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Phenotypic variation and genetic alteration of *Spathoglottis plicata* resulted from in vitro cultured seed irradiated with X-Ray

SUYITNO ALOYSIUS[1,♥], AZIZ PURWANTORO[2], KUMALA DEWI[3], ENDANG SEMIARTI[3]

[1]Department of Biology Education, Faculty of Mathematics and Natural Science, Universitas Negeri Yogyakarta, Karangmalang, Yogyakarta.
[2]Faculty of Agriculture, Universitas Gadjah Mada, Jl. Flora Bulaksumur, Yogyakarta 55281, Indonesia. Phone/Fax: +62- (274) 551228.
[3]Graduate Program in Biology, Faculty of Biology, Universitas Gadjah Mada. Jl. Teknika Selatan, Sekip Utara, Yogyakarta 55281, Indonesia, Tel./fax.: +62-274-580839. ♥email: suyitno_al@uny.ac.id

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Abstract. *Aloysius S, Purwantoro A, Dewi K, Semiarti E.. 2018. Phenotypic variation and genetic alteration of* Spathoglottis plicata *resulted from in vitro cultured seed irradiated with X-Ray. Biodiversitas 19: 1642-1648.* A terrestrial orchid species among genus Spathoglottis as widely cultivated is S. plicata. Variability development of the species through mutation induction has been carried out, but its morphological variations and genetic changes have not been investigated. The purpose of this study is to identify the phenotypic variation and genetic alteration of S. plicata resulted from in vitro cultured seed irradiated with X-ray. Radiation was given at the doses of 0; 6; 12; 18 and 24 rad. The samples were surviving plants resulted from irradiated seeds. Phenotypic variations observed were the number, length and width of the leaf, number of tiller, and flower characteristics. Genetic alteration was detected from DNA homologous POH1, a key gene determining of shoot morphogenesis. Results show that there are variations of leaf color, length and width of the leaf, and the number of the tiller. Plants start to flower at the age of 30 months. The plants flowering reach 64.7% (WT), 50.0% (6 rad), 33.3% (12 rad), 33.3% (18 rad), and 40% (24 rad). Flower color is ranged from white, white slightly purple, purplish white, light purple, reddish purple and purple, found both in mutants and wild-type groups. The alignment result of POH1 homologous DNA obtained from PCR cDNA shows the nucleotide differences at some points between mutants and wild-type that indicate the occurrence of DNA alteration. X-ray induces the changes of POH1 homologous DNA, but it has no obvious relationship to the flower variation.

Keywords: Genetic, phenotypic, POH1 homologous gene, *Spathoglottis plicata*, X-ray

INTRODUCTION

The genus *Spathoglottis* (Orchidaceae) is still unknown status based on the IUCN Red List, but some experts suggest that some species within the genus are vulnerable (Dockrill 1992) or endangered (Murthy et al. 2012). A terrestrial orchid species among *Spathoglottis* as widely cultivated is *S. plicata.* The development of the species through mutation induction was important since the morphological diversity of *S. plicata* was still low (Romeida 2012). The conventional way of cross-breeding produces only limited variation. Mutation induction by radiation with random effects could potentially produce greater variations.

A technique of mutation induction by ionizing rays (X-rays and gamma-rays) was the easiest, useful, and secured way, and it has been widely applied in horticultural crops (Piri et al. 2011), such as *Hordeum* (barley) and *Triticum* (wheat). There were 3278 mutant varieties registered, 561 varieties resulted from X-ray induction, and only one mutant variety in orchids produced but it by another mutagent, those were *Cymbidium* (FAO/IAEA 2018). Seed is the most sensitive stage thus it is appropriately used as target irradiation to obtain new characters (Redei 1992). X-rays irradiation was proven capable of inducing the changes in the structure and color of *Zinnia elegans* flowers (Gultom and Gultom 2015) and many varieties of horticultural crops (van Harten 1998). X-ray irradiation is also expected to be capable of triggering phenotypic variations and its related genes on *S. plicata*. Based on the mutant variety database (FAO/IAEA 2018), there is no mutant variety of orchids produced by X-ray induction. In addition, studies related to the effects of X-ray irradiation on orchid morphogenesis genes have not been reported.

X-rays irradiation has a great ability to penetrate tissues and causes various changes or damages in cells. Ionizing irradiation (X-rays and gamma) triggers excitation and ionization in the material or living cells and causes rupture of chemical bonds in biological systems (Jan et al. 2012). The level of damage by ionizing rays was affected by the type or quality of light, irradiation dose and genotypic factor or sensitivity level of the organism (Hameed et al. 2008; Al-Enezi and Al-Khoyri 2012). The sensitivity of the organism was affected by age, type, genotype, level of activity or physiological conditions and complexity of the tissues (De Micco et al. 2010). Mutations have no necessarily effect directly to the morphological changes in the first generation plants (M1) (van Harten 1998). Mutations may start to appear in one or several offspring generations later on. Mutations might lead to the changes in prominent morphological characters, vague or do not affect morphological changes at all. Based on leaf lobe formation in mutants, mutations are distinguished into mild mutation, moderate mutation and severe mutation (Howell 1998).

The plants might be significantly affected (susceptible), moderately affected or tolerant (unsusceptible) by ionizing radiation. Most of *S. plicata* resulted from seed irradiation have morphological characters similar to wild-type plants (wildtype-like = WT-L). To detect the presence of mutations in a plant that was similar to its wild-type, DNA or RNA analysis was required.

Molecular characterization has been based on gene structure identification, genetic material at the genome (DNA) level, transcription (RNA) or its protein results (de Micco 2010). The POH1 or its homologous genes were members of Knox gene clusters (knotted-like orchid homeobox) which was a key gene in the shoot morphogenesis. From the results of PCR-RAPD, *S. plicata* seedlings experiencing prominent morphological changes show an increase in DNA polymorphism (Aloysius et al. 2017). Whether the orchids *S. plicata* surviving in experimental gardens also undergo genetic changes especially in homologous gene POH1, is an interesting thing to be studied. based on this reason, the purposes of this study were to identify phenotypic variation of *S. plicata* resulted from in vitro cultured seed irradiated by x-rays, and to detect genetic changes in homologous gene POH1 of mutants *S. plicata*.

MATERIALS AND METHODS

Materials

The material used in this study was *S. plicata* orchid plants resulted from acclimatization of plants produced by in vitro cultured seeds irradiated with X-rays. The number of plant samples were given as follows: 18 plants (0 rad); 8 plants (6 rad); 7 plants (12 rad); 6 plants (18 rad), and 10 plants (24 rad).

Orchid planting in the experimental garden

Cultured plants at the age of 8-10 months were acclimatized in stages given as follows: seedlings or plantlets were removed from the bottle, cleanly washed, and then soaked in fungicide (1g /L) for about 5-10 minutes, and then removed to organic fertilizer liquid (2-3 mL/L) for 5-10 minutes. The plants were then put into bottles that have been given cotton moistened with the same fertilizer solution. Furthermore, the bottles were closed using plastic lids with holes to keep connecting with the outside air and finally left them for 1-2 days. The plantlets were then transferred into plastic pots filled with moss which has been soaked in organic fertilizer solution (2-3 mL/L) for 5-10 minutes. The pots were then placed into a plastic lid (1 x 2 m^2) in the experimental garden with 50-75% light penetration. Plants were maintained by spraying water in twice a day. Plants were also sprayed with foliar fertilizer (1-2 mL/L) once in 3 weeks. The surviving plants were then transferred to the soil-manure medium (2 : 1) in plastic pots (8 x10 cm^2). Plants were then moved into a larger plastic pot (12 x 9 cm^2) with the same planting medium. Finally, at the age of 32 months, the plants were moved into a larger pot (23 x 20 cm^2).

Data collecting and processing

Morphological data

Morphological observation of plants includes plant height, number and length of leaf, leaf color, leaf sheet, and clump size (number of tillers). Flower morphology observation includes color and flower diameter, flowering number, length of the flower stalk, and the age of first flowering.

Analysis of POH1 homologous DNA transcript

Detection of genetic changes was focused on the analysis of POH1 homologous DNA transcript of wild-type plants (WT) and irradiated plants. PCR with Reverse Transcript (RT-PCR) was used for the POH1 RNA analysis (gene expression) of WT and mutants resulted from irradiated seeds. Total RNA was extracted and isolated from 0.1 g pieces of shoot bulb (rods) using RNA mini kit (Plant) GeneAid and conducted according to the procedures from the company. Total RNA (1 ug) was used to synthesize cDNA using Transcriptor One-Step RT-PCR kit. Synthesis of cDNA was conducted with PCR via Touch Down using the following condition stages. Phase I: 50º C for 30′ (reverse transcription), 94° C for 7' (initial denaturation), 94° C for 10" (denaturation), 50° for C 30" (annealing), 68º C for 1' (elongation), with 9 time cycles. Phase II: 94° C for 10" (denaturation), 55° C for 30" (annealing), 68º C for 1' (elongation), with 11 time cycles. Phase III: 94° C for 10" (denaturation), 50° C for 30" (annealing), 68º C for 1' (elongation), with 11 time cycles. Finally, 68º C for 7' (final elongation) and 12º C for 7' (hold). DNA complement (cDNA) was used for POH1 homologous DNA amplification.

Amplification of POH1 homologous cDNA uses master mix GoTaqGreen (GTG) (Promega), with POH1 homolog _F1 primer (5'-TAC TTC TAA CAA ATG GTG GGA-3') and' POH1 homolog'_R1 (5'-AAT GCG ATA AGA TAT TGT AGT -3'). The composition of the PCR mix for final volume of 40 µL was 20 µL GTG, 2 µL POH1 homolog_F1 primer and 2 µL POH1 homolog_R1 (10 pg/µL), 1.5 µL DNA template, and 14.5 µL nuclease-free water. PCR was performed in the following stages: pre-denaturation (95º C for 2'), 40 time cycles with stages: denaturation (95° C for 30"), annealing (57º C for 30"), elongation (72° C for 1'.30").Then, the extension phase (72° C for 5') and hold (12º C for 5'). DNA amplicons were checked using electrophoresis on 1% agarose gel which was given 2 ul DNA staining of Florosafe brand, run on 100 V for 55 minutes. DNA ladder of Vivantis brand which provides DNA fragment sizes of 100 - 3000 bp was used as DNA markers. The electrophoresis results were observed under UV transilluminator and photographed.

Moreover, DNA amplicon was sequenced. The alignment was conducted using MEGA7 program. The alignment results were then utilized to identify the differences in nucleotide composition (nucleotide polymorphism) between mutant and WT.

Data analysis

Qualitative data of plant morphology were analyzed using qualitative descriptive analysis. Quantitative data

related to morphological parameters were analyzed using one-way analysis of variance followed by Duncan's multiple range tests with a significance level of 95%. Results of POH1 homologous DNA transcript alignment were identified to find the genetic changes (deletion, insertion or substitution of nucleotide bases).

RESULTS AND DISCUSSION

Identification of morphological variation

Spathoglottis plicata which were successfully acclimatized and survived have no prominent morphological character differences. Plants have enough strong habitus and fresh green linear (lanceolate) leaves. In general, the appearance of the plant habitus (Figure 1) was similar to WT-L plants.

Variations also appear on the characteristics of leaf size, the number of tillers, flower stem length, inflorescence number, and size of flower (Table 1), leaf color, lamina structure and number of tillers or cluster size (Table 2). These results show different influence or response of the plants. As stated by Borzoeui et al. (2010), Jan et al. (2012), and Minisi et al. (2013) that irradiation produces radiation stress as a result of free radicals (ROS) production which was highly toxic and mutagenic and also affect differently towards the morphology, anatomy, biochemistry, and physiology of plants depending on the dose given.

There were variations in the morphological traits, but not specific to a group of irradiated plants and also found in the WT plants group. Plants with yellow-green variegate leaf chimera were found in the WT group (5.5%), 18 rad irradiated plants (16.7%), and 24 rad irradiated plants (10%) and most of the other leaves were green or dark green. Variegated yellow-green leaf chimera was also found in *S. plicata* plants as a result of gamma irradiation (doses of 30-40 Gy) on the plantlets (Romeida 2012). Variations in leaf color were determined by the content or leaf pigment production, especially chlorophyll and xanthophyll. Mueller et al. (2012) stated that the xanthan or chlorine leaf mutants of barley (*Hordeum vulgare*) occur because of the decrease in the production of chlorophyll-a and chlorophyll-b.

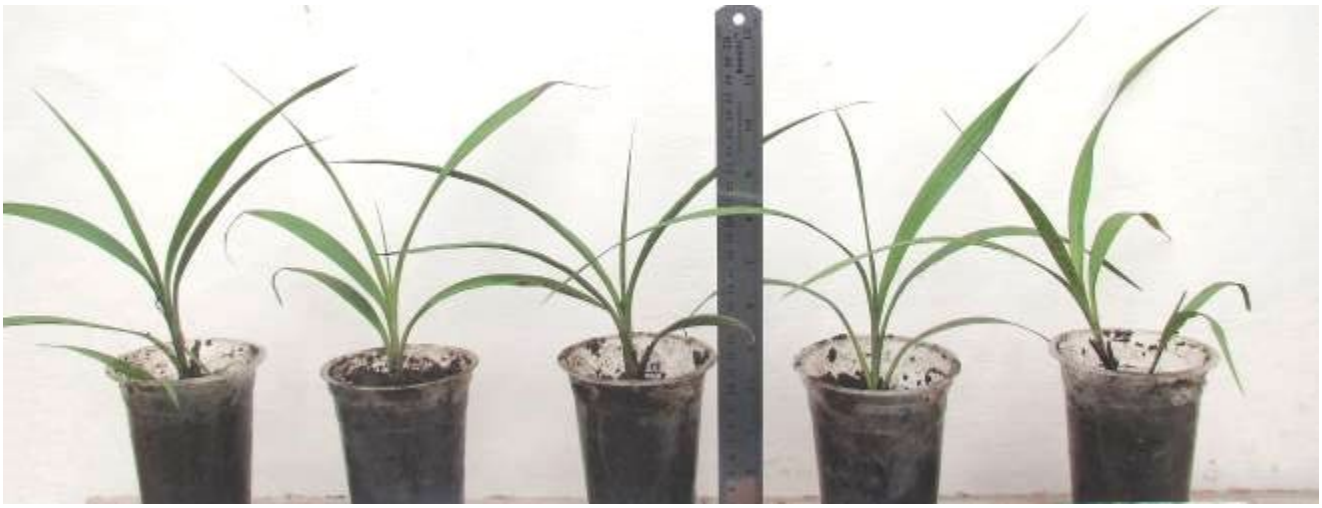


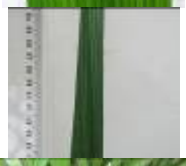
Figure 1. Samples of *Spathoglottis plicata* orchid at the age of 24 months resulted from in vitro culture of irradiated seeds at the doses of 0 (WT), 6, 12, 18, and 24 rad

Table 1. Morphological variation of *S. plicata* orchid resulted from seeds irradiation with X-rays

Doses (rad)	Length of flower stalk*	Flower size (l x w)*	Number of flower bud*	Number of inflorescence ns	Age at the first flowering (months)*	Number of tillers ns	Leaf length (cm)*	Leaf width (cm)ns
0	44.39±7.3b	20.58±2.9 ab	21.8±5.4ab	2.3±0.7 a	33.9±3.9ab	11.5±4.8a	73.3±7.5 a	3.5±0.7a
6	29.44±6.9a	16.35±3.4 a	18.7±10.2ab	3.7±3.6 a	41.5±6.5 b	9.0±3.1 a	58.9±8.4 a	3.6±1.1a
12	37.5±3.4bc	18.5 ±6.1a	15.2±6.6 a	3.3±0.9 a	39.7±1.7ab	13.3±2.0a	68.1±8.3 a	4.6±1.1a
18	49.9±10.9c	24.4±3.0b	17.0±3.3a	2.7±1.7a	46.0±5.1 b	12.0±3.7a	88.3±5.9 b	4.4±0.9a
24	45.7±8.4bc	20.8±3.1ab	28.1±2.3b	3.5±1.5a	31.2±7.3 a	7.5±5.0a	69.4±8.1 a	4.4±1.0a

NB. The same letter beside the average value of each variable shows no significant difference ($p > 0.05$) from the analysis of Duncan's Multiple Range Test (DMRT). * : significantly different

Table 2. Percentage of each morphological character of Spathoglottis plicata resulted from irradiated seeds

Morphological characteristic			Irradiated groups					Average
			0 rad	6 rad	12 rad	18 rad	24 rad	
Leaf color		Yellow-green variegata	5.5	0.0	0.0	16.7	10.0	2.19
		Green	94.5	100	100	83.3	90.0	97.81
Lamina		Open	88.9	100	100	83.3	100	95.92
		Roll/ twist	11.1	0.0	0.0	16.7	0.0	4.06
Clump: many (> 11); few (< 10)		Many tillers	77.78	57.14	100	83.3	60.0	75.51
		Few tillers	22.22	42.86	0.0	16.7	40.0	24.49

Moreover, variations in the structure of lamina (longitudinal roll or open), as well as the size of clusters (many or few tillers), were also found. Morphology variations which were not specified in the treatment groups show that these variations were not a result of irradiation. Yellow striped leaves on some plants may be a chimera. According to Leyser and Day (2003), plants can form two or more tissues that were genetically distinct and display different colors called chimera. For many plants, including orchids group, the difference in genome number between tissues often occurs because of endopolyploid (endoreduplication) events, but morphology differences did not accompany it. In *S. plicata* endopolyploid was found in various organs, except sepal, petal, and ovary (Yang and Loh 2004).

The variations concerning inflorescence and floral, no specific traits in plants irradiated groups were revealed (Table 1). Characteristic variations were found in stem length, flower diameter, number of buds produced, inflorescence number, and age of first flowering. This result indicates that the morphology variations of flowers occurring were also not associated with the given irradiation doses.

Prominent morphological variations were found in the color of flower, but this result was also not specific traits in irradiated plants groups. Flowers were generated in several levels of gradation (Table 3), including : (i) white on the entire piece of flowers, (ii) white with labellum part white purplish, (iii) purplish white, (iv) pink or light purple, (v) reddish purple, and (vi) dark purple. In general, the most emerging flower color was purple (48%), and the fewest was the white flower (7.4%). The same variations also occur in flower's labellum, both of the WT group and irradiated plants.

A similar study with gamma irradiation (20-300 Gy) was conducted by Romeida (2012) on *S. plicata* of Bengkulu accession. The study produces normal *S. plicata* plants with different flower colors from their parents. This study found that there were six color gradations of flowers and the phenotypic variation of flower colors might be formed not only as a result of X-ray irradiation but also because of free segregation during the formation of gametes. The discovery of six flower color gradations in WT plants indicates that multiple genes determined the inheritance of flower color in *S. plicata*.

Table 3. Number (%) of *S. plicata* orchid flowers resulted from irradiated plants

Flower color		Irradiated groups					Average (%)
		0 rad	6 rad	12 rad	18 rad	24 rad	
White		1 (7.69)	0	1 (33.3)	0	0	(7.40)
White, side lobe a bit of purple		1 (7.69)	1 (25.0)	0	0	1 (25.0)	(11.11)
Purplish white		3 (23.07)	0	0	0	0	(11,11)
Light purple		1 (7.69)	0	2 (66.7)	0	0	(11.11)
Red-purple		2 (15.38)	0	0	1 (33.3)	0	(11,11)
Purple		5 (38.46)	3 (75.0)	0	2 (66.7)	3 (75.0)	(48.1)
Total of flowered plant (%)		**13** (48.1)	**4** (14.81)	**3** (11.11)	**3** (11.11)	**4** (14.81)	(100)

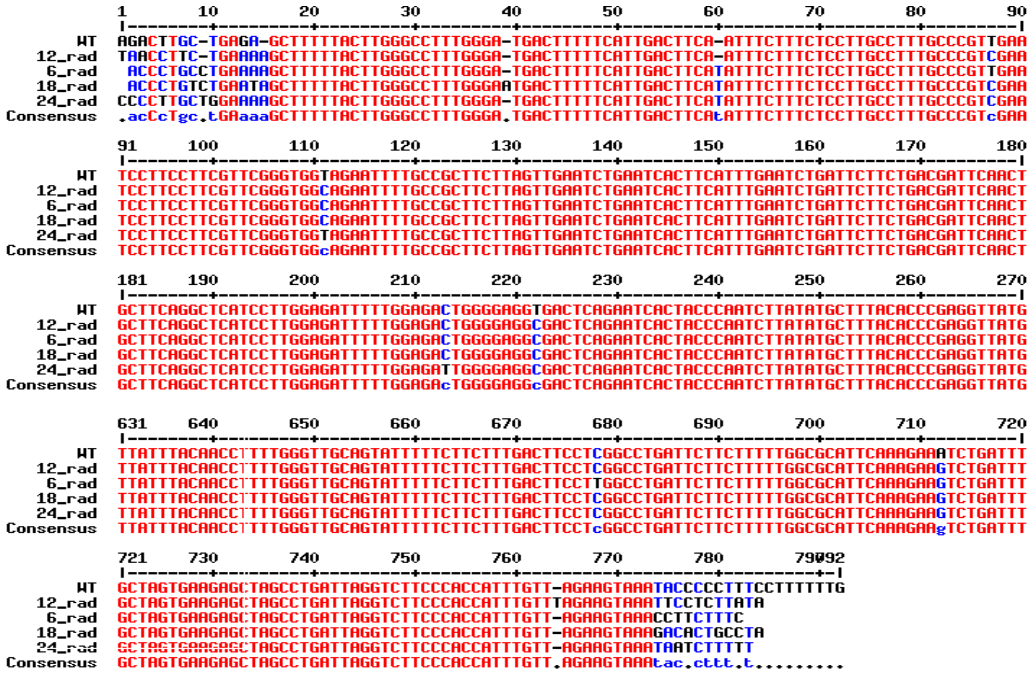


Figure 4. Alignment result of POH1 homologous DNA sequence of WT and irradiated plants

Table 4. Nucleotide changes in complementary DNA (cDNA) of homologous POH1 in irradiated plants.

Doses (rad)	Position of single nucleotide															
	5	**7**	**9**	**10**	**13**	**14**	**15**	**39**	**60**	**87**	**111**	**213**	**222**	**678**	**712**	**764**
WT	T	G	-	T	G	A	-	-	-	T	T	C	T	C	A	-
6	C	G	C	T	A	A	A	-	T	T	C	C	C	T	G	-
12	C	T	-	T	A	A	A	-	-	C	C	C	C	C	G	T
18	C	G	C	T	A	T	A	A	T	C	C	C	C	C	G	-
24	T	G	T	G	A	A	A	-	T	C	T	T	C	C	G	-

Flower color was determined by three main pigments including betalain, carotenoid, and anthocyanin (Grotewold 2006) *in* Lee et al. (2008). According to Lee et al. (2008), the primary pigment determining flower colors of orange, red, purple, dark purple, and purplish blue was anthocyanin. Irradiation of seeds with gamma rays also cause variations in structure and flower color on *Zinnia elegans* (Gultom and Gultom 2015) and *Chrysanthemum* (Lee et al. 2008), changes in leaves and roots morphology of *Dendrobium* (Sulistianingsih 2013), changes in flower color on *S plicata* (Romeida 2012) as well as an increased level of anthocyanin on *Moluccella laevis* (Minisi et al. 2013).

Analysis of POH1 homologous DNA Transcript

Results of POH1 homologous cDNA PCR on WT and irradiated plants at all dose levels show the DNA with a size of about 750 bp (Figure 3). This result suggests that X-rays irradiation does not affect the size of POH1 homologous DNA, let alone chromosome aberration. The sequence of POH1 homologous cDNA and its alignment result (Figure 4) indicates the presence of nucleotide changes in several points (gene mutation, point mutation).

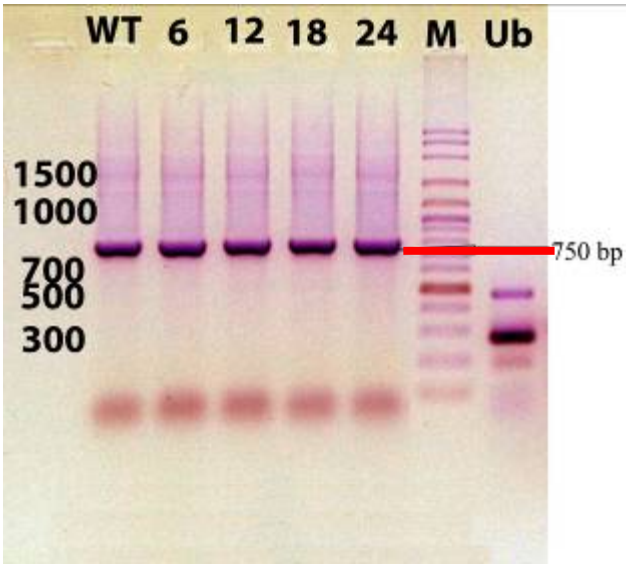


Figure 3. Electropherogram homologous DNA POH1 resulted from PCR cDNA of WT and irradiated plants (6-24 rad) *Spathoglottis plicata*. WT: wild-type, M: DNA Marker, bp: base pair, Ub: Ubiquitin.

Five samples for all level of irradiation dose were morphologically similar, but there were some differences (changes) in the structure of POH1 homologous DNA nucleotides. In POH1 homologous cDNA of irradiated plants, nucleotide substitutions occur in nine points and insertions happen in four points (Table 4). This result suggests that the nucleotide changes are not associated with morphological variations. Nucleotide changes allegedly occur on codon position that has no effect to the amino acid changes or protein produced. According to van Harten (1998), the mutation of nucleotides does not affect gene expression, depending on the changes of codon. Nucleotide changes on the 3rd base of codon do not affect to the changes in amino acid or protein produced.

Results of *S. plicata* from in vitro cultured seeds which were irradiated with X-rays shows phenotypic variations in color, length and structure of leaf lamina, number of tillers, and color of flowers. Some plants have yellow striped leaves, curled leaf, tall plants with few tillers and start flowering at various ages from the age of 30 months. Flower petal colors were graded from white, slightly purple white, light purple, reddish purple, and purple. Based on the gradation of flower color of *S. plicata* was allegedly determined by multiple genes. Based on sequence and alignment results of POH1 homologous DNA transcript, changes in nucleotide composition are found in the form of substitutions and insertions in some points (point mutation) although do not affect clearly on phenotypic changes of *S. plicata*.

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