Conversion of Molecular Markers Linked to *Fusarium* and Virus Resistance in Asiatic Lily Hybrids

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Abstract

Lilies are one of the most economically important monocot flower bulbs. However, lily bulb production faces some challenges such as susceptibility to diseases caused by *Fusarium oxysporum* and Lily Mottle Virus (LMoV). These two are the most important pathogens that cause serious damage. Fortunately, some Asiatic lily hybrids show high level of resistance to *Fusarium* and LMoV. Transmission of these traits into the lily assortment through introgression is possible however combining several loci by conventional breeding will take a long time. Using marker-assisted selection (MAS) could speed up the breeding process considerably. A genetic map of an intraspecific Asiatic backcross population was constructed using three different molecular marker systems (DArT, AFLP and NBS profiling) and four QTLs for *Fusarium oxysporum* and one locus for LMoV were localized on this map. The most tightly linked markers to the most significant two *Fusarium* QTLs in the AA population were converted into more robust PCR markers. Thus, the first markers for MAS breeding in the very huge and highly repetitive genome of lily have been developed.

INTRODUCTION

Disease resistance is a significant feature in ornamental plants. *Fusarium oxysporum* and Lily Mottle Virus are considered as very serious diseases in *Lilium*. The genus *Lilium* belongs to Liliaceae family and comprises of more than 90 species (Asano, 1989), and thousands of cultivars (Int Lily register, 1985-2007). *Lilium* can be taxonomically divided into three main groups: Longiflorum (L), Asiatic (A) and Oriental (O) hybrids. Some cultivars of Asiatic lily hybrids show resistance to diseases caused by *Fusarium oxysporum* and LMoV.

Breeding for resistance in lily is limited by the long juvenile phase (2-3 years) and the fact that selection often takes many cycles of breeding in order to intogress desirable agronomic traits from different parents into a single cultivar. For that, development of molecular markers and using them for molecular assisted breeding are quite worthwhile.

In this study, we focused on converting markers closely linked to *Fusarium* and LMoV resistance into robust PCR markers for use in MAS (marker-assisted selection) breeding.

MATERIALS AND METHODS

Plant Material

A mapping population comprising of 100 backcross progenies of Asiatic hybrids (Straathof et al., 1996) was used for molecular study and QTL mapping. This population is the result of crossing two Asiatic cultivars. ‘Connecticut King’ the partial resistant parent to *Fusarium* and resistant to LMoV was crossed with ‘Orlito’ (resulting from crossing ‘Connecticut King’ with the susceptible parent ‘Pirate’).

Molecular Markers

Three different molecular marker types were produced and used for genetic map of the AA population. In 2002, the first genetic map of this population was published using AFLP marker in which they used two different restriction enzyme combinations (*Pst*I/*Mse*I and *EcoR*I/*Mse*I). Later on, two other sets of molecular markers were produced (NBS-profiling and DArT) and added to this genetic map. The nucleotide-binding site (NBS) profiling were produced according to Van der Linden et al. (2004) using two primers (NBS3 and NBS6) in combination with several restriction enzymes (*Mse*I, *Alu*I, *Taq*I, *Hae*III and *Rsa*I). In total, 278 NBS markers were generated. The other molecular marker set consists of markers using DArT (Diversity Arrays Technology) which is based on micro-arrays that allow screening hundreds of markers simultaneously. The dominant DArT markers were produced in the AA population (Khan, 2009) using two restriction enzyme combinations (*Pst*I/*Mse*I and *Pst*I/*Taq*I). The digested DNA fragments of 'Connecticut King' and 'Orlito' were individualized by insertion into bacterial vector and cloning. A total of 3072 cloned fragments of both parents resulted, 11% of them (338) were polymorphic and were used in mapping. As a result, 33 linkage groups were constructed using JoinMap 3.0 software (Van Ooijen and Voorrips, 2001).

QTL Analysis

Four QTLs for *Fusarium* resistance were identified in the AA genetic map, in addition to single dominant locus for LMoV (van Heusden et al., 2002) using MapQTL Version 4.0 (Van Ooijen et al., 2000). The four QTLs with the level of significance are presented in Table 1.

Conversion of DArT Markers

Colony PCR using forward and reverse primers within the multiple cloning site of the TOPO-vector (Invitrogen) was carried out to sequence DArT markers of interest. The colony PCR reaction 20 µl consists of: 1 µl of culture (liquid media of the DArT clone), 2 µl of each universal forward and reverse primer (10 pmol/µl, M13), 0.8 µl of 5 mM dNTPs, 2 µl of 10× superTaq buffer, 0.08 µl of Taq polymerase (Supertaq) and 12.12 µl H_2O. The next step was to sequence the amplified fragments. The sequence reaction 10 µl contains: 1 µl of DNA fragment, 4 µl BigDye mixA, 0.5 µl of M13 (10 pmol/µl) forward or reverse primer and 4.5 µl of H_2O.

Primers based on DArT marker sequences were tested on 100 individuals from the AA population and the parents. Segregation patterns of the PCR fragment were compared with the scoring of the DArT marker.

RESULTS

Conversion of *Fusarium* QTLs

1. First QTL of *Fusarium* Resistance. The most significant QTL for *Fusarium* resistance (Table 1) is located on linkage group 5 of the AFLP genetic map (van Heusden et al., 2002), and on linkage group 4 of the new generated genetic map after addition of NBS and DArT markers (Fig. 1). This QTL was flanked by two AFLP markers: CKP31M52-12 and CKP31M59-21, and in between three DArT markers (CKPstTaq_73, CKPstTaq_92 and CKPstTaq_205) localized. After running colony PCR and sequencing reaction for CKPstTaq_92 and CKPstTaq_205, we found out that these two fragments have similar sequences with minor indels differing between them. Two different primer combinations were designed based upon these indels and tested in the parents ('Connecticut King' and 'Orlito') and the progeny. The amplified fragments behave like a dominant marker with 1:1 segregation from 'Connecticut King' (Fig. 2). Similarly, CKPstTaq_73 marker was sequenced and many primer combinations were designed and tested. However, only one succeeded in reflecting the segregation pattern of the original DArT marker.

2. Second QTL of *Fusarium* Resistance. A second QTL for *Fusarium* resistance in lily is located on the first linkage group of the AFLP genetic map and on the 7th linkage group of the new map of the AA population (Fig. 3). This QTL was detected with two AFLP markers (CKE40M52A-27 and CKE41M52A-35), and one DArT marker (CKPstTaq_116) localized within this QTL. Our work was focused on the conversion of this DArT marker. The primer combination that was designed according to the sequence of this DArT marker generated monomorphic bands in the parents. For that, the amplified bands in 'Connecticut King' as well as in 'Orlito' were sequenced and some SNPs (single-nucleotide polymorphism) were detected. The SNPs were used to design specific primers for the resistant parent. The new primer combination was able to generate a polymorphic fragment which segregation fits with the segregation of the original CKPstTaq_116 DArT marker.

LMoV Resistance

The resistance to LMoV was found on the top of linkage group 9, and the nearest marker (AFLP marker) was 9cM far away (van Heusden et al., 2002). Later on, this resistance was detected on linkage group 20 in the new genetic map and the nearest marker is a NBS marker (3md34, 7.5cM) (Fig. 4). This NBS marker was segregated in the two parents (3:1 segregation in the population). The marker length was expected to be around 600 bp. In order to produce a PCR marker, this band was excised from the gel for both parents and sequenced. Several SNPs were detected between the parents and they were used to develop a CAPS (cleaved amplified polymorphic sequence) marker. Unfortunately, this effort did not succeed in producing a PCR marker for LMoV.

DISCUSSION

Developing and mapping of new molecular markers in lily is quite important. The new molecular markers techniques (NBS profiling and DArT markers), provided new information and covered more parts of lily's genome. For that, a better quality genetic map was produced which give more possibility to proceed with genetic and breeding work. In the AA population, the first genetic linkage maps were produced using AFLP markers. AFLP technology generates a lot of polymorphism. However, converting and reproducing these markers are time consuming and rather difficult in a genome which has highly repetitive DNA like lily.

NBS-profiling is based on the fact that resistance genes generally have highly conserved motifs, which were used to design primers and amplify candidate genes for resistance in different species (Calenge et al., 2005). In lily, NBS markers generated many polymorphic bands. One of these markers was linked to LMoV resistance. However, the challenging point in this marker is that a considerable part of the obtained sequence consists of a know retro-element. The conversion of this marker was unsuccessful until now mainly because of the repetitive nature of the DNA.

Diversity array technology (DArT) was developed originally in rice (Jaccoud et al., 2001), and has been applied successfully for several crop such as *Sorghum bicolor* (Mace et al., 2008). Production and mapping of DArT markers were of a great help to accomplish the goal of this study. Since DArT markers were ligated and inserted into *E. coli* bacteria, the amplification and sequencing of these markers can be achieved much easily as compared with other marker types such as AFLP and NBS. The bigger length of the DArT fragments (average 1 kb) enhances the chance to find polymorphisms and the hybridization step is likely crucial in selection against highly repetitive sequences.

For two out of the four QTLs for *Fusarium* robust PCR markers were produced. Unfortunately, however, the other two were not associated with any DArT marker. Therefore, no effort was done to convert these QTLs. Later on, more molecular markers will be produced hoping to end up with markers within these two areas as well. The converted markers efficiency will be tested in a wide set of germplasm to validate them, which is the next step before using them in breeding programs.

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Tables

Table 1. List of chromosomal regions with a significant linkage to *Fusarium* resistance in 1992, 1993, 1994 or 1999 (Van Heusden et al., 2002).

Linkage group (and position on map)	1992	1993	1994	1999	Marker in region with highest linkage
Linkage group 1 (17-27 cM)	****	*	-	****	E41M52A-35, E40M52A-27
Linkage group 5 (65-90 cM)	******	****	**	*****	P31M52-12, P31M59-21
Linkage group 13 (35-50 cM)	***	**	**	****	E41M52A-1
Linkage group 16 (0-20 cM)	***	****	*	**	E41M52A-11, P31M55-1

-, *, **, ***, ****, *****, ******, Nonsignificant or significant at P=0.1, 0.05, 0.01, 0.005, 0.001, or 0.0005 respectively by Kruskal-Wallis test.

Figures

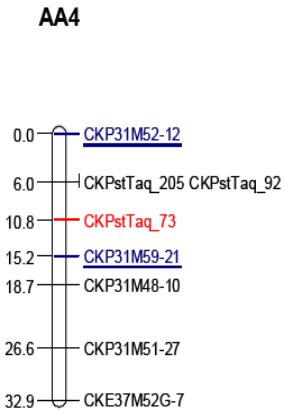

Fig. 1. LG 4 of AA population.

Fig. 2. The segregation of CKPstTaq_205 in some genotypes of AA population.

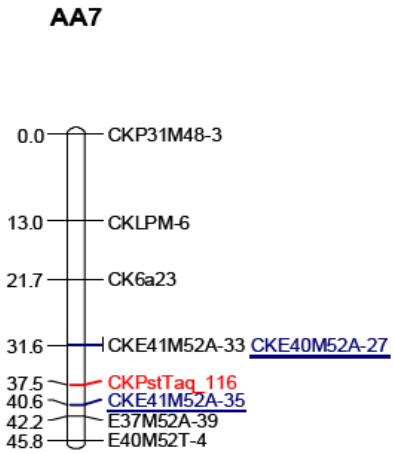

Fig. 3. LG 7 of the AA population.

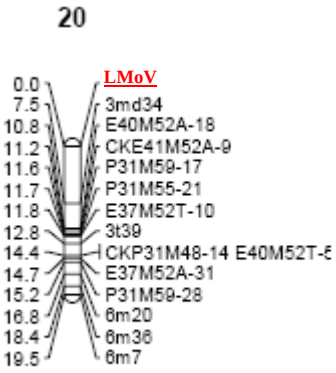

Fig. 4. LG 20 of the AA population.