RESEARCH ARTICLE

Rapid cloning and bioinformatic analysis of spinach Y chromosome-specific EST sequences

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Abstract

The genome of spinach single chromosome complement is about 1000 Mbp, which is the model material to study the molecular mechanisms of plant sex differentiation. The cytological study showed that the biggest spinach chromosome (chromosome 1) was taken as spinach sex chromosome. It had three alleles of sex-related *X*, X^m and *Y*. Many researchers have been trying to clone the sex-determining genes and investigated the molecular mechanism of spinach sex differentiation. However, there are no successful cloned reports about these genes. A new technology combining chromosome microdissection with hybridization-specific amplification (HSA) was adopted. The spinach Y chromosome degenerate oligonucleotide primed-PCR (DOP-PCR) products were hybridized with cDNA of the male spinach flowers in florescence. The female spinach genome was taken as blocker and cDNA library specifically expressed in Y chromosome was constructed. Moreover, expressed sequence tag (EST) sequences in cDNA library were cloned, sequenced and bioinformatics was analysed. There were 63 valid EST sequences obtained in this study. The fragment size was between 53 and 486 bp. BLASTn homologous alignment indicated that 12 EST sequences had homologous sequences of nucleic acids, the rest were new sequences. BLASTx homologous alignment indicated that 16 EST sequences had homologous protein-encoding nucleic acid sequence. The spinach Y chromosome-specific EST sequences laid the foundation for cloning the functional genes, specifically expressed in spinach Y chromosome. Meanwhile, the establishment of the technology system in the research provided a reference for rapid cloning of other biological sex chromosome-specific EST sequences.

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Introduction

Spinach belongs to family Chenopodiaceae, and is a kind of typical XY type sex-determination plants. The composition of chromosome is $2n = 12$ (Bemis and Wilson 1953) in the second stage of the sex chromosome evolution (Ming *et al.* 2007). It has many different sex-expression types including absolute male plant, nutrition male plant, absolute female plant and monoecism (Correns 1929). Before the flowers started to develop, the size of male floral primordium was consistent with that of female floral primordium. After the flowers developed, the development speed of male floral primordia was relatively faster, and no opposite organ in female and male flowers was started. Therefore, there was no trace of opposite organ degradation in the mature unisexual flowers (Sherry *et al.* 1993). Thus, spinach became an ideal research system to study development of functional gene of floral organ. The class B genes *APETALA3* and *PISTILLATA* (Pfent *et al*. 2005), the class C gene *AGAMOUS* (Sather *et al.* 2005) and *AP1/FUL* (Sather and Golenberg 2009) of ABC model in flower development were cloned successively. With the help of RNAi technology, Sather *et al.* (2010) studied the effects of two kinds of MADS box genes including the class B and class C genes on spinach sexual control. The results showed that when the spinach class B genes were inhibited, the anthers would be transferred into the homologous carpel, but the number of perianths in fourth floral whorl would be affected. The female developmental state appeared in the *SpPI* silenced male plants; when the spinach class C floral organ-determining genes (*SpAG*) were inhibited, part of the reproductive organs would lose the functions, the intermediate type of flower would appear, but other sex-specific features or structures would not appear. However, the analysis of genome sequences of *SpAP3* and *SpPI* showed that there was no difference in the alleles between female and male. Thus, these genes were

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not spinach sex-determining genes but only related to sex differentiation.

Spinach has long been used as model material to study sex determination and differentiation. The cytological study showed that the short arms of spinach chromosome 1 (the longest chromosome) had the sex-determining locus (Iizuka and Janick 1962). Lan *et al.* (2006) carried out the chromosome localization analysis on spinach chromosomes using 45S rDNA sequence. There were six 45S rDNA loci on female plant chromosome and five in male plant. Therefore, spinach was XY sex-determining mechanism type in the early period of sex chromosome evolution. The 45S rDNA fluorescence *in situ* hybridization (FISH) results of our research showed that six distinct 45S rDNA hybridization signals appeared on the root tip cell chromosomes of female and male plants. The difference is seen in weak fluorescence signal distribution. In male plants, weak 45S rDNA hybridization fluorescent signal appeared only on one short arm of chromosome in spinach chromosome 1. In female plants, weak 45S rDNA hybridization fluorescent signal existed on one pair of short arms of chromosome in spinach chromosome 1. For the first time spinach chromosome 1 was confirmed as the sex chromosome at the cytological level (Deng *et al.* 2012). With the aid of chromosome microdissection technique, the microdissection and DOP-PCR amplification were successfully carried out on the longest chromosome of spinach in our laboratory, and the amplification products of spinach Y chromosome were successfully identified using the male-specific marker T11A (Onodera *et al.* 2008; Deng *et al.* 2013).

Using 101 AFLP markers and nine SSR markers, the genetic map of spinach backcross populations were successfully constructed. The genetic map was divided into seven genetic linkage groups. The total genetic distance was 585 cM. The region with 1.9 cM distance from SO_4 microsatellite markers was associated with spinach sex determination (Khattak *et al.* 2006). Ten AFLP molecular markers closely linked to the *X*/*Y* locus were identified by separation population grouping analysis. Among them, four markers were coseparated with *Y* gene. These AFLP molecular markers and two known male-specific DNA fragments were located on 13.4 cM region which covered *Y* locus (Onodera *et al.* 2011). These molecular markers laid the foundation for cloning sex-determination gene. The genetic linkage map of spinach male sex control area was drawn by the bulked segregant analysis (BSA) method. The result proved that the linkage of monoecious gene (*M*) and male sex control gene (*Y*) were located on Y chromosome. They were nonallelic genes and the genetic distance was 12 cM (Yamamoto *et al.* 2014). The acquisition of sex control loci closely linked with molecular markers laid a foundation for cloning sex-determining gene. However, the spinach genomic library construction and a lot of screening work are still needed.

The direct alignment method of cloning spinach sex-determining gene was to directly clone functional genes on Y chromosome of spinach. At present, the sex-related functional gene cloning methods included: (i) the cDNA of female and male flower buds or total genomic library was constructed. Then the sex differentiation-related genes were cloned using the differential display technique or suppressive subtractive hybridization technique, so as to obtain the sex-specific markers; (ii) the sex chromosome microdissection and DOP-PCR amplification were performed. The screening of cDNA library or FISH identification was performed with the amplification products as a probe, so as to obtain the specific function sequence of sex chromosome. The positioning analysis was performed in X chromosome; (iii) the sex differentiation-related specific gene was obtained by molecular marker methods (such as RAPD, AFLP and SRAP etc.) (Kejnovský *et al.* 2001). The spinach sex-determining gene was not cloned by the above methods. The single chromosome EST sequence rapid cloning method may become the effective way for cloning spinach sex-determining gene. It was a new technique combining chromosome microdissection with cDNA hybridization-specific amplification. The technology was first established by Zhou *et al.* (2008), and specific expression of 94 EST sequences was successfully cloned in rye 1R chromosome. However, there was no report on successful cloning of the sex chromosome-specific EST sequence.

According to the above issues, the DOP-PCR products of spinach Y chromosome were hybridized with cDNA male spinach in florescence in this study based on the successful acquisition of spinach Y chromosome DOP-PCR amplification products. With female genome and transcriptome as blockage, the cDNA library specifically expressed in Y chromosome was constructed And the cloning, sequencing and bioinformatic analysis of EST sequence in cDNA library were performed. The results of this study would be helpful to clone the functional gene specially expressed in spinach Y chromosome, and provide an effective way for cloning spinach sex-determining gene.

Materials and methods

Plant materials

Spinach (*Spinicia oleracea* L. $2n = 12$) was cultivated in the experimental field of Henan Normal University. The good growth environment was maintained to avoid the biotic or abiotic stress. Japanese big leaf spinach with genetic stability was obtained through selfing.

Microdissection, DOP-PCR amplification and identification of spinach Y chromosome

Referring to the method of Deng *et al.* (2012), after the spinach seeds were germinated in the incubator under the appropriate conditions, the apical meristem chromosome sections in cell metaphase were prepared.

According to the method of Deng *et al*. (2013), spinach Y chromosome microdissection and DOP-PCR

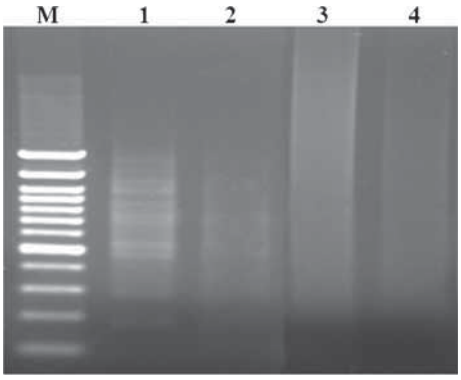

Figure 1. The detection of enzyme digestion efficiency. M, trans DNA marker II; lanes 1 and 3 represent the DOP-PCR products of spinach Y chromosome and the cDNA of male spinach at flowering stage before enzyme digestion, respectively; lanes 2 and 4 represent the DOP-PCR products of spinach Y chromosome and the cDNA of male spinach at flowering stage after enzyme digestion, respectively.

(primer: 5′-CCGACTGATCNNNNNNATGTGG-3′) amplification were performed to obtain the Y chromosome DOP-PCR products. The microdissection Y chromosome amplification products were identified with T11A markers closely linked with spinach male sex control locus (Onodera *et al.* 2008; accession number: E15132; primer pairs: 5′-CCCTAATTAACTCCTCTTTACCCAA-3′ and 5′-TACAAGCCCCATTATCATAACAGTC-3′).

Genomic DNA extraction, mRNA isolation and cDNA synthesis

The male and female spinach genomic DNAs in florescence were extracted using the improved 2× CTAB method. The male plants flower RNA was isolated from spinach with RNApure ultra pure total rapid extraction kit (Aidlab, Beijing, China). Its content was determined and its quality was detected. The male RNA of spinach was reverse-transcribed into double-stranded cDNA with SMARTer^{TM} PCR cDNA Synthesis Kit (Clontech, cat. nos. 634925 and 634926).

Hybridization of Y chromosome DNA and male flower cDNA of spinach

According to the method of Zhou *et al.* (2008), cDNA and DOP-PCR products of spinach Y chromosome were purified before enzyme digestion with DNA coprecipitation agent. Digestion products, 1 μg were taken, 10 U *Sau* 3AI enzyme was added to 25 μL system for enzyme digestion overnight at 37°C. The restriction enzyme activity was inactivated for 15 min at 70°C. The effect of enzyme digestion was detected. 2.5 μL of *Sau* 3AI digested Y DNA or cDNA of spinach, 2 μL (10 μM) of adaptor HSA1 or HSA2, 2 μL 10× T4 ligase buffer (TaKaRa, Dalian, China), 1 μL (3.5 U/μL) T4 ligase and sterile double-distilled water were supplemented to a final volume of 20 μL, kept overnight at 16°C. The DOP-PCR product of spinach Y chromosome was linked with HSA1 (A1L, 5′-GTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3′; A1S, 3′-CCCGTCCACTAG-5′). The total cDNA double chain products in florescence of male spinach were linked with HSA2 (A2L, 5′-TGTAGCGTGAAGACGACAGAAAGGGCGTGGTGCGGAGGGCGGT-3′; A2S, 3′-CCTCCCGCCACTAG-5′). 1 μg blockage DNA (boiling DNA fragment of female spinach genomic DNA which is between 100 and 1000 bp, female genomic DNA enzyme digested with *Sau* 3AI and cDNA of female spinach flowers) was added, alcohol precipitation was performed by chloroform/phenol extraction.

PCR analysis of the ligation efficiency

According to the method of PCR-Select^{TM} cDNA Subtraction Kit User Manual (Clontech, cat. no. 637401), the general housekeeping genes primers actin 01 (5′-CATCAG-

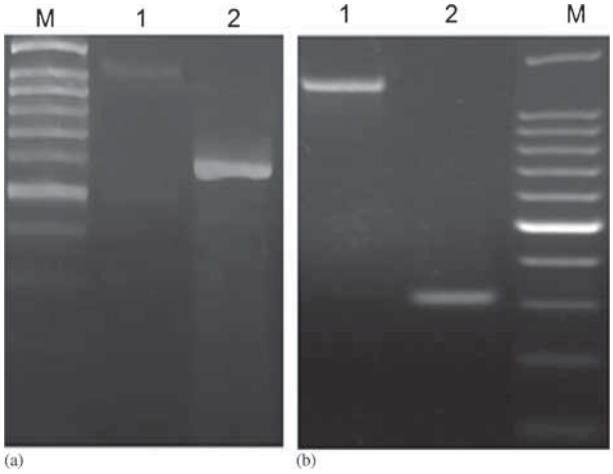

Figure 2. PCR analysis of the ligation efficiency. M, trans DNA marker II; (a) the amplification pattern of the DOP-PCR products of spinach Y chromosome after digestion and ligation with adaptor HSA1, using the primers: lane 1, actin01 and actin02; lane 2, actin02 and P1; (b) the amplification pattern of the cDNA of male spinach at flowering stage after digestion and ligation with adaptor HSA2 using the primers: lane 1: actin01 and actin02; lane 2: actin02 and P2.

GAAGGACTTGTACGG-3′) and actin 02 (5′-GATGGACC-TGACTCGTCATAC-3′) were used. The outside amplification primers P1 (5′-GTAATACGACTCACTATAGGGC-3′) or P2 (5′-TGTAGCGTGAAGACGACAGAA-3′) were used to detect the lingation efficiency of hybridization.

Hybridized DNA treatment with mismatch-sentitive nucleases and PCR amplification of hybridized DNA-cDNA

According to the method of Zhou *et al.* (2008), the male spinach cDNA products in florescence were hybridized with Y chromosomes DOP-PCR products. Every 100 ng hybrid products were treated with 0.1 U mung bean nuclease which digested single stranded DNA (New England Biolabs, Ipswich, USA) for 15 min at 37°C. Then 0.01% SDS was added to inactivate the enzyme. The treated sample was purified by phenol/chloroform extraction. The precipitation was dissolved in 20 μL ultra pure water. Two rounds of PCR amplifications were performed with SSH method. The primers were changed into PN1 (5′-TCGAGCGGCCGCCCGGGCAGGT-3′) and PN2 (5′-AGGGCGTGGTGCGGAGGGCGGT-3′) primers in the second round of amplification. The hybridization efficiency was detected.

Cloning of hybrid PCR products and screening of cDNA sequence specifically expressed in spinach Y chromosome

The second round purified hybridized PCR amplification products were cloned using pMD19-T vector (TaKaRa, code: D102A). Bacteria liquid PCR amplification was performed with M13 forward and reverse primers after blue-white selection. The monoclones above 300 bp were selected and transferred on the new LB ampicillin plate. The inner primer PN1 and PN2 were used to identify the inserts. The inserted fragments above 200 bp were selected. The dot blot hybridization method was used for identifying the source of monoclonal amplification products with DIG-labeled Y chromosome secondary DOP-PCR product, cDNA of male flowers, cDNA of female flowers and spinach female genomic DNA. Meanwhile, cDNA sequence specifically expressed in male spinach sex chromosome was also screened.

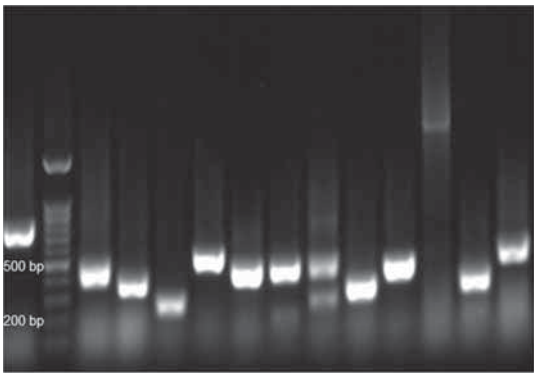

Figure 4. Sampled PCR products released from recombinant clones using PN1 and PN2 as the primers.

Bioinformatic analysis of specific cDNA sequence from male spinach sex chromosome

The positive clones of male spinach sex chromosome specific cDNA sequences were sequenced. Using DNAstar software and Seqman software, the vector and adaptors were removed, and EST sequences with lengths of less than 50 bp were also processed.

Homology searches of all sequences were queried in the GenBank database with BLASTx (homology) and tBLASTx (translation homology) available at the NCBI network service (http://www.ncbi.nlm.nih.gov/BLAST). The sequences of E>1e-10 were taken as the effective alignment sequences. Meanwhile, GO functional annotation was performed on the obtained sequence using Blast2GO software (http://www.blast2go.com/b2ghome).

Results

Detection of enzyme digestion efficiency

The spinach Y chromosome DOP-PCR products and male spinach cDNA purified products in florescence were digested respectively by *Sau* 3AI enzyme overnight. Its enzyme digestion efficiency was detected (figure 1). It can be seen clearly that the main bright band region significantly shifted down

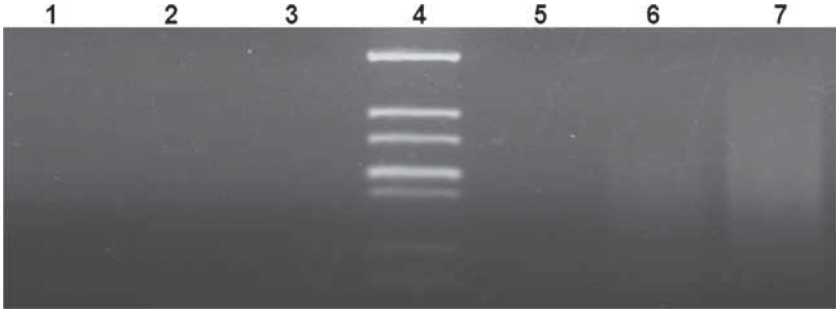

Figure 3. PCR analysis of subtraction efficiency. Electrophoretic patterns of two rounds of suppression PCR with the negative control (water), the hybridized Y chromosome DNA / male flower cDNA, the positive control (the hybridized male genomic DNA / male flower cDNA), as template. Lanes 1, 2 and 3, the primary amplification with double primers P1 and P2; lane 4, molecular weight marker –DL 2000; lanes 5, 6 and 7, the secondary amplification with double primers PN1 and PN2.

after the enzyme digestion and the distribution of bands was more dispersed, suggesting that the samples had been digested into fragments with different sizes by *Sau* 3AI enzyme. The enzyme digestion efficiency was good.

PCR analysis of the ligation efficiency

The spinach Y chromosome DOP-PCR products were linked with the adaptor HSA1. The spinach male flower cDNA was linked with the adaptor HSA2. The primer pair actin 02 and P1 were used to amplify the spinach Y chromosome DNA-linked products. The spinach male flowers cDNA-linked products were amplified using primers actin 02 and P2. The PCR amplification products were compared with that amplified using actin primer (figure 2). The result showed that the brightness of PCR amplification products was significantly higher than that of housekeeping gene, suggesting that the joint efficiency was higher than 25% and the joint efficiency of the connector was higher.

PCR analysis of subtraction efficiency

To detect the subtraction efficiency of spinach Y chromosome DNA and male spinach cDNA, the negative control experiment (hybridization experiment of water and male cDNA) and the positive control experiment (hybridization experiment of male genomic DNA and male cDNA) were established in this study. All through two rounds of PCR amplifications were done (figure 3), the amplification products did not appear in two rounds in negative control experiments, while the amplification products of positive control and the hybridization products with spinach Y chromosome and male cDNA were similar. Therefore, the hybridization and suppression amplification technique can effectively isolate homologous sequences between spinach Y chromosome and male cDNA. The chromosome microdissection technique combined with specific hybridization can effectively obtain the spinach Y chromosome-specific EST sequences.

Cloning and screening of EST sequences specially expressed in spinach Y chromosome

The homologous PCR products after spinach Y chromosome and male spinach cDNA hybridization-specific amplification were linked with pMD19-T vector and transformed into *Escherichia coli* DH5α competent cells. White bacterial colonies 2208 were randomly selected through blue–white selection. The recombinant clones were amplified and screened with adaptor inside primers PN1 and PN2. Fragments 278 with the length of 150 bp and the above single clones were picked out as shown in figure 4. The fragment size was between 150 and 400 bp.

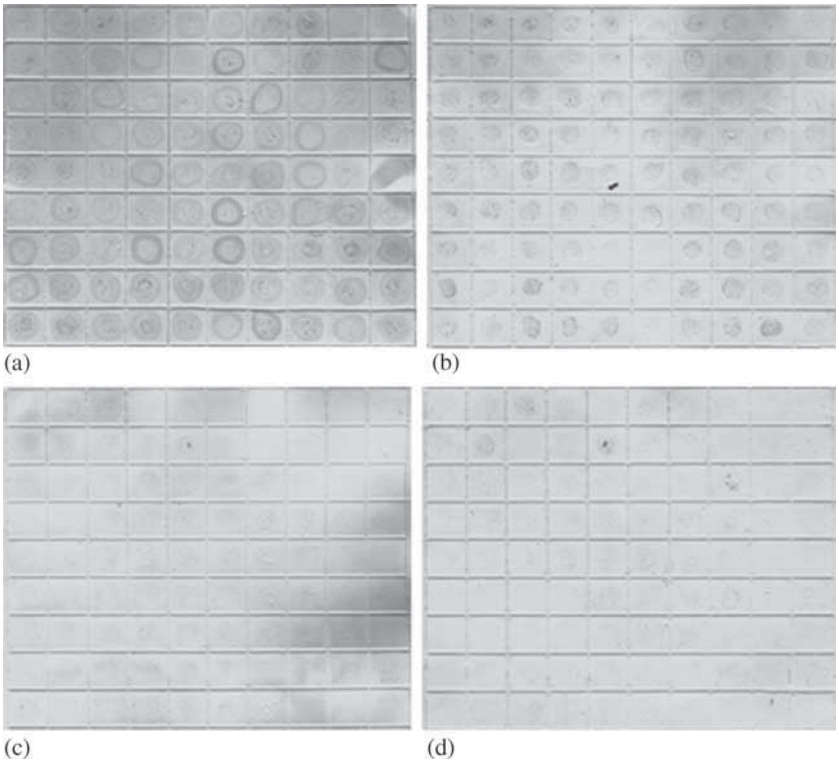

Figure 5. Characterization of a part of selected recombinant clones by dot blot hybridization. Characterization of partially selected recombinant clones by dot blot hybridization with DIG-labelled Y chromosome secondary DOP-PCR product (a), cDNA of male flowers (b), cDNA of female flowers (c) and spinach female genomic DNA (d).

Characterization of recombinant clones by dot blot hybridization

Recombinant plasmid DNAs of randomly selected recombinant clones 278 were hybridized with DIG (digoxigenin)-labelled spinach Y chromosome secondary DOP-PCR products, cDNA of male spinach flower, cDNA of female spinach flower and female genomic DNA, respectively. Nearly all (99%) recombinant plasmid DNAs could be hybridized with Y chromosome DOP-PCR product and cDNA of male spinach flower (figure 5, a&b), indicating that the inserts in recombinant plasmids DNAs are homologous with Y chromosome DNA. Whereas, there were only weak and/or no hybridization signals when the recombinant clones were hybridized with cDNA of female spinach flower and female genomic DNA (figure 5, c&d). The results indicate that the EST sequences especially expressed in spinach Y chromosome were selected. Recombinant clones 150 of EST sequences especially expressed in spinach Y chromosome were obtained and sequencing was performed.

Bioinformatics analysis of spinach Y chromosome-specific EST sequences

The comparison results of BLASTx showed that 48 of 63 EST sequences had no homologous alignment, suggesting that they were new series, while 16 sequences had homologous alignment results (table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). The homologous alignment of BLASTn indicated that 12 EST sequences had homologous sequences of nucleic acids (table 2 in electronic supplementary material).

The term association information analysis of GO level 2 (figure 6) showed that there were 33 GO terms above level 2 in cellular component C. They were cell part (36.37%), protein complex (27.27%), nonmembrane-bounded organelle (21.21%), membrane-bounded organelle (12.12%) and organelle part (3.03%), respectively. There were 60 GO terms above level 2 in the biological process P. Among them, the proportions of metabolic process and cellular process were higher, 23.33 and 18.33%, respectively. There were 50 GO terms above level 2 in molecular function F. Among them, the proportion of associated GO terms which was combined with organic cyclic compound binding, heterocyclic compound binding, small molecule binding and protein binding was the largest i.e. 62.00%. The proportion of enzyme activity associated GO terms which were related with transferase activity and hydrolase activity accounted for 36.00%, while GO terms related to signal transducer activity accounted for 2.00%.

Discussion

Compared with other research methods, a new technical method that combined spinach Y chromosome microdissection

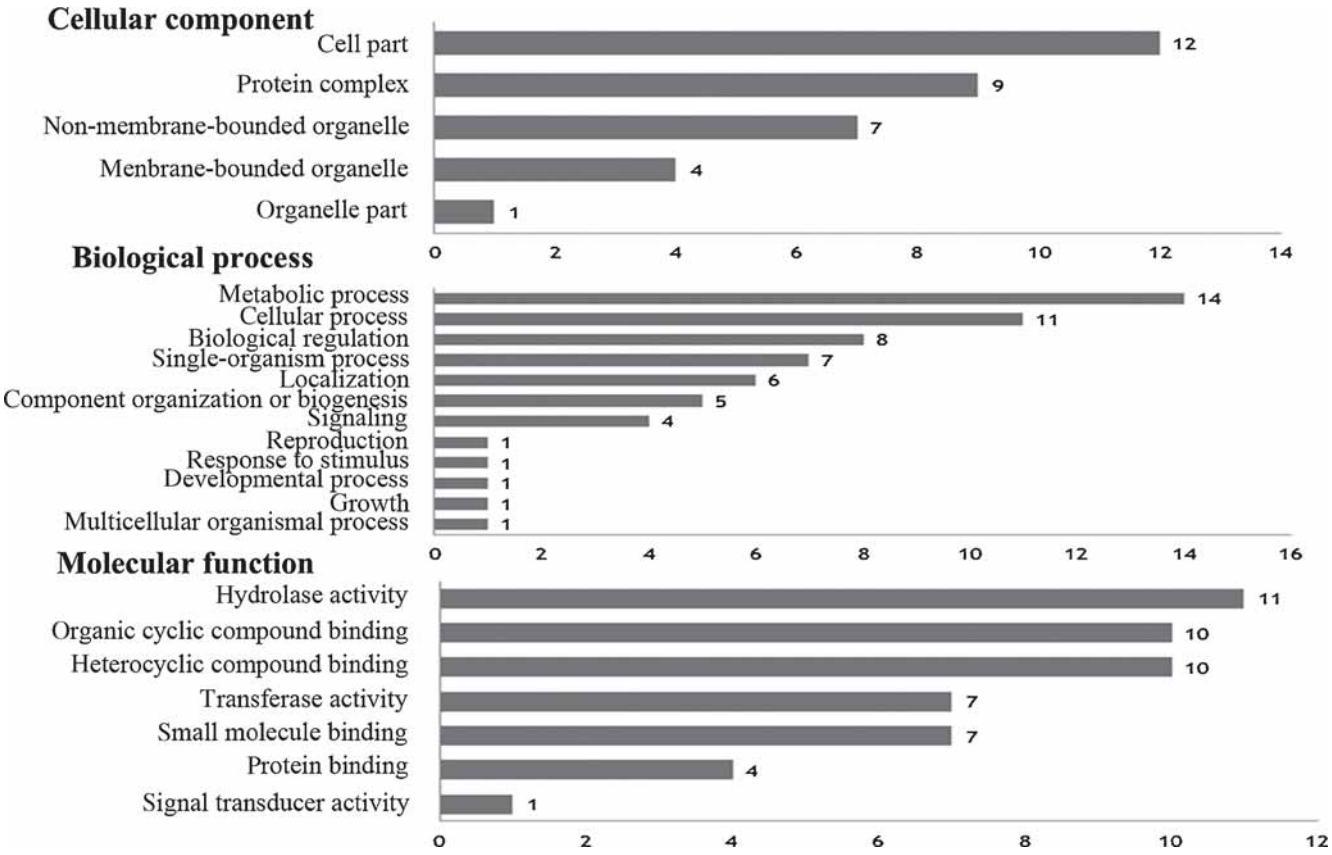

Figure 6. Classification of the ESTs according to Gene Ontology criteria. The Arabic numerals represent the number of GO term at each step of the classification process. The biological process combined graph was made based on ontology level 2.

with HSA technology to study specific EST sequences of spinach Y chromosome was first constructed in this study. HSA technology has been applied in the study of human, animal and plant (Lecerf *et al.* 2001). A large number of valuable EST sequences were obtained. The ESTs of specific chromosome were obtained with chromosome microdissection combined with HSA technology. The specificity of the technology was stronger, the application value was higher and the application scope was wider. Based on spinach Y chromosome DOP-PCR amplification and identification, HSA technical method for rapidly cloning spinach Y chromosome-specific EST sequences was successfully constructed in this study. The establishment of the technical system can lay the foundation for successfully cloning the functional genes of spinach Y chromosome, also provide the reference for cloning specific EST sequences of sex chromosome of other organisms.

Of the 63 EST sequences obtained in this study, BLASTx alignment results showed that 48 sequences had no homologous alignment results, they were newly cloned sequences. The acquisition of these sequences laid the foundation for us to further understand the genomic structure of spinach Y chromosome. Meanwhile, these sequences also helped us to clone the new functional genes. The homologous sequence alignment in nucleic acid database showed that there was 98.60% of similarity between the ESTs SP_{1-60} and peroxidase mRNA gene of spinach, suggesting that the obtained EST sequences did come from spinach species, the synthesis of peroxidase played a certain role in the sex differentiation of male plants, and EST sequence might be the regulation factor related to sex-determining genes. The similarity of SP_{1-62}, SP_{20-26} and microRNA of *Amborella trichopoda* was higher. The microRNA played a decisive role in sex determination of persimmon tree (Takashi *et al.* 2014). Therefore, the function of these microRNAs has to be further studied.

The homologous alignment results of BLASTx and BLASTn showed that EST sequences SP_{2-52}, SP_{3-12}, SP_{4-42}, SP_{5-13} and SP_{5-62} had the alignment results in nucleic acid and protein databases. Both data results showed that the similarity between EST SP_{2-52} and mRNA sequence encoding *Arabidopsis* mitochondrial half-ABC transfer-related proteins was 98.96%. The similarity between EST SP_{3-12} and mRNA sequence encoding *Betula pendula* histidine kinase was 87.64%. The similarity between EST SP_{4-42} and *eDmc1* mRNA sequence in Japanese eel encoding RecA family DMC1 was up to 96.58%. The similarity between EST SP_{5-13} and mRNA sequence encoding castor iron transport protein was up to 99.80%. The similarity between EST SP_{5-62} and *AnSAMS4* mRNA sequence encoding Oceania Atriplex S-adenosyl-L-methionine synthase 4 was 81.88%. It was speculated that five EST sequences might have similar function with its homologous sequence.

ABC transporter was one of the largest protein families discovered in all organisms at present (Henikoff *et al.* 1997). It was derived by the hydrolysis of ATP and it had the output and input functions. The plant genomic encodings were up to 100 ABC transporters. Among them, the corresponding function analysis was performed on 22 of 130 *Arabidopsis* ABC transporters (Verrier *et al.* 2008). ABC transporter was localized in most membrane structures of plant cells, such as plasma membrane, tonoplast, chloroplast, mitochondria and peroxidase, and showed diverse functions. It was initially discovered to participate in the later detoxification period, namely, vacuolar deposition (Martinoia *et al.* 1993). It was found to play a certain role in tissue growth, plant nutrition, plant development, abiotic stress resistance, resistance to pathogen invasion and interactions with the environment later. To exert this effect, they exhibited different basic behaviours, such as depositing lipid on the surface, collecting phytic acid in the seeds and transporting auxin and abscisic acid (Kang *et al.* 2011). *Arabidopsis* gene *STARIK* mutation can alter the morphologies of leaves and nuclei to cause short stature and plant chlorosis. *STARIK* gene encoded *Arabidopsis* half-ABC transporter subfamily mitochondrial ABC transporter Sta1 (Kushnir *et al.* 2001). Therefore, it was speculated that the EST SP_{2-52} sequence of spinach Y chromosome might be involved in the physiological growth, resistance and other related processes, and might play an assistant role in spinach male sex expression process.

DMC1 is a RecA interacting protein and it is an essential interacting protein in meiotic homologous recombination of multiple organisms. It was already detected in protists, fungi, animal and plants so far (Petersen and Seberg 2002). The biochemical and structural analyses showed that *RAD51* and *DMC1* had homologous DNA pairing and single strand exchange, and had a similar effect (Sung 1994; Li *et al.* 1997; Hong *et al.* 2001; Masson and West 2001; Sheridan *et al.* 2008). However, *DMC1* only played a role in meiosis stage, *RAD51* played a certain role both in mitosis and meiosis recombination processes (Aboussekhra *et al.* 1992; Bishop *et al.* 1992; Shinohara *et al.* 1992; Neale and Keeney 2006). Deletion of *RAD51* can cause the obstacles in homologous chromosome pairing and synapsis in meiosis process and the appearance of a large number of chromosome fragments in meiotic pachytene; a distinctly different meiotic phenotype emerged on *dmc1* mutant, chromosome synapsis could lead to the random degradation of single chromosome. Thus, *DMC1* played a major role in the process of *Arabidopsis* meiotic recombination, and *RAD51* played a supplementary role (Da Ines *et al.* 2013). The generation of *DMC1* homologous gene played an important role in occurrence of wheat polyploidy (Wang *et al.* 2012). The male sterile loci of *Dmc1–Dmc1*^{Mei11}, encoded a missense mutation fragment in DNA-binding site of L2, to eliminate the influence of the invasion fragments. The male heterozygote can interfere in the smooth proceeding of meiotic pachytene so as to cause infertility. Its character was that it is incomplete chromosome synapsis without chiasma. Most female oocytes can repair the crossing process of DMC1 defect type, and formed the complete gametes (Bannister *et al.* 2007). *Dmc1* expressed in the initial forming stage of spermatocytes, before meiosis and mitotic phase (Kajiura-Kobayashi

et al. 2005). Thus, *Dmc1* played a very important role in the development process of animal male sex. However, DMC1 was only observed in plants that the DMC1 was closely related with meiosis of plant body, and was an essential interacting protein in the process of meiotic homologous recombination. Whether it was related to sex development has to be verified. It is speculated that EST SP_{4-42} sequences were closely related to meiotic homologous recombination in the formation process of spinach gametophyte, and may also be associated with spinach male sex development. This has to be verified in the later researches.

In plants, trace metal transport of developmental organ is mainly mediated by sieve tube. The ligand composed the complex with the free ions to prevent cell damage. In the castor bean seedlings, all iron ions were basically combined on the proteins of phloem exudate in seedling stage. Inside the sieve tube, TTP in the form of combined iron also combined the free iron ions. This protein which was formed by the bonding of 96 amino acids had the higher similarity with late embryogenesis enriched protein family (Kruger *et al.* 2002). The iron transport protein in rice mitochondria played an essential role in the growth of plants (Bashir *et al.* 2011). Thus, the iron transport protein played a very important role in the growth of plants and embryonic development. The EST SP_{5-13} sequence may play a certain role in the process of growth and development of spinach seedlings and development of embryo. However, whether it was directly related to sex determination has to be proved in the later experimental studies.

In conclusion, the EST sequences especially expressed in spinach Y chromosome were obtained by combining chromosome microdissection and HSA technology in the research. The bioinformatics analysis showed that sequences EST SP_{2-52}, SP_{4-42} and SP_{5-13} might be related to spinach male sex development. However, the function of the EST sequences still needed to be proved in the future studies.

Acknowledgements

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