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Review

Review on the development of virus resistant plants in *Alstroemeria*

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Abstract This review describes the stratagies of development of virus-resistant *Alstroemeria* plants using the genetic modification system. Despite of increasing of its importance in cut flower market, improvements of some horticultuirally important traits such as fragrance, long vase-life, virus resistance and tolerance against abiotic stresses are lack of the breeding program in *Alstroemeria*. Of these traits, virus-resistance is quite difficult to develop in *Alstroemeria* plants due to the limitations of genetic variation in the existed germplasm. To extend the genetic variation, plant biotechnological techniques such as genetic transformation and tissue culture should be combined to develop virus-resistant line in *Alstroemeria*. In this review, several strategies for the generation of virus-resistance by using natural resistance genes, pathogen-derived genes and other sources including pathogen-derived proteins, virus-specific antibodies and ribosome-inactivating proteins are presented. Also, brief histories of breeding, tissue culture, and transformation system in *Alstroemeria* plants are described to inderstand of the application of transgenic approach for the development of virus-resistance in *Alstroemeria* species.

Keywords *Alstroemeria*, Breeding, Meristem culture, Virus-resistance, Transformation

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Introduction

Alstroemeria and its position in the cut flower market in the world

During the last two decades, *Alstroemeria* has been one of the most commercially successful ornamental cut flowers in Japan, the Netherlands, the U.K., and the USA. Especially, characteristics like long vase-life, large color variety and a low energy required during cultivation have stimulated this success. The production of *Alstroemeria* flowers has been rapidly increasing in Europe and other parts of the world (Spence et al. 2000). Up to now, a huge number of cultivars have been released on the commercial market mainly as cut flowers, however, *Alstroemeria* plants are also known as pot and garden plants on a small scale (Van Schaik 1998).

In the year 2004, *Alstroemeria* cut flowers ranked in the 10th position of the sales volume in U.S.A with about 8.6 million stems (http://www.gardenguides.com/86199-top-10-flowers.html). Recently, it has ranked in the 10th position at the auction in Aalsmeer, The Netherlands in 2008 (http://www.floraholland.com).

Breeding history of *Alstroemeria*

Breeding programs of *Alstroemeria* have been focused on the production of cut flowers. In the early 1950s, three *Alstroemeria* species were released into Europe − *A. pelegrina*, *A. ligtu* and *A. aurea*. Since then, the interest in *Alstroemeria* as an ornamental has increased. The commercial quality of this first *Alstroemeria* was poor due to the short flowering period, bad quality of stem and leaf. Nevertheless, these first *Alstroemeria*s were most probably the ancestors of the modern hybrids that were often produced after

crossing with wild species.

Currently, the *Alstroemeria* cultivars can be divided into three types. One of these - the "Orchid type"- has open flowers with a long flowering period. "Orchid type" plants are diploid (2n=2x=16) and almost sterile, whereas they are easily propagated *in vitro* (De Jeu et al. 1992). Crossing Chilean with Brazilian species has created the "butterfly type" of plants. The "butterfly" type is allotetraploid (2n=4X) and produces viable 2X gametes (De Jeu et al. 1992). The "hybrids type" was created by several crossings between various species and cultivars.

Mutation techniques have been used for *Alstroemeria* breeding since 1970 to increase variation in flower color, stripes of the inner petal, flower size and height of plants. After irradiation of actively grown rhizomes with X-rays, a variety of mutants were obtained. Some of these mutants were selected and vegetatively propagated and then developed into a new cultivar (Broertjes and Verboom 1974).

Up till now, more than 60 species/genotypes have been released onto commercial markets by applying conventional breeding techniques. One problem found in conventional breeding is the lack of useful genes in *Alstroemeria* germplasm for use in further breeding. The majority of the *Alstroemeria* cultivars are polyploid, which makes breeding time consuming. However, new cultivars have been produced by using interspecific hybridization in the last decades (De Jeu and Jacobsen 1995). Furthermore, cross-hybridization does not always lead to seed set, although some hybrids were produced by using embryo rescue techniques (Buitendijk 1992). The slow process of breeding delays the introduction of new cultivars to the commercial market.

Overview of genetic modification in *Alstroemeria* and other monocot ornamentals

The first introduction of foreign genes into plants was achieved in the early 1980s. Since then, there have been many revolutionary events in plant genetic engineering and thus transformation is now a routine procedure for over 100 different plant species (Primrose et al. 2001), including commercially valuable crops.

In addition, transgenic genotypes of important crops such as wheat, rice, barley, and potato are grown. Besides these edible crops, plant genetic engineering technologies including genetic modification systems are now also actively used in ornamental species. To date, only a small number of reports on regeneration in *Alstroemeria* have been published. These reported systems could not be readily used for genetic modification. To this end, several approaches were investigated and described in this thesis to try to obtain a more efficient regeneration as well as transformation system in *Alstroemeria*. The one of the main aim of recent improvement of *Alstroemeria* cultivars was to develop a transformation system to produce resistant *Alstroemeria* plants against *Alstroemeria Mosaic Virus* (AlMV), which is one of the most dangerous and endemic viruses in *Alstroemeria*.

Alstroemeria, like other monocot ornamentals have been generally recalcitrant to genetic transformation techniques that are routinely applied in dicotyledonous plants.Before commencing the genetic modification experiments, the development of a regeneration system more efficient than the existing one was demanded. Until now, embryogenic calli were induced from stem segments (Lin et al. 2000a), nodes with axil (Kim et al. 2006) and immature ovule (Van Schaik et al. 1996). By using these embryogenic culture systems, Kim et al. (2005) isolated protoplasts from embryogenic calli of *Alstroemeria* plants and regenerated them into plants. Such as protoplast-based regeneration system can be utilized for somatic hybridization between cross-incompatible species or gene transfer through electroporation (Hoshino 2008a).

FEC induced from stem tissue of seedling plants was transformed with particle bombardment by pAHC18 that contained the luciferase gene as a reporter gene (Lin et al. 2000b). Plants were obtained from 10 independent lines. After 1 year of maintenance, however, only a few plants were still luciferase-positive. Furthermore, FEC was also used to transform using *Agrobacterium tumefaciens* (Van Schaik 1998) however, no transgenic plants were produced. Van Schaik (1998) concluded that FEC might be an alternative source for genetic modification and an ideal explant without severe somaclonal variations provided young FEC was used for transformation. To obtain transgenic *Alstroemeria* plants via either *A. tumefaciens* or particle bombardment, an efficient regeneration system and the optimization of parameters influencing the transformation process should be prepared.

Furthermore, in many monocotyledonous ornamentals, particle bombardment and *Agrobacterium*-mediated transformation have been used for the production of transgenic plants with improved agricultural traits. Particle bombardment has been applied in *Alstroemeria* (Lin et al. 2000b), *Gladiolus* (Kamo et al. 1995, 2000, 2005), *Dendrobium* (Kuehnle and Sugii 1992), *Lily* (Watad et al. 1998), and *Tulip* (Wilmink et al. 1995). *Agrobacterium*-mediated transformation has been applied in *Alstroemeria* (Akutsu et al. 2004a, 2004b; Kim et al. 2007; Hoshino et al. 2008b), *Anthurium* (Chen and Kuehele 1996), *Cymbidium* (Yang et al. 1999), *Iris* (Jeknic et al. 1999), and *Phalaenopsis* (Chai et al. 2002; Belarmino and Mii 2000). However, in spite of several successful reports, *Agrobacterium*-mediated transformation is still cumbersome for quite a number of monocot ornamentals. In the past decades, most of the reports on the transformation of monocot ornamentals used the GUS gene as a reporter gene because of its accuracy, fast and convenient characteristics.

Virus diseases in *Alstroemeria*

Once the new cultivars are developed, these plants should be propagated without loss of quality. However, numerous factors have a negative influence on the quality of *Alstroemeria*. Virus diseases are the most important problem in maintaining a high quality in the plant material. It has become apparent that many serious virus diseases in the world are the direct or indirect result of human activities (Thresh 1982). These activities are the use of monoculture in vast areas, the introduction of virus vectors into new areas, the introduction of new viruses into new areas through travel or transportation, and repeated use of the same field for the same crop (Hull 2002).

Viruses have caused severe problems in *Alstroemeria* plants propagated by rhizome splitting. According to Van Zaayen (1995), different viruses are reported in several European countries such as England (Brunt and Phillips 1981), Italy (Bellardi and Bertaccini 1991) and the Netherlands (Hakkaart and Versluijs 1985). The "butterfly-type" is generally infected with the most problematic virus in the *Alstroemeria* species, *Alstroemeria Mosaic virus* (AlMV). Fig. 1A shows particles of AlMV in the infected *Alstroemeria* plants.

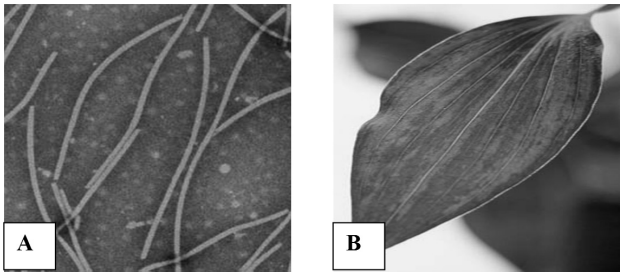

Fig. 1 *Alstroemeria* infected with mosaic virus A) AlMV particles as seen in the TEM (magnification: 31,000×) (B) symptoms on leaves (Kindly provided by Ir. Inge Bouwen, Plant Research International, Wageningen UR, The Netherlands)

In addition, the *Alstroemeria* Carla virus (AlCV) and cucumber mosaic virus (CMV) have been found in the "Aurea-type" *Alstroemeria*. These two viruses have also been observed in other *Alstroemeria* groups. Recently, *Alstroemeria* plants became infected with the Tomato spotted wilt virus (TSWV), and the Impatiens necrotic spot tospovirus (INSV). However, until now, they are not very common in *Alstroemeria* cultivation.

Among several viruses mentioned so far, AlMV is the most common virus in *Alstroemeria* species and belongs to the potyvirus group. *Alstroemeria* Mosaic Virus (AlMV) belongs to the potyviruses, which have flexuous rod-shaped particles and are transmitted by aphids in a non-persistent manner. It causes severe damage to *Alstroemeria*.

Plants infected with AlMV have symptoms such as streaking on the leaves, light green and dark spots (Fig. 1B) and flower-break (Chiari and Bridgen 2002). The biggest problem is that the symptoms in the plants are difficult to assess.

Also, there is substantial variation in symptoms dependent on the cultivar, growing conditions and the time of year (Van Zaayen 1995). This wide range of variation means that more than one potyvirus can exist and infect *Alstroemeria* (Hakkaart and Versluijs 1985). Recently, a new potyvirus was discovered and named the *Alstroemeria* streak virus (AlSV) (Wong et al. 1992). However, Van der Vlugt and Bouwen (2002) have concluded that AlMV and AlSV are strains of the same virus. Until now, unfortunately, there has been little research done on the development of AlMV-resistant lines by using either conventional breeding or genetic modification techniques in *Alstroemeria*. Even though virus-free stocks can be produced by meristem tissue culture, new breeding lines, which carry a virus-resistance,

are still needed. It is because virus-free stock from meristem culture could not maintenance virus-free characteristics during the breeding and culture period in *Alstroemeria*.

Also, one other problem is that conventional breeding has shown its limitations to extend genetic variation in the breeding progam of *Alstroemeria*. Therefore, genetic transformation could be combined with the conventional breeding techniques as well as meristem culture for the development of virus-resistant *Alstroemeria* plants.

Protection strategies against virus diseases can be applied in *Alstroemeria*

In general, strategies for the control of virus diseases in most crops have been focused on methods designed to avoid the virus infection (Fraser 1989), breeding of resistant lines, control of vectors, or production of virus-free stocks through tissue culture (Hull 2002). More interest is being given to a combination of these strategies. However, even this combined strategy has also proven unsuccessful in preventing virus infection or spread in crops. The first virus-free *Alstroemeria* cultivars were obtained mainly by meristem culture (Hakkaart and Versluijs 1985).

Unfortunately, the protocol described by Hakkaart and Versluijs (1985) takes four months to make virus-free stocks and contained little information on factors such as the optimal size of meristem tissues or the best method to confirm the eradication of virus. Recently, Chiari and Bridgen (2002) improved the meristem culture protocol and reported the production of virus-free *Alstroemeria* plants against AlMV. In spite of this effort, however, the meristem culture-derived plant can also be a target for AlMV and therefore become infected in the greenhouse during the culture period as well as on the commercial market due to contact with AlMV-infected sources. A long-term solution to the problems caused by AlMV could be the production of *Alstroemeria* transgenic plants that are genetically resistant or immune to the virus.

Transgenic approaches for the development of virus resistance

With the advent of gene transfer techniques and molecular identification of the virus genome structure, a number of virus-resistant crops have been produced and are in the process of being commercialized (Chowrira et al. 1998). This resistance based on virus-derived transgenes has been known to be effective against various plant viruses (Grumet 1995). However, despite its success in many crops, there are no reports on the production of transgenic virus-resistant *Alstroemeria* plants.

To obtain virus-resistant plants through genetic modification, there are three major sources of transgenes for protecting plants against viruses. The first source is "natural resistance genes" which after identification can be isolated and transferred to plant species using genetic modification. For instance, the *Rx1* gene, which confers strong resistance to PVX, has been isolated from potato and transferred to *Nicotiana benthamiana* and *N. tobacum* (Bendahmane et al. 1999). In the same way, the *N* gene, which gives resistance to TMV, found in *N. glutinosa*, was transferred to tomato (Whitham et al. 1996). In rice, the *N* gene transformed with the particle bombardment showed hypersensitive resistance to *rice hoja blanca virus* as well (Lentini et al. 2003).

The second source is genes derived from viral sequences, also referred to as pathogen-derived resistance (PDR). PDR had developed from the phenomenon of cross-protection, which refers to the resistance of plants to virus infection if plants have a viral transgene (Sijen 1997). It was expected that expression of the pathogen-derived gene could either prevent or inhibit the virus infection and movement process. In PDR, there are two main molecular mechanisms for its operation. One is protein-based and the other is nucleic acid-based protection (Hull 2002). In protein-based protection, coat protein-mediated resistance is the most widely used because the nucleic sequence of many viruses has been identified and cloned. Transforming plants with viral sequences that encode the coat protein of the virus achieve it. When this protein accumulates in uninfected plants, it results in resistance by uncoating the virus particle before translation and replication (Chahal and Gosal 2002). Coat protein-mediated resistance was first described in tobacco for TMV (Power-Abel et al. 1986). Subsequently, coat protein-mediated resistance by genetic modification has been demonstrated successfully in citrus (Febres et al. 2003), papaya (Lines et al. 2002; Kung et al. 2010), potato (Racman et al. 2001), phalaenopsis (Liao et al. 2004), soybean (Wang et al. 2001), squash (Pang et al. 2000),

wheat (Sivamani et al. 2002), gladiolus (Kamo et al. 2010) and watermelon (Yu et al. 2010).

Apart from the coat protein-mediated resistance, virus movement proteins can confer partial resistance (Malyshenko et al. 1993) or protection to other viruses with a similar genome organization (Beck et al. 1994). However, virus movement problems can have a detrimental effect for plant development as was reported by Hou et al. (2000). Another approach based on the protein level is the use of viral replicase proteins. Conclusions from several reports suggest that interaction between replicase proteins and other viral-encoded proteins may affect the process of the replication and cell-to-cell movement, leading to the arrest of the replication procedure (Hull 2002).

RNA-mediated resistance, antisense-mediated, satellite RNA-mediated resistance and ribozymes-mediated resistance are examples of nucleic acid based protection. RNA-mediated, antisense-mediated and satellite RNA-mediated resistance have been widely applied and show successful resistance in several crops. In RNA-mediated resistance, the introduced viral sequences do not produce a protein, thereby the protection is due to the RNA. Unlike coat protein-mediated resistance, the following four features have been reported in this strategy. Pang et al. (1993) found no correlation between the level of resistance and the expression level of the transgene. Secondly, RNA-mediated resistance is not dose-dependent and shows resistance at a high level of inoculum (Hull 2002). Thirdly, the resistance is narrow based and only against viruses, which have a similar virus genome sequence as that of the inserted transgenes (Hull 2002). Finally, transformed viral sequences may be methylated or truncated (Kohli et al. 1999). The molecular mechanism behind RNA-mediated resistance associated with the low steady states of transgene RNA and homology-dependent or post-transcriptional gene silencing might explain the narrow range of resistance. For instance, when the resistance was obtained by the transcript, and not by the protein, or if transgenic plants with a low level of viral transgene expression showed more resistance than did those plants with a high level of transgene expression, it can be assumed that the resistance generated in these cases might be due to homology-dependent gene silencing (Hull 2002).

The antisense-mediated resistance is based on a strategy first developed to control fruit ripening (Smith et al. 1990) and virus resistance (Elmer and Rogers 1990) in tomato. For this, the cDNAs representing viral RNA genomes were cloned in an antisense orientation behind an appropriate plant promoter and transferred to plants. Antisense RNA can control gene expression. RNA production of the coat protein will therefore be inhibited by this antisense sequence, and will arrest the production of new virus particles in plant cells.

Finally, several RNA viruses have small RNA molecules called satellite RNAs, which affect the severity of infection by a virus. These satellite RNAs are entirely dependent on their helper virus for the replication and encapsidation (Kuwata et al. 1991; Simon 1988). Generally, the presence of a satellite RNA can control the severity of infection caused by its helper virus (Tien and Wu 1991), thereby reducing damage, although severe and different levels of damage can be induced in some cases. Using this strategy, Kim et al. (1997) observed that severity of infection was attenuated in the offspring of hot pepper.

The final source of transgenes for protecting plants against viruses is genes from various sources that inhibit or interfere with the target virus. These include pathogen-related proteins, virus-specific antibodies, ribosome-inactivating proteins, antisense to β-1, 3-glucanase. However, some of these sources showed no resistance or only limited application in a small number of crops. Table 1 outlines target genes associated with different strategies. Of all these strategies, coat protein-mediated resistance has been widely applied and is one of the most successful strategies for producing virus-resistant plants (Wilmink 1996).

In the near future, RNA-mediated resistance would be chosen to obtain *Alstroemeria* transgenic plants, which are resistant to ALMV and not a strategy based on coat protein-mediated resistance, because it is then difficult to distinguish between resistance and expression of potyvirus. For the production of AlMV resistant *Alstroemeria* plants by genetic modification, therefore, particle gun-mediated transformation system would be used because particle bombardment is the quite reliable and available transformation system with a high efficiency in *Alstroemeria* at this moment. A DNA vector containing the coat protein gene and the 3'-non translated region sequence was constructed to confer virus-resistance against AlMV. Since there was no ATG codon in the construct, and thus a protein

Table 1 Summary of the various strategies used to obtain virus resistance in plants

Resistance type	Target gene	Reference
Natural	*N* gene	Whitham et al. 1996; Lentini et al. 2003; Fedorowicz et al. 2005
	*Rx1*gene	Bendahmane et al. 1999
PDR (Pathogen-derived resistance)	Coat protein	Powell-Abel et al. 1986; Sivamani et al. 2002; Tripathi et al. 2004; Kamo et al. 2005; Liao et al. 2004; Kung et al. 2010; Yu et al. 2010
	Viral movement protein	Cooper et al. 1996
	Viral replicase	Golemboski et al. 1990; Praveen et al. 2005 Kamo et al. 2010
	RNA-mediated	Reviewed by Prins and Goldbach 1996
		Chen et al. 2004; Higgins et al. 2004
	Antisense RNA	Reviewed by Tabler et al. 1998
	Ribozymes	Reviewed by Tabler et al. 1998
	Satellite-mediated	Harrison et al. 1987
	DI nucleic acid-mediated	Kollar et al. 1993
Other sources	PR protein	Hooft van Huijsduijnen et al. 1986
	β-1,3'glucanase	Beffa et al. 1996
	Virus specific antibody	Hiatt et al. 1989
	Ribosome-inactivating proteins	Reviewed by Wang and Tumer 2000
	Ribonuclease gene pac-1	Watanabe et al. 1995
	2',5'-oligoadenylate synthase	Truve et al. 1993

would not be produced, it was established that resistance would occur at the transcript level by so called RNA-mediated resistance.

In conclusion, we outlined several strategies to develop virus-resistant *Alstroemeria* plants with the help of tissue culture inclduing meristem culture and genetic transformation system. In the near future, virus-resistant *Alstroemeria* plants via genetic transformation will be produced.

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