Mutants of inflorescence development in alfalfa (Medicago sativa L.)

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Alfalfa (Medicago sativa L., Medicago varia Mart., Medicago falcata L.) is a perennial leguminous plant well-known as the queen of forages cultivated all over the world. The general biology and morphology of the plant has been described in detail. The typical inflorescence of the plant is raceme. Due to the multistep inbreeding process in this cross-pollinated species, different mutant forms have been found in inbred progenies. They include long racemes, panicle-like racemes (with fertile and sterile flowers), complicated branched racemes, and fasciated inflorescences. The fasciation trait was discovered first in long racemes and then it was introduced into every mutant inflorescence type by hand pollination. By means of pair hybridization, transitional forms of some mutants were isolated and the new mutant forms combined two or three mutant genes. New gene names are proposed for new duplex and triplex mutant types: lpfas, pi1lpfas, brilpfas. Medicago truncatula is a conventional model species for legume genome research. M. truncatula and alfalfa share highly conserved nucleotide sequences and exhibit nearly perfect synteny between the two genomes. The knowledge about inflorescence development in model M. truncatula plants adds to understanding the genetic nature of mutant inflorescence development in alfalfa; therefore, we compiled the information on the genetic regulation of inflorescence development in M. truncatula. The M. truncatula mutant mtpim has a complicated inflorescence structure resembling panicle-like inflorescence in alfalfa. Presently, it is known that the inflorescence architecture in M. truncatula is controlled by spatiotemporal expression of MtFULc, MtFULc, MtAP1, and SGL1 through reciprocal repression. Some mutants isolated in M. truncatula resemble alfalfa mutants in phenotype. The mutant generated by retrotransposon insertion mutagenesis and named sgl1-1 has a cauliflower-like phenotype looking just like the cauliflower mutant in alfalfa. New data concerning genes regulating inflorescence development in model legumes approach us to understanding the phenomenon of inflorescence mutations in alfalfa. The information of inflorescence mutants in nonmodel crops may augment our knowledge of plant development and help crop improvement.

Key words: Medicago sativa L.; alfalfa; mutants; inflorescences; plant development.

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Мутанты развития соцветий у люцерны (Medicago sativa L.)

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Люцерна (Medicago sativa L., Medicago varia Mart., Medicago falcata (L.)) – многолетнее бобовое растение, известное как «королева кормов» и культивируемое на всем земном шаре. Общая биология и морфология растения описаны в деталях, типичное соцветие люцерны – открытая брахтеозная кисть. В процессе многоступенчатого самоопыления в инбредных поколениях люцерны определены мутантные по строению соцветий формы. Среди них удлиненные, метелковидные (фертильные и стерильные), ветвиство сложного строения, а также соцветия с фасцированным цветеносом. Признак фасциации соцветия выявлен у люцерны среди удлиненных соцветий и далее был введен в каждый мутантный тип соцветия путем скрещивания вручную. Посредством парных скрещиваний созданы переходные гибридные мутантные формы, сочетающие два или три мутантных признака. Новые двойные и тройные мутанты приобрели названия: lpfas, pi1lpfas, brilpfas. Medicago truncatula – классический модельный объект проведения геномных исследований у бобовых. M. truncatula и люцерна посеновая проявляют консервативную нуклеотидную последовательность и обладают высокой степенью синтеза генов. Знание о регуляции развития соцветий у модельного растения M. truncatula полезно для понимания генетической природы мутаций у люцерны, в связи с чем был проведен анализ информации о генетике развития M. truncatula. К настоящему времени известно, что архитектура соцветия у M. truncatula находится под контролем пространственно-временной экспрессии генов MtFULc, MtFULc, MtAP1 и SGL1 посредством обратного подавления. Некоторые мутанты, выделенные у M. truncatula, имеют фенотип, схожий с фенотипом мутантов люцерны. Мутант mtpim M. truncatula обладает сложными соцветиями, напоминающими метелковидные соцветия у мутанта люцерны посевной. Мутант, полученный путем мутагенеза через инсерцию ретротрансзонами, под названием sgl1-1, имел фенотип типа «цветной капусты», присущий мутанту с аналогичным на-
Introduction

In most traditional botanical terms inflorescence is a flowering shoot. Different theories of inflorescence classifications exist, especially in legume. In the present paper for characterization of the mutant inflorescences in alfalfa the terms of the axe of the first order, the axes of the second and higher orders will be used for convenience. Typical inflorescence of alfalfa is an open bracteous compound raceme. Flowering in the wild type inflorescence of alfalfa starts acropetally. In general flowering in angiosperms starts from transition of shoot apical meristem (SAM) to flower apical meristem (FAM). Mutants of inflorescence development in alfalfa demonstrate the wide range of variability of positions of FAM development.

Inflorescence type is one of the main traits in plant taxonomy. The shapes of flowers and their organization into branching systems, called inflorescences, dictate much of plant diversity. Development mutant deviations are good example of possible confusing in species taxonomic attribution using herbarium specimen. For example, panicle-look inflorescence, the most famous spontaneous mutation in alfalfa, transforms the habitus of the plant radically. Teratological events in plants attracted attention of botanists for a long time (Fedorov, 1958), but the genetic nature of some morphological deviations still remains not quite clear.

Alfalfa is a tetraploid cross-pollinated plant, self-pollination in few progenies allows to reveal the hidden polymorphism and sometimes leads to spontaneous mutations. Blossoming and pods setting is the top of individual plant development, the success or failure in ontogenesis is crucial. Inflorescence bearing flowers is the main construction for reproductive mis

Materials and methods

All spontaneous mutants described below were got in VIR during large-scaled population screening in inbred progenies in the field conditions. Plants in individual standing were covered by isolators (one-half of the plant). Flowers under isolators were tripped artificially by hand and self- or cross-pollinated. Self-pollination and crossing were made without castration. Hybridization was made by pollination by pollen of desired parent under the isolators in field. Some material was grown and pollinated in 2017 in greenhouse without isolators in the lack of insects (Pushkin laboratories of VIR). No any chemical or radioactive mutagens were used. Field experiments were conducted in 1981–1992 at former VIR Aral Research Station (Kazakhstan) and in 2009–2011 at VIR Maykop Research Station (Adygeya Republic, Northern Caucasus).

Mutants description

Panicle-like mutants. Typical inflorescences of alfalfa – open bracteose raceme. Most famous spontaneous inflorescence mutation in alfalfa – panicle-like inflorescence, was discovered independently by several breeders (Dudle, Wilsie, 1956, 1957; Bayly, Craig, 1962; Murray, Craig, 1962; Pashenko, Rustamova, 1971; Mariani et al., 1976; Kinosita, Sugino, 1982; Dzyubenko N.I., Dzyubenko E.A., 1992). It is a compound inflorescence with fertile, semi-sterile and totally sterile flowers. On the axil of the first order instead of the FAM the second order axes are formed bearing flowers and pods (Fig. 1, a, Fig. 2, b–c).

This type of mutation was found in VIR in 1981 in self-pollinated progenies from crosses of the plants varieties Ellerslaier and Tibetskaya. The expression of the trait varied widely in the progenies. The mutant plants were divided into four groups depending upon the expression of the trait: a) plants with normal racemes and few panicle-like inflorescences; b) plants with few normal racemes, simple panicles and few large panicles with compound structure forming pods (fertile); c) plants with compound panicle inflorescences only, different violations of some flower structures may be observed, including actinomorphic petals, vestigial generative organs, pod setting decreased to some extent due to the presence of vestigial flowers (semi-fertile, semi-sterile); d) cauliflower-like inflorescences with rudimental flowers not forming pods (sterile).

Undifferentiated flower primordia stopped at their differentiation at the Va stadia of organogenesis (Kuperman, 1984). According to F.M. Kuperman (1984), inflorescence primordia and bracts are developing at the third stage of morphogenesis of higher plants, while differentiation of floral meristems occurs at the fourth stage of morphogenesis. Bracteas in cauliflower-like mutant are well-developed. The cauliflower phenotype appears as a mutation in different plant genera and possible has common genetic regulation.

The panicle-like inflorescence trait is governed by a single recessive gene pi-1 (for M. sativa) and pi-2 (for M. falcata) (Dzyubenko N.I., Dzyubenko E.A., 1992). Z. Bodzon (2016) named this mutation as br and remarked that it enhanced 6–10 times floret number per inflorescence. Sterile panicle-like inflorescence, cauliflower type, was supposed to be controlled...
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by one recessive gene in nulliplex position (Kinoshita, Sugi­nobu, 1982).

**Mutation “Branched raceme”**. Most striking mutation, never found in alfalfa before, characterized by partial replacement of racemes by shoot-looking structures, bearing flowers (and setting pods) on the main axil at the bottom part and forming additional axes of the second order (sometimes even third or fourth orders) bearing flowers in its turn (Fig. 1, b). Flowers in the upper part may have some abnormalities such as flowers fused together or actinomorphic flowers with polymeric gynoecium. Progeny of self-pollinated mutants with branched racemes divided into groups of plants with different phenotypes.

**Phenotype 1.** Dwarf plants up to 30 cm, non-­flowering and non-branching, with fragile shoots with short internodes, with dark green leaves.

**Phenotype 2.** Semi-dwarf plants 30–50 cm high with lonely almost sessile pale flowers, with fragile shoots and dark green leaves. Non-branching plants.

**Phenotype 3.** Plants of common size and dark green leaves and common inflorescences.

**Phenotype 4.** Plants with branched inflorescences. This inflorescence type does not fit any inflorescence type according any botanical classification, including last one suggested for legumes (Sinjushin, 2018). In the upper part of the raceme, the secondary axes are formed instead of flowers, some of them continue to produce axes of the third and higher orders, at the bottom part of the main axe normal flowers are set. Flowering starts acropetally by the bottom flowers at the axe of the first order and by the bottom flowers of the second order axes. Size and branching of axes of the second order demonstrated a large variability (Fig. 3). Mutation was named bri (Dzyubenko N.I., Dzyubenko E.A., 1992–1994, 1998, 2009, 2010). Branched inflorescences were characterized by high pollen and ovules fertility close to norm (Dzyubenko, 1990) and good seed production.

One can supposed that such kind of splitting in the self-­pollinated progenies – presence of branched inflorescences type plants (abundance of SAM and FAM activity) together with the presence of dwarf plants with shortened internodes and dark green leaves (lack of SAM and FAM activity), may be connected with some gene system acting through hormones regulation, as it is described in A. Dalmadi et al. (2008).

**Long inflorescence.** In the population of variety Vela k-42716 some plants of spontaneous mutants with long racemes were revealed. Plants with long racemes were self-­pollinated. After self-pollination in progenies the length of the racemes in plants varied from 18 to 28 cm, amount of flowers per raceme – 10–32 cm. Fallen (un-­pollinated) flowers and buds consisted up to 75 % from initial amount of flowers in some racemes, meanwhile in some racemes pod setting was good, promising for increasing the seed yield in alfalfa (Dzyubenko N.I., Dzyubenko E.A., 1991). Fertility of the pollen was high in most plants with long racemes, fertility of ovules was also close to norm (Dzyubenko, 1990).
Another source of long racemes in alfalfa – so called maloasian alfalfa from Turkey. VIR alfalfa collection was estimated by this trait and promising germplasm with high seed production was isolated (Dzyubenko N.I., Dzyubenko E.A., 1991) (Fig. 4). Other breeders also paid attention to long racemes in alfalfa as a potential for increasing seed yield (Staszewski, 1986; Bodzon, 1998). The \( lp \) trait is controlled probably by one recessive gene inherited in tetrasomic way (Dzyubenko N.I., Dzyubenko E.A., 1991; Bodzon, 1998).

**Fasciation mutants’ development in alfalfa. Broadening phenotypic diversity.** Initially long inflorescences with fasciation were found in the self-pollinated progenies of the alfalfa accessions from Asia Minor from VIR collection. By means of deep inbreeding up to the fourth generations and subsequent selection the plants with wide fasciation at the top of inflorescence were obtained. Fasciation of the peduncle did not affect seriously the structure of the flowers. Nevertheless, up to one-half or the flowers dropped without pod setting. The dropped flowers were analyzed, they represented buds of different age. The amount of dropped buds increased with the extent of fasciation of peduncle. Expression of the trait “fasciated inflorescence” varied, the extreme manifestation was observed as 100% fasciated inflorescences at the plant with up to 7 cm width of peduncles. All buds of the plant dropped at the stage 1–2 mm. Fasciated peduncle may split at the top into some sectors. The expression of the trait in self-pollinated progenies of the mutant plants varies from slight fasciation at the top of inflorescences to totally sterile inflorescences with flat peduncle. The trait “\( fis \)” was easily transferred to other inflorescence mutant forms (Fig. 5), using hand pollination.

In panicle-like mutants, especially in fertile forms, the flat tending of the inflorescence peduncle does not lead to flowers fertility reduction. In branched inflorescences monstrous inflorescences with fasciated peduncles were obtained, semi-fertile,
Discussion

A main factor that shapes inflorescence architecture is the identity of the meristems produced in the inflorescence apex, what determines the relative position where flowers are formed. In Arabidopsis, upon floral transition, the vegetative meristem transforms into inflorescence meristem, which produces floral meristems in its turn. The development of the Arabidopsis inflorescence can be mostly explained by the function and mutual regulation of three genes: TERMINAL FLOWER 1 (TFL1), LEAFY (LFY), and APETALA 1 (API) (Shannon, Meeks-Wagner, 1993; Blazquez et al., 2006). These three genes act as opposing forces maintaining the balance between inflorescence and floral meristem identity at the inflorescence apex (Blazquez et al., 2006). By other definition, at least four genes are necessary for the specification of floral meristem identity in Arabidopsis: LEAFY (LFY), CAULIFLOWER (CAL), APETALA1 (API), and FRUITFULL (FUL) (Weigel et al., 1992; Kempin et al., 1995).

Arabidopsis FAM forms simple racemes, not compound, so the reason for looking the appropriate models for responsible gene net in more relative model legumes plant is evident. Demonstrated macro- and microsynteny between the genomes of the model legume Medicago truncatula and other related species like diploid and tetraploid Medicago sativa (Choi et al., 2004) and pea (Pisum sativum) (Kalo et al., 2004) makes these plants easy targets to reveal gene functions. In Pisum sativum UNI, BROC, and PIM genes all play roles in assigning floral meristem identity to the third-order branch. PIM mutants continue to produce inflorescence branches, resulting in a highly complex architecture and aberrant flowers, uni mutants initiate a whorl of sepals, but floral organogenesis is aberrant beyond that developmental point, and the double mutant uni pim lacks identifiable floral organs. A wild-type phenotype is observed in broc plants, but broc enhances the pin phenotype in the double mutant, producing inflorescences that resemble broccoli. Collectively these genes ensure that only the third-order meristem, not higher- or lower-order meristems, generates floral organs, thus precisely regulating the overall architecture of the plant (Singer, 1999).

Different reverse genetic and genomic tools providing to establish the function of candidate genes, responsible for architectural traits are available in several model and non-model legume species. Medicago truncatula is a classic model species for legumes. Through various international and national genomic initiatives sufficient amount of Medicago truncatula phenotypic

some flowers had polymeric gynoecium, nevertheless most of the plants set the pods. In pea (Sinjushin, 2016) fasciation of the peduncle did not affected the viability of flowers in most cases, too. As a rule, fasciation affects most of SAM and FAM of the plant simultaneously, leading to the peduncles and stem flattening to the different extent. New combinations of mutant inflorescence types with fasciation and their designation are given in the Table 1.

Proliferation

Loss of FAM meristem identity in inflorescences leads to such phenomenon as proliferation with developing of vegetative shoot as prolongation of the inflorescence stalk or the axis of the second order, such cases were revealed in panicle inflorescences and long racemes (Fig. 6).

Mutants defective in their floral meristem identity (FAM) are possible to produce leaves after their transition to reproductive development, so some mechanisms cause “reprograming” of FAM during this transition. Mutants defective in LEAFY/FLORICAULA (LFY/FLO) are available in various angiosperms, including tomato, pea, maize, snapdragon and Arabidopsis, and all show severe defects in flower develop-
mation. For instance, in snapdragon flo mutant flowers are replaced by shoots (Coen et al., 1990).

Table 1. Plan of crosses between plants with different mutant inflorescences and generated double and triple mutant inflorescences in alfalfa

| Crossing variant                        | Raceme with elongated peduncle lp | Raceme with fasciated peduncle fas | Raceme with elongated peduncle lpfas |
|----------------------------------------|----------------------------------|----------------------------------|------------------------------------|
| Raceme with elongated peduncle lp      | lpfas                            |                                  | lpfas                              |
| Panicle-like inflorescence (fertile) pi1| pi1fas                           | pi1lp                            | pi1lpfas                           |
| Cauliflower panicle-like inflorescence (sterile) pi1| pi1fas | pi1lp | pi1lpfas |
| Branched inflorescence bri             | bri                              | bri_lp                          | bri_lpfas                         |

Fig. 6. Proliferation.
a – in cauliflower inflorescence; b – in long inflorescence.
mutants were arisen using methods of forward and reverse genetics. Most used strategies for mutants generation were Tnt1 mutagenesis, TILLING and activation tagging. Both forward and reverse genetics screenings enabled to isolate interesting morphological mutants (extreme dwarf with dark green leaves, mutant with inflorescence and floral organ defects, unifoliate mutant with cauliflower-like inflorescences) (Tadége et al., 2008). Mutant populations generated by the retrotransposons Tnt1 in M. truncatula are routinely used now for identification of mutants of genes of interest through reverse genetics (Cheng et al., 2011). Then, the virus induced gene silencing (VIGS) methods are available in several legume species including M. truncatula (Grönlund et al., 2008).

In M. truncatula the leaf development mutants with four alleles from a M. truncatula mutant collection generated by tobacco Tnt1 retrotransposon insertion mutagenesis were isolated (Tadége et al., 2008). The mutants were named sgl1-1 (single leaves) to sgl1-4, because all adult leaves were simple in these mutants, resembling the first leaf (juvenile leaf) developed in the wild-type plants. Flowers developed in sgl1 mutants were abnormal and infertile, lacking petals and stamina and producing numerous flowers with cauliflower-like morphology. Because of their infertility, the sgl1 mutants were maintained as heterozygotes. Progenies from self-pollination of heterozygous lines segregated wild-type-like and mutant plants in a 3:1 ratio, suggesting that the mutant phenotype was linked to a single recessive locus (Wang et al., 2008).

In pea development of inflorescences and flowers is under the control of few genes. PIM (Proliferating Inflorescence Meristem) was validated by A. Berbel et al. (2001), its homolog in M. truncatula was named mtPIM (Benloch et al., 2006). Corresponding UNI in pea (Hofer et al., 1997), M. truncatula homolog gene is sgl1 (Wang et al., 2008). Homolog to Arabidopsis clve gene LF in pea is If (Foucher et al., 2003), homolog in M. truncatula is unknown. Function of the gene vegetation1 in pea (Berbel et al., 2012) is required for compound inflorescence development. Mutant veg1 forms vegetative shoots instead of inflorescences. A. Berbel et al. (2012) found that genetic network controlling the legume compound inflorescence is distinct from that in grasses and Solanaceae.

Results of expression patterns analyses of TFL1, FUL1, AP1 and SGI in M. truncatula indicated that they play specific role in identity determination of primary inflorescence meristem, secondary inflorescence meristems, floral meristems and common primordia, respectively (Cheng et al., 2018). In M. truncatula mutants ap1 and ap1 sgl1 manifested proliferating inflorescences, double mutant mtap1 sgl1 completely lost floral identity, resembling cauliflower phenotype (Cheng et al., 2018). The conclusion was made that inflorescence architecture in M. truncatula is controlled by spatiotemporal expression of MtTFL1, MtFULC, MtAP1, and SGL1 through reciprocal repression (Cheng et al., 2018). The data about homolog genes and mutants inflorescences in model plants, resembling mutants of M. sativa described above, are given in Tables 2–4.

The most unclear situation in alfalfa mutants arises in case of lp and bri mutants in the lack of resembling mutations within the model plants. Because of the complex segregation pattern of the tetraploid inheritance in M. sativa, geneticists often study the diploid alfalfa species and subspecies belonging to the M. sativa complex, such as diploid M. sativa ancestor Medicago coerulescens and cultivated alfalfa described above. In the nearest future no doubt the researchers will be able to identify genes responsible for these mutations.

### Table 2. Genes influencing compound inflorescence formation in Arabidopsis (flower-inflorescence transition), and their homologs in P. sativum and M. truncatula

| Arabidopsis thaliana | Pisum sativum | Medicago truncatula |
|---------------------|---------------|---------------------|
| LEAFY (LFY)         | LF            |                      |
| (Weigel et al., 1992) | (Foucher et al., 2003) |                      |
| UNIFOLIATA (UNI)    | Sgl           | MtPIM               |
| (Hofer et al., 1997)| (Wang et al., 2008) | (Benloch et al., 2006) |
| APETALA (AP1)       | PROLIFERATING INFLORESCENCE MERISTEM (PIM/PEAM4) |                      |
| (‘Yanofsky, 1995)   | (Hofer et al., 1997; Berbel et al., 2001) |                      |
|                      | VEGETATIVE (VEGI) | (Berbel et al., 2012) |

### Table 3. “Cauliflower” phenotype mutants in Arabidopsis, Pea and Medicago

| Arabidopsis thaliana | Pisum sativum | Medicago truncatula | Medicago sativa |
|---------------------|---------------|---------------------|-----------------|
| ap1 cal             | unipim        | mtap1 sgl1          | aaaa            |
| (Kempin et al., 1995) | (Singer et al., 1999) | (Cheng et al., 2018) | (Kinoshita, Suginobu, 1982) |

### Table 4. Genes families regulating fasciation in Arabidopsis and Pea

| Arabidopsis thaliana | Pisum sativum |
|---------------------|---------------|
| CLAVATA (CLV) and FASCIATA (FAS) gene families | FASCIATA (FA) and FA gene families |
| (Williams, Fletcher, 2005) | (Sinjushin, Gostimskii, 2007) |

### Conclusion

Summarizing achievements in developmental genetics of inflorescence development in model plants relative to M. sativa, we approach to understanding of possible genetic network, regulating the mutant inflorescence deviations in cultivated alfalfa described above. In the nearest future no doubt the researchers will be able to identify genes responsible for these mutations.
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