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A Weak Allele of *FASCIATED EAR 2* (*FEA2*) Increases Maize Kernel Row Number (KRN) and Yield in Elite Maize Hybrids

Khuat Huu Trung ¹, Quan Hong Tran ¹, Ngoc Hong Bui ¹, Thuy Thi Tran ¹, Kong Quy Luu ², Nga Thi Thu Tran ³, Loan Thi Nguyen ¹, Diep Thi Ngoc Nguyen ³, Bach Duong Vu ², Duong Thi Thuy Quan ¹, Diep Thuy Nguyen ¹, Hien Thi Nguyen ³, Cuong Cao Dang ³, Bao Manh Tran ³, Tran Dang Khanh ^{1,4} and Son Lang Vi ^{2,5,*}

¹ Department of Genetic Engineering, The Agricultural Genetics Institute, Km2 Pham Van Dong Street, Hanoi 122000, Vietnam; khuathuutrong@yahoo.com (K.H.T.); quan.th.93@gmail.com (Q.H.T.); buihongngoctb@gmail.com (N.H.B.); thuy80vdt@gmail.com (T.T.T.); nguyenloan0110@gmail.com (L.T.N.); quanthithuyduong01121997@gmail.com (D.T.T.Q.); nguyenthuydiep2002@yahoo.com (D.T.N.); khangkonkuk@gmail.com (T.D.K.)

² Faculty of Biotechnology, Chemistry and Environmental Engineering, PHENIKAA University, Yen Nghia, Ha Dong, Hanoi 12116, Vietnam; luuquycong_s15@hus.edu.vn (K.Q.L.); sutuhadong.2009@gmail.com (B.D.V.)

³ Thai Binh Seed Group Joint Stock Company, 36 Quang Trung Street, Phuong Tran Hung Dao, Thai Binh 410000, Vietnam; tranthunga@thaibinhseed.vn (N.T.T.T.); ngocdiepkhctdk56@gmail.com (D.T.N.N.); hiennt.bvtv@gmail.com (H.T.N.); dangcaocuong@thaibinhseed.vn (C.C.D.); tranmanhbao.tsc@gmail.com (B.M.T.)

⁴ Center for Agricultural Innovation, Vietnam National University of Agriculture, Hanoi 131000, Vietnam

⁵ PHENIKAA Research and Technology Institute (PRATI), A&A Green Phoenix Group JSC, No. 167 Hoang Ngan, Trung Hoa, Cau Giay, Hanoi 11313, Vietnam

* Correspondence: son.vilang@phenikaa-uni.edu.vn; Tel./Fax: +84-90-320-2285

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Abstract: Meristems are central to plant growth and development, yet evidence of directly manipulating this control to improve crop yield is scarce. Kernel row number (KRN) is an important agronomic trait that can directly affect maize (*Zea mays* L.) yield. However, this trait is difficult to select by phenotyping, since it is highly variable in the mixed genetic backgrounds in early selfing generations. This study sought to improve this trait by marker-assisted backcrossing (MABC) of a weak allele of *FASCIATED EAR 2* that is known to affect inflorescence meristem size, but the effect of which on yield is unclear. All of the four introgressed tropical elite inbreds of different heterotic groups, which are homozygous for the *fea2-1328* allele, had 2–5 more KRN compared to their respective recurrent parents. Furthermore, one hybrid made from crosses between two introgressed parents also had KRN increases that resulted in up to 28% yield increase compared to the original hybrid across multiple yield trials. The novel negative effects of the pericentromeric *fea2* and/or its linkage drag effect on plant height, seed weight, and ear length, which could prevent line improvement, were revealed in several genetic backgrounds. Integration of conventional phenotypic selection to overcome these undesirable effects was discussed. This is the first work to demonstrate the possibility to increase yield of maize varieties using a mutation in a meristem size regulator. The crossing, selection strategies, and recombinant lines in this work can be applied to other elite maize hybrids and provide a potentially straightforward, non-transgenic way to improve the yield of an existing variety by 8–28%.

Keywords: maize yield; marker assisted selection; backcross; MAS; kernel row number; KRN; *FEA2*

1. Introduction

Maize (*Zea mays* L.) is one of the most important crops and is providing billions of dollars annually to agriculture, supplying over 30% of the calories consumed by humans [1]. Maize is cultivated in over 165 countries under different complex agroclimatic conditions, with a total global harvest area of approximately 187 million hectares, and producing 1.14 billion tonnes worldwide [1,2]. In the last century, yields of maize and its quality have been significantly increased via conventional and advanced hybrid programs. However, the adverse impacts of climate change on both abiotic and biotic stresses, such as temperature increase, changes in precipitation patterns, and pests/disease infestations, have significantly reduced worldwide maize productivity [3,4]. Notwithstanding, the demand for maize has steadily increased in recent decades and is expected to continue to rise, with estimated maize consumption to double by 2050 [2,5].

In Vietnam, maize is the second biggest annual crop after rice in terms of harvested area and is consumed for animal feed in the livestock and poultry industry. This crop is cultivated in diverse environments of both rainfed upland and irrigated lowland areas in the northeast, northwest, central highlands, and southeast regions [6]. Maize production and harvested areas increased almost 3-fold from 0.43 million ha in 1990 to 1.18 million ha in 2014, with remarkable yield increases from 1.55 to over 4.0 t ha⁻¹ [7]. However, maize yield in Vietnam is still lower compared with global averages due to both breeding and agriculture practices. Since maize is a crop that exhibits strong heterosis, maize breeding often involves developing the best inbred lines with good characteristics and high combining ability to make hybrids [8]. Once a good pair of parents from opposite heterotic groups have been selected, the further improvement of this hybrid will focus on improving each parent/both parents without affecting their combining ability [8]. Therefore, backcross is often used to improve an existing elite maize variety.

Marker-assisted backcrossing (MABC) is a useful strategy to introduce a new trait to a variety [9]. This method uses molecular markers to track a locus of interest so the trait can be selected based on genotype. The loci of interest from a donor parent (DP) are transferred to a recurrent parent (RP) by repeated backcrosses to the RP. This approach has successfully improved several traits, such as protein quality using *opaque 2* and *opaque 16* markers [10,11], beta-carotenoid content using *CrtRB1* and *LcyE* markers [10,12–14], disease resistance [15–17], oil content [18], and drought tolerance [19–21]. For introducing a recessive trait, backcrossing must be carried out in both parents.

Yield of maize is a complex quantitative trait determined by multiple component traits, such as number of ears per plant, kernel weight (KW), kernel row number (KRN), and number of kernels per row [22]. Of these, KRN is one of the most important traits and a key target for breeders [23–25]. QTL (Quantitative Trait Loci) that control KRN and yield have been studied extensively [22,26–30], and some of them have been cloned [23,25].

FASCIATED EAR 2 was cloned from a maize mutant with fasciated ear tips [31]. *FEA2* is homologous to *CLAVATA2* from *Arabidopsis*, and encodes a leucine-rich repeat receptor-like protein (LRR-RLP) that acts as a negative regulator of inflorescences' meristem proliferation and size. Bommert, Nagasawa, and Jackson [23] showed that *FEA2* underlies a KRN QTL in a B73/Mo17 RIL (Recombinant Inbred Line) population. Although null *fea2* mutants have enlarged and deformed ear tips and short ears with disorganized rows, a TILLING (Targeting Induced Local Lesions in Genomes) approach identified several alleles of *FEA2* with normal shaped ears and increased KRN. The *fea2-1328* allele was found to have the strongest effect, with an increase of approximately three KRN, without reducing ear length. However, the impact this allele has on overall yield, or its effect in other genetic backgrounds, is unknown.

The idea of increasing KRN to improve yield is controversial because without increasing overall photosynthesis capability, increasing KRN may lead to a compensatory shortening of ears and/or smaller seeds, without a yield increase. Hence, the impacts on maize yield by favorable KRN QTLs in different genetic backgrounds have remained uncertain. Therefore, we investigated the potential to

increase KRN and yield of elite tropical maize germplasms and their hybrids using a weak *fea2* allele, and showed that indeed, this can lead to yield increases.

2. Materials and Methods

2.1. Plant Materials

LVN10 and VS36 are two elite maize hybrids that are being commercialized in Vietnam [32,33]. Both maize hybrids are well adapted to many cultivating areas of this country and there is a demand to improve their yield. ML10, BL10, MVS36, and BVS36 are maternal parents (ML10, MVS36) and paternal parents (BL10, BVS36) of two elite maize hybrids, LVN10 and VS36, respectively. These parents were generated by Vietnamese researchers via traditional selfing and phenotypic selection methods. ML10, of which a genome has been re-sequenced, has been extensively used in Vietnamese maize breeding programs and to generate a mutation population [32]. The four parental inbreds belong to the different heterotic groups. All *fea2* alleles in W22 background were provided by David Jackson, CSHL, New York, USA [23]. For phenotypic measurements of parental introgression lines (ILs), several homozygous *fea2-1328*-/- BC₃F₃ families were used. For the LVN10 *fea2*-/- hybrid, homozygous parental lines from BC₃F_x IL and BC₃F_x IL families were used to make crosses; with “x” refers to the selfing generation. The hybrid seeds LVN10 *fea2*-/- used in the Thai Binh Spring cropping season in 2017 were ML10-IL1 (BC₃F₃), crossed by BL10-IL1 (BC₃F₃). The seeds used in Thai Binh in Autumn 2017 and Son La in Summer 2018 were ML10 BC₃F₄ (derived from selfed ML10-IL1 (BC₃F₃)) × BL10 BC₃F₄ (derived from selfed BL10-IL1 (BC₃F₃)). The seeds used in Ha Noi and Thai Binh in Spring 2020 were ML10 BC₃F₆ × BL10 BC₃F₆. All materials are available for research and breeding via MTA (Material Transfer Agreement) by directly contacting Son Lang Vi.

2.2. Field Growing Conditions, Agronomic Trait Measurements, and Yield Trial

Maize was grown on fields in Ha Noi (21°06′19.3″ N, 105°49′32.2″ E), Thai Binh (20°33′44.8″ N, 106°24′35.6″ E), and Son La province (21°13′53.8″ N, 103°57′37.8″ E), Vietnam. Spring crop seasons are Feb–Jun; Autumn seasons are Sep–Jan; Summer seasons are May–Sep. Fertilizer was applied at the rate of 150 kg N, 90 kg P₂O₅, and 90 kg K₂O per ha. All P₂O₅ was applied at planting, and then, N and K₂O were applied twice at 20 days (6-leaf stages) and 50 days (before bolting stages) after planting.

Yield trials and phenotypic measurements were carried out following the randomized complete block design. For inbreds and introgression lines, each line was planted using one replicate. For hybrids, each genotype was replicated at least three times. For each replicate, an entry was planted in four-row plots, 5 m long, with 0.75 m spacing between rows and 0.25 m between plants in a row. Only two middle rows were used for measurements of yield and other traits.

For yield calculation: Yield was calculated as: $(P3 \times P1) \times 10,000 / (P2 \times n \times 0.75 \times 0.25)$ kg/ha.

- *n* is the total number of plants in the two middle rows.
- P1 is the sum weight of all the fresh effective ears, which includes both the first and second ears that have more than 20 kernels, from the two middle rows (kg).
- P2 is the sum fresh weight of the ears from sample plants (kg). Twenty plants (ten consecutive plants from the 5th to the 14th from each of the two middle rows) per genotype were taken as the sample plants.
- P3 is the weight of the resulting dry seeds (at 14% humidity) from sample plants (kg).

For plant trait measurements, measurements were performed using sample plants only. Plant height, ear height, tassel branch number, kernel row number, ear length, and 1000 dry seed weight (at 14% humidity) were measured as follows. Plant height—the distance from the base of the plant to the height of the first tassel branch; ear height—the distance from the base of the plant to the node of the highest effective ear; tassel branch number—the number of primary branches; kernel row number—determined at the middle

of the ear. For most entry, at least 20 plants ($n \geq 20$) were used for measurements). For 1000 dry seed weight, measurements were repeated three times on pooled seeds from all sample plants ($n \geq 3$).

2.3. Marker-Assisted Backcrossing

The scheme of applying MABC is shown in Figure 1a. The mutant *fea2-1328* with a W22 inbred background was used as a donor parent (DP) and ML10, BL10, MVS36, and BVS36 inbreds were used as recurrent parents (RPs). Homozygous mutants of *fea2-1328* (W22) were crossed to RP to create F_1 , which were then backcrossed to the corresponding RP to make BC_1F_1 . Genotyped heterozygous BC_1F_1 s that phenotypically resembled the RP the most were backcrossed to the RP to develop BC_2F_1 . This backcross process was repeated to obtain BC_3F_1 . The BC_3F_1 s were selfed to make BC_3F_2 s, which were genotyped to find the homozygous type. Homozygous BC_3F_3 s were selfed for further generations and selected based on the similarity to the RP and homogeneity.

Figure 1. (a) The schematic diagram of developing introgression lines; the averages of kernel row number (KRN) were given in brackets for each line. (b) Genotyping the *fea2-1328* alleles with a dCAPs marker. A F_2 family segregating for *fea2-1328* was analyzed with the dCAPs marker using oSV1 and oSV2 primers. PCR products were cut with PstI and analyzed on 3% agarose gel. This marker clearly distinguished three types of genotypes: wild-type plants (lane number 1, 10, 12, and 14), homozygous mutant plants (lane number 5, 13, and 15), and heterozygous plants (lane number 2, 3, 4, 6, 7, 8, 9, 11, and 16).

In each F_1 generation of a backcross, i.e., BC_yF_1 , 8–20 plants were genotyped for *fea2-1328* alleles. In each F_2 generation of a backcross, BC_yF_2 , 16–40 plants were genotyped for *fea2-1328* alleles. For each RP, at least 2–8 of the best backcross families were used for the next backcross.

At BC_3F_6 , the introgression lines derived from BL10 and ML10 were subjected to genome background checking by SSR (Simple Sequence Repeats) analysis. Two-hundred SSR markers, sequences of which were obtained from www.maizegdb.org, were used to screen for polymorphic markers between BL10, ML10, and DP. Identified polymorphic markers were then used to genotype BL10-IL1(BC_3F_6) and ML10-IL1(BC_3F_6) using pooled DNA of 16 individuals per family. Marker information was provided in Tables S2 and S3.

2.4. Designing New dCAPs Marker, Genotyping for *fea2* Alleles and SSR Analysis

A new dCAPS marker was re-designed based on the nucleotide sequence of the *fea2-1328* alleles published in Bommert, Nagasawa, and Jackson [23]. The information on primers and their sequences for genotyping was listed in the supporting information (Table S1). Primers were ordered from Integrated DNA Technologies Inc. (IDT), Singapore, Republic of Singapore. PCR mix was as follows: 10 μ L vol reaction; PCR buffer 10 $\times$ —1 μ L; dNTPs 10 mM—0.25 μ L; Taq—1 unit; forward and reverse primer 10 μ M—0.4 μ L each; DMSO—1 μ L; DNA template and water to 10 μ L. Thermal conditions were followed for oSV1/oSV2: 94 °C for 3 min, followed 35 cycles of (94 °C: 20 s, annealing temperature: 30 s (62 °C for oSV1 + oSV2; 55 °C for SSR markers), 72 °C: 30 s), completed with 72 °C for 5 min.

For genotyping *fea2-1328*, PCR products were then digested with restriction enzymes PstI-HF (from New England Biolabs Inc, Ipswich, MA, USA) for 2 h at 37 °C, then analyzed on the 3% agarose gel. For SSR analysis, PCR products were analyzed on 6–10% polyacrylamide gel. Example images of genotyping for *fea2-1328* are illustrated in Figure 1b.

2.5. Statistical Analysis

All field data were expressed as mean and standard error of the mean. Chi-square tests and Student's unpaired *t*-tests were applied using Excel, as indicated in the text.

3. Results

3.1. The *fea2-1328* Mutant Allele Caused Increases in KRN in All Four Tested Inbreds in F_2 Generations

Before further introgression, it is useful to predict at the early generation whether the *fea2-1328* mutation expressed in the genetic background of the four inbreds used. Therefore, we analyzed the linkage between the phenotype (KRN) and genotypes in the F_2 generations of the crosses between *fea2-1328* (in W22 background) and the RPs. We found a normal segregation in genotypes, i.e., 1:2:1 ratio, for ML10 and MVS36 (*p*-values in Chi-square test were 0.54 and 0.35, respectively) but a mild distorted segregation in BL10 and BVS (*p*-values in Chi-square test were 0.01 and 0.001, respectively) (Table S4). In three populations with ML10, MVS36, and BVS36, we obtained decent numbers of ears to measure KRN; for the BL10/*fea2-1328* F_2 population, we obtained few ears due to poor plant growth and difficulties in pollinations. We measured all ear-producing plants that were homozygous wild-type and mutant and about 15–32 randomly picked plants that were heterozygous (Figure 2). As seen in the histogram in Figure 2, plants that were homozygous for *fea2-1328* had a higher percentage of harboring a higher KRN than the plants that were homozygous wild-type. The mean KRN of homozygous mutants was larger than that of wild-type plants for all four inbreds' background, as confirmed by Student's *t*-test, with *p*-values equal to 1.1×10^{-5} , 0.023, 0.00054, and 8.6×10^{-6} for the ML10-, BL10-, MVS36-, and BVS36-derived populations, respectively (Table S5). Additionally, statistically comparing the mean KRN of heterozygous plants to wild-type and homozygous mutant plants revealed that *fea2-1328* was likely to be recessive in ML10/W22 and BVS36/W22 F_2 with mixed background, as heterozygous is not different to wild-type, but semi-dominant in BL10/W22 and

MVS36/W22 F₂ with mixed background, as heterozygous plants also showed an increase in KRN over wild-type plants, although to a lesser extent than that of mutant plants (Table S5).

Figure 2. Segregation of phenotypes and genotypes in an F₂ generation in four recurrent parent backgrounds. AA, Aa, aa: plants that are homozygous wild-type, heterozygous, and homozygous mutant for the *fea2-1328* allele, respectively. *n*: number of plants used for measurements. Genotyped plants were picked randomly for phenotyping; the number of plants indicated did not reflect the segregation ratio of different genotypic classes.

Therefore, *fea2-1328* mutation expressed well and caused an increase in KRN in the F₂ generation of all four inbreds and the degree of dominance/recessiveness of *fea2-1328* depended on the genetic background used.

3.2. Marker Assisted Backcrossing

MABC was carried out with foreground selection for the *fea2-1328* marker in each backcross generation, including F₁, BC₁F₁, BC₂F₁, BC₃F₁, BC₃F₂, and BC₃F₃. Several BC₃F₃ introgression lines that were homogeneous and resembled the RPs well were named after their respective RPs, for example, ML10-IL1, ML10-IL2, etc. These were selfed to make BC₃F₄ to BC₃F₆ and crossed to make hybrids which were used for further analysis.

To determine the introgression region in ILs, we screened all the published SSRs available on www.maizegdb.org around *FEA2* and could identify several polymorphic markers between the RP and the DP. Genotyping these markers across DP, RP, and BC₃F₄ ILs (Figure 3) revealed the size of introgression region in ILs. BVS36-ILs had recombinations for *bnlg1755* and *umc2027*; the introgression region was therefore equal or less than 35.4 Mb, the smallest among all four RPs. BL10-ILs had recombinations for *bnlg1755* and *bnlg2291*, the introgression region was equal or less than 51.3Mb. MVS-ILs and ML10-ILs only had recombinations on one side, which was at marker *bnlg1755*, 15.3Mb upstream of *FEA2*. As *FEA2* is in bin 4.05, where the centromere lies, limited recombination prevented us from narrowing down the introgression regions.

Figure 3. Analysis of the introgression region around the *FEA2* loci. SSR (Simple Sequence Repeats) analysis using template DNAs from DP, RP, and corresponding ILs. IL1-1, IL-2, or IL-3 indicated pooled DNA from individuals of the BC₃F₄ family 1, 2, or 3. (a) Polymorphic SSRs between DP and ML10. (b) Polymorphic SSRs between DP and BL10. (c) Polymorphic SSRs between DP and MVS36. (d) Polymorphic SSRs between DP and BVS36. Ladders are O’RangeRuler 20 bp DNA Ladders from Thermo Fisher. (e) Physical map of the SSRs that are close to *FEA2*.

To estimate how much of the genome background was recovered in ILs in BC₃, SSR analysis was performed. Two-hundred SSR markers spreading across ten chromosomes of maize were screened and 28 markers were identified to be polymorphic between ML10 and DP (Table S2); the figure for BL10 was 17 (Table S3). In ML10, apart from chromosome 10 where only one polymorphic marker was found, the SSR markers spread largely, even in other chromosomes (Figure S1 and Table S2). In BL10, only one polymorphic marker was found on chromosome 8 and 10 and no polymorphic markers were found on chromosome 7 and 9 (Figure S2 and Table S3). SSR analysis showed 15 out of the 17 markers and 26 out of the 28 markers were recovered in IL for BL10-IL1 (BC₃F₆) and ML10-IL1 (BC₃F₆), respectively (Figures S1 and S2). These results support the high recovery of background genome in our ILs and provide information for further backcrosses and phenotypic analysis.

3.3. The *fea2-1328* Allele Caused an Increase in KRN in most ILs in BC₃F₃ but also Affected Other Agronomic Traits

We thoroughly compared KRN and other agronomic traits between the ILs and their corresponding RPs. The comparison was carried out in BC₃F₃ generations in order to early select the best lines for further backcrosses or selfing. For each RP, one to three of the best homozygous lines were selected based on their resemblance to the RP and used for detailed analysis and measurements.

In the BC₃F₃ families, the plants were homogeneous (Figure 4). Major agronomic traits, including plant height (PH), ear height (EH) and tassel branch number (TBN), ear length (EL), ear diameter (ED), 1000 seed weight (SW), kernel row number (KRN), and number of kernel per row (KRP) between RPs and (ILs), were compared as shown in Figure 4 and Table 1. It was apparent that KRN significantly increased in six out of seven ILs (Table 1). The strongest increase was observed in MVS36-ILs of 5.6 rows (averaged two BC₃ lines), followed by 4.1 rows in ML10-ILs (averaged two BC₃ lines), 3.1 rows in BL10-ILs, and the weakest increase was in BVS36-ILs of 1.64 rows (averaged two BC₃ lines) compared to respective RPs.

Figure 4. Plants and ears of introgression lines that were homozygous for *fea2-1328* at the BC₃F₃ generation and the corresponding recurrent parents.

Table 1. Measurements of agronomic traits and yield of introgression lines and their corresponding recurrent parents.

Lines	Plant Height cm	Ratio	Ear Height cm	Ratio	Tassel Branch Number Ratio	Ear Length cm	Ratio	Diameter cm	Ratio	Kernel Row Number Row	Ratio	Kernel per Row Kernel	Ratio	Weight of 1000 Seeds Gram	Ratio	Yield ×100 kg/ha	Ratio	<i>n</i>
ML10	155 ± 1.9	1.00	54.1 ± 1.7	1.00	8.5 ± 0.7	13.0 ± 0.4	1.00	2.9 ± 0.1	1.00	11.6 ± 0.3	1.00	24.2 ± 0.8	1.00	225 ± 2.0	1.00	31.5	1.00	16
ML10-IL1	143 ** ± 2.2	0.92	48.4 * ± 1.4	0.90	9.1 ± 0.4	10.8 ** ± 0.2	0.83	3.4 ** ± 0.1	1.17	15.8 ** ± 0.3	1.36	21.3 * ± 0.7	0.88	201 * ± 1.8	0.90	31.0	0.98	30
ML10-IL2	143 ** ± 2.1	0.92	43.3 ** ± 1.6	0.80	7.7 ± 0.3	10.6 ** ± 0.2	0.81	3.3 ** ± 0.1	1.11	15.5 ** ± 0.3	1.33	22.4 ± 0.6	0.92	195 ** ± 1.5	0.87	30.0	0.95	27
BL10	165 ± 3.7	1.00	60.4 ± 1.6	1.00	15.3 ± 3.4	9.6 ± 0.2	1.00	3.0 ± 0.0	1.00	9.5 ± 0.2	1.00	24.8 ± 0.7	1.00	156 ± 0.8	1.00	21.0	1.00	26
BL10-IL1	168 ± 2.8	1.02	58.8 ± 1.5	0.97	14.2 ± 0.4	9.6 ± 0.2	0.99	3.1 ± 0.0	1.02	12.6 ** ± 0.4	1.32	24.0 ± 1.1	0.97	148 ± 1.2	0.94	18.5	0.88	24
MVS36	146 ± 5	1.00	62.9 ± 1.3	1.00	13.0 ± 0.3	9.7 ± 0.3	1.00	3.4 ± 0.1	1.00	13.4 ± 0.3	1.00	20.2 ± 0.8	1.00	234 ± 1.8	1.00	30.1	1.00	29
MVS36-IL1	140 ± 2.4	0.96	63.6 ± 1.4	1.01	13.1 ± 0.3	10.2 ± 0.5	1.05	4.1 ** ± 0.1	1.20	19.0 ** ± 0.5	1.42	18.6 ± 0.9	0.92	175 ** ± 2.5	0.75	31.5	1.04	28
MVS36-IL2	153 ± 3.0	1.05	60.2 ± 2.1	0.96	11.8 * ± 0.3	10.0 ± 0.3	1.03	3.8 ** ± 0.1	1.10	19.0 ** ± 0.4	1.41	21.5 ± 0.9	1.07	151 ** ± 2.2	0.65	29.0	0.96	28
BVS36	135 ± 1.5	1.00	36.3 ± 1.1	1.00	8.8 ± 0.3	10.6 ± 0.4	1.00	3.3 ± 0.0	1.00	11.1 ± 0.3	1.00	20.8 ± 1.0	1.00	228 ± 0.5	1.00	20.4	1.00	23
BVS36-IL1	125 ** ± 2.2	0.93	26.7 ** ± 0.8	0.74	7.0 ** ± 0.2	8.6 ** ± 0.4	0.81	3.9 ** ± 0.1	1.16	14.5 * ± 0.4	1.31	14.0 ** ± 0.9	0.67	281 ** ± 1.5	1.23	22.4	1.10	24
BVS36-IL2	131 ± 2.1	0.97	35.1 ± 1.1	0.97	4.5 ** ± 0.3	8.7 ** ± 0.2	0.81	3.2 ± 0.1	0.97	11.6 ± 0.3	1.04	16.6 ** ± 0.6	0.80	228 ± 1.8	1.00	20.6	1.01	26

Mean value ± standard error of the mean (s.e.m) is shown in real value units; the ratio was calculated over the value of the corresponding parent. *n*—least number of plants used for the measurements except for the weight of 1000 seeds (*n* ≥ 3). *, ** indicate significantly different compared to the recurrent parent (Student's *t*-test, *p*-value < 0.05 and 0.01, respectively). IL—introgression line.

Several agronomic traits differed between RP and ILs, and interestingly, the degree of differences varies among inbreds, suggesting other previously undescribed functions of *FEA2* and a complex interaction between *FEA2* and genetic backgrounds in these different heterotic group parents. For example, TBN was remarkably reduced by 21% and 48% in only BVS36-IL1 and BVS36-IL2 compared to BVS36, but varied only slightly (<10%) and not significantly between other ILs and their corresponding RPs. The EL showed an approximately 20% reduction in BVS36-ILs and ML10-ILs, but was almost unchanged in MVS-ILs and BL10-ILs compared to the RPs. PH was reduced slightly by 8% in both ML10 ILs and 7% in BVS36 IL1, but it was not significantly different in the MVS36 ILs and BL10 ILs as compared to RPs. The averaged SW was reduced by approximately 10% in ML10 and 30% in MVS36 background, increased 23% in BVS36-IL1 but did not differ significantly for BL10. As a combination of changes in KRN, SW, and EL, the yield of ILs however did not differ significantly from the RPs. Therefore, it was concluded that despite an over 30% increase in KRN in six out of seven ILs, the yield of these ILs per se did not increase.

3.4. Potential of Using *fea2-1328* to Increase the Yield of Hybrids

Due to heterosis, hybrids may have more photosynthetic material to fill the extra seeds generated by increased KRN, hence the effect of *fea2-1328* on yield may be different between inbreds and hybrids. To further examine if *fea2-1328* can possibly increase the KRN and yield of hybrids, we made crosses between parental ILs of LVN10 hybrid and conducted a yield trial. We have prioritized the hybrid trial on LVN10 due to LVN10 being a more demanded variety in Vietnam. A yield trial was conducted in field conditions across four cropping seasons and three locations, which, respectively, represent the plateau (Thai Binh, Ha Noi) and mountainous (Son La) growing areas of LVN10 maize variety (Figure 5, Table 2). The introgressed hybrid (LVN10 *fea2*^{-/-}) seeds were made by crossing ML10-IL1 (BC₃F_x) and BL10-IL1 (BC₃F_x), with x referring to the generation of selfing. The LVN10 *fea2*^{-/-} hybrid seeds used in the Thai Binh Spring cropping season in 2017 were ML10-IL1 (BC₃F₃) crossed by BL10-IL1 (BC₃F₃). The LVN10 *fea2*^{-/-} seeds used in Thai Binh in Autumn 2017 and Son La in Summer 2018 were ML10-IL1 (BC₃F₄) × BL10-IL1 (BC₃F₄). The LVN10 *fea2*^{-/-} seeds used in Ha Noi and Thai Binh in Spring 2020 were ML10-IL1 (BC₃F₆) × BL10-IL1 (BC₃F₆). All the introgressed hybrids were homozygous for *fea2-1328*. As shown in Figure 5 and Table 2, introgressed LVN10 (LVN10 *fea2*^{-/-}) had on average 1.1 to 3.6 KRNs more than original LVN10. Interestingly, opposite to the results found in its maternal inbred ML10-ILs, the seed weights of LVN10 *fea2*^{-/-} were not reduced compared to the wild-type. Yield was increased by 8% to 28% depending on generations, testing locations, and seasons. The other agronomic traits (PH, EH, EL, TBN) of the introgressed hybrid were largely similar to that of the original hybrid (Figure 5b, Table 2). These results strongly suggested that *fea2-1328* can increase KRN and yield in maize hybrid LVN10, keeping other agronomic traits mostly unaffected.

Figure 5. Yield trial of the original LVN10 hybrid and the LVN10 hybrid with *fea2*-1328 introgressed (LVN10 *fea2*^{-/-}). (a) Yield trial data. Columns showed yields and diamond dots showed KRN. Unfilled symbols are LVN10 control; black-filled symbols are LVN10 *fea2*^{-/-}. Percentages of yield increase over original LVN10 were showed on top of the columns. Yield trials were carried out in three different locations and four seasons as shown. * and ** symbols indicate significantly different by Student's unpaired *t*-test at $p < 0.05$ and $p < 0.01$, respectively. Error bars are s.e.m, $n \geq 3$ for yield; $n \geq 20$ for KRN. (b) Example images of plants and ears of LVN10 and LVN10 *fea2*^{-/-} taken in Son La in summer 2018.

Table 2. Measurements of agronomic traits and yield of introgressed hybrid (LVN10 *fea2*-/-) and the original hybrids (LVN10) in multiple locations and seasons.

Location-Season- Hybrid	Plant Height		Ear Height		Tassel Branch Number		Ear Length		Diameter		Kernel Row Number		Kernel per Row		Weight of 1000 Seeds		Yield		n
	cm	Ratio	cm	Ratio		Ratio	cm	Ratio	cm	Ratio	Row	Ratio	Kernel	Ratio	Gram	Ratio	×100 kg/ha	Ratio	
TB-Au17-LVN10	213 ± 1.2	1.00	114.3 ± 1.5	1.00	N.D.	-	14.3 ± 0.1	1.00	4.0 ± 0.2	1.00	11.7 ± 0.2	1.00	30.6 ± 1.8	1.00	255 ± 0.5	1.00	63.5 ± 0.6	1.00	20
TB-Au17-LVN10 <i>fea2</i> -/-	219 ± 3.0	1.02	102.8 ± 2.2	0.90	N.D.	-	13.4 * ± 0.2	0.94	4.2 ± 0.2	1.04	13.8 * ± 0.2	1.18	32.1 ± 0.2	1.05	245 ± 0.8	0.96	70 * ± 0.3	1.10	20
SL-Su18- LVN10	196 ± 3.2	1.00	111.8 ± 3.2	1.00	N.D.	-	16.5 ± 0.6	1.00	4.7 ± 0.1	1.00	11.4 ± 0.6	1.00	31.4 ± 2.0	1.00	300 ± 1.8	1.00	63.3 ± 2.8	1.00	20
SL-Su18- LVN10 <i>fea2</i> -/-	209 * ± 1.5	1.06	116.4 ± 2.2	1.04	N.D.	-	16.9 ± 0.5	1.02	5.0 * ± 0.1	1.07	15.0 ** ± 0.6	1.32	39.8 ** ± 1.6	1.27	291 ± 3.2	0.97	81.1 * ± 3.3	1.28	20
HN-Sp20-LVN10	264 ± 1.1	1.00	133.5 ± 1.3	1.00	15.2 ± 0.2	1.00	17.1 ± 0.1	1.00	3.9 ± 0.02	1.00	10.4 ± 0.1	1.00	33.6 ± 0.6	1.00	264 ± 3.2	1.00	75.7 ± 4.1	1.00	60
HN-Sp20-LVN10 <i>fea2</i> -/-	244 ** ± 1.0	0.96	122.0 ** ± 0.9	0.91	13.1 ** ± 0.2	0.86	19.0 ** ± 0.1	1.11	4.2 ** ± 0.03	1.07	12.8 ** ± 0.1	1.23	33.0 ± 0.5	0.98	267 ± 4.3	1.01	96.4 * ± 3.9	1.27	60
TB-Sp20- LVN10	208 ± 2.3	1.00	119.4 ± 1.9	1.00	10.0 ± 0.3	1.00	15.5 ± 0.1	1.00	4.1 ± 0.02	1.00	11.0 ± 0.1	1.00	33.5 ± 0.7	1.00	273 ± 4.1	1.00	75.7 ± 1.7	1.00	40
TB-Sp20- LVN10 <i>fea2</i> -/-	212 * ± 0.8	1.02	117.1 ± 0.6	0.98	9.1 ** ± 0.2	0.91	15.0 ** ± 0.1	0.97	4.3 ** ± 0.01	1.05	13.0 ** ± 0.1	1.18	32.9 ± 0.3	0.98	280 ± 3.4	1.02	83.4 * ± 1.1	1.10	40

Mean value ± standard error of the mean (s.e.m) was shown in real value units; the ratio was calculated over the value of the corresponding parent. N—least number of plants used for the measurements except for the weight of 1000 seeds ($n \geq 3$), for yield $n \geq 3$. N.D.—not determined. *, ** indicate significantly different compared to the original hybrid (Student's *t*-test, *p*-value < 0.05 and 0.01, respectively). Yield trial place, and season: TB-Au17—Thai Binh, Autumn 2017; SL-Su18—Son La, Summer 2018; HN-Sp20—Ha Noi, Spring 2020; TB-Sp20—Thai Binh, Spring 2020.

4. Discussion

In this study, we introgressed a weak allele of *FEA2*, *fea2-1328*, into four Vietnamese elite inbreds and measured phenotype and yield in the ILs as well as the resulting hybrid of one pair of inbreds. Our results indicated that *fea2-1328* can increase from 2 to up to 6 more kernel rows without causing fasciation in all four genetic backgrounds tested, as well as in the LVN10 hybrid. *fea2-1328* improved both the low KRN inbred (like ML10 and BL10, BVS36 with 10–12 KRN) and high KRN inbred (like MVS36 with 14 KRN). These results have reconfirmed the effect of weak *fea2-1328* alleles on KRN as suggested by Bommert, Nagasawa, and Jackson [23] and provided the additional information that this effect can express well in other maize backgrounds with different KRN.

Besides affecting KRN, our detailed characterization of ILs of several genetic backgrounds showed new findings on the potential effect of *fea2-1328* and/or its linkage drag on other traits, notably TBN, PH, EL, and SW. Those effects were found to be dependent on the genetic background of the RP. This not only provides important information for the breeders, but also a basis for further gene function study. For example, *fea2-1328* causes a strong reduction in TBN in the BVS36 background; reduction in TBN can be a target for breeders to divert assimilates to ears rather than tassel for yield improvement. These differences between ILs and their corresponding RPs detailed in Table 1 can be explained by at least three factors: the effect of *fea2-1328* itself, the linked loci to *FEA2*, i.e., the linkage drag, and the other unlinked residual donor genetics remaining in the ILs. Our data suggest *fea2-1328* itself and/or the linked loci are the main causes because of several observations. Firstly, the reduction in EL, PH, and SW can be observed in multiple ILs of the same RP background and is also observed in several RP backgrounds, suggesting it is likely that *fea2* and/or its tightly linked loci rather than the unlinked residual donor loci, which was caused by incomplete backcross, are responsible. Because of limited recombination around *FEA2*, which is in the centromeric region, our results could not discriminate between the effect of *fea2-1328* itself or the linked loci. Our SSR analysis revealed that the introgressed linkage groups were reasonably large (over 35Mb), hence potentially having many genes that contribute to the differences between ILs and RPs. It is noteworthy to mention that our data supported the complex interaction between *fea2-1328* and the genetic background. For example, the deleterious effect (on PH, EL, SW) of *fea2-1328* seemed modest in the BL10 background.

The potentially deleterious effect of *fea2-1328* and its linkage drag on the PH, EL, and SW were not described in the previous report [23] and may have important implications for further use of this allele in maize breeding. When introgressing *fea2-1328* alleles to improve the yield of ILs or at least keep yield unaffected, breeders must consider background genetics' effect and keep the balance between KRN increase, plant vigor, and yield during backcrossing rather than selection based solely on the highest percentage of genomic RP recovery or ILs with the highest KRN increase.

Our results also revealed interesting insights into the different effects of *fea2-1328* in inbreds compared to hybrids. Clearly, the deleterious effects that *fea2-1328* had in the maternal parent ML10-IL1 (on TBN, PH, SW, and EL) were largely lifted in hybrids. We propose that the heterosis and higher photosynthesis capacity of introgressed hybrid plants created enough biomass to fill the extra kernels created by increased KRN, whereas the maternal inbred parents may not have had enough biomass to do this. It has been shown that there is a close relationship among kernel number, kernel weight, and sink/source ratio, which was controlled by the ability of plants to partition the assimilates into kernels during the grain-filling period [34–36]. Our results suggest even if the yield does not increase in both introgressed parental inbreds per se, the resulting introgressed hybrid may still have increased yield.

When using MABC targeting a fully recessive locus, making both parents carry the same locus may reduce the heterosis of hybrids. This concern is particularly relevant in the case of *FEA2*, where the pericentromeric position of *FEA2* can lead to large introgression size, which, when made homozygous in hybrids, could cause reduced heterosis. It was previously proposed that genes in centromeric and pericentromeric regions can be one important contributing factor to heterosis [37–39]. In this study, introgressed LVN10 *fea2*^{-/-} retained full heterosis and had a higher yield than the original

LVN10. This result removes the doubt about reduced heterosis caused by *FEA2*-linked pericentromeric homozygosity in introgressed hybrids, at least in the LVN10 case. Our study presented here may be useful when one is considering MABC of pericentromeric recessive locus in heterosis-sensitive crops.

The potential of using mutations in meristem size regulators of the CLV-WUS signaling pathway to improve fruit size, fruit number, and yield have been shown in several other crop species [40]. In tomato (*Solanum lycopersicum*), *fasciated and branched (fab)*, a mutation in a homolog of *CLV1* [41]; *locule number (lc)*, a mutation in a homolog of *WUSCHEL* [42]; *fasciated (fas)*, a mutation in a homolog of *CLV3* [41]; and *excessive number of floral organs (eno)*, a mutation in an upstream regulator of *WUSCHEL* [43], were all shown to cause enlarged fruits and higher locule numbers. Natural mutations in these genes have played central roles in domestication and breeding, and have contributed to the diversification of fruit size and shape in tomato [44–46]. Point mutation in the *CLV3* homolog in field mustard (*Brassica rapa*) caused an increased number of locules in siliques and had the potential to increase seed yield [47]. In rice (*Oryza sativa*), *CLV1* and *CLV3* homologs were first cloned from mutants with higher floral organ numbers: *fon1-1* and *fon4-1*, respectively [48–50]. These alleles did not produce more flower/seeds per panicle; however, an allelic mutant of *FON1* called *multi grain 1 (mg1)* [51], and an allelic mutant of *FON4* called *twin-grain 1 (tg1)* [52], were later discovered and were both reported to have two florets in the spikelet, hence having the potential to increase grain number per panicle.

Future directions are to expand this study using weak alleles of other meristem size regulators like *TD1* [53], *FEA3* [54], *FEA4* [55], *CT2* [56], and *ZmCLE7* [32,57]. It is of interest and agronomic importance to see how much KRN can increase without affecting other traits, and how much yield can be increased in hybrid using stacks of favorable KRN genes/QTLs. Furthermore, one can use CRISPR-Cas9 to generate weak alleles in meristem size regulators and test their effects on the yield of inbred per se and hybrid [57,58].

5. Conclusions

This study was the first report to demonstrate that *fea2-1328* can be utilized to improve KRN and yield of an existing hybrid and provide a previously undescribed potential function of *FEA2* on TBN, PH, and EL. Our yield-improved tropical maize germplasms are freely available to breeders for their breeding programs. This knowledge can be applied to other elite maize hybrids and provides a simple, non-transgenic (GMO) approach to improve the yield of an existing variety by 8–28%.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/11/1774/s1>, Table S1: Primers used to genotype *fea2-1328* and analyze the introgression region; Table S2: SSR markers that are polymorphic between the ML10 (RP) and *fea2-1328* -/- (W22) (DP); Table S3: SSR markers that are polymorphic between the BL10 (RP) and *fea2-1328* -/- (W22) (DP); Table S4: Genotypic segregation ratio analysis of F2 families of RP/DP; Table S5: The comparison of KRN means of different groups of genotypes in F2 population of RP/RP, Figure S1: Genome background analysis of ML10-IL1 (BC₃F₆) with SSR markers; Figure S2: Genome background analysis of BL10-IL1(BC₃F₆) with SSR markers.

Author Contributions: Designed the research S.L.V., K.H.T., B.M.T.; conducted the experiments S.L.V., K.H.T., Q.H.T., N.H.B., T.T.T., K.Q.L., N.T.T.T., L.T.N., D.T.N.N., B.D.V., D.T.T.Q., D.T.N., H.T.N., C.C.D.; analyzed the data and wrote the manuscript: S.L.V., K.H.T. and T.D.K. All authors have read and agreed to the published version of the manuscript.

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