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Review

Barley Developmental Mutants: The High Road to Understand the Cereal Spike Morphology

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Abstract: A better understanding of the developmental plan of a cereal spike is of relevance when designing the plant for the future, in which innovative traits can be implemented through pre-breeding strategies. Barley developmental mutants can be a Mendelian solution for identifying genes controlling key steps in the establishment of the spike morphology. Among cereals, barley (*Hordeum vulgare* L.) is one of the best investigated crop plants and is a model species for the *Triticeae* tribe, thanks to several characteristics, including, among others, its adaptability to a wide range of environments, its diploid genome, and its self-pollinating mating system, as well as the availability of its genome sequence and a wide array of genomic resources. Among them, large collections of natural and induced mutants have been developed since the 1920s, with the aim of understanding developmental and physiological processes and exploiting mutation breeding in crop improvement. The collections are not only comprehensive in terms of single Mendelian spike mutants, but with regards to double and triple mutants derived from crosses between simple mutants, as well as near isogenic lines (NILs) that are useful for genetic studies. In recent years the integration of the most advanced omic technologies with historical mutation-genetics research has helped in the isolation and validation of some of the genes involved in spike development. New interrogatives have raised the question about how the behavior of a single developmental gene in different genetic backgrounds can help in understanding phenomena like expressivity, penetrance, phenotypic plasticity, and instability. In this paper, some genetic and epigenetic studies on this topic are reviewed.

Keywords: homeotic mutants; *Hordeum vulgare*; spike architecture; genomics

1. The Cereal Spike

A cereal spike is an important plant organ, being the single biggest source of food for humankind. This food and feed source must be further improved; the FAO indicates that a 50% increase of cereal production (from 2.1 to 3 billion tonnes) is needed to meet the demand of the increasing population [1]. This means that the grain number of cereal spikes must be improved in the near future, together with a biomass increase [2]. As discussed by Sreenivasulu and Schnurbusch [3], grain number enhancement can be theoretically obtained through modifications of the spike fertility and morphology. Due to the implications in the grain production and yield, the genetic dissection of the developmental plan of this storage sink is therefore of relevance when designing the cereal for the future. In this frame, collections of morphological barley mutants can help improve the understanding of the cereal spike development

process. Some examples of the use of barley genetic resources to elucidate spike development will be reviewed.

2. Why Barley?

Among cereals, barley is a model organism from both a genetic and genomic point of view: barley is characterized by a high degree of natural variation and by its adaptability to several different cultivation environments. Several morphological and physiological forms have evolved, including winter, spring, two-rowed, six-rowed, awned, awnless, hooded, naked, and covered grain, malting, feed (grain and forage), and food types. Its diploid genome, whose sequence is available [4], and the self-pollinating mating system, together with the availability of genetic and genomic resources, make this plant a reference model. Barley is characterized by strong genetic variability for developmental traits and this characteristic is important in ensuring its broad adaptability.

3. Genetic Dissection of the Key Developmental Trait of Barley Spike

Historically, barley genetic studies have their foundations in Mendelian mutants, characterized by an altered physiology and/or morphology. Starting in the 1920s, thousands of different barley mutants—both natural or obtained via mutagenesis—have been collected world-wide and designed as Barley Genetic Stocks (BGS). The BGS lines are conserved in different gene banks and information about them can be found in the "Barley Genetic Stocks Database" [5] which is illustrated with images. These genetic resources provide one of the most efficient tools to study the developmental process of the spike, to identify the single genes involved, and to understand their regulation and interactions. The collections are not only comprehensive in terms of single Mendelian spike mutants, but with regards to double and triple mutants (Figure 1), derived from crosses between single mutants, as well as near isogenic lines (NILs), which are useful tools for genetic studies.

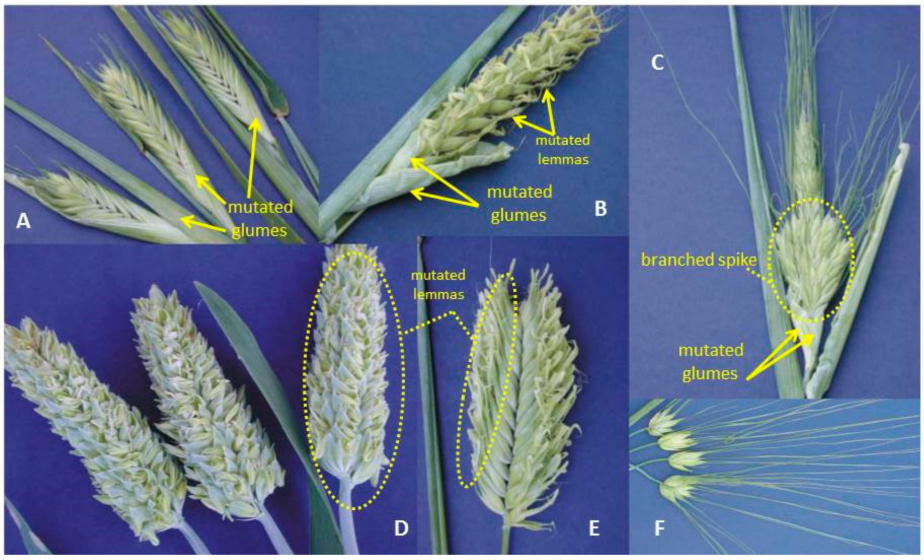

Figure 1. Phenotypes of some double mutants, obtained by crossing single ones: (**A**) = *Awnless/third outer glume:* the double mutant is characterized by the presence of a very large bract (third outer glume) subtending the lowest spikelet, followed by some large glumes; (**B**) = *Hooded/third outer glume*: the lemma develops a trifurcate structure, similar to a "hood", that includes a central deformed floret with two lateral wings. Moreover, large glumes are present at the spike basis; (**C**) = *Branched/third outer glume*: the spike is ramified and large glumes are present at its basis; (**D**) = *Hooded/low number of tillers*; (**E**) = *Hooded/wide outer glume*: the lemmas bear the hoods and the spikes are compact; and (**F**) = *Many glumes on the lateral/wide outer glume:* in the plant population derived from this cross, a mutant characterized by large glumes and "reduced number of internode rachis" (rnir) has been found.

In recent years, the integration of the most advanced omics technologies with historical mutation-genetics research has helped in the isolation and validation of some of the genes involved in spike development. Interestingly, some genes that have been known for a long time to be responsible for the mutant phenotype have recently been cloned and their functions have been elucidated [6]. Several of these genes are involved in mutations that, selected by early farmers, transformed wild plants into domesticated ones, representing an important contribution to the development of the ancient agrarian societies [7,8].

4. The Phytomer Model: Key to Understand the Ontogeny of Barley Inflorescence

The mature inflorescence of cultivated barley (spike or ear or head) consists of the floral stem (rachis) and floral units (spikelets). This indeterminate inflorescence is a raceme in which one central and two lateral spikelets are positioned in groups of three at each rachis internode. Each spikelet is enclosed and consists of a floret subtended by two bracts (outer glumes) that are the upper inner palea and the lower lemma. The top region of the lemma can bear the awn. The floret consists of a carpel with one single ovary, two styles with plumose stigmata, three stamens, and two lodicules [9].

Phytomeric models based on anatomical, histological, and genetic analyses have been proposed by Bossinger et al. [10]. More recently, Forster et al. [11] revised the Bossinger's model, starting from the visual analysis of developmental mutants—already described or induced in "Optic" barley. The disruptive effect of mutations on the metameric structure of this plant organ has been demonstrated to be a powerful tool for understanding a complex structure like the spike. The observations were made for mutants at different growth stages and a simpler explanation has been adopted for the interpretation of each mutant structure [11]. According to both models, central to the grass architecture is the presence and repetition of phytomer units, linked together by nodal structures. Each node is composed of two half nodes. Classically, two types of phytomers are reported: the "vegetative" type 1 phytomer and the "generative" type 2, a special structure present at branching. Additionally, the inflorescence has a phytomeric structure, even if this is particularly difficult to explain. According to Bossinger et al. [10], the restriction of the internode elongation of the rachis and rachilla is a characteristic of barley spike. The first two organs that are positioned on the rachilla axis are the subtending glumes, which Bossinger considered to be a unique organ, corresponding to a type 2 phytomer. The lemma is considered to be a leaf-like structure (type 1 phytomer), whereas palea belongs to a type 2 phytomer. Lodicules and stamens can be reconducted to variants of a type 1 phytomer, whereas the carpel is a special terminal type 1 phytomer. This model has been simplified by Forster et al. [11], who suggested that both vegetative and generative structures can be explained by a single repeating phytomer unit. The organs of the barley plant-and of the spike- can therefore be divided into two different types: single or paired. Specific organs can be derived from the fusion of paired structures. Central in the Forster's model, is therefore the concept that a plant organ can be the result of growth activation/suppression in specific regions of the phytomer, and even the result of the association of linked phytomers. Therefore, the plant development could be led by the positioning of phytomeric units and by switching their growth on and off.

5. The Brittle Rachis

The most important trait selected by humans during the cereal domestication process and related to the evolution of barley spike is the transformation of a brittle spike into a non-brittle spike. In wild spike, at maturity, the formation of "constriction grooves" that result in the disarticulation of each rachis node and the free dispersion of seeds can be seen (Figure 2).

Figure 2. Close-up of a *Hordeum vulgare* spp. *spontaneum* spike disarticulated at maturity. In wild *Hordeum* species, the three spikelets and their slender awns form a light dispersal unit that permits both anemochory and zoochory.

The loss of this natural grain dispersal allows the quantity and quality of harvestable grain to increase. Using classical genetic approaches, two linked loci, mapped on barley chromosome 3H, *brittle rachis 1 (Btr1*) and *brittle rachis 2 (Btr2),* are involved in the mutation from the brittle rachis into a non-brittle phenotype. The conversion is possible in the presence of the dominant alleles of both genes. In major details, non-brittle, domesticated barleys have a 1bp deletion in *Btr1* or an 11bp deletion in *Btr2.* Recently, molecular studies have better elucidated the evolution of this key domestication trait. The paper from Pourkheirandish et al. [12] is a milestone in this direction: the authors hypothesize that the anthropogenic selection operated in favor of the mutated forms of a signal transducing receptor and its protein ligand. The two gene products, BTR1 and BTR2, act together to control the cell wall thickening in the disarticulation zone of the rachis node, through molecular mechanisms that are not fully understood. Moreover, the authors, on the basis of both DNA sequences and archaeo-botanical data, demonstrated the independent origins of barley domestication. By tracing the evolutionary history of allelic variation in both genes, it can be concluded that the *Btr1*- and *Btr2*-type barleys emerged independently in different environments and at different times. According to genetic studies, two "transition zones" were found, characterized by a high level of changes between the *Btr1*- and *Btr2*-types: the area between Iran and Afghanistan, and the Levant and the Southern part of the Mediterranean Sea. According to the archaeological record, the cultivation of wild barley, before its domestication, was present in the Southern Levant area [8]. More recently, Civan and Brown [13] discovered a third type of non-brittle genotype, carrying the change of a leucine into a proline in the BTR1 aminoacid sequence.

Using a strategy based on the development of a high-resolution population, a new locus, *thresh-1*, present in *Hordeum spontaneum* and involved in the threshability phenotype, has been identified and mapped on chromosome 1H [14]. The candidate genes identified control the plant cell wall composition.

6. The Row Number

Triticeae inflorescence bears one-three spikelets with a single flower at each rachis internode. A barley spike is characterized by the development of one central and two lateral spikelets at each rachis internode. The row number of the barley spike depends on the fertility of the lateral spikelets. Six-rowed spikes have fertile lateral spikelets, whereas two-rowed spikes have sterile lateral spikelets.

Six-rowed genotypes produce more grains per spike, compared with two-rowed ones. The two-rowed state is ancestral, being found in the wild progenitor of cultivated barley (*Hordeum vulgare* ssp. *spontaneum*), where the sterile spikelets form part of the seed dispersal mechanism.

Up to now, five independent loci—i.e., *six-rowed spike1* (*vrs1*), *vrs2*, *vrs3*, *vrs4*, and *Intermedium spike-c* (*Int-c*)—have been identified as being involved in the six-rowed phenotype [15]. *Vrs1* encodes a homeodomain-leucine zipper class I transcription factor that is a negative regulator of lateral spikelet fertility [16], whereas *int-c* is an ortholog of the *TEOSINTE BRANCHED1* maize gene. It has been shown that the allelic combinations of both *vrs1* and *int-c* can modify lateral spikelet development. Six-rowed barleys generally bear loss-of-function *vrs1.a*, together with *Int-c.a*. On the contrary, two-rowed phenotypes have a functional *Vrs1.b* accompanied by *int-c.b*. In brief, more than ten different and independent *INT* genes have been identified that can influence the effect of the *Vrs1* locus, determining modulation in the size and fertility of lateral spikelets. Ramsay et al. [17] identified the primary function of *INT* loci as being related to the growth of the axillary organs of the plant. The other three *vrs* loci determine varying levels of lateral spikelet fertility. In particular, *vrs4* mutants show complete lateral spikelet fertility, as well as the possibility to produce additional spikelets and florets. Koppolu et al. [15] characterized *vrs4*, finding orthology with the maize transcription factor RAMOSA2, involved in inflorescence development in grasses. Expression studies indicated that HvRA2 is a central player in inflorescence development, being involved not only in the regulation of triple spikelet meristems determinacy, but also in the control of *Hordeum* specific row type determination. Moreover, *vrs4* regulates the expression of *vrs1*, and therefore, the *Hordeum* specific row type determination, which is either two- or six-rowed.

A third class of row-type is known as *Labile*-barley (*Hordeum vulgare* L. convar. *Labile* (Schiem.) Mansf.) and it was initially considered to be an irregular row-type of Abyssinian accession. The *Labile*-barley has a variable number of fertile spikelets at each rachis internode (zero to three fertile spikelets/rachis internode) and therefore, its phenotype is intermediate between a two- and a six-rowed type. A deep phenotypic description, using scanning electron microscopy, of spikelet fertility in *Labile*-barleys has been presented by Youssef et al. [18]. These authors observed, in *Labile*-barleys, some arrested central floral primordia during the stamen development. The re-sequencing of *vrs1* and *int-c* loci in 219 *Labile* accessions showed that these genotypes have a six-rowed genetic background, but reduced lateral spikelet fertility due to the recessive *labile* (*lab*) locus presence [18]. Recently, Helmy et al. [19] studied how *vrs2*, which encodes a SHORT INTERNODES (SHI) transcriptional regulator, contributes to barley inflorescence and shoot development. *Vrs2* in the floral organ positively regulates auxin (IAA) biosynthesis and indirectly (via IAA) negatively regulates both cytokinins and the conversion of bioactive gibberellic acid forms. There is a gradient of *vrs2* expression along the length of the inflorescence: the highest expression of this gene is detected at the base of the spike and decreases toward the apex. The same gradient is observed for the hormonal level. Therefore, this gene maintains auxin homeostasis and gradients during normal spike development. Moreover, *vrs2* transcripts are abundant at the tips of spikelet and floret primordia, suggesting a further role in the formation of axillary structures from the main shoot. In the *Vrs2.e* mutant, hormone patterns are disrupted, promoting the formation of six-rowed and supernumerary spikelets [20].

7. Branched Spike

A canonical barley spike has a branchless shape. However, mutants characterized by branched spikes (Figure 3) have been described as naturally occurring since ancient times.

A poly-row-and-branched spike (*prbs*) mutation has been described as being involved in the inflorescence differentiation from a panicle into a spike. This mutation can alter the inflorescence morphology in two ways: (a) determining the conversion of the rudimentary lateral spikelets specific to two-rowed genotypes into fertile spikelets; and (b) determining the development of additional spikelets in the middle of the spike, resulting in a branched spike. Shang et al. [21], starting from morphological observations of developing immature spikes of the mutant and descendants with branched spikes,

showed that the *prbs* gene has a key role in the spikelet development at the triple-mound stage. In mutant *Prbs*, new meristems develop at the flanks of lateral spikelets and the meristems located in the middle spikelet were changed into branch meristems, from which branched spikes are formed. The same authors mapped the *prbs* gene to chromosome 3H and demonstrated that this gene is not allelic to *vrs4* [19]. *Vrs4* has also been found to be involved in another mutant phenotype derived from a particular development of the node. Poursarebani et al. [22] demonstrated that *vrs4* is involved in the regulation of *compositum2* expression, a gene found to be orthologous to the *branched head*^{t} (*bh*^{t}) locus regulating spike-branching in tetraploid 'Miracle-Wheat'.

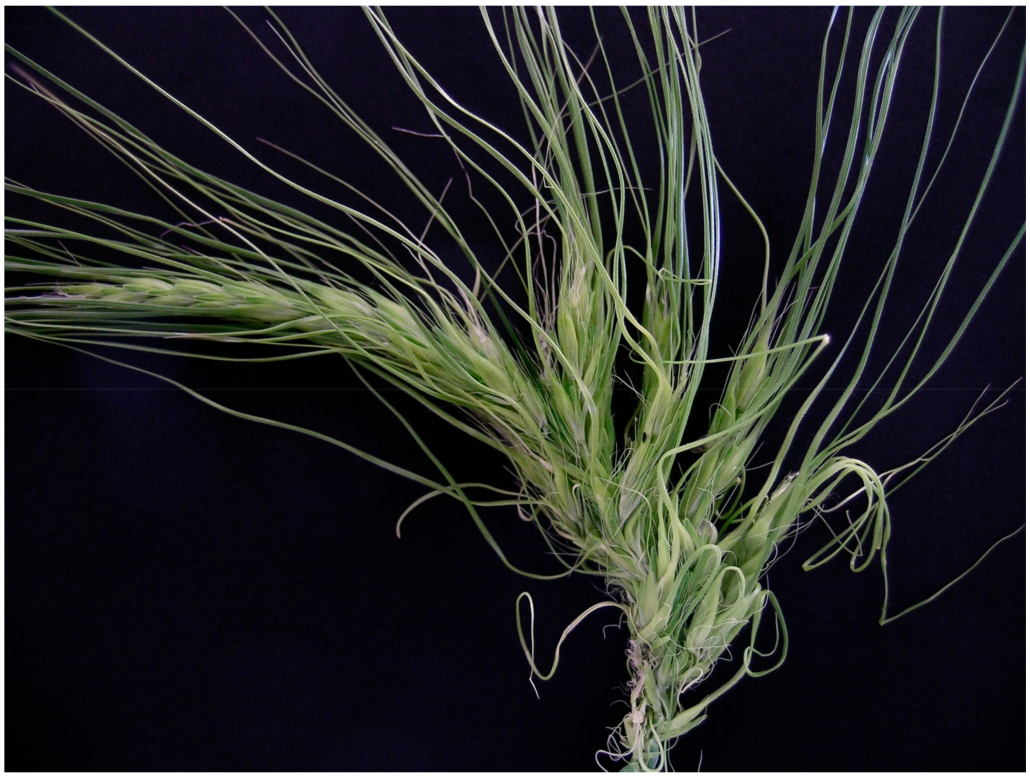

Figure 3. Branched mutant spike.

The branching of the spike can have an epigenetic basis. On this subject, Brown and Bregitzer [23] observed that *Ds-miR172* mutants have an abnormal spikelets development plan, with the conversion of glumes to partially developed florets in apical regions of spikes. The spike basal region has an abnormal branching phenotype, as a result of the irregular development of spikelet meristems. Each branch is formed by multiple, abnormal spikelets and other floral organs, instead of a single spikelet. A similar phenotype was found in maize (*Ts4* mutant), and even in this genotype, the mutation affects an orthologous *miR172*.

8. Spike Density

The reduction or increase of the rachis internode length results in different spike densities (Figure 4). Classical genetic studies have identified several loci as being involved in the modulation of spike density, such as *dense spike*, *zeocriton*, *lax spike,* and *laxatum.* This is a recessive mutation (*lax-a*) with a pleiotropic effect: long rachis internode, large base of lemma awns, and the transformation of lodicules into two additional stamens. Consequently, the *Laxatum* mutant shows five anthers instead of the regular three [13,24]. A panel of major genes is involved in the control of spike density, as demonstrated by the genetic analysis of several morphological mutants. A class of such mutants controls, through *dense spike* (*dsp*) genes, the rachis internode length, resulting in dense or compact

spikes. One of the *dsp* genes (*dsp.ar*) was mapped by high resolution bi-parental mapping to a 0.37 cM interval between markers SC57808 (*Hv_SPL14*)–CAPSK06413 residing on chromosome 7H. This region putatively hosts more than 800 genes, as deduced by a comparison with barley, rice, sorghum, and *Brachypodium* collinear regions [25]. However, the classical map-based cloning of the gene *dsp.ar* is complicated because of the unfavorable relationship between the genetic and physical distances at the target locus [23]. In the same position of *dsp.ar*, Taketa et al. [26] have mapped *dsp.1*. The same authors have positioned *lks.2* (short awns) on chromosome 7. Houston et al. [27]) exploited an allelic series of *Zeo* mutants and demonstrated that dense spikes are largely caused by polymorphisms in the microRNA172 (miR172)-binding site of the *HvAP2* gene. This is an ortholog of an *APETALA2* transcription factor. When examined in detail, the authors demonstrated that HvAP2 turnover driven by microRNA 172 regulates the length of a critical developmental window required for the elongation of the inflorescence internodes. In other words, the increase or decrease of this developmental temporal window can dramatically impact the spike density [27].

Figure 4. Different spike density: lax spike (**left**) and dense spike (**right**).

9. Cleistogamy

Cultivated barley is an autogamous plant. In the barley flower, the stigmas become receptive before anther extrusion and, when the anthers become ready for pollination, the stigmas are able to capture sufficient self-pollen without fertilization by windborne non-self pollen. On the contrary, in some wild accessions, the extrusion of the anthers is so pronounced that the rate of outcrossing is strongly increased in comparison with cultivated barley. There are natural variants of cultivated barley in which it closed flowering can be observed, due to the fact that palea and lemma remain tightly closed throughout the period of pollen release. Such a phenomenon is known as cleistogamy. Cleistogamous flowers typically have smaller lodicules in comparison with the non-cleistogamous types. A canonical barley floret is therefore strictly cleistogamous; however, mutants characterized by a variation in the cleistogamy level have been described and have been the starting point for the identification of the *cly1* gene. This is the same gene as the previously cited *HvAP2,* involved in the spike density phenotype. The cleistogamous state in barley is recessive and is under the control of a single gene at the *cleistogamy 1* (*cly1*) locus, which maps to the long arm of chromosome 2H. Nair et al. [28] have isolated *cly1* by positional cloning. This gene is a transcription factor containing two AP2 domains and a putative

miR172 targeting site, and codes for an AP2-protein that inhibits the development of the lodicule, avoiding the opening of the flower and the subsequent pollen dispersal and cross-pollination. *Cly1* gene expression is therefore epigenetically regulated. In non-cleistogamous barleys, *cly1* mRNA is subjected to a cleavage directed by miR172, resulting in low levels of the AP2 protein and floret opening. On the contrary, in cleistogamous genotypes, *cly1* mRNA is not cleaved and therefore, high levels of the AP2 protein result in the failure of lodicule swelling. However, there are genotypes in which alternative mechanisms of regulation can impact on the cleistogamy level. Wang et al. [29] showed that the cv. SV235 is cleistogamous, and in this genotype, the downregulation of *cly1* is unrelated to miR172-directed mRNA degradation, and is caused by an epiallele that represses transcription.

10. Lemma and Awn

The barley floret is protected by two leafy organs, the lemma and the palea, both considered to be reduced vegetative leaves. The upper part of the lemma forms the awn. Several mutations can perturbate the canonical development of these structures [30]. Among these, there is the dominant *Hooded* (*K*) mutation. In such mutants, a flower develops on the lemma instead of the awn (Figure 5).

Figure 5. *Hooded* spike in comparison with awned and awnless ones.

A duplication of 305 bp in intron IV of the homeobox gene *Bkn3*—belonging to the knox family and well known to have a pivotal role in the development of leaf primordia- is responsible for the *Hooded* phenotype [31]. In a *Hooded* mutant, the *Bkn3* gene is overexpressed. Four proteins that bind the intron-located regulatory element (Kap intron-binding proteins) have been identified by Osnato et al. [32]. Two of these proteins, Barley Ethylene Response Factor1 (BERF1) and Barley Ethylene Insensitive Like1 (BEIL1), should mediate the fine-tuning of *Bkn3* expression by ethylene. In mutagenized *KK* seeds, five genetic loci (*suK*) have been identified as able to suppress *K* expression in transcription, leading to a phenotype characterized by the replacement of the ectopic *K* flower with awns shorter than the wild type [31].

The awn, an apical extension from the lemma of the spikelet, is a relevant photosynthetic organ that plays important roles in determining the grain size and yield. Wide natural variation for the awn length and shape has been observed. More than 700 short awn (*Lks*) and breviaristatum mutants

have been characterized with the tools of classical genetics. Two main groups of awn-mutants can be identified: one characterized by phenotypic variation in the awn only, and the other in which the mutated phenotype is not only restricted to the awn, but also extends to several other plant organs. Among the several loci involved in awn development, You et al. [33] studied the short awn 2 (*lks2*) gene, which produces awns that are about 50% shorter than normal, and this is a natural variant restricted to Eastern Asia. Positional cloning revealed that *lks2* encodes an *SHI*-family transcription factor. Histological observations of longitudinal awn sections showed that the *Lks2* short-awn phenotype resulted from a reduced number of cells.

11. Naked Seed

The great majority of cultivated barleys have covered (hulled) caryopses, in which outer lemma and inner palea are strictly adherent to the pericarp epidermis at maturity. However, few genotypes have free-threshing called naked (hulless) caryopses. Hulled barley is mainly used in animal feed because of its higher yield, mainly due to the fact that the hull protects embryos from damage during mechanical harvesting. Even barley for brewing is covered, because the presence of the glumes provides a filtration medium in the separation of fermentable extract (wort) during malt processing. Obviously, covered grain is an adaptive trait in the wild, but during the domestication process, naked barley has been selected for direct human consumption. Barley domestication has been proposed to have originated more than 10,000 years before present [34], whereas naked barley appeared around 8000 years before present in Neolithic agricultural settlement sites in the Near East and western India, and quickly spread to Europe, Africa, and Asia. Although naked barley is today distributed worldwide, it is more frequent in East Asia, especially in the highlands of Nepal and Tibet. It has recently been used to clone the gene *nud*, which controls the covered/naked caryopsis. The gene has been mapped on chromosome arm 7HL and its greatest level of expression has been localized to the testa. The *nud* gene is homologous to the *Arabidopsis WIN1/SHN1* transcription factor gene, which is involved in the lipid biosynthesis pathway. In barley, the hulled caryopsis is therefore controlled by an *Ethylene Response Factor* (*ERF*) family transcription factor gene regulating the lipid biosynthesis pathway [35]. Briefly, the *nud* gene regulates the deposition of lipids on the epidermis of the pericarp. In covered barleys, this lipid layer is present and favours the adhesion of the hull to the caryopsis surface. On the contrary, in naked barleys, the lipid layer is missing and no adhesion is ensured between the hull and caryopsis, resulting in the free-threshing of the hull at maturity.

12. The Genetic Background Effect

Starting from the observation of barley mutants, new interrogatives have raised the question about how the behavior of a single developmental gene in different genetic backgrounds could help in understanding phenomena like expressivity, penetrance, phenotypic plasticity, and instability. An example of expressivity modulation has been found in genetic materials bearing a lemma mutation. The "*Leafy lemma*" phenotype was isolated in 1990 at the Istituto Sperimentale per la Cerealicoltura (Fiorenzuola d'Arda, Italy), in a plot in which the recessive mutant short awn (*lk2*) was grown. In the mutant, the lemma is transformed in a leaf-like structure. The transition zone of the lemma is particularly similar to the ligule-auricle region. Genetic analysis was carried out by crossing the mutant with several wild type genotypes and the segregation ratio was 15:1 [30]. The *Leafy lemma* mutant is characterized by the transformation of the lemma in a leaf-like structure, with a consequent increase in the seed size and photosynthetically active area (Figure 6).

Classical genetic information is available for the *Leafy lemma* mutant [30], indicating the involvement of two independent genes in the mutant phenotype. In the frame of the CREA-GB's mutant collection, 27 pairs of sister lines (wt/*Leafy lemma*) were generated in a backcross program in which the *lel* mutation has been introgressed in the 'Kaskade' background. These lines carry a *lel* mutation in different genetic backgrounds derived from different combinations of the parental genomes. In the lines carrying the mutation, a wide variation in the size of the mutated lemma can be

observed. In other words, the mutated lemma ranges from a small foliar-like structure to a large one, depending on the genetic background characteristic of each line. Figure 7 reports the mean areas of the mutant lemmas in the 27 sister mutant lines.

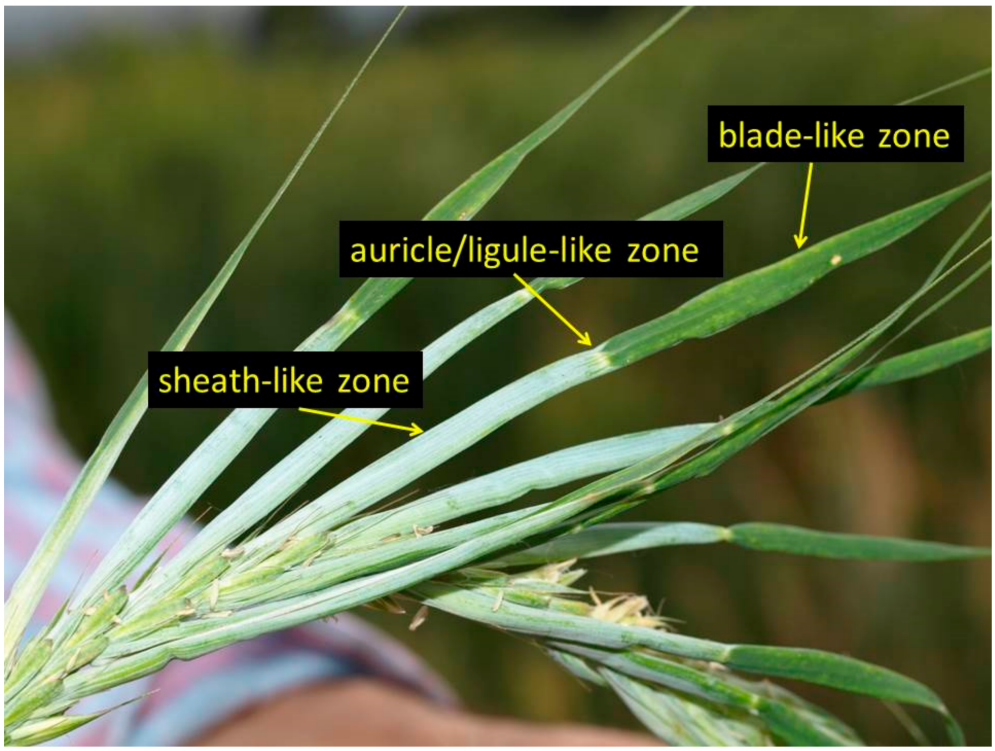

Figure 6. Spike of *Leafy lemma* mutant.

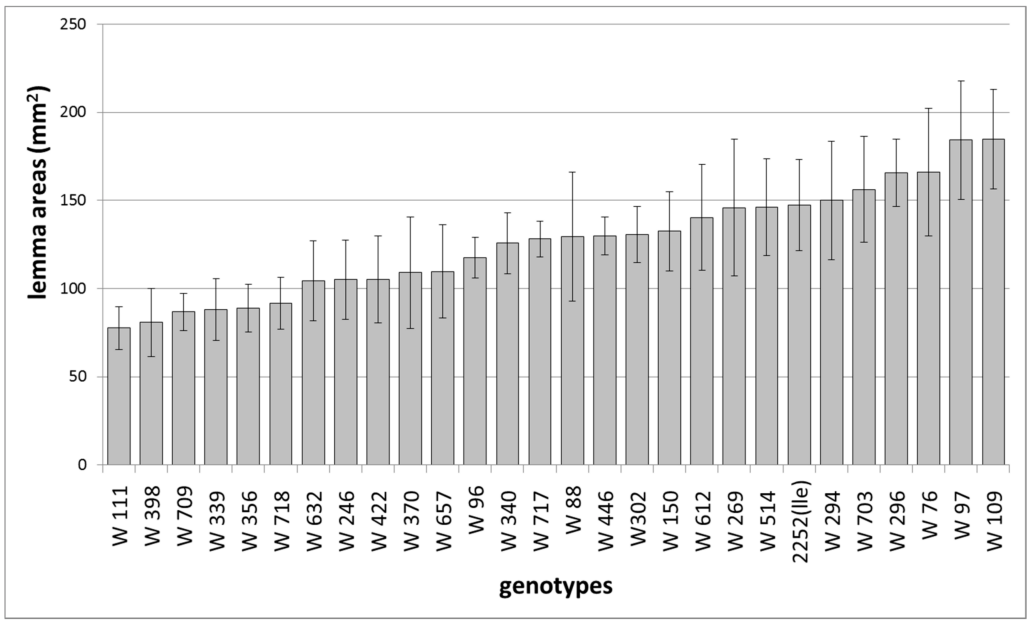

Figure 7. Different expressivity of the mutation—in terms of mutated lemma size—in sister lines derived from the cross between *Leafy lemma* mutant and Kaskade cultivar. The *x*-axis illustrates the labels of the different sister lines, whereas the *y*-axis represents the areas (expressed as mm^2) in which the mutated lemmas are reported.

In this genetic system, the expressivity of *lel* genes are clearly dependent on the genetic background, although the molecular basis of this epistatic effect is totally unclear. It is well known that genetic background effects contribute to the phenotypic consequence of a mutation, even if little is known about how these effects modify genetic systems. In the Drosophila model system, Dworkin et al. [36], working on the background effect on the expressivity of a *Scallopped* mutation, found that the phenotype is mediated through the misregulation of a series of developmental patterning genes, the epistatic interaction between mutated genes is background dependent, and the phenotypic variations correlate with qualitative and quantitative differences in downstream gene expression.

The genetic background—and the interaction among some specific genes—can be responsible not only for phenotypic variations, but also for other complex effects, like phenotypic instability. A very interesting example is presented in the work of Siuksta et al. [37]. Working on barley double mutants, obtained by crossing single ones, these authors focused their attention on *Hv-Hd/tw2* double mutants, characterized by inherited phenotypic instability. Some of the *Hv-Hd/tw2* mutants are phenotypically instable and this phenomenon is evident through several generations. Inflorescence variation in these double mutants includes the development of ectopic flowers, which range from negligible outgrowths to flowers with sterile organs, together with several new features of the spike, such as bract/leaf-like structures and naked gaps in the spike. This same phenomenon of phenotypic instability has been observed in our CREA-GB's collection [38], in which double and triple mutants have been developed by crossing the *Hooded* genotype with other homeotic single mutants. In this collection, rare "*Seeded in Hood*" have been found, in which small seeds in some hoods are developed. *Seeded in Hood* is a spontaneous phenotype that occurred in a plot of the double mutant *deficiens (Vrs1.t) x Hooded (K)*". In the *deficiens* background, the size of the hood is bigger than in the other *Hooded* mutants and, probably due to this characteristic, some fertile florets can develop in the hood (Figure 8).

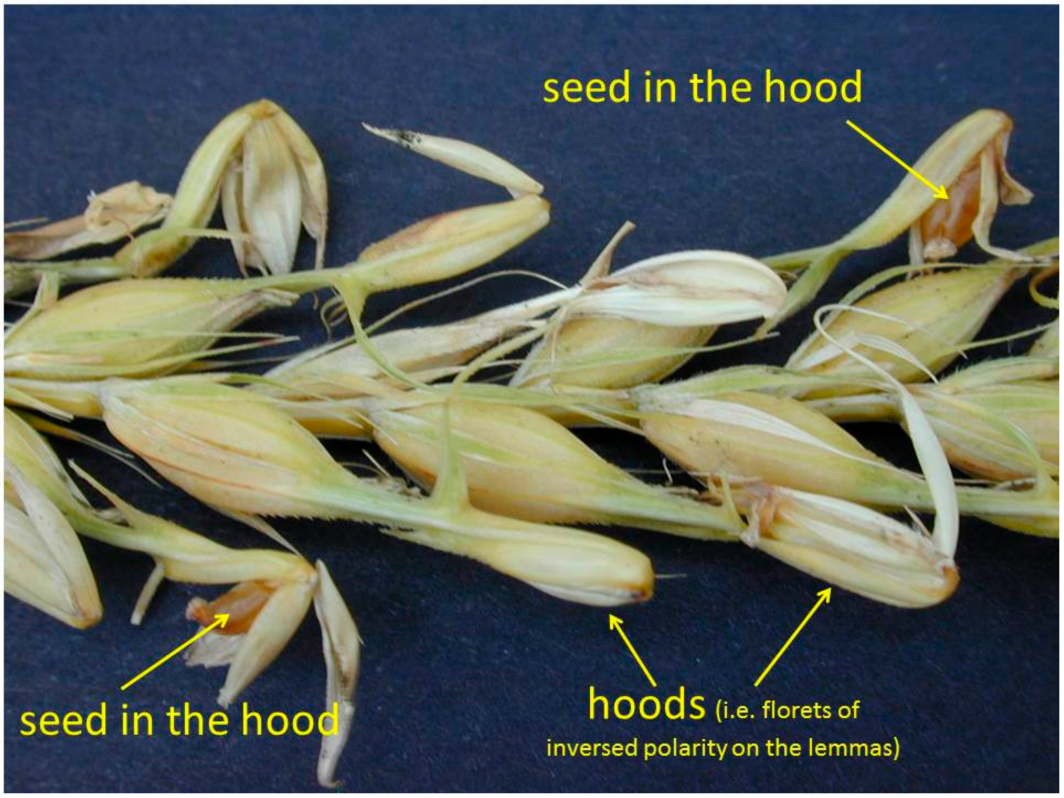

Figure 8. Two small seeds in the hoods of the "*Seeded in Hood*" double mutant, derived from the cross *deficiens/Hooded*.

This fact is evidenciated by the presence of small seeds in the hood. These small seeds have been grown in a glasshouse and the F1 plants grow normally as the *Hooded* phenotype. The F2 seeds derived from the F1 plants, also grown in a glasshouse, generated complex segregant populations, characterized by extreme phenotypic instability, with different *Hooded* spikes, including *Elevated hooded*, *Branched hooded*, and the first stage of hood development (*Trifurcatum*) (Figure 9).

Figure 9. Some phenotypes of the F2 progenies derived from the segregation of the "*Seeded in Hood*" caryopsis. Top left is a hooded branched spike, top right is a trifurcatum spike, and bottom shows hooded and elevated hooded spikes.

Siuksta et al. [37] suggested that unbalanced hormonal pathways can be a key factor to explain such a phenomenon. In particular, auxin interaction with the *Kn1* gene and other homeotic genes can determine an imbalance in auxin distribution, resulting in phenotypic instability. To demonstrate this hypothesis, Siutska et al. [37] treated flower/spike structures of double mutant lines with auxin inhibitors and with 2,4-D. They observed a normalization effect on the phenotype caused by auxin inhibitors—and an opposite effect of 2,4-D-, indicating that ectopic auxin hyper-accumulation probably plays a role. However, there marked variability among different double mutant lines in the response to 2,4-D and auxin inhibitors. This fact can be explained by the role played by the different genetic backgrounds resulting from segregation. In conclusion, double and triple mutants can be a very promising system to elucidate the molecular mechanisms at the basis of phenomena already described by classical genetics, like expressivity, which still remain largely unclear.

13. Conclusive Remarks

The study of barley morphological mutants is useful at two different levels:

- from an applied point of view, to identify loci that control traits of agronomic and qualitative relevance for pre-breeding and breeding programs;

- from a speculative point of view, as unique tools to better understand the developmental plan of a crop and the major forces driving its evolution.

Regarding this last point, it is well known that genetic changes can occur at a nucleotide scale (single-nucleotide variations-SNV-, insertions/deletions—InDels), at a gene scale (e.g., copy number variations-CNVs), and at a chromosomal scale. At a nucleotide scale, for example, a single SNV created the cleistogamous flower type in barley. Structural variations (SV), at chromosomal a scale, are another relevant source of genetic diversity [39] and, among the mechanisms responsible for SV, transposable element (TE) dynamics are relevant. TE jumping can lead to genome rearrangements and changes in the genome size. The activity of TEs, in particular the DNA repair after TE excision, is a known source of mutations, which can accelerate genome evolution [40]. Gene enhancers and promoters can be altered by TE activities, as well as gene coding regions. TEs are highly represented in the barley genome, constituting more than 60% of the whole genome sequence, and are involved in the creation of novel genetic diversity. For example, the duplication of the *HvHox2* gene, due to TE activity, and its neo-funzionalization, created a row-type spike [16]. If TE dynamics can heavily modify the genome structure, it has been suggested that chromatin modifications and, in general, epigenetic regulation, can shape the TE-induced effects [41]. Verisimilarly, a combination of these mechanisms is at the basis of the continuum of morphological variants that can be observed in a barley spike. However, this is only speculation, and needs experimental demonstrations to be proved. To this aim, the recent development of "fast-forward genetics", based on NGS technologies, offers a new and powerful tool to geneticists and developmental biologists. The legacy of barley developmental mutants, obtained by meticulous research over half a century, together with derived mapping populations, can now be exploited in mapping-by-sequencing studies. Some examples of such an approach are already available. Mascher et al. [42], studying the multinoded phenotype, demonstrated that exome sequencing performed on phenotypic bulks of a mapping population is a feasible strategy to identify candidate genes involved in the mutated phenotype. Liu et al. [43] applied genotyping by sequencing (GBS) on a recombinant inbred line population (GPMx) derived from a cross between a two-rowed and a six-rowed barley. These authors identified three Quantitative Trait Loci (QTL) linked to plant height, the first in a region encompassing the spike architecture gene *Vrs1* on chromosome 2H, the second in an uncharacterized centromeric region on chromosome 3H, and the third in a region of chromosome 5H coinciding with the previously described dwarfing gene *Breviaristatum-e* (*Ari-e*).

Even genome-wide reverse genetics in barley open new perspectives to understand the gene-function relationships. New genetic materials can be developed with an alternative approach to the classical isolation of mutants, i.e., new mutations can be created through targeted mutagenesis and genome editing [44]. Even TILLING (Targeting Induced Local lesions IN Genomes) populations can be useful for forward and reverse approaches, and can provide multiple independent alleles of a single gene with varying levels of phenotypic expressivity. TILLING populations have been developed in Barke barley cultivar and have been used on the one hand to screen morphological mutants (forward genetics), and on the other to determine the molecular mutation frequency of candidate genes (reverse genetics) [45]. A detailed TILLING analysis for the vHox1 gene, controlling the row-type morphology, identified a set of nucleotide positions that, if mutated, alters the row spike morphology, indicating the existence of functionally relevant sites of the HvHOX1 protein.

In conclusion, spike morphology is associated with row type, grain density, spike length and grain number, and is a target of central importance in crop improvement. The barley developmental mutants have demonstrated their high value for the identification and functional characterization of key genes that can be important in prebreeding for ideal plant architecture (IPA), as a means to enhance the yield potential of existing elite varieties and to design the plant for the future.

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