Enzyme Polymorphism in *Cymbidium* Orchid Cultivars and Inheritance of Leucine Aminopeptidase

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Abstract. Seventy *Cymbidium* (Swartz.) cultivars were analyzed for isozyme variability in eight enzyme systems by starch gel electrophoresis. All systems studied [aspartate aminotransferase (AAT), malate dehydrogenase (MDH), alcohol dehydrogenase (ADH), phosphoglucosmutase (PGM), glucose phosphate isomerase (GPI), triosephosphate isomerase (TPI), and shikimate dehydrogenase (SKDH)] showed polymorphism. When all enzyme systems were evaluated, 68 of the 70 *Cymbidium* cultivars could be distinguished. Isozymes could not distinguish between the cultivars Golden Star 'Kumamoto' and Golden Star 'Sunrise'. No cultivar showed a single unique pattern, but the TPI system gave one "diagnostic" pattern. Segregation ratios from controlled crosses suggested that LAP-1 is simply inherited and controlled by at least two alleles.

Cymbidium is an important genus in Orchidaceae, with 44 documented species. *Cymbidium* species have been hybridized for over a century to produce plants with flowers of rich texture and color and large size that have formed the basis of a worldwide cut flower and more recently, pot plant industry. *Cymbidium* hybrids are probably the most commercially important group of orchids in cultivation (Du Puy and Cribb, 1988).

As in other orchids, propagation is mostly vegetative and mislabeling can occur during distribution. This is not easily detectable until flowering. The use of morphological traits for cultivar identification is limited by environmental effects on phenotype. In fact, one of the major causes of taxonomic confusion in the genus is the reliance placed on gross morphological characters, especially of the flowers (Du Puy and Cribb, 1988). Furthermore, most *Cymbidium* hybrid cultivars are related and quite difficult to distinguish morphologically before flowering. This, coupled with the fact that *Cymbidiums*, like other orchids, are inherently slow growing, makes the existence of genetic markers for cultivar identification at the vegetative stage very important.

Due to their codominant expression and lack of environmental effects, isozymes provide an objective and reliable method for cultivar identification, if sufficient polymorphism exists. Isozymes have been used to identify cultivars of various plants, including apple, *Malus × domestica* Borkh. (Weeden and Lamb, 1985), pineapple, *Ananas comosus* L. (DeWald et al., 1988), and the purple loosestrife species *Lythrum salicaria* L. and *Lythrum virgatum* L.

(Strefeler et al., 1996). In orchids, isozymes have been used to study gene variation and gene flow in populations (Rossi et al., 1992) and interspecific relationships (Schlegel et al., 1989). We recently reported isozyme variation for in vitro-cultured protocorm-like bodies (PLBs) of some *Cymbidium* cultivars and genetic analysis of AAT-1 and PGM-1 loci (Obara-Okeyo and Kako, 1997).

The objectives of this study were to extend the number of useful isozyme systems in *Cymbidium*, to characterize a wide range of *Cymbidium* cultivars through enzyme polymorphism, and to study the genetic control of observed LAP phenotypes.

Materials and Methods

Healthy, mature leaves of selected cultivars were sampled, and washed and rinsed with distilled water. Samples (1 g) were then ground in a prechilled pestle and mortar with 3 mL extraction buffer before being absorbed in 3 × 6 mm Advantec No. 2 filter paper wicks. The extraction buffer consisted of 50 mM Tris HCl (pH 8.0), 20% glycerol (m/v), 10% polyvinylpyrrolidone (PVP-40), 0.5% Triton X-100, and 14 mM β-mercapto ethanol. The samples were used immediately or stored at -80 °C for later electrophoresis. Storage for up to 40 d had no impact on resolution.

Horizontal starch gel electrophoresis was conducted on 8% hydrolyzed starch (Wako Chemical Co., Osaka, Japan) gels, consisting of one of three gel buffers, at 4 °C. The first buffer system was a lithium-borate Tris-citrate system with 0.016 M LiOH and 0.192 M boric acid (pH 7.5) in the electrode buffer. The gel buffer consisted of 1.6 mM LiOH, 19.2 mM boric acid, 0.007 M citric acid, and 0.046 M Tris, pH 8.0. This system was used for AAT (EC 2.6.1.1), TPI (5.3.1.1), SKDH (EC 1.1.1.25), ADH (EC 1.1.1.1), and GPI (EC 5.3.1.9), MDH (EC 1.1.1.37), and PGM (EC

2.7.5.1) were resolved in the second system, which was a continuous morpholine-citric acid buffer with 0.04 M citric acid in the electrode and 1.6 mM citric acid (both adjusted with morpholine to pH 7.0). The third system was a Tris-citrate pH 7.8 (TC 7.8) buffer of Shields et al. (1983). This buffer was used to study LAP (EC 3.4.11.1).

Electrophoresis was carried out at a constant 200 V until the anodal migration of the bromophenol blue (BPB) marker dye front had reached 10 cm from the origin. After electrophoresis, the gels were sliced and stained for the various enzymes. Isozymes were localized according to Soltis et al. (1983). All stained gels (except for TPI) were washed and fixed in 7% acetic acid. Gel slices for TPI were fixed in 50% methanol.

Zymograms were drawn to scale and the relative mobility (Rf) calculated for each band based on its migration relative to the front. In systems with multiple zones, the zone with the greatest anodal migration was designated 1. Slower migrating zones were assigned progressively higher numbers. All runs were repeated three times to confirm the consistency of the banding patterns.

Progenies of controlled crosses involving several cultivars made in 1993 and 1995 were used in the inheritance studies of LAP.

Results and Discussion

Polymorphism was observed in all enzyme systems for the cultivars studied. Repeated sampling invariably gave the same set of isozyme phenotypes and preliminary studies had shown no intracultivar isozyme variation.

Two zones of activity were observed when gels were stained for AAT. The genetic control of AAT-1 and PGM-1 in *Cymbidium* was reported earlier (Obara-Okeyo and Kako, 1997); AAT-1 and AAT-2 gave three and two phenotypes, respectively.

MDH showed two zones of activity (Fig. 1). A fast single-banded monomorphic zone was seen at Rf 0.72. MDH-2 zone showed three patterns that were either single-banded or three-banded. The fastest band at MDH-2 was at Rf 0.55 and occurred either alone (phenotype B) or in combination with bands at either Rf 0.5 and 0.53 or Rf 0.45 and 0.5 for phenotypes A and C, respectively. MDH is generally dimeric in plants (Arulsekhar et al., 1986b; Mowrey et al., 1990) and its patterns can be difficult to interpret. Its inheritance cannot always be inferred by its phenotype due to post-translational modification, comigrating electromorphs, null alleles, and the presence of interlocus dimers (Arulsekhar et al., 1986a; Cheliak and Pitel, 1985).

Assays of gels for LAP gave one polymorphic zone. The clearly stained bands showed individuals to be either single or double banded, giving three patterns (Figs. 1 and 3C). The rapidly migrating band was at Rf 0.45, while the slower one was at Rf 0.4. *Cymbidium* cultivars expressed the three phenotypes at almost equal frequencies (Table 1).

ADH zymograms gave six different banding patterns composed of one to six bands,

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with the fastest at Rf 0.5 and the slowest at Rf 0.31 (Fig. 1). Except for patterns D and E, which were expressed by only seven and nine individuals, respectively, the remaining phenotypes were evenly distributed (Table 1). This enzyme is dimeric in plants, although assigning homologies among the ADHs from different species without amino acid sequence data is difficult (Gottlieb, 1981). Except for the fact that expected heterodimeric bands were not observed for patterns B and D, the observed phenotypes in *Cymbidium* tend to support the dimeric nature of this enzyme. Torres and Tisserat (1980) also reported lack of an otherwise expected ADH heterodimer.

One zone of activity was observed in gels assayed for PGM. Individuals had the fast Rf 0.42 band either alone or together with the slow Rf 0.35 band (Fig. 2). This enzyme is known to be monomeric in plants (Byrne and Littleton, 1988).

SKDH has been reported to be monomeric in plants (Kephart, 1990; Mangum and Peffley, 1994). In our study, this enzyme system gave four patterns with one to six bands (Fig. 2). *Cymbidium* cultivars gave two well resolved anodal bands at Rf 0.48 and Rf 0.44. Below these were three lightly stained bands Rf 0.34, 0.30, and 0.25 (Fig. 3A).

One zone of activity was observed at GPI. Individuals had either one band or three. In those with three bands, the second one was midway between the fast and the slow for the phenotypes A, D, and E. This banding pattern is suggestive of a dimeric enzyme, consistent with reports for other plants (Goldring et al., 1985; Gottlieb, 1981). Three putative alleles at one locus can be proposed for GPI control in *Cymbidium*.

TPI showed the greatest polymorphism of the systems studied. Seven different banding patterns composed of three to six bands were observed (Figs. 2 and 3B). The most anodal TPI band was at Rf 0.75, while the slowest was at Rf 0.30.

Isozyme variability among cultivars. Of the 70 *Cymbidium* cultivars included in the study, all except one pair could be successfully discriminated when all eight enzyme systems were used (Table 2). AAT, ADH, SKDH, GPI, and TPI were very useful systems for identification due to their high polymorphism. The indistinguishable cultivars were Golden Star 'Kumamoto' and Golden Star 'Sunrise'. The identical patterns of these two cultivars can be explained by common descent. Common descent also can explain the fact that three sets of cultivars differed in only two or three enzymes. Lucky Rainbow 'Lapine Joyner' and Lucky Rainbow 'Sainte Lapine' differed at TPI, AAT-1, and LAP, whereas Sazanami 'Champion', Sazanami 'Harunoumi', and Sazanami 'Otome' differed at SKDH and TPI. Sleeping Beauty 'Golden Bird' differed from Pharaoh 'Tetra High' only at GPI and TPI. Sleeping Beauty is one of the parents of Pharaoh.

None of the patterns observed was unique to any of the cultivars studied. Arbitrary assignment of any phenotype with a frequency of <10% as diagnostic pattern showed that TPI

had one such pattern (A), with only six (8.6%) of the cultivars showing it. This should be useful in identification of *Cymbidium* cultivars. The observed isozyme variability in *Cymbidium* is comparable to other outcrossing plants, such as pineapple (DeWald et al., 1988) and almond (Hauagge et al., 1987).

Inheritance of leucine amino peptidase. Table 3 shows symbols for putative genes coding for LAP in *Cymbidium*, accompanied with corroborating segregation data. When crosses were made, resulting progenies segregated in a Mendelian fashion with conformity to the expected ratios for a simply inherited model. Individuals had either the slow migrat-

ing allele designated "a" or the more anodal allele designated "b." Heterozygous individuals ("ab") display two bands, suggesting a monomeric nature for the enzyme, as reported in other plants (Arulsekhar et al., 1981; Goldring et al., 1987). When tested with the χ^2 goodness of fit, segregation for progenies of the ab x ab crosses showed a good fit to the expected 1:2:1 ratios (Table 3). Segregation for aa x ab and ab x bb both fit the expected 1:1 ratio. In making the aa x ab crosses, however, <1% of the crosses where aa was used as the male parent gave progenies. Results were therefore obtained from a one-way cross for these individuals. The possibility of male sterility was

Fig. 1. Schematic zymograms of representative phenotypes (A, B, C, D, E, F) for AAT, MDH, LAP, and ADH isozymes in *Cymbidium*.

Table 1. Frequencies of phenotypes observed among the 70 *Cymbidium* cultivars analyzed.

Phenotype	AAT-1	AAT-2	MDH-2	LAP-1	ADH	PGM	SKDH	GPI	PI
A	24	34	26	22	13	33	18	16	6
B	30	36	20	24	13	37	23	13	15
C	16	---	24	24	14	---	14	14	11
D	---	---	---	---	7	---	15	12	7
E	---	---	---	---	9	---	---	14	12
F	---	---	---	---	14	---	---	---	10
G	---	---	---	---	---	---	---	---	9

Fig. 2. Schematic zymograms of representative phenotypes (A, B, C, D, E, F, G) for PGM, SKDH, GPI, and TPI isozymes in *Cymbidium*.

Fig. 3. Photograph of banding patterns for SDKH (A), TPI (B), and LAP (C) in *Cymbidium* cultivars.

considered, but the aa individuals gave progenies when selfed (Table 3). Some kind of pollen cross-incompatibility could be responsible, but why no progenies were recovered from these crosses remains largely unexplained.

This study shows that isozymes can be used for identification of *Cymbidium* cultivars at the vegetative stage. This should be useful to breeders, propagators, and germplasm repositories for early detection of mislabeled plants and therefore help maintain the purity of *Cymbidium* stocks. Since most *Cymbidium* cultivars are routinely used as parents in producing new hybrids, the observed variability can be useful in germplasm evaluation. Precise identification of cultivars by isozymes can also be useful for patent purposes. The simple inheritance of LAP-1 makes it suitable as a genetic marker.

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Table 2. Isozyme phenotypes for eight enzyme systems (and nine polymorphic regions) in 70 *Cymbidium* cultivars. Phenotypes correspond to isozyme patterns (Figs. 1-3).

Cultivar	Banding patterns								
	AAT-1	AAT-2	MDH-2	LAP	ADH	PGM	SKDH	GPI	TPI
African Adventure 'The Nile'	A	A	C	B	F	B	D	D	E
Akatuki 'Tokai'	B	A	B	B	B	B	B	B	D
All Star 'Red King'	C	A	C	C	E	A	A	A	F
All Star 'Marrian'	C	A	A	A	E	A	A	D	F
Allegro 'Cherry Blossom'	A	A	B	C	E	A	A	E	F
Angelica 'December Queen'	B	B	C	B	A	B	B	A	F
Atlantic Sun 'Cold Springs'	B	A	A	A	A	A	B	A	B
Autumn King 'Pinky'	B	B	A	A	B	A	A	C	G
Ayako Tanaka 'Tokai'	B	B	C	A	C	A	A	E	B
Babylon 'Castle Hill'	A	B	B	A	F	B	B	E	C
Bethlehem 'Star Light'	A	A	B	B	B	A	C	E	D
Blue Smoke 'Green Meadow'	C	B	C	C	F	B	C	B	A
Celle del Rey 'Jade Queen'	A	A	A	B	A	B	D	B	F
Cariga 'Golden'	A	A	C	B	C	B	B	D	G
Cathy Sue 'Dark Red'	A	B	A	C	C	A	B	D	G
Ceres 'Girrahween, AM/NSW'	A	B	A	A	E	B	B	C	B
Charm 'Elegance'	B	B	B	B	E	A	A	B	B
Cherry Blossom 'Profusion'	B	B	C	A	F	A	B	C	E
Christmas Green 'Holy'	C	A	C	A	F	A	D	C	E
Christine Peak 'Tokai'	B	B	B	A	D	B	D	A	C
Cleopatra 'Tokai'	C	A	B	C	C	B	D	E	B
Cool Call 'Ballad'	A	A	C	C	A	A	B	B	A
Cracker Jack 'Midnight Magic'	C	A	B	C	B	A	C	B	B
Cotton Club 'Tokai'	C	B	C	C	B	B	C	C	C
Early Bird 'Pacific'	A	B	A	A	A	A	A	E	D
Erin Delight 'Kako'	B	B	B	B	B	B	D	A	E
Flower Song 'Spring Dance'	B	B	C	C	C	B	D	C	D
Golden Star 'Kumamoto'	A	A	A	C	C	A	B	A	G
Golden Star 'Sunrise'	A	A	A	C	C	A	B	A	G
Gracious 'Romancing Nile'	C	A	B	C	F	B	A	A	B
Great Flower 'Ballerina'	A	A	B	A	A	A	C	E	E
Green Stone 'Plush'	A	A	A	B	D	A	C	E	D
Hiroshima Angel 'Sylvia'	C	B	C	C	D	A	A	E	E
Hiroshima Lady 'Sophia'	B	B	A	A	D	B	B	E	F
Honey Moon 'Tropical Yellow'	B	A	B	A	E	A	B	E	C
Hunter's Point 'Sunset'	B	B	B	C	A	B	B	D	B
Lady Back 'Calisona'	B	B	A	A	A	B	A	B	B
Latigo 'Yellow Magic'	B	A	B	B	B	B	B	B	C
Liliana 'Pink Champagne'	C	A	C	B	C	A	D	C	G
Louise Stewart 'Snow Flake'	B	B	A	B	E	B	D	C	E
Love Call 'Cupid'	B	A	B	B	E	A	A	E	E
Lovely Bunny 'Juliet'	A	B	B	C	B	A	C	B	G
Lucky Rosanna 'Honey Moon'	A	B	C	A	A	A	C	D	A
Luckybel 'Dream Color'	A	B	C	A	B	B	B	A	G
Lucky Flower 'Amitsuhime'	B	B	C	C	C	B	A	C	C
Lucky Rainbow 'Lapine Joyner'	A	A	C	A	D	B	B	A	C
Lucky Rainbow 'Sainte Lapine'	B	A	C	B	D	B	B	A	E
Mini Dream 'Golden Color'	B	B	A	C	F	B	C	C	F
Peppermint 'Miss Taipei'	A	A	A	B	F	A	B	D	B
Pharaoh 'Tetra High'	A	B	A	B	A	A	D	D	B
Red Start 'Exbury'	C	A	C	A	C	A	C	B	B
Rodco's Creation 'Golden Gate'	B	B	B	A	A	B	A	B	C
Sable Dance 'Kensington'	B	A	B	B	F	B	D	C	D
Sarah Jean 'Ice Cascade'	C	A	C	C	F	A	B	D	E
Sazanami 'Champion'	B	B	A	B	B	B	A	A	D
Sazanami 'Harunoumi'	B	B	A	B	B	B	C	A	G
Sazanami 'Otome'	B	B	A	B	B	B	A	A	A
Sensation 'Mid Night Gem'	C	A	A	A	C	B	B	C	A
Sensation 'Purple Queen'	C	A	A	B	E	A	D	C	C
Showgirl 'Husky Honey'	A	B	A	C	F	B	D	E	B
Sleeping Beauty 'Golden Bird'	A	B	A	B	A	A	D	E	F
Sussex Port 'Peace'	B	B	B	B	B	B	C	C	F
Sweetheart 'Hakuun'	C	A	B	A	C	B	A	A	F
Sylvan Fair 'Princess Kiko'	B	B	A	A	F	B	A	D	B
Sylvan Rainbow 'Himiko'	B	A	C	C	F	B	C	B	B
Valley Flower 'Cherry Ripe'	B	A	C	C	F	B	B	A	C
Valley Promise 'White Ace'	A	A	A	B	A	A	C	B	E
White Christmas 'Grace Kelly'	B	B	C	B	C	B	D	D	E
Wylan 'Sailor Bay'	C	B	C	C	C	A	A	D	C
Yellow Hammer 'Iinuma'	A	B	A	B	D	A	B	B	A

Table 3. Genotypic ratios and χ^2 goodness of fit values for simple inheritance of leucine aminopeptidase (LAP-1) in *Cymbidium*.

Crosses ^z	Segregation classes			Test ratio	χ^2	P value (%)
	aa	ab	bb			
	<i>aa</i> x <i>ab</i> ^y					
Atlantic Sun 'Cold Springs' x Valley promise 'White Ace'	17	19	---	1:1	0.028	80-90
Christmas Green 'Holy' x White Christmas 'Grace Kelly'	18	21	---	1:1	0.103	70-80
Honey Moon 'Tropical Yellow' x Pharaoh 'Tetra High'	14	17	---	1:1	0.129	70-80
	<i>ab</i> x <i>bb</i>					
Pharaoh 'Tetra High' x All Star 'Red King'	---	21	24	1:1	0.089	70-80
Latigo 'Yellow Magic' x Cracker Jack 'Midnight Magic'	---	14	12	1:1	0.038	80-90
	<i>ab</i> x <i>ab</i>					
Valley Promise 'White Ace' x White Christmas 'Grace Kelly'	18	36	16	1:2:1	0.171	90-95
Pharaoh 'Tetra High' x Latigo 'Yellow Magic'	11	21	8	1:2:1	0.55	70-80
	<i>aa</i> x <i>aa</i> ^x					
Atlantic Sun 'Cold Springs' self-pollinated	29	---	---	---	---	---
Sylvan Fair 'Princess Kiko' x Hiroshima Lady 'Sophia'	12	---	---	---	---	---

^zLetters aa, ab, and bb denoting genotypes are assigned to correspond with LAP phenotypes A, B, and C, respectively.^yProgenies were recovered with "aa" as the paternal parent in <1% of the aa x ab crosses.^xReciprocal crosses were not made.

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