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On the Role of Genes *DETERMINATE, LATE FLOWERING* and *FASCIATA* in the Morphogenesis of Pea Inflorescence

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Summary: The effects of mutations *determinate* (*det*), *late flowering* (*lf*), *fasciata* (*fa, fas*), and *nodulation4* (*nod4*) on development of inflorescence in pea were characterized. All listed mutations distort functions of stem apical meristem and influence development of axillary flower-bearing axis (short paracladium) leading to formation of terminal flower and bracts. Structure of flower terminating short paracladium was analyzed and hypothesis on origin of its structure was proposed. Scheme of genetic network in control of inflorescence development in pea was designed.
Key words: bract, compound inflorescence, determinate growth, fasciation, *Pisum sativum* L., terminal flower

Introduction

Increase of productivity of agriculturally valuable plants is achievable through construction of new morphotypes with altered morphology of shoot, inflorescence and flower. In this connection, genetic control of higher plants development represents one of the most interesting problems in both theoretical and applied biology. Studies on the basic principles of this control are traditionally carried out using model objects, namely *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) and *Antirrhinum majus* L. (Scrophulariaceae). These objects however have some limitations: both species possess simple leaf and inflorescence thus complicating direct approximation of obtained results on significant part of plant taxa. Hence, wider spectrum of model species is needed. Garden pea (*Pisum sativum* L., Fabaceae) represents traditional and one of most convenient object in plant biology, the only model species bearing compound leaf and inflorescence (Hofer et al. 2001), nodulation capacity (Borisov et al. 2004) and peculiar flower symmetry, the latter evolving independently from snapdragon clade which also has zygomorphic (monosymmetric) flower (Wang et al. 2008). Given species has outstanding agricultural value and

any data on genetic control of its inflorescence architecture are of significant practical value, since new morphotypes may serve as initial material for breeding of new highly productive cultivars.

Legumes as a whole are characterized by compound inflorescences in which open first order axis is terminated with racemose floral unit; this pattern is repeated in second order axes, short (with limited growth) and long (open) (Weberling 1989). Only two legume species are known forming terminal flower on main axis: *Gleditsia triacanthos* L. and *Gymnocladus dioicus* (L.) K. Koch. (both Caesalpinioideae subfamily). Many legumes possess so-called truncated double inflorescence, in which main axis growth is not limited and flowers are born on short and long paracladia (Fig. 1A). Short paracladia may represent simple or globose (*Trifolium*) racemes, umbels (*Lotus*) or even be reduced until solitary flower (*Caragana, Lathyrus* p.p.). Normally pea produces short paracladia with two flowers with axis terminated with sterile residuum («stub» in terms of Singer et al. 1990), Fig. 1A, 2E).

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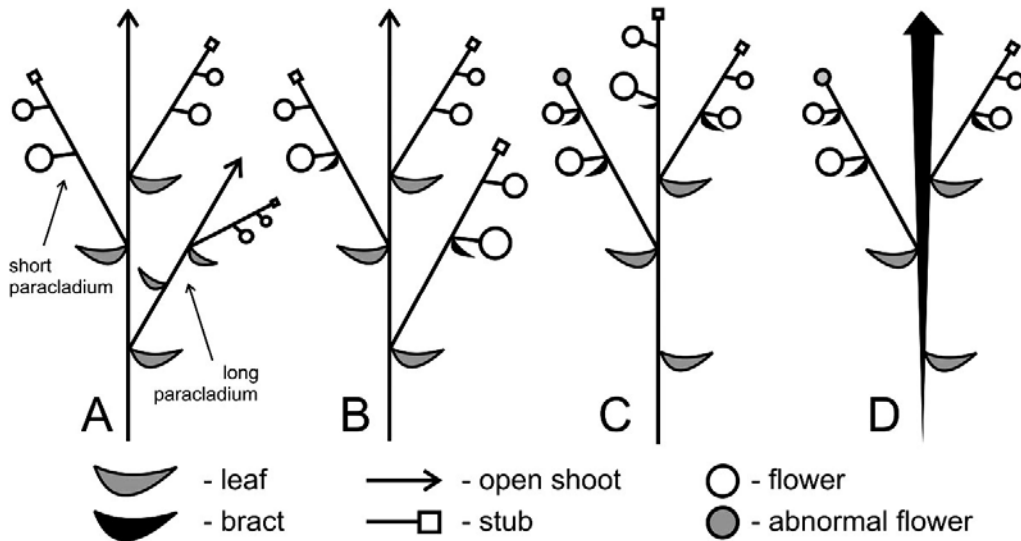e-mail: Sinjushin@pisumsativum.org, asinjushin@mail.ru

Figure 1. Schematic representations of pea inflorescences. A, wild type; B, *lf*; C, *det*; D, *fas*

To date, numerous pea mutants with altered inflorescence structure have been described (Singer et al. 1999, Weller 2007). The significant role in inflorescence ontogeny belongs to genes *DETERMINATE* (*DET*) and *LATE FLOWERING* (*LF*). In *det* mutants, stem apical meristem (SAM) precociously stops proliferation and forms flower-bearing axis identical to axillary ones (Fig. 1C). Mutant *lf* is characterized with earlier flowering as compared with wild-type plants via earlier formation of short paracladia (Fig. 1B). Genes of family *VEGETATIVE*, *VEG1* and *VEG2*, are antagonists of *LF*. Plants with *veg* genotype completely lack short paracladia or exhibit gradual transition between long and short paracladia (Weller 2007). Main axis is terminated with flower in double mutant *det veg1* (Singer et al. 1999) and *stp* (*stamina pistilloida* Taylor et al. 2001). Genes of *FASCIATA* (*FA*, *FA2*, *FAS*) family, *NOD4* and *SYM28* play important role providing SAM stability (Sinjushin & Gostimskii 2007). These genes limit SAM enlargement and mutants are characterized with more or less expressed stem fasciation and indeterminate growth pattern (Fig. 1D).

Forms with anomalous inflorescence features were used as initial material for pea breeding resulting in production of determinate (Determinantnyi VSKhI in Russia) and fasciated (Rosacrone in Germany, Shtambovyi 2 in USSR, Bulawa in Poland etc.) highly productive cultivars.

Genetic control of inflorescence morphogenesis has been studied in *Arabidopsis* most precisely. Undifferentiated state of SAM is supported by few genes, the most important of which is *TERMINAL FLOWER1* (*TFL1*). Mutant *tfl1* produces terminal flower on main axis (Shannon & Meeks-Wagner 1993). Pea genes *DET* and *LF* represent homologs of *TFL1* (Foucher et al. 2003). Maintaining the meristematic condition of SAM is conditioned by activity of gene *WUSCHEL* (*WUS*); negative regulation of *WUS* expression is provided by few gene groups, the most well-known of which are *CLAVATA* (*CLV*) and *FASCIATA* (*FAS*) (Williams & Fletcher 2005). It is problematic to state homology between listed genes in *Arabidopsis* and pea unambiguously. It had been demonstrated however that the orthologous genes (e.g., *CLAVATA*-like) are involved in regulation of processes of control of SAM activity and nodulation in legumes (Searle et al. 2003, Krussel et al. 2011). Combining functions of stem morphogenesis and nodulation control in one gene seems to be typical for legumes only, hence indicating difficulties in transferring data from one model object to numerous non-related taxa.

The given work is aimed at study of interaction and expression features of some genes engaged in inflorescence morphogenesis in pea.
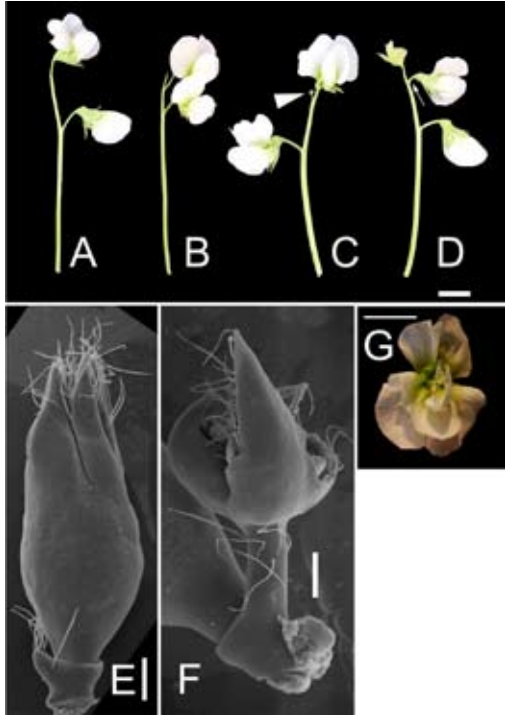
Figure 2. Variability of short paracladia structure in *fas* genotype (mutant "Shtambovyi"): short stub (A, E; trichomes are clearly seen in E), long stub (B), early senescing flower bud (C, arrowhead, and F, SEM microphotograph), well-developed terminal flower (D, G; arrow indicates bract in D). Scale bars: 1 cm (A-D, G), 300 μm (E, F)

Materials and Methods

The following lines of garden pea (*Pisum sativum* L.) were used as material for given work: fasciated lines JI2771 (*fas*), JI5 and JI2671 (*fa*) from John Innes Centre (Norwich, UK); fasciated mutant "Shtambovii" (*fas*) from the collection of Genetics Department of M.V. Lomonosov Moscow State University (Moscow, Russian Federation), line "Lupinoid" (*fa det*) (All-Russian Research Institute of Grain Legumes and Groats Crops, Orel, Russian Federation); fasciated and hypernodulating lines K301 and K507 (*nod4*) (Institute of Cytology and Genetics, Novosibirsk, Russian Federation), P64 (*sym28*) (All-Russian Research Institute of Agricultural Microbiology, Pushkin, Russian Federation). More detailed information on genotypes of lines is presented in work (Sinjushin & Gostimskii, 2007). Except listed lines, donors of mutations *det* (lines DTR and DTR(m) from All-Russian Research Institute of Breeding and Seed Production of Vegetables, Lesnoi Gorodok, Russian Federation, and Genetics Dept. of Moscow State University, respectively) and of "strict allele" *lf^a* (line WL102 from Institute of Genetics of Agricultural Plants, Sweden, bearing numerous morphological mutations) were used. Cultivar Nemchinovskii-766 (initial line for "Shtambovii" mutant) was chosen as wild-type control. Parental forms, hybrids and recombinants were planted in open field conditions on a territory of S.N. Skadovskii
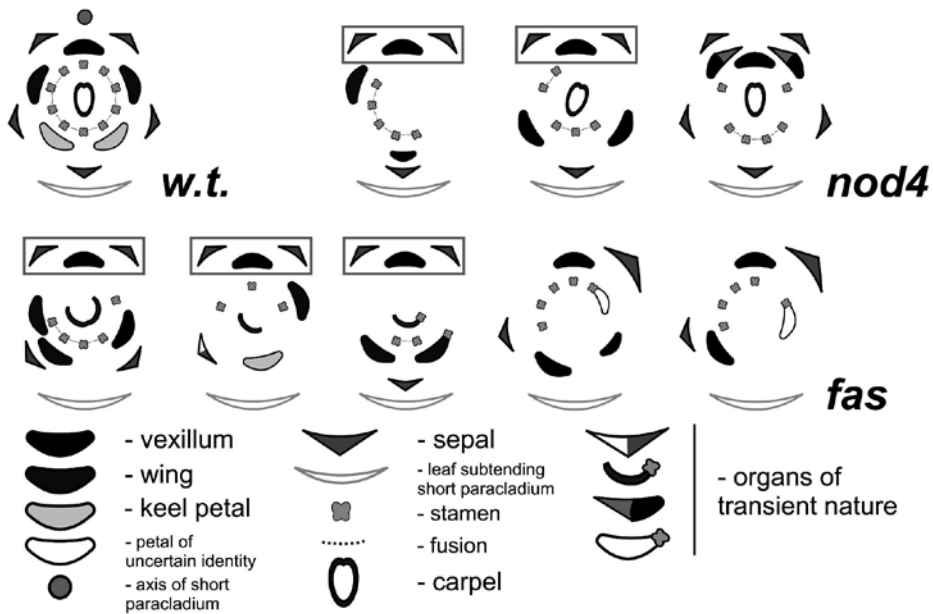

Figure 3. Diagrams of flowers terminating short paracladia in fasciated mutants in comparison with wild type (w.t.). Frame encircles the most stable positions (see text for details)
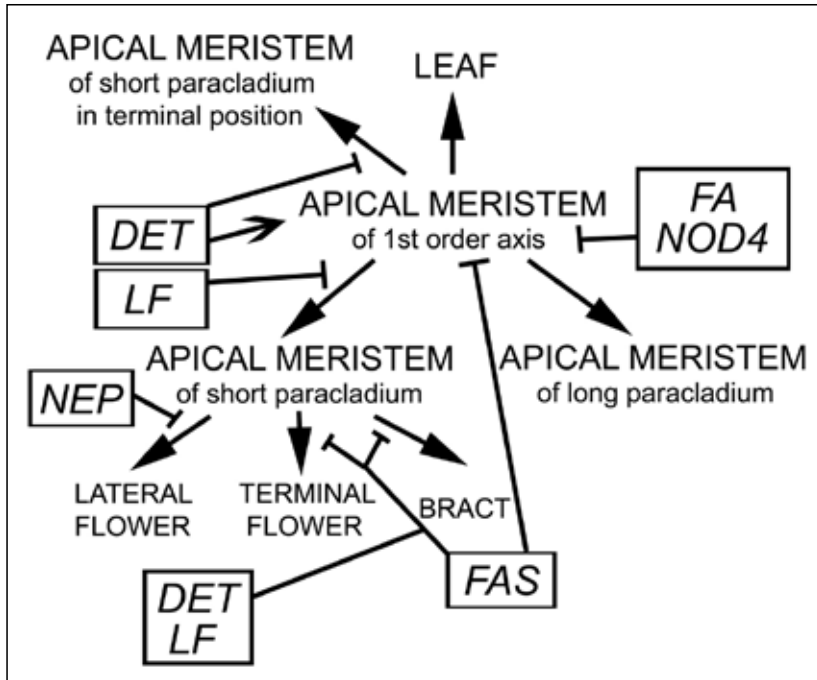
Figure 4. Scheme of relations between stem apical meristem and its derivatives together with genes controlling SAM activity. Genes *FA, FAS* and *NOD4* negatively regulate SAM size; *FAS*, *DET* and *LF* prevent formation of terminal flowers and bracts on short paracladia; *NEP* limits short paracladium proliferation; *DET* also maintains undifferentiated state of SAM. The apical meristem of long paracladium in general has the same developmental pattern as SAM of 1st order axis. →, positive regulation; ⊣, negative regulation.

Zvenigorod Biological Station (Western Moscow region, Russian Federation) during 2006-2010 summer seasons. Studies on flower structure were performed using method of diagrams (Eichler 1875). Preparation for SEM analysis was carried out according to earlier described protocol (Sinjushin & Gostimskii 2008). Plant material was fixed and dissected in 70% ethanol and dehydrated through ethanol series with final dehydration in acetone. The specimens were then critical-point dried, mounted on special pedestals and coated with Au+Pd in sputter coater Eiko IB-3. After this preparation, specimens were visualized with usage of scanning electronic microscope CamScan-S2 (Cambridge Instruments, UK; secondary electron image regime) with accelerating voltage of 20 kV.

Results

Inflorescence structure in wild-type plants and mutants
In wild-type plants (cv. Nemchinowskii-766), formation of normal inflorescence was observed. Flowering began from 14.0 ± 0.82

node (Tab. 1, average ± standard deviation are presented). Frondose laves subtended racemes (short paracladia) bearing one or two flowers and invariably terminating with more or less expresses sterile stub (Fig. 2E). Stub itself produced trichomes which are interpreted as indicator of termination of proliferation by some authors (Singer et al. 1990).

Four types of axillary raceme terminus were observed in most mutant forms: short stub, long stub, early senescing flower bud or terminal flower with well-developed perianth (Fig. 2). Quantitative characteristics of distribution of listed types in studied lines are presented in Table. Terminal flower or bud was second or third if counted from the paracladium base (Tab. 1). Acropetal flowering order remained, i.e. terminal flower was the last to open. True terminal position of abnormal flower is confirmed when analyzing SEM data (Fig. 2F).

Double mutants *fas det* and *fas lf* exhibited somewhat different distribution of frequencies of raceme types. Almost all axillary racemes were terminated with flower bearing well-developed perianth (Tab. 1).

Table 1. Distribution of different anomalies of short paracladium structure in the studied lines

Line (genotype)	No. of plants analyzed	No. of short paracladia	Node of floral initiation (average ± stand. dev.)	Type of terminal structure (%)				Bracts (%)		Serial no. of terminal flower (%)	
				Short stub	Long stub	Senescing flower bud	Flower	Solitary	Paired	Second	Third
Nemchinovskii-766 (control) (standard)	10	48	14.0 ± 0.82	88.0	12.0	-	-	8.0	-	-	-
«Shtambovyi» (fas)	20	121	13.9 ± 0.87	11.6	7.4	60.3	20.7	31.4	26.4	52.1	13.2
JI2671 (fa)	10	64	15.4 ± 0.99	59.4	17.1	18.8	4.7	12.5	-	23.4	-
K301 (nod4)	20	111	14.4 ± 0.69	52.3	5.4	18.0	24.3	2.7	-	42.3	-
DTR (det)	10	30	13.6 ± 1.17	59.9	26.7	6.7	6.7	30.0	3.3	10.0	3.3
DTR(m) (det)	10	20	19.1 ± 2.23	75.0	15.0	10.0	-	-	-	10.0	-
WL102 (lf)	10	52	12.7 ± 2.16	20.8	79.2	-	-	2.1	-	-	-
fas det	10	27	13.8 ± 1.17	11.8	-	29.4	58.8	17.7	17.7	76.5	17.7
fas lf	10	43	12.5 ± 1.38	2.9	6.4	30.2	60.5	25.6	18.6	60.5	30.2

The axillary racemes of most studied mutants were characterized by presence of bracts, usually paired, which are normally absent in pea (Tab. 1). No abnormalities of inflorescence development were observed in the lines bearing *fa* and *sym28* mutation, nor in double mutants *fa det*. The only exception is represented by line JI2671 (*fa*) which developed altered short paracladia only during summer season of 2007 (Tab. 1). Similar flower-like structures terminating paracladium were reported for *lf* forms (Weller 2007).

One more notable peculiarity of mutant *det* was connected with structure of topmost lateral paracladium with subtending leaf which resembled flower and bract, respectively. Leaf structure ranged between normal and strongly reduced (e.g., unifoliolate). Paracladium often remained shortened with fused flowers which sometimes gave rise to twin pods after ripening. The topmost lateral paracladiuim is usually retarded in flowering time in comparison with terminal and the first lateral ones, which flower almost simultaneously.

Structure of flower terminating second-order axis

In cases when terminal flower developed, its morphology was studied precisely. As compared with lateral flowers of normal morphology, number of organs was significantly less (Fig. 3), gynoecium developed rarely. The most stable position was found for two adaxial sepals and adaxial petal (standard or vexillum); number and position of other flower parts varied. Most petals were differentiated in vexillum-like manner, but in some cases their nature was obscure. Sometimes formation of flower parts of hybrid structure was observed; these combined features of sepal and petal, petal and stamen or other structures. Many organs developed with certain distortions; gynoecium was often absent or carpel margins remained free. During all observation period, no fruit formation from terminal flowers was noted.

Discussion

Specificity of terminal flower development

Numerous mutants are known in model plants which form terminal flower on main axis (Coen & Nugent 1994, Bradley et al. 1996). Formation of terminal flowers in racemose inflorescences is of special evolutionary interest (Sokoloff et al. 2006). The flower abnormalities in fasciated pea lines were mentioned by L.N. Kostrikova (1967) for the first time, and later in paper (Ambrose 1993), but without special reference to genotype

and morphology. Legumes are interesting in this connection, as they mostly possess multiaxial (triaxial in pea) inflorescences and zygomorphic flowers. Except this, fasciated mutants provide opportunity to study mass material rather than spontaneously arising terata.

Distortions in structure of the ectopic flower are notable. Properly speaking, obvious difficulties always exist for zygomorphic flower which terminates main axis: asymmetric (monosymmetric) distribution of gene activities can hardly be imagined in this position. Probably this may serve an explanation for fact that terminal flower is actinomorphic (polysymmetric) in *cen* mutants of *Antirrhinum majus* normally having zygomorphic lateral flowers (Bradley et al. 1996). In forms described in given work the abnormal flower is true terminal, and paracladium becomes closed (although flowering order remains acropetal).

To estimate possible reasons underlying distorted morphology in ectopic flowers, one should reveal functional differences in positions of terminal and lateral flowers. The inhibiting influence of surrounding organs has crucial role in flower formation, especially in determination of organ initiation order. As it had been demonstrated in work (Prenner 2004), most legume flowers are characterized by earlier organ initiation from abaxial side, and pea follows this tendency (Tucker 1989). This can be probably explained as a result of inhibition from apex of racemose inflorescence and/or bracteoles. The latter do not initiate in pea but may remain cryptic (i.e. detected on expression level only) and influence flower formation. Bract influence, if present, is less or overlapped by listed factors, as abaxial-to-adaxial organ initiation order remains constant in legumes with both bracteose and bractless inflorescences (Prenner 2004).

In pea flower terminating short paracladia of mutants, distribution of inhibitions may differ. Apex of paracladium has no more influence, while subtending frondose leaf can provide robust inhibiting action. Hence, organs from adaxial side may initiate (or be marked out) first, and their initiation appears to be guaranteed and most stable in development.

Probably it is of certain significance that apical meristem of paracladium tends to reduction (normally exhausting and producing sterile stub) thus becoming less than normal floral meristem. As a result, organs initiate with altered number and position. Alterations in flower morphology seem to be connected

with unusual distribution of inhibitions, rather than with tendency to polysymmetry (that is why organs from adaxial side remain stable). Hence, in our opinion, abnormal flowers in fasciated pea mutants cannot be interpreted as true terminal peloria (in terms of (Rudall & Bateman 2003)).

Further organ differentiation depends on regulatory activity of genes influencing newly initiated primordia. The genes of ABC classes play the most important role in determination of organ type (e.g., Coen & Meyerowitz 1991), and their regulatory fields may overlap abnormally in ectopic flower; this can serve an explanation for organs of hybrid structure.

It should be noted that the whole complex of abnormalities of flower structure in fasciated pea lines cannot be interpreted as flower fasciation. As it had been demonstrated in earlier work (Sinyushin 2010), fasciated flowers of different species are characterized by progressive increase of organ number in every whorl as compared with normal flower. Most flowers of fasciated pea individuals are normal, while terminal ones develop with even less organ number than lateral.

Gene network in inflorescence formation

Studies on *Arabidopsis* developmental genetics revealed tight connection between control of activities of SAM and floral meristem. For example, mutants *clavata* (*clv1, 2* and *3*) possess both flower and stem fasciation (Clark et al. 1993), and such trend is found in many plants. Legumes represent notable exception, as flowers of known fasciated mutants in this group are normal (reviewed in: Sinyushin 2010).

Certain parallels in genetic control of flower formation in *Arabidopsis* and axillary raceme in *Pisum* are notable. SAM of *tfl1* mutant of *A. thaliana* transforms into floral meristem, while SAM in pea mutated in gene *DET* (homolog of *TFL1*) gives rise to terminalized paracladium (Fig. 1C). Another aberration in *tfl1* mutants is earlier flower initiation on main axis. Pea mutants *lf* (gene *LF* represents one more homolog of *TFL1*) are characterized by earlier formation of short paracladia (Fig. 1B). Mutations in *CLV* genes of *Arabidopsis* lead to proliferation of fasciated flower axis (Clark et al. 1993). Similarly, proliferation of paracladium occurs in fasciated pea plants.

As for *det* mutant, it is remarkable as having main shoot (first-order axis) terminalized with short paracladium (second-order axis) and paracladium itself terminalized with flower (third-order axis). One may conclude that function of gene *DET* is to maintain undifferentiated state of apical meristem of n-order axis via prevention of its transformation into axis of (n+1)-order. The same may be noted for *TFL1* in *Arabidopsis* although this plant bears simple inflorescences. Hence, phenotype of *det* and *lf* pea mutants may be explained by original conservation of *TFL1* function in legumes. Bract formation in normally bractless paracladia of studied mutants is unusual and needs further investigation. Tight connection between formation of terminal flower and bracts is however discussed by Penin et al. (2005) as a common rule.

Two types of abnormal paracladium proliferation are known in pea. In one case axis proceeds growing, and additional lateral flowers initiate. As a result, many-flowered short paracladium is formed as, e.g., in *neptune* (*nep*) mutants (Singer et al. 1999) or in many wild species of tribe Fabeae Rchb. Another case is described in given work: flower-bearing axis produces terminal flower. Cessation of short paracladium growth seems to be genetically controlled action. Possibly size of this paracladium meristem is key factor determining its fate. This meristem normally initiates one or two lateral flowers and then diminishes producing "stub" at the end of proliferation. When some size threshold is increased, this meristem keeps proliferating and producing lateral flowers, as in *nep* mutants (Singer et al. 1999). Finally, in combination of superfluous meristem size with specific profile of gene expression floral morphogenesis becomes possible as it does in fasciated mutants.

Conclusions

Summary of revealed interaction of studied genes and their influence on inflorescence development is presented in Fig. 4. The multiple role of some genes (e.g. *NOD4*) regulating SAM size, short paracladium proliferation and nodulation is unique feature which can be probably observed in legumes only. The described peculiarities of mutants may appear promising in designing new morphotypes for pea breeding (e.g., bracts formation can be evaluated as positive feature increasing productivity).

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