Regulated Expression of a Calmodulin Isoform Alters Growth and Development in Potato

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Summary

A transgene approach was taken to study the consequences of altered expression of a calmodulin iso-form on plant growth and development. Eight genomic clones of potato calmodulin (PCM1 to 8) have been isolated and characterized (Takezawa et al., 1995). Among the potato calmodulin isoforms studied, PCM1 differs from the other isoforms because of its unique amino acid substitutions. Transgenic potato plants were produced carrying sense construct of PCM1 fused to the CaMV 35S promoter. Transgenic plants showing a moderate increase in PCM1 mRNA exhibited strong apical dominance, produced elon-gated tubers, and were taller than the controls. Interestingly, the plants expressing the highest level of PCM1 mRNA did not form underground tubers. Instead, these transgenic plants produced aerial tubers when allowed to grow for longer periods. The expression of different calmodulin isoforms (PCM1, 5, 6, and 8) was studied in transgenic plants. Among the four potato calmodulin isoforms, only the expression of PCM1 mRNA was altered in transgenic plants, while the expression of other isoforms was not signifi-cantly altered. Western analysis revealed increased PCM1 protein in transgenic plants, indicating that the expression of both mRNA and protein are altered in transgenic plants. These results suggest that increas-ing the expression of PCM1 alters growth and development in potato plants.

Key words: Solanum tuberosum L., calmodulin, tuberization and transgenic plants.

Introduction

Calmodulin, a Ca^{2+}-binding regulatory protein is known to play a pivotal role in Ca^{2+} signaling in eukaryotes. Calmo-dulin is considered to be multifunctional because of its ability to interact and regulate the activity of a number of other pro-teins (Cheung, 1980; Roberts et al., 1986; Poovaiah and Reddy, 1987; Poovaiah and Reddy, 1993; Hanson et al., 1994). It is well established that many Ca^{2+}-regulated proces-ses are mediated by calmodulin in both plants and animals (Cheung, 1980; Roberts et al., 1986; Paliyath and Poovaiah, 1984; Veluthambi and Poovaiah, 1984; Rasmussen and Means, 1989; Klee, 1991).

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The presence of multiple calmodulin genes has been reported in animals (Nojima, 1987, 1989; Hardy et al., 1988; Fischer et al., 1988). The existence of calmodulin isoforms has been reported in plants (Poovaiah et al., 1992; Perera and Zielinski, 1992; Gawienowski et al., 1993; Botella and Arteca, 1994; Takezawa et al., 1995). However, the role of these isoforms in plant growth and development is not clearly understood. Altering calmodulin levels is known to affect cell growth and development (Chafouleas et al., 1984; Roberts et al., 1986; Davis and Thorner, 1989; Ohya and Anraku, 1989). Furthermore, decreased calmodulin levels by antisense RNA arrested the cell cycle (Rasmussen and Means, 1989).

Calcium and calmodulin are known to influence tuberiza-tion in potato (Balamani et al., 1986; Jena et al., 1989). To study the role of calmodulin isoforms in plant growth and development, several calmodulin genes were cloned and char-acterized (Takezawa et al., 1995). Among these calmodulin genes (PCM1-8), the expression of PCM1 was highest in the

stem and stolon tip. Studies with transgenic plants carrying the PCM1 promoter fused to the GUS reporter gene revealed that promoter activity is highest in the shoot apex and in the tip of the developing tuber, suggesting that PCM1 may play an important role in shoot growth and tuberization (Takezawa et al., 1995). The role of PCM1 in growth and development was studied by using transgenic potato plants carrying sense construct of PCM1 cDNA fused to the CaMV 35S promoter.

Materials and Methods

Production of transgenic plants carrying sense construct of PCM1

The expression vector used for this study was the binary vector Ti-plasmid pGA748 which was derived from pGA643 (An et al., 1988) by replacing the small HindIII-ClaI fragment with a synthetic oligonucleotide containing multiple cloning sites. The EcoRI site at the left border of pGA643 was destroyed by filling in the sticky ends with dNTPs and DNA polymerase Klenow fragment before addition of the oligonucleotide. The resulting plasmid pGA748 carries seven unique restriction sites (HindIII-ScaI-MluI-XhoI-EcoRI-ClaI-BglII) between the CaMV 35S promoter and the terminator of gene 7. We have previously reported the isolation of a cDNA clone of approximately 1.1 kb EcoRI fragment carrying the potato calmodulin cDNA clone, PCM-1 (Jena et al., 1989) that contains the entire coding region as well as the 5' and 3' untranslated regions. The 636 bp ScaI-HpaI fragment containing the calmodulin coding region was used for construction of two plasmids by inserting it into the ScaI site of pGA748. The plasmid pGA924 contains the sense PCM1 ORF. The binary plasmid pGA924 was transferred into Agrobacterium tumefaciens LBA4404 by the direct DNA transfer method (An et al., 1988).

Agrobacterium tumefaciens LBA4404 harboring the binary vector was used for transformation of potato (Solanum tuberosum L. var. Russet Burbank; An et al., 1988). The internode sections from in vitro cultured potato seedlings of Russet Burbank were co-cultivated for three days with A. tumefaciens as described by Sheerman and Bevan (1988). Kanamycin resistant transformants were selected after four to eight weeks. Transgenic and untransformed control plants were moved to the greenhouse to study their growth and development. Tubers from the transgenic and control plants were harvested, stored, and replanted on three successive occasions over a two year period.

Northern analysis

Total RNA was isolated as described by Verwoerd et al. (1989) and 5 µg of the RNA was denatured with glyoxal/DMSO, electrophoresed in 1% agarose in 10 mmol/L sodium phosphate buffer (pH 7.0) and transferred to Zeta-probe membrane (BIO-RAD). The 3' untranslated region of PCM1 and other isoforms (PCM5, 6, and 8) were used as templates to prepare riboprobes specific for the sense strand. Hybridization was performed as described by Sambrook et al. (1989). Following hybridization, the filters were washed at 65 °C in solution containing 0.1× SSC and 0.1% SDS.

Western analysis

Calmodulin isoforms (PCM1 and PCM6) were expressed in E. coli using the pET3b expression vector (Studier et al., 1990). These expressed proteins were purified as described by Fromm and Chua (1992). Plant extract (30 µg) was separated on the 12.5% SDS-PAGE and transferred onto a PVDF membrane (Millipore). Western analysis was carried out using anti-calmodulin monoclonal antibody as described by Jablonsky et al. (1991).


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            * * * * * *
PCM 1            AEQLTEEQIAEFKEAFSLFDKDGDGCITTKELGTVMRSL
PCM 5,6,8        -D----D--S-----------------------------
Arabidopsis CaM-2 -D----D--S-----------------------------
Barley CaM-1     -D----D--------------------------------
Chick            -D---------------------------T---------

            * * * * *
PCM 1            GQNPTEAELQDMISEADADQNGTIDFPEFLNLMARK
PCM 5,6,8        -------------N-V---G----------------
Arabidopsis CaM-2 -------------N-V---G----------------
Barley           -------------N-V---G----------------
Chick            -------------N-V---G---------TM-----

            * * * * * *
PCM 1            MKDTDSEEELKEAFKVFDKDQNGFISAAELRHVMTNL
PCM 5,6,8        --------------R---------------------
Arabidopsis CaM-2 --------------R---------------------
Barley           --------------R---------------------
Chick            ---------IR---R-----G--Y------------

            * * * * *
PCM 1            GEKLTDEEVDEMIREADIDGDGQVNYEEFVRMMLAK
PCM 5,6,8        ----------------V-----I--D---KV-M--
Arabidopsis CaM-2 ---------------K---V-----I--D---KV-M--
Barley           ----------------V-----I--D---KV-M--
Chick            -----------------------------Q--T--
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Fig. 1: Amino acid sequence comparison of different calmodulin genes (PCM1, 5, 6 and 8) from potato with Arabidopsis CaM-2 (Ling et al., 1991), barley CaM-1 (Ling and Zielinski, 1989), and chick calmodulin (Putkey et al., 1983). Asterisks indicate the position of amino acids involved in Ca^{2+}-binding. The sequence in the fourth Ca^{2+}-binding region towards the C-terminus are shown (bottom).
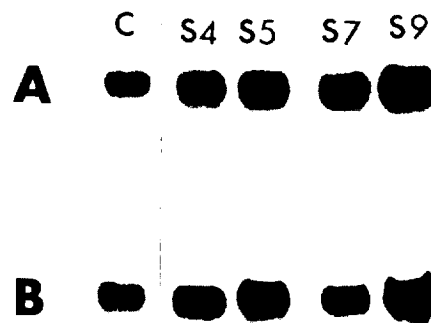

Fig. 2: Northern analysis showing the expression pattern of PCM1 in stem and leaf tissues of transgenic potato plants (Russet Burbank). (A) leaf tissue (B) stem tissue of transgenic plants carrying sense construct of PCM1 fused to the CaMV 35S promoter. Control (C); 4 sense plants (S4, S5, S7, and S9). Since the expression in leaf is much lower than in the stem, the autoradiograph for leaf RNA was exposed for longer periods.

Results and Discussion

Comparison of amino acid sequences of calmodulin isoforms

Comparison of the deduced amino acid sequences of different potato calmodulin genes (PCM1, 5, 6 and 8) with Arabidopsis ACaM-2 (Ling et al., 1991), barley (Ling and Zielinski, 1989), and chicken (Putkey et al., 1983) revealed that PCM1 has several amino acid substitutions, especially in the fourth Ca^{2+}-binding region, suggesting a unique role for this isoform (Fig. 1). In contrast, PCM5, 6 and 8 are closely related and resemble other known plant calmodulins.

Fig. 3: Comparison of growth and tuberization of transgenic plants carrying sense construct of calmodulin driven by the CaMV 35S promoter. (**A**), Control and 3 transgenic plants carrying sense construct. Plant on the left is the control; to the right are the 3 sense plants (left to right: S4, S5 and S7). Comparison of tuber shape and size with control (**B**) and S7 transgenic plant (**C**). Tuberization pattern of S9 transgenic plant expressing the highest amount of PCM1 (**D**), plant on the left is the control. Photograph showing aerial tubers on the S9 transgenic sense plant (**E**).

Altered growth response of transgenic plants carrying the CaMV 35S promoter fused to sense or antisense construct of PCM1

To study the functional role of PCM1 in growth and development, transgenic potato plants carrying sense construct of PCM1 cDNA (Jena et al., 1989) driven by the CaMV 35S promoter were produced. Northern analysis was carried out using total RNA from leaf and stem of transgenic plants to study altered PCM1 mRNA. As shown in Figs. 2 A and 2 B, PCM1 mRNA was increased in sense plants as compared to controls. These transgenic plants showed differences in internode elongation, apical dominance, height, and branching (Fig. 3). These transgenic plants also varied in their tuberization patterns as compared to the control (Fig. 3). Among several transgenic plants, four transformants carrying the sense construct that showed variations in growth and development were studied in detail.

The sense plants were categorized into three groups based on calmodulin mRNA levels. The first group showed no increase in the calmodulin mRNA (S2, S6 and S8). The second group showed a moderate increase in calmodulin mRNA (S4, S5, S7 in Fig. 2), while the third group showed an increase of mRNA of more than five fold higher as compared to the first group and the untransformed controls (S9, Fig. 2). The first group did not show any phenotypic differences as compared to the untransformed controls. Plants in the second group with a moderate increase in calmodulin mRNA were taller and showed increased stem elongation and strong apical dominance as compared to the controls (Fig. 3 A). At the time of harvest, the average height of the control plants was 168 ± 4.15 cm and the average height of the sense plants (S4, S5 and S7) was 185 ± 6.02 cm, 203 ± 6.06 cm, and 203 ± 4.76 cm, respectively (Fig. 3 A). The increased height of the sense plants was primarily due to increased internode elongation. The average internode length of the controls was 3.84 ± 0.09 cm and the average internode length of sense plants (S4, S5 and S7) was 4.77 ± 0.15 cm, 5.07 ± 0.15, and 5.04 ± 0.12 cm, respectively. The average number of internodes in control and sense plants was not significantly different.

Transgenic plants in the second group with a moderate increase in calmodulin mRNA (Fig. 2, S4, S5 and S7) formed elongated tubers (Fig. 3 C). These results indicate a correlation between PCM1 expression and tuber development. However, in transgenic sense plants in group 3 with the highest mRNA (Fig. 2, S9) tuber formation was inhibited while stolon elongation was dramatically increased (Fig. 3 D). Interestingly, when these plants (S9) were allowed to grow for longer periods, they formed aerial tubers (Fig. 3 E). Consistent results were obtained when the tubers from these transgenic plants were successively harvested, stored to break dormancy, and replanted on three separate occasions over a two year period.

Expression of calmodulin isoforms in transgenic plants

Since calmodulin genes are highly conserved, altering the level of PCM1 mRNA could also influence the levels of other isoforms. Therefore, we have studied the expression of other
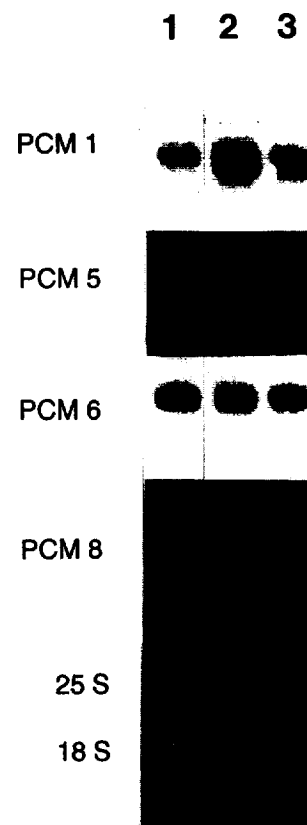

Fig. 4: Northern analysis using gene specific probes showing the expression of different isoforms (PCM1, 5, 6, and 8) in leaf tissues. Control (lane 1), S9 transgenic plant (lane 2) and S7 transgenic plant (lane 3). Ethidium bromide-stained gel showing that similar amounts of RNA were loaded in each lane. 25S and 18S ribosomal RNA bands are shown.

isoforms in transgenic plants (S7 and S9) by northern analysis. The expression of PCM5, 6 and 8 was not significantly altered in these transgenic plants (Fig. 4). These results suggest that the phenotype of sense plants is predominately due to PCM1.

Changes in calmodulin levels in transgenic plants

To correlate the phenotypic effects observed in the transgenic plants to the changes in the protein level, western analysis was performed. To distinguish PCM1 from other calmodulin isoforms, E. coli expressed PCM1 and PCM6 were used as standards. Even though PCM5, 6, and 8 are coded by different genes (Takezawa et al., 1995), their deduced amino acid sequences are identical (Fig. 1). Hence, we have chosen PCM6 as a representative for comparison from this group. PCM1 and PCM6 can easily be distinguished based on their mobility on SDS-PAGE (Fig. 5). Transgenic plants (e.g., S7, S9) showed a three to five fold increase in protein as compared to control plants.

We have previously generated transgenic tobacco plants carrying the potato calmodulin cDNA (PCM1) in sense and
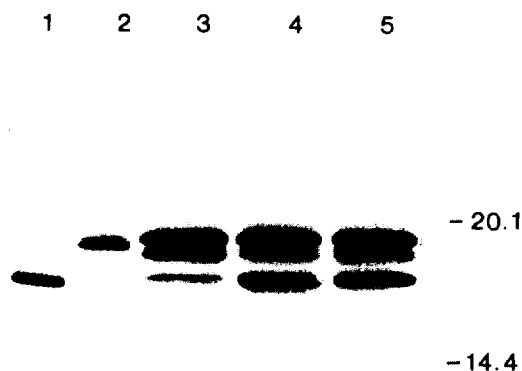
1   2   3   4   5

– 20.1

– 14.4

Fig. 5: Western analysis showing the expression of different calmodulin isoforms in control and transgenic plants. *E. coli* expressed PCM1 (lane 1), PCM6 (lane 2), untransformed control (lane 3), S9 transgenic plant (lane 4) and S7 transgenic plant (lane 5). Molecular weight standards (kDa) are marked.

antisense orientations driven by the CaMV 35S promoter (Poovaiah et al., 1992). These transgenic plants did not reveal significant differences in growth and development. Introduction of barley calmodulin into tobacco did not reveal significant differences in growth and development (Zielinski et al., 1990). Roberts et al. (1992) produced transgenic tobacco plants carrying the methylation mutant (VU-3) calmodulin and normal (VU-1) calmodulin. Transgenic plants expressing the calmodulin methylation mutant (VU-3) showed decreased stem internode, reduced seed and pollen viability, and reduced seed production. However, the transformants expressing (VU-1) were found to be indistinguishable from the control plants. The differences in the transgenic plants carrying VU-3 were attributed to mutant calmodulin. It is evident from these studies that altering the levels of specific isoforms of calmodulin or the post-translational modification of calmodulin can alter growth and development in plants.

Acknowledgments

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