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SHORT COMMUNICATION

Expression pattern of two paralogs of the *PI/GLO*-like locus during *Orchis italica* (Orchidaceae, Orchidinae) flower development

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Abstract The class B MADS-box genes belong to two distinct functional groups: the *AP3/DEF*-like and the *PI/GLO*-like sub-families. In orchids, *AP3/DEF*-like genes are present in four copies, each with a different role in floral organ formation, which is described in the "orchid code" model. Interestingly, the orchid *PI/GLO*-like genes are present in two copies in Orchidinae, whereas they are described as single copy in the other orchid lineages. The two *PI/GLO*-like paralogs have site-specific different selective constraints; in addition, they show relaxation of purifying selection when compared to the single-copy lineages. In this study, we present a comparative analysis of the expression patterns of the two *PI/GLO*-like paralogs, *OrcPI* and *OrcPI2*, in floral tissues of *Orchis italica* in different developmental stages using real-time PCR. The two genes show similar expression profiles in the tissue examined, with differences detectable between immature and mature inflorescence. In all cases, *OrcPI2* is expressed at a higher level than *OrcPI*. Real-time PCR results reveal that the co-expression of the two duplicated loci could have a fully or partially redundant function. The possible evolutionary fate of *OrcPI* and *OrcPI2* is discussed as well as their involvement in ovary development.

Keywords Expression pattern · Gene duplication · MADS-box genes · Real-time RT-PCR

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Introduction

Flower initiation and development are realized through complex networks of exogenous and endogenous signals including light stimuli, hormones, ligand–receptor interactions, signal transduction pathways, and transcription factors cascades. A relevant role in flower formation is played by MADS-box genes, a wide gene family encoding transcription factors able to form homo- and heteromultimeric complexes that can bind DNA. Plant MADS-box genes have evolved mainly through successive gene duplications followed by sub- and/or neo-functionalization, giving rise to five different functional classes (A through E) whose interactions are described by the ABCDE model (Jack 2001; Theissen 2001). In brief, the expression of class A genes drives the development of sepals alone (whorl 1) and, together with the expression of class B genes, of petals (whorl 2); the expression of class C genes determines the development of carpels (whorl 4) alone and, together with the expression of class B genes, of stamens (whorl 3); class D genes are involved in ovule formation; class E genes are necessary for the correct development of all floral organs (Fig. 1a). Even though the ABCDE model, initially developed on *Arabidopsis thaliana*, works well for most eudicots, slight modifications are required to fit the floral organ formation in some monocots, such as lilies and orchids, in which sepals and petals show similar morphology and are termed tepals (Fig. 1b). The expansion of class B genes expression to whorl 1 is thought to have determined the presence of sepaloid petals (tepals) in these plant groups (Kanno et al. 2007).

Orchidaceae is one of the largest families among flowering plants and includes five subfamilies (Apostasioideae, Vanilloideae, Cypripedioideae, Epidendroideae, Orchidoideae) (Fig. 2) and a large number of tribes and subtribes (Gorniak

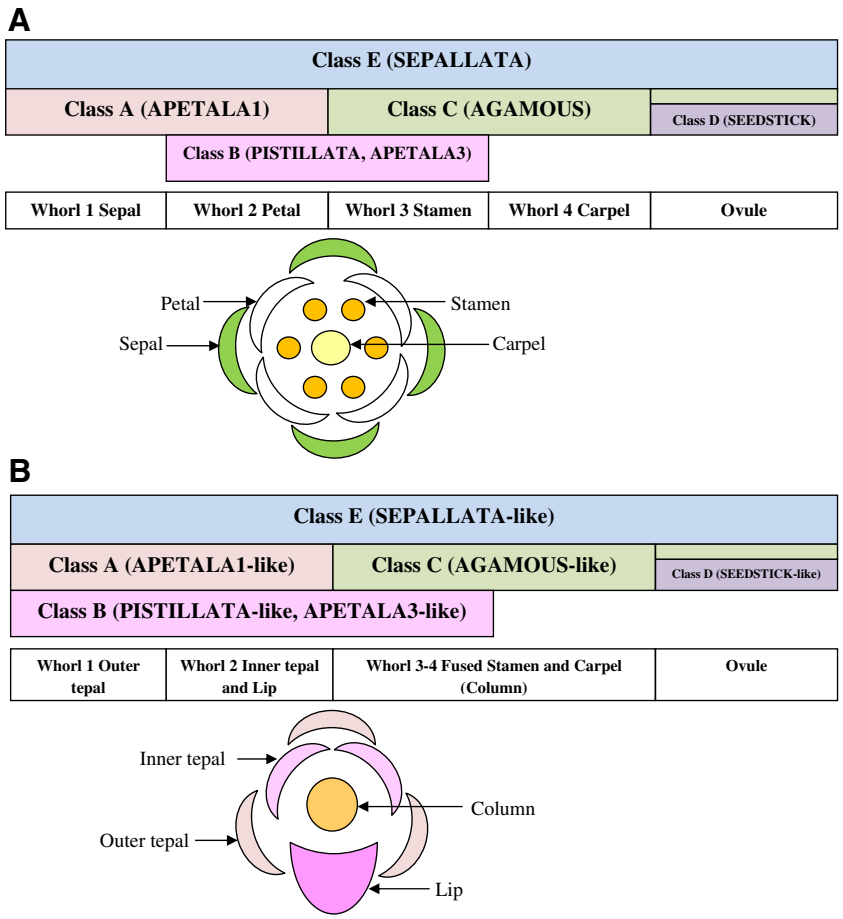

Fig. 1 Diagram of the ABCDE model (**a**) and of the expanded ABCDE model of floral development (**b**)

et al. 2010). This family includes species with greatly diversified and specialized floral morphology, even though a general common structure is detectable. The orchid flower is zygomorphic and includes three outer tepals, two lateral inner tepals, and a median inner tepal (lip or labellum). Male and female orchids reproductive organs are fused into a single structure (column or gynostemium); at the top of the column is the male anther containing packets of pollen (pollinia) and at the base of the column is the ovary.

The recent theory known as "the orchid code" assumes that class B *AP3/DEF*-like genes had a relevant role in the evolution of the orchid perianth. Two main duplication events generated the current class B *AP3/DEF*-like genes in orchids. After the duplications, changes in regulatory and coding regions led to sub- and neo-functionalization of *AP3/DEF*-like genes that are divided into four different clades in orchids, each of which has its own specific expression pattern. High levels of expression of clade-1 and clade-2 *AP3/DEF*-like genes and low levels of clade-3 and clade-4 specify inner lateral tepals, whereas lip development requires low expression levels of clade-1 and clade-2 *AP3/DEF*-like genes and high clade-3 and clade-4 expression levels. In contrast to the *AP3/DEF*-like genes, orchids class B *PI/GLO*-like genes are documented as single-copy loci in almost all the orchid sub-families (Mondragon-Palomino et al. 2009; Tsai et al. 2005; Xu et al. 2006), while two *PI/GLO*-like paralogs have been described in Orchidinae (Kim et al. 2007; Cantone et al. 2011). Evolutionary analysis revealed that the two *PI/GLO*-like

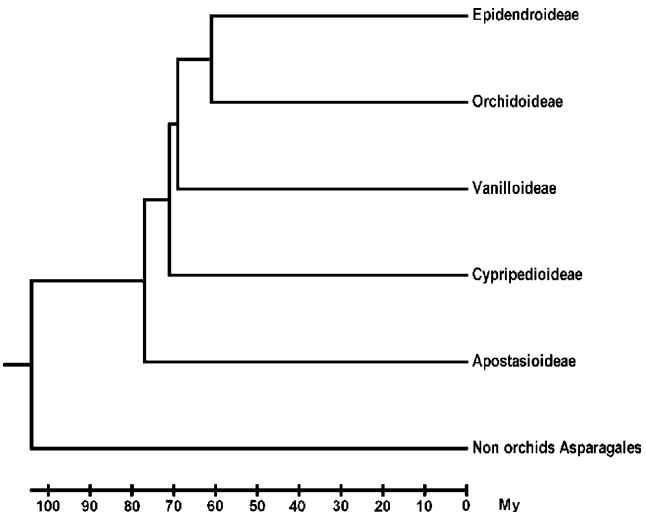

Fig. 2 Time-calibrated simplified phylogeny of the orchid subfamilies, modified from Gustafsson et al (2010). *My* millions of years

paralogs evolved under different selective constraints with a peculiar pattern of purifying selection acting on synonymous sites, suggesting a possible sub-functionalization of these *PI/GLO*-like genes within Orchidinae (Aceto et al. 2007; Cantone et al. 2009, Cantone et al. 2011). To date, the available data that pertain to the expression pattern of the two *PI/GLO*-like paralogs are incomplete and obtained by non-quantitative techniques.

In this study, we compared the tissue expression of the *PI/GLO*-like paralogs in floral tissues of *Orchis italica* (Orchidaceae, Orchidinae) at different developmental stages using real-time RT-PCR to integrate the evolutionary analysis results with the *OrcPI* and *OrcPI2* gene expression analyses.

Materials and methods

Plant material

Outer and inner tepal, lip, column, and not pollinated ovary tissue of *O. italica* were examined at two different developmental stages. The earliest stage corresponds to a young inflorescence before anthesis (~9 mm diameter size) (Fig. 3a), and the latest stage corresponds to a mature inflorescence after anthesis (Fig. 3c).

Fifteen and five florets from a single immature and mature inflorescence, respectively, were dissected and quickly stored in RNA Later (Ambion) at −20°C until processing. In addition, ten ovaries were manually pollinated, collected 3, 7, and 10 days after pollination (dap) and quickly stored as described before. Leaf tissue was also collected and stored under the same conditions.

RNA extraction and real-time PCR experiments

Total RNA was extracted from the *O. italica* tissue samples using TRIzol (Ambion), treated with DNase I (Ambion) following the manufacturer's instructions and quantified by using the BioPhotometer (Eppendorf). Integrity of total RNA was checked by agarose gel electrophoresis.

Based on the cDNA sequence of *OrcPI*, *OrcPI2*, and of the actin gene (*OitaAct*) of *O. italica* (accession number AB094985, AB537504, and AB630020, respectively), primer pairs that selectively amplify a fragment of each cDNA were designed using the software Primer Express v.3.0 (Applied Biosystems). The primer sequences and the amplicon lengths are listed in Table 1.

Total RNA (350 ng) isolated from the different tissues was reverse transcribed using the Advantage RT PCR kit (Clontech) with oligo dT primers. After the reaction, the RT mixture was raised to 35 μl with nuclease-free distilled water. The real-time PCRs were performed using a 7500 real-time PCR System (Applied Biosystems) in the presence of ×1 Power Sybr® Green PCR Master mix (Applied Biosystems) and 0.1 μM of each primer. The thermal protocol was as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. A melting curve of PCR products (95–60°C) was performed to ensure the absence of artifacts. The reactions were conducted using 2 μl of the RT mixture for *OrcPI* and *OrcPI2* gene PCR amplification and 1 μl for *OitaAct* gene amplification. For

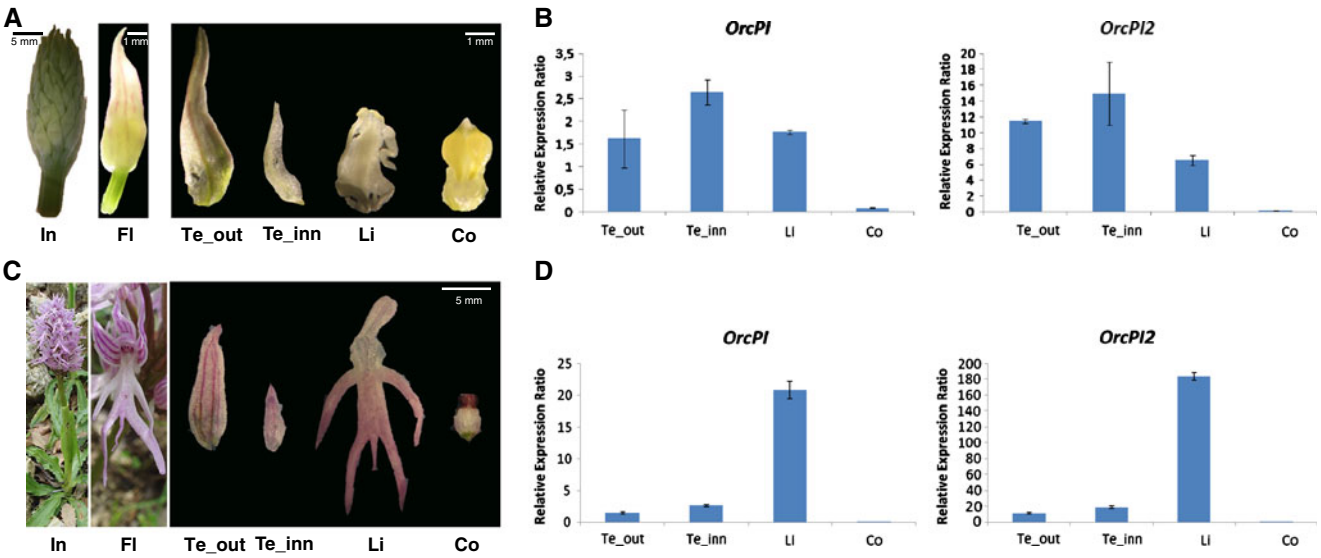

Fig. 3 Floral tissues of *O. italica* and *OrcPI* and *OrcPI2* gene expression patterns. **a**, **c** Immature and mature inflorescence, respectively. **b**, **d** Relative expression ratio of the *OrcPI* and *OrcPI2* genes in floral tissues from immature and mature inflorescence, respectively. *In* inflorescence, *Fl* floret, *Te_out* outer tepal, *Te_inn* lateral inner tepal, *Li* median inner tepal (lip), *Co* column. The *bars* on the graphs represent standard deviation

Table 1 Primer nucleotide sequences used in real-time PCR experiments

Primer name	Sequence (5′–3′)	Amplicon length (bp)
OrcPI-RealF	CCCAGAATATGCGGACCAGATGCC	56
OrcPI-RealR	TGGGCTGGAAAGGCTGCACG	
OrcPI2-RealF	GAGAGTACGCACCGCCACCG	54
OrcPI2-RealR	GCTGGATGGGCTGCACACGA	
OitaAct-RealF	TCGCGACCTCACCAATGTAC	68
OitaAct-RealR	CCGCTGTAGTTGTGAATGAATAGC	

all genes examined, two independent biological samples of each tissue were evaluated in technical triplicates, and negative controls were performed without cDNA in the reaction mixture. Two amplicons for each gene were cloned and sequenced using the BigDye Terminator Cycle Sequencing Kit v. 1.1 (Applied Biosystems). Sequence reactions were run on a 310 Genetic Analyzer (Applied Biosystems).

Data analysis

PCR efficiency and optimal threshold cycle (C_T) for the primer pairs that amplify the target genes, *OrcPI* and *OrcPI2*, and the *OitaAct* endogenous control gene were calculated for each well using the online real-time PCR Miner tool (Zhao and Fernald 2005). In brief, this software defines the exponential phase of each PCR starting from raw fluorescence data and estimates the PCR efficiencies (E) for each well through a nonlinear regression algorithm without the need of a standard curve. The mean PCR efficiency for each gene was used in the subsequent analysis. The relative expression ratios (rER) of the *OrcPI* and *OrcPI2* genes in the different tissues were calculated applying the Gene Expression's C_T Difference (GED) approach (Schefe et al. 2006), using *OitaAct* as the endogenous control gene and leaf cDNA as the reference sample. For each reaction, the amount of the target cDNA (*OrcPI* and *OrcPI2*) relative to the amount of the *OitaAct* cDNA was measured applying the following formula:

$$\mathrm{rER} = \left(1 + E_{\mathrm{target}}\right)^{-\Delta C_{\mathrm{T\ target}}} / \left(1 + E_{\mathrm{actin}}\right)^{-\Delta C_{\mathrm{T\ actin}}}$$

where E is the mean PCR efficiency of the gene, $\Delta C_{T\ target}$ is the difference between the C_T value of the target gene (*OrcPI* or *OrcPI2*) in the tissue of interest and the C_T value of the target gene in the reference tissue (leaf), $\Delta C_{T\ actin}$ is the difference between the C_T value of the *OitaAct* gene (endogenous control) in the tissue of interest and the C_T value of the *OitaAct* gene in leaf tissue. Subsequently, mean rER and standard deviation values were calculated for each triplicate and for the two biological samples.

Differences in the relative expression levels of the *OrcPI* and *OrcPI2* genes between and/or among different samples were assessed by the two-tailed *t* test and ANOVA followed by the Tukey HSD post hoc test, respectively.

Results and discussion

The presence of two transcribed paralogs of the *PI/GLO*-like locus in orchids is restricted to Orchidinae, whereas the other orchids appear to possess a single *PI/GLO*-like gene. The duplicated copies present within Orchidinae evolved under selective constraints that were different from those acting on the single copy *PI/GLO*-like genes of the other orchid lineages. Both paralogs of Orchidinae show relaxation of purifying selection when compared to the single-copy lineages, which is a common pattern after gene duplication events. In addition, a high percentage of sites between the two *PI/GLO*-like paralogs show differences in selective constraints mainly due to a reduced synonymous substitution rate. Evolutionary analyses suggested possible functional divergence of the two *PI/GLO*-like genes in Orchidinae. To clarify the expression pattern of the two paralogs in different floral tissues and in different developmental stages and to compare it to the expression profile of the single-copy gene observed in the other orchid lineages, we performed real-time PCR analysis in *O. italica*.

The melting-curve analysis performed after the real-time PCR amplifications of the *OrcPI*, *OrcPI2*, and *OitaAct* cDNAs showed the effectiveness of the primer pairs used. Sequence analysis of the cloned amplicons confirmed the specificity of the amplified products, corresponding to the selected fragments of the *OrcPI*, *OrcPI2*, and *OitaAct* genes. The mean PCR efficiencies calculated for each gene by real-time PCR Miner tool showed comparable values, ranging from 0.92±0.02 for the *OrcPI2* gene to 1.04±0.02 for the *OitaAct* gene.

Figure 3 shows the relative expression ratio of the *OrcPI* and *OrcPI2* genes in different floral tissues of immature (b) and mature (d) inflorescence. Relative expression analysis revealed the presence of different levels of transcripts of *OrcPI* and *OrcPI2* in the examined floral tissues of *O. italica*, where the *OrcPI2* gene showed a higher expression rate when compared to its paralog, *OrcPI*. The paired

samples *t* test showed this difference was significant for all tissues, with $p \leq 0.030$.

In the immature inflorescence (Fig. 3b), the significantly lowest expression of both genes is detected in the column (*p* values of the Tukey HSD post hoc test ≤0.003) with an expression ratio *OrcPI2*/*OrcPI* of 1.6. The expression level of both genes detected in the outer tepal is not significantly different from that observed in the lip with an expression ratio *OrcPI2*/*OrcPI* of 7.05 in the outer tepal and 3.69 in the lip. The highest expression level of the *OrcPI* and *OrcPI2* genes was detected in the lateral inner tepals. In lateral inner tepal, the expression ratio of *OrcPI2/OrcPI* is 5.65. Even though the difference between this tissue and the outer tepal and lip is not statistically significant for the *OrcPI* gene, the comparison is statistically significant for the *OrcPI2* between the lateral inner tepals and the lip tissue.

In the mature inflorescence (Fig. 3d), the expression pattern of the *PI/GLO*-like paralogs is different from the profile observed in the immature inflorescence tissues. Both genes continue to show the lowest expression in the column (*t* test *p* values ≤0.016) with an expression ratio *OrcPI2*/*OrcPI* of 2.6. The amount of *OrcPI* and *OrcPI2* gene transcripts in the outer and lateral inner tepal is higher than what was detected in the column. However, the difference between the *OrcPI* gene transcript levels in the column and the outer tepal and the difference between the outer and lateral inner tepal are not statistically significant. However, the differences in the *OrcPI2* gene expression levels is statistically significant among all the tissues examined and showed higher transcript levels in lateral inner tepals than outer tepals ($p=0.039$). The expression ratio of *OrcPI2*/*OrcPI* is ~7 in both tissues. The significantly highest expression level of both *PI/GLO*-like genes was in the lip tissue with an expression ratio *OrcPI2/OrcPI* of 8.82.

The pairwise comparison of the *OrcPI* and *OrcPI2* relative tissue expression level between immature and mature inflorescence gave significant results in the lip and column tissues ($p \leq 0.002$), which showed an increase in gene expression for both genes in the lip from immature to mature inflorescence and an opposite trend in the column. Specifically, the expression ratio mature/immature inflorescence is 0.16 in the column and 11.77 in the lip for *OrcPI* and 0.27 in the column and 28.1 in the lip for *OrcPI2*. The expression level of both genes remains approximately the same in the outer and lateral inner tepals with an expression ratio mature/immature inflorescence of ~1.

The relative expression profile detected for the *OrcPI* and *OrcPI2* genes appears to follow a common, co-expression pattern in the different floral tissues and in the different developmental stages examined, which is in general agreement with what was observed in some *PI/GLO*-like single-copy orchid lineages (Mondragon-Palomino and Theissen 2011).

As it was hypothesized the involvement of the *PI/GLO*-like gene *PeMADS6* as negative regulator of the ovary development in the orchid *Phalaenopsis equestris* (Epidendroideae) (Tsai et al. 2005), relative expression analysis of both *OrcPI* and *OrcPI2* was conducted on ovary tissue of *O. italica* before and after pollination. In orchids, ovary development is triggered by pollination and in *O. italica* the complete maturation of the ovary is reached approximately 10 days after pollination (Cozzolino, personal communication). Figure 4 shows the relative expression profile of the *OrcPI* and *OrcPI2* genes in ovary tissue collected from immature and mature inflorescences before pollination and 3, 7, and 10 days after manual pollination. Both genes are weakly expressed in the ovary of the immature inflorescence, whereas significantly high expression is detected in the ovary of the mature inflorescence before pollination. After pollination the expression of both genes rapidly decrease, resembling the expression profile of *PeMADS6* in the ovary of *P. equestris*, which supports the previously hypothesized role of the *PI/GLO*-like genes as negative regulators of ovary development in orchids. As observed in the other floral tissues examined, expression of the *OrcPI2* gene in the ovary is higher than that of the *OrcPI* gene (*p* values ranging from 0.003 to 0.019) with an expression ratio *OrcPI2/OrcPI* of 3.58 in the mature inflorescence ovary.

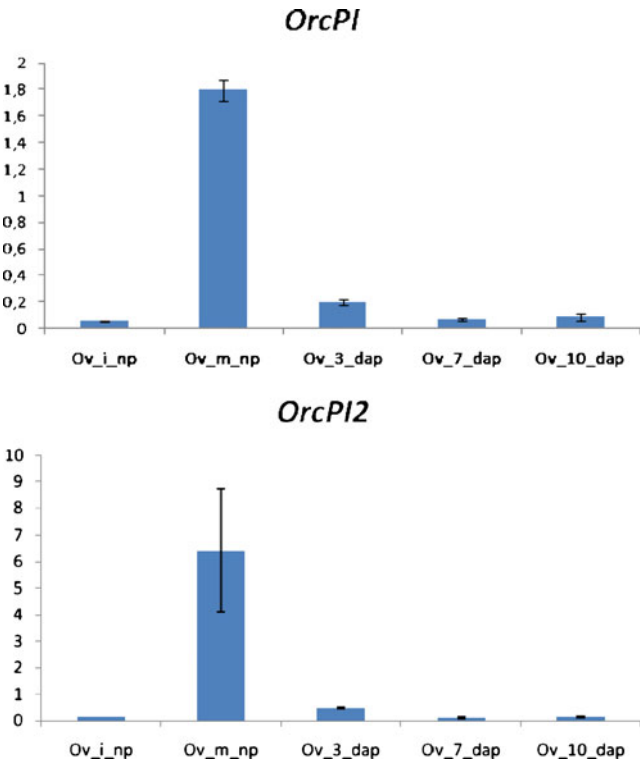

Fig. 4 Relative expression ratio of the *OrcPI* and *OrcPI2* genes in *O. italica* ovary tissue. *Ov* ovary; *np* not pollinated; *dap* days after pollination. The *bars* on the graphs represent standard deviation

The expression analysis of the two *PI/GLO*-like paralogs in *O. italica* revealed that the duplicated genes, *OrcPI* and *OrcPI2*, are co-expressed. However, in all tissues, the amount of *OrcPI2* gene transcripts, ortholog of the single-copy *PI/GLO*-like gene from other orchid lineages, is higher than the *OrcPI* gene transcripts. This transcriptional behavior confirms our previous hypothesis of a recent lineage-specific duplication event within Orchidinae, with a functional role of the duplicated gene *OrcPI* fully or partially redundant relative to *OrcPI2*. Based on the evolutionary and expression pattern of the two paralogs, the fate of the duplicated locus, *OrcPI*, might evolve through sub-functionalization which may result in, for example, a partition between the OrcPI and OrcPI2 protein's capability to bind different products of the four different class-B AP3/DEF-like proteins during the formation of the specific floral quartets, which are necessary for correct floral tissue development.

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Conflict of interests The authors declare the absence of any conflict of interests.

References

Aceto S, Montieri S, Sica M, Gaudio L (2007) Molecular evolution of the OrcPI locus in natural populations of Mediterranean orchids. Gene 392(1–2):299–305. doi:10.1016/j.gene.2007.01.005

Cantone C, Gaudio L, Aceto S (2011) The GLO-like locus in orchids: duplication and purifying selection at synonymous sites within Orchidinae (Orchidaceae). Gene 481:48–55. doi:10.1016/j.gene.2011.04.004

Cantone C, Sica M, Gaudio L, Aceto S (2009) The OrcPI locus: Genomic organization, expression pattern, and noncoding regions variability in *Orchis italica* (Orchidaceae) and related species. Gene 434(1–2):9–15. doi:10.1016/j.gene.2008.12.015

Gorniak M, Paun O, Chase MW (2010) Phylogenetic relationships within Orchidaceae based on a low-copy nuclear coding gene. Congruence with organellar and nuclear ribosomal DNA results. Mol Phylogenet Evol, Xdh. doi:10.1016/j.ympev.2010.03.003

Gustafsson AL, Verola CF, Antonelli A (2010) Reassessing the temporal evolution of orchids with new fossils and a Bayesian relaxed clock, with implications for the diversification of the rare South American genus Hoffmannseggella (Orchidaceae: Epidendroideae). BMC Evol Biol 10:177. doi:10.1186/1471-2148-10-177

Jack T (2001) Relearning our ABCs: new twists on an old model. Trends Plant Sci 6(7):310–316

Kanno A, Nakada M, Akita Y, Hirai M (2007) Class B gene expression and the modified ABC model in nongrass monocots. ScientificWorldJournal 7:268–279

Kim S-Y, Yun P-Y, Fukuda T, Ochiai T, Yokoyama J, Kameya T, Kanno A (2007) Expression of a DEFICIENS-like gene correlates with the differentiation between sepal and petal in the orchid, Habenaria radiata (Orchidaceae). Plant Sci 172(2):319–326

Mondragon-Palomino M, Hiese L, Harter A, Koch MA, Theissen G (2009) Positive selection and ancient duplications in the evolution of class B floral homeotic genes of orchids and grasses. BMC Evol Biol 9:81

Mondragon-Palomino M, Theissen G (2011) Conserved differential expression of paralogous DEFICIENS- and GLOBOSA-like MADS-box genes in the flowers of Orchidaceae: refining the 'orchid code'. Plant J. doi:10.1111/j.1365-313X.2011.04560.x

Schefe JH, Lehmann KE, Buschmann IR, Unger T, Funke-Kaiser H (2006) Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's CT difference" formula. J Mol Med 84(11):901–910. doi:10.1007/s00109-006-0097-6

Theissen G (2001) Development of floral organ identity: stories from the MADS house. Curr Opin Plant Biol 4(1):75–85

Tsai WC, Lee PF, Chen HI, Hsiao YY, Wei WJ, Pan ZJ, Chuang MH, Kuoh CS, Chen WH, Chen HH (2005) PeMADS6, a GLOBOSA/PISTILLATA-like gene in *Phalaenopsis equestris* involved in petaloid formation, and correlated with flower longevity and ovary development. Plant Cell Physiol 46(7):1125–1139

Xu Y, Teo LL, Zhou J, Kumar PP, Yu H (2006) Floral organ identity genes in the orchid Dendrobium crumenatum. Plant J 46(1):54–68

Zhao S, Fernald RD (2005) Comprehensive algorithm for quantitative real-time polymerase chain reaction. J Comput Biol 12(8):1047–1064. doi:10.1089/cmb.2005.12.1047