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Research article

Transcriptome de novo assembly and analysis of differentially expressed genes related to cytoplasmic male sterility in onion

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ABSTRACT

Onion (*Allium cepa* L.) is one of the major vegetables in China and accounts for a large proportion of China's vegetable exports. Onion cytoplasmic male sterility, which is often used in onion breeding, is caused by the interaction between the nuclear genes and the cytoplasm. However, the underlying molecular mechanism of onion cytoplasmic male sterility remains unclear. In this study, we analysed the anther microstructure of the onion cytoplasmic male sterile line SA2 and the onion maintainer line SB2. We found that the pollen abortion in SA2 occurred at the tetrad stage during the microspore development, which was very different from that in SB2. We used the Illumina HiSeq platform to sequence RNA from anthers at the tetrad stage collected from the SA2 and SB2 lines. The RNA sequencing and transcriptome assembly produced 146,413 All-Unigenes. Based on an analysis of the differentially expressed genes, we identified two cytoplasmic control genes, *atp9* and *cox1*, and three nuclear-related genes, *SERK1*, *AG* and *AMS*. These transcriptomic results were also verified by fluorescence quantitative PCR. Our study provides important information about genes related to onion cytoplasmic male sterility, and it will help improve the understanding of the molecular mechanism of onion cytoplasmic male sterility.

1. Introduction

Onion (*Allium cepa* L.) is a biennial herb that originated at the Mediterranean coast alluvial plain (China Agriculture Press, 1984). According to the shape of their bulbs, onions can categorized as *Allium cepa* L., *Allium cepa* L. var. *aggregatum* G. Don and *Allium cepa* L. var. *viviparum* Merg. (Fan et al., 2004). Onion is widely cultivated in China from the north to the south, with still-expanding acreage (Wang et al., 2003).

Cytoplasmic male sterility (CMS) is a maternally inherited trait that prevents a plant from producing functional pollen grains. CMS is extensively used to produce F1 hybrids to increase fruit yield and decrease costs (Budar and Pelletier, 2001). Two types of CMS cytoplasm (CMS-S and CMS-T), along with a normal cytotype, have been previously reported in onions (Jones and Emsweller, 1936; Berninger, 1965). S-cytoplasm (Jones and Emsweller, 1936) was observed in the onion cultivation species 'Italian Red', which cannot be seeded after self-fertilization. The sterility is caused by an interaction between the cytoplasm and single nuclear recovery gene. It can be restored by the dominant allele, Ms, of the nuclear fertility locus (Jones and Clarke, 1943). Male sterile plants have sterile cytoplasm, and the fertility recovery site is a homozygous recessive gene (Smsms). Plants with a common cytoplasm (N) and a recovery site for fertility in the form of a homozygous recessive gene (Nmsms) are maintainer line plants and can be maintained by male sterile lines by crossing the sterile line and the maintainer line. Research indicates that the fact that ms recessive alleles are prevalent in onions and tiller onions suggests that the mutation from Ms to ms happened in the early stages of onion evolution, and this mutation has occurred several times (Little et al., 1944; Davis, 1957). The S-cytoplasm is stable, maintaining its male sterile traits even under different environmental conditions due to its fertility recovery, which is controlled by single-cell nuclear genes. Thus, it is widely used in onion F1 hybrid breeding (Havey, 2000). The T-cytoplasm (Berninger, 1965) was discovered in the European onion cultivation species 'Jaune paille des Vertus'. A genetic analysis showed that the genetic mechanism of the T-cytoplasm nuclear interaction was much more complex than that of the S-cytoplasm, and its sterility is affected by three pairs of nuclear genes: 1 pair of individual genes (A) and 2 pairs of complementary

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genes (B and C) (Schweisguth, 1973). Due to its genetic complexity and the ease with which fertility is restored, it is difficult to use the T-cytoplasm system for applications in production. S-cytoplasm and T-cytoplasm fertility can be restored with the ms locus and A-B-C loci, respectively. Male sterility induced by the CMS-S cytoplasm can be restored by a single nuclear locus (Jones and Clarke, 1943), whereas at least three independent Rf loci may be involved in restoring fertility in CMS-T male sterility plants (Schweisguth, 1973).

The male sterile phenotype of CMS lines arises because of alterations in chimeric open reading frames (ORFs) in the mitochondrial genome (Yamagishi and Bhat, 2014). During the critical period of anther development, a variety of recombinant mitochondrial genes produced by exchanges, deletions, or insertions disrupt normal mitochondrial function, and the mutated mitochondrial genes interact with nuclear genes and affect the development of microspores, leading to male sterility (Wu et al., 2009). The oxidative phosphorylation pathway is carried out in the mitochondria, and cytochrome oxidase and ATP synthase are indispensable enzymes in this pathway. Cytochrome oxidase, the terminal oxidase of the electron transport chain located in the mitochondria, plays an important role in controlling electron transport. ATPase, which is widely distributed in plants, catalyses the release of energy by ATP hydrolysis and is required for metabolic activities such as the transport of various substances, signal transduction and material synthesis (Xie et al., 2006). Changes in the structures of the genes controlling these enzymes affect the oxidative phosphorylation pathway, which in turn affects the normal function of the mitochondria and leads to CMS.

In most cases, CMS is related to the premature degradation of the tapetal cell, a sporogenous tissue that nurtures the pollen mother cells (Hernould et al., 1998). Through cytological morphological observations, it was found that cytoplasmic male sterility in onions was caused by the interaction of the tapetum and microspore and was not the result of the action of any tissue in isolation (Wu et al., 2009). The tapetum is the innermost layer in the anther wall, directly adjacent to the pollen mother cell in the pollen sac. This layer is essential to the normal development of pollen, and any processes that are hindered during the development of the tapetum may lead to male sterility. In recent years, genes related to the development of the tapetum have been found in some plants, such as *BAM1/BAM2* in Arabidopsis (Hord et al., 2006), *YY1/YY2* in rice (Tsuchiya et al., 1992), *TA13* in tobacco (Koltunow et al., 1990), and *MAC1* in maize (Sheridan et al., 1999). The discovery of these genes will help reveal the molecular mechanism underlying the development of plant tapetal cells.

Recently, Illumina sequencing techniques have enabled fascinating discoveries in the life sciences and dramatically improved the efficiency of gene discovery (Zhang et al., 2013). The RNA-seq technique has been useful in the identification of CMS genes in other plant species (Shearman et al., 2014).

To identify candidate genes involved in CMS in onions, we performed RNA-seq using biological material from the anther at the tetrad stage of an onion male sterile line and a maintainer, and we obtained transcriptomic information by the de novo assembly of the onion transcriptome. We identified differentially expressed genes in the two lines and discussed the potential relationship between these genes and male sterility in onions.

2. Materials and methods

2.1. Plant materials

The plant materials used in this experiment were from the onion sterile line SA2 and the maintainer line SB2, which were provided by the onion and garlic research group of Northeast Agricultural University. SA2 (S-cytoplasm) and SB2 have the same genetic background, both derived from the long-type onion higher-generation inbred line 'L1102'. The fertility of SA2 can be restored by SB2, but the offspring are still sterile. In the autumn of 2015, the SA2 and SB2 lines were planted in the Greenhouse Horticulture Station of Northeast Agricultural University, and they were bolted in April of the following year. After flowering, the anthers were collected at four stages: the pollen mother cell stage, the tetrad stage, the free microspore stage and the mature pollen grain stage. The corresponding sizes were 1–1.5 mm (pollen mother cell stage), 1.5–2 mm (tetrad stage), 2–2.5 mm (free microspore stage) and 2.5–3 mm (mature pollen grain stage). We took different anthers from SA2 and SB2 to perform paraffin section microscopic observations at the four stages. The microscopic analysis results proved that the sizes we determined corresponded to the four anther stages. The anthers were immediately frozen in liquid nitrogen after collection and stored at −80 °C. The anther material of each stage was taken from 20 onions and mixed for fluorescent quantitative PCR.

We observed that, in contrast with the maintainer line SB2, pollen abortion occurred at the tetrad stage in the male sterile line SA2. Therefore, the anthers of this stage were selected to be the transcriptome sequencing materials. Two anther samples per onion were taken from ten SA2 onions and ten SB2 onions, and the sample RNA was sequenced by the BGI Company (Shenzhen, China).

2.2. Microscopy

Anthers at the different developmental stages were taken and placed in an FAA fixative solution overnight. They were subsequently dehydrated with an ethanol gradient for 2 h each and finally placed in 95% ethanol overnight. The dehydrated buds were embedded in paraffin, sectioned into 8 μm thick slices, mounted on slides, and stained with safranin solution and solid green. After the slices were dehydrated, they were covered with a drop of neutral resin and placed in a 38 °C drying oven overnight. After drying, they were viewed and pictured under a light microscope (SZX10, OLYMPUS).

2.3. Anther collection and RNA extraction

The flower buds of SA2 and SB2 were measured by a Vernier caliper at four stages (pollen mother cell stage, tetrad stage, microspore stage, mature pollen stage). Anthers were taken from the buds with tweezers, and the buds were put into 1.5 mL centrifuge tubes and then immediately frozen in liquid nitrogen. The samples were subsequently stored in a −80 °C freezer.

The total RNA was extracted and tested by the BGI Company (Shenzhen, China). The RNA concentration was determined by a Qubit Fluorometer, an Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit, Agilent, Cat No.5067-1511), a NanoDrop (NanoDropTM), and a microplate reader; the OD260/OD280 and OD260/OD230 values were determined by the NanoDrop; the 28S/18S, 23S/16S and RIN values were determined by the Agilent 2100 Bioanalyzer. The samples were sequenced after passing the quality tests.

2.4. Sequencing and de novo transcriptome assembly

Sequencing was performed on the Illumina HiSeq platform. Low quality reads, such as those resulting from joint contamination or containing high unknown N contents were first removed, and the filtered reads (clean reads) were then assembled de novo with the Trinity software (to remove PCR repetition and to improve assembly efficiency). The assembled transcripts were then clustered to remove redundant transcripts using TGICL, and the obtained transcripts (Unigenes) were re-clustered to further remove redundant transcripts and to obtain the final Unigenes (All-Unigenes) for the subsequent analysis.

2.5. Gene functional annotation

The All-Unigenes were annotated with seven functional databases