Sex-specific marker and *trans*-zeatin ribosidase in female annual Mercury

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Abstract

Mercurialis annua is dioecious with unisexual flowers lacking vestiges of opposite sex organs. Previously, we showed that the cytokinin *trans*-zeatin (*t*-Z) was correlated with female sex genes whereas *t*-Z production stops at its precursor, the *trans*-zeatin riboside (*t*-ZR) in males. Hoping to further relate sex genes and *t*-Z production, we underwent a search for eventual female-specific DNA and for a specific enzyme producing the feminizing signal. We report the cloning of a female-specific fragment and the identification of a *t*-ZR β-ribosidase. The specific-DNA was isolated by AP-PCR (arbitrary primed-polymerase chain reaction) from a female line. Its sequence shows significant identity with the precursor of the β-subunit of polygalacturonase (PG) isoenzyme1 cDNA expressed during tomato fruit development. A stereospecific *t*-ZR β-ribosidase activity was purified 490-fold from female apices. It belongs to a female non-covalent enzymatic complex which contains a fructose 1-6 biphosphate aldolase and two 14-3-3 proteins (N-terminal sequences). Furthermore, a cytokinin-oxidase(s) and a PG activity were associated to the purified complex. Together, these results suggest that one PG subunit could be the product of the female-specific DNA which, consequently, cannot be a feminizing allele. Complete purification of the *t*-ZR ribosidase will enable us to further investigate female sex genes and female-marker relationships. © 1998 Published by Elsevier Science Ireland Ltd.

Keywords: Sex-specific DNA; Female-enzymatic complex; Cytokinin metabolism; Sex determination; Annual Mercury

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1. Introduction

Plant flowers generally exhibit both types of sexual organs conferring the hermaphroditic condition. In the majority of species with unisexual flowers, the floral meristem is generally considered to be prevented from forming the organs of the opposite sex which remain vestigial, and the flower is functionally staminate or pistillate. In this case, flower development involves the arrest of preformed sexual organs in bisexual floral primordia. This process is considered to be regulated by sex determination genes [1]. Numerous Euphorbiaceae species present unisexual flowers without vestigial organs of the opposite sex. Floral primordia seem to avoid the hermaphrodite condition, suggesting that sex determinants act earlier than the appearance of floral primordia. This is probably the case of the dioecious weed mercury, *Mercurialis annua*, with male and female plants which also differ in general architecture and physiology [2]. Previous correlated genetic and hormonal studies [3,4] have demonstrated that the male sex is determined by three independently segregating genes A, B1 and B2. The dissociation of the dominance of A plus B genes induces the female sex. Genes B1 and B2 or corresponding alleles determine the degree of maleness or femaleness. They are implied in the process of cytokinin metabolites production inducing the male or the female phenotype with probably a direct link between feminizing alleles b1b2 and the female-specific cytokinin metabolite *trans*-zeatin (*t*-Z). In males, the zeatin pathway stops at its immediate precursor, the *trans*-zeatin riboside (*t*-ZR), whereas the *t*-Z mononucleotide accumulates.

Until now, only genes determining the abortion of organs of one sex in originally bisexual flower primordia have been isolated. TASSELSEED2 (TS2) which determines the abortion of pistils and induces a male development in maize tassels has been characterized and cloned. The predicted amino acid sequence of the Ts2 protein shows significant similarity to hydroxysteroid dehydrogenase. The authors [5] think that a gibberellin or a steroid-like molecule might be the substrate for Ts2 action. Also from maize, the anther ear1 gene

An1, has been cloned. This gene induces perfect flowers on normally pistillate ears and is involved in the synthesis of copalyl-diphosphate, the first intermediate in gibberellin biosynthesis [6]. The third species with abortive unisexuality in which male-specific restriction fragments have been identified is *Silene latifolia*. The relative difference analysis method revealed at least three specific markers in relation with sex determining genes (gynoecium suppressor, stamen development, anther maturation). They were assigned to Y-linked loci by comparison with the karyotypes of various flower mutants [7].

To further investigate the female sex development in mercury, we looked for the possible presence of female-specific DNA and for the existence of a female-specific enzyme able to convert *t*-ZR into the specific feminizing cytokinin *t*-Z.

2. Materials and methods

2.1. Plant material

Experiments were performed with the dioecious plant *Mercurialis annua* L., $2n = 16$, Euphorbiaceae. The isolation of female-specific DNA fragments was performed using the isogenic line 19-$_5$ of sex genotype AAb1b1b2b2 [3]. Two male strains of sex genotype Aa,b1b1,B2B2, weak male, Aa,B1B1,B2B2, resistant male, were selected for the degree of resistance to feminization by benzylaminopurine (10^{-7}M three times a day, for 14 days) from 500 wild male plants from the habitual natural population. Ten resistant males and ten males easily feminized (weak), after their return to male state, were selected and submitted again to feminization. Four males of each category were again treated. Finally, two resistant and two weak males were propagated as cuttings. The resistant male R11, and the weak male W23, were used in the experiments.

Wild male and female plants of natural populations from surroundings of Orleans containing characteristic proportions of possible sex genotypes [3] were used for protein isolation and to verify the presence of sex-specific DNA fragments.

Table 1
Purification steps of the active products from female apical shoots

Purification steps	Total proteins (mg)	Total activity (nmol h^{-1})	Specific activity (nmol h^{-1} mg^{-1})	Yield (%)	Purification factor
1. Crude extract	168	521	3.1	100	1
2. 40–70% SO$_4$(NH$_4$)$_2$	71	497	7	95	2.2
3. Sephadex G200 filtration	22	192	8.7	37	2.8
4. DEAE MemSep1010	4	42	10.5	8	3.4
5. Hitrap blue affinity	0.8	36.4	45.5	7	14.7
6. 5′-Amp-sepharose 4B affinity	0.03	45.6	1520	8.7	490

2.2. DNA extraction, blot analyses, isolation and nucleotide sequence of sex-fragments

Genomic DNA was isolated according to Graham and Robert [8]. The sex-specific fragments amplified from Eurogentec arbitrary primers by a PCR standard program were identified on agarose gel migrations. Transfers to Hybond membranes (Amersham) of AP-PCR fragments or of EcoRI and HindIII digests were hybridized with [α^{32}P]dCTP (110 PBq mol^{-1}) labeled probes (prime-a-gene kit of Promega). The DNA of selected fragments, eluted from gel slices by repeated freezing-thawings was purified by classical methods. Cloning was done in the pCR-Script Amp SK$^+$ plasmid. Plasmids containing DNA inserts with their respective primers were completely sequenced on both strands with a DNA sequencer (Pharmacia ALF manager). Among the six reading frames, one, without stop codon was a complete open reading frame. Identity searches were performed according to the program FASTA in the sequence data banks EMBL, Genbank and NBRF and in the protein data bank NBRF. The new sequences are reported in the Genbank under accession number U79772.

2.3. Amino acid sequence and hydrophobic cluster comparisons

The deduced amino acid sequence of isolated mercury DNA and the corresponding part of tomato β-subunit cDNA product were compared by the HCA method. Hydrophobic clusters are determined in a two-dimensional representation of sequences and allow comparison and alignment of patterns. The efficiency of the method has been established for low sequence identities starting at 7% and the range of 12–20% identity is routinely workable [9].

2.4. Isolation of the enzymatic complex and enzymatic assays

Soluble protein extracts were prepared as previously reported [10] from 100 g of frozen wild female shoot apices. Wild male apices were used as control. After homogenization in 100 ml of the optimal buffer for cytokinin enzymes (Tris–HCl 55 mM, DTT 10 mM, EDTA 0.5 mM at pH 7.5), the active proteins were purified as indicated in Table 1. Protein concentration was measured according to the Bradford method [11]. The assay mixture for ZR-β-ribosidase activity was already described [10]. Reaction products, c- or t-Z, adenosine and adenine, were determined and measured by mass spectrometry [4]. The same method was used to demonstrate tomato PG inactivity upon ZR substrates. PG assays were done as described [12] and reducing sugars were measured by the arseniomolybdate method [13] using D-galacturonic acid as a standard.

2.5. Gel electrophoresis and N-terminal sequences

Electrophoreses were performed on the horizontal system Multiphor II (Pharmacia Biotech) using non-denaturing homogeneous 10% acry-

lamide gels (250 × 110 × 0.5 mm 50 mA, 300–900 V, 60 min) or in the presence of 10% mercaptoethanol on 7.5 or 15% acrylamide (Excel-gels 245 × 110 × 0.5 mm Pharmacia. Biotech). SDS-PAGE were done on 8–18% gradient Excel-gels (50 mA; 250–600 V, 80 min) and stained with Coomasie Briant blue or silver. After Coomassie blue staining, the protein stripes were cut out and eluted in 0.1 M sodium acetate at pH 8.5 and SDS 0.1% according to Kurth and Stoffel [14] for one night at 37°C. They were sequenced according to Edman degradation [15] on a Perkin–Elmer microsequencer (Procise 492). Before sequencing, protein products were filtered on a Prospin cartridge (Perkin–Elmer).

3. Results

3.1. Isolation of female-specific genomic DNA, sequence and homology

The PCR-based strategy for amplification and comparison of electrophoretic patterns of short arbitrary stretches of DNA from female and male genomes [16] was chosen because female-specific cDNAs could not be selected by several means [17,18].

After three successive selections among 40 primers, the stable and repetitive amplification of one specific 0.7 kb fragment, from the female line F was identifiable among PCR-products generated by the primer P04, 5′-GTGTCTCAGG-3′ (Fig. 1-I, A and B). The 0.7 kb fragment was not in any of the male strains (R, W) or any wild male individuals (2, 4). With the primer P03, 5′-CTG-ATA-CGC-C-3′, two male-specific fragments (1.1, 1.3 kb) were amplified. The sex-specific fragments and a non-sex-specific P04-fragment (0.9 kb) as positive control were used to confirm the female specificity by sequentially probing transfers of PCR-patterns (Fig. 1-II, III A and B) and of genomic digests (Fig. 1, C-I and -II). Hybridization of PCR transfers with the positive control (Fig. 1-II, A), with the male-specific probe used as negative control (Fig. 1-II B), and with the female probe (Fig. 1-III, A and B) are in complete agreement with the female specificity of the 0.7 kb

fragment. Genomic DNA gel blot analyses (Fig. 1 C-I and -II) of the female line F, and of a mixture of five wild females DNA (lane 5) with the putative female-specific probe, confirm its specificity. The DNA of male strains R, W and of wild males (lane 3) did not produce any signal. Together these results are consistent with the probe being a female-specific fragment represented as a unique sequence in the female genome at least for the tested genotypes.

The 0.7 kb fragment was cloned in the pBluescript plasmid and sequenced (Fig. 2). Adjacent to the cloning site Srf 1, the 5′ end was identified by the presence of the P04-primer and by the predicted amino acid sequence which reveals a 221 amino acids non-interrupted open reading frame determined as described in methods. The complementary sequence of P04 was recognised at the 3′ end.

Database searches indicated that the nucleotide sequence presents the highest similarity with the cDNA of the β-subunit of tomato polygalacturonase (PG) isoenzyme 1 precursor [19]. The female fragment corresponds to nucleotides 906–1569 of tomato cDNA. Extensive identity, 69.9%, spreads out between bases 321 and 658 of mercury and between 1255 and 1563 of tomato. The comparison of predicted amino acid sequences (Fig. 3) reveals 70% identity between amino acid residues 117 and 219 of mercury and 408 and 510 of tomato. After optimal alignment, the total identity is 53.3% and eight repeating motifs of the core consensus sequence FT-NYGxxGNGGxxx, of aromatic amino acid-rich glycoproteins, AroGPs, are superimposed. Zheng and co-authors [19] suggested a possible position for the carboxyl-terminus of the mature β-subunit of tomato near the Met-397. This residue is integrated in a small hydrophobic core (residues 393–399), shown in the hydrophobic cluster analyses of corresponding parts of deduced proteins (Fig. 4). A Met-residue is not present in mercury and the hydrophobic core is replaced by a series of hydrophilic amino acids (residues 101–109). Extensive identity begins after Lys-403 and 404 in tomato and Lys-116 and Arg-117 in mercury. These two basic residues could represent the cleavage sites of mature proteins by comparison
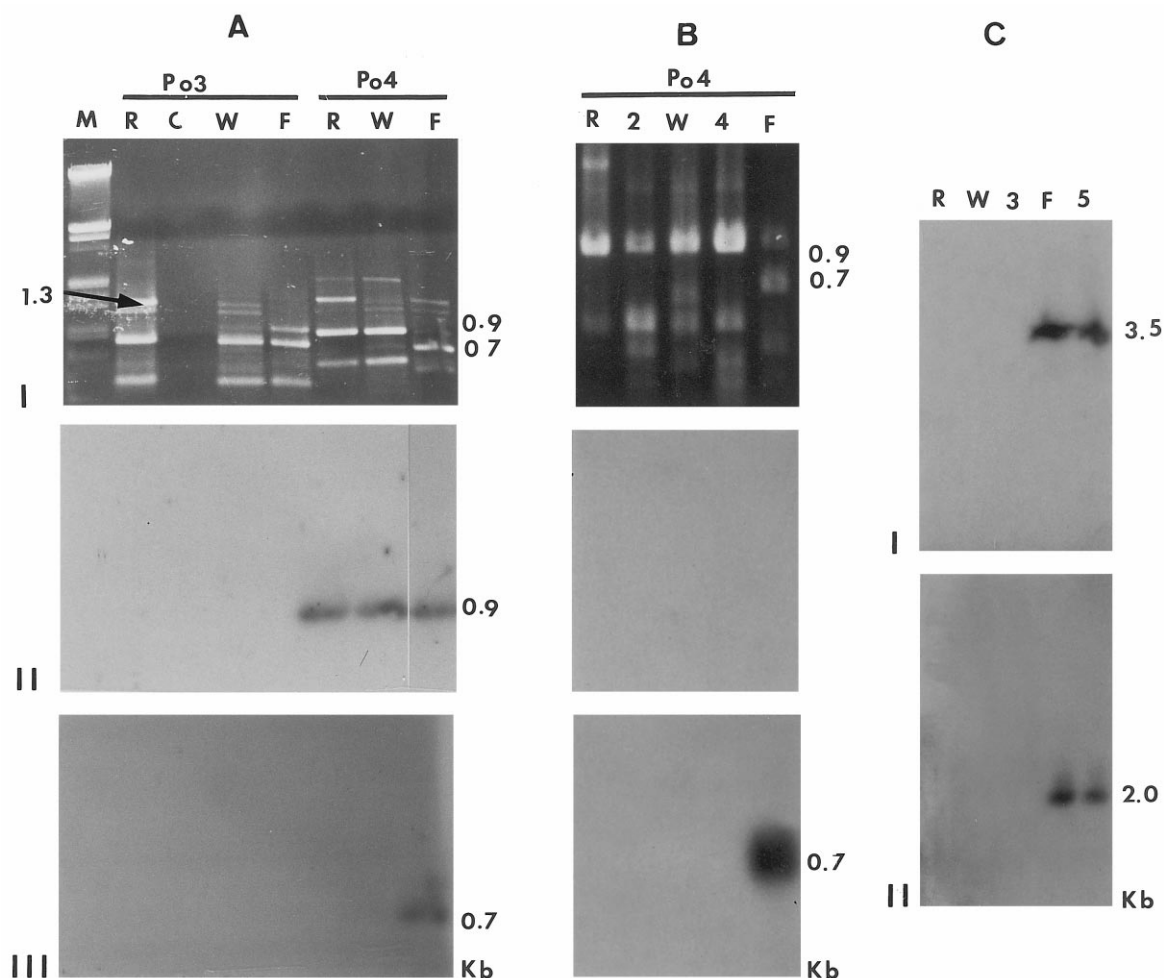
Fig. 1. Specificity of the female DNA fragment by comparison of electrophoretic mobility of AP-PCR fragments from male and female plants (A-I and B-I), by sequential hybridizations of transfers with control probes (A-II and B-II), with the female-specific probe (A-III and B-III) and by Southern blots analyses (C-I and -II). (A-I, B-I). The female-specific fragment 0.7 kb and the positive control 0.9 kb are generated by the P04-primer; two P03-fragments indicated by an arrow appear male-specific. (A-II) the positive control shows identical signals at the 0.9 kb level for male and female P04-fragments. (B-II) No hybridization of the male-specific probe (negative control) with the B-I transfer. (A-III and B-III) The female probe only hybridizes at the level of the female-specific P04-fragment (15 μl PCR-products by lane amplified from 5 μg DNA by the primers carried out into 1% gel, transferred to Hybond N⁺ filters). (C-I and C-II) Analyses of DNA digested by *Eco*RI and *Hin*dIII enzymes (15 μg digestion products by lane). A unique female digest hybridizes with the female-specific probe. (Molecular lengths in kilobases. M, λ size-markers; R, resistant male; C, control without DNA; W, weak male; F, female line 19 _ 5; 2, 4, wild-male individuals; 3, mixture of DNA from five wild females).

with the processing of precursor proteins in animals [20]. In both plants, the putative carboxyl-propeptides of precursor proteins are nearly identical, but the supplementary degenerated FVY motif, (residues 112–115) in mercury and the differences of hydrophobicity at the carboxyl-ends of mature proteins indicate structural differences between these species. Together, these results strongly suggest that the complete female-specific fragment encodes a protein of the AroGPs family.

3.2. Purification of a female enzymatic complex

The hormonal results [4] suggested a specific enzyme able to produce the feminizing signal t-Z, correlated with genes inducing the female sex. Further investigation required its purification and its comparison with the putative product of the female-specific fragment. After three classical purification steps and two successive affinity chromatographies, the activity able to convert the t-ZR to t-Z was enriched 490-fold, as shown in Table 1. The anion-exchange chromatography of step 4 resulted in six peaks of which only the third was active. The Cibacron blue 3GA-sepharose affinity gel bound ≈ 80% of total unwanted proteins, but did not bind the enzyme activity that is fixed onto 5′-AMP sepharose 4B and is eluted with 0.5M NaCl while no activity at all was present in the wash. The total protein composition was analyzed by electrophoresis after each step. At the end of the procedure, the non-dena-

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                                                    10        20         30         40         50        60
                                            MHTKIHLPFC ILLLLLFSLP SFNVVVGGDG ESGNPFTPKG YLIRYWKKQI SNDLPKPWEL
                                               70        80         90        100        110       120
                                            LNKASPLNAA QYATYTKLVA DQNALTTQLH TFCSSANIMC APDLSPSLEK HSGDIH...
                                              130        140        150        160        170       180
                                            SDKN...NYGT NEPGIGWNIE KNYSEGENIP VNS...RYGRG SPRDN...DNY ASDGNVIDQS
                                              190        200        210        220        230       240
                                            ENSYSTSTAG GSGK...NANA NANDNLH...  SXSDQGTGGV QK...SQEA NAGDQ...
                                              250        260        270        280        290       300
                                            GKNGNGANGE ENSYGNDINV IGSI...NYGQ TANGGDQ... SYGENGNVEE N-...GGG

                                                        Mercury NH2- VSQDNEH NI...GTGA
                                                                                                        17
                                              310        320        330         ¤        340        350       360
                                            NGPSEI...NSX RDQEN...GLDT ETTYVK-DANG GEAN...NGQ SENEGTDV...  TXGK-GGNDH
                                            *   * ***   **** ***  *   ***        ** * **    * **           ** **
                                            NTAVDE...SSX RNG...AVGHDS IC...YARNDNA GKVN...NNGK HSTLGNDI...  ...GKGGS-KGTT
                                              362        379         ¤         47         57         ¤  67     77
                                            IN. ...GMN NI...VKDT ATE...HNKT SQVLASIMEV NGGKKVNNRW VEPGKFEREK
                                            ****          ****           ** *  ***             *    ********
                                            TVG...GPD R...ICKG GIE...YINIT TTSGSEVY..  .......KRF VEPGKFERES
                                              87        97        ¤         115        118       128
                                            MLKSGTTMEM PDIKDKMEKR SELPRVIASK LPFSTSKIAE LKKIFHAGGE SQVEKMIGDA
                                            *** ** * *  ** * ****** ***  * *  *  * *** *  * ***** * *  * **
                                            MLKEGNMMM  PDIVDKMEKR SELPLSIVSK LPFSSSNLIE MKKIFHALDD SETERVIVNA
                                              138       148        158        168        178       188
                                            LSECERAPSA GETKRCVNSA EDMIDFATSV LGRNVVVRIT EDTKGSNGNI MIGSVKGING
                                            * **** ***  **** *  **  *******  *            ** ****   ****
                                            LAECERKASQ GETKQCVGSL EDMIDFAVSV LRH 221 ...-COOH
                                              198       208        218
                                              549       559        569        579        589       599
                                            GKVTKSVSCH QTLYPYLLYY CHSVEKVRVY EADILDPNSK VKINHGVAIC HVDTSSWGES
                                              609       619        630
                                            HGAFVALGSG FGKIEVCHWI FENDMIWAIA D
```

Fig. 3. Alignment of the amino acid sequence derived from the mercury female-specific DNA with the complete amino acid sequence encoded by the tomato polygalacturonase β-subunit isoenzyme 1 precursor cDNA [19]. Identical amino acids are indicated by stars. To maximize the identities, a nine amino acid gap has been introduced near the supposed end of the mature mercury PG enzyme. M residue -397 of tomato, absent from mercury is in bold. Potential N-glycosylation sites are indicated by squares. Amino acids shaded in grey represent the first residues of the consensus sequence (14 amino acids) for aromatic amino acid-rich glycoproteins defined in [19].

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ggagcccacogcggtgncggccgctctagcccgtgtctcaggataacccgcataatact   60
     *                                      V S Q D N P H N T
ttcaaaaactacggcacggtgcaaataccgccgtggatagcttctctagctacagaaat  120
F K N Y G T G A N T A V D S F S S Y R N
ggagctaatgttggtcatgactcgtttcagtcgtacgctaggaatacaaatgcaggaaag  180
G A N V G H D S F Q S Y A R N T N A G K
gttaatttcgtaaattatgggaaacattcaacctctggaaatgatacatttaaagaatat  240
V N F V N Y G K H S T L G N D T F K E Y
ggtaaaggttcaaagggtatcacaacggttggtttcaaaactggttacggtccggatcggct  300
G K G S K G I T T V G F K T Y G P D R A
ttcaaagattatattcagaaagtggcacttttgctcggttacaccaacactacaacgact  360
F K D Y I Q K G G T F A G Y T N T T T T
tggcagttttgtatataaaagatttgttgagcctggcaagtttttcaggggagtcaatgag  420
S G S F V T F V K R F R E S M
ttgaaggaaggggaatgttatggtcatgcccgacattgtagataaaatgcccaaaaggtca  480
L K E G N V M V M P D I V D K M P K R S
tttttgcccttatcgattgtctctaaattaaccattttcatcctcgaatcttacggagatg  540
F L P L S I V S K L P F S S S N L T E M
aagaaaaattttcatgcactagatgactcagaaacggagcggcgtgattgtcaacgcgcta  600
K K I F H A L D D S E T E R V I V N A L
gccgaatgcgagcgcgaaaagctagccagggtgagaccaagcagtgcgtgggctcgtttggag  660
A E C E R K A S Q G E T K Q C V G S L E
gatatgatagactttgctgtatacggtootcgagacacgggccggctgtcagg  720
D M I D F A V S V L R H                            *
aattgatgatatcaagcttatcgataccgtcgacctcgagggggggggc
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Fig. 2. Nucleotide and deduced amino acid sequences in the single-letter code of the female-specific DNA. The P04-primer at 5′-position and its inverse at 3′-position are shaded in gray. Both sides of the SrfI cloning site of the pCR-Script TM SK$^{(+)}$ plasmid and the three restriction sites, XmnI and 2 AvaII, of the insert are underlined. The extra-sequences in 5′ and 3′ beyond the SrfI site, are parts of the cloning plasmid in which asteriks indicate two different amino acids (GeneBank U.79772).

turing gel of the active eluate revealed a single silver stained band (Fig. 5A) of isoelectric point 5.4 when compared with commercially available standards on ampholine gradient electrophoresis (Fig. 5B). Although the subsequent steps for further separation led to the loss of activity, the fraction eluted from the 5′-AMP-sepharose chromatography was submitted to electrophoresis in the presence of mercaptoethanol on two different homogeneous gels (Fig. 5D, E). Several silver stained bands appeared, suggesting that to present activity, the enzyme must be associated with other proteins. SDS-PAGE gradient gels (Fig. 5C) and 2D-PAGE confirm a close protein association of at least six bands. Wild male extracts used as

Table 2
N-terminal sequences of three proteins from the female active fraction separated on SDS-PAGE and homologies[a]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
M[1]	X	X	X	A	D	E	L	V	K	T	A	K	T	V	A	X	P
I	S	S	Y	A	D	E	L	V	K	T	A	K	T	V	A	S	P
M[2]	S	P	X	E	A	T	*R*	E	*E*	N	*V*	*Y*	*M*	*A*	*K*	*L*	*A*
II	S	T	A	E	A	T	R	E	E	N	V	Y	M	A	K	L	A
M[3]	S	P	X	E	A	T	*R*	E	*E*	N	*V*	*Y*	*M*	*A*	*K*	*L*	*A*

[a] I, Spinach 1-6-biphosphate aldolase; II, Barley 14-3-3 protein; M[1], mercury protein 36 kD; M[2], 32 kD; M[3], 30 kD. In M[2] and M[3], no amino acid can be determined at the third position, because it is probably modified. Italics: homologies with a 14-3-3 isoform of *Arabidopsis*.

control, produced proteins of same molecular weigth but quantitatively different (Fig. 5 F).

Parallel SDS-PAGE gels stained by the Coomassie Blue revealed three bands corresponding to the most prominent silver stained. Therefore they were eluted from gel slices [14]. Each purified protein was submitted to automated Edman degradation. Protein sequencing without ambiguity yielded the amino-terminal sequences shown in Table 2 which allowed direct identification of the three proteins by additional database searches. The first, 36 kD, shows 93% identity between residues 4 and 17 with the fructose 1,6 biphosphate aldolase of spinach and 67–92% identity with maize, rice and *Arabidopsis* aldolases respectively [21]. The second protein of 32 kD and the third one (30 kD) are 100% identical between residues 4 and 17 with the 14-3-3 protein of barley [22] and present 94% identity with the corresponding protein of rice. Mercury residues 7, 9, and 11–17 correspond to a conserved part of the first exon of the GRF1-GRF14χ gene of the 14-3-3 isoform of *Arabidopsis* [23].

To extend our knowledge of other proteins of the female complex, several possible functions were investigated with the active fraction coming from the 5′-AMP-sepharose column (Table 1 and Fig. 5C, Fig. 2). Table 3 clearly shows the presence of an activity hydrolysing *t*-ZR, with no activity for the *c*-ZR. The stereospecificity is particularly strict. Although adenosine β-glycosidases were removed with the second eluted peak of the anion-exchanger (not shown) at the purification step 4 (Table 1), measurable amounts of adenosine and traces of adenine resulted from all cytokinin assays. This indicates the presence of cytokinin-oxidases cleaving the lateral isoprenoid chain from *t*-ZR to release adenosine [24]. Table 3 also shows parallel results obtained with male extracts. Finally, eventual PG functions were investigated hoping to relate it to the putative product of the female-specific sequence. The mercury fraction was able to hydrolyse the β-glycosidic linkages of polygalacturonic acid, but it was 20-fold less efficient than the commercial purified *Aspergillus* PG (Table 3). As countercheck, no *c*- or *t*-Z was detected in reaction products of active purified tomato PG using *t*-ZR or *c*-ZR as substrates (Table 3). Consequently, two activities able to cleave a β-glycosidic bond are present in the female active fraction, one for *t*-Z production, the second for reducing sugars production. With the three proteins unambiguously identified by microsequencing, at least three enzymatic activities were co-eluted from the 5′-AMP-sepharose column: a strict stereospecific *t*-ZR β-ribosidase, a cytokinin-oxidase(s) and a PG activity. These proteins migrated as a single band at pI 5.4, but were dissociated in denaturing conditions, thus losing activity. To function, they appear to be associated and constitute a non-covalent enzymatic complex.

4. Discussion

Using a female isogenic line and by comparison with male plants of known sex genotypes, a female-specific fragment has been isolated by the AP-PCR method, cloned and sequenced. The suc-
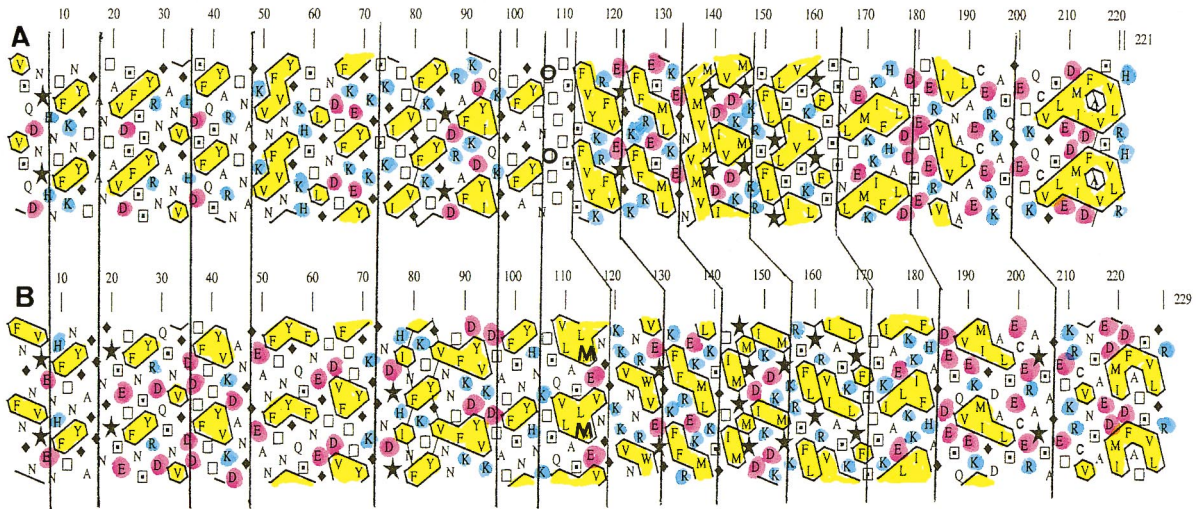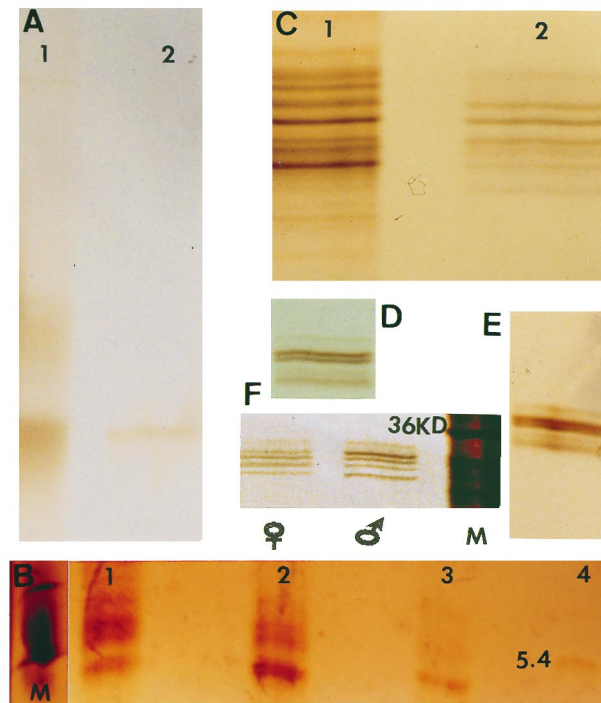
Fig. 4

Fig. 5

Fig. 4. Computer comparison of hydrophobic cluster analyses HCA, of corresponding parts of the deduced amino acid sequences of the female-specific DNA of mercury (A) and of the tomato β-subunit of polygalacturonase isoenzyme 1 cDNA (B). Vertical lines indicate the proposed alignments. Differences are visible near the putative ends of mature proteins. The tomato Met-397 residue is in bold and the corresponding position in mercury is O. Yellow, hydrophobic amino acids; white squares, polar amino acids; red, acidic amino acids; blue, positively charged amino acids; black stars, proline; black diamond, glycine (Software, Ref. [9]).

Table 3
Comparative activity of the female fraction and of tomato polygalacturonases for *t*- and *c*-zeatinribosides[a]

Substrates	Enzymes	Zeatin (%)	Ado	Ade
t-Zr	Mercury	100	100	Tr
c-Zr	Mercury	0	*	*
t-Zr	Male control	25	188	Tr⟩
t-Zr	Tomato PG2	0	*	*
c-Zr	Tomato PG2	0	*	*
t-Zr	Tomato PG1+PG2	0	*	*
c-Zr	Tomato PG1+PG2	0	*	*
		Reducing sugars (%)		
PG acid	Aspergillus	100		
PG acid	Mercury	5		

[a] Detection and measurement of reaction products by mass spectrometry. Comparative activity of the female fracton and of Aspergillus polygalacturonase for polygalacturonic acid. O.D. detection of reducing sugars at 520 nm, arseniomolybdate method. Ado, adenosine; Ade, adenine; *, not studied; Tr, traces.

cess of this technique is difficult to predict and depends on the size of genomes and on the proportion and complexity of sex unique sequences. It has been successfully applied to isolate sex-markers in animals [25]. In mercury, with 16 homomorphic chromosomes inferior to 3 μm, the genome is probably small and of comparable size with castor bean (323 Mbp), a monoecious species of the same Euphorbiaceae subgroup, that is 2-fold that of *Arabidopsis* (145 Mbp, [26]). Because the female line results from seven self-fertilizations [3], it presents a reduced polymorphism that also enhances the possibilities to select differential fragments. Although the segregation of the female-specific fragment has not been established by crossing, its absence from the restricted DNA of all investigated males suggests that it segregates with sex. The existence of male-specific fragments that do not cross hybridize with the female-specific fragment is also reported (not shown). Repeated experiments with the DNA of same strains produced the same sex-specific fragments, furnishing the evidence for the existence of two male-specific fragments and for one cloned female-fragment at least for the tested genotypes. The deduced amino-acid sequence of the female-specific DNA clearly showed a strong identity with the PG1 β-subunit of tomato [19]. We do not know whether the corresponding transcript is expressed in mercury flower. However, we have characterised PG activity in apices bearing unpollinated female flowers (Table 3). This result suggests that the mercury PG mRNA might also be present in female flowers. The fact that the isolated fragment is female-specific and encodes for a potential PG activity was unexpected but agrees with the spatial distribution of the homologous mRNA in tomato[19]. Further investigations are necessary in order to understand the relationships between PG activity and femaleness.

We also report for the first time, the presence of a strict stereospecific *t*-ZR β-ribosidase apt to

Fig. 5. Electrophoreses at various purification steps of the female enzyme complex. (A) After 5′-AMP-sepharose 4B affinity chromatography (lane 2), products resolved as one active band on PAGE electrophoreses in native conditions, no band was present at the level of contaminant proteins visible after Hitrap blue affinity chromatography (lane 1). (B) Disappearance of contaminant proteins through purification on electrophoreses on ampholine gradient pI 3.5-9.5. Only proteins between pI 5 and 6 are presented. After the last chromatography (lane 4), a unique band at pI 5.4 is present. (M, size markers; 1, after gel filtration on Sephadex G200; 2, after DEAE anion-exchanger chromatography; 3, Hitrap Blue products). (C) SDS-PAGE gradient gel (8–18%) of products eluted from the Hitrap blue column (lane 1) and from the 5′-AMP-sepharose column (lane 2). (D, E) Migration in the presence of mercaptoethanol on 7.5 and 15% homogeneous gels respectively. (F) Comparison on SDS-PAGE (8–18% gradient gel) of male and female active fractions from 5′-AMP-sepharose column (M, size markers KD).

produce the female-specific metabolite in young wild female apices as repetitively demonstrated. Without large populations of isogenic lines, unselected populations being mixtures of sex-genotypes [3], we cannot determine if the *t*-ZR ribosidase is a direct product of b1b2 alleles as supposed [4]; it is not encoded by the female-specific DNA. Consequently, the cloned specific-fragment is not a feminizing allele and has to be considered as a sex-specific marker. In contrast, and by comparison with the composition of the active form of tomato PG [19], the mercury PG activity could contain the product of the female-specific DNA as regulatory subunit. Other components of the protein complex also appear to be regulatory. Aldolases occupy a key position in the glycolytic pathway [21] and the duality *t*-ZR-ribosidase/cytokinin-oxidases is generally considered as controlling the levels of active cytokinin metabolites in plants [24]. The plant 14-3-3 proteins have been described as possible components of signal transduction pathways by alterations in their state of phosphorylation by the multifunctional Ca^{2+}-dependent protein-kinases [27]. The fact that electrophoretic bands of same molecular weight (Fig. 5F) and identical enzymatic activities have been repetitively characterised in wild males was unexpected. However, the prominent male bands were different and each electrophoretic pattern appeared sex-specific; furthermore, the *t*-ZR ribosidase and the cytokinin-oxidase(s) showed different affinity according to sex (K_m/t-RZ = 8.47 in females and 13 mM in males; oxidative-activity 2-fold higher in males). Although we do not have the direct proof of a link *t*-ZR ribosidase-feminizing alleles, together these results further indicate that sex alleles and male and female regulatory complex are related. In Mercury, complete investigations of cytokinin production and signalling beetween sexes appear now possible to uncover new receptors and the molecular details of cytokinin transduction pathway. These studies will also shed light on the problem of sex determination, for which the first research to be performed might consist in the complete purification and sequencing of female *t*-ZR ribosidase allowing at first, the isolation of female alleles then of males, and the study of their relations with the sex-specific markers.

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