QUALITATIVE ASPECTS OF EXTRA-MORPHOLOGICAL ACTIVITY ON ANATOMIC SECTIONS OF REVERTED HOMEOTIC SEGREGANTS OF THE WINGED BEAN *PSOPHOCARPUS TETRAGONOLOBUS* (L.) DC UNDER LABORATORY CONDITIONS

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Key words: **Homeotic gene, hyper-anabolic, extra-morphological, extra-rhizogenous, extra-vegetative.**
Gene homeótico, hiper-anabólica, extra-morfológica, extra-rizógena, extra-vegetativa.

Abstract

Laboratory studies on anatomical sections from reverted segregants of the winged bean ***Psophocarpus tetragonolobus*** *(L.) DC, homozygous for a recessive homeotic gene in its activated state, revealed four stages of morphological transformation. Normal floral specimens reverted from the sexual reproductive function to an asexual photosynthetic function with no further activity (previous analysis). In laboratory, reverted specimens could remain inactive or show three types of extra-morphological activity. These types were: extra-vegetative activity (33.71 %), extra-rhizogenous activity (35.96 %), both these forms of extra-morphological activity (30.34 %). This activity, in terms of sites on anatomic sectons, showed significant occurrence at the pre-whorl bract regions, plus two of the four floral whorl regions, i.e. the calyx (sepals) or first whorl and the ovary (carpel) or*

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fourth whorl. Data shows that the one gene already identified with this morphological transformation, besides being homeotic and recessive, is also a "natural morphological mutant" that is nonepigenetic.

Resumo

ASPECTOS QUALITATIVOS DE ATIVIDADE EXTRA-MORFOLÓGICA NAS SECÇÕES ANATÔMICAS DOS SEGREGANTES HOMEÓTICOS DO FEIJÃO-DE-ASA ***PSOPHOCARPUS TETRAGONOLOBUS*** *(L.) DC EM LABORATÓRIO. Análises em laboratório de secções anatômicas originadas dos segregantes revertidos do feijão-de-asa* ***Psophocarpus tetragonolobus*** *(L.) DC, homozigóticos para um gene homeótico recessivo, no seu estado ativado, revelaram quatro tipos de transformação morfológica. Espécimens florísticas normais reverteram da função sexual reprodutiva para uma função fotossintética assexual sem qualquer atividade a mais (análise prévia). Em laboratório, espécimens revertidas ficaram inativas ou mostraram três tipos da atividade extra-morfológica: extra-vegetativa (33,71 %), extra-rizógena (35,96 %) e ambas formas desta atividade extra-morfológica (30,34 %). Esta atividade, em termos das posições nas secções anatômicas, apresentou ocorrências significativas nas regiões pré-verticilares da bráctea e em duas das quatro regiões verticilares florísticas, i.e. a região do cálice (sépalas) ou primeira verticilo floral, e o ovário (carpelo) ou quarto verticilo floral. Os resultados obtidos confirmam que aquele primeiro gene já identificado com esta transformação, além de ser homeótico e recessivo, também é um "mutante morfológico natural" que não é epigenético.*

Introduction

Research over the past eighteen years has revealed the existence of homeotic genes in both plant and animal species (BELL et al., 1999; GEHRING, 1999; NUTT, et al., 1999). A plethora of these genes have been shown to affect flower development as upstream or downstream regulators (BUSCH et al., 1999; WAGNER et al., 1999). Many of these genes affect morphology as inactivator or repressor genes causing specific loss-of-function phenotype, or as meristem identity genes causing specific gain-of-function phenotype (WEIGEL & MEYEROWITZ, 1994) in terms of recognized, defined, confirmed and predictable structures (e.g. floral, reproductive buds having a determinate growth vs. photosynthetic, vegetative "shoot" buds having an indeterminate growth) that may switch or alternate because of expression of the homeotic gene (SCHWARZ-SOMMER et al., 1990). These influences are documented in a broad spectrum of angiosperm species (MENA et al., 1996; WAGNER et al., 1999; WEIGEL & MEYEROWITZ, 1994). However only one "natural morphological mutant" affecting actual alterations in phenotype has thus far been reported

(CUBAS et al., 1999). It is specifically epigenetic in that it causes "...heritable changes in gene expression that occur without a change in DNA sequence." (WOLFFE & MATZKE, 1999).

Flower development seems to occur in progressive stages (COEN et al., 1990; MANDEL et al., 1992) including the four floral whorls (i.e. sepals, petals, stamens and carpels) that compose complete flowers plus the pre-floral (i.e. pre-whorl) lateral organs, the bracts (KRAMER & IRISH, 1999; STERN, 1988). Any phenotypic influence by homeotic genes depends as much on their transcriptional activation or inactivation (BUSCH et al., 1999; WAGNER et al., 1999; WEIGEL & MEYEROWITZ, 1993) as it does on their physical presence in the genome DNA (GRIFFITHS & NEUMANN-HELD, 1999) of the respective species.

Laboratory tests using specimens of anatomic sections (i.e. stems, flowers, fusions, reverted flowers, reverted buds) from reverted segregants of the winged bean *P. tetragonolobus*, homozygous for a recessive, activated, homeotic gene (BENYA, 1995) have shown significant differences in metabolic persistence according to normality of sections (i.e. non-reverted or reverted); purity of water (i.e. deionized or plain) used as a cultural medium, and presence or absence of extra-morphological (i.e. extra-rhizogenous and/or extra-vegetative) activity in those sections (BENYA, 1999). Such extra-morphological activity reflects and is a qualitative indicator of elevated anabolic (i.e. hyper-anabolic) activity associated with segregants containing the gene and showing this type of homeotic, germinal reversion.

Purpose of this research was to observe specific floral and vegetative sections, both normal and reverted, under laboratory conditions, documenting any hyper-anabolic (i.e. extra-morphological) activity in terms of qualitative aspects (i.e. anatomical regions; number of sites at those regions) of extra-rhizogenous and extra-vegetative activity. Researchers then compared presence of such activity on organs (i.e. bracts, ovaries, etc.), organ systems (i.e. buds, flowers) and simple anatomic parts (i.e. stems) of reverted segregants and/or sections of segregants.

Material and Methods

Researchers selected 156 reverted specimens, field grown, representing three basic anatomic and/or organ categories and tested these under controlled laboratory conditions. These three categories were: fused floral and non-floral parts (i.e. "fusions") from reverted segregants, reverted stems, reverted flowers and reverted flower buds.

The 156 specimens were divided into 39 treatments of usually four repetitions per treatment (varying between two and five reps). Each treatment was con-

ducted within individual plastic cups and/or glass test-tubes using plain or deionized water within a double chambered laboratory as previously described (BENYA, 1999). Statistical analyses (i.e. ANOVA, X^2, t-tests,) were used to identify and verify significant differences.

Results and Discussion

Four stages of morphological transformation (a plasticity of phenotype) and three types of extra-morphological activity were observed. Floral structures reverted from the sexual reproductive function to an asexual photosynthetic function but with no further activity. Reverted specimens continued active showing one of three further types of morphological transformation (i.e. hyper-anabolic, extra-morphological activity); extra-vegetative, extra-rhizogenous, or both of these forms.

Of the 156 specimens tested 67 (42.95 %) showed no further activity while 89 (57.05 %) showed some form of hyper-anabolic, extra-morphological activity. Distribution of these 89 active specimens according to the three anatomic categories was: four fusions, six reverted stems and 79 reverted flowers and flower buds. These 89 specimens were classified and assigned "activity values". A specimen showing only extra-vegetative activity was assigned an activity value of "one". A specimen showing only extra-rhizogenous activity was assigned a value of "two". Specimens showing both activities received a value of "three". White extra-morphological activity was assumed to be rhizogenous. Virescent (chlorophyllized) extra-morphological activity was assumed to be vegetative.

Extra-rhizogenous activity was valued above that of extra-vegetative because all three categories of reverted specimens under study were of an aerial, foliar or floral origin from the "above ground body plan" (WAGNER et al., 1999) where extra-vegetative activity should be expected rather than, or at least prior to extra-rhizogenous activity which is usually subterranean in nature. The 89 active specimens were composed of 30 that had an activity value of one ("1"), 32 that had an activity value of two ("2") and 27 that had an activity value of three ("3"). This was in accord with expected proportions of 1:1:1 ($X^2 = 0.3191$, NS).

Five major anatomical regions (i.e. four specifics and one non-specific region) were identified where extra-rhizogenous and/or extra-vegetative activity occurred. The four specific regions were: the calyx, floral stem, bracts, ovary, and the one non-specific region was "all other". These five regions were further specified according to extra-rhizogenous or extra-vegetative activity. Among the 89 specimens showing any type of extra-morphological activity there were actually present a total of 676 possible activity sites (APPENDIX).

Most sites showed no (i.e. zero) activity. A total of 145 active sites at the 676 possible sites (21.45 %) on the 89 specimens showed one or more ex-

tra-morphological structures (rhizogenous and/or vegetative). Analysis of variance (ANOVA) showed that any one of the 145 sites that yielded any extra-morphological structure is statistically significantly distinct ($F = 17.47$; $P < 0.01$) from those sites (i.e. 531) that showed no activity.

Qualitative distribution of the 145 active sites on the 89 specimens ranged between one and four active sites per specimen and showed an overall mean of 1.629 per specimen. Multiple occurrences of active sites (i.e. two or more per specimen) were manifest on 41 of the 89 specimens or 46.07 % of the total. Such multiple occurrences of sites could signify multiple extra-vegetative sites only, multiple extra-rhizogenous sites only or a combination of both. Six active sites on four specimens showed both extra-vegetative and extra-rhizogenous activity at the same site. These six double activity sites were treated as separate (i.e. distinct) entities for purposes of statistical analysis (APPENDIX).

Considering all extra-morphological activity (TABLE I); occurrence of the 145 active sites was significantly high at the bract regions (37 sites on 36 specimens, $P < 0.01$), calyx regions (27 sites on 27 specimens, $P < 0.05$) and ovary regions (38 sites on 35 specimens, $P < 0.01$) of the 89 specimens; which correspond to the pre-floral, first floral and fourth floral whorls respectively. Occurrence was significantly low at the elongation of the ovary-calyx convergence (four sites on four specimens, $P < 0.01$), a subregion of the ovary. Occurrence on the floral stem region (18 sites on 18 specimens) and at the "all other" region (25 sites on 23 specimens) did not vary significantly from the overall distribution. More specific examination of floral stems revealed that, of the 18 specimens that showed activity in that region, 14 had it along their stem shaft region (not statistically significant) while four showed it at their base (significantly low, $P < 0.01$). None showed this activity at both subregions of the stem.

Occurrence of extra-rhizogenous sites (87 sites on 59 of the 89 active specimens; TABLE II) ranged from one to three active sites per specimen on those 59 specimens. These sites occurred at all five major anatomical regions. Their occurrence showed a distribution, in terms of statistical significance, that was high at the bract and calyx regions, not significantly different from the group distribution at the stem regions, but significantly low at the ovary and "all other" anatomical regions.

Occurrence of extra-vegetative sites (58 sites on 57 of the 89 specimens; TABLE III) ranged from one to two active sites per specimen on those 57 specimens. These arose at three of the five major anatomical regions: bracts, ovary (plus the ovary-calyx convergence) and "all other" region. This occurrence was significantly high at the ovary regions, low at the bract regions and ovary-calyx convergence subregions and not significantly different from the distribution for the group at the "all other" region.

A general qualitative gradient was apparent on the 89 active specimens (TABLE I). Extra-vegetative activity was significantly high at active sites from the elongation of the ovary-calyx convergence (i.e. four vegetative) to the ovary itself (35 vegetative and three rhizogenous) (i.e. 39 of 42 sites, or 92.86 %). Ex-

tra-rhizogenous activity significantly dominated at sites from the calyx (27 rhizogenous) to the bracts (36 rhizogenous and one vegetative) and stem (18 rhizogenous) (i.e. 81 of 82 sites or 98.78 %) ($X^2 = 106.7$; $P < 0.01$). The 27 specimens showing both extra-rhizogenous and extra-vegetative sites (i.e. those with an activity value of "three") all failed to develop into photosynthetically self-sustaining autotrophic organisms.

TABLE I - Distribution of the 145 active sites according to anatomical region on the 89 active specimens.

Anatomical region or subregion	Number of sites	Number of specimens	Type	t-value[1]	P
Calyx	27	27	rhizogenous	2.142	< 0.05
Floral stem					
(shaft)	14	14	rhizogenous	1.106	NS
(base)	4	4	rhizogenous	7.032	< 0.01
total	18	18	rhizogenous	0.052	NS
Bracts	36	36	rhizogenous	4.006	< 0.01
	1	1	vegetative	16.803	< 0.01
total	37	36	both	4.214	< 0.01
Ovary	35	35	vegetative	3.799	< 0.01
	3	3	rhizogenous	8.653	< 0.01
total	38	35	both	4.423	< 0.01
Convergence ovary-calyx	4	4	vegetative	7.032	<0.01
All other	22	22	vegetative	0.711	NS
	3	3	rhizogenous	9.397	<0.01
total	25	23	both	1.366	NS
Total =	145				

[1] $\mu = 0.2145$

Lack of any identified extra-morphological activity at the stamens or third floral whorl is notable but not completely surprising. Previous research concerning flowering in other species (COEN et al., 1990; KRAMER & IRISH, 1999; SCHWARZ-SOMMER et al., 1990) suggests a unique complexity of functions involving (or pertaining to) development of this third floral whorl.

Multiple site activity on individual active specimens was common (41 of the 89; 46.07 %) but distribution was heavily skewed toward solely rhizogenous and combined rhizogenous-and-vegetative activity ($X^2 = 23.51$; $P < 0.01$). A total of 27 of the 41 multi-site specimens (65.85 %; all showing activity type three) pre-

sented one or more rhizogenous and vegetative sites; 13 of the 41 (31.71 %) multi-site specimens presented two or more rhizogenous sites only, but only one specimen (2.44 %) presented two vegetative sites solely. Thus 40 of the 41 multi-site specimens (97.56 %) presented extra-rhizogenous activity either solely or in combination with extra-vegetative activity. This predominance suggested an abundance of a morphogen or morphogens responsible for rhizogenous activity.

TABLE II - Distribution of the 87 extra-rhizogenous sites on 59 specimens.

Anatomical region	Number of sites	Number of specimens	t-value[2]	P
Calyx	27	27	2.292	< 0.05
Floral stem				
(shaft)	14	–	0.920	NS
(base)	4	–	6.702	< 0.01
total	18	18	0.223	NS
Bracts	36	36	4.184	< 0.01
Ovary	3	3	8.272	< 0.01
All other	3	3	8.992	< 0.01
Totals =	87	(87 - 28*) = 59 specimens		

[2] $\mu = 0.2067$

* 28 multi-site specimens (22 with two sites each, i.e. double representation, three specimens with three sites each, i.e. triple representation) 22 x 1 = 22
3 x 2 = 6
28

TABLE III - Distribution of the 58 extra-vegetative sites on 57 specimens.

Anatomical region or subregion	Number of sites	Number of specimens	t-value[3]	P
Bracts	1	1	17.882	< 0.01
Ovary	35	35	3.561	< 0.01
Convergence ovary-calyx	4	–	7.581	< 0.01
All other	22	22	0.428	NS
	totals = 58	58 – (1 specimen with two sites) = 57 specimens		

[3] $\mu = 0.2275$

Ubiquity of distribution of extra-rhizogenous sites (occurring at all five anatomical regions) as compared with a more restrictive distribution of extra-vegetative sites (occurring at three of the five regions) suggests different morphogens or at least distinctly different concentrations of the same morphogen to be responsible for such activity. The further occurrence of both extra-rhizogenous and extra-vegetative activity at the same anatomic region (i.e. bracts, ovary and the "all other") even on the same specimen (i.e. six cases) further emphasizes the possibility of at least two morphogens being involved in these phenomena, although the timing associated with appearance and definition of structures may be crucial if only one morphogen is responsible, a topic that falls outside the scope of this report.

Distribution of extra-rhizogenous sites was more variable (i.e. ranging from one to three per specimen, TABLE II) than was the distribution of extra-vegetative sites (i.e. ranging from one to two, and only one multi-site specimen showed two such sites, TABLE III). This may reflect the more normal position of extra-vegetative sites as part of the "above ground body plan" as WAGNER et al. have stated (1999) where more control mechanisms for such growth remain intact even though a specimen has reverted, as compared to an abnormal position of extra-rhizogenous sites which are usually part of the below ground body plan of a plant. Or it may reflect a "default" biological mechanism, inactivator in origen, where a deficiency (of presence or of sufficient concentration) of a specific morphogen or morphogens causes an already florally induced bud to revert to a vegetative function.

Concentrations of extra-rhizogenous activity and extra-vegetative activity were clear and distinct. However they were not exclusive. Leakage or minimal intermixing of the two types occurred at the bract, ovary and the "all other" regions. This structural intermixing at the morphological level suggests a morphogenical intermixing at the physiological level and may well be a major factor explaining why all of the 27 active specimens showing both extra-rhizogenous and extra-vegetative activity failed to develop into self-sustaining organisms. Coordination between the two activities was lacking.

The four stages of morphological transformation and the three types of extra-morphological activity (contained within those stages) represent two principle functions of homeotic genes; inactivation and/or meristematic identity. Reversion of flowers and flower buds from their normal sexual reproductive function to an asexual photosynthetic function with no extra-morphological activity was inactivation; simple loss of function. Further transition of these inactive reverted specimens to extra-morphologically active ones was meristematic; simple gain of function. However the three steps of extra-morphological activity encountered in this data (i.e. extra-vegetative solely, extra-rhizogenous solely, or both) is less than simple. It suggests at least two major possibilities. At least one morphogen is operating at more than one concentration (probably quite specific) in a timing sequence, which is also probably quite specific. Or there may be two or more morphogens operating separately and/or in combination at less specific concen-

trations and/or less crucial timing sequences. Physiological and molecular analysis of these phenomena, plus studies of the timing sequence would be valuable.

Even after reversion, the pre-whorl bracts and the four floral whorls maintained their identity although their forms may drastically have changed. Development of meristematic activity at many of these organs and organ sites in fairly specific types suggests a specificity of function or functions (e.g. synthesis and/or purification and/or storage) for these organs that may provide further information concerning their own anatomical and morphological origin and developmental history.

Conclusions

Although one recessive gene seems responsible for this reversion phenomenon as a whole (BENYA, 1995), the data presented here suggest that a number of other genes may be associated with the four stages of morphological transition and the three steps of extra-morphological activity therein. The concentration of active sites at organ regions (i.e. bract, calyx and ovary; the pre-whorl, first whorl and fourth whorl regions respectively) rather than at strictly anatomical regions (i.e. floral stem, stem base and "all other") or rather than at combined organ and non-organ regions suggests a specificity of function for these organs in producing and/or separating and/or using and/or storing any morphogenic substance or substances (e.g. hormones) responsible for site activation and/or continued activity.

Lack of coordinated development of the 27 reverted specimens showing both extra-vegetative and extra-rhizogenous activity into self-sustaining autotrophs suggests a crucial gap in any governing mechanism associated with this specific type of reversion. Three possibilities must at least be considered. Such a governing mechanism does not exist. At least one governing mechanism does exist but was not activated under laboratory conditions. The governing mechanism exists and was activated but simply was not observed.

The gene already identified with this phenomenon is homeotic, recessive and responsible for natural morphological changes, but it does not appear to be epigenetic. Data thus far recorded suggests that the gene is expressed only in an asexual, photosynthetic phenotype that is exclusively intragenerational. Such expression may permit cloning (perhaps in the future) but by definition it is not heritable, and heritability of genetic expression is an essential of epigenetics. Changeability is heritable through this gene, but not the specific changes themselves. This gene should therefore be classified as a "natural morphological recessive mutant" that is nonepigenetic.

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APPENDIX - Description of treatments

Possible active sites were determined by dividing the 89 active specimens into two groups; those that contained all five of the major anatomical regions where extra-morphological activity was observed (i.e. 83 specimens) and those that did not contain all of those regions (i.e. six specimens). Reverted stems, by definition contained no floral parts. Neither did any of them show any rhizogeny at their stem base region. Thus the only active region pertaining to them was "all other". Fusions by definition contained all five regions (even though they may not be identifiable) as did all reverted flowers and reverted flower buds. Thus multiplying the number of active reverted stems (six) by the number of anatomical regions pertinent to them (one, i.e. "all other") (6 x 1 = 6), one arrives at a total of six possible active regions.

Calyx and floral stem as distinct regions only showed extra-rhizogenous activity. The ovary-calyx conjunction elongation region (a subset of the ovary region) showed only extra-vegetative activity. However depending on any one active specimen, the bracts, ovary and "all other" regions showed either extra-rhizogenous or extra-vegetative activity and sometimes both. They were thus treated as six distinct experimental regions. Total possible active regions was then calculated by multiplying the number of floral, fusion and bud specimens (83) by the number of activity regions (i.e. six: calyx rhizogenous, floral stem rhizogenous, bract rhizogenous, bract vegetative, ovary rhizogenous, ovary vegetative), 83 x 6 = 498. These 83 specimens also contained the two experimental categories "all other rhizogenous" and "all other vegetative". However the total number of specimens for these two categories had to increase to 89 in order to account for the six reverted stems. The anatomical categories "all other rhizogenous" and "all other vegetative" were thus represented on 89 active specimens (not only on 83). This value yielded a further 178 possible active sites (i.e. 89 x 2 = 178) rendering a final total of 676 possible sites where extra- morphological activity could occur (i.e. 498 + 178 = 676).