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Identification and characterization of sex related genes in *Actinidia arguta* by suppression subtractive hybridization

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ABSTRACT

Actinidia arguta, with sweet flavour, high nutritional value and health promoting role, is a typical dioecious plant. But dioecy represents an important constraint in kiwifruit breeding programmes and also requires identification of male and female genotypes before planting an orchard. Therefore, understanding molecular basis of sex determination in *A. arguta* is important for molecular identification of seedling gender at early stage. PCR-based suppressive subtractive hybridization (SSH) was performed to screen genes related to sex determination. A total of 533 and 508 positive clones, from forward and reverse libraries respectively, were selected for sequencing. The clean ESTs were assembled into 309 (forwad) and 369 (reverse) unigenes, and 228 and 299 of them hit known homologous sequences of the NCBI nr database. Based on Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, four unigenes that might be involved in sex determination were selected for validation by qRT-PCR. The expression of *PME* was very strong in male flower at late development stage, with 72 times higher than that of female flower. Reversely, male flower exhibited extremely low expression level of *ACO*, *MAN1* and *MYC2* at late development stage as compared with female flower. The variable expression patterns of these genes between male and female flowers suggested their importance in controlling sex expression. These results advanced our understanding of the molecular mechanism of sex determination in *A. arguta*.

1. Introduction

The genus of *Actinidia* contains over 70 species distributing across a wide natural range from the tropics (lat 0°) to cold temperate regions (lat 50°N) (Huang et al., 2004; Ferguson and Huang, 2007). The most popular and widely grown species from this genus is *Actinidia deliciosa* (A. Chev.) C. F. Liang & A.R. Ferguson. *Actinidia arguta* (Siebold & Zucc.) Planch. ex. Miq., known as hardy kiwifruit, kiwiberry, grape kiwi or baby kiwi, is the second commercially cultivated *Actinidia* species with increasing production worldwide (Li et al., 2013; Wang et al., 2015) attribute in part to its high nutritional value, sweet flavour, thin and green hairless edible skin as well as the role of health promoting. Compared to traditional kiwifruits, *A. arguta* is frost resistant and can be cultivated in cooler regions. Additionally, they are nearly free of pests and diseases, and are perfectly suitable for ecological cultivation (Drzewiecki et al., 2016). *A. arguta*, as a traditional Chinese medicine, was recorded in Compendium of Materia Medica (Bencao Gangmu) by Li Shi-Zhen about 500 years ago (Liu and Liu, 2016). Modern pharmacological studies have also demonstrated that extract from the fruit of *A. arguta* has many beneficial bioactive effects on human health, such as preventing chronic inflammatory diseases, reducing blood glucose potential, antioxidant and anticancer properties (Ravipati et al., 2012; Bae et al., 2016; Nishimura et al., 2016; Wojdyło et al., 2017). Owing to its perfect edible character as well as nutritional and medicinal value, this fruit crop has attracted great interest worldwide especially in Europe and showed promising market prospect.

The vast majority of flowering plants are hermaphrodite and develop bisexual flowers. On the other hand, a small portion of dioecious species with separate male and female individuals are also found accounting for approximately 6% of flowering plants. All members of known species in the genus *Actinidia* are functionally dioecious. Female plants bear flowers that are hermaphroditic in appearance but produce empty pollen, whereas male plant flowers have a rudimentary carpel that aborts before style elongation or ovule initiation (Ferguson, 1984; Zhang et al., 2015). This attribute may have evolved to promote outcrossing, and in some cases appears to allow efficient allocation of

Abbreviations: SSH, suppressive subtractive hybridization; *PME*, pectin methylesterase gene; *MAN1*, endo-beta-mannanase gene; ACC, l-aminocyclopropane-1-carboxylate; *ACO*, ACC oxidase gene

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resources to optimize reproduction (Lebel-Hardenack and Grant, 1997). A proper ratio of female plant to male plant is 8:1 for artificial cultivation of hardy kiwifruit, which can easily achieve pollination. Therefore, female plant of *A. arguta* has a higher economic value than male plant. Moreover, fruit selection is the main work of hardy kiwifruit breeding, but the male plant bears no fruit (Huang, 2002). What is more, the perennial Plant reaches reproductive maturity in 3–5 years, with approximately 50% of male plants (Testolin et al., 1995). This leads to a considerable cost for maintaining unwanted male seedlings before its maturity. A method to identity the gender of seedlings before their flowing is necessary for both artificial cultivation and breeding program of hardy kiwifruit. However, morphological approach for identification the gender of young seedlings is difficult because female and male seedlings are extremely morphologically similar before flowing. Understanding molecular and genetic mechanisms of sex determination and sex differentiation in *A. arguta* is important for agricultural application and will help for molecular identification of seedling gender at early stage. Although it is hypothesized that *Actinidia* genus sex-determining genes are localized in a pair of chromosomes which function like an XX/XY system with male heterogamety (Harvey et al., 1997), the molecular mechanism is still largely unknown due to lack of genomic information, gene discovery and subsequence verification.

Suppression subtractive hybridization (SSH) is a powerful tool for identifying and isolating differentially expressed genes. The SSH technique is believed to generate an equalized representation of differentially expressed genes and enrich rare sequences by over 1000-fold in one round of subtractive hybridization (Diatchenko et al., 1996). In addition, this method leads to fewer false positives compared to the other techniques used to identify differentially expressed genes (Sahebi et al., 2014). SSH has been successfully used to identify genes responsive to plant development (Xu et al., 2007), abiotic/biotic response (Garg et al., 2013), and different phenotypes (Yang et al., 2015). In order to illustrate the molecular basis of sex differentiation in *A. arguta*, SSH method was used in the present study to discover the differentially expressed genes that may be involved in sex determination of *A. arguta*. This approach successfully identified a number of sex related genes and four were further validated by quantitative real time-polymerase chain reaction (qRT-PCR). These results enhanced our understanding of sex determination in *A. arguta*.

2. Materials and methods

2.1. Plant materials

A. arguta (Shining green cultivar; Accession No. AA2009F5) grown under natural condition in Jilin Agricultural University (Jilin Province, China) were used as plant materials. Morphological inspection indicated that both male and female flowers undergone a bisexual period and became unisexual by termination of the development of the androecium or gynoecium. For sampling, flower development was divided into four stages including bud appearing (stage 1), bud expending (stage 2), alabastrum formation (stage 3) and big alabastrum (stage 4) stages. Both male and female flower buds were collected at these four stages and frozen with liquid nitrogen to be preserved at minus 80 ° before total RNA isolation.

2.2. RNA isolation and construction of SSH libraries

Total RNA was extracted from the flower buds of *Actinidia arguta* at four developmental stages using the TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The mRNA was then isolated from total RNA using PolyA Tract mRNA Isolation Systems (Promega, USA) according to the manufacturer's instructions. The integrity of total RNA was examined using 1.2% agarose gel electrophoresis and the concentration and purity of total RNA and mRNA were determined by using micro-volume UV–vis spectrophotometer (Nanodrop, USA) at wavelengths of 230, 260 and 280 nm.

Two subtraction cDNA libraries (forward and reverse) were constructed using PCR-Select cDNA Subtraction Kit (Clontech, USA) according to the manufacturer's instructions. The cDNAs were reverse transcribed from 1 μg of an equal mixture of four developmental stage mRNAs from male or female flower bud respectively. For forward subtraction, cDNA from the female flower bud was used as a "tester", and the male flower bud was used as a "driver". For reverse subtraction, the "tester" cDNA was obtained from the male flower bud while the cDNA from female flower bud acted as a "driver". Both the tester and driver cDNAs were digested with RsaI and ligated to adapters. Afterward, two rounds of subtractive hybridization and suppression PCR amplification were conducted to remove background cDNA and enrich target genes. The PCR products were purified using the Axygen PCR product purification kits (Axygen, USA), and then inserted into the pGEM-T vector (Promega, USA) and transformed into *Escherichia coli* DH-5 α cells. The transformed cells were grown on LB agar containing 100 mg/l ampicillin antibiotic at 37 °C overnight. The white individual clones were randomly selected and colony PCR was carried out to identify target insert fragments. False positives and colony mixture were removed according to the colony PCR result.

2.3. DNA sequencing and analysis

Positive clones from both forward and reverse subtracted libraries were sequenced using an ABI 3730 DNA Analyzer (Applied Biosystems, USA) with the vector specific T7 and SP6 primers. The obtained raw sequence reads were screened and trimmed of the vector and primer sequences using VecScreen tool (www.ncbi.nlm.nih.gov/Vecscreen). The clean ESTs were assembled into contigs and singles using DNASTAR software. All unigenes (contigs and singles) greater than 200 bp were subjected BLASTx (www.ncbi.nlm.nih.gov/) with E-value less than 1e-5 against the NCBI database. Further functional annotation was carried out using BLAST2GO (www.blast2go.com), and KEGG pathway analysis was performed using KOBAS 2.0 software (http://kobas.cbi.pku.edu.cn/).

2.4. Expression analysis by quantitative real-time PCR (qRT-PCR)

According to annotation results, quantitative real-time PCR (qRT-PCR) was employed to analyze the expression of four genes related to sex differentiation. Three biological replicate measurements were performed for each data point and three technical replicates were performed for each sample. The total RNA was isolated from the flower bud at four different developmental stages as detailed above. The total RNA was treated with DNase (Promega, USA), and reverse transcription was performed using a First Strand cDNA Synthesis Kit (Takara, Japan).

Primers (Table 1) for the qRT-PCR were designed using Primer Premier 5.0 software according to the target EST sequences. Before qRT-PCR, semi-quantitative reverse transcriptase-PCR was performed to validate the existence of target genes and test the homogeneity of mRNA abundance. PCR amplification was carried out in a total volume of 20 μl, and the reaction mixture consisted of 0.5 μM of each primer, 30 ng of template cDNA and 10 μl of 2 × Premix Taq (Takara). The amplification profile consisted of one initial denaturation cycle of 5 min at 94 °C, followed by 30 cycles which consist of 94 °C for 10 s, appropriate annealing temperatures for 15 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. qRT-PCR was performed in a thermocycler (BioRAD, USA) using a SYBR *Premix Ex Taq*™ Kit (Takara, Japan). The reaction mixture contained a total volume of 20 μl, including 10 μl of 2 × SYBR Green Master mix reagent, 500 ng template cDNA, and 0.5 of μM of each primer. The reaction condition consisted of the following steps: 95 °C for 5 min; 40 cycles (95 °C for 10 s, 60 °C for 15 s and 72 °C for 30 s). The relative difference in expression level of target genes was calculated using the $2^{-D\Delta Ct}$ method (Livak and Schmittgen, 2001). In