Identification of multiple PEPC isogenes in leaves of the facultative Crassulacean Acid Metabolism (CAM) plant *Kalanchoe blossfeldiana* Poelln. cv. Tom Thumb

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Abstract In the facultative Crassulacean Acid Metabolism (CAM) plant *Kalanchoe blossfeldiana* cv. Tom Thumb, CAM can be induced by short-day treatment or water deficiency stress. From young leaves of well-watered and water-stressed individuals of this plant, cDNA clones coding for a partial sequence of the key enzyme of CAM, phosphoenolpyruvate carboxylase, were isolated after transcription of mRNA. cDNA polymorphism was established by enzyme restriction profiles and sequencing data. Four PEPC isogenes could be shown to exist in *K. blossfeldiana* forming two gene pairs, with 95%–98% homology inside and only 75% between the pairs. One cDNA sequence pair having a length of 1113 bp and an open reading frame of 371 AA was identified as PEPC isoform specific for the C_3 state, whereas the pair having a length of 1116 bp and an open reading frame of 372 AA could be attributed to the CAM state. These results were confirmed by Southern Blot hybridization. (EMBL, accession numbers X 87818, X 87819, X 87820, X 87821.)

Key words: Crassulacean Acid Metabolism (CAM); Gene family; *Kalanchoe*; Phosphoenolpyruvate carboxylase; Phylogenetic tree

1. Introduction

Phosphoenolpyruvate carboxylase (EC 4.1.1.31., PEPC) is a multifunctional enzyme and ubiquitous in plant kingdom. Using biochemical or immunological methods, different isoforms have been separated and shown to be tissue-specific or linked to specific physiological roles [1]. The most extensive studies performed on this enzyme concerned its role as the primary carboxylating enzyme in C_4 photosynthesis and Crassulacean Acid Metabolism (CAM) [2]. More recently, there were attempts to characterize PEPC also on the level of the genes. Up to now, these studies comprise four species of prokaryots and 14 species of higher plants and lead to the view that PEPC is encoded by a multigene family whose polymorphism and likely phylogenetic relations have been described by Lepiniec et al. [3]. Further analyses of PEPC gene structure performed on the C_4 plants *Zea mays, Sorghum vulgare* and *Flaveria trinervia* revealed that in these species three genes exist which were classified as 'housekeeping', root-inherent and light-inducible photosynthetic PEPC isoforms, respectively [4].

As far as CAM is concerned, the only deep-going studies on PEPC gene diversity and expression were conducted in the halophilic facultative CAM plant *Mesembryanthemum crystallinum* which shifts from C_3 photosynthesis to CAM upon the action of a set of environmental or endogenous factors [5]. In this plant, the PEPC gene family was shown to consist of two gene isoforms, with one of that genes (ppc1) being transcribed upon salt stress and supposed to be specifically linked to functioning of CAM [6]. However, aiming to understand whether the existence and expression of specific PEPC genes are prerequisits for the performance of CAM, it is necessary to extend the investigations also on facultative CAM plants other than *M. crystallinum*. Therefore, we have initiated a study on the PEPC genes in *Kalanchoe blossfeldiana* Poelln. (Crassulaceae). Although this plant is actually the first discovered example of a facultative CAM plant [7], amazingly enough it has not yet been included into the investigations of PEPC genes. In *K. blossfeldiana*, CAM can be induced by photoperiod, i.e. by short-day treatment [7,8], ageing of the leaves [9], water deprivation stress [10] and application of external hormones [11]. In any of these modalities, CAM induction was found to be paralleled by increase in PEPC activity due to neosynthesis of the enzyme [12], by accumulation of PEPC-specific mRNA [13] and by transcriptional activation, as established by run-off assays [14]. These findings suggest that also in *K. blossfeldiana* PEPC gene expression is an important step in CAM induction. The present study deals with the diversity of PEPC genes in *K. blossfeldiana* and the question of whether or not in this facultative CAM plant members of the PEPC gene family can be specifically attributed to the C_3 and the CAM modes of photosynthesis, respectively.

2. Materials and methods

2.1. Plant material

K. blossfeldiana (Poelln.) cv. Tom Thumb was propagated via cuttings deriving from the clone described earlier [8]. Plants were cultivated during 2 months on an artifical substrate and optimally watered with standard nutrients. The plants were 2 months old when used in the experiments. To maintain C_3 performance in the young leaves, the experimental plants were cultivated according to [12], under long-day conditions (16 h light/8 h darkness; quantum flux density (PAR) during day of 300 μmol m^{-2} s^{-1}) and with a thermoperiod of 27°/17°C (day–night). Induction of CAM was achieved by totally suppressing watering [12], with otherwise exactly the same conditions as described above. Extraction of RNA or DNA was performed from young leaves (number 2 from the apex) sampled on the droughted or the control plants.

2.2. RNA and DNA isolation

Extraction of total RNA followed the guanidine isothiocyanate method [15]. For final purification, total RNA was centrifuged through a dense cushion of caesium chloride. The RNA pellet was dissolved in

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diethyl pyrocarbonate-treated distilled water, precipitated and stored at −80°C. DNA was isolated after the protocol described by Murray and Thompson [16].

2.3. PCR amplification

A specific PCR amplification was performed using 18–20 bases mixed oligonucleotides corresponding to conserved regions in the 3′ part of PEPC genes from *Sorghum*, *Flaveria*, tobacco and maize (Pepc1 at the 5′ side: TC(CTA)GA(TC)TC(CAT) GG(AC)AA(AG)GA(TC) GC and Pepc2 at the 3′ side: GC(GAT)GC(GAT)AT(GCA)CC (CT)TTCAT(GT)GT). Oligo(dT)-primed single-stranded cDNA was synthesized from 4–6 μg total RNA. Excess oligo(dT) was removed by centrifugation through Microcon filter units (Amicon, Witten, Germany). Amount of 1/20th of the cDNA was PCR amplified in a 100 μl reaction volume containing 10 mM Tris/HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 25 pmol primer and 2.5 U Taq polymerase. A number of 35 cycles were run, each cycle consisting of 30 s of denaturation at 95°C, 45 s of annealing at 55°C and for extension 120 s at 72°C. An aliquot containing 1/10th of the amplified product was analysed by agarose gel electrophoresis. Amplified products were purified by Microcon 100 filter units according to the manufacturer's instruction and blunt end subcloned in pUC18 for further sequence determination.

2.4. Restriction analysis

cDNA fragments obtained by PCR amplification were cloned in pUC18 vector and transformed in *E. coli* DH5α competent cells. Plasmid cDNA from the bacterial positive clones were digested by five restriction enzymes (*Hin*dIII, *Bam*HI, *Pst*I, *Eco*RI, *Sal*I) and analysed on 0.8% agarose gels.

2.5. Hybridization analysis

For Southern blot analysis, 5–10 μg DNA was subjected to electrophoresis in a 0.8% (w/v) agarose gel and blotted for 15 h using 1.5 M NaCl, 0.25 M NaOH as transfer buffer. Prehybridization and hybridization were done in a solution containing 6 × SSC, 5 × Denhardt's and 0.1% SDS with the addition of 1.5×10^6 cpm radiolabeled cDNA probe/ml to the hybridization solution. For each cDNA probe, TM was calculated with 2°C for each A and T nucleotide and 4°C for each G and C nucleotide and hybridization was performed 5°C below TM for 12–18 h. Filters were washed at room temperature once in 6 × SSC for 15 min, at hybridization temperature once in 2 × SSC for 30 min, 2 × SSC, 0.1% SDS for 30 min and finally for 15 min in 0.2 × SSC, 0.1% SDS. (The *Sorghum* probe was a gift from the late Dr. C. Cretin, Université Paris Sud; Orsay, France.)

2.6. DNA sequence analysis

cDNA inserts subcloned in pUC18 were sequenced by the dideoxy chain termination method using with the T7 DNA polymerase and [α-^{35}S]dATP. Both strands of the DNA inserts were sequenced using either pUC18 universal and reversed primers or specific oligonucleotides with length of 18–20 bases derived from previous sequencing data. Sequence similarities of the PEPC-DNA and protein sequences were determined by PC-aided analysis by using the program DNASIS (Hitachi). Phylogenetic relationsships were derived from the aligned amino acid sequence data by the Higgins and Sharp [17] algorithm CLUSTAL 4.

Table 1
Homology values between the four *K. blossfeldiana* PEPC isoforms (ppc Kb1-ppc Kb4) with the corresponding regions of the PEPC isoforms from *Z. mays* (C_4), *S. vulgare* (C_4), *F. trinervia* (C_4), *F. springlei* (C_3), *G. max* (C_3), *Saccharum* hybrid (C_3), *M. crystallinum* (C_3, CAM) and *M. sativa* (C_3).

Plant species		ppc-Kb1 (%)	ppc-Kb2 (%)	ppc-Kb3 (%)	ppc-Kb4 (%)
Z. mays	C_4	73.2	73.8	76.6	76.9
S. vulgare	C_4	71.9	72.4	75.8	76.1
F. trinervia	C_4	76.2	76.8	83.3	83.6
F. springlei	C_3	79.0	79.5	85.2	85.5
G. max	C_3	80.1	80.6	84.6	84.9
Saccharum hybrid	C_3	79.0	78.4	84.3	84.9
M. crystallinum	C_3	79.6	79.8	84.7	85.0
M. crystallinum	CAM	77.9	78.4	82.4	82.4
M. sativa	C_3	79.8	80.3	84.6	84.9

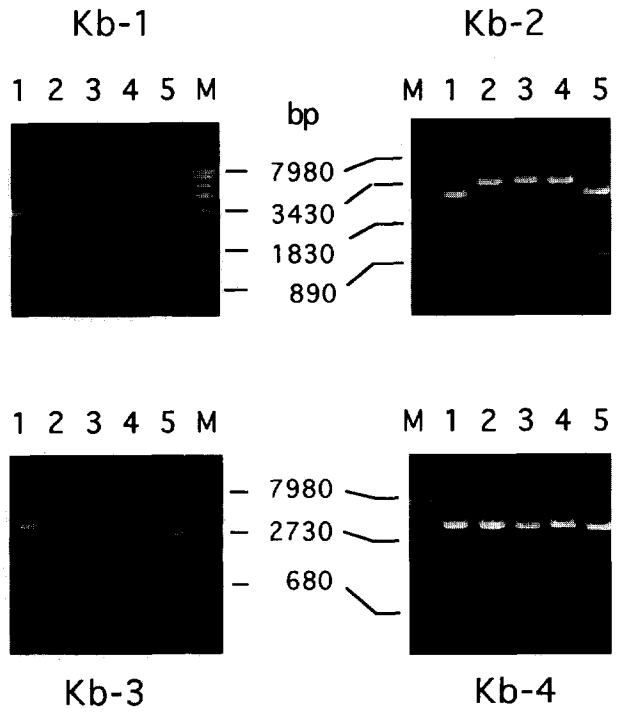

Fig. 1. Agarose gel analyses of the four different PEPC clones (Kb-1–Kb-4) from *K. blossfeldiana*. Lane 1 shows the restriction pattern with *Hin*dIII, lane 2 with *Eco*RI, lane 3 with *Pst*I, lane 4 with *Sal*I and lane 5 with *Bam*HI. M is the molecular standard.

3. Results and discussion

3.1. Identification of multiple PEPC-cDNA clones

A number of 18 positive identified PEPC-cDNA clones of *K. blossfeldiana* after ligation of the ~1100-bp fragment amplified by PCR were investigated by restriction with five different enzymes. Agarose gel analysis showed four different restriction patterns (Fig. 1). The clone Kb-1 (3 of the 18 clones) shows one restriction site for *Hin*dIII and one for *Sal*I (probably, there is one *Bam*HI site). The clone Kb-2 (7 of the 18 clones) shows one restriction site for *Hin*dIII and *Bam*HI. The Kb-3 clone (5 of the 18 clones) shows one restriction site for *Pst*I and *Bam*HI. Finally, the clone Kb-4 (3 of the 18 clones) does not show any site for the five mentioned restriction enzymes.

3.2. Southern hybridization analysis

Southern blot hybridization was carried out to investigate the copy number of genomic sequences encoding the PEPC isoforms in *K. blossfeldiana* genome and corresponding to the four cDNAs obtained. Total genomic DNA was digested by *Bam*HI, *Hin*dIII and *Eco*RI restriction enzymes. The restricted DNA fragments were transfered on Hybond N membrane (Amersham) and hybridized with a specific C_3 type *Sorghum* PEPC probe or with the Kb-1, Kb-2 and Kb-3 cDNA fragments. The hybridization with the C_3 *Sorghum* probe (Fig. 2A) indicates four hybridization signals for the *Eco*RI and the *Bam*HI restriction. Thereby, the first signal in the *Bam*HI restriction is a double band. The restriction with *Hin*dIII shows

only two very strong signals, presumably both are also double signal bands, because the gel run was too short. In Fig. 2B, therefore, a longer gel run is shown, with four different hybridization with radiolabelled probes of *Sorghum*, Kb-1, Kb-2 (*Eco*RI digestion) and Kb-3 (*Bam*HI digestion). The multiple-band patterns observed confirm the existence of the four isogenes encoding PEPC in this plant species. When genomic DNA was digested by *Bam*HI or *Hin*dIII and the fragments hybridized with the Kb-2 clone or with the Kb-3 clone, different patterns could be obtained (data not shown), suggesting that these two latter cDNA can be used as specific probes.

3.3. Sequence analysis

Sequence analysis revealed that the four PEPC-cDNA clones can be separated in two pairs, viz. Kb-1/Kb-2 and Kb-3/Kb-4. Within each pair,the degree of homology was very high, viz. 95% in the case of Kb-1/Kb-2 and 98% in the case of Kb-3/Kb-4. Contrastingly, the degree of homology between the pairs was only 75% (Fig. 3). The alignment shows that in the pair Kb-3/Kb-4 two codons are missing, viz. one methionine (position 117) and one glutamine codon (position 339). Only one alanine codon is missing in the clone Kb-2 (position 242) and one valine codon (position 249) in the clone Kb-1. The restriction maps deduced from the nucleid sequence data show similar profiles between the members of each pair and confirm the experimental results found in the restriction analysis. The *Bam*HI site is confirmed in the Kb-1 clone. The motif **FHGRGGTVGRGGGPTHL** (residues 37–53) is highly conserved among all eukaryotic and prokaryotic PEPC analysed so far and there is a great evidence that this part of the phosphoenolpyruvate carboxylase enzyme is involved in PEP binding and catalytic activity [1,18].

Using the Higgins and Sharp algorithm CLUSTAL 4 [17] (based on progressively aligning sequences according to the branching order in an initial phylogenetic tree), we have compared the amino acids sequences of the PEPC isoforms of *K. blossfeldiana* (derived from the nucleotide sequences) with

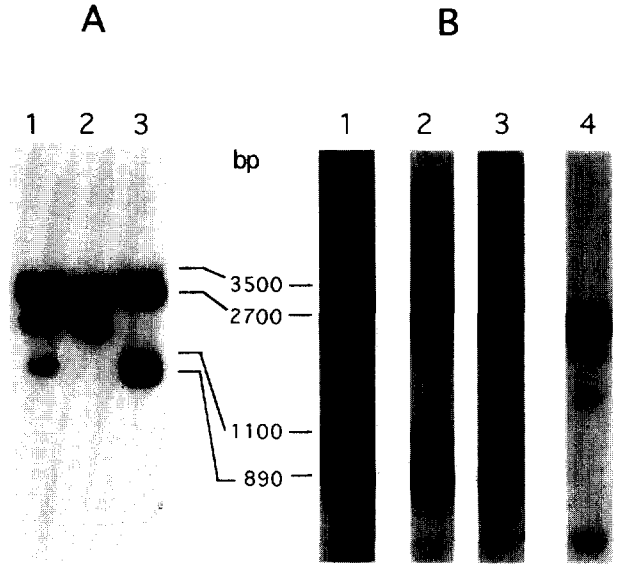

Fig. 2. (A) Hybridization with radiolabelled probe of *Sorghum* C_3. Lane 1 *Bam*HI, lane 2 *Hin*dIII and lane 3 *Eco*RI restriction. (B) Hybridization with radiolabelled probe of *Sorghum* C_3, Kb-1, Kb-2 and Kb-3 cDNA fragment. Lane 1–3 *Eco*RI digestion and lane 4 *Bam*HI digestion.

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ppc-Kb1  SDSGKDAGRLSAAWELYKAEEELIEVAKQHGVKLTMFHGRGGTVGRGGGPTHLAILSQPA   60
ppc-Kb2  SDSGKDAGRLSAAWQLYKAQEELIEVAKQHGVKLTMFHGRGGTVGRGGGPTHLAILSQPA
         ************** **** * *  ***  ****************************
ppc-Kb3  SDSGKDAGRLSAAWQLYKAQEDLVKVAKEFGVKLTMFHGRGGTVGRGGGPTHLAILSQPP
ppc-Kb4  SDSGKDAGRLSAAWQLYKAQEDLVKVAKEFGVKLTMFHGRGGTVGRGGGPTHLAILSQPP

ppc-Kb1  DTIQGSLRVTIQGEVIERSFGEAQLCFKTLQRYTAATLEHGMIPPSSPKQECRACLMDEM   120
ppc-Kb2  DTIQGSLRVTIQGEVIERSFGEAQLCFKTLQRYTAATLEHGMIPPSSPKQEWRACLMDEM
         *** ****** ****** **** *** ************ * ** **  ***  * ***
ppc-Kb3  DTIHGSLRVTVQGEVIEQSFGEEHLCFRTLQRYTAATLEHSMCPPASPEPEWRELL-DEM
ppc-Kb4  DTIHGSLRVTVQGEVIEQSFGEEHLCFRTLQRYTAATLEHSMCPPASPEPEWRELL-DEM

ppc-Kb1  AVVTTERYRSIVFREPRFVEYFRLATPELEYGRMNIGSRPSKRKPSGGIESLRAIPWIFA   180
ppc-Kb2  AVVTTERYRSIVFREPRFVEYFRLATPELEYGRMNIGSRPSKRKPSGGIESLRAIPWIFA
         **  ** ** *** *******   *********** ************************
ppc-Kb3  AVAATEEYRTIVFHEPRFVEYSASATPELEYGRMNVGSRPSKRKPSGGIESLRAIPWIFA
ppc-Kb4  AVAATEEYRTIVFHEPRFVEYSASATPELEYGRMNIGSRPSKRKPSGGIESLRAIPWIFA

ppc-Kb1  WTQTRFHLPVWLGFGEAFRHVIDKDNKNLLMLQQMYNEWPFFRVTIDLVEMVFAKGDPGI   240
ppc-Kb2  WTQTRFHLPVWLGFGEAFRHVIDKDNKNLLMLQQMYNEWPFFRATIDLVEMVFARGNPGI
         *************** ** ****** ******* *** ***** ************ ***
ppc-Kb3  WTQTRFHLPVWLGFGAAFKHVIDKDIKNLLMLQEMYNRWPFFRVTIDLVEMVFAKGDPGI
ppc-Kb4  WTQTRFHLPVWLGFGAAFKHVIDKDIKNLLMLQEMYNRWPFFRVTIDLVEMVFAKGDPGI

ppc-Kb1  AALYDKLL-SEELWPLGEQLRTAYNDTKSYLLKITGHNEILEGDPFLKQRLKVRTAYITT   300
ppc-Kb2  A-LYDKLLVSEELWPLGEQLRTAYNDTKSYLLKITGHNEILEGDPFLKQRLKVRTAYITT
         * ****** ** *** *****  ** **  **   **   *** * * ***  *  ****
ppc-Kb3  AALYDKLLVSEDLWPFGEQLRNNYNETKNLLLQVAGHKDLLEGNPYLRQRLRLRDSYITT
ppc-Kb4  AALYDKLLVSEDLWPFGEQLRNNYNETKNLLLQVAGHKDLLEGNPYLRQRLRLRDSYITT

ppc-Kb1  LNVCQAYTLKRIRDPSYQVPVRPPIAKEIMEGSVSSANQLVKLNPTSEYAPGLEDTLILT   360
ppc-Kb2  LNVCQAYTLKRIRDPSYQVPVRPPIAKEIMEGSVSSANQLVKLNPTSEYAPGLEDTLILT
         ** ************ * * ***   ***** *   *  **********************
ppc-Kb3  LNGCQAYTLKRIRDPNYHVTVRPRLSKEIME-SNKAAAELVKLNPTSEYAPGLEDTLILT
ppc-Kb4  LNACQAYTLKRIREPNYHVTVRPRLSKEIME-SNKAASELVKLNPTSEYAPGLEDTLILT

ppc-Kb1  MKGIAAGMQNTG   372
ppc-Kb2  MKGIAAGMQNTG
         ************
ppc-Kb3  MKGIAAGMQNTG
ppc-Kb4  MKGIAAGMQNTG   371
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Fig. 3. Amino acid sequence alignment of the four PEPc isoforms of *K. blossfeldiana*. Identical amino acid residues between the two isoform pairs are marked by asterisk. Differences inside the two sequence pairs are marked by bold letters. The putative catalytic activity site (residues 37–53) which is found in all so far known PEPC is underlined.

the corresponding regions of the PEPC isoforms from *Z. mays* (C_4), *S. vulgare* (C_4), *F. trinervia* (C_4), *Flaveria springlei* (C_3), *Glycine max* (C_3), *Saccharum* hybrid (C_3), *M. crystallinum* (C_3, CAM) and *Medicago sativa* (C_3). In Table 1, the homology values of all four *K. blossfeldiana* isoforms with the enumarated plant species are shown. The homology ranged between 71–85%. The two Kb-3/Kb-4 isoforms show a higher homology to the selected plant species than the Kb-1/Kb-2 PEPC isoform pair.

To quantitate the relationship between the various above-mentioned PEPC isoforms, a phylogenetic tree was constructed. Fig. 4 shows that the Kb-1/Kb-2 pair seems to be more closely related to the PEPC isoform from *M. crystallinum* supposed to be CAM-specific and the Kb-3/Kb-4 pair to the C_3-specific PEPC isoform of *M. crystallinum* and the other PEPC-C_3 forms. The PEPC isoforms of the monocotyledonous C_4 plants *S. vulgare* and *Z. mays* form a closed cluster at a very early state supporting the general theory of an early separation of the monocot and dicot plants. This reinforces the view that the di- and monocotyledonous C_4 plants evolved separately and independently after the monocot–dicot divergence [19].

4. Conclusion

Considering the high similarity of the nucleotid sequences within the gene pairs, one might argue that the slight deviations have been caused by the PCR procedure. However, accidential generation of new restriction sites in an open reading frame is very unlikely, thus, the above argument can be ruled out as a

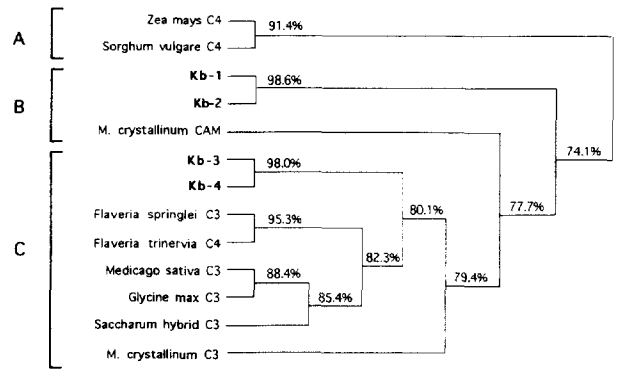

Fig. 4. Putative phylogenetic relationship of the four *K. blossfeldiana* (ppc Kb1-ppc Kb4) PEPC genes to *Z. mays* C_4, *S. vulgare* C_4, *M. crystallinum* C_3 and CAM, *F. trinervia* C_4, *F. pringlei* C_3, *M. sativa* C_3, *G. max* C_3 and *Saccharum* hybrid C_3 PEPC isoforms. (A) Monocot C_4 PEPC forms; (B) CAM PEPC forms; (C) C_3/dicot C_4 PEPC forms. The tree was constructed using the program CLUSTAL 4 of the PC DNASIS software package (Hitachi).

likely explanation. The present results show that PEPC from the CAM plant *K. blossfeldiana* is encoded by four isogenes that can be separated in two pairs. These pairs seem to be specific for the C_3 and CAM mode of photosynthesis, respectively, as suggested by the comparison with the *M. crystallinum* PEPCs. The presence of four isogenes could be due to duplication of two ancestral genes. An alternative hypothesis is that the *Kalanchoe* cultivar used in the present investigation (Tom Thumb) could be a hybrid whose parental genomes are expressed and used. Future studies will show which of the two explanations holds true.

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