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Research article

Cytological, molecular mechanisms and temperature stress regulating production of diploid male gametes in *Dianthus caryophyllus* L.

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ABSTRACT

In plant evolution, because of its key role in sexual polyploidization or whole genome duplication events, diploid gamete formation is considered as an important component in diversification and speciation. Environmental stress often triggers unreduced gamete production. However, the molecular, cellular mechanisms and adverse temperature regulating diplogamete production in carnation remain poorly understood. Here, we investigate the cytological basis for 2n male gamete formation and describe the isolation and characterization of the first gene, *DcPS1* (*Dianthus Caryophyllus Parallel Spindle 1*). In addition, we analyze influence of temperature stress on diploid gamete formation and transcript levels of *DcPS1*. Cytological evidence indicated that 2n male gamete formation is attributable to abnormal spindle orientation at male meiosis II. *DcPS1* protein is conserved throughout the plant kingdom and carries domains suggestive of a regulatory function. *DcPS1* expression analysis show *DcPS1* gene probably have a role in 2n pollen formation. Unreduced pollen formation in various cultivation was sensitive to high or low temperature which was probably regulated by the level of *DcPS1* transcripts. In a broader perspective, these findings can have potential applications in fundamental polyploidization research and plant breeding programs.

1. Introduction

Polyploidy, the possession of more than two sets of chromosomes, is especially widespread and is considered to be a major evolutionary force for driving speciation and also diversification in flowering plants. Up to 70% of angiosperm species are estimated as polyploid (Ramsey and Schemske, 1998; Otto and Whitton, 2000; Adams and Wendel, 2005; Otto, 2007). Polyploids are believed to arise commonly by the formation and fusion of diploid pollen and eggs without reduction of the somatic chromosomes during sexual polyploidization, and also called unreduced gamete or 2n gametes. A wide variety of plant species in natural ecosystems produce 2n gametes, and many species and hybrids of floral crops including *Lilium*, *Rosa*, *Dianthus caryophyllus*, Orchid species, *Begonia* hybrids, *Primula denticulata* also produced 2n gametes (Teoh, 1984; El Mokadem et al., 2002; Crespel et al., 2006; Dewitte et al., 2009; Hayashi et al., 2009; Zhou et al., 2012; Chung et al., 2013). Unreduced gametes are useful in enabling crosses between plants of different ploidy levels and could overcome unbalanced parental contributions in the developing seed (Barcaccia et al., 2003; Carputo et al., 2003; Köhler et al., 2010). Meanwhile 2n gametes from a distantly related hybrid can be a source of intergenomic recombinant chromosomes and lead to higher fitness and heterozygosity in the offspring (Ramanna and Jacobsen, 2003; Chung et al., 2013). Therefore, sexual polyploidization has played a great role in producing new varieties and breeding of polyploid ornamental and horticultural crops.

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Unreduced gametes most commonly arise through meiotic defects. Several cytological mechanisms have been described that generate 2n gametes. These mechanisms include the omission of one of the meiotic cell divisions, alterations in meiosis I or meiosis II spindle morphology, or defects in meiotic cytokinesis (Ramanna and Jacobsen, 2003; De Storme and Geelen, 2013). The predominant mechanism resulting in 2n spore formation is perhaps co-orientation of spindles (parallel spindles or fused spindles) during metaphase II and anaphase II (Mok and Peloquin, 1975; Veilleux, 1985; Bretagnolle and Thompson, 1995; Lopez-Lavalle and Orjeda, 2002). As the normal number of four the second meiotic division (meiosis II) poles is reduced to two, dyads are generated that contain two diploid spores instead of the normal tetrads with four haploid spores (Genualdo et al., 1998; De Storme and Geelen, 2013). Disruption of the spindle orientation in meiosis II in *parallel spindle 1*(*ps1*) mutant *Arabidopsis thaliana* causes the presence of parallel, fused, and tripolar spindles (d'Erfurth et al., 2008). *A. thaliana Parallel Spindles1* (*AtPS1*) contains two highly conserved regions (d'Erfurth et al., 2008). An N-terminal forkhead associated domain (FHA) could be involved in protein–protein interactions (Durocher and Jackson, 2002) and the C-terminal conserved region shows similarity to a PilT N-terminus domain (PINc) that bind RNA (Clissold and Ponting, 2000).

Environmental stress, specifically temperature stress has impacted upon processes in meiotic cell division (Pécrix et al., 2011; De Storme et al., 2012). Heat stress alters specifically male meiotic chromosome behavior, resulting in meiotically restituted dyads and triads that originated from the ectopic induction of parallel and tripolar spindles at metaphase II. Different to heat stress, cold stress significantly alters cell plate formation at the post-meiotic stage and cell wall establishment in process of male meiosis which lead to generate triads, dyads, and monads (De Storme et al., 2012). Genetic studies in *Arabidopsis* have identified three proteins that regulate the orientation of the meiosis II spindle apparatus in male meiosis, namely Arabidopsis FORMIN14 (AFH14), JASON (JAS) and *AtPS1* (d'Erfurth et al., 2008; Li et al., 2010; De Storme and Geelen, 2011). However, because little is known about the impact of temperature on the transcription profile and post-transcriptional processing of these genes, more detailed studies are needed to determine their role in pollen mother cell stress sensitivity.

Carnation (*D. caryophyllus* L.), Caryophyllaceae, is one of the important ornamental crop worldwide. Most carnation cultivars produce 2n gametes (Zhou et al., 2012). Despite the ecological and agricultural significance of polyploids derived from 2n gametes, cytological descriptions and the molecular mechanisms underlying 2n gametes formation of carnation and influence of environmental stress on its related genes are as yet poorly understood. We describe the cytological mechanisms that are involved in the formation of 2n pollen in carnation. The role of *D. caryophyllus Parallel Spindle 1* (*DcPS1*) in the formation of diplogametes is presented by the isolation, characterization, and analysis of expression patterns of *DcPS1* in three cultivars. We revealed that different genotypes produce different frequencies of 2n gametes. Moreover, the effect of temperature could be associated with the formation of 2n pollen in carnation.

2. Material and methods

2.1. Plant material and temperature measurement

The experiments were carried out in seven diploid carnation (2n = 30): 'Promesa', 'Guernse Yellow', 'YunhongErhao', 'Red Barbara', 'L.P. Barbara', 'Nogalte' and 'Arevalo' which were planted in Plastic Shed. During growth, temperature was measured using a Hobo Shuttle Data Transporter (Shuttle, Onset Computer Corporation) and then downloaded to the computer using BoxCar Pro 4.0 (Onset Computer Corporation).

2.2. Pollen grain size analysis

Pollen samples were collected from male fertile plants that flowered in Mar., June, Sept., Dec., 2013. Anthers from at least three opening flowers were collected and squashed in a drop of aceto-carmine on a glass slide and then observed under a Nikon E800 microscope (×100) equipped with Nikon digital camera. Pollen grains were considered viable if they had regular shape and red stained cytoplasm. The size of more than 200 viable pollen grains per individual was measured using the Image-pro-plus 6.0 (ipp 6.0) morphometric system. Because the diameter of 2n pollen grain is about 1.3 times larger than that of reduced n pollen grains. When 1.3 times of mean pollen diameter was exceed, pollen were considered as the 2n pollen grains of cultivars (Ssebuliba et al., 2008). The viability of pollen grains of *D. caryophyllus* cultivars was estimated.

2.3. Cytological analysis of male meiosis

The bud of 'Nogalte' cultivars collected from march to April in 2013 was used for cytological analysis of male meiosis. Anthers were removed from young floral buds collected and fixed in a Carnoy's solution [absolute ethanol/glacial acetic acid (3/1, v/v)] for 24 h at room temperature. The anthers were excised on a glass slide and stained with carbol-fuchsin for 1 h and covered with cover glass. Observations were made under a Nikon E800 microscope at ×1000.

The number of dyads, triads, and tetrads were counted when PMCs were at the tetrad stages. The expected 2n pollen frequency was obtained from the observed number of dyads, triads, and tetrads using the following formula: Expected 2n pollen (%) = $(2 \times Dy + Tr)/(2 \times Dy + 3 \times Tr + 4 \times Te) \times 100\%$, where Dy, Tr, and Te correspond to the numbers of dyads, triads, and tetrads, respectively.

Observation of anther development was also performed on paraffin sections as described previously (Zhou et al., 2013). Anthers under different developmental stages were collected based on the length of bud, and fixed in formalin-acetic acid-alcohol (FAA) for 24 h and dehydrated through a graded series of ethanol solutions, and then embedded in paraffin wax. Sections were cut to 8–12 μm thickness, stained with hematoxylin, and then photographed under the microscope.

2.4. Cloning of a DcPS1 conserved fragment

Genomic DNA was extracted from buds of 'Promesa' using the CTAB method. The forward (VVbase-2086-F) and reverse (VVbase-2363-R) primers were designed from conserved regions of PINc domain by examination of protein multiple alignments using Clustal W (Table S1). PCR products were excised and extracted by QIAquick Gel Extraction Kit (Qiagen) and cloned into the pMD™18-T Vector (TaKaRa, Japan), and clones were sequenced.

2.5. Extension of the original DcPS1 fragment by chromosomal walking

The initial 239 bp *DcPS1* fragment was extended by high-efficiency thermal asymmetric interlaced PCR (Liu and Chen, 2007) with primers used for hiTAIL-PCR (Table S1). Briefly, 3ca-179-R and a LAD primer were applied to the pre-amplification reaction. Then the primary TAIL-PCR is carried out using the nested specific primer 3ca-115-R and AC1. The secondary TAIL-PCR could