Abstract

Andromonoecy is a widespread sexual system in angiosperms, characterized by plants carrying both male and bisexual flowers. Monoecy is characterized by the presence of both male and female flowers on the same plant. In cucumber, these sexual forms are controlled by the identity of the alleles at the M locus. In melon, we recently showed that the transition from monoecy to andromonoecy result from a mutation in 1-aminoacyclopropane-1-carboxylic acid synthase (ACS) gene, CmACS-7. To isolate the andromonoecy gene in cucumber we used a candidate gene approach in combination with genetical and biochemical analysis. We demonstrated co-segregation of CsACS2, a close homolog of CmACS-7, with the M locus. Sequence analysis of CsACS2 in cucumber accessions identified four CsACS2 isoforms, three in andromonoecious and one in monoeic lines. To determine whether the andromonoecious phenotype is due to a loss of ACS enzymatic activity, we expressed the four isoforms in Escherichia coli and assayed their activity in vitro. Like in melon, the isoforms from the andromonoecious lines showed reduced to no enzymatic activity and the isoform from the monoeic line was active. Consistent with this, the mutations leading andromonoecy were clustered in the active site of the enzyme. Based on this, we conclude that active CsACS2 enzyme leads to the development of female flowers in monoeic lines, whereas a reduction of enzymatic activity yields hermaphrodite flowers. Consistent with this, CsACS2, like CmACS-7 in melon, is expressed specifically in carpel primordia of buds determined to develop carpels. Following ACS expression, inter-organ communication is likely responsible for the inhibition of stamina development. In both melon and cucumber, flower unisexuality seems to be the ancestral situation, as the majority of Cucumis species are monoeic. Thus, the ancestor gene of CmACS-7/CsACS2 likely have controlled the stamen development before speciation of Cucumis sativus (cucumber) and Cucumis melo (melon) that have diverged over 40 My ago. The isolation of the genes for andromonoecy in Cucumis species provides a molecular basis for understanding how sexual systems arise and are maintained within and between species.
and identified it as an 1-aminoacyclopropane-1-carboxylic acid synthase (ACS) gene, CmACS-7 [8]. ACS enzymes belong to a multigene family of pyridoxal 5'-phosphate (PLP)-dependent enzymes that catalyze the first committed and generally rate-limiting step in ethylene biosynthesis in higher plants [9]. We showed that CmACS-7 is specifically expressed in carpel primordia, and, in andromonoecious genotypes, a missense mutation leads to loss of enzymatic activity. Phenotypically, melon and monococious (a) gene appears to act similarly to cucumber Monocious (M) gene; in both species, the dominant allele suppresses stamen development in pistillate flowers without affecting male flower formation, whereas the recessive allele “releases” such inhibition, resulting in bisexual flowers instead of female flowers.

In the present study, we investigated whether the cucumber Monocious (M) locus is orthologous to the melon a locus, and encodes for an ACS homologous to CmACS-7. Using a genetic approach we showed that CsACS2, a cucumber ACS highly homologous to CmACS-7, co-segregates with the Monocious (M) locus. CsACS2 like CmACS-7 is specifically expressed in buds determined to develop a carpel in carpel primordia and, like in melon, loss-of-function mutations, located near the active site of CsACS2 are associated with andromonoecy.

Results

Identification of the cucumber orthologue of melon CmACS-7

In our previous study [8], we have shown that the melon and monococious (a) gene encodes CmACS-7, and that andromonoecy results from a mutation in its active site. As the cucumber M locus acts similarly to melon a gene, we asked whether the cucumber ortholog of CmACS-7 could be encoded by the M locus. To test this hypothesis, we designed PCR primers to amplify the CmACS-7 ortholog in cucumber and amplified a 2420-bp genomic fragment. Sequence analysis of the amplified DNA shows that, similar to melon, the cucumber gene contains 3 exons and 2 introns (Figure 1A), and encodes an ACC synthase of 445 amino acids that corresponds to CsACS2 (GenBank accession no. D89732; [10]). Homology analysis showed that the coding regions of CmACS-7 and CsACS2 share 98% identity in their amino acid sequence and only differ in eight residues, all of which are located in non-conserved positions among seed plants (Figure 1B). The other three ACS family members reported in cucumber (CsACS1G and GenBank accessions no. BAA33374 and BAA33375) are more distant, and only share 53–62% amino acid identity with CmACS-7. A phylogenetic tree, representing twelve ACS sequences from different plants were aligned, we observed that the residues G33, P209 and S399 are conserved across all plastid proteomes (Figure 3A; [11]). P209 and S399 residues are located in the conserved box 4 and 7, respectively, in close proximity to invariant residues that are conserved in all aminotransferases (Figure 3A; [12]). Crystallographic studies have determined that residues in the box 4 and 7 are involved in binding of the PLP cofactor and the enzyme substrate S-adenosyl methionine (SAM), respectively. The G33 residue is located nearby the residues P209, V34 and F35 that contribute to the hydrophobic pocket where adenosine ring of SAM is positioned (Figure 3B; [13]).

To determine whether hermaphrodite flowers in andromonoecious and hermaphroditic plants is due to loss of a CsACS2 enzymatic activity, we expressed the four different forms of the protein as six-histidine tag (His6-CsACS2) fusion proteins in Escherichia coli. The purified proteins were assessed for enzymatic activity in vitro by monitoring 5'-methylthioadenosine (MTA) formation at different PLP concentrations. The L294 isoform was found to be totally inactive. The C253 isoform has a reduced activity and no tendency towards saturation was observed up to 200 μM of SAM leading to a 15-fold increase in the Km value compared to CsACS2 isoform (Figure 4, Table 2). The S200 ACS isoform has a reduced enzymatic activity; its Vmax is approximately 17% of the Vmax of CsACS2 (Figure 4, Table 2). Consistent with this, in tomato, a P209 ACS mutants generated by site-directed mutagenesis displayed more than 95% reduction of the enzymatic activity [14]. P209 is a conserved residue of ACS box 4 involved in PLP binding. The decrease in the enzyme activity is likely due to less efficient binding of the PLP cofactor [13]. Taking all these data together and because the three isoforms from the lines homozygote for the m alleles showed reduced to no enzymatic activity and the unique isoform from the lines harbouring the dominant M alleles was active we concluded that the loss of CsACS2 enzyme activity is likely the cause of the apparition of hermaphroditic flowers in cucumber.

Structural and biochemical basis for CsACS2 allelic variations

To understand the basis of allelic differences in the M gene, we compared the sequence of CsACS2 in 28 cucumber accessions (Table 1). In the absence of structured cucumber core collections, accessions were selected based on their sexual types and geographic origin to cover the maximum genetic diversity. All tested monocious, gynoecious and androecious accessions harbouring the dominant M allele presented the same CsACS2 protein sequence. In contrast, hermaphrodite and andromonoecious accessions, harbouring the recessive m allele, revealed three protein isoforms, G33C, P209S and S399L, each differ by a single amino acid mutation of CsACS2 (Figure 1B). When the closest ACS homologous sequences from different plants were aligned, we observed that the residues G33, P209 and S399 are conserved across all plastid proteomes (Figure 3A; [11]). P209 and S399 residues are located in the conserved box 4 and 7, respectively, in close proximity to invariant residues that are conserved in all aminotransferases (Figure 3A; [12]). Crystallographic studies have determined that residues in the box 4 and 7 are involved in binding of the PLP cofactor and the enzyme substrate S-adenosyl methionine (SAM), respectively. The G33 residue is located nearby the residues P209, V34 and F35 that contribute to the hydrophobic pocket where adenosine ring of SAM is positioned (Figure 3B; [13]).

To determine whether hermaphrodite flowers in andromonoecious and hermaphroditic plants is due to loss of a CsACS2 enzymatic activity, we expressed the four different forms of the protein as six-histidine tag (His6-CsACS2) fusion proteins in Escherichia coli. The purified proteins were assessed for enzymatic activity in vitro by monitoring 5'-methylthioadenosine (MTA) formation at different PLP concentrations. The L294 isoform was found to be totally inactive. The C253 isoform has a reduced activity and no tendency towards saturation was observed up to 200 μM of SAM leading to a 15-fold increase in the Km value compared to CsACS2 isoform (Figure 4, Table 2). The S200 ACS isoform has a reduced enzymatic activity; its Vmax is approximately 17% of the Vmax of CsACS2 isoform (Figure 4, Table 2). Consistent with this, in tomato, a P209 ACS mutants generated by site-directed mutagenesis displayed more than 95% reduction of the enzymatic activity [14]. P209 is a conserved residue of ACS box 4 involved in PLP binding. The decrease in the enzyme activity is likely due to less efficient binding of the PLP cofactor [13]. Taking all these data together and because the three isoforms from the lines homozygote for the m alleles showed reduced to no enzymatic activity and the unique isoform from the lines harbouring the dominant M alleles was active we concluded that the loss of CsACS2 enzyme activity is likely the cause of the apparition of hermaphroditic flowers in cucumber.
Figure 1. Sequence analysis of CsACS2. (A) Schematic diagram of CsACS2 gene structure. The numbers indicate the size of the 3 exons (filled boxes) and the 2 introns (black lines) in bps. The grey boxes indicate the 5' and the 3' UTR. (B) Alignment of the melon protein CmACS-7 and cucumber CsACS2. The grey boxes indicate the 8 residues that are polymorphic between CsACS2 and CmACS-7. The blue and red boxes indicate the amino acid changes associated with the andromonoecious phenotype in melon and cucumber, respectively. (C) Relationships between CmACS-7, CsACS2 and ACS from Arabidopsis. CsACS1G, BAA33374 and BAA33375 are ACS from cucumber. The distance tree was produced using ClustalW to align the sequences and using a neighbour-joining algorithm to group them. The length of lines connecting the proteins indicates the mean number of estimated substitutions per site (corrected for multiple substitutions). Scale bar, 0.05 substitution per site.
CsACS2 is expressed in carpel primordia of female and hermaphrodite flowers

In cucumber, a detailed morphological description divided the flower meristem development into 12 distinct stages before anthesis [15]. The critical stage at which development of the inappropriate sexual organ is arrested occurs at stage 6, just after the elaboration of carpel primordia [15]. In situ expression analysis carried out by Saito et al. [16] showed that in gynoecious and monoecious cucumber accessions, CsACS2 mRNA began to accumulate during stage 4 and its expression continues at later stages. The early accumulation of CsACS2 mRNA was localised in the carpel primordia of flower determined to develop as female and not detected in male flower primordia of the monoecious plant. Since the m ms locus with CsACS2 activity accounts for the functional variation between the M and m alleles (Figure 4), we therefore concluded that CsACS2-mediated ethylene production in the carpel primordia likely prevents the development of the stamina in female flowers, but is not required for carpel development, since both mm and MM plants develop a functional carpel.

Discussion

In melon and cucumber, flower unisexuality seems to be the ancestral situation, as the majority of the Cucumis species are monoecious [18]. Based on our previous studies and the present work, we have demonstrated that the ancestor gene of CmACS-7/CcACS2 performed the stamen suppressing activity before speciation of Cucumis sativus and Cucumis melo. These two species, differing in chromosome number and probable geographic origin, belong to two different clades in the Genus Cucumis and were estimated to have diverged over 40 My ago [19,20]. Variants with pistillate-bisexual flowers appeared much later: the point mutations that reduced ACS activity are different in the two species. Interestingly, the same gene has been independently selected, in both clades, to “engineer” a bisexual flower. Andromonoecy is a major sexual system that has evolved independently numerous times [21] and is found in ~4,000 species in 33 angiosperm families [22]. It has been suggested that andromonoecy is selected to permit flexibility in resource allocation to male and female function, and is thus found in species where fruit set is resource-
Yamasaki et al. [7] showed that
[4,7,26]. The action of
development and inhibit stamen formation in the pistillate flowers
that are required to perform a dual action: promote pistil
the stamens, ethylene-insensitive. It remains, however, unclear why exogenous
prevent the synthesis of receptors, rendering the particular tissue
Therefore, lack of ethylene production at a critical site could
respond to ethylene by increasing their transcript levels [17].
biosynthesis and sensing genes such as
stamen elongation, and reduced induction of several ethylene
ethylene; the assays included inhibition of hypocotyl elongation, of
[28]. The above models suggests
femaleness increases along the shoot and can ''diffuse'' from an
rootstock to an
plants does not cure their deficiency [7];
possibly, the developmental window for the receptors to respond or
or ACC to act [29] is very narrow. The application of ethylene or
its precursor on the whole plant could have a pleiotropic effect that
masks the direct effect of ethylene on m-mediated sexual function.

The expression patterns of CsACS2 were shown to correlate with
femaleness: CsACS2 transcripts increased when monoecious plants entered the female phase [7,10,30,31], or were exposed to short
photoperiods that enhance femaleness [31]. Spatial expression patterns of CsACS2 were reported by Yamasaki et al. [30] and by
Saito et al. [16] to be closely related to the fate of specific flower
buds. CsACS2 mRNA was strongly localised in the carpel
primordium of flower determined to develop as female and not
detected in male flower primordia. By quantitative RT-PCR we
showed that CsACS2 is mainly expressed in female and
hermaphrodite flowers. These data are similar to those we recently
reported in melon for CmACS-7 [8].

The observation that the ethylene signal required to inhibit the
stamens is generated in a different tissue, the carpel primordium,
and must be translocated to the target tissue, is puzzling. Ethylene
has a dual role and is also required at the same time for carpel
development; applying exogenous hormone, or engineering
melons to over-express ACS, resulted in increased femaleness
[32]. Nevertheless, melon and cucumber plants expressing inactive

<table>
<thead>
<tr>
<th>Accession</th>
<th>Source</th>
<th>Sex type</th>
<th>Genotype</th>
<th>Amino acid 33</th>
<th>Amino acid 209</th>
<th>Amino acid 399</th>
<th>Bisexual flowers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erez</td>
<td>Zeraim Gedera</td>
<td>androecious</td>
<td>MM ff aa</td>
<td>G</td>
<td>P</td>
<td>S</td>
<td>no</td>
</tr>
<tr>
<td>PI 369717</td>
<td>Poland</td>
<td>androecious</td>
<td>MM ff aa</td>
<td>G</td>
<td>P</td>
<td>S</td>
<td>no</td>
</tr>
<tr>
<td>249-2</td>
<td>Hazera Genetics</td>
<td>gynoecious</td>
<td>MM FF AA</td>
<td>G</td>
<td>P</td>
<td>S</td>
<td>no</td>
</tr>
<tr>
<td>Elem Female</td>
<td>Zeraim Gedera</td>
<td>gynoecious</td>
<td>MM FF AA</td>
<td>G</td>
<td>P</td>
<td>S</td>
<td>no</td>
</tr>
<tr>
<td>Beth Alfa</td>
<td>Zeraim Gedera</td>
<td>monoecious</td>
<td>MM FF AA</td>
<td>G</td>
<td>P</td>
<td>S</td>
<td>no</td>
</tr>
<tr>
<td>CGN22246</td>
<td>Czechoslovakia</td>
<td>monoecious</td>
<td>MM FF AA</td>
<td>G</td>
<td>P</td>
<td>S</td>
<td>no</td>
</tr>
<tr>
<td>ECD-mono.</td>
<td>Hannover Univ.</td>
<td>monoecious</td>
<td>MM FF AA</td>
<td>G</td>
<td>P</td>
<td>S</td>
<td>no</td>
</tr>
<tr>
<td>ED-mono.</td>
<td>Hannover Univ.</td>
<td>monoecious</td>
<td>MM FF AA</td>
<td>G</td>
<td>P</td>
<td>S</td>
<td>no</td>
</tr>
<tr>
<td>Market More 76</td>
<td>Toronto Univ.</td>
<td>monoecious</td>
<td>MM FF AA</td>
<td>G</td>
<td>P</td>
<td>S</td>
<td>no</td>
</tr>
<tr>
<td>Oman</td>
<td>Kew Gardens UK</td>
<td>monoecious</td>
<td>MM FF AA</td>
<td>G</td>
<td>P</td>
<td>S</td>
<td>no</td>
</tr>
<tr>
<td>Poinsett 76</td>
<td>Indam Seeds</td>
<td>monoecious</td>
<td>MM FF AA</td>
<td>G</td>
<td>P</td>
<td>S</td>
<td>no</td>
</tr>
<tr>
<td>Shimshon</td>
<td>Zeraim Gedera</td>
<td>monoecious</td>
<td>MM FF AA</td>
<td>G</td>
<td>P</td>
<td>S</td>
<td>no</td>
</tr>
<tr>
<td>Wrd-mono.</td>
<td>Hannover Univ.</td>
<td>monoecious</td>
<td>MM FF AA</td>
<td>G</td>
<td>P</td>
<td>S</td>
<td>no</td>
</tr>
<tr>
<td>Ames 3950</td>
<td>Australia</td>
<td>andromonoecious</td>
<td>mm ff AA</td>
<td>C</td>
<td>P</td>
<td>S</td>
<td>yes</td>
</tr>
<tr>
<td>CGN21634</td>
<td>Nigeria</td>
<td>andromonoecious</td>
<td>mm ff AA</td>
<td>C</td>
<td>P</td>
<td>S</td>
<td>yes</td>
</tr>
<tr>
<td>CGN22937</td>
<td>Australia</td>
<td>andromonoecious</td>
<td>mm ff AA</td>
<td>C</td>
<td>P</td>
<td>S</td>
<td>yes</td>
</tr>
<tr>
<td>PI 292012</td>
<td>Israel</td>
<td>andromonoecious</td>
<td>mm ff AA</td>
<td>C</td>
<td>P</td>
<td>S</td>
<td>yes</td>
</tr>
<tr>
<td>PI 618932</td>
<td>China</td>
<td>andromonoecious</td>
<td>mm ff AA</td>
<td>C</td>
<td>P</td>
<td>S</td>
<td>yes</td>
</tr>
<tr>
<td>SGN22933</td>
<td>USA</td>
<td>andromonoecious</td>
<td>mm ff AA</td>
<td>C</td>
<td>P</td>
<td>S</td>
<td>yes</td>
</tr>
<tr>
<td>319H</td>
<td>Michigan State Univ.</td>
<td>hermaphrodite</td>
<td>mm FF AA</td>
<td>G</td>
<td>S</td>
<td>S</td>
<td>yes</td>
</tr>
<tr>
<td>CGN22938</td>
<td>URSS</td>
<td>hermaphrodite</td>
<td>mm FF AA</td>
<td>G</td>
<td>S</td>
<td>S</td>
<td>yes</td>
</tr>
<tr>
<td>ECD-hermaph.</td>
<td>Hannover Univ.</td>
<td>hermaphrodite</td>
<td>mm FF AA</td>
<td>G</td>
<td>S</td>
<td>S</td>
<td>yes</td>
</tr>
<tr>
<td>ED-hermaph.</td>
<td>Hannover Univ.</td>
<td>hermaphrodite</td>
<td>mm FF AA</td>
<td>G</td>
<td>S</td>
<td>S</td>
<td>yes</td>
</tr>
<tr>
<td>PI 351139</td>
<td>Former soviet union</td>
<td>hermaphrodite</td>
<td>mm FF AA</td>
<td>G</td>
<td>S</td>
<td>S</td>
<td>yes</td>
</tr>
<tr>
<td>PI 356809</td>
<td>Former soviet union</td>
<td>hermaphrodite</td>
<td>mm FF AA</td>
<td>G</td>
<td>S</td>
<td>S</td>
<td>yes</td>
</tr>
<tr>
<td>PI 370643</td>
<td>Former soviet union</td>
<td>hermaphrodite</td>
<td>mm FF AA</td>
<td>G</td>
<td>S</td>
<td>S</td>
<td>yes</td>
</tr>
<tr>
<td>Wrd-hermaph.</td>
<td>Hannover Univ.</td>
<td>hermaphrodite</td>
<td>mm FF AA</td>
<td>G</td>
<td>S</td>
<td>S</td>
<td>yes</td>
</tr>
<tr>
<td>CGN 22961</td>
<td>Former soviet union</td>
<td>hermaphrodite</td>
<td>mm FF AA</td>
<td>G</td>
<td>S</td>
<td>S</td>
<td>yes</td>
</tr>
</tbody>
</table>

Table 1. Cucumber germplasms used in this study.

doi:10.1371/journal.pone.0006144.t001
CsACS2 isoforms are not compromised in carpel formation, suggesting that other ACS isoforms provide the carpel-promoting function. Thus, CsACS2 is dedicated to stamen arrest, whereas other ACS isozymes that act in the carpel, such as the F-locus that encodes CsACS1G, cannot affect the stamens [33–35]. Such specificity of enzymes that produce a gaseous end-product could seem counter-intuitive. It implies that ethylene synthesis and perception are regulated in spatially restricted patterns that change during development and among sex types. The pistil-located ACS could export ACC towards the stamen, where ethylene will eventually form by a local ACC oxidase. The other ACS isoforms present in the carpel could be expressed at different stages or tissues, such that ACC export does not occur at the critical time. This could ensure that the inhibitory signal is only activated in pistillate flowers that express CsACS2, and not in male flowers. This provides a molecular explanation to the differential action of M on male versus female flowers.

Methods

Plant material and segregating populations

Cucumber genotypes of different sex types used for this study are listed in Table 1. To perform linkage analysis of sex determining genes, three segregating populations were prepared. A common monoeocious accession, Cucumis sativus var. hardwickii from Holofer, Oman (“Oman”), accession 0092760, Kew Botanical Gardens, UK was crossed with cultivated cucumber lines to maximize the level of polymorphism between the partners and facilitate mapping. For analyzing the segregation of the Female (F) gene, a gynoeccious line, Elein Female (FFMMLL) was crossed with the monoeocious genotype, “Oman” (fMMLA), the F1 was self-fertilized and an F2 population was produced. For the androecious (a) gene segregation, “Oman” was crossed with an androecious line, Erez (fMMaa), and the F1 was selfed to produce the F2 population. To analyze the segregation of the Male (M) gene, “Oman” was crossed with an hermaphrodite line 319H, genotype FFmmAA, and
the F1 hybrid was back-crossed to 319H to produce a BC1 progeny. The rest of the genotypes in Table 1 served for the accession comparison of the CsACS2 sequences.

Plants were grown in the greenhouse in the spring and summer in 10 L pots under standard agronomic conditions and evaluated for the sex of their flowers at each node along the main stem and side branches 2–3 times, for at least 30 nodes of the main stem. Individual plants' leaves were sampled and DNA was extracted according to Baudracco-Arnas [37].

**Table 2.** The kinetic parameters, Km and Vm, measured for CsACS2, S209 and C33 protein isoforms.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Km (µM)</th>
<th>Vm (µmol.min⁻¹.mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsACS2</td>
<td>7 ± 1.5</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>S209</td>
<td>13 ± 3</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>C33</td>
<td>104 ± 3</td>
<td>2.0 ± 0.3</td>
</tr>
</tbody>
</table>

L399 isoform was not included in this table as it is completely inactive. doi:10.1371/journal.pone.0006144.t002

**Plant genotyping**

To identify plants carrying recombination events, plant NA was extracted from each individual of the three segregating populations. Determination of the sexual phenotype was performed for all plants. For genotyping, a 302 pb NA fragment of the CsACS2 gene was amplified with primers listed in Table S2 and sequenced. Polymorphisms within this fragment were used to determine recombinant alleles.
Quantitative RT-PCR and RACE-PCR experiments

Total RNA was extracted from frozen leaves, stems and flowers using the Trizol reagent (Invitrogen). To avoid sampling contamination and to accurately assay the CsACS2 mRNA expression, we used cucumber accessions bearing only one flower type: the male, and bisexual flower buds were collected from the gynoecious line Elem Female, and from the androecious line, Erez, respectively. Contaminating DNA was removed by NaseI treatment (Invitrogen). First-strand cDNA was synthesized from 2 μg of total RNA using the SuperScript® III reverse transcriptase (Invitrogen). Primer design was performed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers sequences used are listed in Table S2. To check the specificity of the designed primers, all amplicons were sequenced and blasted against NCBI database. Polymerase chain reactions were performed in an optical 384-well plate with an ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems) apparatus, using qPCR MasterMix Plus for SYBR® Green I w/o UNG (Eurogentec) and according to manufacturer’s instructions. Cycling conditions were as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min. PCR amplification specificity was verified by a dissociation curve (55°C to 95°C). A negative control without cDNA, technical replicates on three independent cDNA samples (derived from the same RNA sample), and three independent biological experiments were performed in all cases. Data were analysed using the S S 2.0 software (Applied Biosystems). To compare data from different PCR runs and cDNA samples, Ct values for CsACS2 were normalized to the Ct value of CsACTIN2 (primers shown in Table S2). CsACS2 relative expression was determined as described in Czechowski et al. [30].

Bacterial strains, plasmids and chemicals

The BL21 (DE3) pLYS cells transformed with CsACS2 protein expression constructs were incubated in 25 ml of Luria-Bertani medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) supplemented with ampicillin and chloramphenicol (50 μg/ml each) and incubated overnight at 37°C. This pre-culture was used to inoculate 2 L of the same medium supplemented with ampicillin (50 μg/ml), and the cells were grown at 37°C in a shaking incubator at a speed of 180 rpm until O.D.600 = 0.6. IPTG was added (0.5 mM) to induce protein expression and cells were grown for 5 hours at 25°C, harvested by centrifugation and kept overnight at −45°C. The frozen cells were resuspended in 15 ml of 50 mM TRIS pH 7.9 and 500 mM NaCl buffer, disrupted on ice by sonication in the presence of the protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and aprotinin (10 μg/ml each). The cell debris were removed by centrifugation at 13000 g for 15 min and the supernatant was immediately used for enzyme purification.

Purification of recombinant CsACS2

The supernatant was applied to a Ni-NTA 15 ml column (Sigma) equilibrated with 30 mM Tris at pH 8, 500 mM NaCl buffer. The column was washed with 50 mM Tris at pH 8, 500 mM NaCl buffer supplemented with 10 mM imidazole until no protein was detected in the flow-through. Wild type or mutant forms of CsACS2 were eluted using the same buffer supplemented with 100 mM imidazole and dialyzed against 50 mM K-Phosphate buffer supplemented with 10 mM imidazole and dialyzed against 50 mM K-Phosphate buffer at pH 8.5 in the absence of PLP, and then concentrated using a Millipore Amicon® Ultra device (5,000 MWCO). The concentrated fraction (20 mg/ml) of CsACS2 was aliquoted and stored at −45°C in the presence of 25% glycerol until use. Protein purification was performed by capillary electrophoresis on an Experion® device (Bio Rad), using PRO200 chips, according to the manufacturer’s instructions.

Partial purification of Adenosine Deaminase

Aspergillus adenosine deaminase purchased from Sigma was further purified by ethanol fractionation according to Wollenden et al. [39]. Specifically, 5 g of lyophilized deaminase powder were suspended in 90 ml of cold water in a glass beaker and 47 ml of acetone were added. The mixture was stirred at 4°C for 5 min then centrifuged at 2000 g for 1 min. The resulting pellet was mixed with 33 ml of water and stirred for 5 min, centrifuged at 2000 g for 5 min and the pellet was discarded. Ten ml of ethanol were added to the supernatant, the mixture was stirred at 4°C for 5 min and centrifuged again. Twenty ml of Ethanol were added to the supernatant and gently stirred at 4°C for 3 hours. The alcoholic mixture was centrifuged for 5 min at 7000 g and the pellet resuspended with 6 ml of water. The protein solution was then dialyzed against a 5 mM solution of sodium acetate at pH 5.3 for at least 24 hours. The resulting solution was concentrated using a Millipore Amicon® Ultra device (5,000 MWCO) in the presence of glycerol (20%) to 5 mg/ml of protein, aliquoted and stored at −45°C until use. The ethanol precipitated deaminase was routinely found to be 1000 times more active than the original powder (activity measured in 100 mM HEPES, pH 5.5 buffer using 5’AMP as a substrate, data not shown).
ACS activity assays

ACC synthase activity was determined by monitoring MTA formation by differential spectroscopy, recorded on an Uvikon 940 spectrophotometer (Biotek-Kontron Instruments) according to White et al. [40]. Specific activity measurements were performed in triplicate using 3 different enzyme preparations. We incubated 60 µM SAM in 100 mM K-Phosphate buffer, pH 8.5 (0.2 ml), and adenosine deaminase (8 µg for 0.2 ml) in the absence or presence of PLP (ranging in concentration from 0 to 100 µM) in a quartz cuvette for 3 min at 25°C, after addition of the purified enzyme (1 to 2 µg/l). The conversion of the MTA produced by ACS into an imine derivative was monitored at 256 nm (ε MTA = –7740 M⁻¹ cm⁻¹) and specific activity was expressed as mol of MTA formed per min per mg of protein. The same protocol was followed for Vm and Km determination with a concentration of SAM ranging from 1 to 100 µM.

Protein structure modeling

The CaACS2 three-dimensional structures were generated using the Geno3D server (http://geno3d.pdb.cgl.ucsf.edu). Superposition of the tomato D ACS structure (1HAY.pdb) determined by x-ray crystallography [13], and our three models of CaACS2, was carried out and visualized using the Chimera server (http://www.cgl.ucsf.edu/chimera).

Phylogenetic analysis

Multiple sequence alignment of full-length protein sequences was performed using the ClustalW (http://www.ebi.ac.uk/Tools/clustalw2). Phylogenetic trees were constructed by MEGA4 (http://www.megasoftware.net/index.html) based on the Neighbor-Joining method.

Supporting Information

Table S1 Segregation analysis of andromonoecious (m) locus with CaACS2. Detailed scores for the parental lines, and 91 backcross-1 individual progeny, are displayed for the sex phenotype (Mm, monoecious, mm, hermaphrodite) and for 3 SNPs in the CaACS2 genomic sequence. SNP C1391T causes the amino acid P2098 substitution that causes the allelic M/m difference. Found at: doi:10.1371/journal.pone.0006144.s001 (0.06 MB XLS)

Table S2 Primers used in this study

Found at: doi:10.1371/journal.pone.0006144.s002 (0.03 MB XLS)

Acknowledgments

We thank P. Audigier and C. Lepage for plant care and A. Martin, C. Clepet and A. Winger for discussions and comments on the manuscript.

Author Contributions

Conceived and designed the experiments: AB RPT AB. Performed the experiments: AB CT IK MAS RPT. Analyzed the data: AB CT MAS RPT AB. Wrote the paper: AB RPT AB.

References