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***Short communication***

# Association of putative viral RNA sequences to flower colour variegation in Japanese primrose (*Primula sieboldii* E. Morren)

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## ABSTRACT

**Flower colour variegation is characteristic symptom of viral infections in ornamentals such in the case of camellia, tulip, petunia, and daffodil. This is also seen in different cultivars of Japanese primrose like 'Shichikenjin', 'Sotorihime' and 'Isaribi', signifying a possible viral origin. Putative viral RNA sequences were first identified in the *Primula sieboldii* databases. Total RNA was then isolated and reverse transcriptase polymerase chain reaction was done using virus-specific primers to identify the association between the presence of putative viral RNA sequences and flower colour variegation. Five putative viral RNA homologous sequence groups were identified, with a possible virus identity of *Cycas necrotic stunt virus*, *Lettuce big-vein associated virus* and *Rosa multiflora cryptic virus*. Results showed that instances of amplifications were seen in both variegated and non-variegated within and across cultivars denoting the presence of putative viral RNA and a possible viral morbidity in the amplified cultivars. Instances wherein amplification of non-variegated flowers, while no amplification of variegated flowers within the same cultivar were also observed. Variegated plants of cultivars which had variegated and non-variegated flowers had a weak correlation. Thus, no definite association could be deduced from the presence of putative viral RNA and flower colour variegation. It is also recommended that flower tissue should be used for gene expression analysis to avoid error in differential gene expression.**

**Key words:** Flower colour variegation, *Primula sieboldii*, viral RNA, gene expression.

Petals for cut-flowers and flowering pot plants, such as *Primula sieboldii*, are highly significant as they dictate the commercial value of these crops; consequently, the petal's form and colour became the target characteristic for breeding various ornamental crops. Several studies also show that virus infection also causes the difference in flower colour patterns in different flowers like in the case of camellias (Plakidas, 6), lily (Dekker *et al.*, 2), tulips (Morikawa *et al*., 5). On the other hand, flower colour breaks are also seen in different cultivars of Japanese primrose cultivars like 'Shichikenjin', 'Shibori-Tatuta' and 'Kogarashi'. Apart from this, in the analysis of RNA sequence expressed in Japanese primrose by using next generation sequencing, it was found out that the cDNA library of *P. sieboldii* was composed of one percent putative viral cDNA (Aoki *et al*., 1).

Thus, there is a possibility of symptomatic flower colour variegation on different Japanese primrose cultivars based on viral morbidity. This study will help elucidate the possible cause of flower colour variegations in Japanese primrose. The general objective was to conduct a molecular approach in determining the possible association between the presence of putative viral RNA sequence(s) and flower colour variegation in Japanese primrose cultivars with variegated and non-variegated flowers.

The plant materials consisted of 15 variegated and 3 non-variegated, wild-type *P. sieboldii* varieties. Using Expressed Sequence Tag (EST) viewer, a Basic Local Alignment Search Tool (BLAST) search with the Keyword "virus" was done on two *P. sieboldii* databases gathered from Next Generation Sequencing (NGS) data of the University of Tsukuba. Sequence groups with possible viral origin and greater than 45% value of positives were retained and five primers were made based from these sequences using Primer 3. Total RNA was isolated from the leaf samples using the Promega Reliaprep™ RNA tissue miniprep system (Promega, WI, USA), followed by cDNA synthesis using Takara Primer Script™ RT Reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan). Polymerase chain reaction (PCR) of the cDNA was performed using the custom-made primers, followed by gel electrophoresis and, consequently, UV visualization. Finally, cluster analysis and correlation assessment between variegation and amplification per homologous sequence group was done to assess association.

The BLAST search and screening showed five possible non-homologous sequence groups with a possible virus identity of *Cycas necrotic stunt virus, Lettuce big-vein associated virus* and *Rosa multiflora*

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*cryptic virus*. Results showed that most putative viral sequences are amplified and present in more than one cultivar (Table 1). More than one virus is probably infecting the primrose cultivars and could have cumulative symptoms. Viral morbidity is therefore present in both variegated and non-variegated types of the same cultivar. Instances of amplifications were seen in both variegated and non-variegated within and across cultivars (Table 1). As an example, variegated and non-variegated Odamaki shows same amplification pattern all throughout. Furthermore, instances wherein amplification of non-variegated flowers, while no amplification of variegated flowers within the same cultivar were also seen (Table 1). It can also be observed that some have no amplification on cultivars having variegated flowers only. Thus, no definite pattern and relationship could be deduced from the virus-specific amplification data. In contrast with Hunter *et al*. (2), variegated flowers showed 100% amplification using virus-specific primers, while non-variegated flowers showed otherwise.

On the other hand, variegated plants of cultivars which contain variegated and non-variegated flowers do not exhibit 100% amplification. Calculated correlation coefficients between variegation and amplication were 0.21, 0.21, 0.11, 0 and -0.11, for homologous sequence groups 1, 2, 3, 4 and 5, respectively. This denotes a weak correlation between variegation and presence of putative viral RNA sequences. Thus, in a per distinct putative viral RNA sequence basis, no definite association could be deduced from the virus-specific amplification data. This aside, wild type plants, with non-variegated flowers, show absence of amplification and could indicate no virus infection (Table 1).

Since no definite pattern can be deduced by singly observing the amplification data and expression of virus symptoms could have resulted from cumulative effects of viruses infecting a plant, cluster analysis will group the observations into clusters with relatively homogeneous cases of amplification. A virus could play a role in the phenotype as part of a virus complex (Martin *et al*., 3). In cluster I, variegated and non-variegated 'Asukagawa' is present. In cluster II, non-variegated 'Tamakujaku' is clustered with the variegated 'Isaribi'. On the other hand, in

**Table 1.** Amplification of putative viral RNA from leaf tissues of variegated and non-variegated Japanese primrose flowers

| Cultivar | Virus group | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | | 4 | | 5 | |
| | Yes | No | Yes | No | Yes | No | Yes | No | Yes | No |
| Asukagawa | - | - | - | - | - | - | + | + | - | - |
| Isaribi | - | - | - | - | - | - | + | - | + | + |
| Odamaki | + | + | + | + | + | + | + | + | - | - |
| Karakoromo | + | - | + | - | + | - | - | + | - | + |
| Shichikenjin | + | + | + | + | - | + | + | + | - | - |
| Tamakujaku | - | - | - | - | - | - | + | + | + | + |
| Sotorihime | + | + | + | + | + | + | - | + | - | - |
| Hanachiru-Sato | - | - | - | - | - | - | + | + | - | - |
| Myochiriki | - | - | - | - | - | - | + | + | - | + |
| Dairiki-Muso | + | - | + | - | + | - | - | - | + | - |
| Momozono* | - | | - | | - | | + | | + | |
| Miyako-Asobi* | - | | - | | - | | + | | + | |
| Tennyo* | - | | - | | - | | + | | + | |
| Tsuki-No-Utage* | - | | - | | - | | + | | + | |
| Fujikoshi* | - | | - | | - | | + | | + | |
| Wild type 1 | | - | | - | | - | | - | | - |
| Wild type 2 | | - | | - | | - | | - | | - |
| Wild type 3 | | - | | - | | - | | - | | - |

Legend: (+) amplification; (-) no amplification; Yes = variegated; No = non-variegated; *absence of non-variegated clone

cluster V, variegated and non-variegated 'Odamaki' was clustered with the non-variegated 'Sotorihime' (Fig. 1). At 81% coefficient of similarity, clusters can contain both variegated and non-variegated varieties within and across cultivars; hence, no definite relationship can be determined, based on the general amplification data, across all the putative virus RNA specific primers used.

Knowing the cause of Japanese primrose flower colour variegation will help in identifying appropriate breeding methods for flower colour variegation. If variegation is set to be caused by virus infection, asexual propagation of crops can be done to conserve the phenotype of the flowers. Also, viruses can be considered for broader applications to boost ornamental qualities. From here, if a plant species is known to exhibit flower variegation due to viral infection, inoculation to produce variegation could be done to its susceptible varieties to produce variegated flowers. This also will aid in selection. If the industry demands for full-colored flowers, selecting non-infected plants would be essential. Thus, breeding for novel full-coloured flowers should only consider non-variegated/non-infected flowers as breeding materials. Breeding for variegated flowers, on the other hand, could also involve breeding for virus-susceptible varieties. Moreover, early selection of variegated-flower-producing plants can also be done by screening plants with viral RNA sequences.

No definite pattern or association between presence of putative viral RNA sequences and flower colour variegation could be deduced from the virus-specific amplification data. Although virus infection is systemic, inconclusive results were obtained as it is suspected that gene expression could vary depending on the plant part and growth stage. Flower tissue, acquired at the same growth stage, should be used in tandem with qPCR methods in order to quantify the virus gene expression and relate it to the intensity of variegation. On the other

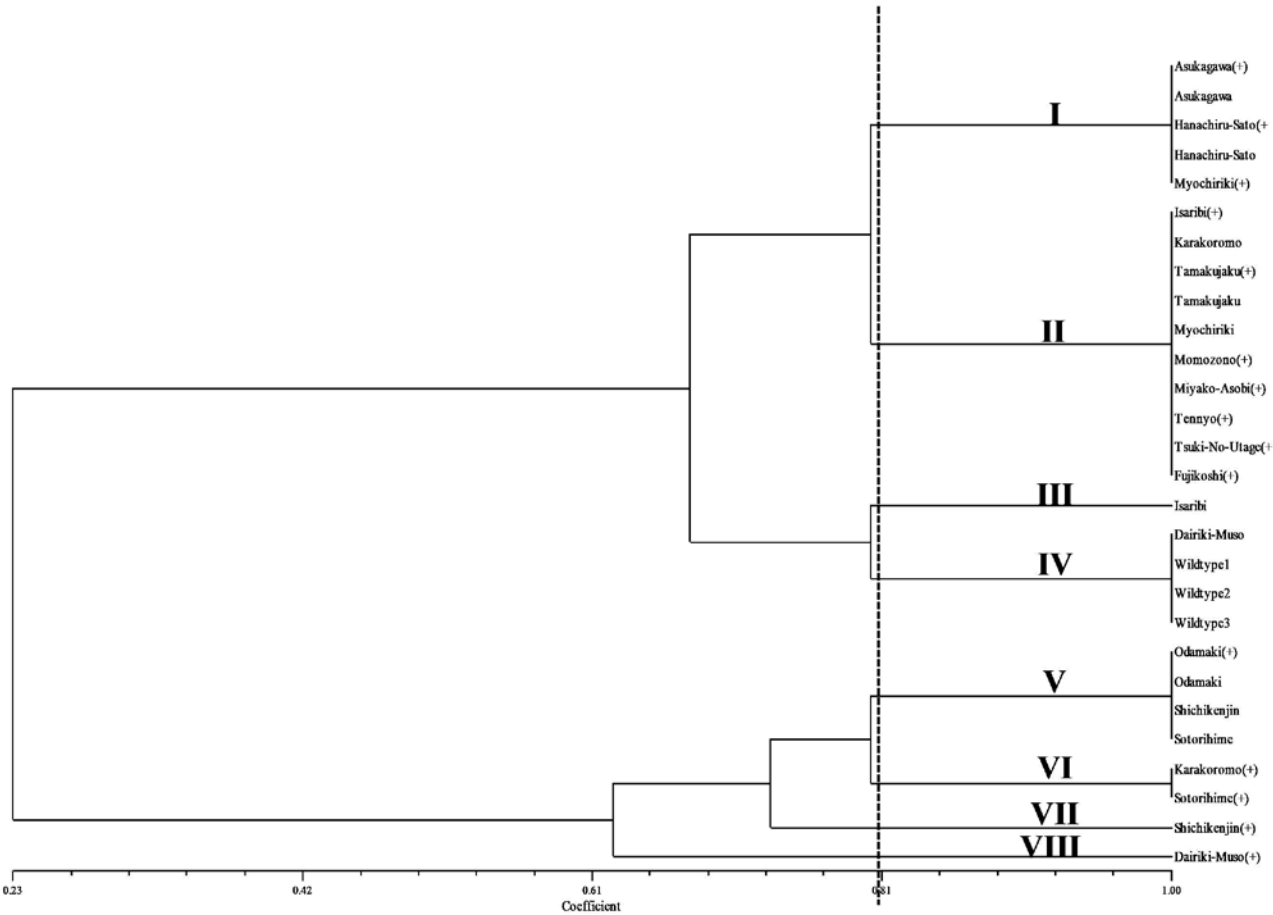


**Fig. 1.** Dendogram of the 28 variegated and non-variegated Japanese primrose plants constituted by 18 cultivars. (+) indicates presence of variegation. The numerical scale indicates the similarity of incidence of distinct putative viral RNA presence in Dice's similarity coefficient. The vertical line cuts the dendogram at 0.81 similarity coefficient.

hand, wild types can also be utilized in inoculation studies to determine expression of symptomatic flower color variegation upon rubbing with the sap from a variegated flower.

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