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Inheritance Analysis and Marker Development of White Rust Resistance in Chrysanthemum

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ABSTRACT

The main aims of this study were to understand the inheritance mode of hexaploid chrysanthemum (random or preferential chromosome pairing) and to develope molecular markers to enhance breeding efficiency for the white rust resistance in chrysanthemum. A segregation analysis using simple sequence repeat (SSR) markers was carried out to clarify the inheritance mode of chrysanthemum. Through genotyping the pseudo-F_1 testcross population derived from a cross between 'Dancer' and 'Puma White' using 49 polymorphic SSRs, 65 informative SSR marker alleles were analyzed. Of them, 33 marker alleles showed a good fit to the expected segregation ratio for the hexasomic inheritance, whereas 24 marker alleles showed for the disomic inheritance supporting the autopolyploid segregation mode in chrysanthemum. The observed ratio of non-simplex to simplex markers, 25 versus 99, indicated the hexasomic inheritance mode. In addition, random marker allele assortment showed in the six markers gave a conclusive evidence for the hexasomic inheritance mode of these loci. After

revealing the inheritance mode of chrysanthemum, genetic analysis of white rust resistance was carried out. A total of 188 pseudo-F_1 testcross progenies were inoculated with *Puccinia horiana* isolates, then 161 progenies were identified as the resistant and 27 as the susceptible. This result gave a good fit to a ratio of 4:1 (P = 0.05327, α = 0.05) for resistance and susceptibility, suggesting that a single dominant gene controls resistance and 'Dancer' carries two dominant alleles coding the resistance gene. To improve breeding efficiency for white rust resistance in chrysanthemum, molecular markers were developed through the bulked segregant analysis. A total of 280 random amplified polymorphic DNA (RAPD) and 256 amplified fragment length polymorphism primers were screened. Then, OPI-13_{520} RAPD marker was presumed as the linked marker to the white rust disease resistance. The OPI-13_{520} marker was verified in 188 pseudo-F_1 testcross progenies and just six off-springs were found as the recombinants. Based on the expected phenotypic segregation ratios in a pseudo-F_1 testcross population, a duplex type of white rust resistance alleles and a duplex type of OPI-13_{520} markers in 'Dancer' were on the same chromosome and hence linked in coupling phase. The genetic distance between white rust resistance gene and OPI-13_{520} marker was determined to be 4.0 cM. The OPI-13_{520} marker was successfully converted into sequence characterized amplified region (SCAR) marker.

Keywords: chrysanthemum, inheritance mode, marker development, *Puccinia horiana*, resistance, sequence characterized amplified region

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LIST OF ABBREVIATIONS

AFLP	amplified fragment length polymorphism
BSA	bulked segregant analysis
cDNA	complementary DNA
EST	expressed sequence tag
FAM	6-carboxy-fluorescine
FISH	fluorescence *in situ* hybridization
HEX	hexachloro-6-carboxy-fluorescine
ISSR	inter-simple sequence repeat
MADCE	microsatellite allele dose and configuration establishment
PCR	polymerase chain reaction
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RH	relative humidity
ROX	6-carboxy-X-rhodamine
RV	ratio value
SCAR	sequence characterized amplified polymorphism
SSR	simple sequence repeat
QTL	quantitative trait loci

GENERAL INTRODUCTION

Chrysanthemum white rust, caused by *Puccinia horiana* Henn., is one of the most destructive disease in the production of chrysanthemum through the world. Under humid conditions in greenhouses, it spreads rapidly, resulting in considerable economic losses each year (Dickens, 1990; Firman and Martin, 1968). Infection causes pale green to yellow spots up to 4 mm diameter on the adaxial leaf surface, then forms raised buff or pinkish pustules on the abaxial leaf surface as shown in Fig. 1 (Baker, 1967). In extensively infected plants, infection can occur even in the stems, bracts, flower buds, and florets depending on susceptibility of cultivars (Dickens, 1970). For reducing damages and economic losses, fungicides are regularly sprayed on chrysanthemum plantations even though symptoms are not shown because eradicating pathogens is difficult if the plants are infected (Dickens, 1990; Stapel and Guerrand, 2012). However, considering the cost and the environmental concerns over fungicide applications, genetic resistance to white rust could be an efficient approach for controlling white rust disease in chrysanthemum cultivation (Park et al., 2014).

Till now, a lot of sources of resistance to white rust have been identified in chrysanthemum species and cultivars (Dickens, 1968; Martin and Firman, 1970; Park et al., 2014; Yamaguchi, 1981; Zeng et al., 2013). In addition, white rust resistance has been suggested to be controlled by a single dominant gene based on the segregations in hexaploid chrysanthemums for resistance (De Jong and Rademaker, 1986). Despite of much effort on chrysanthemum breeding for resistance to white rust, several complicating factors such as its polyploidy, high

Fig.1. Occurrence of white rust disease in chrysanthemum cultivation (A) and typical disease symptoms (B).

heterozygousity, and self-incompatibility make exploiting genetically-determined resistance difficult. Molecular markers tightly linked to the resistance gene can be used for overcoming these obstacles, as has already been demonstrated in several crop species (Heusden et al., 2002; Malek et al., 2000; Ukoskit and Thompson, 1997). However, in chrysanthemum, only a few genetic studies have been performed to develop molecular markers.

Most of the cultivated chrysanthemums have been found to be predominantly hexaploid (2n = 6x = 54), however some found somatic chromosome numbers indicating aneuploidy (Dorwick, 1953). Although the origin of its polyploidy and the genome constitution still remain uncertain, the most generally accepted hypothesis is that the cultivated chrysanthemums are allohexaploids derived from complex interspecific hybridization including *Chrysanthemum indicum*, *C. makinoi*, *C. japonense*, *C. ornatum*, and *C. zawadskii* (Ackerson, 1967; Anderson, 2006; Dowrick, 1953). Cytological studies of meiotic behavior and physical mapping of 45S rDNA loci by fluorescence *in situ* hybridization (FISH) supported the allohexaploid hypothesis (Abd El-Twab and Kondo, 2003; Dorwick, 1953; Li et al., 2011; Watanabe, 1977). Furthermore, F_1 seedling segregations for white rust resistance by De Jong and Rademaker (1986) often fitted to disomic (preferential pairing) inheritance rather than hexasomic (random pairing) inheritance.

There are several reports supporting hexasomic inheritance hypothesis. Cytological evidence that meiotic behaviors expected in the allopolyploid found in induced colchiploids derived from *C. japonense* (2n = 6x) and *C. boreale* (2n = 2x), even though they possess complete homologous chromosome sets was

suggested (Watanabe, 1983). In addition, genetic evidence that the segregations for carotenoid pigmentation gave good fits to the expected ratios for hexasomic inheritance was also provided (Langton, 1989). Most recently, Klie et al. (2014) suggested that chrysanthemum should be classified as segmental allohexaploid because of the mixed inheritance shown in the mainly dominantly scored genetic markers from their segregating population.

Therefore, firstly, this study was carried out to discriminate whether the inheritance mode of hexaploid chrysanthemum is hexasomic, disomic, or mixed through mining simple sequence repeats (SSR) markers from the expressed sequence tags (ESTs) available in the public domain and analyzing segregation in a pseudo-F_1 testcross population resulting from a cross between two hexaploid chrysanthemum cultivars. Secondly, molecular markers linked to white rust resistance gene were intended to develop using bulked segregant analysis with random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers. These experiments will provide molecular evidence that contributes to the discussion on the inheritance mode of chrysanthemum and breeding strategy that improve breeding efficiency based on marker assisted selection for white rust resistance in chrysanthemum.

LITERATURE REVIEW

Origin of *Chrysanthemum* species

Chryasnthemums are herbaceous flowering plants of the genus *Chrysanthemum* in the family Asteraceae. They were first described in Chinese literature as early as the 15th B.C. and have been cultivated as ornamental plant for more than 3,000 years. In addition to aesthetic value, chrysanthemums have been used for food and medicine. Flower petals are eaten in salads or teas and young shoots are steamed for pharmaceutical purposes (Woolman, 1953).

Historically, about 200 *Chrysanthemum* species were classified into the genus *Chrysanthemum*, but the majority of them have been subdivided into 38 genera of the chrysanthemum complex by Anderson (1987). Since then, taxonomic classifications of chrysanthemum complex have changed repeatedly because the classification systems of chrysanthemums are incomplete and unsystematic. At present, taxonomic classification is determined by embryo sac development, cypselar anatomy, plant habit, phytochemical characteristics, and molecular markers (Borgen, 1972; Harling, 1951; Liu et al., 2012).

The genus *Chrysanthemum* comprises about 40 species mainly found in East Asia. In this genus, the considerabe variations in morphology and ploidy have been exhibited. Especially, with centered in China, the diversification and speciation have been progressing during long cultivation history through hybridization and adaption. Then, many *Chrysanthemum* species have been spread to south-east Asian and European countries from China.

Chrysanthemum was first introduced out of Asia via the Netherlands in 1688,

then spread to England in 1754 and USA in 1798 (Clark, 1962; Dorwick, 1953). Although many cultivars bred in and spread from China and Japan having long chrysanthemum breeding history, the hybridization and selection of chrysanthemum were first officially recorded in 1827 by M. Bernet. New cultivars with small-flowered 'Pom-pom' and incurved types grown today have been developed and used widely in hybridization. Indeed, these few genotypes were known as the progenitors of the small-flowered modern cultivars (Emsweller et al., 1937).

Japan has also played an important role for the center of cultivation and breeding of modern chrysanthemum cultivars. Around the 4th century, chrysanthemums were first introduced to Japan via Korea from China (Anderson, 2006). At that time, most of chrysanthemums were likely similar to *C. indicum*, having many flowers with single-type bloom simultaneously, and were grown in the garden (Dowrick, 1953). However, these cultivars were hybridized into many other species and cultivars with different flower colors, shapes, and growing habits. Then, modern chrysanthemum cultivars specialized for cut-flowers, potted plants, and garden mums have been bred in Japan and spread worldwide.

Chrysanthemum, *Dendranthema* × *grandiflorum* (Ramat.) Kitamura (syn. *C. morifolium* (Ramat.)), is one of the most important floricultural crops in the world. The chrysanthemums, used as cut flowers or potted plants, are mostly grown in plastic tunnels or greenhouses and mainly produced in China, Japan, the Netherlands, Italy, and Colombia (AIPH, 2013; De Backer, 2012; Spaargaren, 2002). It is cultivated on an area of more than 20,000 ha, and more than 5 billion stems and pots are marketed annually in EU and Asian flower auctions (AIPH,

2013). In Korea, chrysanthemum is also economically important ornamental crop after rose. It was cultivated on an area of 489 ha, and 233 million stems and 11 million pots were marketed in Korean flower auctions in 2013. Moreover, chrysanthemum is one of Korea's main exported floricultural crops. The value of exports, which totaled only 2.1 million US dollars in 1999 when the export of the cut flowers of chrysanthemum to Japan began in earnest, hit 10 million US dollars in 2010 (MAFRA, 2014).

Inheritance mode of chrysanthemum

Chrysanthemums are native species in the Mediterranean region (centered in Algeria and the Canary Islands) and Asia (centered in China, Korea, and Japan) (Dowrick, 1952). Genetic diversity of chrysanthemums has been originated in these centers by interspecific hybridization, polyploidization, mutation, and others (Anderson et al., 1992; Dorwick, 1952, 1953; Wasscher, 1956). Species collected in Mediterranean region are mostly diploid (2n = 2x = 18), but some are decaploid (2n = 10x = 90). Otherwise, ploidy range of species colleted in Asia is much greater from diploid to decaploid. Based on these, Dorwick (1952) reported that most of diploid species occur in the center of origin, the Mediterranean, while polyploidy are increased depending on increasing latitude.

Today, most of the cultivated chrysanthemums have been found to be hexaploid (2n = 6x = 54) whose basic choromosome number is 9, although botanical species for the garden have been found to be diploid (Dowrick, 1952, 1953). However, some cultivars have been found as aneuploidy that somatic chromosome numbers are ranging from 2x = 47-63 (Dorwick, 1953). Meiosis in

hexaploid *Chrysanthemum* species is more complex compared to meiosis in diploid species, because homo(eo)logous chromosome pairing is altered depending on the genome consititution. In an allopolyploid species, such as rose and wheat, preferential chromosome pairings among the homologous chromosomes occur (Nybom et al., 2004). Thus, the genetics of those species is similar to diploids. In autopolyploid species, the chromosomes pair randomly with their homoeologous chromosomes during meiosis (Ripol et al., 1999).

In general, polyploids derived from interspecific hybridization predominantly formed bivalents at meiosis, although univalents have been observed (Bleier, 1934). In addition, bivalents formation is associated with preferential chromosome pairings among the homologous chromosomes (Watanabe, 1977, 1983). The origin of polyploidy and the genome constitution in chrysanthemums still remain uncertain. However, the most generally accepted hypothesis is that the cultivated chrysanthemums are allohexaploids, because the hexaploid chrysanthemums have been derived from complex interspecific hybridization including *C. indicum*, *C. makinoi*, *C. japonense*, *C. ornatum*, and *C. zawadskii* (Ackerson, 1967; Anderson, 2006; Bleier, 1934; Dowrick, 1953).

Cytological studies have supported the allohexaploid hypothesis. Dorwick (1953), Watanabe (1977), and Li et al. (2011) reported that bivalent are normally formed and multivalent are rare in various polyploid *Chrysanthemum* species during meiosis. And physical mapping of 45S rDNA loci by FISH also showed that polyploidy of *Chrysanthemum* species might be derived from allopolyploidization (Abd El-Twab and Kondo, 2003).

Segregation analysis of white rust resistace preformed by De Jong and

Rademaker (1986) submitted a genetic evidence(s) for the allohexaploid hypothesis. They described four types of disease reactions between host and pathogen; resistance, imcomplete resistance, resistance with necrosis, and susceptibility. They also found that F_1 seedling segregations for white rust resistance expected to be controlled by single dominant gene often fitted to disomic (associated with preferential chromosome pairing) inheritance mode rather than hexasomic (associated with random chromosome pairing) inheritance.

Furthermore, inheritance of anthocyanins and carotenoids in 'Vulcan' chrysanthemum cultivar with orange flower and its orange-flowered offsprings also fit a disomic inheritance model. Anthocyanins might be controlled by a single dominant gene responsible for transposable element expression of red pigments overlaid on carotenoids (Teynor et al., 1989b). Segregation of carotenoid pigment controlled by a single dominant allele I (inhibitor) showed best fit in an 1:1 expected ratio, thus supported the allohexaploid hypothesis (Teynor et al., 1989a)

However, the diploid-like meiotic behaviors do not reflect that the chromosomes of chrysanthemum pair with their homologous chromosomes preferentially owing to structural differences or genetic controls suppressing homoeologous pairing (Cifuentes et al., 2010). Watanabe (1983), who had stated that chrysanthemum is allopolyploidy, reported that a higher than expected frequency of bivalent formation was found in induced colchiploids derived from *C. japonense* (2n = 6x) and *C. boreale* (2n = 2x), even though they possess complete homologous chromosome sets. Therefore, gradual reduction in number of zygomeres, and differentiation in homology recognition and regulation system

during evolution are presumably suppress multivalent formation in polyploidy chrysanthemum and thus enabling diploidization behavior.

In addition, Langton (1989) provided genetic evidence that the segregations for carotenoid pigmentation gave good fits to the expected ratios for hexasomic inheritance. Self-compatible genotypes, nulliplex for the dominant allele I responsible for inhibition of carotinoid expression, were crossed with pink-flowered ‘Pink Marble’ and ‘Pollyanne’ chrysanthemum cultivars which have dominant allele I. Segregation analysis in the progenies of ‘Pollyanne’ crosses gave extremely good fits to a 19:1 ratio which would be expected for crosses between triplex and nulliplex of autohexaploid species. Under the assumption of disomic inheritance, simplex genotypes selected from ‘Pink Marble’ F_1 progenies were selfed to obtain duplex genotype with paring dominant alleles on pairing homologues. However, subsequent crosses with the nulliplex ‘Canary’ gave good fits to a 4:1 ratio which would be expected for crosses between duplex and nulliplex of autohexaploid species, providing evidence for a hexasomic inheritance mode.

Klie et al. (2014) suggested that chrysanthemum should be classified as segmental allohexaploid because of the mixed inheritance shown in the mainly dominantly scored genetic markers from their segregating population. However, because of the difficulty of obtaining distinct segregation ratios in polyploid chrysanthemums and the lack of large numbers of well-defined and broadly useable markers, evidence towards the mixed inheritance hypothesis is still scarce.

Chrysanthemum white rust caused by *Puccinia horiana*

Rust disease in general

The plant rusts, caused by Basidoiomycetes of the order Uredinales, are destructive diseases on grains such as wheat, oats, barley, vegitables such as bean and asparagus, ornamentals such as chrysanthemum, carnation, and snapdragon, and trees such as pine, apple, and coffee. They cause tremendous economic losses and famines worldwide. The rust fungi infect mostly leaves and stems and cause numerous rusty, orange, yellow, or even white-colored spots erupting through the epidermis, but some rusts form swellings or galls (Agrios, 2005; Kolmer et al., 2009).

There are approximately 7,000 species of rust fungi in the monophyletic order Uredinales. Most of rust fungi are specialized obligte parasites (biotrophs) in nature and different rust species infect different crops or cultivars. In the case of rust fungi that morphologically identical but infect different hosts, they were classified into special forms (*formae specialis*), for example, *Puccinia graminis* f. sp. *tritici* on wheat, *P. graminis* f. sp. *hordei* on barley, etc. (Agrios, 2005; Kolmer et al., 2009).

In disease cycle, most rust fungi have five different spore stages, which are teliospores, basidiospores, pycniospores, aeciopores, and uredospores, in that order. And these rusts are called macrocyclic or long-cycled rusts. Rusts that produce only teliospore and basidiospore are classified into microcylic or short-cycled rusts. On infection, in general, the basidiospores germinate and penetrate the plant epidermis or through stomata. Then, the basidiospores initiate to produce haploid mycelium that forms spermagonia containing spermatia and

receptive hyphae. After fertilization of spermatia with receptive hyphae, dikaryotic mycelium and spores are produced, then aeciospores, uredospores, and teliospores are produced, subsequently (Agrios, 2005; Kolmer et al., 2009).

Rust fungi disperse mostly by wind, although transinfection can also occur by insects, animals, rain, etc. Zandvoort et al. (1968) reported that the spores of the chrysanthemum white rust can spread by wind over a distance of at least 700 meters, and Baker (1967) also supposed that the white rust is transported by wind over several kilo-meters.

Chrysanthemum white rust disease

Chrysanthemum white rust, caused by *P. horiana*, is one of the most destructive fungal diseases in chrysanthemum. *P. horiana* was first reported in 1895 in Japan, and first identified by Hennings in 1901 (Hiratsuka, 1957). In 1963, chrysanthemum white rust was first discovered in England, on stocks imported from Japan. Since then, chrysanthemum white rust was found in most countries where chrysanthemums were commercially cultivated. By the late 1960s, white rust was found in many other European countries including Denmark, Norway, and the Netherlands (Baker, 1967). The disease has also been detected in New Zealand, USA, Canada, and Mexico (EPPO/CABI, 2004; Water, 1981).

P. chrysanthemi has been repoted to be another causal agent of white rust in chrysanthemum, although it is not a major pathogen (Punithalingam, 1968a). *P. chrysanthemi* could be easily distinguished from *P. horiana* by mycological characteristics. Unlike *P. chrysanthemi*, *P. horiana* does not produce uredinal

stage on infection. Teliospores of *P. horiana* are oblong in shape and yellow in color, while those of *P. chrysanthemi* are round and brown (Punithalingam, 1968a, b).

For infection, relative humidity (RH) and temperature conditions are important on basidiospore dispersal, germination, and infection. High RH > 96%, and temperature range, 17 to 24°C, are necessary for sporidia development from teliospores of *P. horiana* on chrysanthemum leaves. Given optimum condition, 5 hours are sufficient to infect adjacent plants (Park and Kim, 1993; Whipps, 1993). Infection causes pale green to yellow spots up to 4 mm diameter on the adaxial leaf surface, then forms raised buff or pinkish pustules on the abaxial leaf surface (Baker, 1967). In extensively infected plants, infection can occur even in the stems, bracts, flower buds, and florets depending on susceptibility of cultivars (Dickens, 1970).

For reducing damages and economic losses, fungicides are regularly sprayed on chrysanthemum plantations, even though symptoms are not shown because eradicating pathogens is difficult if the plants are infected (Dickens, 1990; Stapel and Guerrand, 2012). Several cultural methods have also been proposed to control white rust disease in chrysanthemums. Heat treatment of infected plants at 37-40ºC for 20 h or the dipping of cuttings in water at 45ºC for 5 min have been reported to reduce chlorosis and sporulation (Zadoks et al., 1968). Furthermore, the sporidia have been reported to be sensitive to desiccation at 90% RH that an exposure to 80% RH for 5 min or 90% RH for 1 h eradicates 100% of the sporidia (Firman and Martin, 1968). However, use of these methods is practically difficult, because heat treatments can cause the death of terminal buds

or whole plants and the malformation of plants. Furthermore, control over RH in large greenhouses is costly.

Pathotypes of chrysanthemum white rust

In view of the interactions between hosts and parasites, differential interactions have been demonstrated to exist between *P. horiana* isolates and *Chrysanthemum* species (De Backer et al., 2011; Velasco et al., 2007; Yamaguchi, 1981). Variations in resistance depending on isolates were observed from the inoculation study on 40 cultivars with six typical white rust isolates collected from different regions of Japan (Yamaguchi, 1981). Among six *P. horiana* isolates, some showed wide range of infections, while others showed medium or narrow range of infections, suggesting remarkable differences in pathogenicity among *P. horiana* isolates.

Velasco et al. (2007) studied the pathogenic and molecular variability. Sixteen isolates of *P. horiana* collected from three different states of Mexico were inoculated in five chrysanthemum cultivars, thus the isolates exhibited different virulence depending on their geographical origin. In addition, three groups in the virulence were corresponded exactly to molecular variability which was analyzed through the DNA polymorphisms detected by RAPD, microsatellite primed polymerase chain reaction, and randomly amplified microsatellite polymorphism.

De Backer et al. (2011) also confirmed the presence of pathotypes in *P. horiana* and tried to explain this through gene-for-gene type of resistance decribed by Flor (1956). For covering a broad geographical rang on a large set of

cultivars, they collected 22 single-pustule isolates of *P. horiana* originated from three different continents over four different years and these individual isolates were inoculated in 36 selected cultivars. Among them, 24 cultivars showed a differential interaction phenotypic profile. All isolates infected a minimum 4 to a maximum 19 differential cultivars, thus their unique profile demonstrated the highly complex race structure in this pathosystem. For detecting genetic diversity of *P. horiana*, De Backer (2012) identified 33 polymorphisms, 32 single nucleotide polymorphisms and 1 SSR, for *P. horiana* through seqeuncing 25 loci. Using these markers, 25 genotypes of *P. horiana* isolates collected from different continents were discriminated. Based on these results, pathotype diversity of *P. horiana* was found to be much greater than genotype diversity. They explained the reason that more than seven ressitance genes in *Chrysanthemum* species corresponded to avirulence genes in *P. horiana*.

Identification of germplasms resistant to chrysanthemum white rust

Since white rust was first reported in England in 1963, outbreaks occurred in all over in England and Wales. Subsequently, infection was seen on a number of commercial cultivars. However, several cultivars were observed free of infection although they were grown near the infected plants (Baker, 1967). Based on these observations, Dickens (1968) investigated the susceptibility of various *Chrysanthemum* species and their cultivars, and determined the majority of the *Chrysanthemum* species tested appear to be resistant to white rust except only the Asian hybrids. Martin and Firman (1970) evaluated the level of resistance in 270 cultivars of the florists' chrysanthemum. They observed that 208 cultivars

were rated as immune (showed no symptoms) or resistance (necrotic reaction or discrete telia developed), whereas only 62 cultivars were rated as susceptible. Yamaguchi (1981) also screened 19 *Chrysanthemum* species and 250 cultivars for resistance to white rust. Remarkable differences in white rust resistance were determined among *Chryansthemum* species and cutivars tested.

De Jong and Rademaker (1986) described four types of reactions of the chrysanthemum cultivars to *P. horiana*; (1) complete resistance, no symptoms visible, (2) incomplete resistance, few pustules developed slowly and sporulted limitedly, (3) necrosis, necrotic reaction around the growing rust colonies and completely inhibited sporultion, (4) susceptible, many rust pustules developed quickly and sporulated abundantly. Then, they classified chrysanthemum cultivars depending on the reaction to *P. horiana*. In the case of complete resistance, they suggested that a single dominant gene controls white rust resistance in hexaploid chrysanthemums based on the segregation analysis. However, resistant cultivars which showed incomplete resistance or necrosis did not carry a monogenic resistance factor.

Furuta et al. (2004) suggested that an intergeneric somatic hybrid by electofusion of protoplasts between chrysanthemum and wormwood (*Altemisia sieversiana*) has been shown to be more resistant than the chrysanthemum progenitor. Zeng et al. (2013) reported the variation for resistance to white rust in *Ajani*a and *Chrysanthemum* species, and suggested interspecific hybridizaiotn between commercial chrysanthemum and various *Ajani*a and *Chrysanthemum* species might have considerable potential for white rust resistance breeding.

Molecular markers in chrysanthemum

Genetic studies of chrysanthemums have been hampered owing to the genome complexity, various levels of polyploidy, high level of heterozygosity, and the occurrence of both inbreeding depression and self-incompatibility (Anderson and Ascher, 2000; Dowrick, 1952; Huang et al., 2000; Zagorski et al., 1983). Therefore, chrysanthemums still lag far behind many other self-pollinated crops in using advanced molecular techniques. Nevertheless, molecular techniques have been attempted in chrysanthemum studies for a wide range of purposes from detecting genetic diversity of chrysanthemums, estimating relationships between chrysanthemum species, identifying cultivars or species, constructing genetic linkage map, and discovering genes.

Wolff and Peters-van Rijn (1993) detected genetic variability in chrysanthemum using RAPD markers. Wolff et al. (1994) developed restriction fragment length polymorphism (RFLP) probe and primers, then identification of chrysanthemum cultivars and stability of DNA fingerprint pattern were analyzed using these RFLPs (Wolff et al., 1995). Especially, the results obtained by Wolff et al. (1994, 1995) were used to distinguish bud sports (mutants derived vegetatively from a elite cultivar but differ from it in some characteristics) or progenitor themselves reliably.

Dia et al. (1998) used RAPD profiles to infer the origin of the cultivated chrysanthemums and Lema-Ruminska et al. (2004) used RAPD markers to detect polymorphisms and validate hybridity in *Chrysanthemum*. Miao et al. (2007) developed inter-simple sequence repeat (ISSR) markers to estimated genetic relationshpip of 85 chrysanthemums cultivars. Wang et al. (2007)

evaluated the genetic stability of regenerants of *in vitro* cultured chrysanthemum 'Jinba' using ISSR markers. In addition, AFLP markers have been also used to estimate genetic relationship among *Chrysanthemum* species (Zhou and Dai, 2002).

The first preliminary genetic linkage map of the hexaploid chrysanthemum cutivars, 'Yuhualuoying' and 'Aoyunhanxiao', were constructed with a combination of RAPD, ISSR, and AFLP markers by using the double pseudo-testcross mapping strategy (Zhang et al., 2010). Thereafter, a marker-trait association analysis of two chrysanthemum flowering traits, initial blooming time and the duration of flowering, were carried out using sequence-related amplified polymorphism markers (Zhang et al., 2011a, b). And single-locus quantitative trait loci (QTL) with additive effect and epistatic QTL associated with plant architectural trait in chrysanthemum (erect or creeping type) were detected and mapped (Zhang et al., 2012). Most recently, based on the genetic analysis of branching trait in spray chrysanthemum, 16 additive QTLs including five QTLs for primary branch number, each four QTLs for branch height and branch angle, and three QTLs for primary branch length were identified (Peng et al., 2015).

The ESTs were reported by Chen et al. (2009). A complementary DNA (cDNA) library was constructed from the inflorescence of the chrysanthemum cultivar 'Zhongshanzigui' with anemone flower. In total, 7,307 clones were sequenced and 4,563 unique sequences were identified. By the comparison to the GenBank, 2,608 unique sequences showed homology to genes of known function of other organisms, and 1,217 unigenes were clustered in the 23

categories by orthologous group analysis. Based on these results, they obtained numerous transcripts responsible for flower development and flower pigmentation (Chen et al., 2009).

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CHAPTER I

Analysis of Inheritance Mode in Chrysanthemum Using Expressed Sequence Tag-derived Simple Sequence Repeat Markers

ABSTRACT

To study the inheritance mode of hexaploid chrysanthemum (random or preferential chromosome pairing), a segregation analysis was carried out using simple sequence repeat (SSR) markers derived from chrysanthemum expressed sequence tags (ESTs) in the public domain. A total of 248 EST-SSR primer pairs were screened in chrysanthemum cultivars 'Dancer' and 'Puma White', of which 49 EST-SSRs were selected as polymorphic and informative markers. These polymorphic markers were used for genotyping a pseudo-F_1 testcross population derived from a cross between the two cultivars. The 49 EST-SSRs detected 210 marker alleles with an average number of 4.29 marker alleles per locus. For 180 of these polymorphic SSR marker alleles, segregation could be estimated using a χ^2 goodness of fit test ($\alpha = 0.05$) with the expected segregation ratios for hexasomic or disomic inheritance. For 65 SSR marker alleles the segregation ratio was informative for the type of inheritance, 33 marker alleles gave a good fit to the expected segregation ratio for hexasomic inheritance, whereas 24 marker alleles gave a good fit for disomic inheritance showing a higher number

of marker alleles supporting autopolyploid segregation in chrysanthemum. In addition, the observed ratio of non-simplex to simplex markers was 20:80 (25 versus 99) supported hexasomic inheritance. Furthermore, random marker allele assortment was found within the six fully informative markers giving conclusive evidence for hexasomic inheritance in chrysanthemum at these chromosomal regions.

Keywords: allele dosage, allopolyploidy, autopolyplidy, *Chrysanthemum* × *morifolium*, genotyping, segregation type

INTRODUCTION

Chrysanthemum (*Chrysanthemum* × *morifolium* (Ramat.)), mainly used as cut flower or potted plant, is one of the most important floricultural crops in the world. In spite of its economic importance, only a few genetic studies have been performed to understand the inheritance mode of chrysanthemum which is an important feature in polyploid genetics.

Most of the cultivated chrysanthemums have been found to be predominantly hexaploid (2n = 6x = 54), but some found somatic chromosome numbers indicated aneuploidy can occur (Dorwick, 1953). Although the origin of its polyploidy and the genome constitution still remain uncertain, the most general accepted hypothesis is that the cultivated chrysanthemums are allohexaploids derived from complex interspecific hybridization including *C. indicum*, *C. makinoi*, *C. japonense*, *C. ornatum*, and *C. zawadskii* (Ackerson, 1967; Anderson, 2006; Dowrick, 1953). Cytological studies of meiotic behavior showed that bivalent are normally formed and multivalent are rare in polyploid *Chrysanthemum* species which is considered as supporting the allohexaploid hypothesis (Dorwick, 1953; Li et al., 2011; Watanabe, 1977). Physical mapping of 45S rDNA loci by fluorescence in situ hybridization (FISH) also showed that polyploidy of *Chrysanthemum* species might be derived from allopolyploidization (Abd El-Twab and Kondo, 2003). Furthermore, De Jong and Rademaker (1986) found that F_1 seedling segregations for white rust resistance often fitted to disomic (preferential pairing) inheritance rather than hexasomic (random pairing) inheritance.

However, the diploid-like meiotic behaviors do not reflect that the chromosomes of chrysanthemum pair with their homologues preferentially owing to structural differences or genetic controls suppressing homoeologous pairing (Cifuentes et al., 2010). Watanabe (1983) reported that a higher than expected frequency of bivalent formation was found in induced colchiploids derived from *C. japonense* (2n = 6x) and *C. boreale* (2n = 2x), even though they possess complete homologous chromosome sets. Therefore, the possibility was suggested that gradual reduction in number of zygomere, and differentiation in homology recognition and regulation system during evolution might suppress multivalent formation in polyploidy chrysanthemum thus enabling diploidization behavior. Furthermore, Langton (1989) also provided genetic evidence that the segregations for carotenoid pigmentation gave good fits to the expected ratios for hexasomic inheritance. Most recently, Klie et al. (2014) suggested that chrysanthemum should be classified as segmental allohexaploid because of the mixed inheritance shown in the mainly dominantly scored genetic markers from their segregating population. However, because of the difficulty of obtaining distinct segregation ratios in polyploid chrysanthemums and the lack of large numbers of well-defined and broadly useable markers, evidence towards the mixed inheritance hypothesis is still scarce. Thus, more controlled genetic studies using preferably gene based co-dominantly scorable markers are required to further investigate the mode of inheritance.

Owing to their multi-allelic nature and co-dominant inheritance, simple sequence repeats (SSRs) have been used as markers for plant genetics, breeding applications and evolutionary studies in a large number of plant species (Ellis

and Burke, 2007; Varshney et al., 2005) as well as for inheritance studies in complex polyploids (Nybom et al., 2004). In recent years, a tremendous number of DNA sequences including expressed sequence tags (ESTs) have been made available in public databases. Therefore, EST-derived SSR markers (EST-SSRs) can be rapidly and inexpensively developed (Pashley et al., 2006).

For discriminating whether the inheritance mode of hexaploid chrysanthemum is hexasomic (random chromosome pairing), disomic (selective chromosome pairing) or mixed, SSR markers have been mined from ESTs available in the public domain and used them for segregation analysis in a pseudo-F_1 testcross population resulting from a cross between two hexaploid chrysanthemum cultivars. These experiments will provide molecular evidence that contributes to the discussion on the inheritance mode of chrysanthemum, representative for the situation in more polyploid ornamentals with a complex cultivation history.

MATERIALS AND METHODS

Plant material and DNA extraction

From a pseudo-F_1 testcross population, resulting from a cross between two hexaploid chrysanthemum cultivars, maternal 'Puma White' and paternal 'Dancer', 94 randomly chosen progenies were used for SSR genotyping. The direction of the cross was chosen because 'Puma White' has anemone type flowers that hardly produce pollens and thus the chance of selfing was minimized ('Dancer' has single flowers). Total genomic DNA was extracted from young leaf tissues using the DNeasy® plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction with minor modifications in adding 2% polyvinylpyrrolidone (Sigma-Aldrich, St. Louis, MO, USA) to the lysis buffer. The quality and quantity of the DNA were determined by using a NanoVue™ spectrophotometer (GE Healthcare Bioscience, Pittsburgh, PA, USA) and by visual inspection after electrophoresis on an 1% agarose gel.

Detection of SSRs in ESTs

EST sequences for chrysanthemum were retrieved from the public database, the European Molecular Biology Laboratory (EMBL) nucleotide sequence database (http://www.ebi.ac.uk/embl/index.html in August 2010), using a Sequence Retrieval System. The obtained EST sequences were scanned using MISA (MIcroSAtellite; freely available at http://pgrc.ipk-gatersleben.de/misa/download/misa.pl) to detect SSR-containing sequences with or without assembly using Mimicking Intelligent Read Assembly (MIRA; freely available at

http://sourceforge.net/projects/mira-assembler/) (Chevreux et al., 2004; Thiel et al., 2003). Criteria for the SSR search were a minimum of eight repeat units for dinucleotide SSRs and five repeat units for tri-, tetra-, penta-, and hexa-nucleotide SSRs excluding mononucleotide SSRs to increase the chance of selecting polymorphic markers (Smulders et al., 1997).

Polymorphism analysis of EST-SSR markers

Primer pairs were designed in the flanking regions of the simple sequence repeats using PRIMER3 (freely available at http://biotools.umassmed.edu/bioapps/primer3_www.cgi), and then screened for polymorphism on a 6% polyacrylamide gel using the two parental cultivars, 'Dancer' and 'Puma White' and silver staining for amplicon detection. For the EST-SSR markers selected from parental screening, the universal tail polymerase chain reaction (PCR) method as described by Schuelke (2000) with some modifications was used to genotype F_1 progenies as well as parents). For the PCR analysis, the forward specific primer for each SSR locus was designed with the addition of the universal tail sequence (5'-AAC AGG TAT GAC CAT GA-3') at the 5' end. The universal tail primer for second round PCR was labelled with 6-carboxy-fluorescine, hexachloro-6-carboxy-fluorescine, or 6-carboxy-X-rhodamine at the 5' end. Reverse primers were designed in such a way that a GTTT tail at 5' end was present to reduce stutters (Brownstein et al., 1996). PCR was performed in two steps. In the first PCR, the forward and reverse primers of an EST-SSR marker were used to incorporate the universal tail sequence into the PCR products. In the second PCR, diluted PCR products (1:100) were amplified with

a fluorescently labelled universal tail primer and the specific reverse primer of each EST-SSR marker. Both the first and second PCR amplification were performed in a total volume of 20 μL containing 2 μL of template DNA (10 ng/μL) or diluted PCR product, 1.0 μL of each primer (10 pmol/μL), 2.0 μL of 10× PCR buffer, 2.0 μL of dNTP (2 mM) and 0.1 μL DreamTaq-polymerase (5 U/μL) (Fermentas, Vilnius, Lithuania). PCR reactions were run as follows: initial denaturation at 95°C for 5 min, then 25 cycles each of 95°C for 30 s, T_m for 30 s and 72°C for 1 min, and a final extension at 72°C for 10 min (Veriti™ Thermal Cycler, Applied Biosystems, Foster, CA, USA). The final products were run on an ABI PRISM 3730 DNA Analyzer (Applied Biosystems) using the internal-lane size standard, GeneScan-500 LIZ. Genotyping data were analyzed using GeneMapper software v4.0 (Applied Biosystems).

EST-SSR marker analysis

Electropherogram data from the ABI PRISM 3730 DNA Analyzer were visualized by GeneMapper 4.0 software and peaks corresponding to each SSR marker allele were converted into binary form (presence = 1, absence = 0) depending on product size. All the detected SSR marker alleles were classified by their presence in the maternal parent, in the paternal parent, or in both. SSR marker allele configuration of the parents, 'Dancer' and 'Puma White', was estimated by analyzing the observed segregation ratio of each peak in the 94 pseudo-F_1 progenies, according to the expected segregation ratio (Table I-1) of the two inheritance models, hexasomic (random pairing) and disomic (preferential pairing), as described by Langton (1980). For all markers, a χ^2 test

Table I-1. Expected segregation ratios (presence:absence) for a single locus dominant gene in hexaploid chrysanthemum, assuming that bivalents are formed at meiosis with either random (hexasomic) or preferential (disomic) pairing.

Cross combination	Random pairing (hexasomic)	Preferential pairing (disomic)
Simplex × Nulliplex	1:1	1:1
Duplex × Nulliplex	4:1	3:1*
Triplex × Nulliplex	19:1	7:1*
Simplex × Simplex	3:1	3:1
Duplex × Simplex	9:1	7:1*
Triplex × Simplex	39:1	15:1*
Duplex × Duplex	24:1	15:1*
Triplex × Duplex	99:1	31:1*
Triplex × Triplex	399:1	63:1*

*With non-paring dominant alleles, otherwise expected segregation ratio would be 1:0 (this latter segregation type has not been found).

($\alpha = 0.05$) was carried out to infer their goodness-of-fit to the expected segregation ratio in each model.

SSR marker allele configuration using the 94 pseudo-F_1 progenies was analyzed by the microsatellite allele dose and configuration establishment (MADCE) procedure with a minor modification (van Dijk et al., 2012). Using a simplex marker allele that segregates in a ratio of 1:1 as reference allele, the peak area of each SSR marker allele relative to the peak area of the reference allele, the ratio value (RV), was calculated. Based on the SSR marker allele dose of the parents assessed by segregation analysis, correction factor of RVs was identified between a SSR allele and the reference allele. Then, the SSR allele dosages and configuration of 94 pseudo-F_1 progenies were estimated by examining the RVs.

Proportion of non-simplex to simplex EST-SSR markers

The proportion of non-simplex to simplex markers was used to discriminate the type of polyploidy, autopolyploidy vs. allopolyploidy, in *Saccharum robustum* (Al-Janabi et al., 1994) and *Ipomea batatas* (Kriegner et al., 2003; Ukoskit and Thompson, 1997). Only simplex, duplex, and triplex markers (in conjunction with nulliplex in other parent) can generate polymorphism in a hexaploid plant while alleles with higher dose produce monomorphic markers when scored in a dominant way. The expected frequencies for non-simplex markers are estimated by adding up the probability of transmission of an absent allele for duplex and triplex markers. Under the assumption of autohexaploidy, 25% (1/5 for duplex marker + 1/20 for triplex marker) of all segregating markers

are expected as non-simplex and 75% as simplex. In allohexaploidy, the expected frequency for non-simplex markers is 37.5% (1/4 for duplex marker + 1/8 for triplex marker) and 62.5% for simplex markers. For polymorphic SSR marker alleles, observed segregation ratio of non-simplex to simplex marker alleles was tested for goodness-of-fit by a χ^2 test ($\alpha = 0.05$) with the expected segregation ratio for each inheritance model.

RESULTS

Detection of SSRs in ESTs

In total, 8,358 chrysanthemum ESTs, registered in EMBL, were used for this study, and these represented 4,467 kb of chrysanthemum transcriptome. For searching SSRs, these ESTs were mined using MISA after sequence assembly with MIRA to remove redundancy. Among them, 5,485 ESTs were assembled to 1,039 consensus sequences and 58 SSRs in 52 of those consensus sequences were detected. Due to the low efficiency of SSR detection in the assembled sequences, the database was also mined without prior assembly, and then 385 SSRs including the previously identified 58 SSRs were detected in 362 ESTs, suggesting an average frequency of one SSR per 11.6 kb or per 21.7 EST sequences analyzed in the chrysanthemum transcriptome. Because these SSRs were detected from a redundant EST database, cluster analysis was performed using DNASTAR SeqMan (Lasergene, Madison, WI, USA) to reduce overestimation. Of the 362 ESTs, as a result, a total of 278 non-redundant sequences, which consisted of 242 ESTs revealed as singletons and 36 consensus sequences clustered from 120 ESTs, were identified to contain 285 SSRs (Fig. I-1).

Of the 285 non-redundant SSR, trinucleotide repeats (TNRs) were the most abundant group with 228 (80.0%), followed by 43 (15.1%) dinucleotide repeats (DNRs), while tetranucleotide repeats (TTNRs) and hexanucleotide repeats (HNRs) were rarely found (< 3.0%). Among the TNRs, the motif ACC/GGT (24.6%) was the most frequently found, followed by AAC/GTT (17.6%) and

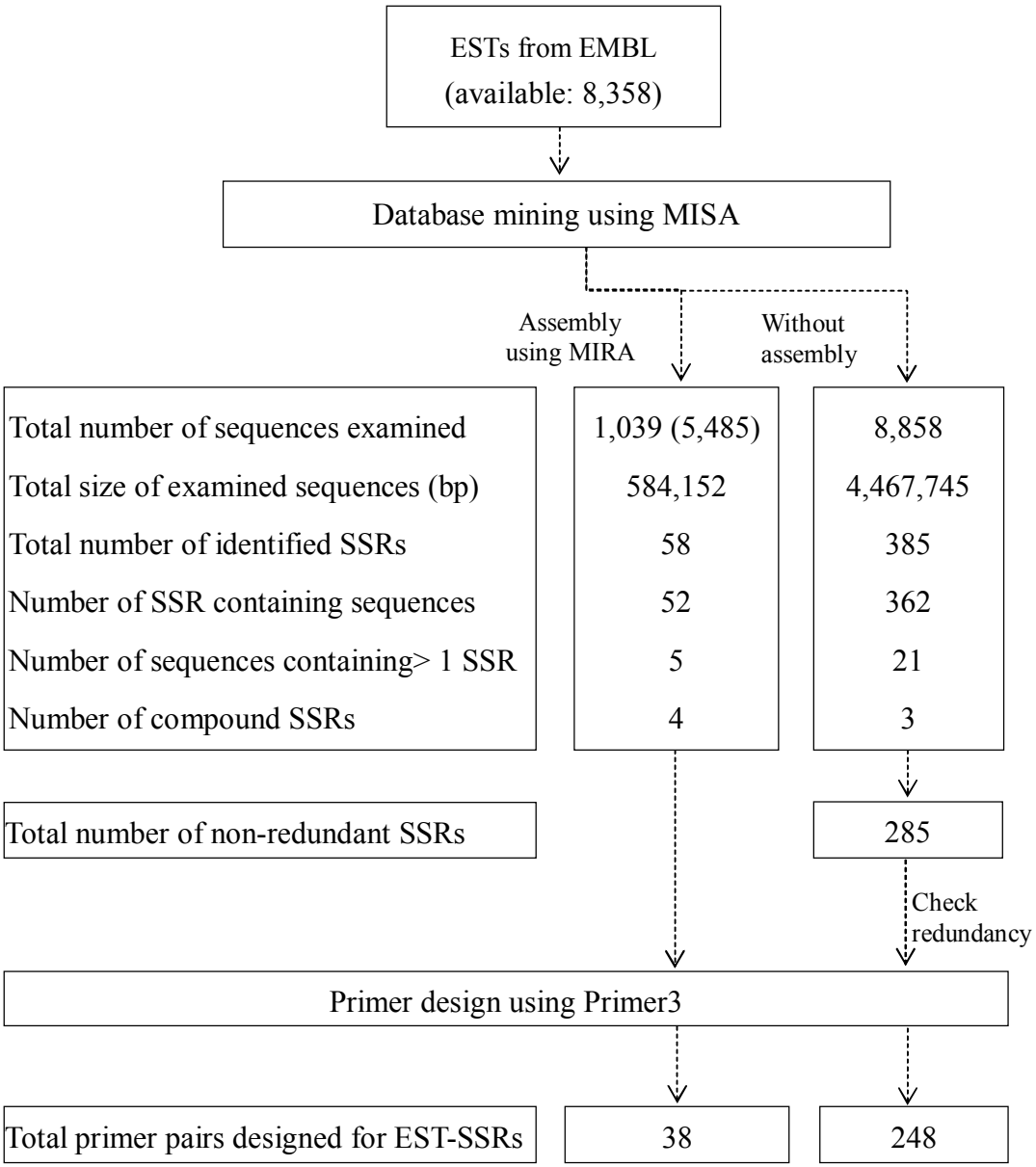

Fig. I-1. Scheme used for database mining and development of SSR markers from chrysanthemum ESTs registered in EMBL.

AGT/ACT (14.9%), while the motifs ACG/CGT and AGG/CCT were rarely found (3.1%). Among the DNRs, the AC/GT motif was most frequently (58.1%) found, followed by AG/CT (34.9%) and AT/TA (7.0%), while the motif CG/CG was not found (Table I-2).

Selection of EST-SSR primer pairs

From the 285 non-redundant SSRs, 248 primer pairs were designed using PRIMER3. For the pre-selection of primer pairs, all primer pairs were screened for polymorphism on polyacrylamide gel using the two parental cultivars, 'Dancer' and 'Puma White'. A total of 142 primer pairs, of which 90 were polymorphic and 52 were monomorphic, amplified amplicons around the expected size, while the remaining 106 primer pairs did not amplify or yield any meaningful amplicons (i.e., spurious banding patterns). Based on the number of bands and band patterns amplified, 49 of the 90 polymorphic EST-SSRs were finally selected as for SSR genotyping in the F_1 population (Supplentary table 1).

Polymorphism analysis of EST-SSR markers

Using the selected EST-SSR primer pairs, SSR genotyping was performed by the two-step nested PCR method. As a result, a total of 210 SSR marker alleles were detected in the 49 loci (4.29 alleles per locus), and of these, 180 marker alleles (85.7%) were polymorphic in the F_1 population whereas 30 alleles (14.3%) were present throughout (Table I-3). All found alleles were accounted for by the parental genotypes. Based on analyzing the observed segregation ratio of each peak in 94 F_1 progenies and the RV, the dose of the 180 polymorphic

Table I-2. Frequency and distribution of different types of SSRs identified in the chrysanthemum ESTs.

Repeat motif	Number of repeat units												Total
	5	6	7	8	9	10	11	12	13	14	15	>15	repeats
AC/GT	-	-	-	12	3	3	3	1	1	1	1		25
AG/CT	-	-	-	6	4	2	-	1	-	1	-	1	15
AT/AT	-	-	-	-	-	-	-	1	-	-	-	2	3
AAC/GTT	23	7	3	4	3	-	-		-	-	-		40
AAG/CTT	15	9	-	2	-	-	-		-	-	-		26
AAT/ATT	12	7	3	2	-	-	-	1	-	-	-	1	26
ACC/GGT	37	8	8	2	1	-	-	-	-	-	-	-	56
ACG/CTG	6	1	-	-	-	-	-	-	-	-	-	-	7
ACT/ATG	9	4	2	-	-	-	-	-	-	-	-	-	15
AGC/CGT	5	2	1	-	1	-	-	-	-	-	-	-	9
AGG/CCT	6	1	-	-	-	-	-	-	-	-	-	-	7
AGT/ATC	21	11	1	1	-	-	-	-	-	-	-	-	34
CCG/CGG	7	1	-	-	-	-	-	-	-	-	-	-	8
AAAC/GTTT	1	-	-	-	-	-	-	-	-	-	-	-	1
AAAG/CTTT	1	2	-	-	-	-	-	-	-	-	-	-	3
AAAT/ATTT	1	-	-	-	-	-	-	-	-	-	-	-	1
AACT/ATTG	-	-	-	-	-	-	-	-	-	-	-	-	0
AAGG/CCTT	1	-	-	-	-	-	-	-	-	-	-	-	1
AAGT/ATTC	1	-	-	-	-	-	-	-	-	-	-	-	1
AGGT/ATCC	-	-	-	1	-	-	-	-	-	-	-	-	1
AACCCT/ATTGGG	1	-	-	-	-	-	-	-	-	-	-	-	1
AAGTAG/ATCTTC	1	-	-	-	-	-	-	-	-	-	-	-	1
ACCACT/ATGGTG	1	-	-	-	-	-	-	-	-	-	-	-	1
ACCATC/AGTGGT	2	-	-	-	-	-	-	-	-	-	-	-	2
ACGTCC/AGGTGC	1	-	-	-	-	-	-	-	-	-	-		1
NN(MNR)	-	-	-	18	7	5	3	3	1	2	1	3	43
NNN(DNR)	141	51	18	11	5	-	-	1	-	-	-	1	228
NNNN(TNR)	5	2	-	1	-	-	-	-	-	-	-	-	8
NNNNNN(HNR)	6	-	-	-	-	-	-	-	-	-	-	-	6

Table I-3. Classification of 210 SSR alleles detected in 49 SSR loci depending the χ^2 goodness of fit test ($\alpha = 0.05$, df = 1) using the expected segregation ratios of hexasomic and disomic inheritance.

	Autohexaploid (hexasomic)				Allohexaploid (disomic)			
	No. of alleles present in 'Dancer'	No. of alleles present in 'Puma White'	No. of alleles present in both parents	Total	No. of alleles present in 'Dancer'	No. of alleles present in 'Puma White'	No. of alleles present in both parents	Total
Monomorphic	1	1	28	30	1	1	28	30
Polymorphic								
Simplex × Nulliplex	56	43	-	99	56	43	-	99
Duplex × Nulliplex	8	11	-	19	6	7	-	13
Triplex × Nulliplex	2	4	-	6	4	3	-	7
Simplex × Simplex	-	-	16	16	-	-	16	16
Duplex × Simplex	-	-	8	8	-	-	4	4
Unidentified dose	-	-	26	26	-	4	30	34
Distorted at <0.05	2	4	-	6	2	5	-	7
Total	69	63	78	210	69	63	78	210

marker alleles was estimated by χ^2 goodness of fit test ($\alpha = 0.05$) to the appropriate expected segregation ratios of hexasomic and disomic inheritance, respectively.

Among the 180 marker alleles, 99 uni-parental and 16 bi-parental simplex marker alleles were not affected by the type of inheritance (hexasomic vs. disomic), segregating in a ratios of 1:1 and 3:1 in the F_1 population, respectively. However, segregation of the other 65 SSR marker alleles depended on the type of inheritance. Under the assumption of hexasomic inheritance, 19 and 6 uni-parental SSR marker alleles gave fits to 4:1 and 19:1 segregation ratios expected for duplex and triplex marker alleles, respectively. Only 6 SSR marker alleles from 5 different markers showed distorted segregation at $\alpha < 0.05$ under this hexasomic inheritance assumption. In the case of bi-parental SSR marker alleles; while 8 SSR marker alleles gave fits to 9:1 segregation ratio's expected for duplex-simplex alleles, the dose of 26 marker alleles could not be identified owing to the multiple fits to different types of segregation ratio's. None of the alleles were distorted at $\alpha < 0.05$. On the other hand, under the assumption of disomic inheritance, 13 and 7 uni-parental SSR marker alleles gave fits to 3:1 and 7:1 segregation ratios expected for duplex and triplex marker alleles, respectively, and 4 bi-parental SSR marker alleles gave fits to 7:1 segregation ratios expected for duplex-simplex alleles. Seven SSR marker alleles were distorted at $\alpha < 0.05$ and the dose of 34 marker alleles could not be identified (Table I-3).

Proportion of non-simplex to simplex EST-SSR markers

Of the 130 segregating uni-parental SSR marker alleles, 99 were determined as simplex marker alleles by segregation analysis. However, depending on the type of polyploidy, 25 and 24 were determined as non-simplex (duplex or triplex) marker alleles, and 6 and 7 were distorted at $\alpha < 0.05$ under the assumption of autopolyploidy and allopolyploidy, respectively. The observed ratios of non-simplex to simplex marker alleles were estimated to be around 0.20 to 0.80. Based on the χ^2 test ($\alpha = 0.05$) for their goodness-of-fit to the expected ratio of autopolyploid and allopolyploid, the observed ratio supported an autopolyploid nature of chrysanthemum (Table I-4).

Transmission and segregation patterns of SSR marker alleles

Through the segregation analysis, SSR marker allele configuration of the parents in each SSR locus could be estimated. For example, for marker ChSSR-061 six different marker alleles were detected (noted as A_1 to A_6), which consisted of three paternal, one maternal, and two bi-parental alleles (Fig. I-2). Among them, three uni-parental marker alleles, A_2, A_4, and A_6 that segregated 1:1 and a bi-parental marker allele, A_3, that segregated 3:1 were identified as simplex markers and not being affected by the type of inheritance. A uni-parental marker allele, A_1, that showed segregation fitting both 4:1 for a hexasomic or 3:1 for a disomic segregation was identified as duplex marker. Bi-parental marker allele, A_5, was present in the whole F_1 offspring and assigned as duplex-triplex marker allele based on other allele dosages and the maximum of six alleles present in hexaploid chrysanthemum (Table I-5). Furthermore, the MADCE analysis using the RVs in the 94 individual progenies reflected the expectation

Table I-4. Observed proportion of non-simplex to simplex alleles and χ^2 goodness of fit test ($\alpha = 0.05$, df = 1) using the expected segregation ratios of hexasomic and disomic inheritance.

Marker type	Autohexaploid				Allohexaploid			
	Observed		Expected		Observed		Expected	
	Number	Ratio	Number	Ratio	Number	Ratio	Number	Ratio
Total	130				130			
Sub-total	124	1.000	124	1.00	123	1.00	123	1.000
Simplex	99	0.798	93	0.75	99	80.4	76.8	0.625
Non-simplex	25	0.202	31	0.25	24	19.6	46.2	0.375
P-value ($\alpha = 0.05$)			0.213				0.000	
Distorted at $\alpha < 0.05$*	6				7			

*Distorted alleles presence absence segregation indicates non-simplex dosage.

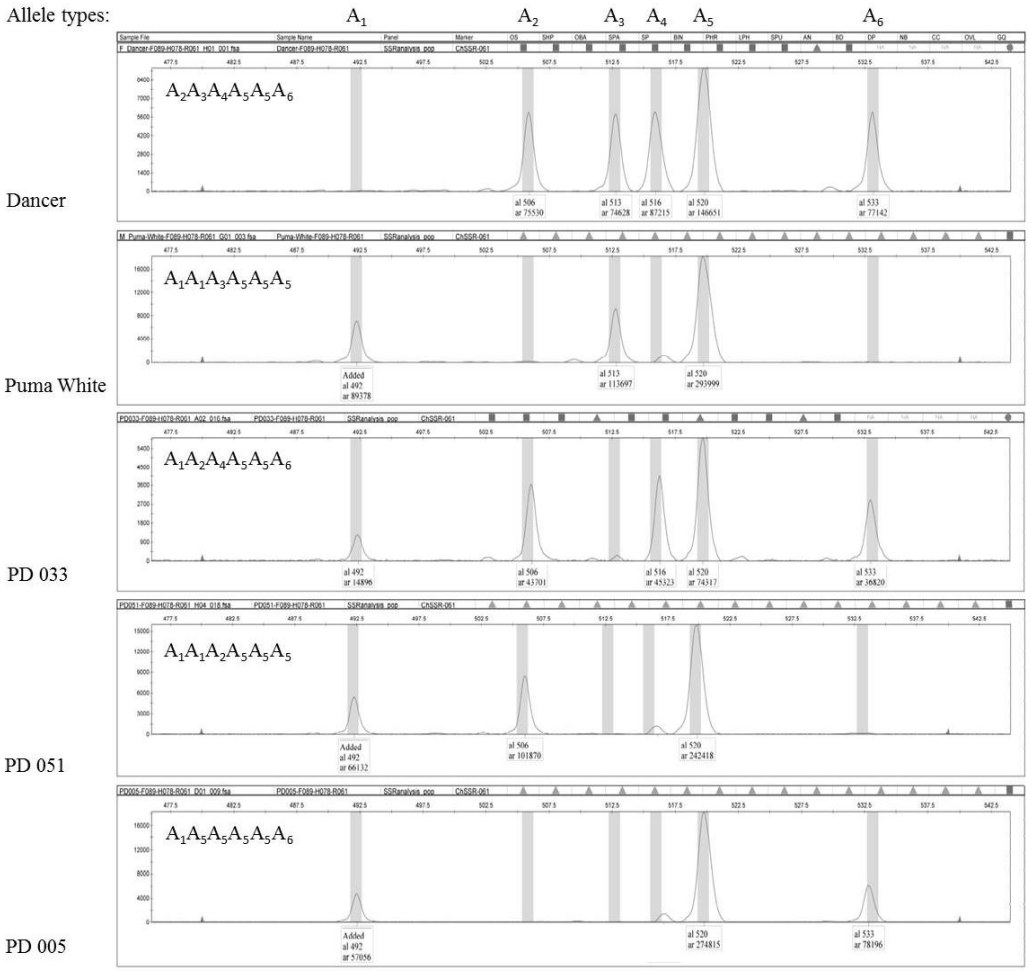

Fig. I-2. Genotype analysis of ‘Dancer’, ‘Puma White’, and their three progenies, ‘PD-033’, ‘PD-051’ and ‘PD-005’, in ChSSR-061 locus.

Table I-5. Allele dose and genotypic segregation ratio of each allele in ChSSR-061, and χ^2 goodness of fit test ($\alpha = 0.05$) using the expected segregation ratios of hexasomic and disomic inheritance.

Allele type	Allele dose of		Observed number of individual depending on allele dose							Expected ratio		Probability of χ^2 test
	Dancer	Puma White	6	5	4	3	2	1	0			
ChSSR-061_A_1	0	2					19	54	17	hexasomic	1:3:1	0.946
										disomic	1:2:1	0.158
ChSSR-061_A_2	1	0						50	40	hexasomic	1:1	0.292
										disomic	1:1	0.292
ChSSR-061_A_3	1	1					16	48	26	hexasomic	1:2:1	0.270
										disomic	1:2:1	0.270
ChSSR-061_A_4	1	0						44	46	hexasomic	1:1	0.833
										disomic	1:1	0.833
ChSSR-061_A_5	2	3		2	8	28	44	8	0	hexasomic	1:12:37:37:12:1	0.157
										disomic	1:5:10:10:5:1	0.004
ChSSR-061_A_6	1	0						44	46	hexasomic	1:1	0.833
										disomic	1:1	0.833

for six marker allele dose of ChSSR-061 well. In the scattered diagram shown in Fig. I-3, allele dose of uni-parental simplex alleles A_2, A_4, and A_6, was clustered into two groups, bi-parental simplex allele A_3, and uni-parental duplex allele A_1, into three groups, and bi-parental duplex-triplex allele A_5, into five groups.

Based on the segregation patterns of allele dose of ChSSR-061 in the 94 F_1 progenies, the six marker alleles were estimated by the χ^2 goodness of fit test ($\alpha = 0.05$) to the appropriate expected segregation ratios of hexasomic and disomic inheritance, respectively. In four of these marker alleles segregation was not affected by the type of inheritance, the other two marker alleles gave fits to 1:3:1 and 1:12:37:37:12:1 segregation ratios expected for autohexaploid. Based on the SSR genotyping of the ChSSR-061 locus, the genotypes of 'Dancer' and 'Puma White' were determined as $A_2A_3A_4A_5A_5A_6$ and $A_1A_1A_3A_5A_5A_5$, respectively (Table I-5, Fig. I-2). Subsequently the segregation behavior of alleles can be studied in the offspring. The genotypes of two informative F_1 progenies 'PD-033' and 'PD-051' were determined as $A_1A_2A_4A_5A_5A_6$ and $A_1A_1A_2A_5A_5A_5$, respectively. It means that alleles $A_2A_4A_6$ of 'PD-033' and alleles $A_2A_5A_5$ of 'PD-051' were derived from 'Dancer'. Given these results and when chromosomes would preferentially pair with their homologues during meiosis, the A_2 allele would pair with A_3 allele, A_4 with A_5, and A_6 with A_5 in 'Dancer' (Fig. 4). However, for example in F_1 progeny 'PD-005', the genotype found was $A_1A_5A_5A_5A_5A_6$, in which the A_2 and A_3 alleles are both missing. Taking the previous results into account, this genotype is not possible under disomic segregation (preferential pairing) because homologous chromosomes should segregate from each other at meiosis and inherit to their

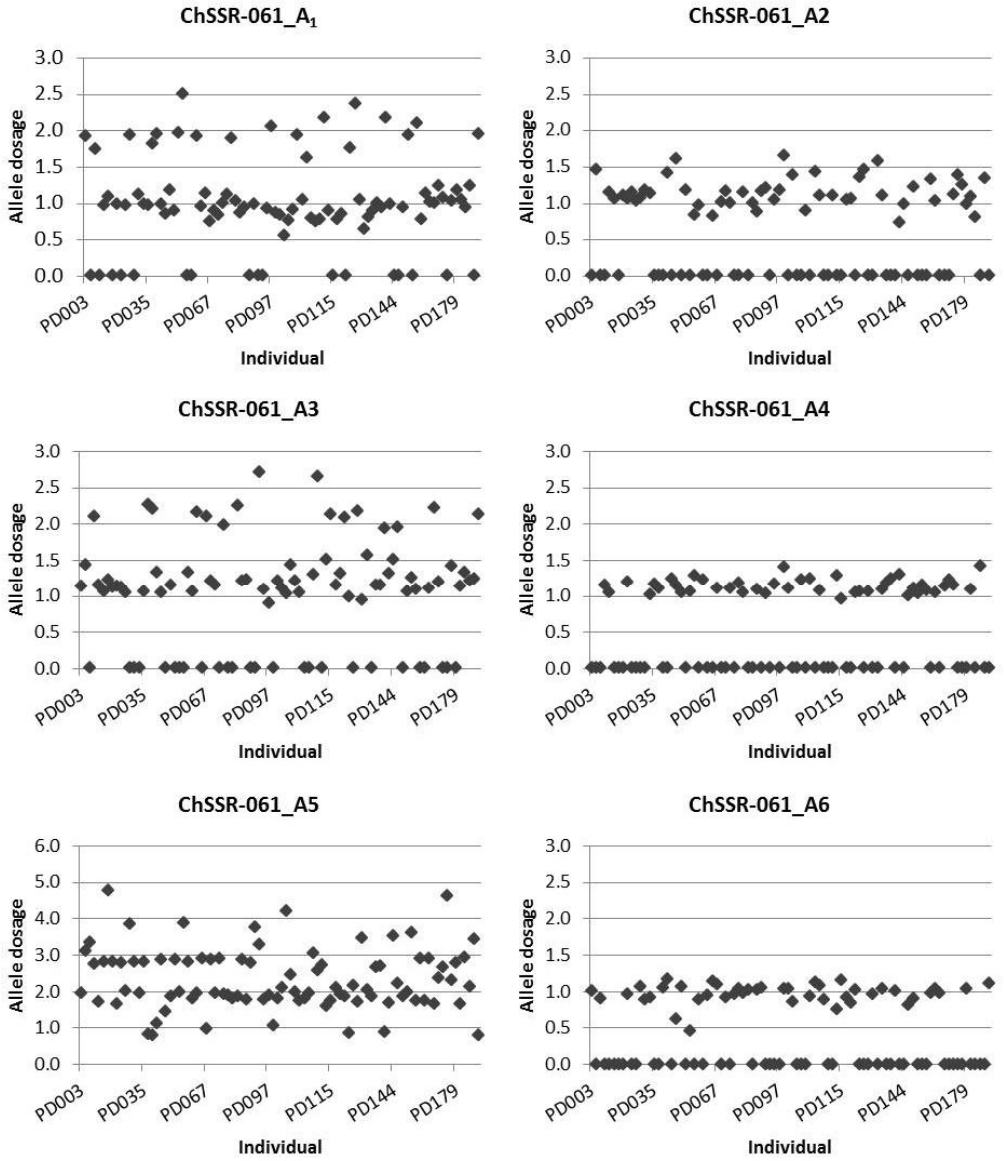

Fig. I-3. Scattered diagrams of 94 individuals in ChSSR-061 depending on allele dose. Each dot represents a single individual, names of 7 individuals have been indicated at the x-axis as examples.

Null hypothesis: If chrysanthemum is allohexaploidy,

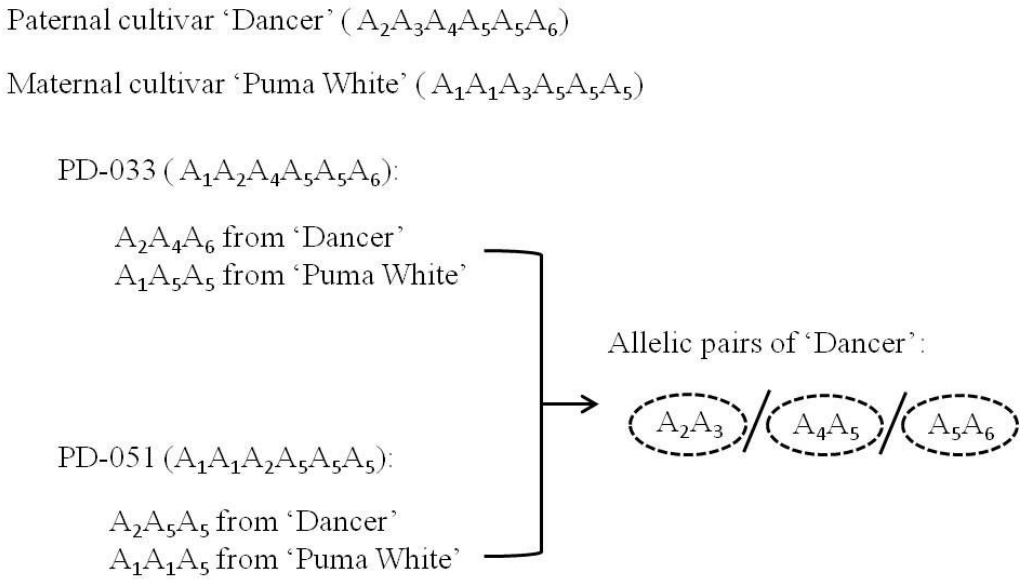

Fig. I-4. Diagram of possible chromosome pairing of 'Dancer' on the assumption of disomic mode of inheritance.

progenies. For this informative SSR locus thus no preferential allele combinations were observed, all six alleles randomly assorted in the 94 F_1 progenies, as would be expected in autopolyploid. Similarly further evidence of random assortment indicating an autopolyploid segregation type for all 5 other fully informative SSR loci (Supplementary tables 3 - 7, Supplementary figs. 1 - 5; ChSSR-134, ChSSR-157, ChSSR-164, ChSSR-213, and ChSSR-239) was found. Preliminary data on mapping, using simplex markers, showed that these in total 6 informative SSR loci map to three different chromosomes.

DISCUSSION

Genetic studies in cultivated chrysanthemum (2n = 6x = 54) are difficult due to its polyploidy (Dowrick, 1952), high level of heterozygosity (Huang et al., 2000) and self-incompatibility (Zagorski et al., 1983). Two hypotheses, allopolyploid and autopolyploid, have been proposed for chrysanthemum, however the question on inheritance mode remained uncertain as data were largely lacking or inconclusive (De Jong and Rademaker, 1986; Langton, 1989). Hexaploid chrysanthemums are generally thought to be derived from complex interspecific hybridizations among multiple chrysanthemum species (Ackerson, 1967; Anderson, 2006; Dowrick, 1953). The hexaploid genome of chrysanthemum might be derived from allopolyploidization based on their observations of pairing behavior during meiosis in several species (Dorwick, 1953; Li et al., 2011; Watanabe, 1977) and on the physical mapping results of 45S rDNA loci by FISH (Abd El-Twab and Kondo, 2003). However, these results are inconclusive and insufficient to determine that chrysanthemum will behave like an allopolyploid in meiosis. For instance, prevalent bivalent formation during meiosis were found in *Lotus corniculatus* (2n = 4x = 48) and *Tulipa chysantha* (2n = 4x = 48), and in *Phleum paratense* (2n = 6x = 42) which show tetrasomic and hexasomic inheritance, respectively (Darlington and Mather, 1949; Dawson, 1941; Nordenskiold, 1953). In addition, bivalents were normally formed in the autotetraploid and autodecaploid chrysanthemum induced by colchicine, suggesting that all homo(eo)logous chromosomes could replace each other at meiosis (Watanabe, 1983) and bivalent formation may be under genetic control

(Watanabe, 1977). Thus, this study was carried out to determine whether the inheritance mode of hexaploid chrysanthemum is disomic (selective chromosome pairing) or hexasomic (random chromosome pairing) through analyzing segregation ratios and patterns of multi-allelic and co-dominant EST-SSR markers in a pseudo-F_1 testcross population and extend on the suggestion of Klie et al. (2014) that chrysanthemum may be a segmental allopolyploid.

The segregation analysis for SSR marker alleles in 94 pseudo-F_1 progenies was more indicative to hexasomic inheritance although also signals for disomic inheritance were found as well. Excluding 115 of 180 polymorphic SSR marker alleles which were not affected by the type of inheritance, 33 marker alleles gave good fit to the expected segregation ratio for the hexasomic inheritance and only 6 marker allele were distorted at a level of $\alpha < 0.05$ while 24 gave good fit for the disomic inheritance and 7 were distorted. In addition, the proportion of non-simplex to simplex markers of 20.2% versus 79.8% supported an autopolyploid nature of chrysanthemum. Although the proportion of non-simplex to simplex markers and the observed segregations for EST-SSR alleles indicated hexasomic inheritance in chrysanthemum, they may not be conclusive. De Jong and Rademaker (1986) investigating segregations of the single dominant *Ph* gene for white rust resistance often found a better fit to the disomic inheritance in crosses between resistant cultivars. However, the cross between the resistant '80211' and the susceptible 'IVT 80058-1' gave an excellent fit to a ratio of 4:1 for duplex × nulliplex segregation in hexasomic inheritance. Jordan and Reimann-Philipp (1983) investigating segregation of dominant allele A for anthocyanin pigmentation also found a good fit to the hexasomic inheritance in the cross

between anthocyanin absent ‘Heyday’ and anthocyanin present ‘T72/17’, but another cross between ‘T72/17’ and anthocyanin absent ‘T71/181/16’ gave a good fit to a 7:1 ratio indicating triplex × nulliplex segregation with disomic inheritance. These conflicting results make it difficult to conclude on a general mode of inheritance in chrysanthemum. The reason why Klie et al. (2014) suggested chrysanthemum should be classified as segmental allohexaploid is also that mixed inheritance was shown in the genetic data of their chrysanthemum population. Whereas segregation data from dominant markers and the conclusions that are based upon marker frequency calculations from these data may suffer from a number of assumptions, the best assessment of inheritance mode can be obtained from linkage analysis and co-dominant marker segregation data. In both cases in Klie et al. (2014), these data (absence of repulsion phase markers and random pairing in 3 SSR markers) directed towards random pairing.

Similarly, the analyzed population in this study also showed more evidence for polysomic rather than disomic inheritance. Transmission patterns of alleles in fully informative co-dominant SSR loci gave clear evidence for random chromosome assortment and polysomic inheritance and as such linked this specific inheritance mode to the chromosomal regions where these markers reside. Given the sometimes contradicting results from previous studies segmental allopolyploidy cannot be ruled out for crosses with another genetic background or even for chromosomal segments not targeted with the current set of informative SSR markers in the population. Further studies are needed towards the extent to which preferential pairing may occur in chrysanthemum in different genetic backgrounds. Because the EST-SSR markers are single locus and based on genes

they can be expected to be easily transferable to other mapping populations to study the inheritance in a broader germplasm. However, to sample all chromosomal regions for detailed segregation analysis much higher marker densities are needed. For these single nucleotide polymorphism markers being co-dominant would be best suited as they can be easily be obtained in high throughput with the current sequencing and genotyping technologies. In combination with the set of SSR markers described in this study this would allow construction of a high density map and thus also enable to shed further light on the inheritance type of chrysanthemum genome wide.

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CHAPTER II

Identification of Genetic Resources Resistant to White Rust Caused by *Puccinia horiana* and Genetic Analysis of White Rust Resistance in Chrysanthemum

ABSTRACT

To identify genetic resources resistant to white rust disease caused by *Puccinia horiana*, a total of 179 commercial cultivars consisting of 27 standard and 153 spray chrysanthemums were inoculated with *P. horiana* isolates collected from Muan and Jeonju. Of those, 41 spray cultivars without visible disease symptoms were rated as resistant. Nine standard and twenty-eight spray cultivars, for which few but limitedly enlarged chlorotic lesions and pustules were found on leaves, were rated as moderately resistant. The remaining 18 standard and 83 spray cultivars were rated as susceptible with a high level of infection. Based on the results of screening white rust resistance, nine pseudo-F_1 testcross populations between susceptible cultivars, susceptible and resistant cultivars, and resistant cultivars were generated to analyze inheritance of the white rust resistance in hexaploid chrysanthemum. Two hundred and twenty-five F_1 progenies of two crosses between susceptible cultivars, 'Puma White' × 'Sei-Alps' and 'Puma White' × 'Chunilbeon', were assessed to be all susceptible to white rust. The cross between the resistant 'Bongan' and the susceptible 'Chunilbeon' segregated

in an 1:1 ratio for resistance (R) and susceptibility (S). The cross between resistant cultivars, ‘Kokka Akafuji’ and ‘Bongan’ segregated in a 3:1 ratio for R:S, suggesting that white rust resistance in hexaploid chrysanthemum was controlled by a single dominant gene. The crosses between the resistant ‘Dancer’ and susceptible cultivars, ‘Puma White’, ‘Sei-Alps’ and ‘Universe’, segregated in a 4:1 ratio. In addition, the crosses between the resistant ‘Dancer’ and resistant cultivars, ‘Bongan’ and ‘Biaritz’, segregated in a 9:1 ratio. These segregations indicated that ‘Dancer’ carries two dominant alleles coding for white rust resistance, thus is identified as a duplex. Based on the χ^2 goodness of fit test (α = 0.05) to the expected segregation ratios of preferential pairing and random pairing, respectively, the segregation for white rust resistance in hexaploid chrysanthemum often fit the random chromosome pairing hypothesis (hexasomic inheritance), rather than preferential chromosome pairing hypothesis (disomic inheritance).

Keywords: disease reaction, pathogenicity, pseudo-F_1 testcross, resistant breeding, segregation ratio

INTRODUCTION

Chrysanthemum (*Dendranthema* × *grandiflorum* (Ramat.) Kitamura), mainly used as cut flower or potted plant, is one of the most important floricultural crops in the world. It is cultivated on an area of more than 20,000 ha, and more than 5 billion stems and pots are marketed annually in EU and Asian flower auctions (AIPH, 2013). In Korea, chrysanthemum is also economically important ornamental crop after rose and one of Korea's main exported floricultural crops. The value of exports, which totaled only 2.1 million dollars in 1999 when the export of the cut flowers of chrysanthemum to Japan began in earnest, hit 10 million dollars in 2010 (MAFRA, 2014). Recently, however, as plant quarantine in Japan has been strengthened, chrysanthemum exports have been threatened because sufficient quantities to sustain the trade have declined due to chrysanthemum white rust disease.

Chrysanthemum white rust, caused by *P. horiana* Henn., is the most destructive fungal disease of chrysanthemum in Korea (Park and Kim, 1993). Infection mainly starts at the upper surface of the leaf and causes pale green to yellow spots (up to 4 mm in diameter) and then forms raised buff or pinkish pustules on the lower surface of the leaf (Baker, 1967). Additionally, in extensively infected plants, infection can occur even in the stems, bracts, flower buds, and florets (Dickens, 1970). To prevent damages and economic losses, fungicides are regularly sprayed on chrysanthemum plantations before the plants show symptoms of the disease (Dickens, 1990; Stapel and Guerrand, 2012).

Several cultural methods also have been proposed to control white rust disease

in chrysanthemums. Heat treatment of infected plants at 37-40ºC for 20 h or the dipping of cuttings in water at 45ºC for 5 min have been reported to reduce chlorosis and sporulation (Zadoks et al., 1968). In addition, the sporidia have been reported to be sensitive to desiccation at 90% relative humidity (RH) that an exposure to 80% RH for 5 min or 90% RH for 1 h eradicates 100% of the sporidia (Firman and Martin, 1968). However, these methods are difficult to be practically, because heat treatments can cause the death of terminal buds or whole plants and the malformation of plants. Furthermore, control over RH in large greenhouses is also costly. Therefore, the use of resistant cultivars could be one of the most efficient approaches for controlling white rust disease in chrysanthemum cultivation.

Differences in disease resistance against *P. horiana* among chrysanthemum species and cultivars were reported up until the 1980s. Dickens (1968) investigated the susceptibility of various chrysanthemum species, and Martin and Firman (1970) evaluated the level of resistance in 270 chrysanthemum cultivars. Yamaguchi (1981) also screened 250 chrysanthemum cultivars for resistance to white rust. In addition, De Jong and Rademaker (1986) described four types of reactions of the chrysanthemum cultivars to *P. horiana*. They suggested that a single dominant gene controls resistance in hexaploid chrysanthemums based on the segregations for resistance. In most recent, Zeng et al. (2013) reported the variation for resistance to white rust in chrysanthemum species. Using these resistance resources, many commercial cultivars are being intensively bred worldwide. Despite of many breeding programs for white rust resistance, only a few genetic studies have been done due to several complicating factors such as its

polyploidy (Dowrick, 1952), high level of heterozygosity (Huang et al., 2000), and self-incompatibility (Zagorski et al., 1983). In addition, highly complex pathosystem such as the differential interactions between *P. horiana* isolates and chrysanthemums (De Backer, 2012; De Backer et al., 2011; Yamaguchi, 1981), and genetic and physiological diversity of *P. horiana* (Alaei et al., 2009; Dickens, 1971; Martin and Firman, 1970) make genetic studies difficult because multi-genes might be involved in host and parasite interaction.

For exploiting genetically-determined resistance in hexaploid chrysanthemum, the genetic basis of the resistance should be understood. This study is intended to identify genetic resources, to analyze the genetic inheritance of white rust resistance using several crosses between resistant and susceptible cultivars, and to utilize it in breeding program and marker development for white rust resistance in chrysanthemum.

MATERIALS AND METHODS

Plant materials

A total of 179 commercial cultivars of 27 standard and 153 spray chrysanthemums from the National Institute of Horticultural and Herbal Science (NIHHS), Suwon, Korea were used to screen for resistance against white rust. Crosses were made between susceptible cultivars, susceptible and resistant cultivars, and resistant cultivars. Cuttings (5 cm in length) with one fully expanded leaf were harvested from each progeny line and rooted in a 128-plug tray containing a mixture of peat base compost (Sunshine Mix No. 4, SunGro Horticulture, Bellevue, WA, USA). Then, the rooted cuttings were planted in distance of 10 × 10 cm in a experimental greenhouse of NIHHS. The greenhouse was maintained with temperatures ranging from 18-25°C and 12-18°C for day and night, respectively.

Inoculations

In preliminary experiments, different inoculation methods such as placing diseased plants with sporulating lesions among the plants (non-quantitative inoculation) and spraying spore suspension over the plants (semi-quantitative inoculation) were tested (De Backer et al., 2011; De Jong and Rademaker, 1986; Takatsu et al., 2000; Yamaguchi, 1981; Zandvoort et al., 1968). According to the results, non-quantitative inoculation method was easier and more stable, although these two methods were effective to screen the disease resistance against *P. horiana* in chrysanthemum.

To ensure reproducibility, the resistance was screened three times from 2009 to 2011. Six plants from each accession were evaluated in each screening and 'Dancer' and 'Puma White' were used as the resistant and the recessive control, respectively. *P. horiana* isolates were collected from Muan in 2009 and Jeonju in 2010 to 2011, and inoculated in each year when those isolates were collected. In a month after planting, non-quantitative inoculation was performed at different growing stages of chrysanthemum by placing heavily infected plants with distances of 40 cm among the test plants (Fig. II-1A, B, C). For 1 week after inoculation, all test plants were sprayed with water and covered with polyethylene plastic film in the evening to maintain RH close to 100% (Fig. II-1D). The night temperature was maintained at > 15ºC during inoculation (Firman and Martin, 1968).

Evaluation of resistance

The test plants were scored at 3 to 5 weeks after inoculation. On the basis of the development and progression of pustules in the most severely infected leaves, the disease index was divided into six levels: 0, no visible symptoms; 1, < 5 developed pustules but sporulated limitedly; 2, < 20 developed pustules and sporulated slowly; 3, < 50 developed pustules; 4, < 100 developed pustules; and 5, > 100 developed pustules. The resistance levels were determined according to the average disease index as follows: 0.0, no visible symptom, was rated as resistant (R); 0.0-1.0, a few pustules developed slowly and sporulated limitedly, as moderately resistant (MR); 2.0-5.0, many pustules developed quickly and sporulated abundantly, as susceptible (S).

Fig. II-1. Disease inoculation on chrysanthemum in 32-plug tray (A), pot (B), and field (C). All inoculated plants were covered with plastic film to maintain relative humidity close to 100% (D).

Data anaysis

The inheritance of resistance was determined by analyzing the phenotypic segregation ratio in the pseudo-F_1 progenies. Expected ratios for a proposed inheritance models, random pairing (hexasomic inheritance) and preferential pairing (disomic inheritance) were confirmed using χ^2 goodness-of-fit test (α = 0.05) (Table II-1).

Table II-1. Expected segregation ratios (resistance:susceptibility) for a single locus dominant gene in hexaploid chrysanthemum, assuming that bivalents are formed at meiosis with either random (hexasomic) or preferential (disomic) pairing.

Cross combination		Expected segregation ratio (resistance:susceptible)	
		Random pairing (hexasomic)	Preferential pairing (disomic)
Susceptible × Susceptible	Nulliplex × Nulliplex	0:1	0:1
Resistance × Susceptible	Simplex × Nulliplex	1:1	1:1
	Duplex × Nulliplex	4:1	3:1*
	Triplex × Nulliplex	19:1	7:1*
Resistance × Resistance	Simplex × Simplex	3:1	3:1
	Duplex × Simplex	9:1	7:1*
	Triplex × Simplex	39:1	15:1*
	Duplex × Duplex	24:1	15:1*
	Triplex × Duplex	99:1	31:1*
	Triplex × Triplex	399:1	63:1*

*With non-paring dominant alleles, otherwise expected segregation ratio would be 1:0.

RESULTS

Identification of chrysanthemums resistant to white rust

A total of 179 commercial cultivars, consisting of 27 standard and 152 spray types of chrysanthemum, were evaluated for their levels of resistance to white rust. Of the 27 standard chrysanthemums, there was no resistant cultivar. Although ‘Kokka Akafuji’, ‘Otomezakura’, ‘Otomezakura Orange’, ‘Otomezakura Pink’, and ‘Suishin’ which were the most resistant to white rust sometimes showed free of infection, slight necrotic flecks or small pustules were visible on the leaves. ‘Seikonomakoto’, ‘Seikonomakoto III’, and ‘Seinonami’ showed a few chlorotic lesions and pustules on the leaves, but those were developed slowly and sporulated limitedly. Therefore, these nine cultivars were evaluated as moderately resistant. However, the other 17 cultivars including ‘Baekma’, ‘Bayakko’, ‘Iwanohakusen’, and ‘Jinba’ which are widely cultivated in Korea were evaluated as susceptible with a very high level of infection. Many pustules developed on the leaves very quickly and sporulated abundantly (Table II-2, Supplementary table 2).

Compared to the standard type, the levels of resistance appeared to be higher in the spray type. Among the 152 spray chrysanthemums, 41 cultivars including ‘Akira Kazaguruma’, ‘Biarittz Yellow, ‘Bongan’, ‘Kingfisher’, ‘Dancer’, and ‘Quinty’ were evaluated as resistant to white rust and were completely free of infection. Additionally, 28 cultivars, such as ‘Antigua’, ‘Bacardi’, ‘Husky’, ‘Panama’, and ‘Sei Soul Yellow Red’, exhibited low levels of infection to white rust and were evaluated as moderately resistant. The remaining 83 cultivars, such as ‘Chopin’, ‘Feeling Green’, ‘Hunt’, ‘Puma White’, ‘Sei Alps’, and ‘Universe’

Table II-2. Classification of 27 standard and 153 spray chrysanthemum cultivars based on resistance level against white rust caused by *P. horiana*.

Type	Disease response[z]	No. of cultivars	Cultivar
Standard	MR	9	Kokka Akafuji, Otomezakura, Otomezakura Orange, Otomezakura Pink, Otomezakura Yellow, Suishin, Seikonomakoto, Seikonomakoto III, Seinonami
	S	18	Baekma, Byakko, Iwanohakusen, Jinba, Kiranouma, Kokka Shunko, Seifu, Seiko no aki, Seikokaike, Seikoshinnen, Seikoumangetu, Seinoisse, Seinokoto, Seinokyoku, Seiun, Shuhonochikara White, Shuhonochikara Yellow, Yongma
Spray	R	41	Akira Kazaguruma, Albert heijn, Annecy, Ansella, Bacchus, Biarittz Yellow, Crocodile, Dalma, Dancer, Dark Westland, Dutchy, Eunhasu, Flush, Hambaek, Handsome, Jeanny, Jerry, Kingfisher, Kingfisher Cherry, Lexy Red, Marikazaguruma, Melody, Merida Splendid, Morning, Pink Elisa, Quinty, Quinty Pink, Quinty Red, Recharles, Refondo, Rodis White, Rodis Yellow, Roma, Sei Amelie, Sei Energy, Sei Falcao, Sei Mini, Sei Sanp, Sei Soul, Taiyo no Koigokoro, Tobago
	MR	28	Antigua, Arctic Queen, Bacardi, Bennie Jolink, Biarittz Pink, Bongan, Cassa, Champy, Fly Catcher, Gama, Gibaeg, Hebo, Husky, Hyangro, Ilweol, Marabou, Panama, Penny Lane, Piaget, Ping Pong White, Ping Pong Yellow, Sei-Rosa, Sei-Soul Yellow Red, Vesuvio, Vesuvio Yellow, White King, White Marble, Winia

Table II-2. (continued)

Type	Disease response[z]	No. of cultivars	Cultivar
	S	83	Accent, Anastasia, Anastasia Green, Ardilo, Argus, Art Yellow, Artist Yellow, Bijux, Boris Becker Yellow, Bradford, Buffy, Charming Eye, Chopin Dark Pink, Chopin Orange Pink, Chopin White, Chunilbeon, Coral Marble, Cosmos King, Creado, Dash, Deliah Cream, Delilah Yellow, Deliwind Yellow, Dinar, Euro White, Euro Yellow, Feeling Green, Ford, Froggy, Garcia, Golden Kent, Golden PangPang, Green brid, Hunt, Ibis Lime, Ibis Sunny, Inga, Kinkazaguruma, Kumsu, Lineker Salmon, Marscort, Mona Lisa, Mona Lisa Pink, Mona Lisa Splendid, Mona Lisa White, Mona Lisa Yellow, Moonlight, Namba, Nice, Noa, Noeul, Paco, Patra, Peace Green, Pelican, Pink PangPang, Plaisir d'Amour, Puma Sunny, Puma White, Puma Yellow, Pure Angel, Relance, Salinas, Samos, Sei Agness, Sei Alps, Sei Elza, Sei Mariah, Sei Monaco, Sei Night, Sobaek, Stailion, Swan, Text, Tokyo, Topic, Tumaco, Universe, Weldon Dark, Wembley, Whitney PangPang, Zembla, Zembla Lime

[z]Disease reponses were determined according to the average disease index as follows: 0.0 was rated as resistant (R); 0.0-1.0 as moderately resistant (MR); 2.0-5.0 as susceptible (S).

which were easily infected with white rust, were rated as susceptible. The infected leaves of these cultivars were quickly covered with numerous pustules. Moreover, in extensively infected cultivars, pustules formed even on the stems, bracts, and flower buds (Table II-2, Supplemtary table 2).

Genetic analysis of white rust resistance

For genetic analysis of white rust resistance, the hexaploid chrysanthemums 'Dancer', 'Bongan', 'Biaritz', and 'Kokka Akafuji' which were resistant to white rust and 'Puma White', 'Sei-Alps', 'Chunilbeon', and 'Universe' which were susceptible to white rust were selected as parents for crossing. A total of nine crosses were made between susceptible cultivars, susceptible and resistant cultivars, and resistant cultivars.

In progenies derived from the crosses among these parental cultivars, the disease reactions were relatively well discriminated between resistance and suscpetible (Fig. II-2). Two hundred and twenty-five F_1 progenies of two crossing populations between susceptible cultivars, 'Puma White' × 'Sei-Alps' and 'Puma White' × 'Chunilbeon', were shown high level of infection and assessed to be all susceptible to white rust. A number of chlorotic lesions and pustules developed and covered on the leaves with very quickly. With 40 resistant versus 39 susceptible plants, the population derived from the cross between the resistant 'Bongan' and the susceptible 'Chunilbeon' displayed a 1:1 segregation ($P = 0.910$, $\alpha = 0.05$) for resistance (R) and susceptibility (S), indicating that white rust resistance in the hexaploid chrysanthemums was controlled by a single dominant gene.

Fig. II-2. Difference of disease reactions between resistant and susceptible lines of the pseudo-F_1 testcross population, ‘Puma White’ × ‘Dancer’.

The crosses of the resistant ‘Dancer’ with susceptible cultivars, ‘Puma White’, ‘Sei-Alps’, and ‘Universe’, segregated in a 3:1 or 4:1 ratio for R:S. The 3:1 or 4:1 segregation of these crosses indicated that the resistant cultivar ‘Dancer’ carried two dominant alleles coding for white rust resistance. The crosses between the resistant cultivars, ‘Dancer’ × ‘Biaritz’ and ‘Dancer’ × ‘Bongan’, segregated in a 7:1 or 9:1 ratio for R:S. And the cross between ‘Kokka Akafuji’ and ‘Bongan’ segregated in a 3:1 ratio for R:S. These segregations confirmed that ‘Dancer’ carried a duplex type of resistance and ‘Kokka Akafuji’ and ‘Bongan’ carried a simplex type of resistance (Table II-3).

Table II-3. F_1 progeny segregation for reaction to white rust caused by *P. horiana* in crosses between susceptible cultivars, resistant and susceptible cultivars, and resistant cultivars in 2010 ($\alpha = 0.05$).

Cross combination	No. of progenies			Ratios expected in progenies			
	Total	R	S	Selective paring		Random paring	
				R:S	*P*	R:S	*P*
Susceptible × Susceptible							
Puma White × Sei-Alps	122	0	122	0:1	1.000	0:1	1.000
Puma White × Chunilbeon	103	0	103	0:1	1.000	0:1	1.000
Susceptible × Resistant							
Puma White × Dancer	57	46	11	3:1	0.320	4:1	0.895
Universe × Dancer	54	44	10	3:1	0.271	4:1	0.786
Resistant × Susceptible							
Dancer × Sei-Alps	96	72	24	3:1	1.000	4:1	0.221
Bongan × Chunilbeon	79	40	39	1:1	0.910	1:1	0.910
Resistant × Resistant							
Bongan × Dancer	36	33	3	7:1	0.450	9:1	0.739
Biaritz × Dancer	14	13	1	7:1	0.545	9:1	0.722
Kokka Akafuji × Bongan	44	35	9	3:1	0.486	3:1	0.486

DISCUSSION

Chrysanthemum white rust, caused by *P. horiana* Henn., is the most destructive fungal disease in chrysanthemum. In spite of serious damages and economic losses, white rust disease is difficult to prevent because the curtain for shortday treatment of summer cultivation make environmental conditions inside greenhouse suitable for disease infection (Firman and Martin, 1968). For white rust resistance breeding, until recently, the variation for resistance to white rust in various *Chrysanthemum* species and the differential interactions between *P. horiana* isolates and *Chrysanthemum* species have been investigated. However, so far, there is no report on the genetic resources resistant to domestic isolates of *P. horiana*. In this study, white rust resistance of wild chrysanthemum species and commercial cultivars against domestic isolates were identified.

Among the 179 chrysanthemum cultivars tested in these experiments, 41 spray types including ‘Akira Kazaguruma’, ‘Biarittz Yellow, ‘Bongan’, ‘Kingfisher’, ‘Dancer’, and ‘Quinty’ were identified as resistant. And nine standards including ‘Kokka Akafuji’, ‘Otomezakura’, ‘Otomezakura Orange’, ‘Otomezakura Pink’, and ‘Suishin’ and 28 sprays including ‘Antigua’, ‘Bacardi’, ‘Husky’, ‘Panama’, and ‘Sei Soul’ were evaluated as moderately resistant. This high frequency of resistance to white rust indicated that the resistance, reported to be controlled by a single dominant gene, might have been stably inherited by the next generation (De Jong and Rademaker, 1986). Therefore, these genetic resources are expected to be helpful for crossbreeding programs to breed new cultivars resistant to white rust disease in chrysanthemum.

However, differential interactions between *P. horiana* isolates and chrysanthemum cultivars, which have been proposed recently, indicate that more than two types of white rust resistance gene might exist in chrysanthemums (De backer, 2012; De Backer et al., 2011; Yamaguchi, 1981). In this study two kinds of isolates collected from Muan and Jeonju were inoculated, but pathogenicity or virulence did not show any significant difference between them (Supplementary table 2). Thus, to identify the diversity in pathogenicity various isolates of *P. horiana* need to be collected from different location based on main production area and inoculated in a subset of chrysanthemum cultivars chosen based on the results of these experiments. These data are used to identify resistance genes against different pathotypes and pyramid those genes for improving white rust resistance in chrysanthemums.

The overall genetic segregations provided good evidence that a single dominant gene controlled white rust resistance in hexaploid chrysanthemums. De Jong and Rademaker (1986) also proposed that a single gene, symbolized *Ph*, was responsible for white rust resistance although deviation from the expected segregation could be explained by the existence of other mechanisms combined with monogenic resistance. Yamaguchi (1981) also suggested the existence of one major resistance gene through analyzing variation in white rust resistance in F_1 hybrids among chrysanthemum cultivars with various level of resistance.

In the crosses using the resistant cultivars ‘Dancer’ and ‘Bongan’, no maternal effect was found in white rust resistance, but correlation between the level of resistance was observed though both resistance and moderate resistance were inherited by a single dominant gene. This was corresponded with the

reports that no difference was in white rust resistance between reciprocal crosses (Yamaguchi, 1981).

Based on the χ^2 goodness of fit test ($\alpha = 0.05$) to the expected segregation ratios of selective pairing and random pairing, respectively, the segregation for white rust resistance in chrysanthemum often fit the random pairing hypothesis (Table II-3). This was corresponded with the results of Chapter I.

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CHAPTER III

Identification of Molecular Markers Linked to White Rust Resistance in Chrysanthemum Using Bulked Segregant Analysis and Conversion into a Sequence Characterized Amplified Region Marker

ABSTRACT

Genetic anaysis of white rust resistance in chrysanthemum was studied in 188 pseudo-F_1 progenies derived from the cross between the susceptible parent 'Puma White' and the resistant parent 'Dancer'. The segregation ratio based on disease response was fitted to a ratio of 4:1 (resistance to susceptibility). Based on this phenotypic data, developing molecular markers linked to white rust resistance was carried out using random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) via bulked segregant analysis (BSA). 46 polymorphic bands in BSA-RAPD and 10 polymorphic bands in BSA-AFLP were detected between the two bulked-DNA samples. Of them, primer OPI-13_{520} produce a polymorphic band in the individuals of resistant bulk but not in the individuals of susceptible bulk, suggesting a linkage of coupling phase. Using primer OPI-13_{520}, genotyping of 188 pseudo-F_1 testcross progenies was performed, then genotypes of just six off-springs did not correspond with phenotypes. The value of recombination fraction obtained by

successive trials and second derivative of log likelihood was 0.0383 ± 0.0271 and the genetic distance between white rust resistance gene and OPI-13_{520} marker determined to be 4.0 cM. Based on the DNA sequence of OPI-13_{520}, OPI-13_{520} marker was converted into sequence characterized amplified region (SCAR) marker and verified in pseudo-F_1 testcross progenies derived from 'Puma White' × 'Dancer'. The results showed SCAR marker could be used in breeding programs for selection of white rust resistant plants in chrysanthemum breeding.

Keywords: breeding, genetic distance, linkage map, molecular marker, recombination fraction

INTRODUCTION

Chrysanthemum white rust, caused by *Puccinia horiana* Henn., is one of the most destructive diseases in the production of chrysanthemum through the world. Under humid conditions in greenhouse it spreads rapidly, resulting in considerable economic losses each year (Dickens, 1990; Firman and Martin, 1968). Infection causes pale green to yellow spots up to 4 mm diameter on the adaxial leaf surface, then forms raised buff or pinkish pustules on the abaxial leaf surface (Baker, 1967). In heavily infected plants, infection can occur even in the stems, bracts, flower buds, and florets depending on susceptibility of cultivars (Dickens, 1970). For reducing damages and economic losses, fungicides are regularly sprayed on chrysanthemum plantations even though symptoms are not shown because eradicating pathogens is difficult once the plants are infected (Dickens, 1990; Stapel and Guerrand, 2012). However, considering the cost and the environmental concerns over fungicide applications, genetic resistance to white rust could be one of the most efficient approaches for controlling white rust disease in chrysanthemum cultivation (Park et al., 2014).

Till now, a lot of resistance sources to white rust have been identified in chrysanthemum species and cultivars (Dickens, 1968; Martin and Firman, 1970; Yamaguchi, 1981; Zeng et al., 2013; Park et al., 2014). In addition, white rust resistance is controlled by a single dominant gene based on the segregations in hexaploid chrysanthemums for resistance (De Jong and Rademaker, 1986). Despite much efforts of chrysanthemum breeding for resistance to white rust, several complicating factors including the genome complexity, various levels of

polyploidy, high levels of heterozygosity, and the occurrence of both inbreeding depression and self-incompatibility make exploiting genetically-determined resistance difficult (Anderson and Ascher, 2000; Anderson et al., 1992; Dowrick, 1952; Huang et al., 2000; Zagorski et al., 1983). Molecular markers tightly linked to the resistance gene can be used for overcoming these obstacles, as has already been demonstrated in several crop species (Ukoskit and Thompson, 1997; Malek et al., 2000; Heusden et al., 2002).

Chrysanthemums still lag far behind many other selfing-crops in using molecular markers. Nevertheless, molecular markers have been used in chrysanthemums for a wide range of purposes such as detecting genetic diversity of chrysanthemums (Wolff and Peters-van Rijn, 1993), estimating genetic relationships (Dia et al., 1998; Zhou and Dai, 2002; Miao et al., 2007), identifying cultivars or species (Wolff et al., 1994), distinguishing bud sports derived vegetatively from a elite cultivar (Wolff et al., 1995), and validating hybridity (Lema-Ruminska et al., 2004). The preliminary genetic linkage map of the hexaploid chrysanthemum was also constructed with a combination of ramdom amplified polymorphic DNA (RAPD), inter-simple sequence repeat, and amplified fragment length polymorphism (AFLP) markers (Zhang et al., 2010). A marker-trait association analysis of flowering traits, initial blooming time and the duration of flowering were carried out using sequence-related amplified polymorphism markers (Zhang et al., 2011a, b). And single-locus quantitative trait loci (QTL) with additive effect and epistatic QTL associated with plant architectural trait in chrysanthemum were detected and mapped (Peng et al., 2015; Zhang et al., 2012).

So far, however, there is no information on developing molecular markers for white rust resistance in chrysanthemum. Therefore, this study was carried out to develop molecular markers linked to white rust resistance gene using bulked segregant analysis (BSA) with RAPD and AFLP marker. Then, for improving the specifity and better assessing segregation of markers linked to white rust resistance, the less-specific RAPD and AFLP markers converted into highly specific sequence-characterised amplified region (SCAR) marekrs (Paran and Michelmore, 1993). These experiments is expected to improve breeding efficiency based on marker assisted selection for white rust resistance in chrysanthemum.

MATERIALS AND METHODS

Plant material and DNA extraction

From a pseudo-F_1 testcross population, resulting from a cross between two hexaploid chrysanthemum cultivars, 'Puma White' as maternal and 'Dancer' as paternal parent, 188 progenies were used for developing molecular marker linked to white rust resitance. The direction of the cross was chosen because 'Puma White' has anemone type flowers that hardly produce pollen and thus the chance of selfing was minimized ('Dancer' has single flowers) (Fig. III-1). Total genomic DNA was extracted from young leaf tissue using the DNeasy® plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction with a minor modification in adding 2% polyvinylpyrrolidone (Sigma-Aldrich, St Louis, MO, USA) to the lysis buffer. The quality and quantity of the DNA was determined by using a NanoVue™ spectrophotometer (GE Healthcare Bioscience, Pittsburgh, PA, USA) and by visual inspection after electrophoresis on a 1% agarose gel.

Inoculation assay and disease assessment

P. horiana isolates were collected from Jeonju in 2010 to 2012, and inoculated in each year as those isolates were collected. In a month after planting, non-quantitative inoculation was performed in different growing stages of chrysanthemum by placing heavily infected plants with distances of 40 cm among the test plants tested (De Jong and Rademaker, 1986; Zandvoort et al., 1968). For 1 week after inoculation, all the test plants were sprayed with water

and covered with polyethylene plastic film in the evening to maintain a relative humidity close to 100%. And the night temperature was maintained at > 15ºC during inoculation (Firman and Martin, 1968). The test plants were scored 3 to 5 weeks after inoculation. On the basis of the development and progression of pustules in the most severely infected leaves, the disease index was divided into six levels: 0, no visible symptoms; 1, < 5 developed pustules but sporulated limitedly; 2, < 20 developed pustules and sporulated slowly; 3, < 50 developed pustules; 4, < 100 developed pustules; and 5, > 100 developed pustules. The resistance levels were determined according to the average disease index as follows: 0.0 was rated as resistant (R); 0.0-1.0 as moderately resistant (MR); 2.0-5.0 as susceptible (S).

Bulked segregant analysis

Based on the results of screening white rust resistance, the ten most resistant and ten most susceptible progeny lines were selected among 188 progenies of 'Puma White' × 'Dancer'. Then, the resistant and susceptible bulked segregants were generated by combining equal amounts of the respective DNA samples. These two bulked segregants and two parental cultivars, 'Dancer' and 'Puma White', were used for screening primers.

Random amplified polymorphic DNA assay

For BSA with RAPD markers, 280 decamer primers (Operon Technologies, Alameda, CA, USA) were screened for polymorphism among 'Dancer', 'Puma White', and two sets of bulks. PCR amplification were carried out in a total

Fig. III-1. Characteristics of parental lines. (A), the flower of ‘Dancer’; (B), the flower of ‘Puma White’; (C), disease resistance of ‘Dancer’ and ‘Puma White’; (D), disease reaction of ‘Dancer’; (E) disease reaction of ‘Puma White’.

volume of 20 μL containing 2 μL of template DNA (10 ng/μL), 1.0 μL of each primer (10 pmol/μL), 2.0 μL of 10× PCR buffer, 2.0 μL of dNTP (2 mM) and 0.1 μL DreamTaq-polymerase (5 U/μL) (Fermentas, Vilnius, Lithuania). PCR reactions were run as follows: initial denaturation at 95°C for 5 min, then 25 cycles each of 95°C for 30 s, 37°C for 30 s and 72°C for 1 min, and a final extension at 72°C for 10 min (Veriti™ Thermal Cycler, Applied Biosystems, Foster, CA, USA). PCR amplification products were electrophoresed in a 1.0% agarose gel, stained with GelRed™ and photographed under UV light.

Amplified fragment length polymorphism assay

The AFLP analysis was performed as described by Vos et al. (1995) previously with minor modifications. For each sample, 500 ng of genomic DNA was digested with 5 units of *Eco*RI and 4 units of *Mse*I (both NEB) at 37°C for 3 h. After verifying complete digestion on 1.0% agarose gel, *Eco*RI and *Mse*I adaptors (0.1 mM and 1 mM, respectively) were ligated at 37°C for 3 h using 1 unit of T4 DNA ligase and 1× ligation buffer. The pre-amplification was performed with specific primers that had A and C as a pre-selective base at the 3' end of *Eco*RI and *Mse*I adaptors, respectively (Table III-1). Pre-amplification were carried out as follows: initial denaturation at 95°C for 5 min, then 25 cycles each of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min, and a final extension at 72°C for 10 min. Then, selective amplification was performed with *Eco*RI and *Mse*I primers with two extra selective bases at 3' end (Table III-1). The selective amplification was carried out as follows: initial denaturation at 95°C for 5 min, then 8 cycles each of 95°C for 30 s, 65 to 56°C for 30 s (ramp 1°C/cycle)

Table III-1. Restriction enzymes, adaptors, and primer sequences used for AFLP analysis.

Adapter/primer	Sequence
*Eco*RI adapter	5‘-CTCGTAGACTGCGTACC-3' 3'-CATCTGACGCATGGTTAA-5'
*Eco*RI + 1 primer	5'--AGACTGCGTACCAATTC-A-3'
*Eco*RI + 3 primer	5'--AGACTGCGTACCAATTC-ANN-3'
*Mse*I adapter	5‘-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'
*Mse*I + 1 primer	5'-GATGAGTCCTGAGTAA-C-3'
*Mse*I + 3 primer	5'-GATGAGTCCTGAGTAA-CNN-3'

and 72°C for 1 min, 24 cycles each of 95°C for 30 s, 56°C for 30 s and 72°C for 1 min, and a final extension at 72°C for 10 min. The amplified products were electrophoresed on a 6% polyacrylamide gel and visualized by silver staining for amplicon detection.

Cloning and sequencing of the DNA fragment

The DNA band expected to be linked to the white rust resistance gene was extracted from agarose gel and purified using the QIAquick gel extraction kit® and QIAquick PCR purification kit® (Quiagen, Hilden, Germany) according to the manufacturer's instruction. The purified DNA was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) according to the manufacturer's instruction. The cloned fragments were transformed to One Shot® TOP 10 competent cell (Invitrogen, Burlington, Ontario, Canada), using the heat shock transformation method (Sambrook et al., 1989). The transformed cells were plated on LB medium containing ampicillin (0.1 mg/mL), IPTG (200 mg/mL) and X-GAL (20 mg/mL) and incubated at 37°C for 12 h. Colonies containing recombinant plasmids were identified by white color and were transferred to tubes containing 3 ml of LB medium with ampicillin (0.1 mg/mL) and incubated at 37°C for 12 h, under constant agitation (250 rpm). To confirm successful transformation, plasmid DNA was amplified, using primers M13F and M13R. The nucleotide sequence of the insert was determined in a automated sequencer ABI 3730 DNA Analyzer (Applied Biosystems, Foster, CA, USA) using the BigDye Terminator Cyclic Sequencing.

Data analysis

Expected ratio for proposed genetic model and test of linkage were confirmed using a χ^2 goodness-of-fit test. The recombination fraction (r) was calculated by the maximum likelihood method (Mather, 1964).

The likelihood equation is shown as follows:

$$\theta = (n!/a_1!a_2! \ldots a_j!) \times (m_1)^{a1} (m_2)^{a2} \ldots (m_j)^{aj}$$

where θ = likelihood function, a_j = observed number, m_j = expected number, and $n = a_1+a_2+ \ldots +a_j$

RESULTS

Segregation for white rust resistance

As described in Chapter II, the 57 progenies of 'Puma White' × 'Dancer' displayed a clear ratio of 4:1 (P = 0.8946, α = 0.05) with 46 resistant versus 11 susceptible individuals in 2010, suggesting white rust resistance was controlled by a single dominant gene under the assumption of hexasomic inheritanc mode (Table II-3 in Chapter II). To ensure reproducibility, the number of progeny lines was increased up to 188 and screenings of resistance were conducted two times more in 2011 and 2012. The white rust disease responses of 188 progeny lines were shown in Table III-2. The disease response was clear and uniform with in the progeny lines of 'Puma White' × 'Dancer'. The 188 progeny lines displayed a ratio of 4:1 (P = 0.0533, α = 0.05) with 161 resistant versus 27 susceptible individuals whereas a ratio of 3:1 under the assumption of disomic inheritance was distorted at $\alpha < 0.05$ (Table III-3). This indicated that 'Dancer' have a duplex type of resistance and chrysanthemum could be a autohexaploidy plant associated with random chromosome pairing.

BSA with RAPD and AFLP markers

To identify molecular markers closely linked to the white rust resistance gene, a bulked segregant analysis was performed. Based on the result of the resistance screening in 188 pseudo-F_1 testcross progeny lines, the ten most resistant progenies (PD6, PD31, PD45, PD51, PD76, PD79, PD148, PD160, PD172, and PD184) and the ten most susceptible (PD18, PD42, PD66, PD67, PD75, PD120,

Table III-2. White rust disease reactions of 188 progenies derived from 'Puma White' × 'Dancer'.

Line	Inoculation[z]			Line	Inoculation			Line	Inoculation			Line	Inoculation		
	1st	2nd	3rd		1st	2nd	3rd		1st	2nd	3rd		1st	2nd	3rd
Dancer	R	R	R	PD48	R	R	R	PD96	R	R	R	PD145	-	R	R
Puma	S	S	S	PD49	R	R	R	PD97	R	R	R	PD146	-	R	R
PD1	R	R	R	PD50	R	R	R	PD98	R	R	R	PD147	-	S	S
PD2	-	R	R	PD51	-	R	R	PD99	R	R	R	PD148	-	R	R
PD3	S	S	S	PD52	-	R	R	PD100	R	R	R	PD149	-	R	R
PD4	R	R	R	PD53	-	S	S	PD101	R	R	R	PD150	-	R	R
PD5	R	R	R	PD54	R	R	R	PD102	S	S	S	PD151	-	R	R
PD6	R	R	R	PD55	-	R	R	PD103	R	R	R	PD153	-	R	R
PD7	R	R	R	PD56	-	R	R	PD104	S	S	S	PD154	-	R	R
PD8	R	R	R	PD57	-	R	R	PD105	R	R	R	PD155	-	R	R
PD9	R	R	R	PD58	-	R	R	PD106	R	R	R	PD156	-	R	R
PD10	R	R	R	PD59	-	S	S	PD107	R	R	R	PD157	-	R	R
PD11	R	R	R	PD60	-	R	R	PD108	-	R	R	PD158	-	R	R
PD12	R	R	R	PD61	-	R	R	PD109	S	S	S	PD159	-	R	R
PD13	R	R	R	PD62	-	R	R	PD110	R	R	R	PD160	-	R	R
PD14	R	R	R	PD63	R	R	R	PD111	-	R	R	PD161	-	R	R
PD15	-	R	R	PD64	R	R	R	PD112	-	S	S	PD162	-	R	R
PD16	R	R	R	PD65	R	R	R	PD113	-	R	R	PD163	-	R	R
PD17	-	R	R	PD66	S	S	S	PD114	R	R	R	PD164	-	R	R
PD18	S	S	S	PD67	S	S	S	PD115	R	R	R	PD165	-	R	R
PD19	R	R	R	PD68	-	R	R	PD116	R	R	R	PD166	-	R	R
PD20	R	R	R	PD69	R	R	R	PD117	-	R	R	PD167	-	R	R
PD21	-	R	R	PD70	R	R	R	PD118	-	R	R	PD168	-	R	R
PD22	S	S	S	PD71	S	S	S	PD119	-	R	R	PD170	-	S	S
PD23	R	R	R	PD72	-	R	R	PD120	-	S	S	PD171	-	R	R
PD24	R	R	R	PD73	R	R	R	PD121	-	S	S	PD172	-	R	R
PD25	R	R	R	PD74	R	R	R	PD122	-	R	R	PD173	-	R	R
PD26	R	R	R	PD75	S	S	S	PD123	-	R	R	PD174	-	S	S
PD27	R	R	R	PD76	R	R	R	PD124	-	R	R	PD175	-	R	R

Table III-2. (continued)

Line	Inoculation[z]			Line	Inoculation			Line	Inoculation			Line	Inoculation		
	1st	2nd	3rd		1st	2nd	3rd		1st	2nd	3rd		1st	2nd	3rd
PD28	R	R	R	PD77	R	R	R	PD125	-	R	R	PD176	-	R	R
PD29	R	R	R	PD78	R	R	R	PD126	-	R	R	PD177	-	R	R
PD30	R	R	R	PD79	R	R	R	PD127	-	R	R	PD178	-	R	R
PD31	R	R	R	PD80	R	R	R	PD128	-	R	R	PD179	-	R	R
PD32	R	R	R	PD81	R	R	R	PD129	-	R	R	PD180	-	R	R
PD33	R	R	R	PD82	R	R	R	PD130	-	R	R	PD181	-	R	R
PD34	R	R	R	PD83	R	R	R	PD132	-	S	S	PD182	-	R	R
PD35	R	R	R	PD84	R	R	R	PD133	-	S	S	PD183	-	R	R
PD36	R	R	R	PD85	R	R	R	PD134	-	R	R	PD184	-	R	R
PD37	R	R	R	PD86	R	R	R	PD135	-	R	R	PD185	-	S	S
PD38	-	R	R	PD87	R	R	R	PD136	-	R	R	PD186	-	R	R
PD39	R	R	R	PD88	R	R	R	PD137	-	R	R	PD187	-	R	R
PD41	R	R	R	PD89	R	R	R	PD138	-	R	R	PD188	-	S	S
PD42	S	S	S	PD90	R	R	R	PD139	-	R	R	PD189	-	R	R
PD43	R	R	R	PD91	R	R	R	PD140	-	R	R	PD190	-	R	R
PD44	-	R	R	PD92	R	R	R	PD141	-	R	R	PD191	-	R	R
PD45	-	R	R	PD93	R	R	R	PD142	-	R	R	PD192	-	R	R
PD46	S	S	S	PD94	R	R	R	PD143	-	R	R	PD193	-	S	S
PD47	-	R	R	PD95	R	R	R	PD144	-	R	R	PD194	-	S	S

[z]Inoculation was performed each year from 2010 to 2012.

Table III-3. Analysis of inheritance mode for white rust disease resistance in chrysanthemum.

Population	No. of lines	Segreagtion		Expected ratio	*P* ($\alpha = 0.05$)
		Resistant	Suceptible	Resistant:Susceptible	
Puma White × Dancer	188	161	27	3 : 1 (Disomic)	0.0000
				4 : 1 (Hexasomic)	0.0533

PD121, PD170, PD185, PD187) were selected. Then, the resistant and susceptible bulked segregants (R-bulk and S-bulk, respectively) were generated (Fig. III-2).

The pair of bulks with two parents, 'Dancer' and 'Puma White' were screened for 280 RAPD primers (10-mer). The BSA screening resulted in 25 polymorphic bands from 21 primers which were present in 'Dancer' and R-bulk but not in 'Puma White' and S-bulk. 22 polymorphic bands from 18 primers which were present in 'Puma White' and S-bulk but not in the others were also selected. These 47 polymorphic bands were verified in ten resistant and ten susceptible individuals. As a result, only primer OPI-13_{520} produced a polymorphic band in the individuals of resistant bulk but not in the individuals of susceptible bulk whereas the remaining bands were turned out to be false positive (Fig. III-3). A total 256 AFLP primer combinations (*Eco*RI + ANN/*Mse*I + CNN) were also screened using BSA. A total of 1,779 polymorphisms were generated including 852 'Dancer' specific bands and 927 'Puma White' specific bands and the average polymorphic bands per primer combination was 6.95. Among these, 6 bands from 5 primers which were linked to resistance, whereas 4 bands from 4 primers which were linked to susceptibility were selected (Fig. III-4A). These resistant or susceptible specific bands were screened in ten resistant and tne susceptible individuals, but no bands were closely linked to the resistance (Fig. III-4B).

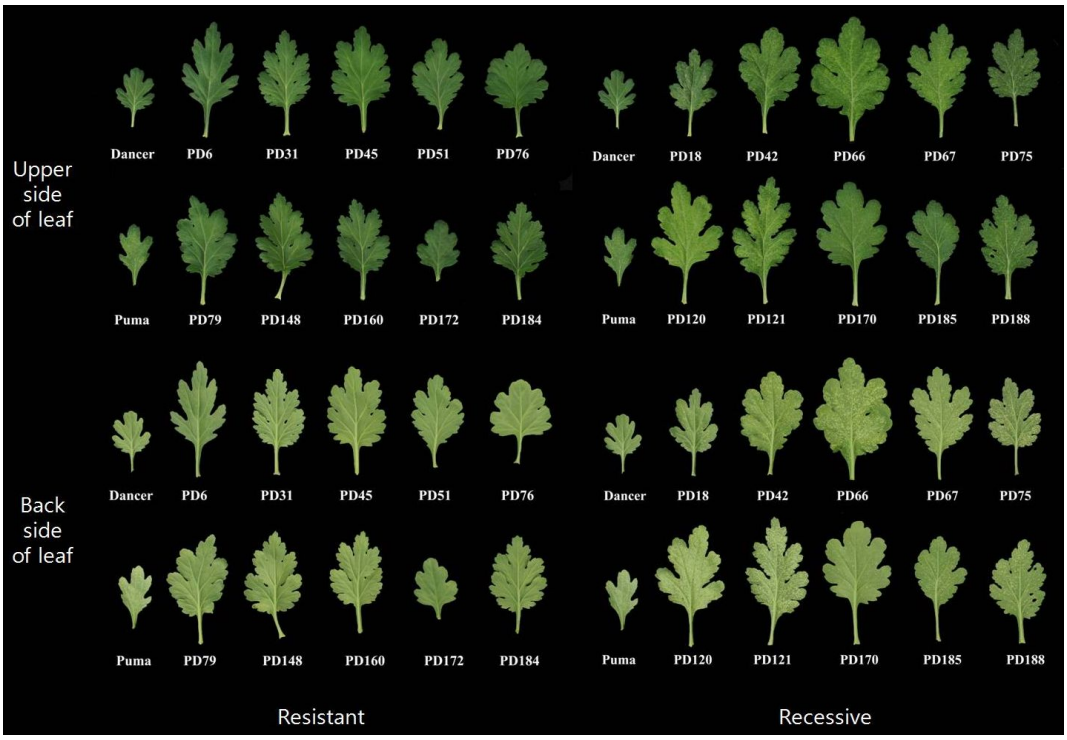

Fig. III-2. White rust disease reactions of resistant ‘Dancer’, susceptible ‘Puma White’, ten resistant individuals selected as R-bulk, and ten susceptible individuals selected as S-bulk among 188 ‘Puma White’ × ‘Dancer’ population.

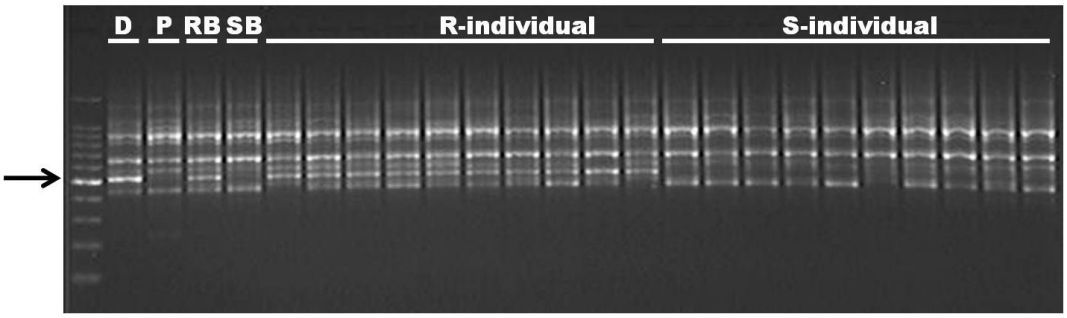

Fig. III-3. RAPD analysis of ‘Dancer’, ‘Puma White’, R-bulk, S-bulk, ten resistant individuals, and ten susceptible individuals with OPI-13 operon primer. D, ‘Dancer’ as resistant parent; P, ‘Puma White’ as susceptible parent; RB, resistant bulk; SB, susceptible bulk; R-individual, ten resistant individuals selected for R-bulk; S-individual, ten susceptible individuals selected for S-bulk.

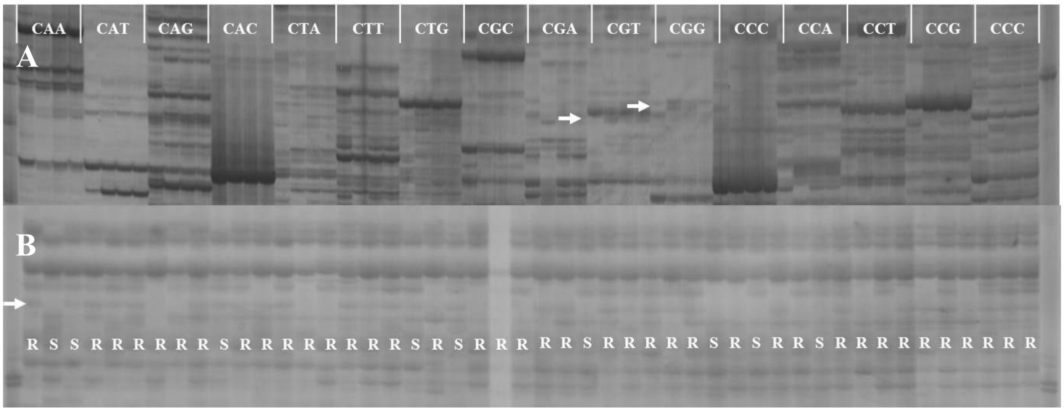

Fig. III-4. (A) BSA-AFLP analysis with *Eco*RI + ATA and 16 different *Mse*I + CNN primer combinations. Red arrows indicate the selected ones as ‘Puma White’ specific bands. (B) A part of segregation patterns of *Eco*RI + AAT/*Mse*I + CAG on parents and 94 progenies of ‘Puma White’ × ‘Dancer’. Red letters indicate recombinant progenies.

Estimation of recombination fraction and linkage analysis

To estimate the genetic distance between the OPI-13_{520} marker and the white rust resistance gene, linkage analysis was carried out with 188 pseudo-F_1 testcross progeny lines. For OPI-13_{520} marker presumed as the closely linked marker to the white rust resistance, the presence or absence of the 520 bp polymorphic bands in resistant parent 'Dancer', susceptible parent 'Puma White', and their progeny lines are shown in Fig. III-5. Among 188 F_1 progeny lines, just six progeny lines did not correspond with phenotypic data. Based on expected phenotypic segregation ratios in the pseudo F_1 progenies shown in Table III-4., it was assumed that a duplex type of white rust resistance in 'Dancer' (RRrrrr) were in combination with a duplex type of OPI-13_{520} marker (MMmmmm). As a result of χ^2-test of independence between resistance gene and OPI-13_{520} marker, χ^2 score is 76.08 and probability is 2.13×10^{-16}. This indicated that the white rust resistance gene and OPI-13_{520} marker were assumed to be linked in coupling phase. The recombination fraction (r) was also determined by the maximum likelihood equation with random chromosome pairing by using general phenotypic proportion and the observed numbers in Table III-4.

The maximum value of θ is obtained by differentiating and equating to 0. For ease of differentiation, the likelihood equation is transformed to log likelihood (L):

$$L = \log(188!/159!1!5!23!) + 159\log(0.8-0.4r+0.2r^2) + 1\log(0.4r-0.2r^2) + 5\log(0.4r-0.2r^2) + 23\log(0.2-0.4r+0.2r^2)$$

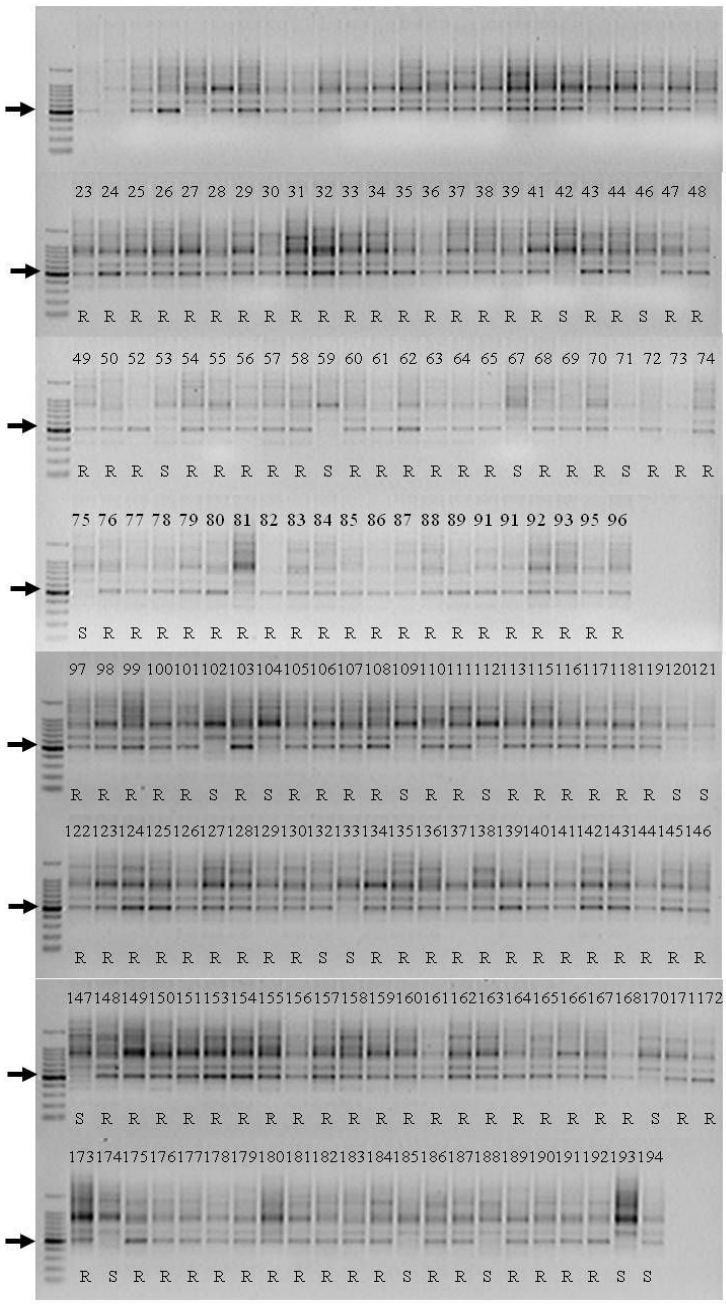

Fig. III-5. Segregation pattern of OPI-13_{520} on 188 progenies of 'Puma White' × 'Dancer'.

Table III-4. Expected phenotypic segregation proportion and general gametic proportions in F_1 single cross population for linkage or independence between white rust resistance gene and a duplex marker based on random chromosome pairing.

Progeny phenotype[z]	General phenotypic proportion			Observed No.
	Coupling	Coupling & repulsion	Double repulsion	
RM	$4/5–2/5r^y + 1/5r^2$	$13/20 – 1/20r^2$	$3/5 + 1/15r + 1/30r^2$	159
Rm	$2/5r – 1/5r^2$	$3/20 + 1/20r^2$	$1/5 - 1/15r - 1/30r^2$	1
rM	$2/5r – 1/5r^2$	$3/20 + 1/20r^2$	$1/5 - 1/15r - 1/30r^2$	5
rm	$4/5 – 2/5r + 1/5r^2$	$1/20 – 1/20r^2$	$1/15r + 1/30r^2$	23

[z]R, resistant; r, susceptible; M, band present; m, band absent.

[y]r = recombination fraction

$$dL/dp = 159/(0.8-0.4r+0.2r^2) - 1/(0.4r-0.2r^2) - 5/(0.4r-0.2r^2) + 23/(0.2-0.4r+0.2r^2) = 0$$

A rearrangement of this equation gives:

$$188r^5 – 1128r^4 + 2537r^3 + 2628r^2 +1148r - 48 = 0$$

The recombination fraction (r) was obtained by successive trials and the associated standard error was estimated by second derivative of log likelihood. The value of r was 0.03832 ± 0.0271

LOD score was calculated as:

$$LOD = \log_{10}(\text{likelihood } r = r') – \log_{10}(\text{likelihood } r = 0.5) = 37.047$$

Such pairwise estimates were input into Joinmap 4.1 software for establishing linkage map. The genetic distance between OPI-13_{520} marker and white rust resistance gene was 4.0 cM (Fig. III-6).

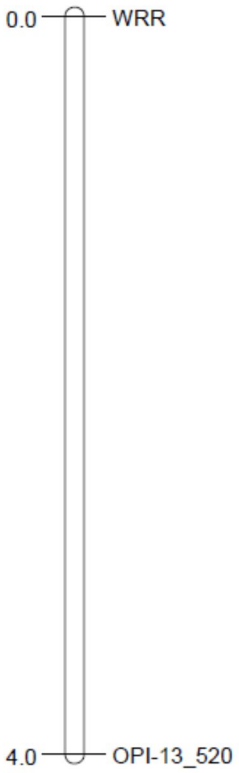

Fig. III-6. Genetic linkage map between white rust resistance gene and OPI-13_{520}.

Conversion of OPI-13_{520} marker to a SCAR marker

To improve the specifity and the reproducibility of the less-specific RAPD markers, the OPI-13_{520} marker was converted into the sequence-characterised amplified region (SCAR) marekr. The basic procedure of the marker conversion is presented in Fig. III-7. The 520 bp fragment of OPI-13_{520} RAPD marker was successfully cloned and sequenced (Fig. III-8). Based on the sequence information, two forward primers and three reverse primers with 20-22 nucleotides were designed for specific amplification of the OPI-13_{520} marker (Table III-5). For the development of SCAR marker, five pairs of SCAR primers were tested on the resistant parent 'Dancer', the susceptible parent 'Puma White', and two bulks (R-bulk and S-bulk). Among the tested primer pairs, OPI-13seqF1/OPI-13seqR1 and OPI-13seqF1/ OPI-13seqR2 generated amplicons of expected size in 'Dancer' and the resistance bulk, whereas the remaining primer pairs generated amplicons in both resistance and susceptibility lanes (Fig. III-9). Among two primer pairs, OPI-13seqF1/OPI-13seqR1 was finally selected for SCAR marker due to better amplication of the marker fragment.

Linkage analysis of OPI-13_{SCAR} was also carried out with 188 pseudo-F_1 testcross progeny lines. The presence or absence of the polymorphic bands in resistant parent 'Dancer', susceptible parent 'Puma White', and their progeny lines are shown in Fig. III-10. Although there was difference in the recombinant progeny lines for OPI-13_{520} and OPI-13_{SCAR}, the number of recombinants was the same showing a genetic distance 4.0 cM to white rust resistance gene.

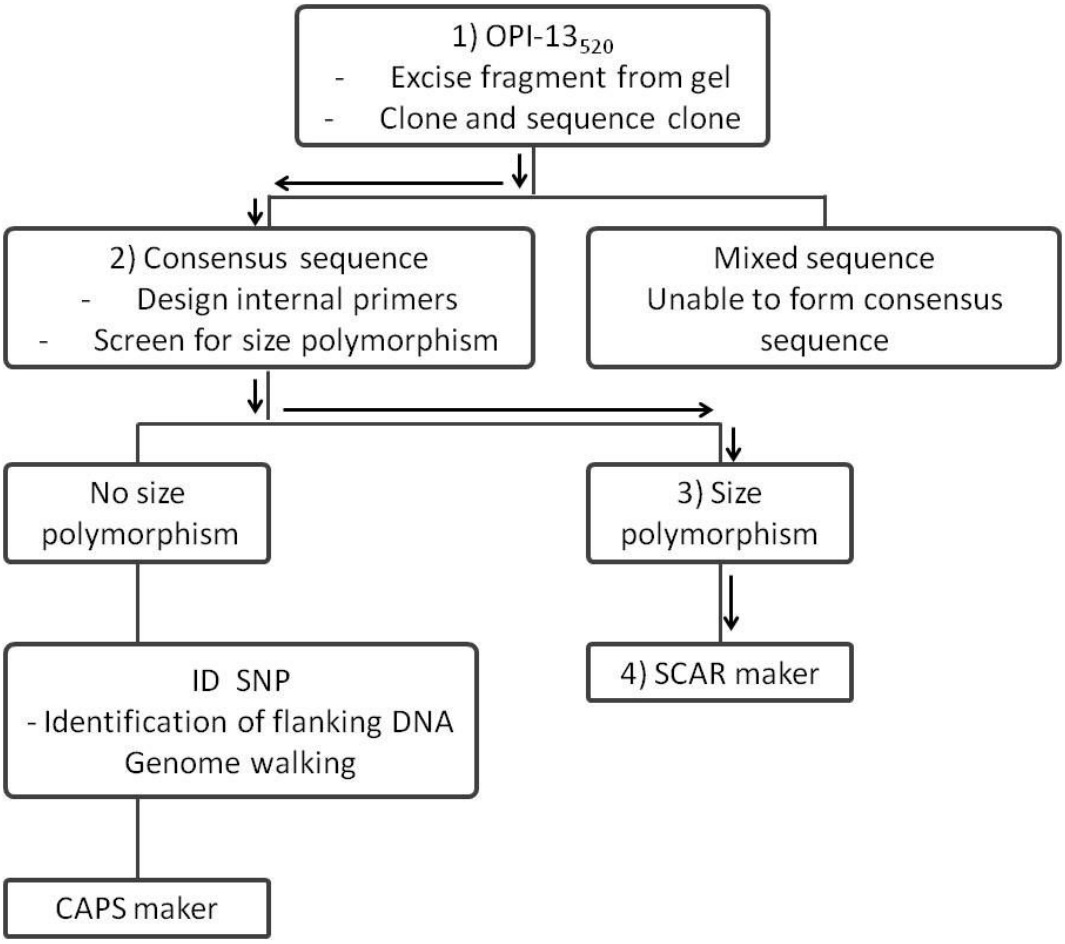

Fig. III-7. Procedure for conversion of OPI-13_{520} into SCAR marker (McNeil et al., 2011).

1	CTGGGGCTGAATAATCCGGTGAAAGTACGAGATCTACTCCACCAAGAAAA	50
51	CAACGAGGCCCAATTTTTCTATTTTATGACCCCAAGCTTGTTGAACATAC	100
101	ATGATTTCACTCGGGTTCCAAAACAAAGGGAAGATGGATGGATGGAGATT	150
151	CAAACGTGGAAATTTGACTCAACTCATGAATTTAAGGATGACTCTCTTTC	200
201	TATTGATATGAAATTTACAAGTCATCAAGGACCTATGTCTGGTCTCATTG	250
251	TTTGTGGCCTTGAGTTTCGGCCAATGTAAAAGAATCATTTAAAAAAAAGG	300
301	TTTTGAAGACAAAATAAAGCTTGCGGTTGTGTTTTAAAATCTCAAACTTC	350
351	TAATTAACAACTCAAGCCAATAACATAACAGAATTGTACCATAGGTACCC	400
401	CTACCGTGATATATGTTTGTATTGCGATAACGAACTAGCCCTTGACGGCG	450
451	TCACTTGATACTAGTAACAGGCCCTGCTAGCCGATCACCACTAGTCTCAA	500
501	CATAGCTAGATCAGCCCCAG	520

Fig. III-8. Sequence information on the desired fragment of OPI-13_{520} RAPD marker at 520 bp.

Table III-5. Primer sequences used for the development of SCAR markers.

Primer		Sequence (5' → 3')
Forward	OPI-13seq F1	GGCTGAATAATCCGGTGAAA
	OPI-13seq F2	TTTCACTCGGGTTCCAAAAC
Reverse	OPI-13seq R1	GGGGCTGATCTAGCTATGTTG
	OPI-13seq R2	TGAGACTAGTGGTGATCGGCTA
	OPI-13seq R3	CACGGTAGGGGTACCTATGG

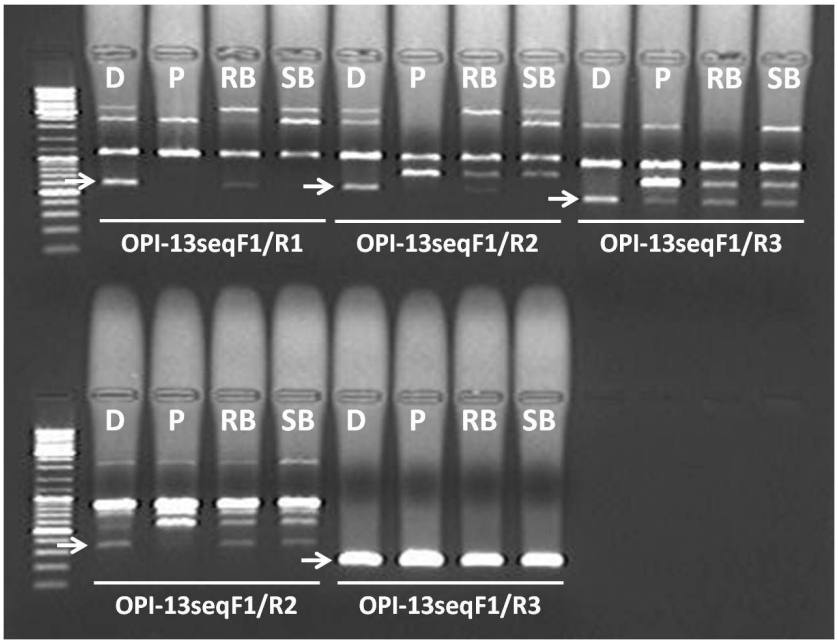

Fig. III-9. Amplification patterns of five primer pairs used for the development of SCAR marker in resistant 'Dancer' (D), susceptible 'Puma White' (P), resistant bulk (RB), and susceptible bulk (SB).

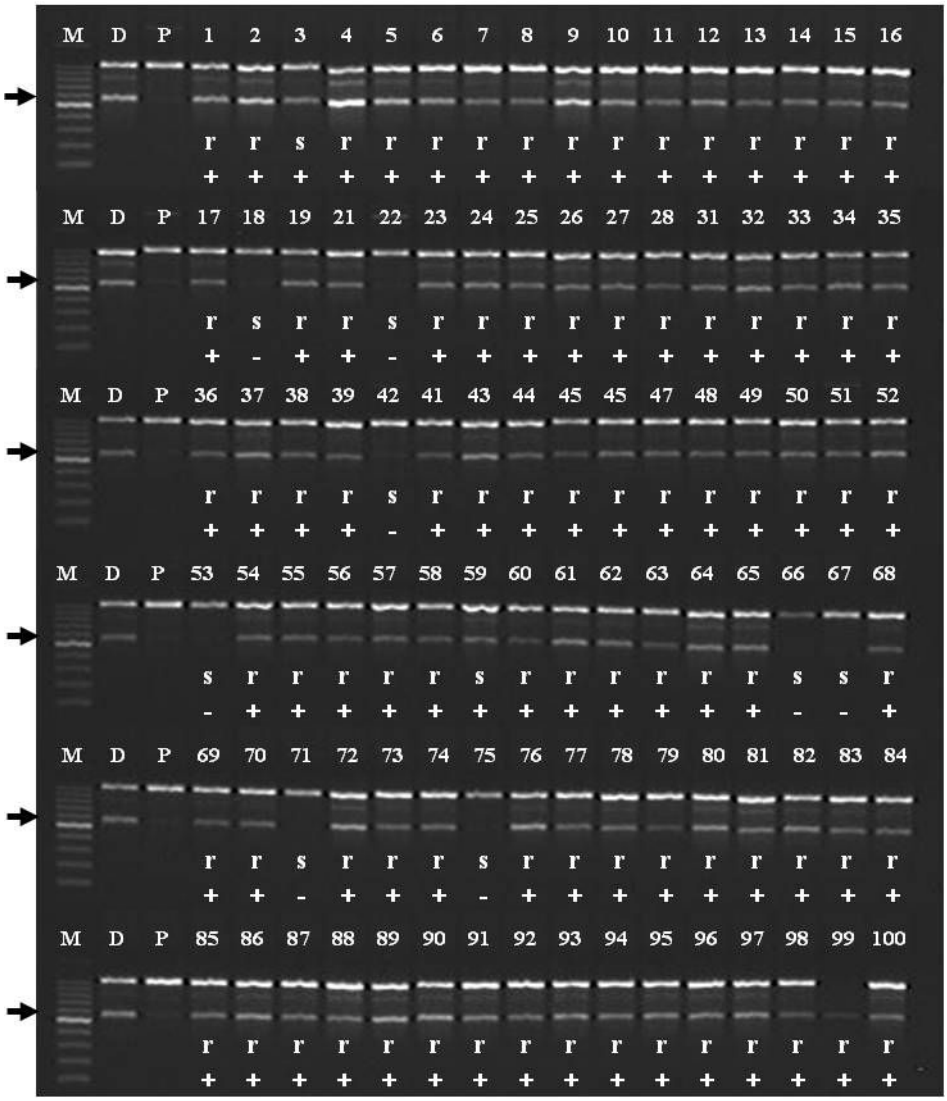

Fig. III-10. Segregation patterns of SCAR marker, OPI-13_{SCAR}, on resistant 'Dancer' (D), susceptible 'Puma White' (P), and 188 their progeny lines.

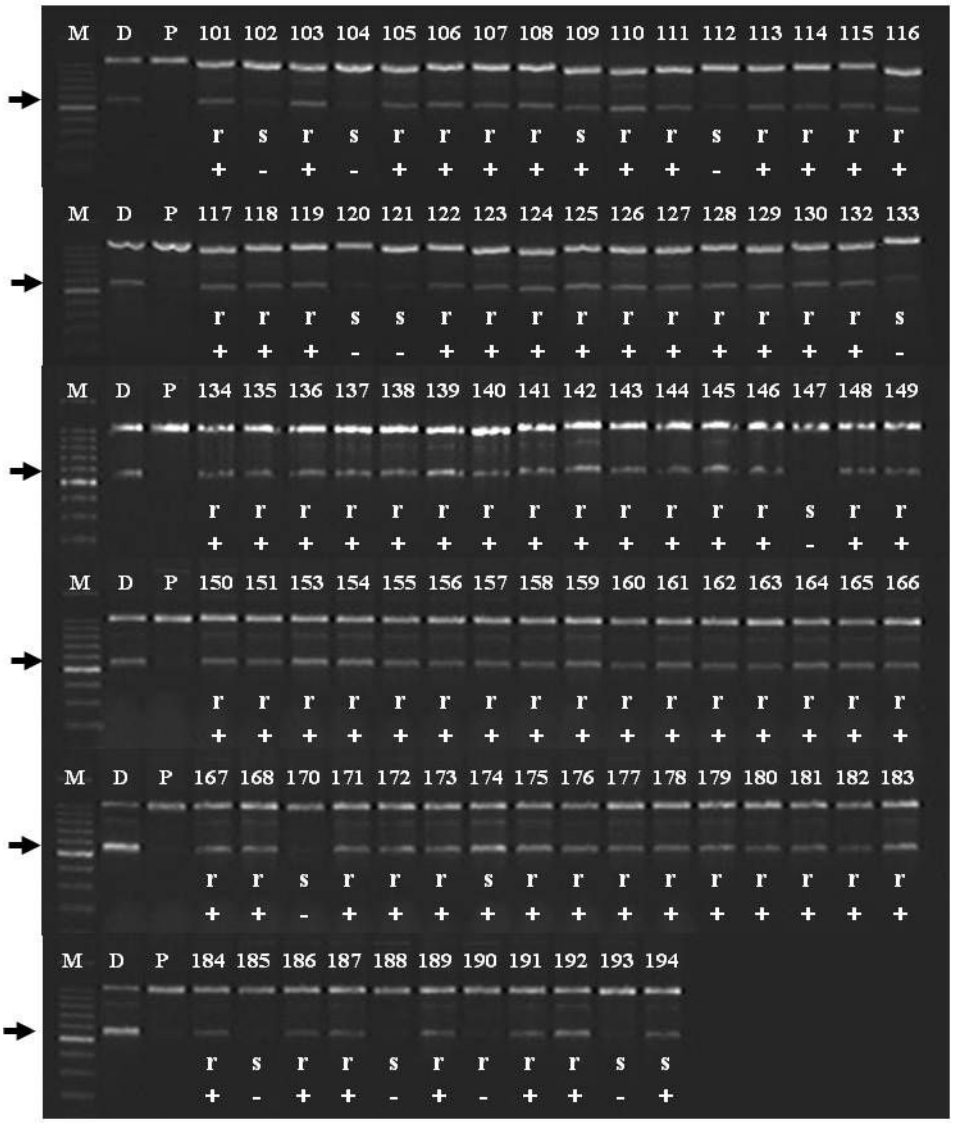

Fig. III-10. (continued).

DISCUSSION

Genetic studies in cultivated chrysanthemums, of which most have been found to be hexaploid (2n = 6x = 54), have been hampered owing to high level of heterozygosity, self-incompatibility, and inbreeding depression (Anderson and Ascher, 2000; Dowrick, 1952; Huang et al., 2000; Zagorski et al., 1983). However, especially on disease resistance in chrysanthemum, a few genetic analyses suggested the white rust resistance might be governed by simple genes, including a single dominat in the type of complete resistance, and a single dominant with minor genes (De Jong and Rademaker, 1986; Yamaguchi, 1981). Most of rust fungi are very specialized pathogens which attack only certain crops or certain varieties, so there are many pathogenic races in nature (Agrios, 2005). The existence of physiological races of *P. horiana* was also demonstrated in chrysanthemum species (De Backer et al., 2011; Velasco et al., 2007; Yamaguchi, 1981). De Backer et al. (2011) tried to explain these differential interactions through gene-for-gene model decribed by Flor (1956). The chrysanthemum cultivar 'Dancer', used as resistant parent in this study, was also shown complete resistance against *P. horiana* and found to have a duplex type of resistant allele (RRrrrr) based on the segregation analysis under the assumption of the random pairing inheritance mode.

Application of molecular markers for chrysanthemum breeding seems to be a little behind many other diploid and selfing crops due to polyploidy and complex genome constitution. Nevertheless, molecular markers tightly linked to the resistance gene can be used for overcoming these obstacles, as has already been

demonstrated in several crop species (Heusden et al., 2002; Malek et al., 2000; Ukoskit and Thompson, 1997). Molecular markers in chrysanthemum have been used for a wide range of purposes from detecting genetic diversity, estimating genetic relationships, identifying cultivars or species, constructing genetic linkage map, and discovering genes (Chen et al., 2009; Dia et al., 1998; Miao et al., 2007; Wolff and Peters-van Rijn, 1993; Wolff et al., 1994, 1995; Zhang et al., 2010; Zhou and Dai, 2002). However, this is the first report identifying molecular markers linked to white rust resistance gene in chrysanthemum. Using a bulked segregant analysis, OPI-13_{520} RAPD marker was found to be linked to the white rust resistance with a genetic distance of about 4.0 cM.

RAPD is widely used for developing molecular markers because it is easy, cheap, and quick. Howerver, RAPD often generates too many non-specific amplicons and has low reproducibility. Conversion of RAPD into SCAR marker is one of the alternatives to overcome this disadvantage (Paran and Michelmore, 1993). In this study, OPI-13_{520} RAPD marker linked to the white rust resistance gene in hexaploid chrysanthemum cultivar 'Dancer' was developed. Furthermore, this marker was successfully converted into a SCAR marker, OPI-13_{SCAR}. Among the primer pairs tested for the SCAR marker, OPI-13seqF1 and OPI-13seqF2 primer pairs produce polymorphic band of expected size. The OPI-13_{SCAR} marker clearly discriminated the presence and absence of the polymorphic band in parental lines and progenies. However, due to the difference of the priming sites between primers of OPI-13_{520} and OPI-13_{SCAR} marker, a few polymorphic bands did not match in same plants.

Developing molecular marker, especially PCR-based SCAR marker, makes it possible to use marker-assisted selection for white rust resistance in chrysanthemum. Selection through molecular markers linked to resistance genes rather than disease screening could significantly speed up the introgression of resistance gene (R-gene) into cultivars. However, due to the diverse genome consititution of chrysanthemum, the marker could not be applicable to wide range of chrysanthemum cultivars. Unfortunately, resistance governed by simple R-genes is often rapidly overcome by new physiological races of the pathogen, in crops grown in large monocultures such as chrysanthemum (McDonald and Linde, 2002). To avoid these outbreaks, pyramiding using molecular markers linked to different R-genes will be effective to keep the durable resistance.

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CONCLUSIONS

Through these experiments, hexaploid chrysanthemums are revealed as autohexaploid showing the hexasomic inheritance mode. Although two hypotheses, allopolyploid and autopolyploid, have been proposed for chrysanthemums, it is generally believed that hexaploid chrysanthemums might be allopolyploid because they are derived from complex interspecific hybridizations among multiple chrysanthemum species. Many cytological studies supported that the hexaploid genome of chrysanthemum might be derived from allopolyploidization based on their observations of pairing behavior during meiosis in several species. However, the segregation analysis for SSR marker alleles in 94 pseudo-F_1 progenies was indicative to the hexasomic inheritance. Excluding 115 of 180 polymorphic SSR marker alleles which were not affected by the type of inheritance, 33 marker alleles gave a good fit to the expected segregation ratio for the hexasomic inheritance and only 6 marker allele were distorted at a level of $\alpha < 0.05$ whereas 24 gave a good fit to the disomic inheritance and 7 were distorted. In addition, the proportion of non-simplex to simplex markers of 20.2% versus 79.8% supported an autopolyploid nature of chrysanthemum. In addition, transmission patterns of alleles in fully informative co-dominant SSR loci gave clear evidence for random chromosome assortment and polysomic inheritance and as such linked this specific inheritance mode to the chromosomal regions where these markers reside.

Based on the result of hexasomic inheritance mode, the white rust resistance in hexaploid chrysanthemum was governed by simple genes. Through resistance screening to white rust caused by *Puccinia horiana* in 179 commercial chrysanthemum cultivars, three resistant cultivars, 'Dancer', 'Bongan', 'Biaritz', and 'Kokka Akafuji', and three susceptible cultivars, 'Puma White', 'Sei-Alps', 'Chunilbeon', and 'Universe', were selected as parents for crossing. A total of nine crosses were made between susceptible cultivars, susceptible and resistant cultivars, and resistant cultivars. Whereas all the progenies were susceptible in the crosses between susceptible cultivars, segregation ratio of the crosses between resistant and susceptible cultivars were 1:1 or 4:1 for resistant (R) versus susceptible (S), indicating white rust resistance in the hexaploid chrysanthemums was controlled by a single dominant gene. Especially, the crosses of the resistant 'Dancer' with susceptible cultivars, 'Puma White', 'Sei-Alps', and 'Universe', segregated in a 3:1 or 4:1 ratio for R:S. And the crosses between the resistant cultivars, 'Dancer' × 'Biaritz' and 'Dancer' × 'Bongan', segregated in a 7:1 or 9:1 ratio for R:S. These results indicated that the chrysanthemum cultivar 'Dancer', used as resistant parent, was shown complete resistance against *P. horiana* and found to have a duplex type of resistant allele (RRrrrr) coding for white rust resistance.

Finally, molecular marker linked to white rust resistace in chrysanthemum was developed and converted into PCR-based sequence characterized amplified region (SCAR) marker for marker-assisted breeding. Using a bulked segregant analysis in the pseudo-F_1 testcross population of 'Puma White' × 'Dancer', the random

amplified polymorphic DNA (RAPD) marker, OPI-13_{520}, was developed and found to be linked to white rust resistance with a genetic distance of about 4.0 cM. Then, OPI-13_{520} RAPD marker converted into SCAR marker, OPI-13_{SCAR} which gives relatively clear result to discriminate between the presence and absence of polymorphic band rather than OPI-13_{520} RAPD marker. Marker-assisted selection using OPI-13_{SCAR} marker could make it possible to speed up the introgression of white rust resistance gene and enhance the efficiency of disease resistant breeding in chrysanthemum.

APPENDIX

Supplementary talbe 1. SSR primer information used for genotyping of the chrysanthemum pseudo-F_1 testcross population.

Designated SSR marker ID	Sequence/ SSR motif consensus ID	Forward primer (5'->3')	Reverse primer (5'->3')	T_m (℃)	Expected product size (bp)
ChSSR-006	DK938159	$(TCT)_5$	AGTGTCAAAACAAAATGGCT	gtttCATCTCTGGCGTTAGGTAAG	55	293
ChSSR-009	DK940806	$(AC)_6$	TCACACACACTGACACAACA	gtttGTGATACGGATTAGAACCCA	55	247
ChSSR-041	DK939533	$(ACA)_5$	CGGCTAAAGAGTTCGTTAAA	gttTGTTTTTGGTTCGAGTTTCT	55	197
ChSSR-043	DK940230	$(CAA)_7$	ATGTTTCTACGGGTTTACGA	gTTTCATTTCCTTGTTGGTTT	55	147
ChSSR-046	DK938512	$(CAC)_5$	CCACTTTTCTAGTGGTGCTC	gttTATATGTCTCCTGGTGGACC	55	384
ChSSR-047	DK941844	$(CCT)_5$	ACAAATCATGCCCACTAATC	gtttCTCACCACCTTACTGTCCTC	55	214
ChSSR-056	DK937181	$(ACA)_7$	TCTATTAATGGACCTCACCG	gtttGGATTCAGATGAGTGTTGGT	55	118
ChSSR-061	DK939513	$(GGT)_5$	TTCTCTAGTGGTGCTCACCT	gtttACAAATCATGCCCACTAATC	55	498
ChSSR-067	DK936872	$(ATA)_5$	AAAGAATTCGGTCATGCTAA	gttTAGCATCATGCATTACTTGC	55	322
ChSSR-070	DK936996	$(AAT)_5$	CATTTTCATCGCCTTCTAAC	gtttAGCTCAAAATACCTCCAACA	55	201
ChSSR-073	DK936630	$(CAC)_5$	CATGTTGAAAACACACCTCA	gtttCGCTTAGAACAAAAACGTTT	55	183
ChSSR-076	DK939046	$(GGT)_7$	TCCTCACTCACTCACTCACA	gtttCCACCACCATTACTATCCAC	55	387
ChSSR-078	DK938159	$(TCT)_5$	AGTGTCAAAACAAAATGGCT	gttTAGGTAAGTCCCAAGCCATA	55	280
ChSSR-088	DK939768	$(CAA)_5$	CCATTCATATGCATTTTCCT	gttTACAACACTTGGTCTCACCA	55	276
ChSSR-089	DK941527	$(TGG)_5$	TGGTGAGACCAAGTGTTGTA	gtttCGGCTATATCTCAACCGTAC	55	395
ChSSR-090	DK936951	$(AGC)_5$	AGGTATCAAACGCCAAACTA	gtttCAGCAGCTTCTTCTTCATCT	55	256
ChSSR-104	DK942673	$(TAC)_6$	TCAACCGTACCTTCACTTCT	gtttATCGGAAAGCAGAGTGATTA	55	173
ChSSR-110	DK942774	$(CCG)_5$	CTGTGTGTAAACCAAACCCT	gtttCAGAGCCATCAGAAGAACTAA	55	190
ChSSR-111	DK942743	$(GGC)_5$	TCCCTTTTCAACATATCCAC	gttTGTTGCTGAAAATTAGGGTT	55	237
ChSSR-124	DK942296	$(CAT)_5$	GAATTCCAAAGAGATCCATG	gttTCGCATCCTCAACTTATTCT	55	223
ChSSR-134	DK942760	$(CTG)_6$	TTTTATGATGGGATTATGGC	gttTGTCCCAACCACCTTACTTA	55	298
ChSSR-142	DK940792	$(GCC)_5$	AATAAACATAAAGACCGCCA	gtttACGTGAAAACTTGAAAAGGA	55	312
ChSSR-145	DK940870	$(ATT)_5$	AGGGTTTTGTTTCAGCATAA	gtttATCGGAAAGCAGAGTGATTA	55	264

Supplementary talbe 1. (continued)

Designated SSR marker ID	Sequence/ consensus ID	SSR motif	Forward primer (5'->3')	Reverse primer (5'->3')	T_m (°C)	Expected product size (bp)
ChSSR-151	DK937366	$(ATA)_5$	GAGAAGCTGATGAGCAGATC	gtttCAAAGGAAGCTTCTCTCTCA	55	348
ChSSR-156	DK938485	$(CCA)_6$	TTAATCATCCCAAAACCATC	gtttGCTTTGTGTTGGTTGGATAT	55	211
ChSSR-157	DK938251	$(AACCCT)_5$	CTACTTCTGCCACATATGCA	gttTCCCATTACCATTTTCAATC	55	371
ChSSR-160	DK940238	$(CAA)_6$	AAACGTTCTGCACCTCTAGA	gttTGATGGTTGACTGAAATTGA	55	138
ChSSR-161	DK940238	$(GAA)_5$	TCAGAAGATGACCAACAACA	gtttATGACTCATTAGGGTTTTGG	55	121
ChSSR-164	DK940191	$(GT)_{10}$	CCCCGCTTCTATTCTATTCT	gtttAGGAATTGGTGAATTTGTTG	55	159
ChSSR-167	DK937424	$(AAG)_6$	ACAGTCATGGTAGCTCCATC	gtttGCCATTCATATCAGGCTTAG	55	138
ChSSR-170	DK940714	$(CAA)_5$	GAGGGCTCCTTTTACACTTT	gtTTGTTTGATCAGGTTTGTGA	55	190
ChSSR-185	DK936833	$(ACC)_6$	ATAGGCACAGGCAGTGTAGT	gtttAACAAACGTAGTCGCTTAGC	55	233
ChSSR-189	DK937368	$(AAG)_6$	AGAACCAGAACCACAACAAC	gtttCAGCTATCTTCTCAACGCTT	55	314
ChSSR-209	DK936909	$(CAC)_5$	CTAACCACCGTCGTCATAAT	gtttGTAGTGGTAGTGGGGTTTCA	55	111
ChSSR-212	DK939519	$(CAA)_8$	AACACACGGTAACCAACAAT	gtttATGGTGATGGTGTAAGGGT	55	124
ChSSR-213	DK939519	$(CAC)_5$	ATTCCACCATTGCAATTG	gtttGTGTTTGGGATTTAGGGTTA	55	128
ChSSR-214	DK940235	$(CATCAC)_5$	ATTCAAACAAACCACCATTG	gtttGGAATTGAGGTGTTTGGATA	55	151
ChSSR-217	DK938496	$(CAA)_5$	TTGGGTAGAAAACCATCATC	gtttAACATCGTTACGGATGAGTC	55	174
ChSSR-218	DK942439	$(ACA)_5$	GTTAACGAAAACATGGCTTC	gttTCAAATTTGGGCTTAACTGT	55	287
ChSSR-231	DK942332	$(ATC)_5$	TCAACAAGATCATGTGCTGT	gtttCTTGTTCTTGTGGTTGTGTG	55	352
ChSSR-232	DK937051	$(ATC)_6$	GCGGCTTATACATCATCTTC	gtttATACGTTGACGAAGAAGCAT	55	260
ChSSR-239	DK942155	$(ACC)_5$	CGCTACAATTCAAACAAACA	gttTAGTAGTCGCTCCACCACTT	55	284
ChSSR-242	DK939975	$(CCA)_5$	ATATCCTGTTCTTCACACCG	gtttAGCCATGGTTACTTGAGAAA	55	259
ChSSR-244	DK936905	$(CAG)_6$	CCTAGAAGACCCCTCATCAT	gtttACGGAAACTGAACTTCTTGA	55	133
ChSSR-247	DK936801	$(ACA)_9$	TTCCTTCATGGGTTTTCTTA	gtttAGATCCGACAGATATTGGTG	55	327
ChSSR-252	DK937106	$(ATC)_5$	GAGAGAGAAGGTAAAAATGGC	gtttAAAGCTTCTTCGGATTTCTT	55	177
ChSSR-253	DK943261	$(GCA)_5$	ATCACGATGACGATGTCAA	gtttAACAGCGACTCCAGAAATAA	55	243
ChSSR-254	DK941412	$(TCA)_5$	GGAAGGGTATGGAAGATTTC	gtttAAGTTGGAGAAATTGACGAA	55	128
ChSSR-258	DK943237	$(TTC)_5$	TGTTTGGACAGAGACACAAA	gtTTGAGAAACAAGAGGTCGTT	55	289

Supplementary table 2. Chrysanthemum cultivars tested for resistance to *P. horiana* and their disease responses.

Type	Accession No.[z]	Cultivar or line	1st inoculation (2009)[y]	2nd inoculation (2010)	3rd inoculation (2011)	Total	Response[w]
Standard	IT244120	Kokka Akafuji	0.00±0.00	0.16±0.40	0.16±0.40	0.11±0.32	MR
	IT244130	Otomezakura	0.16±0.40	0.16±0.40	0.00±0.00	0.11±0.32	MR
	IT244131	Otomezakura Orange	0.00±0.00	0.00±0.00	0.16±0.40	0.05±0.23	MR
	-	Otomezakura Pink	0.00±0.00	0.16±0.40	0.33±0.51	0.16±0.38	MR
	IT234194	Otomezakura Yellow	0.16±0.40	0.20±0.44	0.16±0.40	0.17±0.38	MR
	IT234192	Suishin	0.16±0.40	0.33±0.51	0.16±0.40	0.22±0.42	MR
	IT245131	Seikonomakoto	0.50±0.54	0.83±0.75	0.33±0.51	0.55±0.61	MR
	IT244981	Seikonomakoto III	0.40±0.54	0.50±0.54	0.66±0.51	0.52±0.51	MR
	IT234189	Seinonami	0.33±0.51	0.66±0.51	0.66±0.81	0.55±0.61	MR
	IT232551	Baekma	3.66±0.51	3.16±1.16	4.33±0.81	3.72±0.95	S
	IT234136	Byakko	4.66±0.51	3.00±0.00	3.16±0.75	3.61±0.91	S
	IT232534	Iwanohakusen	2.50±0.83	2.16±0.75	2.16±0.40	2.27±0.66	S
	IT232525	Jinba	4.83±0.40	4.60±0.54	4.50±0.83	4.64±1.24	S
	FCH0203	Kiranouma	3.00±0.00	2.83±0.40	2.66±1.50	2.83±0.85	S
	-	Kokka Shunko	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	S
	FCH0110	Seifu	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	S
	-	Seiko no aki	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	S
	FCH0194	Seikokaike	1.50±0.54	1.16±0.40	1.83±0.75	1.50±0.61	S
	FCH0196	Seikoshinnen	2.33±0.51	2.16±0.40	1.83±0.75	2.11±0.58	S
	FCH0179	Seikoumangetu	2.00±0.00	2.33±0.51	2.16±0.75	2.16±0.51	S
	IT234188	Seinoisse	1.50±0.83	1.16±0.40	1.50±0.54	1.38±0.60	S
	FCH0201	Seinokoto	3.16±0.40	3.00±0.00	2.83±0.40	3.00±0.34	S
	IT234190	Seinokyoku	0.83±0.40	1.50±0.54	1.00±0.70	1.11±0.63	S
	FCH0197	Seiun	4.33±0.51	4.00±0.00	3.83±0.98	4.05±0.63	S
	IT244128	Shuhonochikara White	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	S
	IT244129	Shuhonochikara Yellow	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	S
	IT232548	Yongma	4.50±0.83	3.00±1.54	4.60±0.54	4.00±1.55	S

Supplementary table 2. (continued)

Type	Accession No.[z]	Cultivar or line	1st inoculation (2009)[y]	2nd inoculation (2010)	3rd inoculation (2011)	Total	Response[w]
Spray	IT245424	Akira Kazaguruma	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	FCH0193	Albert-heijn	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	FCH0215	Annecy	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Ansella	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Bacchus	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	IT244119	Biarittz Yellow	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Crocodile	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	IT232543	Dalma	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	IT244123	Dancer	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Dark Westland	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Dutchy	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Eunhasu	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Flush	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	IT232553	Hambaek	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	FCH0176	Handsome	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	FCH0140	Jeanny	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Jerry	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	IT245133	Kingfisher	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Kingfisher Cherry	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Lexy Red	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	IT245425	Marikazaguruma	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Melody	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Merida Splendid	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Morning	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Pink Elisa	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	IT244984	Quinty	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Quinty Pink	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	IT245139	Quinty Red	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Recharles	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Refondo	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	FCH0183	Rodis White	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	FCH0184	Rodis Yellow	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R

Supplementary table 2. (continued)

Type	Accession No.[z]	Cultivar or line	1st inoculation (2009)[y]	2nd inoculation (2010)	3rd inoculation (2011)	Total	Response[w]
	FCH0167	Roma	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Sei Amelie	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	FCH0192	Sei Energy	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Sei Falcao	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Sei Mini	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Sei Sanp	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	IT244133	Sei Soul	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Taiyo no Koigokoro	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Tobago	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	R
	-	Antigua	0.50±0.83	0.50±0.54	0.66±0.81	0.55±0.70	MR
	IT245422	Arctic Queen	0.40±0.54	0.83±0.75	0.50±0.83	0.58±0.70	MR
	IT234150	Bacardi	0.50±0.83	0.60±0.89	1.16±0.98	0.76±0.89	MR
	-	Bennie Jolink	0.66±0.81	0.33±0.51	0.66±0.51	0.55±0.61	MR
	IT244118	Biarittz Pink	0.00±0.00	0.00±0.00	0.16±0.40	0.05±0.23	MR
	-	Bongan	0.16±0.40	0.00±0.00	0.00±0.00	0.05±0.23	MR
	-	Cassa	0.16±0.40	0.00±0.00	0.16±0.40	0.11±0.32	MR
	-	Champy	0.66±0.51	1.00±0.00	0.83±0.40	0.82±0.42	MR
	FCH0175	Fly Catcher	0.00±0.00	0.00±0.00	0.33±0.81	0.11±0.47	MR
	IT232559	Gama	0.16±0.40	0.00±0.00	0.33±0.51	0.16±0.38	MR
	IT232544	Gibaeg	0.00±0.00	0.00±0.00	0.16±0.40	0.05±0.23	MR
	IT232533	Hebo	0.66±0.81	0.50±0.54	0.50±0.54	0.55±0.61	MR
	-	Husky	0.20±0.44	0.50±0.83	0.83±0.75	0.52±0.70	MR
	IT232550	Hyangro	0.16±0.40	0.00±0.00	0.50±0.83	0.22±0.54	MR
	IT232555	Ilweol	0.50±0.54	0.33±0.51	0.83±0.40	0.55±0.51	MR
	FCH0161	Marabou	0.50±0.54	0.50±0.54	0.66±1.03	0.55±0.70	MR
	-	Panama	0.66±0.81	0.33±0.51	0.66±0.51	0.55±0.61	MR
	IT234103	Penny Lane	0.83±0.98	0.50±0.83	0.83±0.40	0.72±0.75	MR
	-	Piaget	0.50±0.54	0.40±0.54	0.83±0.98	0.58±0.70	MR
	-	Ping Pong White	0.33±0.51	1.00±0.89	0.33±0.51	0.55±0.70	MR
	-	Ping Pong Yellow	0.50±0.54	0.60±0.54	1.00±0.63	0.70±0.59	MR

Supplementary table 2. (continued)

Type	Accession No.[z]	Cultivar or line	1st inoculation (2009)[y]	2nd inoculation (2010)	3rd inoculation (2011)	Total	Response[w]
	-	Sei-Rosa	0.50±0.54	0.33±0.51	1.00±0.89	0.61±0.69	MR
	-	Sei-Soul Yellow Red	0.20±0.44	0.00±0.00	0.66±0.51	0.29±0.46	MR
	FCH0005	Vesuvio	0.16±0.40	0.00±0.00	0.66±0.51	0.27±0.46	MR
	-	Vesuvio Yellow	0.00±0.00	0.16±0.40	0.50±0.54	0.22±0.42	MR
	-	White King	0.00±0.00	0.00±0.00	0.83±0.40	0.27±0.46	MR
	-	White Marble	0.60±0.54	0.66±0.81	0.50±0.83	0.58±0.70	MR
	-	Winia	0.33±0.51	0.40±0.54	0.83±0.98	0.52±0.70	MR
	-	Accent	3.66±0.81	4.66±0.51	3.83±0.75	4.05±0.80	S
	-	Anastasia	3.00±0.63	3.83±1.47	3.50±1.76	3.44±1.33	S
	IT232558	Anastasia Green	4.33±0.51	4.33±1.21	4.00±1.67	4.22±1.16	S
	FCH0085	Ardilo	2.00±0.63	1.66±0.51	2.16±1.16	1.94±0.80	S
	IT234117	Argus	3.16±0.40	4.00±1.09	3.33±1.21	3.50±0.98	S
	-	Art Yellow	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	S
	-	Artist Yellow	5.00±0.00	4.16±0.40	4.66±0.51	4.61±0.50	S
	FCH0033	Bijux	2.66±0.81	3.16±0.98	3.00±0.63	2.94±0.80	S
	-	Boris Becker Yellow	1.50±0.83	0.50±0.83	1.33±1.21	1.11±1.02	S
	IT245421	Bradford	4.00±0.63	3.16±1.32	5.00±0.00	4.00±1.43	S
	-	Buffy	3.66±1.03	4.16±0.75	3.33±0.81	3.72±0.89	S
	IT232568	Charming Eye	2.16±0.40	1.66±0.51	2.66±0.51	2.16±0.61	S
	-	Chunilbeon	3.16±0.40	3.00±0.00	4.00±0.00	3.38±0.50	S
	FCH0112	Chopin Dark Pink	2.83±0.40	2.33±0.51	3.16±0.40	2.77±0.54	S
	-	Chopin Orange Pink	2.20±0.44	2.00±0.00	3.40±0.54	2.50±1.06	S
	FCH0113	Chopin White	4.00±0.63	2.50±1.22	4.66±0.81	3.72±1.27	S
	-	Coral Marble	3.16±0.40	4.16±0.40	2.50±0.54	3.27±0.82	S
	-	Cosmos King	2.16±0.98	0.50±0.54	2.80±0.44	1.76±1.23	S
	-	Creado	3.16±0.40	3.00±0.00	3.66±1.21	3.27±0.75	S
	-	Dash	4.83±0.40	4.16±0.40	4.16±0.40	4.38±0.50	S
	FCH0153	Deliah Cream	4.00±0.00	3.16±0.40	4.00±0.00	3.72±0.46	S
	IT244137	Delilah Yellow	3.33±0.51	1.66±0.81	3.33±0.51	2.77±1.00	S
	IT245137	Deliwind Yellow	5.00±0.00	4.83±0.40	4.66±0.51	4.83±0.38	S
	-	Dinar	2.33±0.51	2.00±0.00	3.00±0.00	2.44±0.51	S

Supplementary table 2. (continued)

Type	Accession No.[z]	Cultivar or line	1st inoculation (2009)[y]	2nd inoculation (2010)	3rd inoculation (2011)	Total	Response[w]
	FCH0114	Euro White	1.83±0.40	0.83±0.75	1.60±0.89	1.41±0.84	S
	IT234128	Euro Yellow	2.00±0.00	2.00±0.63	1.83±0.75	1.94±0.53	S
	FCH0094	Feeling Green	5.00±0.00	4.80±0.44	5.00±0.00	4.94±1.18	S
	IT244138	Ford	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	S
	IT245135	Froggy	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	S
	FCH0096	Garcia	2.33±0.51	1.33±0.51	2.16±0.40	1.94±0.63	S
	-	Golden Kent	1.50±0.83	2.40±1.51	3.66±0.81	2.52±1.46	S
	IT246016	Golden PangPang	2.60±0.54	3.33±1.63	4.33±1.03	3.47±1.52	S
	IT234161	Green Bird	2.00±0.00	2.66±0.51	2.83±0.75	2.50±0.61	S
	-	Hunt	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	S
	-	Ibis Lime	3.00±0.00	1.83±0.40	2.00±0.00	2.27±0.57	S
	FCH0204	Ibis Sunny	2.66±0.51	3.16±0.40	2.83±0.40	2.88±0.47	S
	IT234180	Inga	2.80±0.44	1.83±1.16	3.16±1.60	2.58±1.38	S
	IT244122	Kinkazaguruma	1.20±0.44	1.50±1.04	1.33±0.51	1.35±0.75	S
	IT232542	Kumsu	4.16±0.40	3.16±0.75	5.00±0.00	4.11±0.90	S
	-	Lineker Salmon	2.16±0.75	2.16±1.47	1.50±1.37	1.94±1.21	S
	-	Marscort	2.00±0.00	1.83±0.40	2.33±1.21	2.05±0.72	S
	-	Mona Lisa	3.50±1.04	5.00±0.00	5.00±0.00	4.50±0.92	S
	-	Mona Lisa Pink	2.50±1.22	1.00±0.63	1.66±0.81	1.72±1.07	S
	IT245138	Mona Lisa Splendid	2.16±0.40	1.83±0.40	2.00±0.00	2.00±0.34	S
	IT234168	Mona Lisa White	1.83±0.75	0.50±0.54	1.20±0.83	1.17±0.90	S
	IT234166	Mona Lisa Yellow	2.00±0.70	0.60±0.54	1.50±0.83	1.37±0.94	S
	IT244132	Moonlight	3.33±0.51	2.33±0.81	2.40±0.89	2.70±1.04	S
	FCH0159	Namba	2.33±0.51	1.83±0.40	2.66±0.81	2.27±0.66	S
	-	Nice	4.66±0.51	4.83±0.40	5.00±0.00	4.83±0.38	S
	IT234119	Noa	4.16±0.40	5.00±0.00	4.16±0.75	4.44±0.61	S
	IT232541	Noeul	1.16±0.40	1.40±0.54	1.20±0.83	1.25±0.67	S
	-	Paco	1.66±1.75	0.33±0.51	1.33±0.81	1.11±1.23	S
	-	Patra	3.00±0.00	2.00±1.26	2.40±0.89	2.47±1.08	S
	-	Peace Green	4.00±1.26	2.16±0.40	4.00±0.89	3.38±1.24	S
	-	Pelican	1.83±0.75	1.00±0.89	2.00±0.00	1.61±0.77	S

Supplementary table 2. (continued)

Type	Accession No.[z]	Cultivar or line	1st inoculation (2009)[y]	2nd inoculation (2010)	3rd inoculation (2011)	Total	Response[w]
	IT232545	Pink PangPang	2.83±0.40	1.40±0.89	2.00±0.00	2.11±0.78	S
	IT232565	Plaisir d'Amour	3.00±0.00	2.16±0.40	3.00±0.00	2.72±0.46	S
	FCH0086	Puma Sunny	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	S
	IT234093	Puma White	3.83±0.40	3.50±1.22	4.50±1.22	3.94±1.05	S
	FCH0045	Puma Yellow	4.83±0.40	5.00±0.00	5.00±0.00	4.94±0.23	S
	IT232575	Pure Angel	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	S
	IT245134	Relance	3.00±0.00	3.00±0.00	3.00±0.00	3.00±0.00	S
	FCH0171	Salinas	5.00±0.00	4.00±1.54	4.33±1.21	4.44±1.14	S
	-	Samos	2.33±0.51	2.16±0.40	2.16±1.16	2.22±0.73	S
	FCH0187	Sei Agness	2.16±0.40	2.16±1.16	3.33±1.75	2.55±1.29	S
	-	Sei Alps	3.50±0.54	3.33±0.81	3.33±0.81	3.38±0.69	S
	FCH0189	Sei Elza	5.00±0.00	5.00±0.00	5.00±0.00	5.00±1.17	S
	-	Sei Mariah	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	S
	-	Sei Monaco	2.33±1.03	2.83±0.98	2.33±1.50	2.50±1.15	S
	FCH0186	Sei Night	4.00±1.54	4.66±0.81	4.50±1.22	4.38±1.19	S
	IT232549	Sobaek	2.00±0.00	2.66±0.81	2.16±1.94	2.27±1.17	S
	FCH0060	Stailion	3.16±0.40	2.50±0.54	3.00±0.00	2.88±0.47	S
	IT234172	Swan	4.83±0.40	5.00±0.00	4.33±0.81	4.72±0.57	S
	FCH0205	Text	1.83±0.98	2.00±0.63	1.16±1.32	1.66±1.02	S
	-	Tokyo	1.83±0.75	4.66±0.51	2.66±1.36	3.05±1.51	S
	-	Topic	3.00±0.00	2.83±0.40	3.33±1.03	3.05±0.63	S
	IT234181	Tumaco	0.16±0.40	4.83±0.40	1.66±0.81	2.22±2.07	S
	-	Universe	3.83±0.98	4.00±0.00	5.00±0.00	4.27±0.75	S
	-	Weldon Dark	1.33±0.51	1.16±0.40	2.00±0.00	1.50±0.51	S
	-	Wembley	5.00±0.00	4.80±0.44	5.00±0.00	4.94±1.18	S
	IT232564	Whitney PangPang	1.83±0.40	3.16±0.98	0.66±0.81	1.88±1.27	S
	-	Zembla	0.83±0.40	1.83±0.75	2.00±1.26	1.55±0.98	S
	-	Zembla Lime	1.00±0.00	1.00±0.89	1.66±1.03	1.22±0.80	S

[z]Registration number in Rural Development Administration (RDA) Gene Bank Center in Korea.

[y]The year when inoculation was performed.

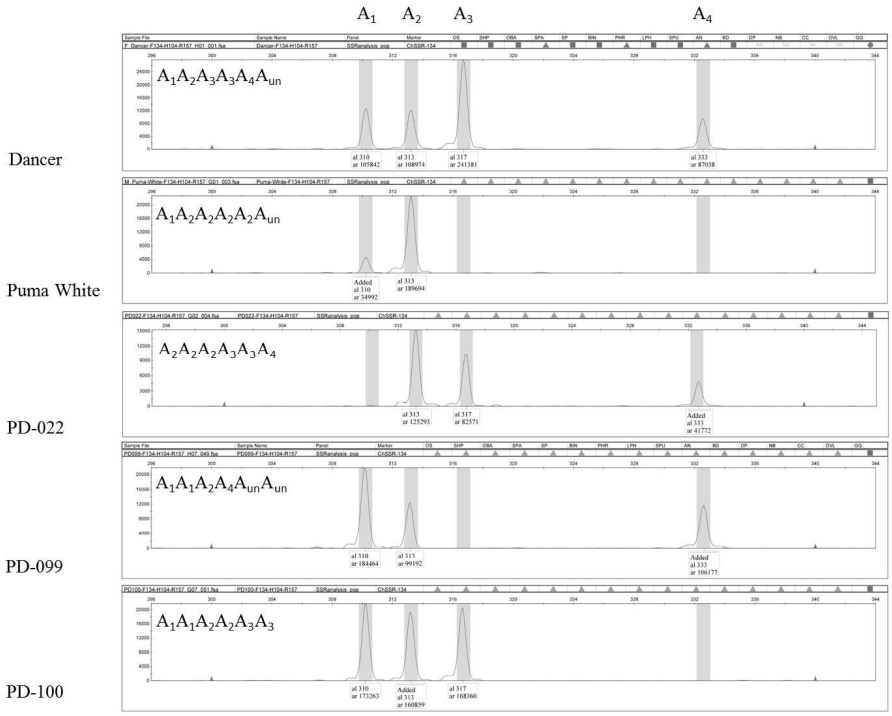
[x]Mean±SD

[w]The response was determined according to the average disease index as follows: 0.0, resistant (R); 0.0-1.0, moderately resistant (MR); 2.0-5.0, susceptible (S).

Supplementary table 3. Analysis of genotypic segregation ratio of each allele in ChSSR-134, and χ^2 goodness of fit test ($\alpha = 0.05$) using the expected segregation ratios of hexasomic and disomic inheritance.

Allele type	Presence/absence of allele		Observed number of individuals		Expected allele dosage		Expected ratio		Probability of χ^2 test
	Dancer	Puma White	No. of present	No. of absent	Dancer	Puma White			
ChSSR-134_A_1	+	+	67	26	1	1	hexasomic	3:1	0.510
							disomic	3:1	0.510
ChSSR-134_A_2	+	+	93	0	1	4	hexasomic	1:0	1.000
							disomic	1:0	1.000
ChSSR-134_A_3	+	-	72	21	2	0	hexasomic	4:1	0.534
							disomic	3:1	0.590
ChSSR-134_A_4	+	-	37	56	1	0	hexasomic	1:1	0.049
							disomic	1:1	0.049

A



B

Null hypothesis: If chrysanthemum is allohexaploidy,

In case of ChSSR-134:

Paternal cultivar 'Dancer' ($A_1A_2A_3A_3A_4A_{un}$)

Maternal cultivar 'Puma White' ($A_1A_2A_2A_2A_2A_{un}$)

PD-022 ($A_2A_2A_2A_3A_3A_4$): $A_3A_3A_4$ from 'Dancer', $A_2A_2A_2$ from 'Puma White'

PD-099 ($A_1A_1A_2A_4A_{un}A_{un}$): $A_1A_4A_{un}$ from 'Dancer', $A_1A_2A_{un}$ from 'Puma White'

→ Allelic pairs of 'Dancer': A_1A_3 / $A_3\_$ / $A_4\_$

However, PD-100 ($A_1A_1A_2A_2A_3A_3$): $A_1A_3A_3$ from 'Dancer', $A_1A_2A_2$ from 'Puma White' → Reject the null hypothesis

Supplementary fig. 1. Genotype analysis of 'Dancer', 'Puma White', and their three progenies, 'PD-022', 'PD-099', and 'PD-100', in ChSSR-134 locus (A) and diagram of possible chromosome pairing of 'Dancer' on the assumption of disomic mode of inheritance (B).

Supplementary table 4. Analysis of genotypic segregation ratio of each allele in ChSSR-157, and χ^2 goodness of fit test ($\alpha = 0.05$) using the expected segregation ratios of hexasomic and disomic inheritance.

Allele type	Presence/absence of allele		Observed number of individuals		Expected allele dosage		Expected ratio		Probability of χ^2 test
	Dancer	Puma White	No. of present	No. of absent	Dancer	Puma White			
ChSSR-157_A$_1$	-	+	39	54	0	1	hexasomic	1:1	0.120
							disomic	1:1	0.120
ChSSR-157_A$_2$	-	+	83	10	0	2	hexasomic	4:1	0.026
							disomic	3:1	0.002
ChSSR-157_A$_3$	-	+	50	43	0	1	hexasomic	1:1	0.468
							disomic	1:1	0.468
ChSSR-157_A$_4$	+	+	92	1	3	1	hexasomic	19:1	0.379
							disomic	7:1	0.039
ChSSR-157_A$_5$	+	-	73	20	2	0	hexasomic	4:1	0.717
							disomic	3:1	0.436

B **Null hypothesis: If chrysanthemum is allohexaploidy,**

In case of ChSSR-157:

Paternal cultivar 'Dancer' ($A_4A_4A_4A_5A_5A_{un}$)

Maternal cultivar 'Puma White' ($A_1A_2A_2A_3A_4A_{un}$)

PD-007 ($A_2A_2A_3A_4A_4A_5$): $A_4A_4A_5$ from 'Dancer' + $A_2A_2A_3$ from 'Puma White'

PD-023 ($A_1A_2A_3A_4A_4A_5$): $A_4A_4A_5$ from 'Dancer' + $A_1A_2A_3$ from 'Puma White'

→ Allelic pairs of 'Puma White': A_1A_2 / A_2_ / A_3_

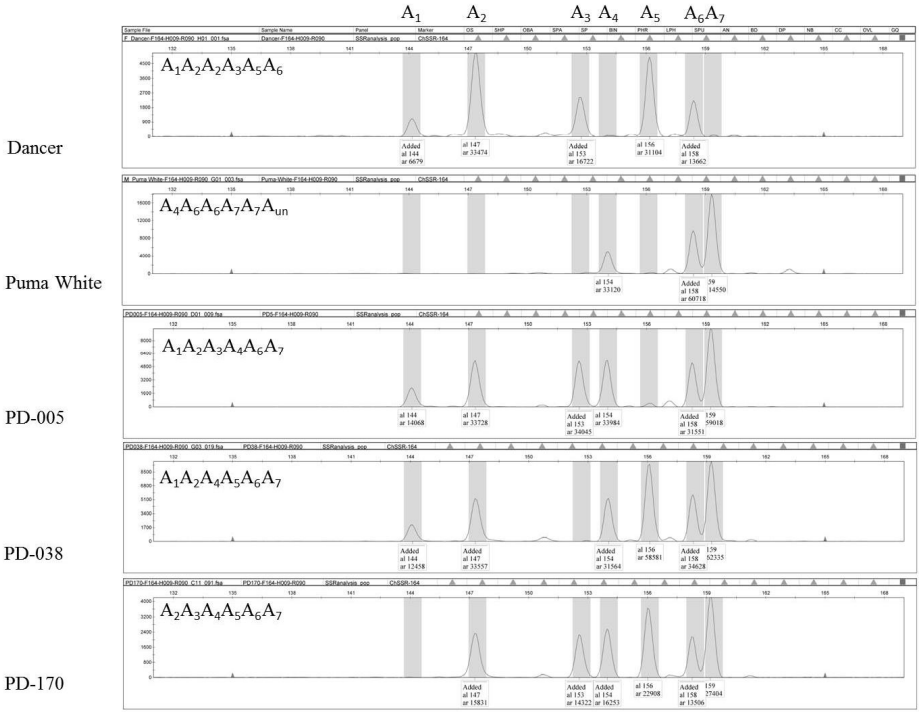
However, PD-149 ($A_1A_2A_2A_4A_5A_5$): $A_4A_5A_5$ from 'Dancer' + $A_1A_2A_2$ from 'Puma White' → Reject the null hypothesis

Supplementary fig. 2. Genotype analysis of 'Dancer', 'Puma White', and their three progenies, 'PD-007', 'PD-023', and 'PD-149', in ChSSR-157 locus (A) and diagram of possible chromosome pairing of 'Puma White' on the assumption of disomic mode of inheritance (B).

Supplementary table 5. Analysis of genotypic segregation ratio of each allele in ChSSR-164, and χ^2 goodness of fit test ($\alpha = 0.05$) using the expected segregation ratios of hexasomic and disomic inheritance.

Allele type	Presence/absence of allele		Observed number of individuals		Expected allele dosage		Expected ratio		Probability of χ^2 test
	Dancer	Puma White	No. of present	No. of absent	Dancer	Puma White			
ChSSR-164_A$_1$	+	-	47	46	1	0	hexasomic	1:1	0.917
							disomic	1:1	0.917
ChSSR-164_A$_2$	+	-	83	10	2	0	hexasomic	4:1	0.026
							disomic	3:1	0.002
ChSSR-164_A$_3$	+	-	48	45	1	0	hexasomic	1:1	0.756
							disomic	1:1	0.756
ChSSR-164_A$_4$	-	+	59	34	0	1	hexasomic	1:1	0.010
							disomic	1:1	0.010
ChSSR-164_A$_5$	+	-	47	46	1	0	hexasomic	1:1	0.917
							disomic	1:1	0.917
ChSSR-164_A$_6$	+	+	87	6	1	2	hexasomic	9:1	0.254
							disomic	7:1	0.078
ChSSR-164_A$_7$	-	+	76	17	0	2	hexasomic	4:1	0.678
							disomic	3:1	0.134

A



B **Null hypothesis: If chrysanthemum is allohexaploidy,**

In case of ChSSR-164:

Paternal cultivar 'Dancer' ($A_1A_2A_2A_3A_5A_6$)

Maternal cultivar 'Puma White' ($A_4A_6A_6A_7A_7A_{un}$)

PD-005 ($A_1A_2A_3A_4A_6A_7$): $A_1A_2A_3$ from 'Dancer' + $A_4A_6A_7$ from 'Puma White'

PD-038 ($A_1A_2A_4A_5A_6A_7$): $A_1A_2A_5$ from 'Dancer' + $A_4A_6A_7$ from 'Puma White'

→ Allelic pairs of 'Dancer': $A_1\_$ / $A_2\_$ / A_3A_5

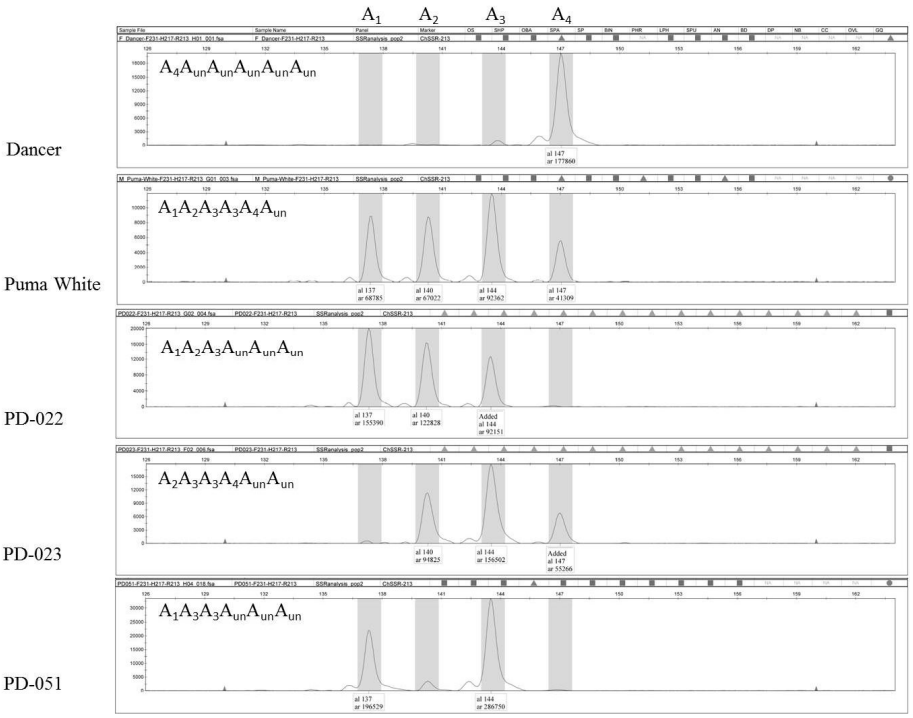
However, PD-170 ($A_2A_3A_4A_5A_6A_7$): $A_2A_3A_5$ from 'Dancer' + $A_4A_6A_7$ from 'Puma White' → Reject the null hypothesis

Supplementary fig. 3. Genotype analysis of 'Dancer', 'Puma White', and their three progenies, 'PD-005', 'PD-038', and 'PD-170', in ChSSR-164 locus (A) and diagram of possible chromosome pairing of 'Dancer' on the assumption of disomic mode of inheritance (B).

Supplementary table 6. Analysis of genotypic segregation ratio of each allele in ChSSR-213, and χ^2 goodness of fit test ($\alpha = 0.05$) using the expected segregation ratios of hexasomic and disomic inheritance.

Allele type	Presence/absence of allele		Observed number of individuals		Expected allele dosage		Expected ratio		Probability of χ^2 test
	Dancer	Puma White	No. of present	No. of absent	Dancer	Puma White			
ChSSR-213_A_1	-	+	48	44	0	1	hexasomic	1:1	0.677
							disomic	1:1	0.677
ChSSR-213_A_2	-	+	46	46	0	1	hexasomic	1:1	1.000
							disomic	1:1	1.000
ChSSR-213_A_3	-	+	74	18	0	2	hexasomic	4:1	0.917
							disomic	3:1	0.229
ChSSR-213_A_4	+	+	67	25	1	1	hexasomic	3:1	0.630
							disomic	3:1	0.630

A



B

Null hypothesis: If chrysanthemum is allohexaploidy,

In case of ChSSR-213:

Paternal cultivar 'Dancer' ($A_4A_{un}A_{un}A_{un}A_{un}A_{un}$)

Maternal cultivar 'Puma White' ($A_1A_2A_3A_3A_4A_{un}$)

PD-022 ($A_1A_2A_3A_{un}A_{un}A_{un}$): $A_{un}A_{un}A_{un}$ from 'Dancer', $A_1A_2A_3$ from 'Puma White'

PD-023 ($A_2A_3A_3A_4A_{un}A_{un}$): $A_4A_{un}A_{un}$ from 'Dancer', $A_2A_3A_3$ from 'Puma White'

→ Allelic pairs of 'Puma White': A_1A_3 / $A_2\_$ / $A_3\_$

However, PD-051 ($A_1A_3A_3A_{un}A_{un}A_{un}$): $A_{un}A_{un}A_{un}$ from 'Dancer', $A_1A_3A_3$ from 'Puma White' → Reject the null hypothesis

Supplementary fig. 4. Genotype analysis of 'Dancer', 'Puma White', and their three progenies, 'PD-022', 'PD-023', and 'PD-051', in ChSSR-213 locus (A) and diagram of possible chromosome pairing of 'Puma White' on the assumption of disomic mode of inheritance (B).

Supplementary table 7. Analysis of genotypic segregation ratio of each allele in ChSSR-239, and χ^2 goodness of fit test ($\alpha = 0.05$) using the expected segregation ratios of hexasomic and disomic inheritance.

Allele type	Presence/absence of allele		Observed number of individuals		Expected allele dosage		Expected ratio		Probability of χ^2 test
	Dancer	Puma White	No. of present	No. of absent	Dancer	Puma White			
ChSSR-239_A$_1$	+	+	86	4	2	2	hexasomic	24:1	0.830
							disomic	15:1	0.479
ChSSR-239_A$_2$	+	-	44	46	1	0	hexasomic	1:1	0.833
							disomic	1:1	0.833
ChSSR-239_A$_3$	+	-	49	41	1	0	hexasomic	1:1	0.399
							disomic	1:1	0.399
ChSSR-239_A$_4$	-	+	47	43	0	1	hexasomic	1:1	0.673
							disomic	1:1	0.673
ChSSR-239_A$_5$	+	-	45	45	1	0	hexasomic	1:1	1.000
							disomic	1:1	1.000
ChSSR-239_A$_6$	-	+	42	48	0	1	hexasomic	1:1	0.527
							disomic	1:1	0.527
ChSSR-239_A$_7$	+	-	47	43	1	0	hexasomic	1:1	0.673
							disomic	1:1	0.673
ChSSR-239_A$_8$	-	+	45	45	0	1	hexasomic	1:1	1.000
							disomic	1:1	1.000

A

B

Null hypothesis: If chrysanthemum is allohexaploidy,

In case of ChSSR-239:

Paternal cultivar 'Dancer' ($A_1A_1A_2A_3A_5A_7$)

Maternal cultivar 'Puma White' ($A_1A_1A_4A_6A_8A_{un}$)

PD-004 ($A_1A_1A_2A_3A_5A_8$): $A_2A_3A_5$ from 'Dancer', $A_1A_1A_8$ from 'Puma White'

PD-045 ($A_1A_3A_5A_6A_7A_{un}$): $A_3A_5A_7$ from 'Dancer', $A_1A_6A_{un}$ from 'Puma White'

→ Allelic pairs of 'Dancer': A_2A_7 // A_3_ // A_5_

However, PD-128 ($A_1A_1A_2A_3A_4A_7$): $A_2A_3A_7$ from 'Dancer', $A_1A_1A_4$ from 'Puma White' → Reject the null hypothesis

Supplementary fig. 5. Genotype analysis of 'Dancer', 'Puma White', and their three progenies, 'PD-004', 'PD-045', and 'PD-128', in ChSSR-239 locus (A) and diagram of possible chromosome pairing of 'Dancer' on the assumption of disomic mode of inheritance (B).

ABSTRACT IN KOREAN

본 연구는 6배체 국화 재배종의 유전양상을 이해하고, 국화의 흰녹병 저항성 품종 육종효율 증진을 위한 분자표지를 개발하는데 목적을 두었다. 6배체(2n = 6x = 54) 국화는 배수성과 높은 이형접합성, 자가불화성 등으로 인해 유전 연구가 어려운 작물이다. 심지어는 국화의 염색체 구성이 이질배수성인지 동질배수성인지 대한 논의 조차도 아직 명확하게 밝혀지지 못하고 있는 실정이다. 따라서 본 연구에서는 simple sequence repeat (SSR) 마커를 이용하여 국화의 기본 유전 양상을 구명하고자 하였다. 총 49개의 다형성 SSR 마커를 이용하여 국화 'Dancer'와 'Puma White' 품종의 pseudo-F_1 교잡 후대집단의 유전자형을 분석하였고, 210개의 다형성 마커를 확인할 수 있었다. 이 중, 이질배수성과 동질배수성을 구별할 수 있는 유전분리비를 나타내는 65개 마커의 후대분석을 수행한 결과, 33개의 마커는 동질배수성의 유전분리비에 더 적합하였고, 24개는 이질배수성의 유전분리비에 적합하여 국화가 동질배수성일 가능성이 더 높게 분석되었다. Simplex 마커에 대한 non-simplex 마커의 비율도 80:20으로 동질배수성의 이론적 비율과 일치하였다. 결정적으로는 전체 대립유전자에 대한 유전자형 정보를 확인한 6개의 SSR 마커에서 SSR locus 내 대립유전자들이 감수분열 시 임의로 대합하여 유전되는 양상 통해서 국화가 동질배수성임을 확인하였다. 국화의 기본 유전양상을 기반으로, 국화 흰녹병에 감수성인 'Puma White'와 저항성인 'Dancer' 품종을 각각 모본과 부본으로 교잡한 pseudo-F_1 후대집단을 이용하여 국화 흰녹병 저항성에 대한 유전분석을 실시하였다. 총 188계통에 대하여 포자비산법을 이용한 국화 흰녹병 저항성 검정을 수행한 결과, 저항성과 감수성이

각각 161계통과 27계통으로 분리되었다. 저항성과 감수성의 4:1 (P = 0.05327, $\alpha = 0.05$) 분리비를 통해 동질배수성 국화의 흰녹병 저항성이 단일우성유전자에 의해 지배되며, 저항성 부본인 'Dancer' 품종의 경우 두 개의 우성대립유전자를 보유하고 있는 저항성 품종으로 분석되었다. 국화 흰녹병 저항성 품종의 육종효율을 증진하기 위한 분자표지는 bulked segregant analysis 방법을 이용하여 개발되었다. Pseudo-F_1 188계통의 국화 흰녹병 저항성 검정 결과를 바탕으로 저항성 10계통 및 감수성 10계통을 선발하고 각각의 DNA를 혼합하여 resistant bulk (R-bulk)와 susceptible bulk (S-bulk)를 만들고 교배양친 'Dancer', 'Puma White'와 함께 280개의 random amplified polymorphic DNA (RAPD), 256개의 amplified fragment length polymorphism 마커에 대한 프라이머 스크리닝을 수행하였다. 그 결과, 국화 흰녹병 저항성과 연관된 OPI-13_{520} RAPD 마커를 개발하였다. 선발된 OPI-13_{520} 마커에 대하여 pseudo-F_1 교잡후대집단 188계통에 대한 다형성을 확인한 결과, 병 저항성 검정 결과와 일치하지 않는 6개의 재조합 계통을 확인할 수 있었다. 동질6배체의 pseudo-F_1 교잡후대집단에서의 기대분리비를 기반으로 duplex 타입의 저항성(RRrrrr) 유전자가 duplex 타입의 OPI-13_{520} 마커(MMmmmm)와 상인연관($\chi^2 = 76.08$, $P = 2.13 \times 10^{-16}$)되어 있으며, 재조합가는 0.0383 ± 0.0271, 유전적 거리는 4.0cM으로 확인되었다. 국화 흰녹병 저항성 육종효율 증진을 위하여 OPI-13_{520} 마커는 유전자 클로닝 및 시퀀싱을 통해 얻어진 염기서열 정보를 바탕으로 sequence characterized amplified region 마커로 최종 전환되었다.