different antisense plants as seen in table 2, but their level was increased compared with the wild-type plants. The increase in one of the polyamines such as putricine in all the antisense plants suggests that either the ornithine decarboxylase level changed to make more putricine or the level of spermidine synthase decreased so that putricine accumulated and spermidine decreased. Whether the changes observed in the *EhCaBP* antisense plants were due only to the alteration in hormonal levels and polyamines cannot be said with certainty; however, this could be one of the factors.

One of the calcium-binding proteins, CaM, has been shown to affect a number of processes in plants (Trewavas and Malho 1997; McAnish and Hetherington 1998). Earlier reports have shown that over-expression of barley *CaM* led to an altered morphology whereas no phenotypic changes were observed in plants transformed with an antisense *CaM* construct (Zielinski *et al* 1990). However, potato CaM

antisense plants showed highly reduced growth whereas plants containing the sense construct exhibited strong apical dominance, produced elongated tubers and were taller than the wild-type (Poovaiah *et al* 1996). To check if the changes observed in *EhCaBP* antisense transgenics are due to changes in CaM, western blot analysis using anti-CaM antibodies was done. There was no significant change in the level of CaM protein. To confirm this, *CaM* antisense transgenic tobacco plants were raised. Again, no alteration in any phenotypic characters was observed. This further implied that it was only the *EhCaBP* antisense construct that led to an increase in chlorophyll and leaf thickness in tobacco plants. At present, we do not know the proteins or pathways that are affected in *EhCaBP* antisense plants; however, it seems that the altered morphology and increased chlorophyll were not observed in the CaM antisense transgenic plants.

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References

Albrecht V, Weinl S, Blazevic D, D'Angelo C, Batistic O, Kolukisaoglu U, Bock R, Schulz B, Harter K and Kudla J 2003 The calcium sensor CBL1 integrates plant responses to abiotic stresses; *Plant J.* **36** 457–470

Arnon D I 1949 Copper enzymes in isolated chloroplasts: polyphenol oxidase in *Beta vulgaris; Plant Physiol.* **24** 1–15

Bajaj S and Rajam M V 1996 Polyamines accumulation and near loss of morphogenesis in long-term callus cultures of rice: restoration of plant regeneration by manipulation of cellular polyamine levels; *Plant Physiol.* **112** 1343–1348

Batistic O and Kudla J 2004 Integration and channeling of calcium signaling through the CBL calcium sensor/CIPK protein kinase network; *Planta* **219** 915–924

Cheong Y H, Kim K N, Pandey G K, Gupta R, Grant J J and Luan S 2003 CBL1, a calcium sensor that differentially regulates salt, drought, and cold responses in *Arabidopsis*; *Plant Cell* **15** 1833–1845

Cheong Y H, Pandey G K, Grant J J, Batistic O, Li L, Kim BG, Kudla J and Sheng Luan 2006 Two calcium sensors and their interacting kinase regulate transpiration and potassium uptake in *Arabidopsis*; Submitted to *Plant J.*

D'Angelo C, Weinl S, Batistic O, Pandey G K, Cheong Y H, Schultke S, Albrecht V, Ehlert B, Schulz B, Harter K, Luan S, Bock R and Kudla J 2006 Alternative complex formation of the Ca-regulated protein kinase CIPK1 controls abscisic acid-dependent and independent stress responses in *Arabidopsis*; *Plant J.* **48** 857–872

Deswal R, Pandey G K, Chandok M R, Yadava N, Bhattacharya A and Sopory S K 2000 A novel protein kinase from *Brassica juncea* stimulated by a protozoan calcium binding protein: purification and partial characterization; *Eur. J. Biochem.* **267** 3181–3188

Fridborg I, Kuusk S, Mortiz T and Sundberg E 1999 The *Arabidopsis* dwarf mutant shi exhibits reduced gibberellin responses conferred by over expression of a new putative zinc finger protein; *Plant Cell* **11** 1019–32

Gong D, Gong Z, Guo Y, Chen X and Zhu J K 2002a Biochemical and functional characterization of PKS11, a novel *Arabidopsis* protein kinase; *J. Biol. Chem.* **277** 28340–28350

Gong D, Zhang C, Chen X, Gong Z and Zhu J K 2002b Constitutive activation and transgenic evaluation of the function of an *Arabidopsis* PKS protein kinase; *J Biol. Chem.* **277** 42088–42096

Guo Y, Qiu Q S, Quintero F J, Pardo J M, Ohta M, Zhang C, Schumaker K S and Zhu J K 2004 Transgenic evaluation of activated mutant alleles of SOS2 reveals a critical requirement for its kinase activity and C-terminal regulatory domain for salt tolerance in *Arabidopsis thaliana*; *Plant Cell* **16** 435–449

Guo Y, Xiong L, Song C P, Gong D, Halfter U and Zhu J K 2002 A calcium sensor and its interacting protein kinase are global regulators of abscisic acid signaling in *Arabidopsis*; *Dev. Cell* **3** 233–244

Hajdukiewicz P, Svab Z and Maliga P 1994 The two RNA polymerases encoded by the nuclear and the plastid compartments transcribe distinct groups of genes in tobacco plastids; *Plant Mol. Biol.* **25** 989–994

Halfter U, Ishitani M and Zhu J-K 2000 The *Arabidopsis* SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3; *Proc. Natl. Acad. Sci. USA* **97** 3747–3752

Hansen C E, Wenzler H and Frederick-Meins J R 1984 Concentration gradients of trans-zeatin riboside and trans-zeatin in the maize stems; *Plant Physiol.* **75** 959–963

Harding S A, Oh S H and Roberts D M 1997 Transgenic tobacco expressing a foreign calmodulin gene shows an enhanced production of active oxygen species; *EMBO J.* **16** 1137–1144

Harmon A C, Gribskov M and Harper J F 2000 CDPKs: a kinase for every Ca^{2+} signal?; *Trends Plant Sci.* **5** 154–159

Heo W D, Le S H, Kim M C, Kim J C, Chung W S, Chun H J, Lee K J, Park C Y, Park C H, Choi J Y and Cho M J 1999 Involvement of specific calmodulin isoforms in salicylic acid independent activation of plant disease resistance responses; *Proc. Natl. Acad. Sci. USA* **96** 766–771

Horsch R B, Fry J E, Hoffmann N L, Eicholtz D, Rogers S G and Fraley R T 1985 A simple and general method for transferring genes into plants; *Science* **227** 1229–1231

Hwang Y S, Bethke P C, Cheong Y H, Chang H S, Zhu T and Jones R L 2005 A gibberellin-regulated calcineurin B in rice localizes to the tonoplast and is implicated in vacuole function; *Plant Physiol.* **138** 1347–1358

Jefferson R A 1987 Assaying chimeric genes in plants: the GUS fusion system; *Plant Mol. Biol. Rep.* **5** 387–405

Karnovsky M J 1967 The ultrastructural basis of capillary permeability studied with peroxidase as a tracer; *J. Cell Biol.* **35** 213–236

Kim K N, Cheong Y H, Grant J J, Pandey G K and Luan S 2003 CIPK3, a calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in *Arabidopsis*; *Plant Cell* **15** 411–423

Kim K N, Lee J S, Han H, Choi S A, Go S J and Yoon I S 2003 Isolation and characterization of a novel rice Ca^{2}+-regulated protein kinase gene involved in responses to diverse signals including cold, light, cytokinins, sugars and salts; *Plant Mol. Biol.* **52** 1191–1202

Kolukisaoglu U, Weinl S, Blazevic D, Batistic O and Kudla J 2004 Calcium sensors and their interacting protein kinases: genomics of the *Arabidopsis* and rice CBL-CIPK signaling networks; *Plant Physiol.* **134** 43–58

Kudla J, Xu Q, Harter K, Gruissem W and Luan S 1999 Genes for calcineurin B-like proteins in *Arabidopsis* are differentially regulated by stress signals; *Proc. Natl. Acad. Sci. USA* **96** 4718–4723

Kusubas S, Kano-Murakami Y, Matsuoka M, Tamaoki M, Sakamoto T, Yamaguchi I and Fukumoto M 1998 Alteration of hormone levels in transgenic tobacco plants overexpressing the rice homeobox gene *OSH1*; *Plant Physiol.* **116** 471–476

Li Y, Hagen G and Guilfoyle T J 1992 Altered morphology in transgenic tobacco plants that overproduce cytokinins in specific tissues and organs; *Dev. Biol.* **153** 386–439

Li L, Kim B G, Cheong Y H, Pandey G K and Sheng L 2006 A Ca^{2+} signaling pathway regulates a K^{+} channel for low-K response in *Arabidopsis*; *Proc. Natl. Acad. Sci. USA* **103** 12625–12630

Liu J and Zhu J-K 1997 An *Arabidopsis* mutant that requires increased calcium from potassium nutrition and salt tolerance; *Proc. Natl. Acad. Sci. USA* **94** 14960–14964

Luan S, Kudla J, Rodriguez-Concepcion M, Yalovsky S and Gruissem W 2002 Calmodulins and calcineurin B-like proteins: calcium sensors for specific signal response coupling in plants; *Plant Cell* **14** S389–S400

Mahajan S, Sopory S K and Tuteja N 2006 Cloning and characterization of CBL-CIPK signalling components from a legume (*Pisum sativum*); *FEBS J.* **273** 907–925

Mayer K *et al* 1999 Sequence analysis of chromosome 4 of the plant *Arabidopsis thaliana*; *Nature* **402** 769–777

McAnish M R and Hetherington A M 1998 Encoding specificity in calcium signaling systems; *Trends Plant Sci.* **3** 32–36

Noodén L D 1988 The phenomenon of senescence and aging; in *Senescence and aging in plants* (eds) L D Noodén and A C Leopold (San Diego: Academic Press) pp 330–386

Pandey G K 1999 *Presence and role of homologues of EhCaBP* (*E. histolytica Calcium binding protein*) *in higher plants and characterization of a novel protein kinase from Brassica juncea*, PhD thesis, Jawahar Lal Nehru University, New Delhi

Pandey G K, Veena, Deswal R, Pandey S, Tewari S B, Tyagi W, Reddy V S, Bhattacharya A and Sopory S K 2001 Calcium signaling: downstream components in plants; in *Signal transduction in plants: current advances* (eds) S C Maheshwari, R Oelmuller and S K Sopory (New York: Kluwer Academic Publishers) pp 125–136

Pandey G K, Reddy V S, Reddy V S, Deswal R, Bhattacharya A and Sopory S K 2002 Transgenic tobacco expressing *Entamoeba histolytica* calcium binding protein exhibits enhanced growth and tolerance to salt stress; *Plant Sci.* **162** 41–47

Pandey G K, Reddy M K, Sopory S K and Singla-Pareek S 2002 Calcium homeostasis in plants: role of calcium binding proteins in abiotic stress tolerance; *Indian J. Biotechnol.* **1** 135–157

Pandey G K, Veena, Deswal R, Reddy V S, Bhattacharya A and Sopory S K 2003 Development of stress tolerance by manipulating the expression of calcium binding proteins; in *Biotechnology in sustainable biodiversity and food security* (ed.) B N Prasad (Enfield (NH), USA: Science Publishers) pp 42–49

Pandey G K, Cheong Y H, Kim K N, Grant J J, Li L, Hung W, D'Angelo C, Weinl S, Kudla J and Luan S 2004 The calcium sensor calcineurin B-like 9 modulates abscisic acid sensitivity and biosynthesis in *Arabidopsis*; *Plant Cell* **16** 1912–1924

Pandey G K, Cheong Y H, Grant J J, Kim B-G, Li L and Sheng Luan 2006 CIPK9, a calcium sensor-interacting protein kinase required for low-potassium tolerance in *Arabidopsis*; *Cell Res.* (in press)

Pandey S, Tewari S B, Upadhyaya K C and Sopory S K 2000 Downstream component in Ca^{2+} signaling; *Crit. Rev. Pl. Sci.* **19** 291–318

Pardo J M, Reddy M P, Yang S, Maggio A, Huh G-H, Matsumoto T, Coca M A, Paino-D'urzo M, Koiwa H, Yun D-J, Watad A A, Bressan R A and Hasegawa P M 1998 Stress signaling through Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin mediates salt adaptation in plants; *Proc. Natl. Acad. Sci. USA* **95** 9681–9686

Poovaiah B W, Takezawa D, An G and Han T J 1996 Regulated expression of a calmodulin isoform altered growth and development in potato; *J. Plant Physiol.* **149** 533–558

Prasad J, Bhattacharya S and Bhattacharya A 1992 Cloning and sequence analysis of a calcium binding protein gene from a pathogenic strain of *Entamoeba histolytica*; *Cell Mol. Biol. Res.* **39** 167–173

Reddy A S N 2001 Calcium: silver bullet in signaling; *Plant Sci.* **160** 381–404

Rudd J J and Franklin-Tong V E 2001 Unravelling response-specificity in Ca^{2+} signalling pathways in plant cells; *New Phytol*. **151** 7–33

Sanders D, Brownlee C and Harper J F 1999 Communicating with calcium; *Plant Cell* **11** 691–706

Sanders D, Pelloux J, Brownlee C and Harper J F 2002 Calcium at the crossroads of signaling; *Plant Cell* **14** S401–S417

Sambrook J, Fritsch E F and Maniatis T 1989 *Molecular cloning, a laboratory manual* (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory) 2nd edition

Sopory S K and Munshi M 1998 Protein kinases and phosphatases and their role in cellular signaling in plants; *Crit. Rev. Plant Sci.* **17** 245–318

Thimann K V 1985 The interaction of hormonal and environmental factors in leaf senescence. *Biol. Plant* **2** 83–91

Trewavas A J and Malho R 1997 Signal perception and transduction: the origin of the phenotype; *Plant Cell* **9** 1181–1195

Xu J, Li H D, Chen L Q, Wang Y, Liu L L, He L and Wu W H 2006 A protein kinase, interacting with two calcineurin B-like

proteins, regulates K^+ transporter AKT1 in *Arabidopsis*; *Cell* **125** 1347–1360

Yadava N, Chandok M R, Prasad J, Bhattacharya S, Sopory S K and Bhattacharya A 1997 Characterization of EhCaBP a calcium-binding protein *Entamoeba histolytica* and its binding proteins; *Mol. Biochem. Parasitol.* **84** 69–82

Zhu J K 2002 Salt and drought stress signal transduction in plants; *Annu. Rev. Plant Biol.* **53** 247–273

Zielinski R E, Ling V and Perera I 1990 Plant protein phosphorylation, protein kinases, calcium and calmodulin; in *Current topics in plant biochemistry and physiology 9* (Columbia, MO: Interdisc. Plant Biochem. Physiol. Prog. Univ., Missouri) pp 141–152

Zielinski R E 1998 Calmodulin and calmodulin-binding proteins in plants; *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49** 697–725

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