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The establishment of solid mutant line from somaclonal variation generated through mature petal cultures of chrysanthemum

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Abstract The mature petals from ray florets of 'Polaris Pink' chrysanthemum were cultured *in vitro* on Murashige and Skoog (MS) medium containing various combinations and concentrations of BA and 2,4-D. The MS medium supplemented with 1 mg/L BA and 1 mg/L 2,4-D yielded the highest efficiency of callus formation after 2 months of culture. The calli were then subjected to shoot regeneration on MS solid medium containing various concentrations of kinetin for shoot induction. The highest number of regenerated shoots was obtained on MS medium supplemented with 2 mg/L kinetin. Using MS medium supplemented with 2 mg/LBA and 0.5 mg/L 2,4-D resulted in somaclonal variation induction. The flower color of the variant line changed from light pink to light purple and the tip of petal was rounder than that of control. The solid variant lines were obtained through several generations of *in vitro* single node cutting. The SRAP showed that the primer pair Me1/Em10 exhibited the polymorphic band between the mother plant and variant plant.

Keywords: Micropropagation, Petal culture, Callus induction, Solid mutant, SRAP marker

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Introduction

Chrysanthemum (*Dendranthema morifolium* (Ramat.) Hemsl.; Asteraceae) is one of the top five most sold cut flowers (by ranking; rose, chrysanthemum, tulip, gerbera and lily) in Holland in 2019 with a revenue of €328 million (van Gelder, 2020). Breeders focused on enhancing of the chrysanthemum ornamental value by improving of flower color, size and form, vegetative height, growth form and sensitivity to light quality/quantity which a variety of breeding techniques have been developed for this plant. However, due to its nature of being a complex hexaploid (2n=6x=54), with heterozygosity, large genome, and self-incompatibility, the cultivated chrysanthemum improvement is mostly successful by mutation technique (Eeckhaut and Van Huylenbroeck, 2011; Teixeira da Silva, 2004; Kumari *et al*., 2019).

The techniques are used for inducing mutation and somaclonal variation to yield the mutant lines are very useful for creating the desired characteristics that may not be found in nature. The mutation is a result of the exposure to physical (such as X-rays, gamma-rays and neutrons) or chemical mutagens (such as ethyl-methane-sulphonate and sodium azides) (Kapadiya *et al*., 2004; Teixeira da Silva *et al*., 2015; Yoosumran *et al*., 2018; Asoko *et al*., 2020). *In vitro* propagation techniques provide the ability for efficient multiplying and maintaining large numbers of elite characteristics. However, plant regeneration *via* callus or cell suspension can lead to a genetic variation with new characteristics that are different from the donor plant. This variation was termed somaclonal variation (Larkin and Scowcroft, 1981).

The somaclonal variation can be induced by *in vitro* stresses which cause a mutagenesis in the regenerated plant. The genetic and morphological changes in a regenerated plant depend on the plant's genotype, explant type, age of the donor plants, the number of subcultures, and the composition of the medium, especially plant growth regulators (PGRs) of which auxin and cytokinin are the two most important PGRs (Gao *et al*., 2010; Bairu *et al*., 2011). This technique is useful and successful for plant breeding as reported in several plants such as *Freesia hybrida* Klatt (Gao *et al*., 2010), *Caladium* 'Pink Cloud' (Ahmed *et al*., 2004), *Coffea arabica* L. (Etienne and Bertrand, 2003) *Petunia hybrida* (Abu-Qauad *et al*., 2010) and chrysanthemum (Vilasini and Latipah, 2000; Nahid *et al*., 2007; Thangmanee and Kanchanapoom, 2011; Miler and Zalewska, 2014).

The somaclonal variation technique is used as a tool for creating new varieties of chrysanthemum *via* tissue culture that is carried out with different sources of explants including leaves (Miler and Zalewska, 2014), stem

segments (Jevremovic *et al*., 2012; Rivai *et al*., 2015), and petals (Vilasini and Latipah, 2000; Nahid *et al*., 2007; Thangmanee and Kanchanapoom, 2011). In 2006, Kumar and Kanwar documented that the gerbera plants regenerating from petal explants exhibited a high level of mutation.

The advantages of mutation breeding are the high heterozygosity of chrysanthemum which increase the apparent mutation rate and produce many excellent mutation types in a short period (Su *et al*., 2019). However, the major disadvantage of somaclonal variation is the chimera characteristic in which the cells in the same plant possess a genetic variation leading to a genetic instability of mutant plants (Jain, 2001). The chimera occurrence of mutation happens within the meristem. In the chrysanthemum leaf, chimera characteristic originates from L1, L2 and L3 layers, and while in the flower, it originates from L1 and L2 layers. The mutagenesis in the L1 layer along with the L2 layer leads to changes in the flower color while the mutagenesis in the L2 layer alone results in changes of the flower's shape (Vilasini and Latipah, 2000). Interestingly, a periclinal chimera, which occurs from a mutated epidermis and unmutated inner layers, mainly causes the mutagenesis in the chrysanthemum. In order to resolve the issue of chimerism, taking a tissue culture has been shown to be a promising technique. The plantlets can be proliferated *in vitro* from both axillary buds and adventitious shoots and thus sequential subcultures can eliminate the chimeric plant and a high percentage of true to type plant (Plader *et al*., 1998; Ahloowalia and Maluszynski, 2001; Peredo *et al*., 2006).

To confirm the solid mutants, typically, regenerated plantlets must typically be physiologically characterized. However, molecular marker analysis techniques can be used in genetic characterization. Sequence-related amplified polymorphism (SRAP) is one of the techniques that effective for cultivar identification and DNA fingerprinting (Mokhtari *et al*., 2013). The SRAP technique is simple and easy to perform and preferentially amplify open reading frames (ORFs) or ORFs related sequences (Li and Quiros, 2001). It is easy to use polymerase chain reaction (PCR) technique and requires no plant genetic information (Guo *et al*., 2009). The SRAP marker is commonly used to study genetic diversity in several plants, such as brassica (Li and Quiros, 2001), cucumbers (Ferriol *et al*., 2003), mulberry (Zhao *et al*., 2009), tomato (Comlekcioglu *et al*., 2010), broad beans (Alghamdi *et al*., 2012), potato (Yanping *et al*., 2014), Siberian apricot (Li *et al*., 2014), and tree peony (Guo *et al*., 2009). There were reports that SRAP markers have been used to screen certain specific characters in chrysanthemum, such as plant architectural traits, flowering traits and biotic and abiotic stress related traits (Su *et al*., 2019).

This investigation was undertaken to produce somaclonal variation in chrysanthemum through plant regeneration from a mature petal culture and the extraction of a genetic stability line by several steps of *in vitro* single node cutting. Thereafter, the detection of the DNA polymorphism between the mother plant and the variant lines were carried out by SRAP technique to confirm the mutation.

Materials and methods

Plantlet regeneration from mature ray florets

Callus induction

The mature ray florets of chrysanthemum cultivar 'Polaris Pink' from a full bloom flower were surface sterilized with 70% ethanol for one minute followed by 5% Haiter® solution (6% sodium hypochlorite) with two drops of detergent for 15 minutes, and then washed thoroughly in sterile water twice, for five minutes each time. The apical and basal parts of the petal were excised and 1.0 cm of the middle part of the petal was used for callus induction. The explants were cultured on a solid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0, 0.1, 0.5, or 1.0 mg/L of 2, 4–dichlorophenoxyacetic acid (2,4-D) and 0, 1, 2, or 3 mg/L of 6-benzyladenine (BA) added with 30 g/L sucrose. The pH of the medium was adjusted to 5.7 prior to solidifying with 7.5 g/L agar. The explants were incubated for eight weeks at 25 ± 2 ℃ and a 16 hr photoperiod with a light intensity of 50 μmol/m^2/s. The explants were transferred to fresh media once at the fourth week.

Plantlet regeneration

The shoot induction was performed by cultivating 0.5x0.5 cm of callus clumps on solid MS medium supplemented with 0, 1, 2, or 3 mg/L of kinetin and with 30 g/L sucrose. The pH of the medium was adjusted to 5.7 prior to solidifying with 7.5 g/L agar. The culturing condition was 25 ± 2 ℃, with a 16 hr photoperiod and a light intensity of 50 μmol/m^2/s. The callus clumps were transferred to fresh media every four weeks for 12 weeks.

Root induction and ex vitro transplanting

The regenerated shoots were transferred to PGR-free solid MS medium containing 7.5 g/L agar and 30 g/L sucrose (pH = 5.7) for four weeks for root induction. Rooted plantlets were transferred to the seedling tray containing sterilized peat moss as planting media and placed under high humidity for four

weeks to harden. The plantlets were then transplanted to the experimental plot at the farmer's commercial field in Thai Shamakhi sub-district, Wang Nam Khiao District, Nakhon Ratchasima, Thailand. The variant plants were selected mainly by flower morphology at the blooming stage.

The establishment of the solid mutant by in vitro single node cutting

A single branch of field-grown variant plant was selected and cultivated *in vitro*. Lateral bud explants were surface sterilized with 20 and 10% of Haiter® solution (6% sodium hypochlorite) with two drops of detergent for 10 minutes each. Thereafter, the explants were washed with sterilized water twice. The explants were cultured on PGR-free solid MS medium containing 7.5 g/L agar and 3 g/L sucrose (pH = 5.7). After a one month, the single node explants were dissected and transferred onto new fresh media.

The *in vitro* single node cutting was repeated eight times. The *in vitro* healthy plants of the 8th generation were transplanted to the same experimental plot as the above described technique. The selection and evaluation based mainly on flower characters were performed at the blooming stage.

One of the most uniform plants among the variant lines was selected from the first field grown and was subjected to propagation by *in vitro* single node cuttings as described above until another eight generations were obtained. Plantlets were transplanted for the second field-grown at the same field plot. Plants at the full bloom stage were subjected to genetic stability evaluation. The most uniform plant was also selected for molecular analysis using SRAP markers.

Data analysis

Data from each study was presented as the mean ± standard error. The statistical significance of the difference between the means of individual groups was assessed using the R Foundation for Statistical Computing Platform version 3.6.1 program.

Molecular analysis by SRAP markers

One of the most uniform plants among the variant lines and a control plant were selected from the field grown plot and their genomic DNA was extracted from leaf tissues using the modified Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1990). The DNA products were resuspended in sterile water and were treated with 2 µl of 10 mg/L RNase A (US Biological, USA) at 37 ℃ for 1 hr.

To detect SRAP, the PCR were performed in 10 µl reaction volume containing 1x *Taq* buffer [$(NH_4)_2SO_4$], 2.5 mM $MgCl_2$, 200 µM dNTP, 1 U *Taq* DNA polymerase (5U/µl) (Thermo Scientific), 0.5 µM of each primer (Table 1), and 25 ng of template DNA. The amplification regime was as follows: 94 ℃ for 5 min followed by 5 cycles of 94 ℃ for 1 min, 35 ℃ for 1 min and 72 ℃ for 1 min, subsequently followed by 35 cycles at 94 ℃ for 1 min, 50 ℃ for 1 min and 72 ℃ for 1 min with a final extension step at 72 ℃ for 7 mins. A total of 10 primer pairs were used in this experiment.

PCR products were electrophoresed through 1.5% agarose gel with 0.5X TBE buffer by comparing with GeneRuler 100 bp plus DNA ladder (Thermo Scientific, USA). The DNA profiles were then compared between the control and the variant lines.

Table 1. The SRAP primer combinations for polymorphic detection in control and variant lines of 'Polaris Pink' chrysanthemum

No.	Forward primer	Reverse primer
1	Me1: TGAGTCCAAACCGGATA	Em10: GACTGCGTACGAATTATG
2	Me7: TGAGTCCAAACCGGACT	Em2: GACTGCGTACGAATTTGC
3	Me18: GAGCGTCGAACCGGATG	Em3: GACTGCGTACGAATTGAC
4	Me21: GTACATAGAACCGGAGT	Em12: GACTGCGTACGAATTGTC
5	Me24: GACCAGTAAACCGGATG	Em3: GACTGCGTACGAATTGAC
6	Me24: GACCAGTAAACCGGATG	Em14: GACTGCGTACGAATTCAG
7	Me24: GACCAGTAAACCGGATG	Em11: GACTGCGTACGAATTTCG
8	Me20: GAGTATCAACCCGGATT	Em4: GACTGCGTACGAATTTGA
9	Me20: GAGTATCAACCCGGATT	Em12: GACTGCGTACGAATTGTC
10	Me12: TGAGTCCAAACCGGGAT	Em4: GACTGCGTACGAATTTGA

Results

The callus induction from the mature petal

The callus induction from the mature ray floret on MS media supplemented with various concentrations of BA and 2,4-D resulted in developing, in the beginning, a light green compact callus (Figure 1A). Differences in the ability to form callus were observed in different concentrations of BA and 2,4-D that were tested. The MS media containing combinations of BA and 2,4-D led to a callus formation, while no callus was observed on MS media adding with BA or 2,4-D alone (Figure 2). The

maximum weight of callus was observed when the petal of the ray floret was cultured on the solid MS medium supplemented with 1 mg/L BA plus 1 mg/L 2,4-D.

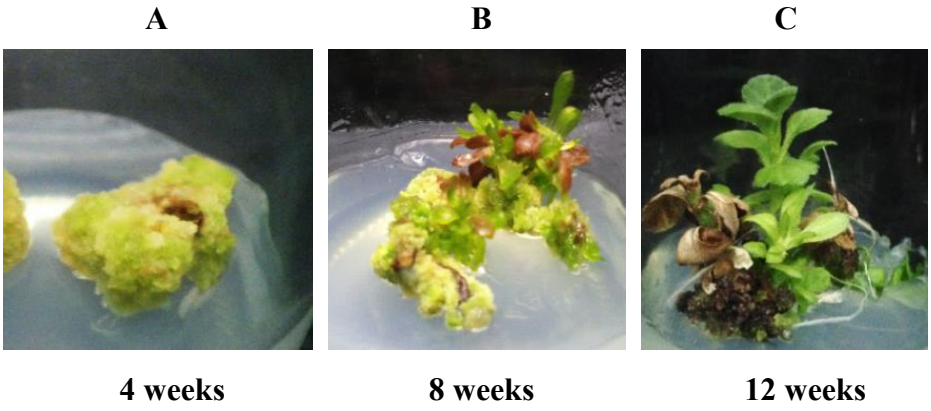

Figure 1. The mature petal of 'Polaris Pink' chrysanthemum forming callus on MS medium supplemented with 2 mg/L BA and 1 mg/L 2,4-D after cultured for 8 weeks (A) and the adventitious shoots induced on MS medium supplemented with 2 mg/L kinetin for 8 (B) and 12 (C) weeks. The culture condition was 50 µmol/m^2/s light, 16 hr photoperiod and 25±2°C

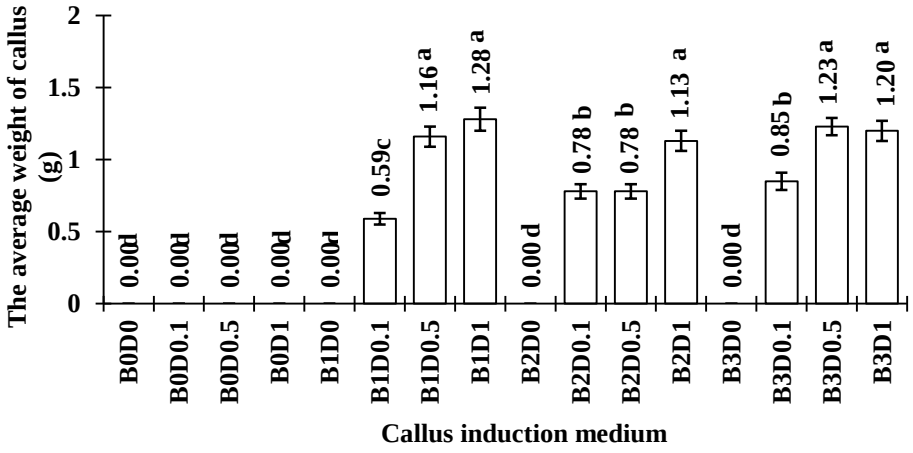

Figure 2. The average callus weight (±SE) of 'Polaris Pink' chrysanthemum derived from mature ray floret that cultured on MS media supplemented with various concentrations of 2,4-D and BA for 8 weeks. The culture condition was 50 µmol/m^2/s light 16 hr photoperiod and 25±2°C. Means followed by the same letter are not significantly different as indicated by DMRT ($p \leq 0.05$). The letters 'B' and 'D' on the x axis represent BA and 2,4-D, respectively

Plantlet regeneration and root induction

The callus was transferred to the solid MS medium supplemented with various concentrations of kinetin for shoot induction. The result revealed that callus clumps began to develop the shoots and small leaves after cultivation for eight weeks on five callus induction media (Figure 1B, 1C). High kinetin concentrations were effective for shoot induction. The highest number of shoots per callus was found on callus induced by MS medium supplemented with 2 mg/L of BA and 1 mg/L of 2,4-D and transferred to MS medium supplemented with 2 mg/L and 3 mg/L of kinetin for shoot induction (Figure 3).

All regenerated shoots were transferred to MS medium without PGRs for root induction and were grown in the field condition. All propagated plants showed true-to-type leaf form and growth pattern. Interestingly, the number of three variant lines showed the difference in petal color and petal shape of the ray florets. The fully developed ray florets changed from pink to light purple and a petal tip became rounder as compared with the normal plant (Table 2, Figure 4).

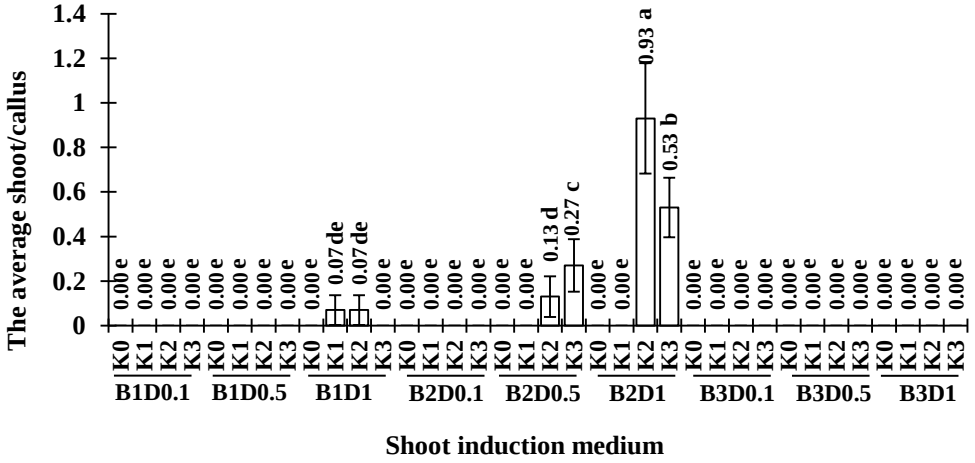

Figure 3. The average number (±SE) of shoots per callus clump of 'Polaris Pink' chrysanthemum induced from the callus that culture on MS media supplemented with various concentrations of 2,4-D and BA for 8 weeks and then subjected to shoot induction on solid MS media supplemented with 0, 1 and 2 mg/L kinetin for 12 weeks. The culture condition was 50 μmol/m^2/s light, 16 hr photoperiod and 25±2°C. Means followed by the same letter are not significantly different as indicated by DMRT ($p \leq 0.05$). The letters 'B', 'D' and 'K' on the x axis represent BA, 2,4-D and kinetin, respectively

Table 2. The number of 'Polaris Pink' chrysanthemum variant lines exhibiting the changes in flower color. Plantlets obtained from callus induced on callus induction media (CI: MS media containing various concentration of 2, 4-D and BA) followed by shoot induction on shoot induction media (SI: MS media containing various concentrations of kinetin

CI (MS +BA+2,4-D) (mg/L)	SI (MS+kinetin) (mg/L)	No. of shoot	No. of plantlet in field-grown	No. of plantlets	
				Control (Pink petal)	Variant (Purple petal)
MS (1+1)	1	1	2	2	0
	2	1			
MS (2+0.5)	2	2	6	3	3
	3	4			
MS (2+1)	2	14	22	22	0
	3	8			
Total number		30	30	27 (90%)	3 (10%)

The establishment of the solid mutant by in vitro single node cutting

The first field grown evaluation of the V8 generation of *in vitro* single node cutting indicated that all of the variant lines showed uniformity in growth characters, leaf shape, and the petal color of the ray florets in fully developed inflorescences. The control and variant lines were mainly different in inflorescence characters (Figure 4 A and B). The inflorescence of the variant line was a spray type with a decorative type of flower. The variant lines had significantly (p<0.05) shorter plant height at four months old in the flowering stage compared with the control line but they had a greater number of flowers per stem (Table 3).

In the second field grown evaluation, the data of V16 progeny indicated the reverse trend in plant height and number of flowers per stem compared with the first field grown (Table 3). At four months old, the control plant was significantly higher (p<0.05) than those of the variant lines; however, the number of flowers also tended to decrease, even it was not a significant difference. The flower shape and color were clearly differed from the control (Figure 4) and 100% of the genetic stability of the variants was also confirmed for the second time (Table 3).

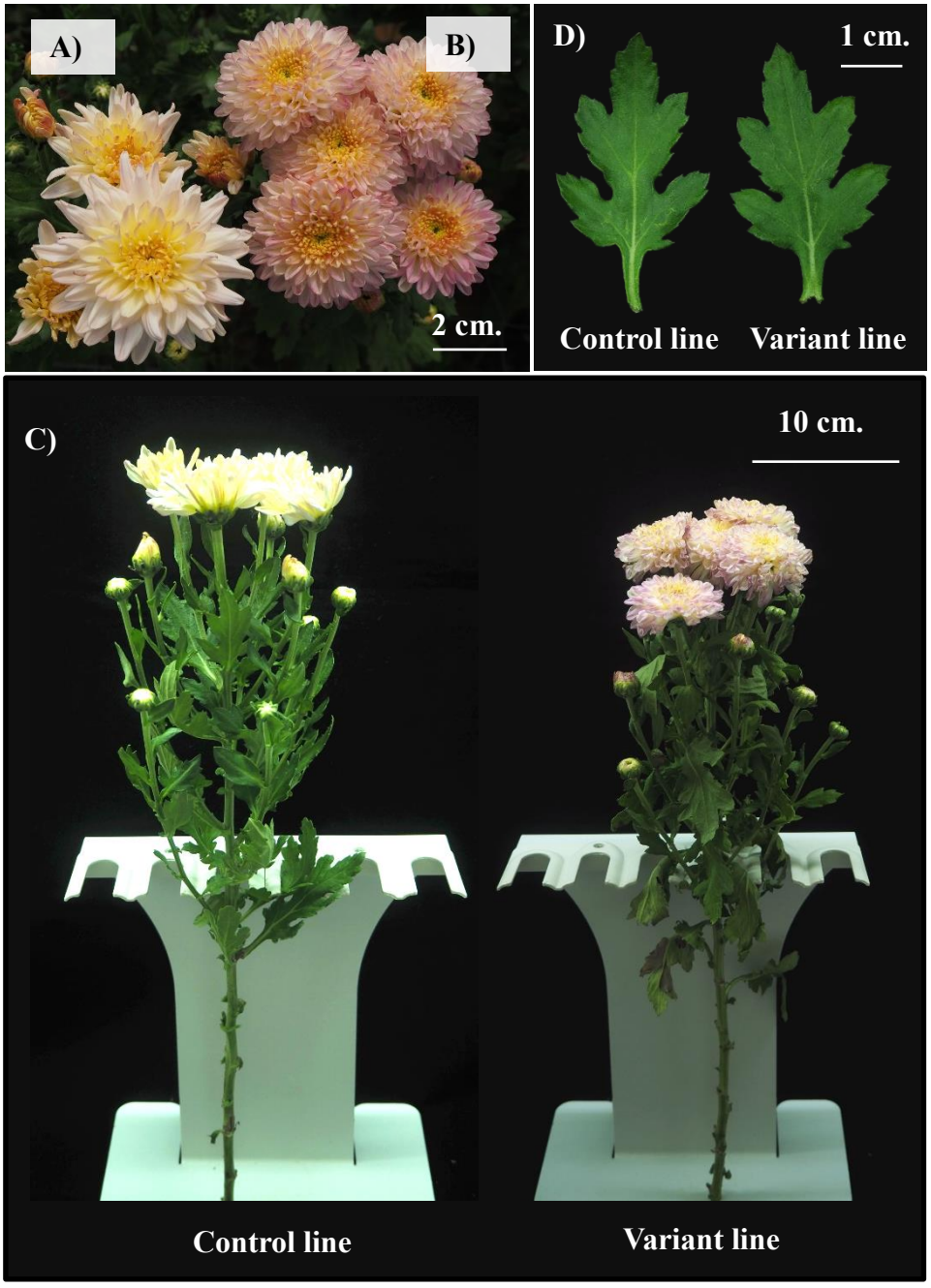

Figure 4. The flowers of control (A) and variant (B) lines of 'Polaris Pink' chrysanthemum obtained from the second field grown. The characters of inflorescence (C) and leaf (D) at the blooming stage was also compared

Table 3. The morphological characteristic and percentage of true type in V8 and V16 progenies of 'Polaris Pink' chrysanthemum after 8 and 16 generations of *in vitro* single node cutting. The plantlets were then transplanted to the experimental plot at Thai Shamakhi sub-district, Wang Nam Khiao District, Nakhon Ratchasima, Thailand (GPS location: 14.0181504, 99.9784448)

Field grown	Line	No. of plants	Plant height (cm)		No. of flowers per stem	No. of true type plants (%)
			2 months	4 months		
1st Field grown (V8)	Control	25	62.20 ±1.68	81.92 ±1.10	4.80 ±0.16	100
	Variant	25	78.00 ±1.24	100.52 ±0.87	5.60 ±0.21	100
	T-test		*	*	*	
	%CV		10.44	5.43	17.88	
2nd field grown (V16)	Control	15	74.40 ±1.91	98.20 ±0.78	5.73 ±0.41	100
	Variant	15	69.13 ±1.13	83.20 ±0.73	5.00 ±0.37	100
	T-test		*	*	ns	
	%CV		8.19	3.24	28.35	

Note * significantly different at $p \leq 0.05$ and ns is non-significantly different at $p \leq 0.05$.

Analysis of genetic variation using SRAP marker

Ten primer combinations were selected for evaluating the difference in DNA profiling of the selected control and variant plants that were planted in the second field cultivation (V16 progeny). The results showed one specific PCR fragment of approximately 850 bp was produced by the primer pair Me1/Em10 on the control line (pink flower) and was absent in the variant line (light purple flower) (Figure 5). The rest of the primer pairs generated no polymorphic DNA bands.

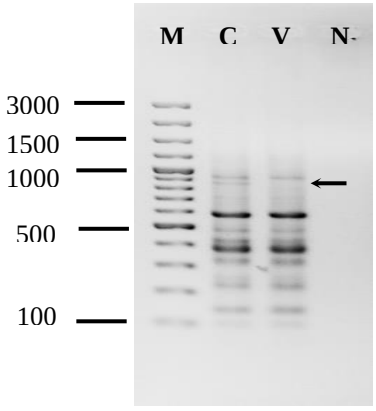

Figure 5. SRAPs amplification by primers combination of Me1/Em10 in control (lane C) and variant (lane V) chrysanthemum lines. The arrow indicates the missing DNA band in the variant line compared with the control line. Lane M is GeneRuler 100 bp plus DNA ladder and lane N is negative control

Discussion

The callus induction from the mature petal

In this study, the mature petal cultured on basal MS media supplemented with 2,4-D or BA alone could not induce callus proliferation. However, the mature petal cultured on MS media supplemented with 2,4-D and together with BA can induce callus proliferation. This result confirmed that the type and concentration of plant growth regulators, specifically auxins and cytokinins and also the ratio of auxin/cytokinin, are important factors that regulate callogenesis. Nasri *et al*. (2018) reported that the leaves of chrysanthemum 'Homa' and 'Delkash' cultured on the MS medium supplemented with BA alone did not enhance callus development. Thangmanee and Kanchanapoom (2011) reported that the combination of BA and 2,4-D induced the best response for callus induction from the ray floret of chrysanthemum which is possibly due to the difference in endogenous levels of growth regulators or a difference in sensitivity in the plant.

However, by exploring at the trend of callus formation in this study, an increasing callus weight associated with an increase in 2,4-D concentration was observed. It was found that higher concentrations of 2,4-D enhanced callus proliferation. Mani and Senthil (2011) reported that the presence of 2,4-D in petal cultures was desirable for callus induction of chrysanthemum. Furthermore, Obukosia *et al*. (2005) reported that the concentration of 2,4-D affected the callus induction of pyrethrum.

Auxin and cytokinin incorporation related to the development of organogenesis through plant tissue culture while using auxin or cytokinin alone may not affect organ development (Song *et al.*, 2011; Kumar *et al*., 2017). Auxin promotes growth, cell division, and elongation of callus, whereas cytokinin affects the shoot induction more than the cell division and elongation (Phillips and Garda, 2019). Thus, the tissue cultured on medium supplemented with BA or 2,4-D alone did not successfully induce callus development in this study. Similarly, Naing *et al.* (2013) reported that the petal culture of chrysanthemum on MS medium containing only 2,4-D did not develop a somatic embryo. However, the development of organogenesis depends on both the concentration of PGRs and plant cultivars. Each cultivar has a different response and uses different PGRs. Naing *et al*. (2013) documented that 'Baeksun' chrysanthemum petal cultured on MS medium supplemented with 1 mg/L 2,4-D and 3 mg/L BA was suitable for embryogenesis (56.3 embryos/explant after 5 weeks of culture). Thangmanee and Kanchanapoom (2011) report that *Chrysanthemum x grandiflorum* was cultured by ray florets

on MS medium supplemented with 13.3 µM BA and 0.5 µM 2,4-D. Miler and Muszczyk (2014) report that the best medium for callus formation from ovules and ovaries of 'Capitola' chrysanthemum was MS medium supplemented with 1 mg/L 2,4-D and 2 mg/L BA.

Plantlet regeneration and somaclonal variation

The shoot or plantlet regeneration depends mainly on the concentration and type of cytokinins in which kinetin was reported to be the most effective cytokinin for regenerating shoots from the callus of *Chrysanthemum morifolium* (Nahid *et al.*, 2007; Thangmanee and Kanchanapoom, 2011). Normally, the plant regeneration through induction media containing auxin and cytokinins results in the difference characters of organogenesis. Shoot formation occurs in the culture medium with a high concentration of cytokinins and a low concentration of auxins, while root formation occurs in culture medium with a high concentration of auxins and a low concentration of cytokinins (Tymoszuk and Zalewska, 2014). Xu *et al.* (2008) reported that the cytokinin plays an important role in the regeneration of shoots from callus in *in vitro* conditions. However, the optimum PGR concentration for adventitious shoot production depends on plant cultivars and responses (Nahid *et al.*, 2007). Nahid *et al.* (2007) report that the shoot induction of chrysanthemum '89' was observed on MS medium supplemented with 2 mg/L BA and 0.1 mg/L kinetin, whereas in the line '4037', shoot induction was successfully obtained from MS medium supplemented with 2 mg/L BA, 0.1 mg/L kinetin, and 0.1 mg/L NAA. Thangmanee and Kanchanapoom (2011) documented that the MS medium supplemented with 9.3 µM kinetin and 4.9 µM IBA was optimal for shoot induction.

In general, the plant tissue culture technique provides an efficient multiplication and maintains a large number of elite genotypes. However, there is sometimes a phenotype that is different from the mother plant. Larkin and Scowcroft (1981) reported that plant cell culture itself generates genetic variability which has been defined as somaclonal variation.

Zalewska *et al.* (2007) cultured the leaf explant of 11 cultivars in chrysanthemum on MS medium supplemented with 2.0 mg/L indoleacetic acid (IAA) and 0.6 mg/L BA, and thus obtained the different color and shape appearances of flowers of the plantlets. Kengkarj *et al.* (2008) studied somaclones from seven commercial cultivars of chrysanthemum that were obtained through the petal segments' culture and found that the color and inflorescence shape change was only present in one cultivar where the petal color changed from magenta to red. Miler and Jedrzelczyk (2018) cultured the

ovaries of chrysanthemum on MS medium supplemented with BAP and 2,4-D at 1 mg/L each of PGRs and obtained different leaf and petal appearances in regenerants. Jin *et al.* (2008) reported that MS media supplemented with a 2,4-D and kinetin combination stimulated a higher genetic variance of cotton tissue than MS media supplemented with IBA and kinetin combination, which 2,4-D, as a result, increased the genetic variability.

The mutation or variation resulted from tissue culture that was attributed by numerous factors, including explant source, the concentration of PGRs (auxin and cytokinin), length of culture period, and number of subculture cycles (Roy *et al*., 2010; Krishna *et al*., 2015). However, the PGRs have an effect on the increase in rate of somaclonal variation and disturb the genetic stability of new regenerants (Abu-Qaoud *et al*., 2010: Lema-Ruminska and Sliwinska, 2015).

In general, plant cells exhibit some plasticity depending upon growth stages and cell fate. Young tissue has higher plasticity than the mature tissue and that makes it easy to reactivate and reprogram the relatively undifferentiated cells, thus making it easy to induce regeneration using a low concentration of PGRs. In contrast, mature tissue can also go through this process, but with a higher concentration of PGRs applied. Thus, high concentration of PGRs leads to somaclonal variation (Gao *et al*., 2010; Bairu *et al*, 2011). In this study, the mature petals were used as the explants and thus the mature cells needed reprogramming from the fully differentiated cells to form callus which was an undifferentiated cell prior to regenerating into plantlets. These favor the occurrence of somaclonal variation. The new variant plants possibly occur from chimera tissue due to the fact that the petals obtain the L1 and L2 layers, with the L1 determining the color change and the L2 determining floral shape (Vilasini and Latipah, 2000).

The establishment of the solid mutant in chrysanthemum by in vitro cutting node

Datta and Chakrabarty (2009) documented that the suitable technique for *in vitro* management of chimera is the tissue culture technique that regenerates plants from stem internodes, stem nodes, shoot tips, and ray florets. However, the characteristic of explant tissue also affects the genetic stability of the variant line depending on the age, type, and genotype of the tissue along with the optimum culture condition. In addition, the tissue in the variant line is probably a periclinal chimera tissue type because this tissue type is the most genetically stable tissue compared to the mericlinal and sectorial chimera (Dowrick and El-Bayoumi, 1965). Ahloowalia and Maluszynski (2001)

reported that several subcultures of tissue led to genetic stability starting from the V3-V4 generation. Plader *et al.* (1998) reported that the somaclonal variation *via* several subcultures of the axillary shoot can lead to more genetic stability of the plant than adventitious shoot used. Ngezahayo and Liu (2004) also documented that the axillary bud proliferation is the most used and is also the most suitable to assure genetic stability for the regenerated plants obtained.

The axillary bud has been successfully used in propagating many plants such as in the hops plant developed by somaclonal variation in internodal segments and a three-fine subculture that presented a genetic stability plant. Panda *et al*. (2007) cultured an axillary bud of *Curcuma longa L.* over 26 months and evaluated the genetic stability of the Random Amplified Polymorphic DNA (RADP) technique which resulted in no genetic variation of *Curcuma longa L.* Some chrysanthemum varieties developed by somaclonal variation possess high genetic stability such as 'Red Reagan' which is a potted plant generated by an axillary bud and several subcultures. Zoghlami *et al.* (2012) cultured an axillary-branch of *Opuntia ficus-indica* (L.) Mill. by subculture several times and studied the genetic stability by RADP technique. The result showed that the increase in the culture period promotes the occurrence of genetic stability due to four polymorphic bands observed at the 2^{nd} subculture cycle, but no polymorphic band was revealed at the 8^{th} and 16^{th} subcultures.

From this experiment, the somaclonal variant lines of 'Polaris Pink' chrysanthemum showed genetic stability since the first field-grown (of the 8^{th} generation) as all variant plants showed similar phenotypes and the result was also confirmed in the second field-grown of the 16^{th} generation in which all variant plants were true to type. However, the plant height and number of flowers per stem in these two field grown were different. These two field evaluations were performed in two different seasons (summer and winter) and this contributed to the phenotypic changes. The environments of both seasons, mainly temperature and light, affected the genotype by environment interactions (GxE) and thus brought about the changes in the varietal performance. Similar phenomenon was reported in rose by Gitonga *et al.* (2014).

Analysis of genetic variation using SRAP marker

The SRAP technique is one that is important to use in agriculture in order to identify the DNA of different plant genotypes, like floral color or shape and type, hybrid identification, sex determination, gene tagging, and genetic diversity analysis. The primer pair Me1/Em10 could identify *Paeonia*

suffruticosa cultivars according to Guo *et al.* (2009), who detected a polymorphic band of cultivar 'Shen Dan Lu' (pink) from 16 varieties of *P. suffruticosa*. Su *et al.* (2019) documented the intensive used of SRAP markers to study several traits in chrysanthemum, such as plant architectural traits, inflorescence traits, leaf traits, flowering time, and certain biotic and abiotic stress traits.

The SRAP primers were selected followed Guo *et al*. (2009) who reported that the primer pair Me1/Em10 and Me7/Em2 detected a different color genotype of *Paeonia suffruticosa*. The Me1/Em10 detected the 'Shen Dan Lu' cultivars which have pink characteristics while Me7/Em2 detected the multicolor character that showed polymorphic bands in the 'Er Qiao' cultivar. In addition, the other eight primer combinations were reported as successfully detecting the QTL of *Rayln1, Rayln2, Rayln3,* and *Rayln4,* which associated with the ray floret layer number in *Dendranthema morifolium* as Zhang *et al*. (2011) reported. In this study, 10 primer pairs were used in SRAP to study DNA polymorphism in the solid variants obtained from 'Polaris Pink' chrysanthemum and only one primer pair, Me1/Em10, revealed a single DNA band missing in the variant line. Meanwhile, nine primer combinations revealed no polymorphic band between the variant line and control line. However, the result of investigation should be further studied in order to determine the genes in the DNA polymorphic band. This can be done by DNA sequencing and comparing the DNA sequences with the NCBI database.

In conclusion, the new variety of 'Polaris Pink' chrysanthemum was successfully established by culturing a mature ray floret on a callus induction medium which was MS medium containing 2 mg/L BA and 0.5 mg/L 2,4-D, followed by shoots regeneration on MS medium containing 2 or 3 mg/L kinetin. Subsequently, the solid variant lines were obtained through several generations of *in vitro* single node cutting. The primer pair Me1/Em10 exhibited the polymorphic band between the mother plant and variant plant.

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