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ORIGINAL PAPER

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Molecular characterization and isolation of the *F/f* gene for femaleness in cucumber (*Cucumis sativus* L.)

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Abstract The biological processes leading to sex expression in plants are of tremendous practical significance for fruit production of many agricultural and horticultural crops. Sex-expression studies in cucumber showed that the different sex types are determined by three major genes: *M*/*m*, *F*/*f* and *A*/*a*. The *M*/*m* gene in the dominant condition suppresses stamina development and thus leads to female flowers. The *F*/*f* gene in the dominant condition shifts the monoecious sex pattern downwards and promotes femaleness by causing a higher level of ethylene in the plant. To investigate the molecular character of the gene *F*/*f*, we used nearly isogenic gynoecious (*MMFF*) and monoecious (*MMff*) lines (NIL) produced by our own backcross programme. Our investigations confirmed the result of other groups that an additional genomic ACC synthase (key enzyme of ethylene biosynthesis) sequence (*CsACS1G*) should exist in gynoecious genotypes. A linkage was also verified between the *F*/*f* locus and the *CsACS1G* sequence with our plant material. After the exploration of different Southern hybridization patterns originating from different *CsACS1* probes, a restriction map of the *CsACS1* locus was constructed. By using this restriction map, the duplication of the *CsACS1* gene and following mutation of the *CsACS1G* gene could be explained. The promoter regions of the genes *CsACS1G* and *CsACS1* were amplified in a splinkerette PCR and sequenced. An exclusive amplification of the new isolated sequence (*CsACS1G*) in gynoecious (*MMFF*) and sub-gynoecious (*MMFf*) genotypes confirmed that the isolated gene is the dominant *F* allele.

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Introduction

The biological processes leading to sex in plants are of tremendous significance for plant breeding as well as for evolution studies. The cucurbit family includes a number of economically important crops, e.g. cucumber (*Cucumis sativus* L.) and melon (*C. melo* L.). The cucumber, with its diverse sex types, represents a perfect model plant for studying the molecular and physiological basis of sex expression. All floral buds contain staminate and pistillate primordia at early stages of their development; however, abortion of either staminate or pistillate parts results in female or male flowers, respectively, while in hermaphrodite flowers the staminate and pistillate primordia mature (Galun 1961). The distribution of these three different flower types (staminate, pistillate and hermaphrodite) on the plant results in the different sex types of cucumber (Table 1). These different sex types are mainly determined by three major genes *F*/*f*, *M*/*m* and *A*/*a* (Galun 1961; Kubicki 1969a–d; Shifriss 1961; Kubicki 1972; Robinson et al. 1976).

The gene *F* promotes femaleness on the whole plant level while the gene *M* determines whether flowers are unisexual [*M*_ or bisexual (*mm*), Table 1]. Thus, the genotype *M*_*FF* is gynoecious; the genotype *M*_*Ff* sub-gynoecious due to the incomplete dominance of *F*; and the genotype *mm F*_ is hermaphrodite. If *F*/*f* is homozygous recessive, the sex expression is influenced by the gene *A*/*a*: *MM ff A*_ genotypes are monoecious; *MM ff aa* genotypes are androecious (Table 1).

Although the three major genes determine the basic regulation of sex types, the sex expression is influenced by additional modifying genes and environmental factors such as photoperiod, temperature and irradiation (Atsmon 1968; Vice-Pruce 1975; Frankel and Galun 1977; Cantliffe 1981; Takahashi et al. 1983; Yamasaki et al. 2003).

The development of four different sex types in cucumber (gynoecious, monoecious, hermaphroditic and andromonoecious) was explained by the one-hor-

Table 1 Major genes controlling sex expression in *Cucumis sativus* (Galun 1961; Kubicki 1969a, c–e; Shifriss 1961)

	F (*A* or *a*)	*f*	
		A	*a*
M	Gynoecious	Monoecious	Androecious
m	Hermaphroditic	Andromonoecious	Androecious

mone hypothesis (Yin and Quinn 1995). It assumes that one hormone—ethylene in cucumber—inhibits maleness and induces femaleness by influencing two different ethylene receptors independently. The hormone concentration and different sensitivity levels of the receptors interact to regulate sex expression. The one-hormone hypothesis is based on a number of investigations with cucumber that have clearly demonstrated that ethylene is the adjusting sex hormone (Galun 1961; Rudich 1985; Trebitsh 1987; Malepszy and Niermirowicz-Szczytt 1991; Trebitsh et al. 1997).

During ethylene biosynthesis, the enzyme ACC (1-aminocyclopropane-1-carboxylic acid) synthase plays a decisive regulatory role. Cloning of ACC synthase genes from different plant species revealed a multi-gene family (Rottmann et al. 1991; Liang et al. 1992; O'Neill et al. 1993; Destefano-Beltran et al. 1995). Trebitsh et al. (1997) isolated a partial sequence of the gene *CsACS1* from cucumber and postulated the existence of an additional genomic ACC synthase sequence in gynoecious plants, designated *CS-ACS1G*, since an additional hybridization fragment was detected in restriction fragment length polymorphism (RFLP) analysis. Using segregating F_2 populations, Trebitsh et al. (1997) showed by mapping that this RFLP marker cosegregates with the dominant *F* gene. However, only a partial sequence of the gene *CsACS1* was isolated (GenBank accession number U59813, Trebitsh et al. 1997). In another study, while Shiomi et al. (1998) isolated the complete mRNA sequence homologous to *CsACS1* gene and designated this *CsACS3* (GenBank accession number AB006805), the postulated additional sequence *CS-ACS1G*, which most probably represents the *F* gene, could not be isolated.

Kamachi et al. (1997) found differences in expression patterns of another ACC synthase (*CSACS2*) gene between non-isogenic gynoecious and monoecious lines. The female line, unlike the monoecious line, showed a stronger and an earlier expression of the *CsACS2* gene.

Furthermore, Kamachi et al. (2000) examined the expression of the genes *CsACS1* and *CsACS2* at the apices of nearly isogenic gynoecious (*FF*) and monoecious (*ff*) cucumber lines. The transcripts of *CsACS2* were detected at the apices of gynoecious and monoecious sex types. However, the *CsACS1* gene was detected only at the apices of the gynoecious line. In these experiments, Kamachi et al. (2000) used *CsACS1* as a probe for the detection of transcripts by Northern hybridization. However, this *CsACS1* probe hybridized to both the *CsACS1* and *CsACS1G* genes (Trebitsh et al. 1997). Therefore, it is not clear whether the additional transcript detected from Kamachi et al. (2000) in *FF* apices is the product of *CsACS1* or the product of the postulated *CsACS1G*.

To distinguish the genes *CsACS1* and *CsACS1G*, we report herein an RFLP map, using different parts of the gene *CsACS1* as probes. This map confirmed the existence of the two duplicated genes *CsACS1* and *CsACS1G* and located the difference between them in the 5′ region. The isolation of the unknown 5′ regions of both genes was achieved using an adaptor ligation-based PCR-mediated walking strategy. The newly isolated promoter sequence of the gene *CsACS1G* was then used to develop a sequence characterized amplified region (SCAR) marker that amplifies the expected fragment in plants with the dominant *F* allele only.

Materials and methods

Plant materials

Nearly isogenic *MMFF* and *MMff* lines were drawn from a backcross programme with three genetically different *FF* lines as recurrent parents (Tatlioglu 1983). Between *ff* and *FF* lines, at least ten backcrosses were carried out. Thus, *FF* and *ff* lines with about 99.9% identical genes were available for the studies described herein with three genetic backgrounds WrD, ED and ECD. The plants were grown in pots (filled with Terreau Professional Gepac Einheitserde Typ T) at day/night temperatures of 24°C/18°C and 16 h of assimilation light (natural light and additional light from Son-T Agro 400, Philips Licht, Hamburg).

DNA extraction, digestion and blotting

Purified total DNA was isolated according to a protocol from Bentzen et al. (1990) modified by Engelke and Tatlioglu (2000). Twenty micrograms DNA from gynoecious and monoecious plants were digested using 30 U of the restriction enzymes *Msp*I, *Hin6*I, *Mva*I and *Mbo*I (MBI Fermentas) according to the instructions of the manufacturer. The restriction fragments were separated by flatbed electrophoresis, using 0.8% agarose gels in 1× TAE buffer for 14–16 h (2 V/cm length of the gel). The DNA was cracked by soaking the gel in 0.25 M HCl for 10 min and then transferred to Hybond-N^+ nylon membranes (Amersham) in 0.4 M NaOH, using a vacuum-system (Pharmacia: 60 mbar, 45 min). The membranes were rinsed in 2× SSC and air-dried.

Amplification of the probes, hybridization and autoradiography

A number of different probes were amplified from the *CsACS3* mRNA sequence (GenBank accession number

AB006805, Shiomi et al. 1998) that reflects the transcript of *CsACS1* (see 'Introduction'). Two of them are given in Fig. 2, representing the restriction sites of *Mbo*I upstream and downstream regions (for the remaining probes and the *Hin6*I and *Mva*I restriction sites, see Mibus 2003).

The probe *cs-acs1.1* was amplified using the primers 5′-CACAAAAGCCACGTGCAATT-3′ (GenBank number AB006805, position 212–231) and 5′-CGGGTAAGCCGTGGTAATCT-3′ (position 446–465); the probe *cs-acs1.2*, using the primers 5′-CGCTCTCTTCCAAGATTACCA-3′ (position 434–454) and 5′-CGGTTCTCCATTTCAAATCTC-3′ (position 642–663). PCR was performed using 15 ng total DNA of *FF* plants in a final volume of 20 µl, containing 0.25 µM of each primer, 150 µM of each dNTP and 0.25 U of Dynazyme in the reaction buffer as recommended by the supplier (Biometra). The reaction mixture was incubated in a thermocycler (Biometra) for 2 min at 94°C, followed by 40 cycles: 1 min at 94°C, 1 min for 58°C, 2 min at 72°C. PCR products were separated by flatbed electrophoresis, using 1.5% agarose gels in 1× TAE buffer.

Radioactive labelling of the probes *cs-acs1.1* and *cs-asc1.2* was achieved by random priming with an Oligolabelling kit from GIBCO, using 50 ng of each probe and 50 μCi $\alpha[^{32}$ P]-dCTP (3,000 Ci/mmol, Amersham). Hybridization and autoradiography was done as previously described (Engelke and Tatlioglu 2000).

Adaptor ligation-based PCR-mediated walking

The isolation of the unknown 5′ DNA promoter regions of *CsACS1* and *CsACS1G* were achieved using an adaptor ligation-based PCR-mediated walking strategy. This splinkerette allows the amplification between a primer annealing in a known part of the sequence and a nearby restriction site laying out of the known part of the sequence (Devon et al. 1995). The protocol has been modified from its original form; in that way that the restriction enzyme *Mbo*I was used to create an overhang for ligation of the corresponding splinker.

Annealing of these splinkers was achieved by heating proper amounts of the primer splinktop (5′-CGAATCGTAACCGTTCGTACGAGAATTCGTACGAGAATCGCTGTCCTCTCCAACGAGCCAAGA-3′) with splnk *Mbo*I (5′-GATCTCGTTGGCTCGTTTTTTTTTGCAAAAA-3′) (150 µg/ml each) in 20 µl Tris (pH 7.4) and 5 mM $MgCl_2$ at 90°C and cooling on the bench top for 20 min. During the following PCR, the free 3′ end of the bottom strand will flip back on itself to form a hairpin and begin elongation. The resulting double-stranded structure is stable and functionally removed from further reaction. The specificity of the PCR reaction is enhanced by this method, since end-repair priming and non-specific annealing is decreased in comparison with vectorettes (Devon et al. 1995).

A volume of 5 µg total DNA was digested in a 20-µl reaction volume with 10 U of *Mbo*I restriction enzyme for 3 h at 37°C, using supplier-recommended buffer (MBI Fermentas). The reaction was terminated by heat inactivation for 10 min at 65°C. Two microlitres of the digested DNA (0.5 µg) and 6 µl of the annealed splinker were mixed with 200 U T4 DNA ligase in the supplier-recommended buffer (New England Biolabs) in a final volume of 20 µl and incubated overnight at 15°C for ligation.

A nested PCR was performed for amplification of the desired fragments. The primer splk0 (5′-CGAATCGTAACCGTTCGTACGAGAA-3′) was used in the first PCR, which annealed to the complement of the longer strand that was generated in the first PCR cycle by priming of a gene-specific primer, splinkACS1.1 (5′-CGGCAAGACCCATCTGAATGATG-3′, derived from position 336-314, GenBank accession number AB006805, Shiomi et al. 1998). The primer splk1 (5′-TCGTACGAGAATCGCTGTCCTCTCC-3′, annealing: 55°C) was used in the second PCR, together with the nested gene-specific primer splinkACS1.2 (see Fig. 3: 5′-GTAGGAGGAATCTTGACCGTGGG-3′, derived from position 254–232, GenBank accession number AB006805). PCR was done as described above, with an annealing temperature of 55°C.

The obtained amplicons were separated by flatbed electrophoresis, using 1.5% agarose gels in 1× TAE buffer and the sizes estimated by comparison to a 100-bp ladder (1 µg, Amersham Pharmacia Biotech). The cloning of PCR products was facilitated by using a TOPO TA Cloning Kit (Invitrogene). Plasmids were recovered using a QuiaPrep-Mini kit (Quiagen) and sequencing was accomplished by MWG-Biotech. The isolated sequences were analysed by using the Clustal W programme, European Bioinformatics Institute (Higgens et al. 1994) and the BLASTn programme, National Center for Biotechnology Information [(NCBI) Altschul et al. 1997].

Results

Southern blot hybridization and construction of a restriction map

In order to distinguish between *CsACS1* and the propagated *CsACS1G*, i.e. the *F* gene, we used different parts of *CsACS1* as probes and different restriction enzymes for Southern blot hybridization (Mibus 2003). Figure 1 shows hybridization patterns of digested (*Hin6*I, *Mva*I and *Mbo*I) DNA of *FF*, *Ff* and *ff* genotypes by using the probe *cs-acs1.1*. After the digestion of the genomic DNA with *Hin6*I, the probe detected a hybridization fragment of 3.9 kb in all genotypes and an additional fragment of 3.0 kb in *FF* and *Ff* (Fig. 1, arrowhead 1), but not in *ff* genotypes. The digestion of the DNA with *MvaI* yielded a fragment of 4.0 kb in all genotypes and 1.7 kb in *FF* and *Ff* (Fig. 1, arrowhead 2), but not in *ff* genotypes. Digestion with *Mbo*I yielded a fragment of 0.9 kb in all genotypes and a fragment of 1.1 kb in *FF* and *Ff* (Fig. 1, arrowhead 3), but not in *ff* genotypes.

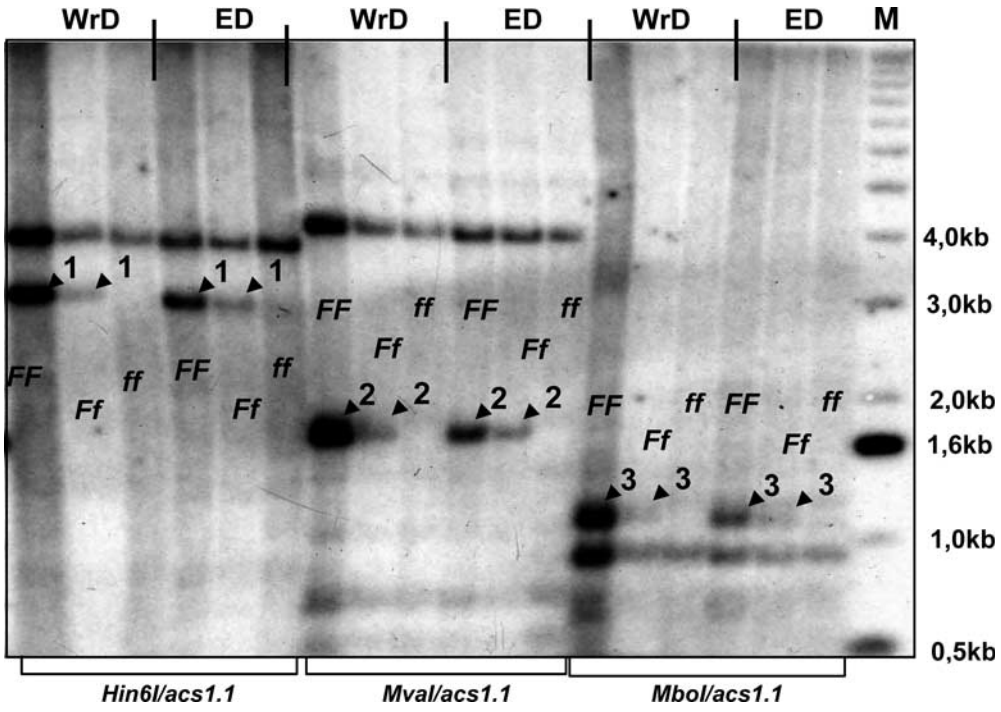

Fig. 1 Southern blot hybridization analysis of genomic DNA isolated from gynoecious (*FF*), sub-gynoecious (*Ff*) and monoecious (*ff*) cucumber with the genetic backgrounds WrD and ED. Each *lane* contains ca. 20 µg DNA. The DNA was digested with the restriction enzymes *Hin*6I, *Mva*I or *Mbo*I. The blot was probed with *cs-acs1.1*. The *arrowheads 1* indicate the additional hybridization signals in *FF* and *Ff* genotypes, 1:3.2 kb after restriction with *Hin*6I, 2: 1.7 kb after restriction with *Mva*I, 3:1.1 kb after restriction with *Mbo*I

The additional hybridization signals in *FF* and *Ff* genotypes confirm the existence of the postulated locus *CsACS1G* (Trebitsh et al. 1997) in our nearly isogenic lines with both genetic backgrounds investigated (WrD and ED). Thus, a linkage is verified between the dominant *F* gene and the postulated *CsACS1G* sequence with our plant material.

Furthermore, the additional fragment (postulated *CsACS1G*) of the *FF* genotypes shows a stronger signal than the *Ff* genotypes (Fig. 1, in particular genetic background ED). These results are consistent with the assumption that the postulated *CsACS1G* sequence and the dominant *F* gene are identical (Fig. 1). Thus, it is possible to distinguish the first-time *FF* and *Ff* with an RFLP marker. However, RFLP markers with different intensity are difficult to handle for breeders, because an accurate DNA concentration is necessary, and RFLP analyses are time consuming and costly. To get more information we started to investigate the *CsACS1* locus with additional different Southern hybridization studies.

By using the probe *cs-acs1.2* (homologous to the 3′ part), identical hybridization patterns were detected in *FF*, *Ff* and *ff* genotypes (Fig. 2a, 643-bp). Thus, additional hybridization signals in *FF* and *Ff* genotypes only occur when the probe *cs-acs1.1* (homologous to the 5′ part) is used. (Fig. 2a, 1,100-bp fragment in addition to the 900-bp fragment; see also Fig. 1, arrowheads 1, 2 and 3).

In order to explain these results a restriction map was constructed (Fig. 2b). The hybridization signal of 643-bp, if probed with *cs-acs1.2*, is consistent with the *Mbo*I restriction sites of the known gene sequence *CsACS1* (Fig. 2b). The 900-bp band that hybridizes independently from the genotype when probed with *cs-acs1.1* represents the 5′ region of the gene *CsACS1*. Thus, the additional 1,100-bp band in *FF* and *Ff* genotypes has to be originated from a changed *Mbo*I restriction site in the propagated it *CsACS1G*, upstream to the known sequence. This 1,100-bp signal was detected in genotypes with a dominant *F* allele only (*FF* and *Ff*), and thus, it is plausible that the 1,100-bp band represents the promoter sequence of the gene *CsACS1G*, and the 900-bp band represents the promoter sequence of the gene *CsACS1* that is present in all genotypes. These assumption were confirmed with other restriction enzymes and probes (see Mibus 2003; data not shown).

Promoter amplification of the genes *CsACS1* and *CsACS1G*

The different promoter sequences of the loci *CsACS1* and *CsACS1G* were isolated by splinkerette PCR with a *Mbo*I-specific anchor primer (splink *Mbo*I). The splinkerette for the 5′ region resulted in ca. 940-bp and 730-bp amplicons, respectively.

Figure 3 shows the alignment of the homologous and heterologous parts of the promoter sequences of the genes *CsACS*1 and *CsACS1G*. The sequence alignment confirmed the size difference of ca. 200-bp detected in Southern hybridization (Fig. 2a) as well as the restric-

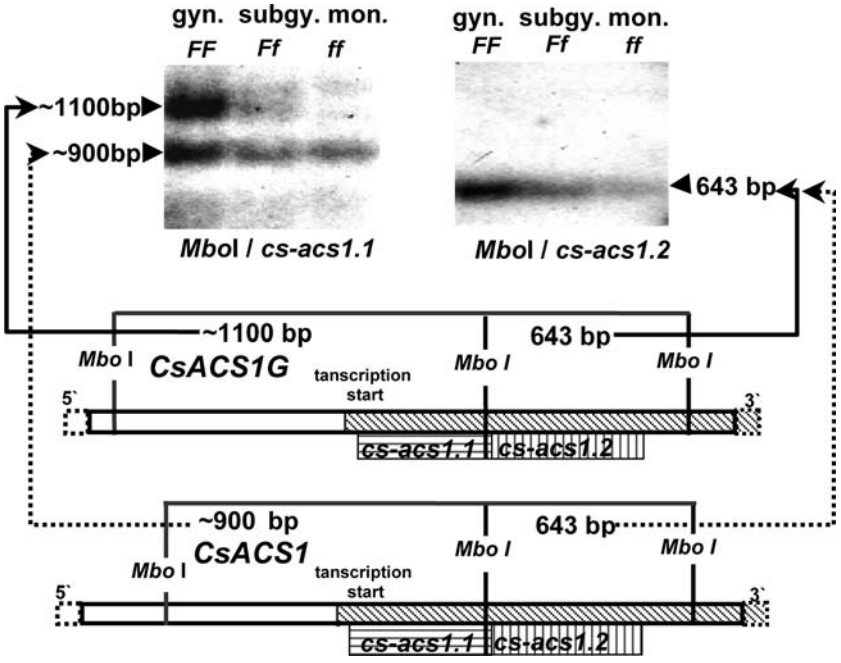

Fig. 2 **a** Southern blot hybridization analysis of genomic DNA isolated from *FF*, *Ff* and *ff* cucumber. Each *lane* contains 20 µg DNA, restricted with *Mbo*I. The blot that was probed with *cs-acs1* shows two bands, 1,100-bp and 900-bp, for *FF* and *Ff* and one 900-bp band for *ff* genotypes, respectively. The blot that was probed with *cs-acs1.2* shows one 640-bp band for all genotypes. *gyn* Gynoecious, *supgy* subgynoecious, *mon* monoecious. **b** Restriction map with identical and different *Mbo*I restriction sites of the loci *CsACS1* and *CsACS1G*, respectively. The identical *Mbo*I restriction sites of the genes *CsACS1* and *CsACS1G* result in the 643-bp signal. The variable *Mbo*I restriction site in the promoter leads to the 900-bp and 1,100-bp band, respectively. The *upper part of the graphic* shows the *Mbo*I restriction sites of the locus *CsACS1G* and the *bottom part of the graphic* shows the *Mbo*I restriction sites of the locus *CsACS1*. *Plain box* Formerly unknown promoter sequence, *box with vertical lines* probe *cs-acs1.1*, *box with horizontal lines* probe *cs-acs1.2*, *box with diagonal lines* known sequence of the gene *CsACS1*

tion map (Fig. 2b). Both sequences include typical promoter *cis*-elements like a TATA box and a CAAT box. Moreover, a putative auxin-inducible *cis*-element (TGTCT) was detected in the promoter region of the gene *CsACS1* as well as *CsACS1G*.

Conversion of the RFLP in a SCAR marker by amplifying the gene *CsACS1G*, i.e. the *F* gene

A SCAR marker was developed by using the differences of the 5′-flanking regions of the genes *CsACS1* and *CsACS1G* for primer design. The sense primer CsACS1.2s: 5′-TTAACTACTTCGGACGGGCATAG-3′ was derived from this heterologous region of the *CsACS1G* sequence. The second sense primer CsACS1.1s 5′-TACCTGCTCTGGTCGGAGACACT-3′ and the antisense primer CsACS1/5′as: 5′-AGGTGTTCAGCAAACATAGGGTG-3′ were derived from the homologous part of the genes *CsACS1* and *CsACS1G* (Fig. 3). PCR amplification was tested in the three genetic backgrounds: WrD, ED and ECD. Thirty *FF*, 40 *Ff* and 15 *ff* genotypes of different segregating offspring were tested with the new SCAR marker. The results show an exclusive amplification of the expected 430-bp fragment in *FF* as well as in *Ff* but missing in *ff* genotypes (Fig. 4). These results confirmed that the isolated sequence *CsACS1G* exists only in genotypes with dominant *F* alleles (Fig. 4).

Discussion

The Southern hybridization experiments described here using probes derived from the gene *CsACS*1 are consistent with previous results (Trebitsh et al. 1997). Trebitsh et al. (1997) reported that *FF* genotypes, in contrast to *ff* genotypes, have an additional restriction fragment in Southern hybridizations if DNA were digested by *Msp*I or *Xba*I and probed with *CsACS*1. Thus, Trebitsh et al. (1997) postulated an additional copy of *CsACS1* in the *FF* genotype and designated this gene *CsACS1G*. The existence of an additional hybridization fragment in genotypes with dominant *F* alleles (*FF* and *Ff*) was confirmed herein by using three additional restriction enzymes (*Hin6I*, *Mbo*I and *Mva*I) (Fig. 1).

However, no complete genomic sequence of *CsACS1* exists in databases, and the existence of the postulated *CsACS1G* was not proved. Thus, the partial genomic sequence *CsACS1* (GenBank accession number U59813, Trebitsh et al. 1997) and the *CsACS1* homologous mRNA *CsACS3* (GenBank accession number AB006805, Shiomi et al. 1998) were used for primer design in order to amplify different parts of this gene that are subsequently used as probes in Southern hybridization. Newly discovered restriction sites (*Mbo*I, *Hin*6I and *Mva*I) as well as the formerly described restriction sites (*Msp*I and *Xba*I, Trebitsh et al. 1997)

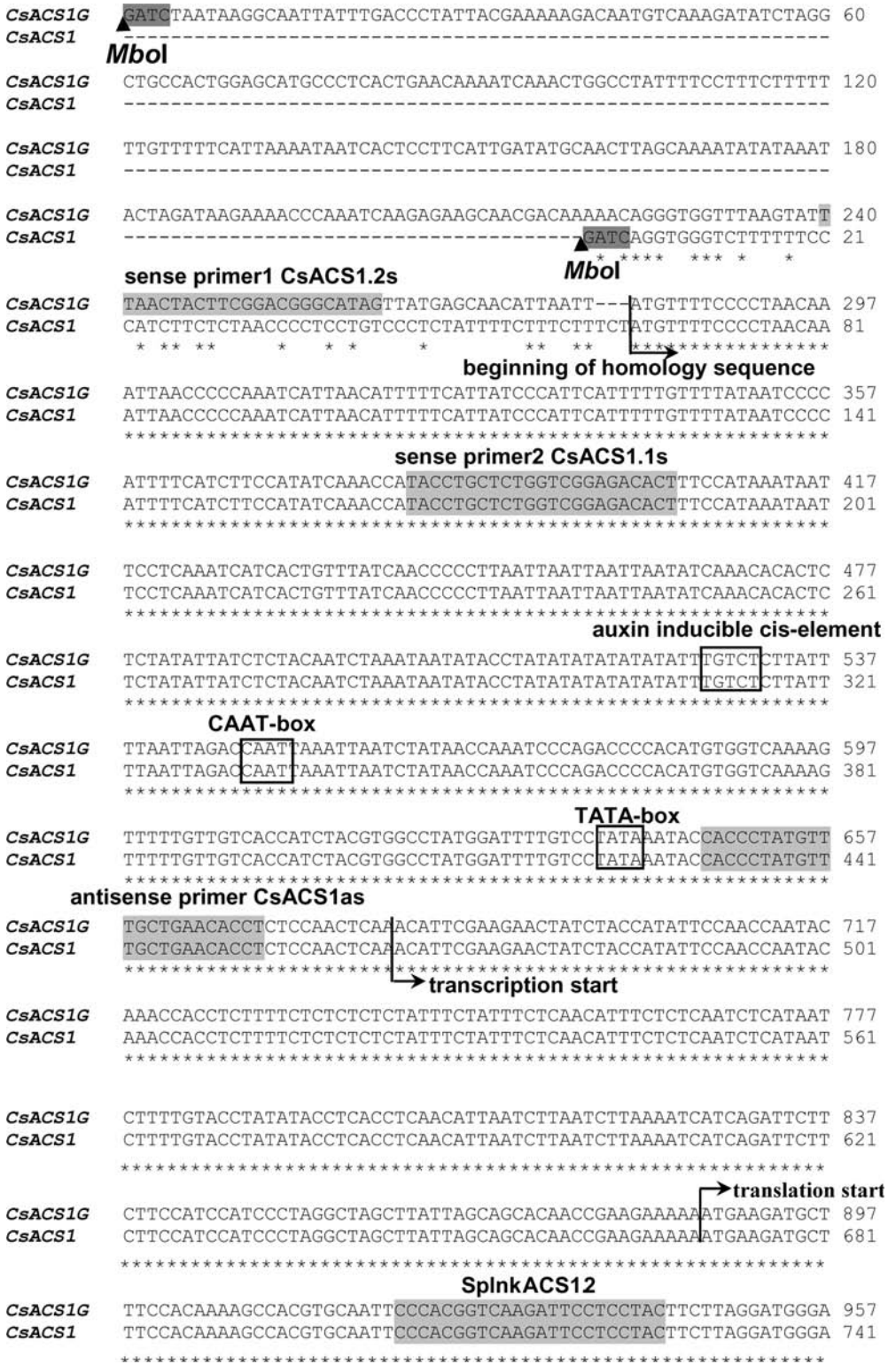

Fig. 3 Alignment of the promoter sequences of the genes *CsACS1* and *CsACS1G*. *Asterisks* represent identical nucleotides, *dashed lines* indicate missing nucleotides, *light-grey shading* primer, *box* putative promoter *cis* sequences (Buchanan et al. 2000), *dark-grey shading**Mbo*I restriction site, *box containing TGTCT sequence* putative auxin-inducible *cis*-element (Guilfoyle and Hagnen 2001). The sense and antisense primers for subsequent PCR amplification of *CsACS1G* are marked. *transcription start* Previously known mRNA sequence (GenBank accession number AB006805, Shiomi et al. 1998)

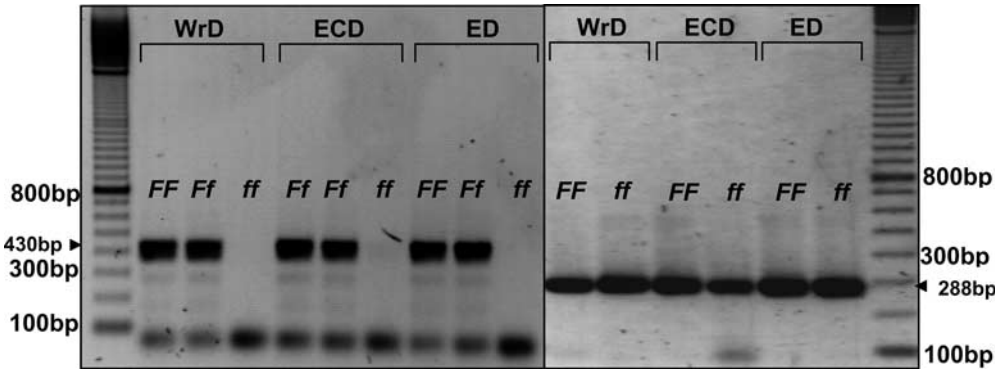

Fig. 4 **a** Specific amplification of the *CsACS1G* sequence in *FF* and *Ff* genotypes within the genetic backgrounds WrD, ED and ECD. The *arrowhead* indicates the amplicon of 430-bp [sense primer1 CsACS1.2 s, Fig. 3, (*CsACS1G*)]. **b** Amplification of the *CsACS1* and *CsACS1G* sequence, respectively, in *FF* and *ff* genotypes within the genetic backgrounds WrD, ED and ECD. The *arrowhead* indicates the amplicon of 288-bp [sense primer2 CsACS1.1 s, Fig. 3 (*CsACS1 or CsACS1G*)]

were integrated in a restriction map (Mibus 2003; see Fig. 3 for *Mbo*I). This map revealed a difference between *CsACS1* and *CsACS1G* in the 5′ region of the genes.

The formerly unknown 5′ regions of both genes were isolated by using splinkerette PCR (Fig. 2), using the restriction map for choosing a suited restriction enzyme, in this case *Mbo*I. Amplicon sizes of 640-bp for the *CsACS1* promoter and 440-bp for the *CsACS1G* promoter were predicted from the restriction sites of *Mbo*I in the map (Fig. 2) and realized with the splinkerette PCR (Fig. 3; Mibus 2003).

The sequence alignment of the two different promoter regions (*CsACS1* and *CsACS1G*) shows complete sequence identity beginning 409-bp upstream from the starting point of the transcription. Within this homologous sequence different promoter, specific *cis*-elements like a TATA box and CAAT box were detected (Fig. 3). Furthermore, a putative auxin-inducible *cis*-element (TGTCT; Guilfoyle and Hagnen 2001) was found in this region and may explain the previously described auxin-induced expression of the gene *CsACS1* in *ff* as well as in *FF* plants (Trebitsh et al. 1997). The 5′ regions of the promoters lying upstream to the 409 identical bases (count from transcription initiation) are heterologous between *CsACS1* and *CsACS1G*. These sequence differences lead to the different *Mbo*I restriction sites and probably further upstream to the different restriction sites of *Xba*I, *Msp*I, *Hin6*I and *Mva*I. By means of these different restriction sites, we expected upstream of the 5′ end a heterologous sequence above 2,500-bp (Mibus 2003). However, further investigations of the promoter sequences of the genes *CsACS1* and *CsACS1G*, respectively, will probably confirm this assumption. The isolated heterologous sequence has no homology to already known sequences in the NCBI database (http://www.ncbi.nlm.nih.gov).

The developed SCAR marker amplifying the promoter sequence of the gene *CsACS1G* shows the exclusive amplification of the expected 430-bp fragment in *FF* and *Ff* genotypes, but no amplification in the monoecious (*ff*) genotypes in all three genetic backgrounds investigated (Fig. 4a). These results confirmed that the isolated promoter sequence of the gene *CsACS1G* is missing in genotypes without a dominant *F* allele (Fig. 4a). The homologous promoter sequence of the genes *CsACS1* and *CsACS1G*, however, is amplified independently of the sex genotype (Fig. 4b; Mibus 2003). Therefore, the gene *CsACS1* exists in all genotypes, and the RFLP results were confirmed. In Northern hybridization, Kamachi et al. (2000) could not detect *CsACS1* transcripts in apices of an *ff* line, but transcripts were evident at the apices of an isogenic *FF* line. Furthermore, the quantity of the detected *CsACS1* transcript at the apices of an *Ff* line was one half of that at the apices of an isogenic *FF* line (Kamachi et al. 2000). However, the coding sequence of *CsACS1* used as probe hybridizes to both *CsACS1* and *CsACS1G* genes (compare RFLP results in Fig. 1 with results from Trebitsh et al. 1997). Thus, the transcript differences found by Kamachi et al. (2000) may be due to the gene *CsACS1G* that represents the dominant *F* gene. These results along with the results presented herein suggest that the different promoter sequences of the genes *CsACS*1 and *CsACS1*G cause an exclusive expression of the gene *CsACS1G* in *FF* and *Ff* plants. Thus, maybe no expression of the gene *CsACS1* occurs in these genotypes as was evident in *ff* genotypes.

According to the presented results the evolutionary development of the gynoecious sex type in *C. sativus* can be explained as follows: In the monoecious wild form of cucumber, *CsACS1* solely exists—a gene that is not expressed in the apices. The duplication of this locus led to the expression of the new gene *CsACS1G*. It is likely that the gene *CsACS1G* is in new genomic surroundings, which leads to the different promoter sequence and results in expression.

The increase of the ethylene concentration in the apices of this genotype results in the development of a sub-gynoecious sex type. Genotypes with the homozygous mutated *CsACS1G* locus develop a higher ethylene concentration and thus, a gynoecious sex expression (Atsmon and Tabbak 1979). These assumptions are in accordance with the previously described results, espe-

cially from Trebitsh et al. (1997) and Kamachi et al. (2000).

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