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Molecular characterization of *Dendrobium* 'Earsakul' mutants from *in vitro* selection for black rot resistance

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Abstract

In vitro chemical mutagenesis of *Dendrobium* 'Earsakul' protocorm-like bodies (PLBs) followed by *in vitro* selection with *Phytophthora palmivora* culture filtrate (CF) generated several putative mutants potentially resistant to black rot. The objectives of this study were to evaluate the black rot resistance levels of these putative mutants, to estimate the genetic variability among them and non-mutagenized controls using inter-simple sequence repeat (ISSR) analysis, and to identify candidate markers with significant associations to black rot resistance. When 3 non-mutagenized control lines and 8 putative mutants, derived from ethyl methane sulfonate (EMS) or sodium azide (NaN_3) induced mutation, followed by *in vitro* selection were evaluated for black rot resistance using detached leaf assay with a virulent isolate NK-53-9, it was found that all controls were susceptible to black rot, but the resistance levels of putative mutants varied. Four of eight putative mutants were moderately resistant or resistant to the disease, suggesting their usefulness in the breeding program. ISSR analysis of these controls and putative mutants with 7 ISSR primers yielded 7 to 29 reproducible bands per primer, ranging in size from 170 to 2100 bp. A total of 114 amplified ISSR fragments were obtained, 53 of which were polymorphic (46.5%). All controls have the same DNA patterns, while all 8 putative mutants showed altered genetic profiles compared to controls and were identified as mutants. The mutant SUT13E18-A appears to have distinct genetic profile compared to others as well as high level of resistance to black rot. Moreover, five ISSR markers significantly associated with black rot resistance were identified. These results suggest that ISSR analysis is efficient for mutant identification and characterization, and *in vitro* chemical mutagenesis followed by *in vitro* selection with *P. palmivora* CF provides a useful tool for future improvement of black rot resistant *Dendrobium*.

Key words: *Dendrobium* 'Earsakul', ethyl methane sulfonate (EMS), inter-simple sequence repeat (ISSR), mutagenesis, *Phytophthora palmivora*, sodium azide (NaN_3)

Introduction

Orchid is economically important in the international floriculture industry, both as cut flowers and potted plants. The wide range of its characteristics in flower shapes, sizes and colours has generated great interests among consumers and collectors (Kuehnle, 2007). *Dendrobium* has become an increasingly popular commercial orchid, and it is also the major cut-flower orchid exported from Thailand. However, its cultivation usually faces numerous disease problems including black rot (*Phytophthora palmivora*). Therefore, new varieties with improved quality and yield as well as resistance to diseases are preferred, which have been produced through hybridization or mutation.

Mutation can add desirable trait(s) into an otherwise excellent cultivar without altering its entire genetic makeup as well as enhance genetic variability, therefore, it is a useful breeding tool for several crops including *Dendrobium* (Khosravi *et al.*, 2009; Khatri *et al.*, 2011; Kumar *et al.*, 2011; Wannajindaporn *et al.*, 2016). Physical or chemical mutagens can be used in mutagenesis to enhance the frequency of mutation above the normal rates (Kodym and Afza, 2003). In various plants, chemical mutagens such as ethyl methane sulfonate (EMS) and sodium azide (NaN_3) are efficient for inducing mutations that may modify the function of proteins and alter phenotypes (Caldwell *et al.*, 2004; Al-Qurainy and Khan, 2009; Arisha *et al.*, 2014).

The traditional selection of mutants by using morphological characteristics is subject to environmental effects, time-consuming and may require a lot of efforts. To circumvent these limitations, the selection at the molecular level based on DNA markers have been utilized. The identification of mutants/somaclonal variants were accomplished by several types of DNA markers, for example amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), and inter-simple sequence repeat (ISSR) (Barakat *et al.*, 2010; Kuchma *et al.*, 2011; Wannajindaporn *et al.*, 2014). In orchids, ISSRs have been used to assess the level of genetic diversity within an endangered lady's slipper orchid (*Cypripedium japonicum*) populations (Qian *et al.*, 2014). Moreover, we also successfully identified suitable ISSR primers for evaluation of sodium azide-induced *Dendrobium* 'Earsakul' mutants (Wannajindaporn *et al.*, 2014) that are useful for molecular characterization of putative *Dendrobium* mutant populations. Recently, several putative black rot resistant *Dendrobium* 'Earsakul' mutants were obtained through *in vitro* mutagenesis of protocorm-like bodies (PLBs) using EMS or NaN_3, followed by *in vitro* selection with *P. palmivora* culture filtrate (CF) (A. Khairum and P.A. Tantasawat, unpublished data). In this study, we characterized these mutants based on the levels of black rot resistance and the genetic variability using ISSR analysis. The potential use of the ISSR markers in the detection of black rot resistance gene(s) was then evaluated using regression analysis.

Materials and methods

Plant materials: Eleven *Dendrobium* 'Earsakul' lines were used for the experiment; three randomly selected non-mutagenized *Dendrobium* 'Earsakul' controls (SUT13C0-1, SUT13C0-2 and SUT13C0-3) and eight putative black rot resistant mutants from chemical mutagenesis and 3 cycles of *in vitro* selection with 30-50% (v/v) CF of *P. palmivora*. Of these eight putative mutants, three were mutagenized with 0.1 mM NaN_3 (SUT13N01-a, SUT13N01-b and SUT13N01-e) according to Wannajindaporn *et al.* (2016), and five were mutagenized with 1.8% (w/v) EMS (SUT13E18-A, SUT13E18-D, SUT13E18-E, SUT13E18-F and SUT13E18-I). Briefly, *Dendrobium* 'Earsakul' PLBs were treated with 1.8% (w/v) EMS for 4 h or 0.1 mM NaN_3 for 1 h, transferred onto VW1 medium (Tantasawat *et al.*, 2015) for 2 months, and transferred onto MS2 medium (Tantasawat *et al.*, 2015) for 2 more months before *in vitro* selection. The putative mutant PLBs were transferred into liquid PSB medium (12.5% (w/v) pea and 1% (w/v) sucrose) supplemented with *P. palmivora* CF at 30-50% (v/v) concentrations for 21 days. The survived PLBs were then transferred to VW1 for recovery for 1 month. Selection was repeated for 3 cycles to ensure efficient resistant screening. The putative mutants were then *in vitro* multiplied up to vM_4 generation before being evaluated in the detached leaf assay and ISSR analysis.

Detached leaf assay for black rot resistance: Inoculum of *P. palmivora* isolate NK-53-9, the most virulent isolate, was prepared according to Khairum *et al.* (2016) and adjusted to 1×10^6 zoospores mL^{-1}. Leaves were wounded with pins and placed on water agar (1% (w/v) agar). Three µL of zoospore suspension was inoculated onto each wound and the leaves were incubated at 25°C in the dark for 5 days. Four replicates were used for each *Dendrobium* line. Severity of symptom scores were attributed according to the following scale: 0, no symptom; 1, very small localized lesions; 2, yellow around lesions; 3, yellow around lesions, hyphae was revealed; 4, brown lesions, hyphae was expanded; 5, brown lesions, hyphae was expanded outside the area covered (Khairum *et al.*, 2016). The resistance levels were classified into 3 groups based on severity scores: resistant, severity scores of 0-1.7; moderately resistant, severity scores of 1.8-3.4; susceptible, severity scores of 3.5-5.

DNA extraction and ISSR analysis: Young leaves were harvested from 8 putative mutants and 3 controls and frozen in liquid N_2. DNA extraction was performed by the cetyl trimethyl ammonium bromide (CTAB) method according to Wannajindaporn *et al.* (2014). DNA was quantified by spectrophotometry using a ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and adjusted to the final concentration of 40 ng μL^{-1} for future use in polymerase chain reaction (PCR) analysis. Seven ISSR primers homologous to microsatellite repeats and containing additional selective anchor nucleotides that were developed from University of British Columbia were chosen for the ISSR analysis (Table 1). These primers are homologous to microsatellite repeats (AC, AG, CA, GA, or TG) anchored at the 3′ end by 1-2 nucleotides, most of them have been used successfully in wild *Cymbidium goeringii* (Xiaohong *et al.*, 2007) and *Dendrobium* 'Earsakul' (Wannajindaporn *et al.*, 2014, 2016). Two of these had an AG repeat motif, two had an AC repeat motif, and one each had either GA, CA or TG repeat motif (Table 1). Each 20-µL PCR mix contained 80 ng genomic DNA template, 1X buffer (75 mM Tris-HCl (pH 9.0), 50 mM KCl, 20 mM $(NH_4)_2SO_4$), 2.5 mM $MgCl_2$, 200 µM of each dNTP, 1 U Geneaid DNA polymerase (Geneaid Biotech Ltd., Taipei, Taiwan), and 4 µM of each ISSR primer. The PCR mixes were amplified with initial denaturation at 94°C for 5 min; 45 cycles of denaturing at 94°C for 45 s, annealing at 53°C for 45 s, extension at 72°C for 90 s; and a final extension at 72°C for 7 min in a TC-PLUS thermal cycler (Bibby Scientific Limited, Staffordshire, UK). The amplified DNA fragments were revealed on 6% (w/v) denaturing polyacrylamide gel followed by silver nitrate staining according to Wannajindaporn *et al.* (2014). Molecular weights of the DNA bands were estimated using 100 bp plus DNA ladder (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA) as standards.

A clearly amplified band was coded as 0 or 1 for its absence or presence, respectively. Similarity coefficients between various putative mutants and controls, in a pairwise comparison, were computed using Jaccard's coefficient, and the resulting similarity matrix was further analyzed using the unweighted pair group method with arithmetic average (UPGMA) clustering algorithm; the computations were carried out using NTSYSpc version 2.2 (F.J. Rohlf, Exeter Software, NY, USA). The goodness of fit of the putative mutants and controls to a specific cluster in the UPGMA

Table 1. ISSR primer sequences, annealing temperature, number of total scorable DNA bands, number of polymorphic DNA bands, percentages of polymorphism and amplified band size for each ISSR primer used for the analysis of 8 putative *Dendrobium* 'Earsakul' mutants and 3 non-mutagenized controls

Primers	Primer sequences	Annealing temperature (°C)	Number of total bands	Number of polymorphic bands	Polymorphism (%)	Amplified band size (bp)
807	$(AG)_8T$	53	23	11	47.8	170-1900
818	$(CA)_8G$	53	17	12	70.6	210-2100
825	$(AC)_8T$	53	7	2	28.6	390-2100
827	$(AC)_8G$	53	7	3	42.9	240-1500
829	$(TG)_8C$	53	16	4	25.0	220-1700
835	$(AG)_8YC$ [a]	53	29	19	65.5	200-2100
840	$(GA)_8YT$	53	15	2	13.3	240-1100
Total			114	53		
Average			16.3	7.6	42.0	

[a] Y, pyrimidines (C, T)

cluster analysis was determined by the Mantel correlation test (Mantel, 1967).

Association analysis: The association between ISSR markers and black rot resistance was evaluated by simple linear regression analysis using SPSS version 14.0 (R. Levesque, SPSS Inc., NY, USA) where resistance trait was treated as a dependent variable, while the ISSR marker was treated as an independent variable following Tantasawat *et al.* (2012). The association of markers with black rot resistance was assessed by testing the level of significance using the Student *t*-test.

Results and discussion

Detached leaf assay showed that black rot symptoms (brown lesions, covering with hyphae) were clearly observed in some *Dendrobium* lines at 5 days after inoculation with the most virulent *P. palmivora* isolate NK-53-9. Disease severity scores were significantly different ($P<0.01$) among the eight putative mutants and three non-mutagenized controls. When the resistance levels were classified based on disease severity, all three controls were found to be susceptible to *P. palmivora* (severity scores of 5). Putative mutants varied widely for the black rot resistance levels from resistant to susceptible. All three NaN_3-mutagenized mutants (SUT13N01-a, SUT13N01-b, SUT13N01-e) were classified as susceptible (severity scores of 3.5-5), however, only one of five EMS-mutagenized mutants (SUT13E18-D) was susceptible (severity score of 3.5). The remaining four EMS-mutagenized mutants were either resistant (SUT13E18-A, SUT13E18-E and SUT13E18-I; severity scores of 0-0.75), or moderately resistant (SUT13E18-F; severity score of 2) to the disease, suggesting the effectiveness of EMS in generating black rot resistant mutants (Table 2). However, whether these mutants maintain their resistance to black rot under greenhouse conditions remain to be determined.

To evaluate the genetic variability of these eight putative *Dendrobium* mutants varied in resistance levels to black rot, compared to non-mutagenized controls, seven ISSR primers were used for ISSR analysis, yielding a total of 114 fragments across all lines, of which 53 fragments were polymorphic, giving

Table 2. Disease severity scores and black rot resistance levels of putative *Dendrobium* 'Earsakul' mutants (SUT13E18-A, D, E, F, I and SUT13N01-a, b, e) and non-mutagenized controls (SUT13C0-1, SUT13C0-2 and SUT13C0-3)

Lines	Disease severity scores [a]	Black rot resistance levels [c]
SUT13C0-1	5.00 ± 0.00 a [b]	Susceptible
SUT13C0-2	5.00 ± 0.00 a	Susceptible
SUT13C0-3	5.00 ± 0.00 a	Susceptible
SUT13E18-A	0.00 ± 0.00 d	Resistant
SUT13E18-D	3.50 ± 0.87 b	Susceptible
SUT13E18-E	0.75 ± 0.25 d	Resistant
SUT13E18-F	2.00 ± 0.00 c	Moderately resistant
SUT13E18-I	0.00 ± 0.00 d	Resistant
SUT13N01-a	4.25 ± 0.75 ab	Susceptible
SUT13N01-b	5.00 ± 0.00 a	Susceptible
SUT13N01-e	3.50 ± 0.87 b	Susceptible

[a] Severity scale: 0, no symptom; 1, very small localized lesions; 2, yellow around lesions; 3, yellow around lesions, hyphae was revealed; 4, brown lesions, hyphae was expanded; 5, brown lesions, hyphae was expanded outside the area covered

[b] Data are presented as means ± SE. Means in the same column with different letters are significantly different ($P<0.05$) based on Duncan's multiple range test (DMRT).

[c] Resistance levels: resistant, severity scores of 0-1.7; moderately resistant, severity scores of 1.8-3.4; susceptible, severity scores of 3.5-5

a polymorphism percentage of 46.5%. Seven to 29 reproducible bands (average 16.3) were amplified per primer, and each primer produced 2 to 19 polymorphic fragments (average 7.6). The length of amplified ISSR fragments ranged from 170 bp (ISSR 807) to 2100 bp (ISSR 818, 825 and 835). ISSR 818 gave the highest percentage of polymorphism (70.6%), followed by ISSR 835 (65.5%) and ISSR 807 (47.8%) (Table 1). ISSR 818 also gave high percentages of polymorphism among NaN_3-induced *Dendrobium* mutants in our previous work (42.9 to 69%), suggesting its usefulness in *Dendrobium* mutant identification (Wannajindaporn *et al.*, 2014, 2016). All the mutants can be differentiated by using only 7 ISSR primers, substantiating the effectiveness of using ISSR to select for mutants thereby allowing earlier selection and reduction of mutant population size.

A total of 53 polymorphic fragments were used for constructing a similarity matrix (Table 3) and a dendrogram (Fig. 1) based

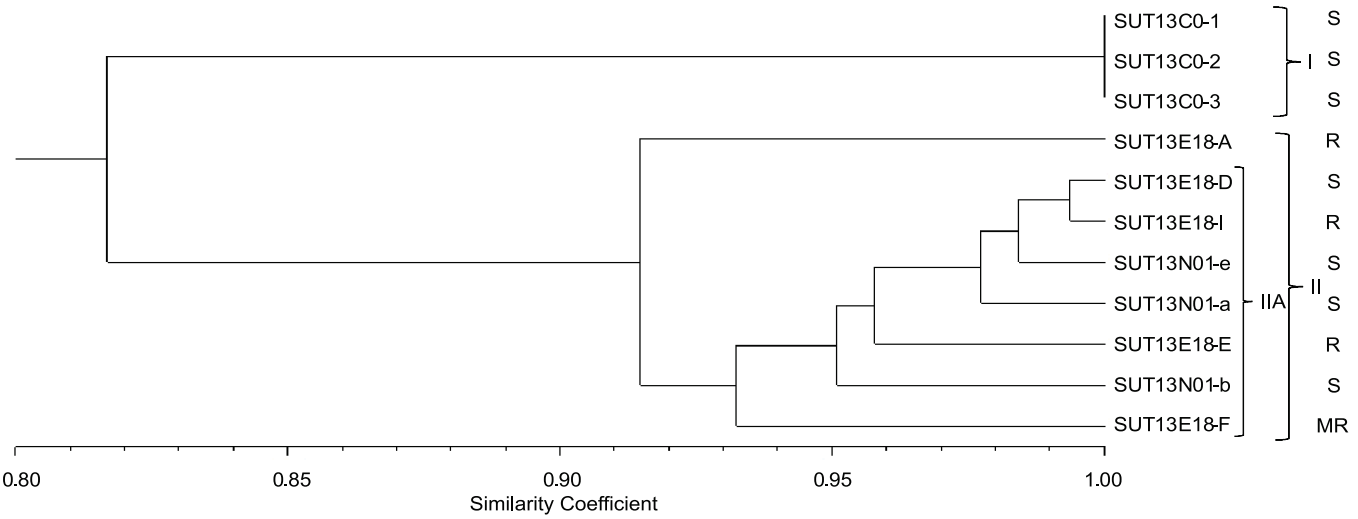

Fig. 1. ISSR-derived dendrogram of 8 putative *Dendrobium* 'Earsakul' mutants (SUT13E18-A, D, E, F, I and SUT13N01-a, b, e) and 3 non-mutagenized controls (SUT13C0-1, SUT13C0-2 and SUT13C0-3). S, susceptible; MR, moderately resistant; R, resistant.

on cluster analysis using UPGMA. The Mantel test with a cophenetic correlation coefficient value of 0.99 indicated that data in the similarity matrix were well represented by the dendrogram. All 3 controls gave the same DNA profiles at all 53 loci, suggesting no evidence of somaclonal variation in the absence of chemical mutagenesis. DNA patterns of all eight putative mutants differed from that of controls, confirming that they are mutants and suggesting that *in vitro* mutagenesis with both EMS and NaN_3 can induce genetic alteration in *Dendrobium*. The altered DNA profiles may result either from the loss/gain of primer binding sites as a result of EMS or NaN_3 induced changes in the nucleotide sequences or changes that alter the size or prevent the successful amplification of a target DNA (Wannajindaporn *et al.*, 2014, 2016). The dendrogram constructed based on ISSR data separated the 11 lines into two clusters at a similarity level of 0.91. Cluster I consisted of all three controls (SUT13C0-1, SUT13C0-2 and SUT13C0-3), while all eight mutants (SUT13E18-A, SUT13E18-D, SUT13E18-E, SUT13E18-F, SUT13E18-I, SUT13N01-a, SUT13N01-b and SUT13N01-e) constituted cluster II. Most mutants clustered together in one large subcluster IIA except SUT13E18-A. No relationship was found between the genetic similarity/ distance and the levels of resistance (Fig. 1).

The Jaccard's coefficients of genetic similarity among the pairwise combinations of eight mutants ranged from 0.886 (SUT13E18-A and SUT13N01-b) to 0.994 (SUT13E18-D and SUT13E18-I), revealing genetic diversity in the mutant population (Table 3). Previous researches also revealed genetic diversity among mutants/ somaclones when analyzed by molecular markers (Ramkrishna *et al.*, 2006; Santos *et al.*, 2008; Barakat *et al.*, 2010). The mutant SUT13E18-A was found to be the most dissimilar from the control (genetic similarity coefficient = 0.779), followed by SUT13N01-a (0.813), and SUT13E18-E (0.818) (Table 3). It is interesting to note that not only SUT13E18-A appears to have distinct genetic profile, it is also resistant to *P. palmivora* isolate NK-53-9 and has been shown to be resistant to four other *P. palmivora* isolates (Khairum *et al.*, 2016). These mutants will be further evaluated for desirable traits and multiplied for future use.

Simple linear regression analysis was performed to determine the association of these ISSR markers with black rot resistance. A summary of simple linear regression, beta, *t*-test, and R^2 for black rot resistance of 5 polymorphic ISSR markers with significant associations to the resistance ($P < 0.05$) is shown in Table 4. The 818_{1748}, 818_{1000} and 835_{501} markers showed negative associations with black rot resistance ($R^2 = 0.374$ to 0.545, $t = -2.317$ to -3.283, $P = 0.009$ to 0.046) and standardized beta coefficients of -0.611 to -0.738, suggesting that they were associated with black rot resistance. By contrast, the 835_{259} and 835_{214} markers showed positive associations with black rot resistance with R^2 values of 0.429 to 0.443 ($t = 2.600$ to 2.677, $P = 0.025$ to 0.029) and standardized beta coefficients of 0.655 to 0.666. Hence, these markers were associated

Table 4. Simple linear regression analysis on ISSR markers and resistance to black rot in 8 putative *Dendrobium* 'Earsakul' mutants and 3 non-mutagenized controls. Only markers significantly associated with black rot resistance are presented.

Markers	Black rot resistance			
	Beta	*t* value	P value	R^2
818_{1748}[a]	-0.611	-2.317	0.046	0.374
818_{1000}	-0.738	-3.283	0.009	0.545
835_{501}	-0.681	-2.791	0.021	0.464
835_{259}	0.655	2.600	0.029	0.429
835_{214}	0.666	2.677	0.025	0.443

[a]The number preceding the subscript (size of ISSR marker in bp) refers to the primer used to generate the marker.

Table 3. Similarity matrix of 8 putative *Dendrobium* 'Earsakul' mutants and 3 non-mutagenized controls based on ISSR analysis

Lines	Controls			Putative mutants							
	SUT13C0-1	SUT13C0-2	SUT13C0-3	SUT13E18-A	SUT13E18-D	SUT13E18-E	SUT13E18-F	SUT13E18-I	SUT13N01-a	SUT13N01-b	SUT13N01-e
SUT13C0-1	1.000										
SUT13C0-2	1.000	1.000									
SUT13C0-3	1.000	1.000	1.000								
SUT13E18-A	0.779	0.779	0.779	1.000							
SUT13E18-D	0.823	0.823	0.823	0.922	1.000						
SUT13E18-E	0.818	0.818	0.818	0.929	0.969	1.000					
SUT13E18-F	0.820	0.820	0.820	0.917	0.932	0.938	1.000				
SUT13E18-I	0.830	0.830	0.830	0.929	0.994	0.963	0.938	1.000			
SUT13N01-a	0.813	0.813	0.813	0.910	0.975	0.944	0.920	0.981	1.000		
SUT13N01-b	0.827	0.827	0.827	0.886	0.951	0.957	0.933	0.945	0.939	1.000	
SUT13N01-e	0.825	0.825	0.825	0.910	0.988	0.957	0.933	0.981	0.975	0.963	1.000

with susceptibility to black rot. Note that these five markers were amplified from only 2 primers (ISSR 818 and 835), allowing simple and convenient screening. However, the putative associations between these ISSR markers and black rot resistance need to be verified with larger mutant populations before their subsequent use in the future.

Our results indicate that ISSRs are efficient for mutant identification and characterization in *Dendrobium*. All eight putative mutants, resulting from *in vitro* EMS or NaN_3 induced mutagenesis followed by *in vitro* selection with *P. palmivora* CF, were genetically altered, compared to non-mutagenized controls, suggesting that they are mutants and implicating the effectiveness of this systematic approach. Variation in black rot resistance levels as well as genetic variability was observed among the eight mutants. At least four out of eight mutants (50%) from *in vitro* selection were found to be resistant or moderately resistant to black rot in the detached leaf assay. These mutants are very useful for future *Dendrobium* improvement for black rot resistance and will be further evaluated for their horticultural traits. Black rot resistance may be identified by 5 ISSR markers, 818_{1748}, 818_{1000}, 835_{501}, 835_{259}, and 835_{214}, with the percentage of phenotypic variance explained by the marker (R^2) of 37.4, 54.5, 46.4, 42.9 and 44.3%, respectively, suggesting the possible use of ISSR markers for the assessment of black rot resistance in *Dendrobium*, which may facilitate future breeding programs.

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