HortScience 58(12):1465–1473. 2023. https://doi.org/10.21273/HORTSCI17350-23

Sequence-related Amplified Polymorphism and Target Region Amplification Polymorphism Markers-based Profiling of Sodium Azide and Ethyl Methanesulfonate-derived Black Rot-resistant *Dendrobium* sp. 'Earsakul' Mutants from In Vitro Mutagenesis and Selection

Witsarut Chueakhunthod
School of Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, 111 University Avenue, Muang District, Nakhon Ratchasima 30000, Thailand

Apinya Khairum
Department of Agriculture, Faculty of Science and Technology, Thepsatri Rajabhat University, Lopburi Province 15000, Thailand

Piyangkoon Jaukwon, Theerawat Chantakot, Tanandorn Woramit, Sasitorn Hualsawat, and Piyada A. Tantasawat
School of Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, 111 University Avenue, Muang District, Nakhon Ratchasima 30000, Thailand

Keywords. black rot resistance, *Dendrobium* sp. 'Earsakul', ethyl methanesulfonate-induced mutants, sequence-related amplified polymorphism, sodium azide-induced mutants, target region amplification polymorphism

Abstract. **To reveal the applicability of sequence-related amplified polymorphism (SRAP) and target region amplification polymorphism (TRAP) markers to mutant genotyping and marker identification for resistance to black rot (*Phytophthora palmivora*) in orchids (*Dendrobium* sp. 'Earsakul'), we fingerprinted four nonmutagenized controls and 12 black rot-resistant mutants obtained with in vitro sodium azide (NaN_3) and ethyl methanesulfonate (EMS) mutagenesis and in vitro selection using culture filtrate of *P. palmivora*. Each of the 20 SRAP and TRAP primer combinations yielded 375 and 384 scorable DNA bands, respectively, of which 94 (24.42%) and 88 (22.91%) were polymorphic, respectively. Mantel's test cophenetic correlation coefficients of SRAP, TRAP, and SRAP/TRAP were 0.750, 0.921, and 0.861, respectively, indicating the efficiency of these markers, especially TRAP and SRAP/TRAP, for *Dendrobium* sp. 'Earsakul' mutant characterization. Moreover, the correlations between the matrices of cophenetic correlation values for the dendrograms of SRAP with TRAP, SRAP with SRAP/TRAP, and TRAP with SRAP/TRAP were 0.399, 0.566, and 0.793, respectively, and the dendrograms based on SRAP vs. TRAP and SRAP vs. SRAP/TRAP, with lower correlations, had more variations, i.e., the number of clusters, the members of clusters, and the placement of the materials, than the ones based on TRAP vs. SRAP/TRAP. Among the three dendrograms, all nonmutagenized controls were clustered together, whereas all the highly resistant and the most resistant mutants were distributed separately as individuals. Interestingly, the four SRAP and TRAP markers were significantly associated with black rot resistance. Overall, our results will be useful for facilitating future *Dendrobium* sp. 'Earsakul' breeding programs.**

Orchids are the most exported and valuable cut flower plants in Thailand, and they generate an annual income of more than $58 million from several countries (i.e., Japan, Vietnam, the United States, China, Italy, South Korea, and others). The Ministry of Commerce of Thailand is focusing on increasing the exportation of cut flower plants to the Association of Southeast Asian Nations countries, hence the increased expansion of exports, with an average rate of increase of 4.41% per year. *Dendrobium* orchids are the most exported orchids because of their high-growing and long-lasting blooms, as well as distinctive flower characters, especially those of *Dendrobium* sp. 'Earsakul', which has purple–white petals and white lips in a triangular shape. However, *Dendrobium* production is largely affected by plant diseases, particularly black rot (*Phytophthora palmivora*). Thus, the development of disease-resistant orchid cultivars is an effective method for controlling disease outbreaks.

Improvement of *Dendrobium* orchids with crossbreeding has several drawbacks, including limited germplasm and the gene pool available for desirable traits, troubles of sexual compatibility, low propagation rate, and long juvenile periods and reproductive cycles. Mutation breeding is an alternative for breeders to develop new cultivars through induction either of radiation or of chemical mutagens because they generate genetic variations or even novel alleles, which may be associated with desirable traits (i.e., disease resistance). Sodium azide (NaN_3) and ethyl methanesulfonate (EMS) are popular chemical mutagens that are available for purchase and highly effective (Arisha et al. 2014; Kumar et al. 2011; Savita et al. 2017; Yoosumran et al. 2018), and they can be successfully used to induce disease resistance in economically important crops (Chen et al. 2014; Lo et al. 2022). Generally, both mutagens induce mutations by base substitution, leading to incorrect base-pairing, and they also induce chromosomal abnormalities. Moreover, mutation breeding in combination with tissue cultures (mutagenesis) provides a feasible method of speeding breeding programs (Constantin 1984).

An array of molecular marker systems, especially arbitrarily amplified DNA markers, i.e., random amplified polymorphic DNA and inter-simple sequence repeat (ISSR), can be commonly used to detect and characterize mutant and somaclonal variants (Bello-Bello et al. 2014; Savita et al. 2017). For target-specific breeding purposes, markers designed to uncover genetic variability should be involved with functional genes because they may reflect functional polymorphisms (Andersen and Lübberstedt 2003). Unlike conventional random markers, i.e., random amplified polymorphic DNA and ISSR, functional diversity markers that are in physical association with coding regions are obtained by designing primers from open reading frames (ORFs) and annotated expressed sequence tags (ESTs) from published sequences of candidate genes (Hu et al. 2005; Li and Quiros 2001).

Sequence-related amplified polymorphism (SRAP) and target region amplification polymorphism (TRAP) markers are commonly used functional markers for molecular analyses with the advantages of simplicity, multilocus nature, high reproducibility, and feasibility of subsequently sequencing their desirable products (Poczai et al. 2013). SRAP markers were first introduced by Li and Quiros (2001) to target the amplification of ORFs by using two primers, a forward primer (with CCGG motif) specific to the exon region combined with a reverse primer (with AATT motif) specific to promoters, introns, and spacers. TRAP markers were developed by Hu and Vick (2003) based on candidate gene-specific detection by using a

fixed primer designed against known ESTs relevant to desirable traits paired with an arbitrary primer of forward or reverse primers of SRAP markers. Currently, there are several ESTs responsible for biosynthesis of secondary metabolites, carbohydrate metabolism and regulatory function, transportation, and stress responses (Tan et al. 2005). They are generally available at OrchidBase (http://orchidbase.itps.ncku.edu.tw/). Plants possess defense responses that begin at the pathogen infection site and subsequently extend to the surrounding cells, thereby activating defense signals and further displaying defense responses throughout the plant, termed systemic acquired resistance (Kamle et al. 2020). Systemic acquired resistance can be triggered by most pathogens in distal/uninfected plant tissues following salicylic acid and pathogenesis-related protein accumulation. We recently found that some unique protein bands of pathogenesis-related proteins, i.e., chitinase and β-1,3-glucanase, were induced in both distal and infected leaves of some *Dendrobium* sp. 'Earsakul'-resistant mutants (Hualsawat et al. 2022; Khairum et al. unpublished data). Therefore, we can design fixed primers of the TRAP markers based on these candidate proteins. To reveal the effectiveness of both marker systems on molecular research, Mirajkar et al. (2017) was able to identify sugarcane mutant germplasm developed through gamma radiation-induced in vitro mutagenesis using SRAP and TRAP markers. El-Shahed et al. (2017) also identified somaclonal variants of micropropagated banana using both marker systems. Moreover, they were successfully used to evaluate genetic variability in orchid species (Feng et al. 2014, 2015). Our experiment aimed to use SRAP and TRAP markers for mutant characterization among four nonmutagenized controls and 12 black rot-resistant mutants of *Dendrobium* sp. 'Earsakul', compare the potential of two marker systems for measuring the levels of genetic variability and mutant identification, and investigate the association of the SRAP and TRAP markers with black rot resistance genes using a regression analysis and Bonferroni correction.

Materials and Methods

Plant materials. Sixteen *Dendrobium* sp. 'Earsakul' lines provided by Hualsawat et al. (2022) and Khairum et al. (2022) were used in our experiment: four nonmutagenized controls and 12 black rot-resistant mutants obtained with in vitro NaN_3 and EMS mutagenesis and three in vitro selection cycles with 30%-30%-40%, and 50%-50%-60% (volume/volume) culture filtrate of *P. palmivora*. Information about the plant materials is presented in Table 1. Briefly, protocorm-like bodies were exposed with 0.5 mM NaN_3 for 1 h or 1.8% (weight/volume) EMS for 4 h and then transferred onto VW1 and then MS2 media (Tantasawat et al. 2015) for 2 months and 2 more months, respectively. The putative mutant protocorm-like bodies were transferred to liquid pea sucrose broth (PSB) medium containing *P. palmivora* culture filtrate at 30% to 60% (volume/volume) for 21 d (Khairum et al. 2018). Protocorm-like bodies withstanding the culture filtrate were recovered on VW1 for 1 month. Selection was repeated for three cycles. The putative mutants were subjected to in vitro multiplication until M_1V_4 generation before the present molecular analysis.

Table 1. Severity scores of symptoms and disease response at 5 d after challenge with *Phytophthora palmivora* isolate NK-53-9 in four nonmutagenized controls and 12 resistant mutants (Hualsawat et al. 2022; Khairum et al. 2022).

Lines	Mutagens	Severity scores of symptoms[i]	Disease response[ii]
SUT16C003[iii]		4.33	S
SUT16C007[iii]		5.00	S
SUT16C008[iii]		5.00	S
SUT16C014[iii]		5.00	S
SUT17N05304	0.5 mM NaN_3	0.00	HR
SUT17N05305	0.5 mM NaN_3	0.67	R
SUT17N05308	0.5 mM NaN_3	0.00	HR
SUT13E18301	1.8% EMS	0.00	HR
SUT13E18303	1.8% EMS	0.83	R
SUT13E18304	1.8% EMS	1.33	R
SUT13E18305	1.8% EMS	0.00	HR
SUT17E18303	1.8% EMS	1.33	R
SUT17E18311	1.8% EMS	0.50	HR
SUT17E18316	1.8% EMS	1.17	R
SUT16E18502	1.8% EMS	0.67	R
SUT16E18503	1.8% EMS	1.33	R

[i] The severity scores were determined using three plants per three replicates. Three leaves were used for one plant.

[ii] The disease responses determined by severity scores at 5 d after pathogen infection were as follows: highly resistant (HR) = 0.00 to 0.50; resistant (R) = 0.51 to 1.50; moderately resistant (MR) = 1.51 to 2.50; moderately susceptible (MS) = 2.51 to 3.50; and susceptible (S) = 3.51 to 5.00.

[iii] Nonmutagenized controls.

EMS = ethyl methanesulfonate.

To isolate their DNA from fresh young leaves, the cetyltrimethyl ammonium bromide method of Zhang et al. (2009) was performed. The quality and quantity of extracted DNA were determined by an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA).

SRAP analysis. Twenty SRAP primer combinations derived from each of the five forward and reverse primers (Table 2) were used. Polymerase chain reaction (PCR) was conducted as stated by Feng et al. (2014) in 20 μL of PCR mix containing 40 ng of template, 1X buffer concentration, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1U Tag DNA polymerase (Vivantis, Selangor Darul Ehsan, Malaysia), and 0.4 μM of each forward and reverse primer. An amplification program was as follows: 94 °C for 5 min, followed by five cycles of 94 °C for 1 min, 35 °C for 1 min, and 72 °C for 1 min; then, an additional 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min were performed. The final extension was performed at 72 °C for 7 min in a T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

TRAP analysis. The fixed primers were designed according to the protocol described by Hu and Vick (2003). Nucleotide sequences of four genes including acidic endochitinase 1 (CHIB1), beta-1,3-glucanase 2 (BGL2), mitogen-activated protein kinase kinase kinase 16 (MAPKKK16), and phenylalanine ammonia-lyase 2 (PAL2) were obtained from Orchidaceae Floral Transcriptome (http://orchidbase.itps.ncku.edu.tw/est/ home2012.aspx), and 20 combinations with the SRAP reverse primers were used (Table 3). The PCR amplification was based on the work by Feng et al. (2015). The final PCR reaction volume was 20 μL of reaction mix that contained 40 ng of template, 1X buffer concentration, 2.5 mM $MgCl_2$, 0.3 mM dNTPs, 1U Tag DNA polymerase (Vivantis, Selangor Darul Ehsan, Malaysia), and 0.4 μM of each TRAP forward and SRAP reverse primer. The PCR program was as follows: 94 °C for 5 min, followed by five cycles of 94 °C for 1 min, 35 °C for 1 min, and 72 °C for 1 min; then, an additional 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min were performed. The final extension was performed at 72 °C for 7 min in a T100™ Thermal Cycler (Bio-Rad Laboratories, Inc.).

The PCR products were displayed on 6% (weight/volume) denaturing polyacrylamide gel run for 70 min at a constant voltage of 200V and stained by $AgNO_3$ according to Sambrook and Russell (2001). Molecular weight markers of 100 bp (Invitrogen, CA, USA) were added to each gel for comparison of the scorable band size.

Data scoring, cluster analysis, and principal component analysis. The absence of a clear band was noted with 0 and the presence was marked with 1 across both marker systems. Polymorphism information content (PIC) was calculated using the formula of Smith et al.

Received for publication 14 Jul 2023. Accepted for publication 13 Sep 2023.
Published online 31 Oct 2023.
This work received the support from the SUT Research and Development Fund. We thank Peter Charles Bint for language editing.
P.A.T. is the corresponding author. E-mail: piyada@sut.ac.th.

Table 2. Sequence-related amplified polymorphism (SRAP) markers for analyzing four nonmutagenized controls and 12 resistant mutants.

Primer combinations					
Forward primers	Reverse primers	Total number of bands	Number of polymorphic bands	Polymorphic bands (%)	Polymorphism information content
Me1	Em1	16	5	31.25	0.442
	Em2	22	7	31.82	0.249
	Em3	15	2	13.33	0.168
	Em4	19	5	26.32	0.192
	Em5	18	8	44.44	0.344
Me2	Em1	14	3	21.43	0.185
	Em2	17	2	11.76	0.436
	Em3	18	4	22.22	0.332
	Em4	15	2	13.33	0.117
	Em5	24	5	20.83	0.213
Me3	Em1	18	6	33.33	0.323
	Em2	18	5	27.78	0.344
	Em4	17	3	17.65	0.310
Me4	Em1	23	5	21.74	0.323
	Em3	13	3	23.08	0.383
	Em4	19	6	31.58	0.281
Me5	Em1	15	3	20.00	0.203
	Em2	28	8	28.57	0.300
	Em4	23	5	21.74	0.295
	Em5	23	7	30.43	0.185
Total		375	94		
Mean		18.75	4.70	24.63	0.281

(1997). Genetic similarity was estimated based on Jaccard's coefficients; the dendrogram was generated by the genetic similarity and unweighted pair group method with an arithmetic average clustering algorithm among the plant genotypes studied. Mantel's test of each marker and comparisons between markers in the cluster analysis were also estimated (Mantel 1967). A principal component analysis (PCA) to represent the multiple dimensions of the *Dendrobium* sp. 'Earsakul' line distributions was also conducted. All of these analyses were implemented using NTSYSpc version 2.2 (Rohlf 1998).

Association analysis. The association between the SRAP and TRAP markers and severity scores of black rot symptoms was determined by a simple linear regression analysis using SPSS version 16.0 (Levesque and SPSS Inc. 2006); the markers were fixed as an independent variable, and black rot disease severity was fixed as a dependent variable according to Tantasawat et al. (2012). The level of significance was tested using the Student *t* test to determine the association. To further reduce the possibility of declaring false-positive results, the *P* value threshold of a stringent $\alpha = 0.05$ with Bonferroni correction (0.05/number of total polymorphic markers) was applied to determine highly significant associations. This test allowed us to reduce the number of significant markers (Holm 1978).

Results and Discussion

SRAP polymorphisms. A total of 20 SRAP primer combinations were used to attempt to amplify DNA bands from bulked DNA of each *Dendrobium* sp. 'Earsakul' nonmutagenized control and resistant mutant (Table 2), producing 375 scorable bands ranging in length from ~200 to 1500 bp. Of these, 94 were found to be polymorphic (24.42%). When compared with our previous works using ISSR markers for characterizing NaN_3 and EMS-induced *Dendrobium* sp. 'Earsakul' mutants from in vitro selection for black rot resistance, the extent of the found polymorphisms was lower than that found by Khairum et al. (2022) and Tantasawat et al. (2017) (28.02%–46.50%), but it was higher than that found by Hualsawat et al. (2022) (22.67%). The polymorphism percentage is largely dependent on the types of markers and the types and number of genotypes being studied. The SRAP markers only amplify

Table 3. Target region amplification polymorphism (TRAP) markers for analyzing four nonmutagenized controls and 12 resistant mutants.

Primer combinations					
Forward primers	Reverse primers	Total number of bands	Number of polymorphic bands	Polymorphic bands (%)	Polymorphism information content
Acidic	Em1	20	3	15.00	0.242
endochitinase 1	Em2	14	2	14.29	0.246
(CHIB1; C)	Em3	14	5	35.71	0.213
	Em4	19	2	10.53	0.219
	Em5	18	2	11.11	0.273
Total		85	14		
Mean		17.00	2.80	17.33	0.239
Beta-1,3-glucanase	Em1	21	10	47.62	0.295
2 (BGL2; B)	Em2	17	2	11.76	0.117
	Em3	32	15	46.88	0.157
	Em4	19	7	36.84	0.250
	Em5	24	5	20.83	0.195
Total		113	39		
Mean		22.60	7.80	32.79	0.203
Mitogen activated	Em1	22	3	13.64	0.276
protein kinase	Em2	19	1	5.26	0.375
kinase kinase 16	Em3	17	0	0.00	0.000
(MAPKKK16;	Em4	19	2	10.53	0.297
M)	Em5	15	4	26.67	0.230
Total		92	10		
Mean		18.40	2.00	11.22	0.236
Phenylalanine	Em1	17	7	41.18	0.320
ammonia-lyase	Em2	20	3	15.00	0.380
2 (PAL2; P)	Em3	17	3	17.65	0.185
	Em4	17	3	17.65	0.311
	Em5	23	9	39.13	0.240
Total		94	25		
Mean		18.80	5.00	26.12	0.287
Grand total		384	88		
Grand mean		19.20	4.40	21.86	0.241

the target region of ORFs, whereas the ISSR markers are ubiquitously scattered across the genome, thereby revealing the variations of the entire genome. Although Hualsawat et al. (2022) used the ISSR markers, their work included a lower number of mutant lines (seven lines) than our SRAP analysis (16 lines). Moreover, the polymorphism percentage of the found polymorphisms was also lower than that found by Feng et al. (2014) (97.00%) using a SRAP analysis of several *D.* species collected from several locations in China. The total number of scorable bands detected with each primer combination ranged from 13 (Me4Em3) to 28 (Me5Em2) (average, 18.75), and each primer combination produced two (Me1Em3, Me2Em2, and Me2Em4) to eight (Me1Em5 and Me5Em2) polymorphic bands (average, 4.70). The polymorphism percentage ranged from 11.76% (Me2Em2) to 44.44% (Me1Em5) (average, 24.63%). Theoretically, the dominant markers, i.e., SRAP, TRAP, or ISSR markers, have the highest PIC value of 0.5. The range of PIC values computed as a mean over all the lines was 0.117 (Me2Em4) to 0.442 (Me1Em1) (average, 0.281), which was higher than that of our previous work using ISSR markers (0.190) (Khairum et al. 2022). The higher PIC values found during this study using the SRAP markers may have resulted from the discriminatory power of this marker system by specifically detecting ORFs of some candidate genes possibly associated with black rot resistance among the resistant and susceptible lines studied, suggesting the potential of this marker system for mutant selection, thereby leading to earlier selection and reduction of the population size during mutation breeding programs. Ma et al. (2021) also found that the PIC revealed by the SRAP markers (0.270) in diverse populations of black cardamom was slightly higher than that of the ISSR markers (0.232).

TRAP polymorphisms. Twenty TRAP primer combinations were created using the four candidate gene sequences (CHIB1, BGL2, MAPKKK16, and PAL2) to detect polymorphisms of these genes among the 16 *Dendrobium* sp. 'Earsakul' lines (Table 3). A total of 384 scorable bands from ~200 to 1500 bp in length were observed; of these, 88 were polymorphic (22.91%). The polymorphism of the TRAP markers was lower than that of the SRAP markers. This is possibly because the TRAP polymorphic loci are specifically relevant to the four candidate gene regions, whereas those of the SRAP markers are obtained directly from coding sequences of any genes complementary with sequences of the SRAP primers. Each primer combination generated an average of 19.20 bands, and 4.40 bands of these were polymorphic. A higher number of bands were generated by the BGL2-based markers (113 scorable bands) with 34.51% polymorphism (39 polymorphic bands) compared with the markers based on PAL2 (94 scorable bands), MAPKKK16 (92 scorable bands), and CHIB1 (85 scorable bands) with 26.60%, 10.87%, and 16.47% polymorphisms, respectively (25, 10, and 14 polymorphic bands, respectively). The polymorphism percentage ranged from 0.00% (M/Em3) to 47.62% (B/Em1) (average, 21.86%). The polymorphism percentage of the markers based on BGL2 ranged between 11.76% and 47.62% (average, 32.79%), whereas the ranges for CHIB1, MAPKKK16, and PAL2 were 10.53% to 35.71% (average, 17.33%), 0.00% to 26.67% (average, 11.22%), and 15.00% to 41.18% (average, 26.12%), respectively. The PIC values ranged from 0.000 (M/Em3) to 0.380 (P/Em2). The overall average PIC of all 20 primer combinations was 0.241, which was moderately informative compared with the ones associated with the coding regions of more genes using the SRAP markers (0.281). Similarly, da Silva et al. (2016) observed that the PIC of the SRAP markers (0.35) was higher than that of the TRAP markers (0.33) when the genetic variability of complex polyploid guarana plant was evaluated.

Genetic diversity and relationships among nonmutagenized controls and resistant mutants. Two unweighted pair group methods with arithmetic average dendrograms of the 16 *Dendrobium* sp. 'Earsakul' lines were constructed using 94 and 88 polymorphic loci of the SRAP and TRAP markers, respectively. We also generated another dendrogram from the combined SRAP/TRAP data with 182 polymorphic loci (Fig. 1). The pairwise genetic similarity coefficients across all lines ranged from 0.698 to 0.922 (average, 0.806) for SRAP, 0.713 to 0.962 (average, 0.855) for TRAP, and 0.743 to 0.935 (average, 0.832) for SRAP/TRAP. However, our previous works found relatively higher similarity levels of 0.897 to 0.908 across these NaN_3 and EMS-induced mutants using the ISSR markers (Hualsawat et al. 2022; Khairum et al. 2022). These levels may have resulted from the higher number of polymorphic bands synthesized by the SRAP, TRAP, and SRAP/TRAP markers (94, 88, and 182 bands, respectively) compared with the ISSR markers (51 and 44 bands, respectively), or as a result of the differences in the nature of the markers. Among the three dendrograms, all nonmutagenized controls were allocated together in cluster I, but their genetics were not completely identical; the levels of their genetic similarity ranged from 0.908 to 0.944, demonstrating evidence of somaclonal variation without NaN_3 and EMS treatments. All the highly resistant and the most resistant mutants were distributed separately as individuals (Fig. 1A–C), possibly demonstrating that mutations in these mutants occurred in different loci. These mutations may affect not only black rot resistance but also other characteristics. The presence or absence of SRAP and TRAP fragments may stem from either the gain or the loss of primer hybridization sites because of changes in the oligonucleotide sequences, i.e., point mutations or changes regarding the alteration of size or hampering of the amplification of a target DNA. NaN_3 created point mutations via a predominant transition of A/T to C/G (86%) and transversion of C/G to A/T (14%) (Olsen et al. 1993; Talebi et al. 2012) and induced chromosome damage and polyploidization (Khan et al. 2009). EMS also induced transitions and transversions (70%–99%) and caused chromosome damage like NaN_3, which differed in the lower frequency of the mutagenic effect on chromosomes (Goldhaber-Pasillas et al. 2014; Serrat et al. 2014). We hypothesized that these different mechanisms of the two mutagens and their varying frequencies that caused random variations may be connected to the differences in genetic makeup and/or genomic features of individual resistant mutants because most of them were distributed separately and not clustered in groups according to the type of mutagens. The genetic distances between all the nonmutagenized controls and all the resistant mutants were 0.205 for SRAP, 0.144 for TRAP, and 0.170 for SRAP/TRAP. Moreover, the levels of the genetic distance between the controls and the NaN_3-induced mutants compared to those of the controls and the EMS-induced mutants across these three data sets were 0.165 vs. 0.218 for SRAP, 0.140 vs. 0.145 for TRAP, and 0.142 vs. 0.179 for SRAP/TRAP. The clear differentiation between these *Dendrobium* sp. 'Earsakul' controls and resistant mutants induced with NaN_3 and EMS was also observed using the ISSR markers and morphological characterization, i.e., increased number of nodes, leaves, and roots (NaN_3 and EMS), increased (EMS) and decreased (NaN_3) node length, decreased leaf length (NaN_3), and increased leaf width (EMS) (Hualsawat et al. 2022; Khairum et al. 2022). These results indicated the potential of both chemical mutagens, especially EMS, to induce genetic variability through mutations under in vitro conditions, and they would be useful for a *Dendrobium* breeding program. Mahpara et al. (2023) observed the efficiency of EMS over NaN_3 by providing more variants and altering the morphological and yield characteristics in chili.

A good fit of the clusters to genetic similarity was indicated by high cophenetic correlation coefficients based on TRAP and SRAP/TRAP from the Mantel's test (0.921 and 0.861, respectively; $P < 0.01$), whereas SRAP had a lower cophenetic correlation (0.750; $P < 0.01$). The SRAP and TRAP markers resulted in different dendrograms in which all the lines were separated into three clusters with eight individuals (Fig. 1A) and four clusters with six individuals (Fig. 1B), respectively. The dendrogram based on both marker systems showed three clusters with eight individuals (Fig. 1C), like the SRAP data, but different for the members of cluster II and the pattern of mutant line clustering. The differences between SRAP-based and TRAP-based dendrograms may be attributable to the polymorphisms detected, again reinforcing the necessity of their target sites for obtaining reliable estimates of genetic relationships among genotypes. Therefore, genetic profiles of these *Dendrobium* sp. 'Earsakul' mutants could not be explained by their real relationship with only one marker system. As evident from the dendrograms, cluster II in SRAP included SUT17N05305(R) and SUT17E18303(R), whereas SRAP/TRAP SUT 17E18303(R) was clustered together with SUT17E18316(R), as also seen in cluster II in TRAP. In SRAP, SUT17N05305(R) was the most divergent with the least similarity (83.20%) among the NaN_3-induced mutants.

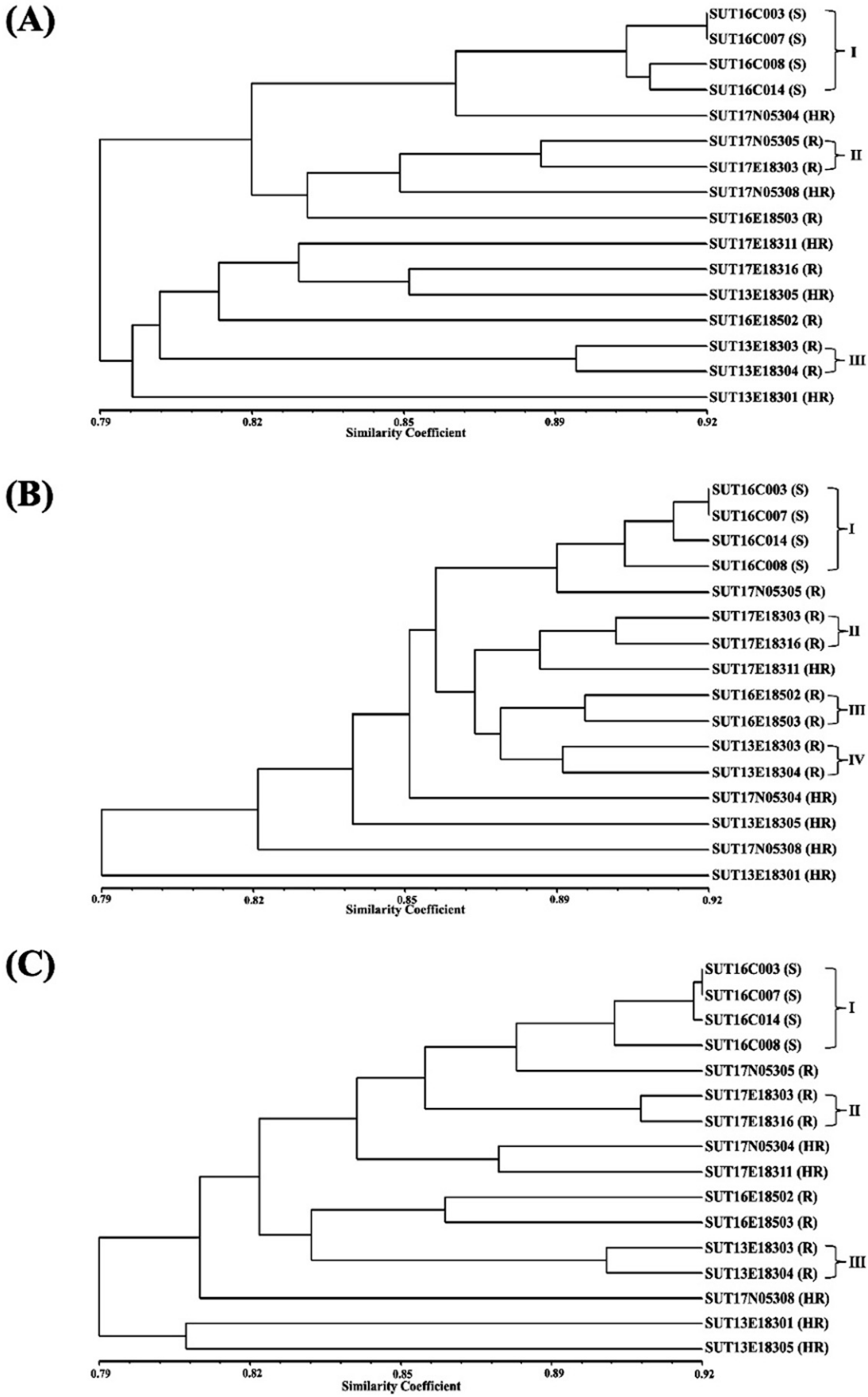

Fig. 1. (**A**) Sequence-related amplified polymorphism (SRAP)-derived, (**B**) target region amplification polymorphism (TRAP)-derived, and (**C**) combined SRAP/TRAP-derived dendrograms of four nonmutagenized controls, SUT16C003 (S), SUT16C007 (S), SUT16C008 (S), and SUT16C014 (S), and 12 resistant mutants, SUT17N05304 (HR), SUT17N05305 (R), SUT17N05308 (HR), SUT13E18301 (HR), SUT13E18301 (R), SUT13E18301 (R), SUT13E18301 (HR), SUT17E18303 (R), SUT17E18311 (HR), SUT17E18316 (R), SUT16E18502 (R), and SUT16E18503 (R).

Hualsawat et al. (2022) also found that this mutant was the most genetically distant and had the maximum genome size and DNA content compared with other NaN_3-induced mutants after ISSR analysis and flow cytometry, respectively. The SRAP markers may amplify more functional regions of its large genome possibly shared in common with other mutants, i.e., SUT17E18303(R), which also had a comparatively large genome (Khairum et al. 2022). Although the TRAP markers only gather information associated with expressed regions that are more conserved and that may not group SUT17N05305(R) with SUT17E18303(R), SUT13E18303(R), and SUT13E18304(R), they were allocated in the same clusters across all three data sets with more than 89.30% similarity (cluster III for SRAP and SRAP/TRAP and cluster IV for TRAP). In TRAP, there was one more cluster (cluster III) that consisted of SUT16E18502(R) and SUT16E18503(R) with 92.10% similarity. However, Khairum et al. (2022) revealed that these four mutants did not form any cluster using ISSR markers. We speculated that the polymorphisms of these *Dendrobium* sp. 'Earsakul' mutants in the intragenic regions were larger, but they were smaller in the vicinity of the microsatellites, which displayed a dynamic genome composition, as also observed by Ruibin et al. (2017). Based on all the molecular markers, the NaN_3-induced mutant SUT17N05305(R) was found to be the most genetically similar to all the nonmutagenized controls (88.20% similarity). On the contrary, two EMS-induced mutants, SUT13E18301(HR) and SUT13E18305(HR), and one NaN_3-induced mutant, SUT17N05308(HR), were the most distantly related to all the controls (76.50, 79.20, and 80.30% dissimilarity, respectively). The genetic relationships of these highly resistant mutants as well as SUT17N05305(R) with all the controls were evident in the TRAP-based and SRAP/TRAP-based dendrograms.

PCA with three-dimensional plots based on all the polymorphic loci revealed that the three coordinates accounted for 21.54, 13.16, and 10.49 (total variance 45.19), respectively, for SRAP, 18.96, 15.53, and 14.45 (total variance 48.94), respectively, for TRAP, and 19.37, 13.37, and 11.25 (total variance 43.99), respectively, for SRAP/TRAP. As with the unweighted pair group method with an arithmetic average cluster analysis using the SRAP, TRAP, and SRAP/TRAP loci, PCA differentiated all the nonmutagenized controls in cluster I from the resistant mutants (Fig. 2). In the TRAP-based and SRAP/TRAP-based PCA, the resistant mutant SUT17N05305(R) was the most closely related to all the controls, and the three highly resistant mutants SUT13E18301(HR), SUT13E18305(HR), and SUT17N05308(HR) appeared to be more distinct than all the controls and other resistant mutants (Fig. 2B and C). Because these highly resistant mutants are quite genetically diverse, they may be used as parents for orchid improvement, and we intend to study them for their resistance mechanisms against black rot disease using a transcriptome analysis in the future. Moreover, the resistant mutants that formed clusters on the TRAP-based and SRAP/TRAP-based dendrograms, including SUT17E18303(R) and SUT17E18316(R) (cluster II for TRAP and SRAP/TRAP), SUT13E18303(R) and SUT13E18304(R) (cluster IV for TRAP and cluster III for SRAP/TRAP), and SUT16E18502(R) and SUT16E18503(R) (cluster III for TRAP) were closely allocated in the TRAP-based and SRAP/TRAP-based PCA. Nevertheless, the first four of these resistant mutants were separated as individuals rather than delineated into the clusters on the SRAP-based dendrogram (Fig. 2A).

Association analysis. A simple linear regression analysis revealed significant associations between 12 SRAP and TRAP polymorphic loci and severity scores of black rot symptoms ($P < 0.05$) (Table 4). Five SRAP loci, $Me1Em1_{470}$, $Me3Em2_{221}$, $Me3Em2_{600}$, $Me4Em1_{350}$, and $Me4Em1_{358}$, and three TRAP loci, $C/Em5_{210}$, $M/Em4_{223}$, and $P/Em3_{275}$, showed negative associations with the severity scores of black rot symptoms. On the contrary, three SRAP loci, $Me1Em1_{270}$, $Me1Em1_{279}$, and $Me3Em1_{261}$, and one TRAP locus, $P/Em5_{426}$, showed positive associations with severity scores of black rot symptoms. After Bonferroni correction ($P < 2.75 \times 10^{-4}$), four markers remained significant. Three markers with negative associations, $Me3Em2_{221}$, $C/Em5_{210}$, and $M/Em4_{223}$ ($R^2 = 0.687$–0.933; $t = -5.538$ to -14.008; $P = 0.0000$), and beta coefficients of -0.829 to -0.966 were associated with black rot resistance. One marker with positive associations, $Me3Em1_{261}$ ($R^2 = 0.953$; $t = 15.557$; $P = 0.0000$), and a beta coefficient of 0.976 was associated with black rot susceptibility. However, these markers should be validated with larger mutant populations for their associations with black rot resistance before further application.

Comparison between marker systems. Mantel's test was used again to calculate and compare the similarity and cophenetic matrices for SRAP, TRAP, and SRAP/TRAP. There were significant correlations between the pairwise comparisons of SRAP with TRAP, SRAP with SRAP/TRAP, and TRAP with SRAP/TRAP (0.475, 0.741, and 0.878, respectively; $P < 0.01$), indicating concordance between these markers for the assessment of genetic diversity and relationships among all the *Dendrobium* sp. 'Earsakul' nonmutagenized controls and resistant mutants. It should be noted that the goodness of fit of TRAP and SRAP/TRAP for cophenetic correlation values of 0.921 and 0.861, respectively, were higher than those of SRAP (0.750), suggesting the suitability of TRAP and SRAP/TRAP over SRAP for the assessment of genetic diversity and relationships among our orchid genotypes. Although the correlations between the matrices of cophenetic correlation values for the dendrograms of SRAP with TRAP, SRAP with SRAP/TRAP, and TRAP with SRAP/TRAP were significant (0.399, 0.566, and 0.793, respectively; $P < 0.01$), there were some variations in the dendrograms. The dendrograms based on SRAP vs. TRAP and SRAP vs. SRAP/TRAP, which had lower correlations, had more variations (i.e., the number of clusters, the members of clusters, and the placement of the materials) than the ones based on TRAP vs. SRAP/TRAP. A possible explanation for the differences in the resolution of SRAP and TRAP may stem from the origin of polymorphic fragments (ORFs and ESTs). We designed the fixed primers of TRAP using ESTs or genes relevant to disease resistance to evaluate the genetic profiles of the *Dendrobium* sp. 'Earsakul' nonmutagenized controls and resistant mutants, and a higher polymorphism was obtained from using the fixed primer designed using BGL2. Our other works found that BGL2 and CHIB1 were associated with antifungal activities during the infection of *P. palmivora* in these black rot-resistant mutants (Hualsawat et al. 2022; Khairum et al. unpublished data). However, the polymorphism revealed by the fixed primer designed from CHIB1 was low. Moreover, the TRAP fragments amplified from the fixed primer designed from BGL2 were not associated with black rot resistance (Table 4). Although the fixed primers were created from sequences of interesting ESTs or genes, it was difficult to decide which DNA fragments were associated with target sequences and how the low polymorphism was obtained. As described by Hu et al. (2005), mismatching between TRAP primers and the target sequences from a low annealing temperature (35 °C) during the first five cycles of the PCR led to ~1% of the amplified TRAP fragments from the desirable genes. It is possible to postulate that most of the polymorphic TRAP fragments derived from nonspecific amplification. Several reports also revealed that only a small number of TRAP fragments had homology to the targeted DNA sequences relevant to the traits of interest (Hu et al. 2005; Liu et al. 2005; Qiao et al. 2007). In addition to TRAP, variations in a target sequence for SRAP detection does not necessarily imply a phenotypic effect. Nevertheless, TRAP and SRAP were more specific than the molecular techniques using only arbitrary primers because we found some fragments associated with black rot resistance, and a cluster analysis based on these markers could differentiate the resistant mutants and all the nonmutagenized controls. In the future, sequencing the amplified fragments associated with black rot resistance should be performed to indicate whether the amplified fragments are from the intended loci originally selected or random fragments from many parts of the genome. We noticed that the fixed and forward primers of the TRAP and SRAP markers have a major role in generating the amplification profiles because the same arbitrary primer synthesized totally different amplification profiles when combined with different fixed primers. Because the TRAP and SRAP reactions generated abundant markers through using a single fixed/forward primer with numerous interchangeable arbitrary primers, they proved to be applicable for genotypic profiling, constructing a linkage map, and locating quantitative trait loci, as performed by Chueakhunthod et al. (2020), Ghanbarnia et al. (2012), Liu et al. (2005), and Xiao et al. (2010). It should be noted that increasing the initial annealing temperature would possibly result in higher specificity and fewer amplified fragments, which may be applicable for targeting

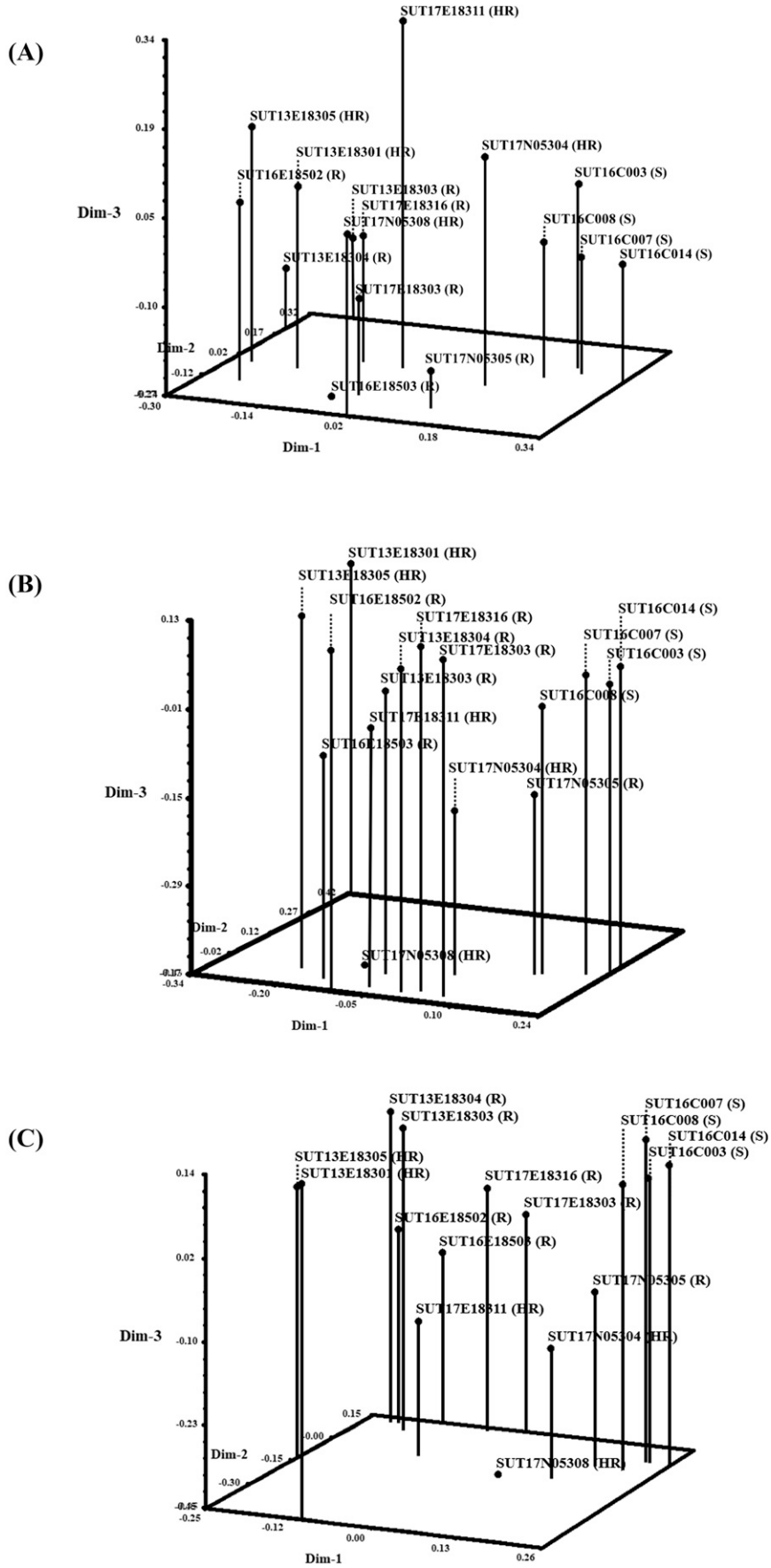

Fig. 2. (**A**) Sequence-related amplified polymorphism (SRAP)-derived, (**B**) target region amplification polymorphism (TRAP)-derived, and (**C**) combined SRAP/TRAP-derived principal component analysis (PCA) plots based on the first three components of four nonmutagenized controls, SUT16C003 (S), (S), SUT16C008 (S), and SUT16C014 (S), and 12 resistant mutants, SUT17N05304 (HR), SUT17N05305 (R), SUT17N05308 (HR), SUT13E18301 (HR), SUT13E18301 (R), SUT13E18301 (R), SUT13E18301 (HR), SUT17E18303 (R), SUT17E18311 (HR), SUT17E18316 (R), SUT16E18502 (R), and SUT16E18503 (R).

Table 4. Simple linear regression analysis between the sequence-related amplified polymorphism (SRAP) and target region amplification polymorphism (TRAP) markers and scores of black rot symptoms in four nonmutagenized controls and 12 resistant mutants.

Markers	Beta	*t* value	*P* value[i]	R^2
$Me3Em1_{261}$[ii]	0.976	15.557	0.0000	0.953
$Me3Em2_{221}$	−0.966	−14.008	0.0000	0.933
$C/Em5_{210}$[iii]	−0.829	−5.538	0.0000	0.687
$M/Em4_{223}$[iv]	−0.966	−14.008	0.0000	0.933

[i] Bonferroni correction threshold (α/number of markers) $0.05/182 = 2.75 \times 10^{-4}$.
[ii] The number preceding the subscript (sizes of SRAP and TRAP markers in bp) refers to the primer combination for generating the marker.
[iii] C = forward primer CHIB1.
[iv] M = forward primer MAPKKK16.

specific genomic regions. However, the potential of rapidly generating abundant markers would be reduced.

Conclusion

We proved the effectiveness of the TRAP and SRAP markers-based profiling of black rot-resistant *Dendrobium* sp. 'Earsakul' mutants induced with NaN_3 and EMS under in vitro mutagenesis and followed by in vitro selection with *P. palmivora*. Their intragenic-specific and multilocus nature as well as simplicity and low cost through using a single forward primer with numerous interchangeable reverse primers make them very attractive for molecular research (i.e., genotyping and genetic diversity and relatedness evaluations, trait mapping, gene tagging, and marker-assisted selection). The SRAP and TRAP profiles that revealed the delineation of the resistant mutant from the nonmutagenized controls and the functional polymorphisms among the resistant mutants can be used as criteria for the selection of parents to increase genetic variability in future breeding programs and for further evaluation of their horticultural traits and black rot resistance before distribution. Moreover, the four SRAP and TRAP markers that were identified as significantly associated with black rot resistance may also be used to initially validate black rot resistance in *Dendrobium* orchids.

References Cited

Andersen JR, Lübberstedt T. 2003. Functional markers in plants. Trends Plant Sci. 8:554–560. https://doi.org/10.1016/j.tplants.2003.09.010.

Arisha MH, Liang BK, Muhammad Shah SN, Gong ZH, Li DW. 2014. Kill curve analysis and response of first generation *Capsicum annuum* L. B12 cultivar to ethyl methanesulfonate. J Genet Mol Res. 13:10049–10061. https://doi.org/10.4238/2014.November.28.9.

Bello-Bello JJ, Iglesias-Andreu LG, Avilés-Viñas SA, Gómez-Uc E, Canto-Flick A, Santana-Buzzy N. 2014. Somaclonal variation in habanero pepper (*Capsicum chinense* Jacq.) as assessed ISSR molecular markers. HortScience. 49:481–485. https://doi.org/10.21273/HORTSCI.49.4.481.

Chen YE, Zhang YH, Yuan S, Liu HM, Zeng XY, Zhang HY. 2014. Ethyl methane sulfonate induces disease resistance in *Begonia* × *hiemalis* Fotsch. Hortic Environ Biotechnol. 55:498–505. https://doi.org/10.1007/s13580-014-0053-2.

Chueakhunthod W, Jinagool W, Meecharoen K, Khwanman R, Pattanaram P, Jantarat N, Palaphon P, Ngampongsai S, Tantasawat PA. 2020. Genetic relationship of mungbean and blackgram genotypes based on agronomic and photosynthetic performance and SRAP markers. Not Bot Horti Agrobot Cluj-Napoca. 48:1845–1861. https://doi.org/10.15835/nbha48412114.

Constantin MJ. 1984. Potential of in vitro mutation breeding for the improvement of vegetatively propagated crop plants. IAEA. 59–77.

da Silva EF, Sousa SB, Silva GF, Sousa NR, Nascimento Filho FJ, Hanada RE. 2016. TRAP and SRAP markers to find genetic variability in complex polyploid *Paullinia cupana* var. *sorbilis*. Plant Gene. 6:43–47. https://doi.org/10.1016/j.plgene.2016.03.005.

El-Shahed AA, Abdellatif KF, Ibrahim IA, Mohamed AM, Abdelsalam IZ, Elsehrawy OA. 2017. Efficiency of sequence related amplified polymorphism (SRAP) and target region amplified polymorphism (TRAP) markers in detecting banana somaclonal variants. Afr J Biotechnol. 16:879–888. https://doi.org/10.5897/AJB2017.15893.

Feng SG, Lu JJ, Gao L, Liu JJ, Wang HZ. 2014. Molecular phylogeny analysis and species identification of *Dendrobium* (Orchidaceae) in China. Biochem Genet. 52:127–136. https://doi.org/10.1007/s 10528-013-9633-6.

Feng S, He R, Yang S, Chen Z, Jiang M, Lu J, Wang H. 2015. Start codon targeted (SCoT) and target region amplification polymorphism (TRAP) for evaluating the genetic relationship of *Dendrobium* species. Gene. 567:182–188. https://doi.org/10.1016/j.gene.2015.04.076.

Ghanbarnia K, Lydiate DJ, Rimmer SR, Li G, Kutcher HR, Larkan NJ, McVetty PBE, Dilantha Fernando WG. 2012. Genetic mapping of the *Leptosphaeria maculans* avirulence gene corresponding to the *LepR1* resistance gene of *Brassica napus*. Theor Appl Genet. 124:505–513. https://doi.org/10.1007/s00122-011-1724-3.

Goldhaber-Pasillas GD, Mustafa NR, Verpoorte R. 2014. Jasmonic acid effect on the fatty acid and terpenoid indole alkaloid accumulation in cell suspension cultures of *Catharanthus roseus*. Molecules. 19:10242–10260. https://doi.org/10.3390/molecules190710242.

Holm S. 1978. A simple sequentially rejective multiple test procedure. Scand J Stat. 6:65–70.

Hu J, Vick BA. 2003. Target region amplification polymorphism: A novel marker technique for plant genotyping. Plant Mol Biol Rpt. 21: 289–294. https://doi.org/10.1007/BF02772804.

Hu J, Ochoa OE, Truco MJ, Vick BA. 2005. Application of TRAP technique to lettuce (*Lactuca sativa* L.) genotyping. Euphytica. 144:225–235. https://doi.org/10.1007/s10681-005-6431-1.

Hualsawat S, Khairum A, Chueakhunthod W, Tharapreuksapong A, Tantasawat PA. 2022. Profiling of black rot resistant *Dendrobium* 'Earsakul' induced by in vitro sodium azide mutagenesis. Eur J Hortic Sci. 87:1–13. https://doi.org/10.17660/eJHS.2022/020.

Kamle M, Borah R, Bora H, Jaiswal AK, Singh RK, Kumar P. 2020. Systemic acquired resistance (SAR) and induced systemic resistance (ISR): Role and mechanism of action against phytopathogens, p 457–470. In: Hesham AL, Upadhyay RS, Sharma GD, Manoharachary C, Gupta VK (eds). Fungal biotechnology and bioengineering. Fungal Biology. Springer, Cham, Switzerland. https://doi.org/10.1007/978-3-030-41870-0_20.

Khairum A, Poolsawat O, Pornbungkerd P, Tharapreuksapong A, Wongkaew S, Tantasawat PA. 2018. Effects of culture media on Phytophthora palmivora growth, α-elicitin production and toxicity to *Dendrobium*. Not Bot Horti Agrobot Cluj Napoca. 46:630–638. https://doi.org/10.15835/nbha 46211076.

Khairum A, Hualsawat S, Chueakhunthod W, Tharapreuksapong A, Tomsantia B, Yenchon S, Papan P, Tantasawat PA. 2022. Selection and characterization of in vitro–induced mutants of *Dendrobium* 'Earsakul' resistant to black rot. In Vitro Cell Dev Biol Plant. 58: 577–592. https://doi.org/10.1007/s11627-022-10261-0.

Khan S, Al-Qurainy F, Anwar F. 2009. Sodium azide: A chemical mutagen for enhancement of agronomic traits of crop plants. Environ We Intl J Sci Technol. 4:1–21.

Kumar H, Anubha, Vishwakarma M, Lal J. 2011. Morphological and molecular characterization of *Brassica rapa* ssp. *yellow sarson* mutants. J Oilseed Brassica. 2:1–6.

Levesque R, SPSS Inc. 2006. SPSS programming and data management (3rd ed). SPSS Institute, Somers, NY, USA.

Li G, Quiros CF. 2001. Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: Its application to mapping and gene tagging in *Brassica*. Theor Appl Genet. 103:455–461. https://doi.org/10.1007/s001220100570.

Liu ZH, Anderson JA, Hu J, Friesen TL, Rasmussen JB, Faris JD. 2005. A wheat intervarietal genetic linkage map based on microsatellite and target region amplified polymorphism markers and its utility for detecting quantitative trait loci. Theor Appl Genet. 111:782–794. https://doi.org/10.1007/s00122-005-2064-y.

Lo KL, Chen YN, Chiang MY, Chen MC, Panibe JP, Chiu CC, Liu LW, Chen LJ, Chen CW, Li WH, Wang CS. 2022. Two genomic regions of a sodium azide induced rice mutant confer broad-spectrum and durable resistance to blast disease. Rice (N Y). 15:2. https://doi.org/10.1186/s12284-021-00547-z.

Ma M, Wang T, Lu B. 2021. Assessment of genetic diversity in *Amomum tsao-ko* Crevost & Lemarié, an important medicine food homologous crop from Southwest China using SRAP and ISSR markers. Genet Resources Crop Evol. 68:2655–2667. https://doi.org/10.1007/s10722-021-01204-6.

Mahpara S, Fatima L, Gul A, Ullah R, Muneer M, Bhatti MA, Iqbal J, Ahmed A, Fatima T, Ijaz MU, Alharbi SA, Alrasheed WA, Ullah MI, Rauf A, Nasir MW, El Enshasy HA. 2023. Exploring the efficiency of ethyl methane sulfonate (EMS) and sodium azide (SA) to induce mutation in chili (*Capsicum annuum* L.) germplasm. J King Saud Univ Sci. 35:102507–102514. https://doi.org/10.1016/j.jksus.2022.102507.

Mantel N. 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. 27:209–220.

Mirajkar SJ, Rai AN, Vaidya ER, Moharil MP, Dudhare MS, Suprasanna P. 2017. TRAP and SRAP molecular marker-based profiling of radiation induced mutants of sugarcane (*Saccharum officinarum* L.). Plant Gene. 9:64–70. https://doi.org/10.1016/j.plgene.2017. 01.002.

Olsen O, Wang X, von Wettstein D. 1993. Sodium azide mutagenesis: Preferential generation of A•T —>G•C transitions in the barley *Ant18* gene. Proc Natl Acad Sci USA. 90:8043–8047. https://doi.org/10.1073/pnas.90.17.8043.

Poczai P, Varga I, Laos M, Cseh A, Bell N, Valkonen JPT, Hyvönen J. 2013. Advances in

plant gene-targeted and functional markers: A review. Plant Methods. 9:6. https://doi.org/10.1186/1746-4811-9–6.

Qiao L, Liu H, Sun J, Zhao F, Guo B, Weng M, Liu T, Dai J, Wang B. 2007. Application of target region amplification polymorphism (TRAP) technique to *Porphyra* (Bangiales, Rhodophyta) fingerprinting. Phycologia. 46:450–455. https://doi.org/10.2216/07-08.1.

Rohlf FJ. 1998. NTSYSpc numerical taxonomy and multivariate analysis system version 2.2 user guide. Applied Biostatistics Inc., Setauket, NY, USA.

Ruibin J, Xudong X, Xiaoming L, Zhishan D, Bo J. 2017. Analysis of the genetic diversity of *Dendrobium candidum* by ISSR, SRAP, and ScoT. J Biobased Mater Bioenergy. 11:1–8. https://doi.org/10.1166/jbmb.2017.1720.

Sambrook J, Russell DW. 2001. Molecular cloning: A laboratory manual (3rd ed). Cold Spring Harbor Laboratory Press, New York, NY, USA. A9.5-A9.7.

Savita S, Pati PK, Virk GS, Nagpal A. 2017. In vitro selection of resistant/tolerant mutants lines of *Citrus jambhiri* Lush. using crude culture filtrate of *Phytophthora parasitica* and their randomly amplified polymorphic DNA analysis. J Phytopathol. 165:771–781. https://doi.org/10.1111/jph. 12617.

Serrat X, Esteban R, Guibourt N, Moysset L, Nogués S, Lalanne E. 2014. EMS mutagenesis in mature seed-derived rice calli as a new method for rapidly obtaining TILLING mutant populations. Plant Methods. 10:5. https://doi.org/10.1186/1746-4811-10-5.

Smith JSC, Chin ECL, Shu H, Smith OS, Wall SJ, Senior ML, Mitchell SE, Kresovich S, Ziegle J. 1997. An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): Comparisons with data from RFLPS and pedigree. Theor Appl Genet. 95:163–173. https://doi.org/10.1007/s001220050544.

Talebi AB, Talebi AB, Shahrokhifar B. 2012. Ethyl methane sulphonate (EMS) induced mutagenesis in Malaysian rice (cv. MR219) for lethal dose determination. Am J Plant Sci. 3:1661–1665. https://doi.org/10.4236/ajps.2012.312202.

Tan J, Wang HL, Yeh KW. 2005. Analysis of organ-specific, expressed genes in *Oncidium* orchid by subtractive expressed sequence tags library. Biotechnol Lett. 27:1517–1528. https://doi.org/10.1007/s10529-005-1468-8.

Tantasawat PA, Poolsawat O, Prajongjai T, Chaowiset W, Tharapreuksapong A. 2012. Association of RGA-SSCP markers with resistance to downy mildew and anthracnose in grapevines. Genet Mol Res. 11:1799–1809. https://doi.org/10.4238/2012.July.2.1.

Tantasawat PA, Khairum A, Arsakit K, Poolsawat O, Pornbungkerd P, Kativat C. 2015. Effect of different culture media on growth and proliferation of *Dendrobium* 'Earsakul' protocorm-like body. HortTechnology. 25:681–686. https://doi.org/10.21273/HORT TECH.25.5.681.

Tantasawat TP, Khairum A, Tharapreuksapong A, Poolsawat O, Tantasawat PA. 2017. Molecular characterization of *Dendrobium* 'Earsakul' mutants from in vitro selection for black rot resistance. J Appl Hortic. 19:130–134. https://doi.org/10.37855/jah.2017. v19i02.23.

Xiao Y, Liu W, Lu YY, Gong WB, Bian YB. 2010. Applying target region amplification polymorphism markers for analyzing genetic diversity of *Lentinula edodes* in China. J Basic Microbiol. 50:475–483. https://doi.org/10.1002/jobm.201000018.

Yoosumran V, Ruamrungsri S, Duangkongsan W, Kanjana S. 2018. Induced mutation of *Dendranthemum grandiflora* through tissue culture by ethyl methanesulphonate (EMS). Agric Technol Thail. 14:73–82.

Zhang MB, Pan LJ, Fan GQ, Chen JM, Cheng P. 2009. Study on DNA isolation from polysaccharides-rich transgenic *Dendrobium*. Mol Plant Breed. 7: 209–214.