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QUARTERLY

RFLP analysis of 1-aminocyclopropane-1-carboxylate synthase *ACC2* and *ACC4* genes from Polish cultivars of tomato

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An important trait of tomato is the rate of fruit ripening, strongly dependent on ethylene production. The ripening-related ethylene synthesis in tomato is controlled mainly by 1-aminocyclopropane-1-carboxylate synthase LE-ACS2 and LE-ACS4 isoenzymes (Rottmann *et al.*, 1991, *J. Mol. Biol.* 222: 937; Lincoln *et al.*, 1993, *J. Biol. Chem.* 268: 19422; Barry *et al.*, 2000, *Plant Physiol.* 123: 979). In spite of numerous reports on the *LE-ACS2* and *LE-ACS4* gene expression, only ones considered the genomic organisation each of these genes (Rottmann *et al.*, 1991; Lincoln *et al.*, 1993) reported one copy of each of these genes in tomato cv VF36. In this article we suggest that the genomic organisation of *LE-ACS2* and *LE-ACSS4* genes may depend on tomato cultivars and may differ from that described by the above authors. The results of Southern analyses of genomic DNAs from 17-day old seedlings (cultivars Jaga, Halicz, Betalux, New Yorker) imply that the genomic organisation of *LE-ACS2* and *LE-ACS4* genes in Polish cultivars differs from that reported for cv VF36.

Ethylene controls many aspects of plant development. Enhanced rates of ethylene production are observed in response to biotic and abiotic stress and in climacteric plants during fruit ripening and flower senescence. Higher plants produce ethylene from *S*-adenosyl-L-methionine (SAM) *via* 1-aminocyclopropane-1-carboxylic acid (ACC). ACC synthase (*S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14), the enzyme catalysing the conversion of SAM to ACC (the most important step in ethylene synthesis) is encoded by a highly divergent multigene family in a number of plant species (Huang *et al.*, 1991; Rottmann *et al.*, 1991; Liang *et al.*, 1992; Botella *et al.*, 1993; Lincoln *et al.*, 1993; Destefano-Beltran *et al.*, 1995; Subramanian *et al.*, 1996; Oetiker *et al.*, 1997; Zarembinsky

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Abbreviations: ACC, 1-amino-cyclopropane-1-carboxylic acid; ACS, 1-aminocyclopropane-1-carboxylate synthase; SAM, *S*-adenosyl-L-methionine.

& Theologis, 1997; Bui & O'Neill, 1998; Shiu *et al.*, 1998; Barry *et al.*, 2000; Bekman *et al.*, 2000; Ishiki *et al.*, 2000). Enhanced ethylene synthesis is usually correlated with an elevated level of 1-aminocyclopropane-1-carboxylate synthase mRNA, but regulatory mechanisms at the posttranscriptional level are also involved.

In tomato, nine members of the LE-ACS multigene family have been reported (Rottmann *et al.*, 1991; Lincoln *et al.*, 1993; Oetiker *et al.*, 1997; Shiu *et al.*, 1998; EMBL GeneBank Database 2002). Three of these genes, *LE-ACS1A, LE-ACS2* and *LE-ACS4*, show ripening-related increase in expression. The level of their transcripts is low or undetectable in mature green fruit and increases at the breaker stage (Barry *et al.*, 2000). *LE-ACS6* transcripts are present in mature green fruit but decline as ripening is initiated. When ripening is in progress, the levels of the most abudant ripening-related *LE-ACS2* and *LE-ACS4* transcripts continue to rise, and ethylene has an autostimulatory effect on their transcription. Both transcripts are superinduced by wounding of the pericarp tissue. Contrary to *LE-ACS4* transcripts which are specific only for ripening fruit, *LE-ACS2* trancripts have been found after pathogen attack, in flooded roots, in elicited tomato suspension culture, in mature and senescent anthers, and in fully senescent petals (Rottmann *et al.*, 1991; Oetiker *et al.*, 1997; Llop-Tous *et al.*, 2000).

The chromosomal localisation of *LE-ACS2* and *LE-ACS4* genes was established for tomato derived from the interspecific cross *Lycopersicon esculentum* cv VF36-Tm2a x *Lycoperscon pennelli* LA716. The two species used in this cross have the same chromosome constitution (2n = 24), (Rottmann *et al.*, 1991; Tanksley *et al.*, 1992). *LE-ACS2* gene resides on chromosome 1 and *LE-ACS4* gene on chromosome 5. The copy number described for cultivar VF36 for each of these two genes was one per a haploid genome (Rottmann *et al.*, 1991; Lincoln *et al.*, 1993).

Such an essential trait of tomato as the rate of fruit ripening is developmentally regulated and strongly depends on *LE-ACS2* and *LE-ACS4* gene expression. We therefore decided to investigate the *LE-ACS2* and *LE-ACS4* gene organisation in tomato cultivated in Poland.

MATERIALS AND METHODS

Plant material. We used the following cultivars of tomato: Jaga, Halicz and Betalux – cultivars brought up in Poland; New Yorker – cultivar brought up in the U.S.A. from crosses: (Geneva11 x Rhode Island) x Fireball. The New Yorker has been imported to Poland thirty years ago and since then cultivated and improved by breeders. All of these cultivars differ from each other in morphology. Results of the RAPD analysis have confirmed also some genetic differences between these cultivars (unpublished results). The plants used for Southern analysis were grown in pots as 17-day old seedling under 16 h daylight and at 25^{o}C.

DNA isolation. DNA extraction from young seedlings was performed according to the method of Junghans & Metzlaff (1990). DNA concentration was measured spectrophotometrically at 260 nm.

Southern analysis of genomic DNA. Approximately 10 μg of total genomic DNA was digested with a tenfold excess of restriction enzyme in a buffer supplied by the enzyme manufacturer (Fermentas) and following electrophoresis in 0.8% agarose gel, transferred to Hybond-N Plus membranes (Amersham).

Gene-specific 3′probe for *LE-ACS2* represented the last exon of the gene from nucleotides 534 through 1553 (according to cDNA GenBank X59145). It was PCR-generated using T1 and T2 primers and total DNA cv Jaga. The PCR amplified fragment was cloned and sequenced to confirm its authenticity. Gene-specific 3′ probe for *LE-ACS4* was a PCR amplified fragment representing the last

exon of the gene. The 3′ probe from nucleotides 528 through 1444 (according to cDNA GenBank X59146) was generated using AS1 and AS2 primers and total DNA cv Betalux. The amplified fragment of *LE-ACS4* was cloned and sequenced before using it as a hybridisation probe to confirm its authenticity. Hybridisation and washes were carried out at high stringency according to Amersham protocols for Hybond-N nylon membrane. The probe was labelled by the random hexamer priming method using the Promega system. Sequencing was performed using the f-mol sequencing kit from Promega.

Primers. PCR primers used for the analysis were designed from sequences deposited within databases.

T1, 5′ gggaattcactaatgcAGATTTAACAGAG 3′ (562), small letters – nucleotides complementary to the part intron 3, capital letters – to the sequence of exon 4; numbering according to cDNA (GenBank X59145).

T2, 5′ (1553) AAGTCTAGACGAGCTCATGGTGAGGGAGG 3′ (1525)

AS1, 5′ (528) ATTTAATAGGGACCTAAGGTGG 3′ (549)

AS2, 5′ (1462) ACATTTTCATCGTACTCCCCATTTG 3′ (1444)

The PCR conditions were: predenaturation step at 95°C, 4 min, followed by 5 cycles, each consisting of a denaturation step of 1 min at 94°C, an annealing step of 2 min at 55°C and extension of 2 min at 72°C; the next 35 cycles were: 1 min at 94°C, 2 min at 57°C and 2 min at 72°C.

RESULTS AND DISCUSSION

LE-ACS2 gene expression has been investigated by many authors but its organisation was reported only by Rottmann *et al.* (1991), who analysed five overlapping EMBL-lambda genomic clones containing the *LE-ACS2* gene and the flanking regions.

LE-ACS4 gene expression has been characterized by several authors but the organisation of the gene was reported by one research group only for cultivar VF36. Rottmann *et al.* (1991) and Lincoln *et al.* (1993) investigated the 17 kb fragment of the tomato nuclear DNA from EMBL-3-lambda clone containing *LE-ACS4* gene and the total genomic DNA from cultivar VF36.

Genomic Southern analysis of *LE-ACS2* gene organisation

The Southern analysis was performed for the total genomic DNA from 17-day old seedlings. The 3′ probe was a PCR-generated fragment corresponding to the last exon of the *LE-ACS2* gene which contained about 70% of the coding region. The restriction fragments hybridising with the 3′ probe were identical for all cultivars analysed (Fig. 1). There were five *Eco*RI strongly hybridising fragments: 1.7 kb; 2.5 kb; 2.7 kb; 3.2 kb and 3.6 kb and a few weakly hybridising fragments. There was one strongly hybridising 4.2 kb *Hin*dIII fragment and a few weakly hybridising fragments smaller than 4.2 kb. The 3′ probe hybridised with the high molecular mass fragments greater than 10 kb in the case *Eco*RI and *Hin*dIII digested DNA, probably because of the presence of the highly methylated undigested genomic DNA.

In summary, our results do not correspond strictly to the *LE-ACS2* gene organisation reported by Rottmann *et al.* (1991), and may suggest a polymorphic character of *LE-ACS2* genes or more than one copy of *LE-ACS2* in the analysed tomato cultivars.

Genomic Southern analysis of *LE-ACS4 organisation*

Southern analysis was performed for the total genomic DNA from 17-day old seedlings of different cultivars (Jaga, Halicz, Betalux, New Yorker). We used the same *Eco*RI and *Hin*dIII digested DNAs which were used in *LE-ACS2* analysis. The hybridisation 3′ probe was a PCR-generated fragment representing the

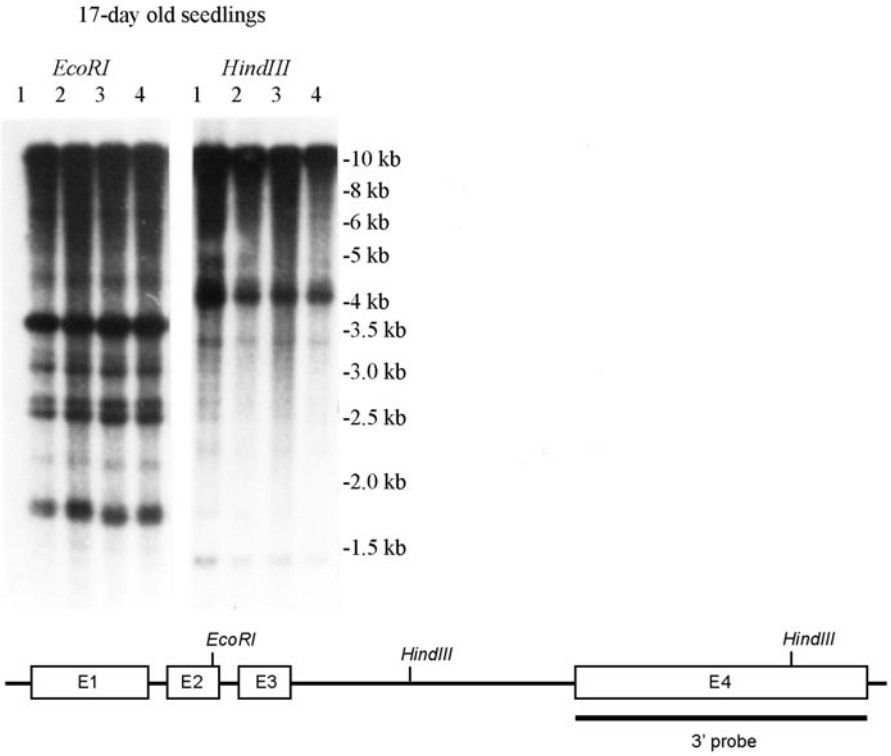

Figure 1. Southern analysis of DNA isolated from 17-day old seedlings.

Tomato genomic DNA from cultivars Jaga (1), Halicz (2), Betalux (3) and New Yorker (4) were digested with *Eco*RI and *Hin*dIII. The 3′ probe was the ^{32}P-labelled fragment representing the fourth exon of the *LE-ACS2* gene. *LE-ACS2* organisation and the partial restriction map (E, H – *Eco*RI and *Hin*dIII, respectively) was shown according to Rottmann *et al.* (1991). Each lane contains 10 μg of digested genomic DNA. The size markers shown on the right is the 1 kb DNA ladder (Fermentas).

last exon of *LE-ACS4* gene which contained about 70% of the coding region. The 3′ probe strongly hybridised with 4.0 kb and 5.0 kb and weakly with 2.25 kb and 2.5 kb *Hin*dIII fragments and with the single 1 kb *Eco*RI fragment (Fig. 2).

The results of Southern analysis of *LE-ACS4* gene were the same for all cultivars analysed but different from those published by Lincoln *et al.* (1993) for cultivar VF36.

Sequence analysis of the last exon of *LE-ACS4* gene from the cultivar Betalux

Rottmann *et al.* (1991) reported that the cloned *LE-ACS4* gene from cv VF36 and *LE-ACS4* cDNA from ripening tomato of a unknown cultivar differed at eight positions. We have cloned PCR-generated fragments corresponding to the last exon of *LE-ACS4* gene from cultivar Betalux. The region of *LE-ACS4* from 550 nt to 982 nt of each clone was sequenced (numbering according to *LE-ACS4* cDNA GenBank/EMBL Data Bank accession number X59146). The nucleotide sequence of the last exon of *LE-ACS4* gene from among the seven Betalux clones analysed was consistent with the published *LE-ACS4* cDNA sequence from an unknown cultivar of tomato at the four controversial positions of this region (*LE-ACS4* cDNA and Betalux *LE-ACS4* gene T_{741}, A_{798}, C_{805}, G_{936}; numbering according to cDNA). This strongly suggests that the form of the *LE-ACS4* gene from cv VF36 reported by Rottmann *et al.* (1991) and by Lincoln *et al.* (1993) does not occur in cultivar Betalux and probably also in the other cultivars analysed. The nucleotide sequence of *LE-ACS4* gene found in cultivar Betalux is the same at the controversial positions as that

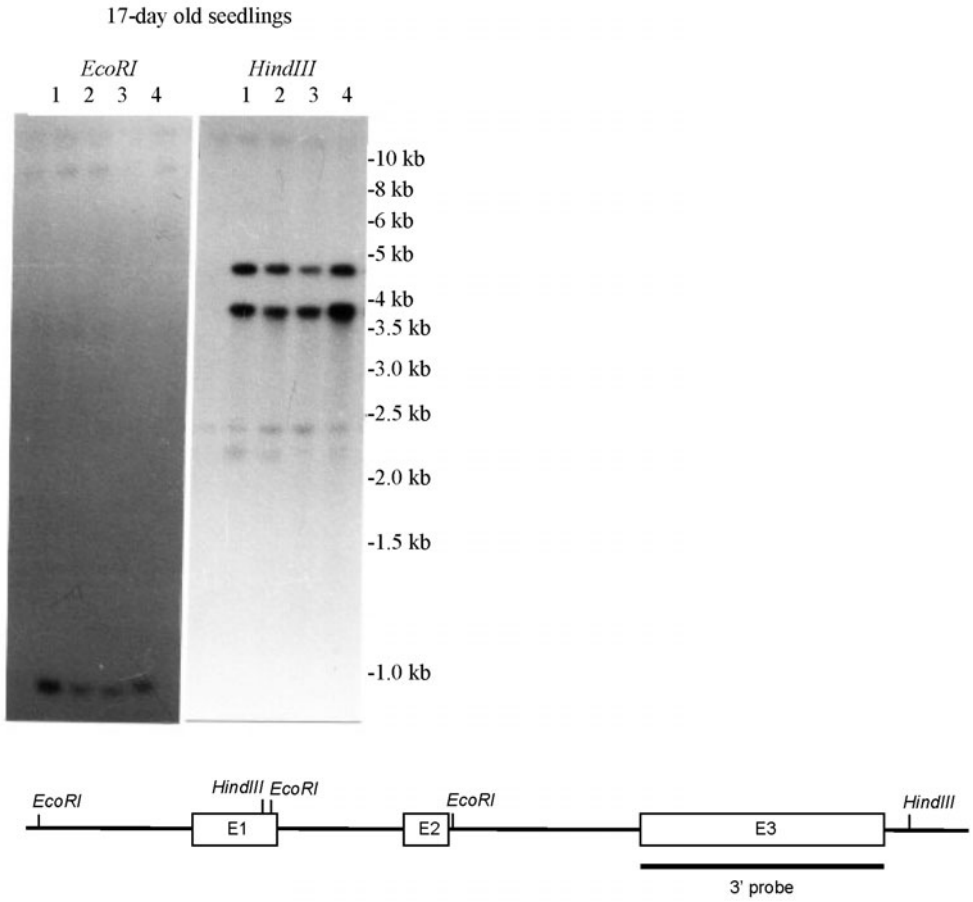

Figure 2. Southern analysis of DNA isolated from 17-day old seedlings.

The 3′ probe was the ^{32}P-labelled fragment representing the last exon of the *LE-ACS4* gene. *LE-ASC4* organisation and the partial restriction map was shown according to Lincoln *et al.* (1993). Each lane contains 10 μg of digested genomic DNA. All designations and the size markers as in Fig 1.

in the reported *LE-ACS4* cDNA sequence. This result may suggest that the transcriptionally active form of the whole *LE-ACS4* gene has not been characterised till now.

The breeders interest is to generate the quality features associated with the growth and ripening of tomato fruits. Probably, the reported organisation and copy number of *LE-ACS2* and *LE-ACS4* genes is best adapted to the local climate requirements and was fixed in the genetic pool of tomato cultivars in Poland.

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