Europ.J.Hort.Sci., **79** (4). S. 233–239, 2014, ISSN 1611-4426. © Verlag Eugen Ulmer KG, Stuttgart

Pyramiding Multiple Genes for Resistance to PVY, TSWV and PMMoV in Pepper Using Molecular Markers

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Summary

Pepper (*Capsicum annuum* L.) is one of the most important vegetables cultivated worldwide. Many pests and pathogens cause economic yield losses in pepper. Potato virus Y (PVY), Tomato spotted wilt virus (TSWV) and Pepper mild mottle virus (PMMoV) are considered among the most destructive viruses affecting pepper in the world. Because chemical treatments have limited success for managing PVY, TSWV and PMMoV, resistant varieties are considered to be the most effective means of controlling these viruses. In this study, resistance genes to these viruses were successfully transferred to the superior sweet Charleston pepper line 'Y-CAR' using molecular markers and biological assays. As a result, a new line which is resistant to PVY, TSWV and PMMoV was developed. The results also showed the applicability of a pyramiding strategy for breeding multiple virus resistance in pepper.

Key words. *Capsicum annuum* – marker – pyramiding – resistance – vegetable – virus

Introduction

Pepper (*Capsicum annuum* L.) is one of the most widely grown and economically important vegetables in the world. Its production is approximately 30 million tons per year worldwide. Turkey ranks third in annual pepper production with 1.9 million tons (FAO 2011). Several viruses affecting pepper cause yield losses. Potato virus Y (PVY), Tomato Spotted wilt virus (TSWV) and Pepper mild mottle virus (PMMoV) are considered as the most problematic viruses in pepper growing areas (Kim et al. 2008; Janzac et al. 2009; Scholthof et al. 2011).

Potato Virus Y (PVY) is the type member of the genus *Potyvirus* and is one of the most important viruses infecting pepper. This virus is transmitted by various aphid species, but the green peach aphid, *Myzus persicae* (Sulzer), is generally considered to be the most important vector (Janzac et al. 2008, 2009). PVY has three pathotypes, designated PVY-0, PVY-1, and PVY 1–2 according to their virulence on pepper genotypes (Kyle and Palloix 1997; Caranta et al. 1999). Since no chemical treatments exist to limit losses caused by PVY infections, one way to control the disease is the use of PVY resistant varieties. Although seven potyvirus resistance genes have been described in pepper, one locus called Pvr4 has been reported to confer dominant inheritance of resistance to all three pathotypes of PVY (Dogimont et al. 1996). This resistance gene was derived from 'Criollo de Morelos 334' (CM334) and has been transferred into many pepper varieties by breeding programs (Janzac et al. 2009).

Tomato spotted wilt virus (TSWV) attacks pepper and tomato especially in vegetable growing areas where the vector species *Frankliniella occidentalis* (Western flower thrips) is widespread (Pappu et al. 2009). The single dominant gene controlling resistance in accessions PI 152225 and PI 159236 of *Capsicum chinense* is designated by the symbol Tsw. This gene can control systemic spread of the virus by a hypersensitive response (Moury et al. 1997, 2000; Suzuki et al. 2003). This gene has been introgressed into some cultivated pepper varieties such as 'Mostar', 'Mertcan', 'Samuray', etc. (Yuksel Seed).

Pepper mild mottle virus belongs to the tobamovirus group and is transmitted by hand, equipment (Matsunaga et al. 2003), irrigation (Choi et al. 2004), contaminated soil (Pares and Gunn 1989) and seed (Ikegashira et al. 2004; Kim et al. 2008). This virus is divided into two pathotypes, $P_{1.2}$ and $P_{1.2.3}$, according to symptomatic reactions (Boukema 1980). Since chemical, physical and cultural methods have limited success for controlling PMMoV, resistant varieties are considered to be the most

effective means of control. The L loci carry resistance genes to PMMoV. Genes L3 and L4 have been widely used to control PMMoV in pepper breeding programs. Plants bearing the L3 gene are resistant to pathotype $P_{1.2}$, but susceptible to $P_{1.2.3}$ (Matsunaga et al. 2003). On the contrary, plants with the L4 gene are resistant to both pathotypes (Matsunaga et al. 2003; Kim et al. 2008). Pepper varieties carrying this gene (L4 gene) were bred and are recently become commercially available.

Virus screening tests for pepper resistance breeding are useful tools. However, they are time-consuming, expensive, laborious, and much space is required to test plants. These drawbacks can be overcome by the use of molecular markers for resistance genes. Molecular markers can detect the desired alleles at an early stage of the plant life cycle and reduce time for phenotypic assessment. As many molecular markers are co-dominant, they can distinguish homozygous and heterozygous resistant plants and also present an opportunity for efficient pyramiding of resistance genes in plants. Pyramiding of resistance genes in plants has been reviewed with molecular markers and biological assay (Ye and Smith 2008).

PVY, TSWV and PMMoV are all present in pepper production areas and together cause important yield losses in the world. Therefore, development of resistant commercial varieties is required for controlling these viruses. The aim of the present study was I) to use molecular markers closely linked to Pvr4, Tsw and L4 loci in pepper breeding program and II) to pyramid and transfer the three genes into a superior breeding line.

Materials and Methods

Plant Material

A pepper (*Capsicum annuum* L.) breeding line, 'Y-CAR', previously obtained from a pedigree program carried out at Yuksel Seed Ltd (Antalya, Turkey) was used as recipient in the backcross program. 'Y-CAR' is a Charleston type sweet pepper with superior agronomic characters but no resistance to PVY, TSWV or PMMoV. 'LET-1', a sweet Charleston type; 'ENT-1', a white Hungarian type and 'RAZ-1', a sweet lamuyo type, were used as resistance sources for TSWV, PMMoV and PVY, respectively (Fig. 1A, B, C). Seedlings of pepper breeding lines were grown in controlled seedling nursery. The seedlings were transplanted from nursery to insect-proof greenhouse and grown for harvest. The pepper plants were planted within the rows 50 cm and between the rows 125 cm wide. Disease-pest control and irrigation were carried out according to practice.

Virus Isolates

Isolates of PVY pathotype 1–2, TSWV and PMMoV pathotype 1.2.3 were kindly provided by Eric Verdin (INRA-PACA-France).

Biological Assay

The isolate of PVY pathotype 1–2 was multiplied in *Capsicum annuum* line 'Y-CAR' according to previous methods (Boiteux et al. 1996; Dhawan et al. 1996; Echer and Costa 2002). Isolates of TSWV and pathotype 1.2.3 of PMMoV were propagated in *Nicotiana benthamiana* (Jahn et al. 2000; Suzuki et al. 2003; Gilardi et al. 2004). A total of 2000 pepper seedlings were tested by these virus isolates during this study.

Inoculum of the three viruses was prepared by homogenizing infected leaves in 0.01 M phosphate buffer (pH 7.0) containing 0.2 % sodium sulfite. Then, 600 mesh carborundum was added. Cotyledons of test plants were inoculated at the cotyledonary to two true leaf stage (Moury et al. 1997, 1998; Kim et al. 2008; Janzac et al. 2009). After inoculation, plants were kept in a growth chamber at 22 °C with a 16 h photoperiod. Inoculations were repeated 3–7 days later.

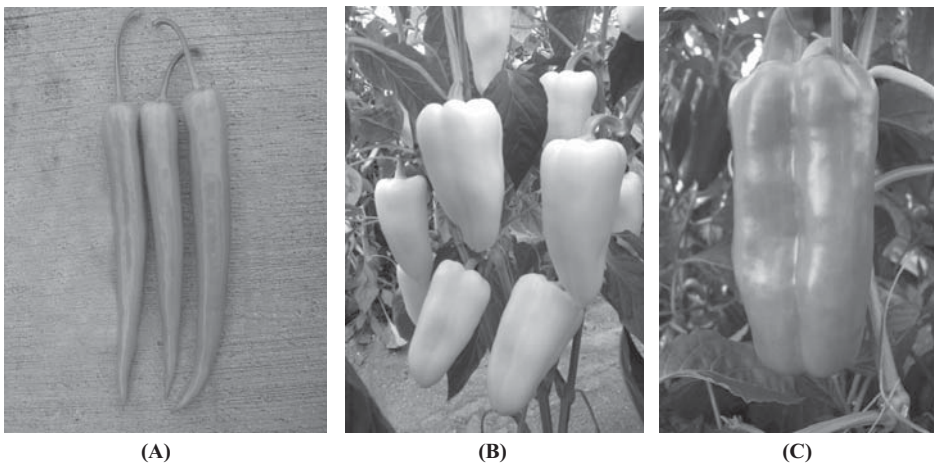


Fig. 1. Pepper lines used as resistance sources for TSWV, PMMoV and PVY. A: LET-1; B: ENT-1; C: RAZ-1.

Test plants were evaluated for symptom expression for 3–4 weeks after inoculation. Inoculated plants showing diseases symptoms on their uninoculated leaves were rated as susceptible. Plants without any symptoms on the uninoculated leaves were accepted phenotypically resistant. Resistance of those plants was confirmed by DAS-ELISA (Clark and Adams 1977).

Molecular Analysis

DNA Isolation. Genomic DNA was isolated from young fresh leaves of pepper by using the Wizard Magnetic Kit (Promega) following the manufacturer's instructions.

Molecular Markers. PCR primer sequences are listed in Table 1. All PCR reactions were set up in a total volume of 25 µl containing 20 ng of genomic DNA, each forward and reverse primer at 0.4 µM, 1xPCR Buffer, 2 mM $MgCI_2$, 0.4 mM dNTPs and 1 U of Taq DNA polymerase (Vivantis) and performed in the thermocycler (PTC-200, MJ Research, USA). The cycling conditions were: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 54 °C for 30 s (CSO and $SCAC_{568}$), 56 °C for 30 s (AP-7/AP-8) and 72 °C for 1 min, and 72 °C for 7 min as a final extension. PCR products were separated on a 2 % agarose gel containing TAE buffer at 110 V for 2 h, and visualized under UV light after staining with ethidium bromide.

PCR products of $SCAC_{568}$ and CSO were digested by XbaI and AlwNI restriction enzymes following manufacturer's instructions, respectively. Electrophoresis was conducted on a 2.5 % agarose gel.

Results

In order to screen the Pvr4 gene, the marker developed by Caranta et al. (1999) was tested, however the marker data were not in accordance with the results of biological assays. Therefore, inoculation tests were performed to determine the PVY resistance of plants. Since the results for molecular markers linked to the L4 and Tsw genes agreed with bioassay tests, they were used to screen PMMoV and TSWV resistance in the pepper lines. PCR results of molecular markers linked to Pvr4, Tsw and L4 genes are shown in Fig. 2–4.

In autumn 2008, superior line 'Y-CAR' was separately crossed with donor resistant parents 'ENT-1' 'LET-1' and 'RAZ-1', and F_1 plants were obtained (Fig. 5). A total of 24 F_1 plants were analysed by molecular markers for the L4 and Tsw genes. Another 24 F_1 plants resistant to PVY were also tested by inoculation with PVY in spring 2009. All plants were genetically heterozygous and resistant to PMMoV, TSWV and PVY. The resistant F_1 plants were backcrossed with the recurrent parent (Fig. 5).

In 2009, BC_1F_1 lines were grown and tested with the TSWV isolate and the resistant lines were selected. These lines were also confirmed by molecular marker to be heterozygous for the resistant locus. BC_1F_1 lines were inoculated with PMMoV and the resistant ones were selected. The same lines were verified for the L4 resistance gene with respect to molecular markers. BC_1F_1 lines were inoculated with PVY, and the resistant ones were determined. All testing results were analysed according to chi-square test and fit the expected Mendelian ratios (data not show). Since the PVY resistance source, 'RAZ-1', is morphologically different from a Charleston type of pepper, the BC_1F_1 lines were backcrossed to 'Y-CAR' to obtain lines which are similar to the desired type (Fig. 5). Two BC_2F_1 lines, which were both resistant to PVY and similar to 'Y-CAR' for agronomic characters, plant and fruit type were selected. BC_1F_1 lines, which were resistant to TSWV and PMMoV were selected and crossed with each other. Two double resistant lines carrying L4 and Tsw were chosen during harvest (Fig. 5).

In spring 2010, BC_1F_1 seedlings obtained from the two selected lines carrying the L4 and Tsw resistance genes were grown and genotyped using the appropriate molecular markers. Double resistant lines for L4 and Tsw were chosen. These lines were selfed, and four cross combinations including lines broadly similar in agronomic, plant and fruit characteristics to 'Y-CAR' were selected. Furthermore, BC_2F_1 lines were grown and inoculated with PVY,

Table 1. Primer sequences used for PCR amplification of the Pvr4, Tsw and L4 loci.

Primer name	Primer sequences (5–3)	PCR product (bp)	Locus	Restriction enzyme	References
CSO	CGAAGAGAGAAGGTC TCAGGGTAGGTTATT	458	Pvr4	AlwNI	Caranta et al. 1999
$SCAC_{568}$	GTGCCAGAGGAGGATTTAT GCGAGGTGGACACTGATACT	568	Tsw	XbaI	Moury et al. 2000
AP-7/AP-8	CGTACTGTGGCTCAAAACTC ATTCGCACCGTTTAGCCCGT	1400	L4	–	Matsunaga et al. 2003

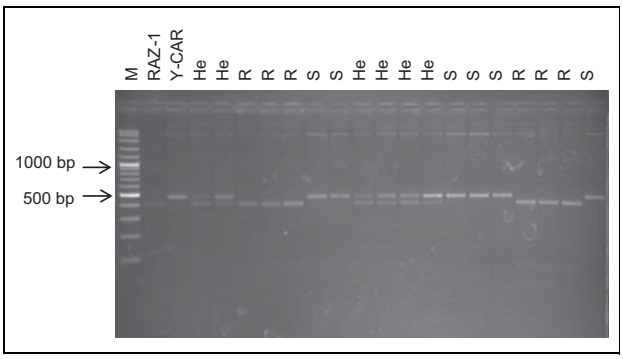


Fig. 2. Digestion of PCR products obtained CSO primer by AlwNI restriction enzyme. M: Molecular marker; RAZ-1: Resistant parent to PVY; Y-CAR: Susceptible parent to PVY; S: Homozygous susceptible; R: Homozygous resistant and He: Heterozygous resistant.

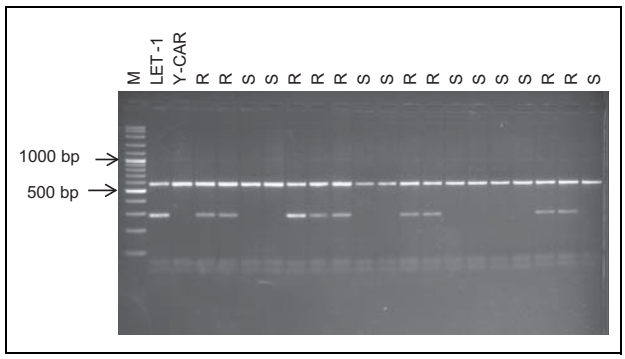


Fig. 3. Digestion of PCR products obtained $SCAC_{568}$ primer by XbaI restriction enzyme. M: Molecular marker; LET-1: Resistant parent to TSWV; Y-CAR: Susceptible parent to TSWV; S: Homozygous susceptible; R: Homozygous or heterozygous resistant.

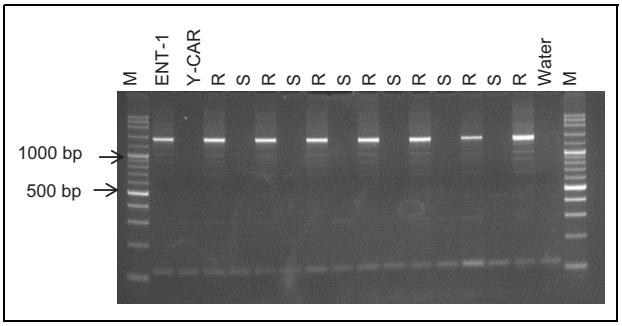


Fig. 4. Digestion of PCR products obtained AP-7/AP-8 primers. M: Molecular marker; ENT-1: Resistant parent to PMMoV; Y-CAR: Susceptible parent to PMMoV; S: Homozygous susceptible; R: Homozygous or heterozygous resistant.

and the resistant lines selected were selfed in the greenhouse. Then, the PVY resistant lines were evaluated for plant and fruit properties, and two lines, which were similar to 'Y-CAR' were selected (Fig. 5).

In autumn 2010, the lines derived from the four cross combinations were screened with molecular markers. Based on these results, the lines with L4 and Tsw were selected. For PVY, the lines derived from two cross combinations were tested by biological assay and the resistant ones to PVY were determined. Lines harbouring both L4 and Tsw, and also ones resistant to PVY were grown in the greenhouse. Lines similar to 'Y-CAR' were identified after three or four fruits developed. Twenty PVY resistant and five L4 and Tsw resistant lines were selected and used as mother and father lines, respectively. They were crossed with each other, and lines resistant to PMMoV, TSWV and PVY were developed. Two cross combinations were selected from the lines which were similar to 'Y-CAR' (Fig. 5).

The selected two lines resistant to PMMoV, TSWV and PVY were selfed and selected from spring 2011 to 2013. In order to evaluate homozygosity, 24 plants from each one of the selected lines were tested for PVY, and screened via markers for Tsw and L4, simultaneously. The homozygous plants for PVY, Tsw and L4 were chosen (Fig. 5). In this way, Charleston type multivirus resistant lines were developed (Fig. 6).

Discussion

The use of genetic resistance is the most effective and economic way of controlling virus diseases in plants such as pepper. Many viruses infecting peppers cause economically important yield losses. Therefore, commercial pepper varieties should bear more than one resistance gene for controlling these pathogens. Gene pyramiding strategies can allow accumulation of resistance genes in a single genotype. The strategy can be accomplished by using major genes and different or the same alleles of one gene (Tan et al. 2010). Pyramiding strategies have been used for resistance to pathogens in several crops. Barloy et al. (2006) showed that pyramiding resistance genes CreX and CreY gave a higher level of resistance against cereal cyst nematodes in wheat. Several studies were reported on pyramiding of bacterial blight resistance genes in rice (Huang et al. 1997; Hittalmani et al. 2000; Singh et al. 2001; Zhang et al. 2006). Gene pyramiding has also been used as an effective approach to achieving multiple and durable resistance to various viruses (Joshi and Nayak 2010). In the present study, the genes, which confer resistance to PVY, TSWV and PMMoV were successfully combined in superior pepper lines using molecular marker and virus resistance assays.

PVY is one of the most common viruses infecting peppers. PVY resistance genes named "*pvr*" have been described in pepper. Although there are many genes for controlling PVY, the Pvr4 locus confers resistance to the three pathotypes of potato virus Y and also to pepper mottle virus (PepMoV). In order to use this gene in a

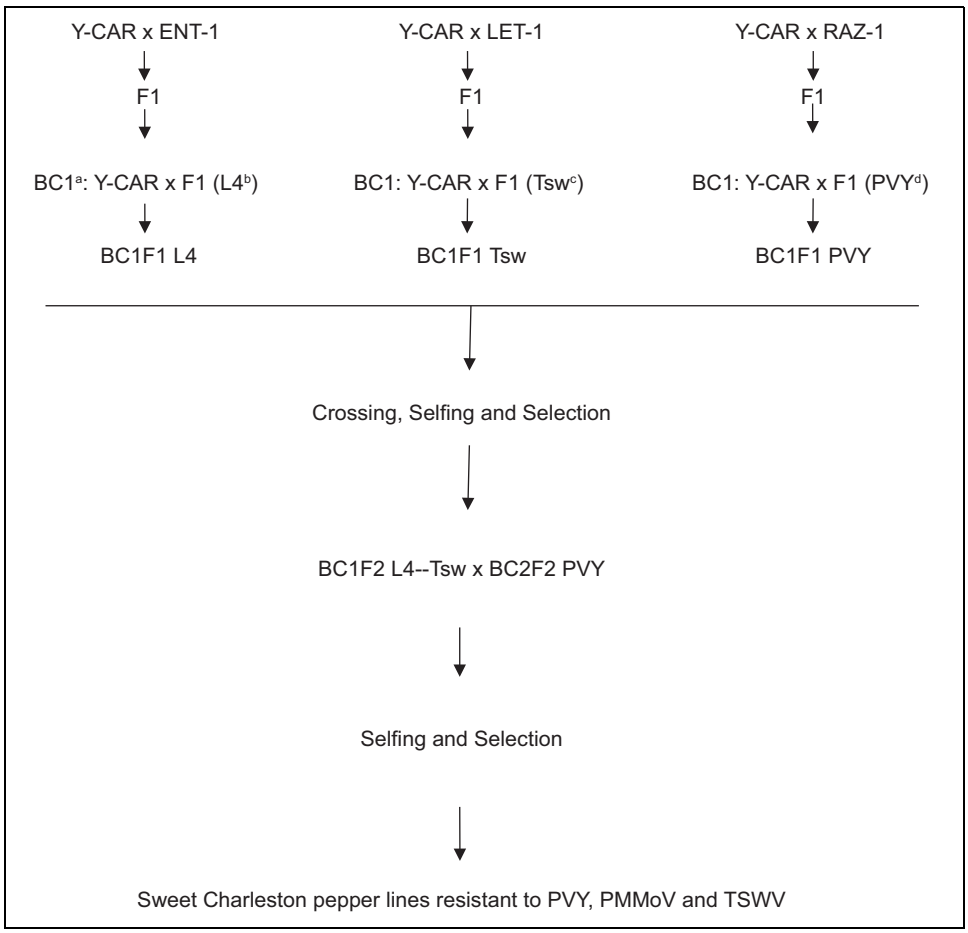


Fig. 5. Pyramiding of genes conferring resistance to PMMoV, PVY and TSWV in sweet Charleston pepper lines ([a]BC: Backcrossing; [b]L4: The gene conferring resistance to PMMoV pathotype 1.2.3; [c]Tsw: The gene conferring resistance to TSWV; [d]PVY: Potato Virus Y pathotype 1–2).



Fig. 6. New line resistant to PVY, PMMoV and TSWV.

marker-assisted selection program, a co-dominant molecular marker, which is linked to PVY resistance was developed (Caranta et al. 1999). The marker was used in parental lines and some commercial varieties for optimization but molecular marker results were not in accordance with inoculation tests. It is supposed that this disagreement could be due to recombination between the marker and Pvr4 locus. Therefore, this marker was not used for screening of breeding lines and plants were tested with a biological assay for PVY resistance. The bioassay results were in accordance with the expected genetic data according to chi square analysis.

The Tsw gene conferring resistance to TSWV was derived from *Capsicum chinense* and introgressed into cultivated peppers (Costa et al. 1995). It is a single dominant gene and confers hypersensitive resistance to TSWV. The screening of breeding lines with a bioassay for TSWV presents some difficulties. But these problems can be overcome by using molecular markers tightly linked to the Tsw gene for marker-assisted selection (Moury et al. 2000). Since this marker indicates if the resistance gene exists in different pepper sources, it was used for screening of resistance of segregating progenies during the breeding process. Both the molecular marker and inoculation tests were used at different stages of breeding and their results agreed with each other.

The gene L4 confers resistance to both $P_{1.2}$ and $P_{1.2.3}$ pathotypes of PMMoV (Matsunaga et al. 2003). Dominant and co-dominant molecular markers linked to the locus of the L4 gene have been developed (Matsunaga et al.

2003; Kim et al. 2008; Yang et al. 2009). Co-dominant markers are more informative than dominant markers in breeding programs. However, dominant marker was used for analysis of the L4 gene in this study. Our molecular marker results were in accordance with bioassay results. Since co-dominant marker for screening of L4 gene did not show accordance with bioassay assay in our previous studies (unpublished data), the marker was not used in this study.

Some commercial pepper cultivars carry resistance to PMMoV, TSWV and PVY. In general, one parent of hybrid cultivars has only one or two and rarely three virus or disease resistances in pepper. Crossing of two parents combines multivirus resistance in one F1 cultivar. In our study, resistance genes for PMMoV, TSWV and PVY were genetically combined in one superior pepper line by pyramiding via marker assisted backcross selection and resistance assays.

In conclusion, a new line resistant to PVY, TSWV and PMMoV was developed. Molecular markers and biological assays are routinely used for many crop species by public and private breeders. The employment of this system in our pepper breeding program will allow the efficient development of new virus resistant pepper varieties.

Acknowledgments

We gratefully acknowledge the financial support of the Scientific and Technological Research Council of Turkey by its TEYDEP foundation (Project 3080171). We would like to acknowledge Anne Frary (İzmir Institute of Technology, İzmir, Turkey) for many comments and critical reading of the manuscript.

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Received 10/07/2013 / Accepted 03/31/2014

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