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Some characteristics of genetic control of *Fagopyrum esculentum* flower development

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Summary: In this work we present a study of the genetic control of flower development in common buckwheat (*Fagopyrum esculentum*) based on the expression analysis of *FesAG, FesLFY* and *FesAP1* – genes orthologous to those controlling flower development in *Arabidopsis thaliana* – in wild type buckwheat and in mutants *fagopyrum apetala, tepal-like bract, green perianth*. In the *fagopyrum apetala* mutant, characterised by the conversion of tepals into carpelloid organs, the expression of *FesAG* is increased what is consistent with the current knowledge based on the studies of model plant species. In another mutant – *green perianth* – characterised by partial conversion of flower into inflorescence, we revealed an increased expression of *FesAP1*. The latter is an unexpected result contradicting current models based on the study of *Arabidopsis thaliana*. Our data suggest a significant difference between the genetic control of flower development in *Fagopyrum* and in *Arabidopsis*.

Keywords: Polygonaceae, *Fagopyrum esculentum*, flower development, developmental genetics, ABC model, orthologs, mutants

Current progress in the understanding of the genetic control of flower development is due mostly to the studies of the model plant species, *Arabidopsis thaliana* (L.) Heynh. However, it has been stressed that for a better understanding of plant morphological evolution new model systems representing different lineages of the angiosperms should be selected (Baum et al. 2002; Nutt et al. 2006). In this work we demonstrate the difference in the genetic control of flower development between *Fagopyrum esculentum* Moench. (common buckwheat) and *Arabidopsis*. This is inferred from the study of gene expression in buckwheat mutants with altered flower and inflorescence development and their morphological analysis. This difference is interesting for developmental and evolutionary framework and makes *Fagopyrum* a promising candidate for being one of these new model systems.

Fagopyrum esculentum possesses the advantages of a classical model species (short life cycle, high seed productivity) but it has some particular morphological features which are not present either in *A. thaliana* or in the other classical model plant species (for comparison of *Fagopyrum* and *Arabidopsis* see Figure 1 a–c). Buckwheat inflorescence has a more complex structure than the one of *Arabidopsis* – it is a raceme consisting of cymose partial inflorescences (Quinet et al. 2004, Fig. 1a). The bract of the first flower in this monochasium partially retains photosynthetic capacity whereas the bracts of flowers of higher orders are reduced to non-photosynthetic thin membranous sheath-like structures. Flowers consist of five tepals, eight stamens arranged in two whorls and three fused carpels: unlike most eudicots, in buckwheat the perianth is not differentiated into sepals and petals. Buckwheat is a member of the family Polygonaceae representing an isolated and insufficiently explored group within the order Caryophyllales, the so called 'non-core' Caryophyllales (Cuènoud et al. 2002). Recently phylogenetic analyses of complete chloroplast genomes provided support for the hypothesis that both, core and non-core Caryophyllales, are

sister to the group of orders called 'asterids' (Logacheva et al. 2008b). *A. thaliana*, the classical model species, belongs to a different group called 'rosids'. In the asterids there are several model species (*Antirrhinum majus, Solanum tuberosum, Petunia hybrida*). Although they are not as extensively explored as *Arabidopsis* the information acquired from the studies on these species can provide a framework for comparative analysis.

Materials and methods

Plant material and morphological analysis: In this work we used *F. esculentum* plants: wild type and *fagopyrum apetala (fap), tepal-like bract (tlb)* and *green perianth (gp)* mutants from the collection of All-Russia Research Institute of Legumes and Groat Crops (Orel, Russia). Plants were grown in a greenhouse at 20–25°C and a relative humidity of 80% under long day (16 hours light / 8 hours dark) conditions. To assess number of floral organs 50 flowers from 5 plants of each genotype were analysed. Flower and inflorescence close-up views were obtained as described earlier (Logacheva et al. 2008a).

Analysis of gene expression: RNA was extracted from inflorescences of two developmental stages: 1) with floral buds no longer than 6 mm, 2) with one opened flower using RNeasy Plant Mini Kit (Qiagen, Germany). To avoid genomic DNA contamination, treatment with DNase using RNase-Free DNase Set (Qiagen, Germany) was performed. The quality of RNA was evaluated using agarose gel electrophoresis; quantification of RNA was performed with Nanodrop 1000 (Thermo Scientific, USA). Reverse transcription was carried out using cDNA Synthesis Kit (first strand) with 24T primer (Silex, Russia). Real-time RT-PCR reactions were run on thermocycler ANK-32 (Syntol, Russia) using the Eva Green master mix (Syntol, Russia) in a reaction volume of 50 µl. Thermal cycling conditions were 95°C for 5 minutes followed by 40 cycles of 95°C for 15 seconds and 62°C for 45 seconds. Each reaction was run in triplicate. For each reaction a melting curve analysis was performed; also all amplification products were run on agarose gel to make sure the absence of primer dimerisation and / or non-specific PCR products. Amplicifation efficiency was calculated using a cDNA dilution series and only those pairs of primers working with efficiency more than 1.95 were used in experiment. To evaluate the relative quantity of each PCR product we used comparative 2-ΔΔCT method (Livak & Schmittgen 2001). *ADENINE PHOSPHORIBOSYLTRANSFERASE 1* (*APT1*) gene was used as a reference for normalization. The sequences of primers used in this study are listed in Table 1.

Results and discussion

Carpel formation and termination of flower meristem: a case of separation of functions

Basic mechanisms of the genetic control of flower development are described by the so called 'ABC model' (Coen & Meyerowitz 1991). This model was inferred from the studies of homeotic

Table 1. Primers used for gene expression analysis.

Gene	Forward primer	Reverse primer
FesAG	GAATTGTCTGTTCTTTGTGATGCTGA	GATTGTTGTTGTGCAGTTCGATTTC
FesLFY	GCAACCGCCGCTACATCTCTCAAC	TGCGTCAATGTCCCAACCTT
FesAP1	TGAAGAAAGCACATGAAATTTCTGTTC	GGTTTCTCTCGAGAAGTTCAATCTT
FesAPT1	CCTCCTGTTGCTTTGGCCCTCG	CACTACTTCAACTCCAACACGCTCA

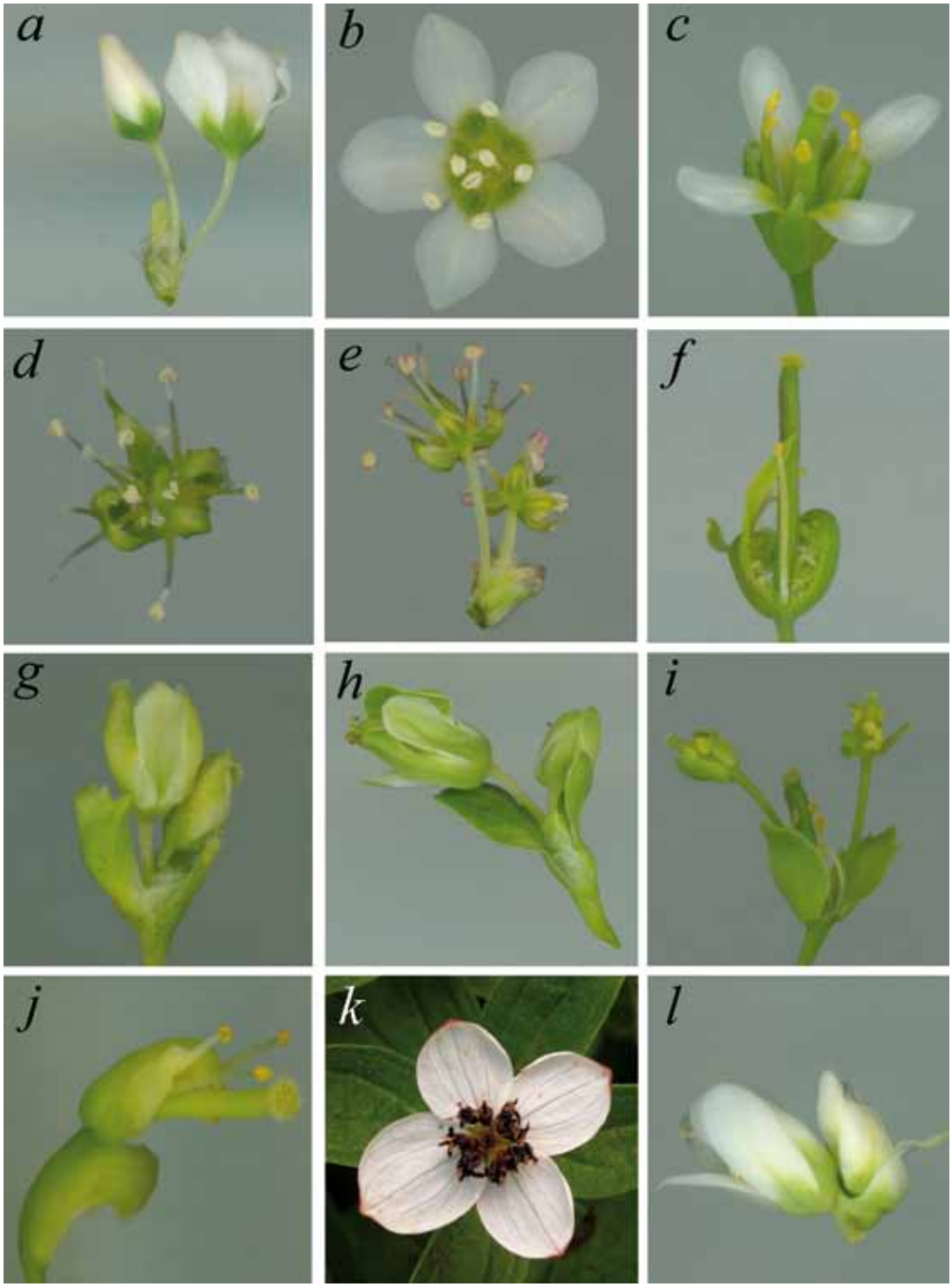

Figure 1. *Fagopyrum esculentum*, wild type: a) partial inflorescence, b) flower; c) *Arabidopsis thaliana*, wild type flower; *F. esculentum*, *fagopyrum apetala* mutant: d) flower, e) partial inflorescence; f) *A. thaliana, apetala2-14* mutant, flower; *F. esculentum*, green perianth mutant: g) flower (note the secondary flowers developed in the axils of leaf-like tepals), h) partial inflorescence; *A. thaliana* mutants: i) *apetala1-20*, j) *leafy-5*, k) *Cornus suecica* L., inflorescence, l) *F. esculentum* tepal-like bract mutant, flower.

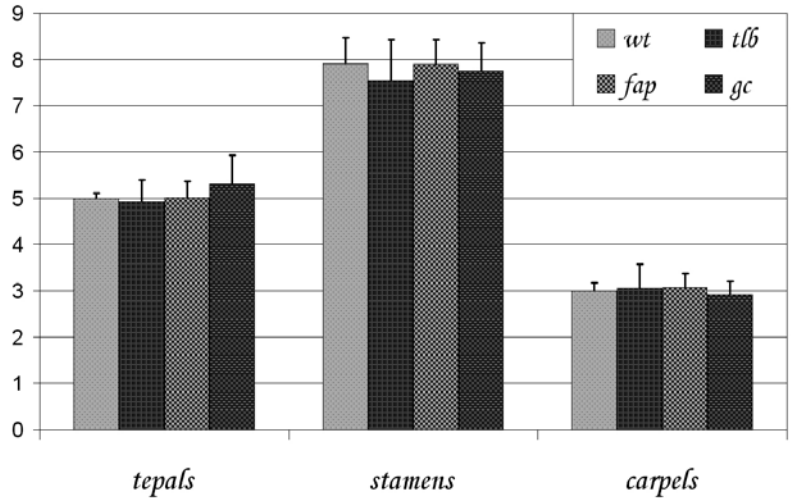

Figure 2. Number of organs in wild type, *fap*, *gp* and *tlb* flowers.

mutants of two model species – *Arabidopsis thaliana* (L.) Heynh. and *Antirrhinum majus* L. The flowers of these plants, as well as most other eudicots, consist of four types of organs: sepals, petals, stamens and carpels. Genes determining floral organ identity are referred to three classes: A – *APETALA1* (*AP1*), *APETALA2* (*AP2*), B – *APETALA3* (*AP3*), *PISTILLATA* (*PI*), and C – *AGAMOUS* (*AG*) (Haughn & Somerville 1988; Coen & Meyerowitz 1991; Weigel & Meyerowitz 1994). All these genes encode transcription factors. The expression of A class genes specifies the sepal identity; the expression of C class genes specifies the carpel identity. The combined action of A and B class genes specifies petals (second whorl); C and B class specifies stamens (third whorl) (Bowman et al. 1989; Coen & Meyerowitz 1991). Later this model was extended to include more genes: D class genes controlling ovule development (Colombo et al. 1995; Angenent et al. 1995) and E class genes that are required for proper development of petals, stamens and carpels (Pelaz et al. 2000).

One of the most important points to understand the genetic basis of flower diversity is the mechanism which is responsible for the development of reproductive organs and the termination of floral meristem. The studies on *Arabidopsis* evidence that at least in this plant these processes are interconnected. The key regulator of both of these processes is the gene *AG* (in terms of the ABC model, the C class gene). It regulates carpel and stamen development; but it also controls floral meristem termination by repressing expression of the gene *WUSCHEL* (*WUS*) which is required for the maintenance of stem cells activity (Lenhard et al. 2001; Mizukami & Ma 1997; Sun et al. 2009).

The study of *fap* mutant allows analysing the association of these two functions in buckwheat. This mutant is characterised by the carpelloidy of the perianth (Fig. 1 d, e). While in wild type plants tepals are petaloid, in mutants they are similar to carpels in colour, shape and structure. The average number of these carpelloid tepals is the same as normal tepals in wild type and equals to five (Fig. 2) (Logacheva et al. 2008a). Similar phenotypes are characteristic for *A. thaliana* mutants in genes which negatively regulate *AG*, e.g. *AP2* (Kunst et al. 1989; Fig. 1 f), and for transgenic plants which ectopically express *AG* or its orthologs from other species (Mizukami &

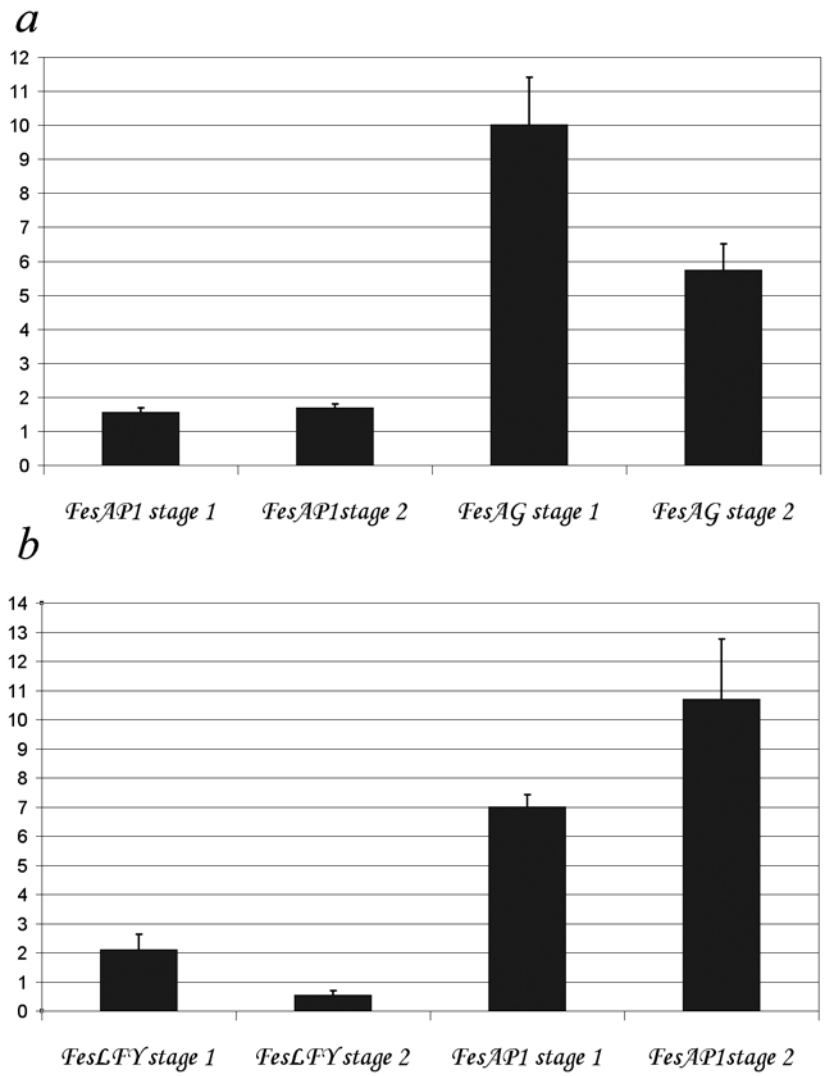

Figure 3. Comparison of expression level in developing flowers and inflorescences of wild type *Fagopyrum esculentum* and mutants: a) *fagopyrum apetala*, b) *green corolla*. Level of gene expression in wild type is taken as a unit.

Ma 1992; Tsuchimoto et al. 1993; Rigola et al. 2001). In all these cases the carpelloidy is mediated by the ectopic expression of *AG* or its functional equivalent. Thus, it was suggested that the molecular basis of *fap* phenotype is the ectopic expression of the *AG* ortholog. Quantitative analysis of the expression of *FesAG* supports this hypothesis – in *fap* its expression is four-fold increased (Fig. 3 a). However, in *Arabidopsis* carpelloidy is always correlated with the reduction of floral organ number that is mediated by the second function of *AG* – the repression of stem cell activity. This can be illustrated by the mutants in *AP2* gene. This phenotype is usually interpreted as a result of homeotic transformation of sepals into carpels and petals into stamens (Bowman et al. 1991). But in strong alleles (*ap2-9*, *ap2-7*, *ap2-14*) the perianth (consisting of four sepals and four petals in wild type) is represented only by 2–4 carpelloid organs (Kunst et al. 1989; Fig. 1 f). Sometimes it is completely absent and the flower consists of two carpels and few stamens. In contrast, in *fap* the number of floral organs is not reduced. Thus, we suggest that C-class genes in

Fagopyrum (in contrast to C-class genes in *Arabidopsis*) do not perform a function of repressing the stem cell activity.

The repression of *WUS* expression by C class genes appeared to be a general mechanism for all angiosperms and was hypothesized to play a major role in the evolutionary origin of flower, conferring determinacy to a shoot bearing microsporophylls and megasporophylls (Baum & Hileman 2006). Recently it was reported that expression of the *AG* ortholog in *Impatiens* is not sufficient to specify meristem determinacy to flower (Ordidge et al. 2005). After more detailed studies of flower development and gene expression patterns the authors suggested that in *Impatiens* the function of the repression of stem cell activity is probably transferred from genes controlling carpel development (i.e. *AG* ortholog) to the genes controlling ovule development (i.e. D class genes). They associate this difference in the genetic control with a difference that exists in placentation type and ovule position between *Impatiens* and *Arabidopsi*s and stresses full understanding of gynoecium development. Therefore a range of plants representing different placentation types should be studied (Chiurugwi et al. 2007). Buckwheat represents a group with a very unusual position of ovules – there is a single ovule that occupies basal position in the cavity of the ovary (Laubengayer 1937). Thus, the supplement of existing data on the genetic control of floral meristem termination with those from buckwheat can greatly expand our knowledge on the genetic basis of flower diversity.

Genetic control of uniseriate perianth: B or not B

In contrast to most of the other core eudicots, having a perianth differentiated into sepals and tepals *Fagopyrum* species have a uniseriate perianth. In *F. esculentum* as well as in most other species of the genus tepals are petal-like, white or pinkish. In the species having sepal-like tepals (e.g. *F. tataricum*) tepal surface micromorphology does not differ from those with petal-like perianth (Hong et al. 2001). This suggests that the genetic control of perianth organ identity is similar in different species of the genus.

As mentioned above, according to the ABC model, sepal identity is caused by the expression of A class genes; and its combination with B class gene expression confers petal identity to floral organs. Further studies on model and non-model plant species, including analyses of gene expression patterns and transgenic plants, allowed to expand and supplement the ABC model and made it applicable to a wider range of species (for review see Krizek & Fletcher 2005). The genes with high sequence similarity to *Arabidopsis* A, B and C class genes and with ability to complement *Arabidopsis* mutations in corresponding genes are found in many angiosperm species which may indicate a generality of the mechanisms responsible for floral organ identity. However, by date there is a lot of evidence that plant development does not always follow the general rules of the ABC model. The mechanisms controlling perianth development are found to be especially diverse. In many species, primarily in monocots, perianth is not differentiated into sepals and petals as in *Arabidopsis*, but consists of identical organs called tepals. They can be either petal-like or sepal-like. To explain this morphology, the so called 'modified ABC model' was formulated. It states that petaloidy of tepals in monocots is mediated by the expansion of B class genes expression (Van Tunen et al. 1993). This suggestion is corroborated by the experimental studies on many monocot species. For example, in tulips (*Tulipa* sp.), plants having flowers with two identical whorls of petal like tepals, expression of B class genes is found in both whorls (Kanno et al. 2003). The same was shown for another monocot species with petaloid perianth: *Crocus*

sativus (Kalivas et al. 2007) and *Agapanthus praecox* (Nakamura et al. 2005). In species having a perianth differentiated into petals and sepals, like Commelinaceae, the expression of B class genes was found only in the second whorl of organs (Ochiai et al. 2004) what is also congruent with the modified ABC model. But this congruence is not characteristic for all studied monocots. In *Asparagus officinalis* B class genes are expressed only in the second perianth whorl besides the morphological similarity of first and second whorls (both of them are petaloid) (Park et al. 2003; Park et al. 2004). Then the genetic control of floral organ identity differs even within monocots. The studies on expression patterns of genes orthologous to ABC genes in basal angiosperms have revealed more complex patterns. For example, in *Persea* (Lauraceae) and *Illicium* (Illiciaceae) perianth besides A and B class genes (that is not unexpected for petaloid tepals found in these species) C class gene orthologs are also expressed. B class genes expression domain is also often expanded in basal angiosperms and includes carpels and sometimes even leaves (Kim et al. 2005; Chanderbali et al. 2009). It was suggested that the variability of gene expression profiles in petals (or petaloid tepals) might reflect the complexity of their evolutionary origin – they are thought to be derived either from bracts (bracteopetals) or from stamens (andropetals) (Kramer & Irish 2000; Kramer & Jaramillo 2005).

The study of buckwheat mutants allows corroborating the suggestion on the genetic control of perianth organ identity in this species. As mentioned above, *fap* mutants have carpelloid perianth and, at the same time, it overexpresses the *AG* ortholog. In case if B class genes were expressed in perianth, it would be staminoid, not carpelloid.

Another mutant with alterations in perianth development – *gp* – is characterised by the phenotype similar to *ap1* and *lfy Arabidopsis* mutants (Fig. 1 g–j). *LFY* and *AP1* encode transcription factors that control floral meristem identity and transition to flowering (Weigel et al. 1992; Weigel & Meyerowitz 1993). *AP1* also takes part in the determination of sepal and petal identity (Irish & Sussex 1990). The mutation in any of these genes leads to a partial or complete loss of perianth organ identity and conversion of flower into inflorescence or vegetative shoot. Similarly tepals are leaf-like in *gp* flowers and sometimes bear secondary shoots in their axils what is typical for the inflorescence (Fig. 1 g). The number of floral organs is not changed (Fig. 2). So we suggest that this phenotype is mediated by the decrease of *AP1* and / or *LFY* orthologs expression. However, the analysis of gene expression in wild type and *gp* mutant does not reveal any alteration in *FesLFY* expression and – more strikingly – reveals strong increase of *FesAP1* (Fig. 3 b). Thus, the phenotype in buckwheat beeing similar to a loss-of-function phenotype in *Arabidopsis* is correlated with the increase of gene expression. These data are congruent with the analysis of transgenic buckwheat plants expressing an ortholog of *AP1* from rice. These plants, when overexpressing this gene, showed delayed flowering and increased branching. In case of overexpression of this gene in antisense orientation (leading to the inactivation of homologous genes) plants were smaller in size and branching was repressed (Kojima et al. 2000). This was an unexpected result because the overexpression of *AP1* or its orthologs in *Arabidopsis* results in the opposite phenotypes: when expressed in the sense orientation, *AP1* accelerates flowering and represses branching (Mandel & Yanofsky 1995). In combination with our results on the increase of *FesAP1* expression in *gp* mutant this supports the hypothesis that in buckwheat *AP1* ortholog takes part in the development of flower and inflorescence but functions in a different way than *Arabidopsis AP1*. This may evidence the different origin of perianth in *Arabidopsis* and in Polygonaceae.

Homeotic transformation as a possible mechanism of formation of novel morphological traits

The structures of flowers and inflorescences in buckwheat differ from those in classical model species, and make it possible to study the genetic control of characters which are absent in these species. One of the morphological novelties which have to be explained is the petaloidy of floral and non-floral organs such as the petaloid bracts in some species of dogwoods (*Cornus*) (Fig. 1 k) or enlarged showy calyx-lobes (calycophylls) in *Mussaenda* (Rubiaceae). According to the ABC model, petal identity requires the function of A and B class floral homeotic genes. In transgenic plants of *Arabidopsis thaliana* which ectopically expressed B class genes in the first whorl of floral organs these organs were petaloid (Krizek & Meyerowitz 1996). Ectopical expression of both A and B class genes caused the transformation of leaves into petals (Pelaz et al. 2001). Presumably, similar mechanism is responsible for the development of petaloid bracts. This is supported by the fact that the expression of the homologs of A and B class genes has been found in petaloid bracts of some species of *Cornus* (Maturen et al. 2005). Among the buckwheat mutants we studied there is one which also has petaloid bracts. It is called *tepal-like bract* (*tlb*) (Fesenko et al. 2005; Logacheva et al. 2008a); the only difference between *tlb* and wild type (on the level of flower and inflorescence structure) is the petaloidy of bracts (Fig. 1 l) The phenotype of *tlb* mutant evidences that the mutation in a single locus can result in a transition to petaloidy. However, as inferred from the analysis of *fap* mutant, the B class genes are not likely to play role in determination of tepal identity in buckwheat. Our preliminary data on the expression of B class genes orthologs also indicate that *tlb* phenotype is not correlated with the increase of B class genes activity. Thus, the molecular basis of petaloidy of bracts in *tlb* is an other than the expansion of B class genes. This suggests that the development of homologous structures does not necessarily involve homologous genes. Comparative study of expression patterns of homologous genes in different species is one of prevailing approaches in plant evo-devo (Soltis et al. 2002). Our results indicate that the power of this approach may be limited and that it should be complemented with the genetic studies including mutant analysis.

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