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Development of high-protein quality leafy maize materials for forage breeding

Chi Thanh Nguyen[1], Xuan Thang Nguyen[1*], Viet Long Nguyen[2], Thi Kim Chung Nguyen[1], Thi Bich Thao Doan[1]

[1]Maize Research Institute, 229 Nguyen Thai Hoc Street, Phung Town, Dan Phuong District, Hanoi, Vietnam
[2]Vietnam National University of Agriculture, Trau Quy Town, Gia Lam District, Hanoi, Vietnam

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Abstract:

The development of high-protein quality leafy maize materials holds promise in maize breeding specialised for forage and silage. In this study, leafy maize materials were developed using conventional breeding and the application of simple sequence repeats (SSR) markers. Results from phenotyping 20 maize lines indicated that PM3, PM7, PM8, PM9, PM10, PM12, PM14, and PM17 have average grain yields equivalent to the check CML161 and significantly higher than CML165. Using primers *phi112* and *phi057,* the *opaque-2* gene was found in 10 lines. The protein content of the lines ranged from 8-11%, making them suitable for breeding quality protein maize (QPM). Notably, PM7, PM8, PM10, PM12, PM14, PM15, PM16, and PM17 have a quality index (QI) above the minimum of 0.8 and are considered QPM lines. Our findings confirmed that lines PM7, PM8, PM10, PM12, and PM17 could initially be evaluated as the most promising for QPM development. By crossing lines carrying the *opaque-2* gene with the leafy maize line (Leafy5), 11 leafy materials (LM) were successfully developed. Specifically, LM6 and LM10, with 10-12 leaves above the uppermost ear and good agronomic traits, were selected as segregating populations for background selection using SSR markers to identify the *opaque-2* gene for developing QPM leafy maize in the future.

Keywords: **forage, leafy maize, maize materials, quality protein maize, simple sequence repeat markers.**

Classification numbers: **3.1, 3.5**

1. Introduction

Maize (*Zea mays* L.) is an important crop for food security, nutrition, and global economic development [1-3]. Additionally, maize is considered a potential crop for green forage and silage due to its superior nutritional energy value and high biomass [3-5]. Using grain-purpose maize varieties with high whole-plant yield is not ideal for forage and silage because the selection of traits for grain is opposite to that for forage and silage maize hybrids [6]. Therefore, to meet the requirements of forage and silage maize, it is necessary to research and develop specialised maize hybrids with high biomass, dry matter yield, good quality, and high digestibility [6-9].

For forage and silage maize breeding purposes, the development of maize hybrids with a higher leaf number above the primary ear is important [6, 9], as the genetic correlation between the leafy trait and increased biomass is positive [10-12]. Scientists have also shown that the leafy trait in maize helps improve the quality of forage and silage, particularly by increasing digestible matter content [9, 10] and carbohydrates [12, 13] while reducing lignin [9]. Current studies elucidate that the *Leafy1 (Lfy1)* gene, which regulates this trait in maize, is considered a dominant gene [7] or an incompletely dominant gene [14].

In addition to high productivity, maize varieties specialised for green forage and silage must also have good quality, particularly in terms of protein. Protein content in maize kernels and 45% grain silage are only 10 and 9% of total dry matter, respectively [15], and essential amino acids in kernels are deficient [16, 17], with tryptophan averaging only 0.4-0.5% of the total protein available in the kernel endosperm [18, 19]. This leads to a decrease in protein quality and nutritional deficiency in food. The digested protein in conventional maize reaches only about 40% of the necessary biological value compared with milk [20, 21]. Therefore, to improve the nutritional value of forage maize, it is essential to enhance protein quality by increasing the content of essential amino acids, such as tryptophan and lysine. For lowland tropical germplasm to be considered QPM, tryptophan and lysine contents in seeds should range from 0.5-1.1% and 2.7-4.5% of total protein, respectively, with a quality index (QI) higher than 0.8 [19].

[*]Corresponding author: Email: nxthangnmri@gmail.com

It is confirmed that the recessive gene *opaque-2* (o_2) is responsible for changing protein compositions and conferring higher lysine and tryptophan contents in the kernel endosperm compared to normal maize carrying the dominant allele (O_2) [19, 22]. The better quality of endosperm protein controlled by the o_2 gene is due to increased lysine and tryptophan contents [16, 20]. Other genes that enhance essential amino acid content in maize endosperm include *floury1* (fl_1), *floury2* (fl_2), *floury3* (fl_3), *opaque-5* (o_5), *opaque-6* (o_6), *opaque-7* (o_7), *opaque15* (o_{15}), and others [23, 24]. Recently, the recessive gene *opaque-16* (o_{16}) has been discovered to have higher tryptophan and lysine contents than o_2-based maize [24-26]. These findings provide a scientific basis for QPM maize breeding.

Thus, to successfully research forage and silage-specific maize, it is essential to develop high-protein quality leafy maize materials. Based on this scientific basis, our study focused on the development of maize materials that have (i) at least eight leaves above the primary ear, high stay-green, (ii) high protein quality, and (iii) good tolerance to common maize diseases and pests, resistance to root and stalk lodging, for breeding forage maize hybrids that are highly adaptable to climate change and meet the requirements for excellent feed quality.

2. Materials and methods

2.1. Inbred lines

Twenty-three inbred lines were used in this study. Among these, twenty inbred lines designated as PM1 to PM20 were collected, selected, and developed from QPM germplasm by the Maize Research Institute, Vietnam. Two QPM lines, namely CML161 and CML165 (the female and male lines of Maize Hybrid HQ2000, respectively), derived from the Centre for International Maize and Wheat Research, were used as control checks. Additionally, one leafy line with more than eight leaves above the uppermost ear, named Leafy5, was selected and developed by Maize Research Institute, Vietnam.

2.2. Phenotyping maize lines

In Spring 2022, trials were conducted to evaluate agronomic characteristics (maturity, morphological traits of leaves, stalk, and grain yield) and tolerance of maize lines, with checks of CML161 and CML165. These trials followed a randomised complete block design (RCBD) according to the Centre for International Maize and Wheat Research's guidelines (1985) [27]. The trials were planted in four rows with three replications, with a plot size of 14 m² (5.0 m row length, 0.7 m distance between hills, and 0.25 m between plants). The trial was carried out at Maize Research Institute, Vietnam's experimental fields under irrigated conditions and on alluvial soil. Morphological and grain yield data were measured from ten plants in the two middle rows of each plot.

2.3. Sampling leaves

In Spring 2022, leaf samples from lines with good tolerance to diseases and pests were collected when the plants were approximately three weeks old for genotyping with *opaque-2* gene-specific SSR markers *phi057* and *phi112*, as applied by R. Babu, et al. (2005) [28] and according to Centre for International Maize and Wheat Research's guidelines (2008, 2016) [19, 29].

2.4. Identifying opaque-2 gene using simple sequence repeats markers

The presence of the *opaque-2* (o_2) gene regulating the trait of QPM was identified using primers *phi057* and *phi112* (Table 1). Detailed information on these markers is available in the maize database (http://www.maizegdb.org).

Table 1. Simple sequence repeat markers used in identifying *opaque-2* gene of maize lines.

SSRs	Forward sequence (5'-3')	Reverse sequence (3'-5')
phi112	TGCCCTGCAGGTTCACATTGAGT	AGGAGTACGCTTGGATGCTCTTC
phi057	CTCATCAGTGCCGTCGTCCAT	CAGTCGCAAGAAACCGTTGCC

DNA extraction was carried out according to the cetyltrimethylammonium bromide (CTAB) method [30]. The PCR reaction was performed on an Eppendorf X50S machine at the laboratory of Maize Research Institute, Vietnam, using the following programme: initial denaturation at 94°C for 2 minutes, denaturation at 94°C for 30 seconds, annealing at 56°C for 1 minute, extension at 72°C for 1 minute, repeating the second step 29 times, final extension at 72°C for 5 minutes, and maintaining at 4°C. The amplified product was subjected to electrophoresis on 2% agarose gel.

2.5. Analysing protein content and quality

Kernel samples of the studied maize lines were collected for analysing total protein, tryptophan, and lysine contents according to official standard methods of TCVN 4328:2007, TCVN 5283:2007, and AOAC994.12, respectively, at the Centre for Food Quality and Safety Research (CEFORES), Vietnam. The protein quality in maize was measured using the QI, which is the percentage of tryptophan content compared to the total protein of the sample. A QI of at least 0.8 is considered indicative of QPM maize [19].

2.6. Developing leafy maize materials with elevated protein content

Based on the results of evaluating the agronomic characteristics and tolerance of these lines combined with identifying the *opaque-2* gene using SSR markers, 10 *opaque-2* gene-carrying lines and CML161 were crossed with the line Leafy5 in Spring 2022. Eleven leafy maize

materials were developed and individually selected for forage maize breeding with high protein quality, named LM1 to LM11. In the Autumn-Winter 2022 crop season, with the aim of phenotypically selecting individuals of each material with a higher leaf number above the primary ear (at least eight leaves), these maize materials were planted with commercial maize hybrids NK7328 and LCH9 as checks and tested for morphological and tolerance traits according to Vietnam's National Standard TCVN 13381-2:2021. Special attention was given to the criteria on leaf number above the primary ear [6, 9] and stay-green ranking [31], which are important morphological characteristics in breeding maize specifically for forage and silage. Individual plants with at least eight leaves above the uppermost ear, good growth, good tolerance to common maize diseases, pests, root, and plant lodging were selected for self-pollination and development of F_2 segregating populations used for background selection with SSR markers to identify the presence of the *opaque-2* gene among leafy individuals for the development of high-protein quality leafy maize.

2.7. Screening opaque endosperm maize kernels on a light table

Light tabling was used to phenotypically select maize kernels carrying the o_2o_2 genotype based on the degree of their opaqueness. For conventional maize breeding, this method is significantly important in successfully developing maize germplasm at the segregating generations of F_2 families [19, 29, 32]. In Spring 2023, F_2 seeds of leafy maize materials were screened and around 50% of opaque kernels (score 3) were selected according to the instructions by B.S. Vivek, et al. (2008) [19].

2.8. Analysing data

Phenotypic data were statistically processed with Microsoft Excel 2016, and the analysis of variance (ANOVA) on grain yield was carried out using IRRISTAT5. Differences in mean grain yield were separated by the least significant difference test ($LSD_{0.05}$) when the F-test was significant ($P \leq 0.05$).

3. Results

3.1. Agronomic and tolerant traits of studied maize lines

The testing results showed that the maturity of studied lines ranged from 116 to 121 days and was comparable to the checks (CML161 and CML165). All lines had 5-7 leaves above the uppermost ear, average plant and ear height, and good tolerance (score 1-2). Lines PM7, PM8, and PM17 were highly tolerant to corn borer (*Ostrinia furnacalis*) (score 1), less infected with sheath blight (*Rhizoctonia solani*), and resistant to lodging (score 1), whereas others, including PM2, PM11, PM13, and PM20, exhibited higher infection levels. Regarding grain yield components, the average weight of 1,000 kernels of the lines varied broadly from 246.4 g (PM2) to 289.9 g (PM10), while the average number of kernel rows per ear ranged from 12.6 to 15.8 rows. There was variation in the grain yield of the studied lines, ranging from 2.26 tons ha^-1 (PM20) to 3.26 tons ha^-1 (PM10), with lines PM3, PM7, PM8, PM9, PM10, PM12, PM14, and PM17 being equivalent to CML161 (3.24 tons ha^-1) and significantly higher than CML165 (2.34 tons ha^-1) (Table 2).

Table 2. Major agronomic characteristics of maize lines.

Name of lines	Maturity (days)	PH (cm)	EH (cm)	LA (leaves)	Corn borer (score)	Sheath blight (score)	Root lodging (score)	P1000 (gram)	KRE (rows)	Grain yield (tons.ha^{-1})
PM1	117	164.7	45.2	5-6	1	1	1	252.1	13.0	2.54
PM2	118	160.6	70.2	5-6	2	2	2	256.0	14.2	2.97
PM3	119	159.8	73.9	5-6	1	1	2	246.4	14.8	3.03
PM4	120	182.6	80.8	5-6	1	1	2	251.8	14.2	2.56
PM5	117	148.7	53.5	5-6	1	1	2	252.5	14.6	2.89
PM6	117	169.5	55.1	6-7	2	1	1	252.6	15.4	2.83
PM7	120	167.5	65.0	6-7	1	1	1	257.6	15.6	3.10
PM8	117	169.4	74.2	6-7	1	1	1	258.6	15.6	3.11
PM9	119	167.2	55.7	5-6	2	1	1	260.8	15.4	3.23
PM10	119	165.8	82.1	5-6	2	1	1	289.9	15.4	3.26
PM11	117	177.5	64.6	5-6	2	1	2	253.9	15.8	2.78
PM12	118	174.0	83.2	5-6	1	1	1	260.7	15.6	3.22
PM13	119	148.8	56.7	5-6	2	2	2	256.0	12.6	2.46
PM14	119	147.6	67.2	5-6	1	1	2	253.9	14.2	3.00
PM15	116	141.1	61.0	5-6	1	1	1	248.1	14.6	2.47
PM16	118	145.5	48.5	5-6	1	1	2	251.5	13.2	2.52
PM17	117	146.5	64.4	5-6	1	1	1	255.6	15.0	3.01
PM18	116	136.7	54.7	5-6	1	1	1	247.4	13.8	2.42
PM19	117	144.5	51.5	5-6	2	1	1	248.1	12.8	2.65
PM20	121	152.3	54.6	5-6	2	1	2	249.2	12.6	2.26
CML161	119	156.1	68.6	5-6	2	1	1	260.1	14.8	3.24
CML165	120	145.0	59.3	5-6	2	1	2	191.4	12.8	2.34
CV%										*5.3*
$LSD_{0.05}$										*0.247*

PH: Plant height, EH: Ear height, LA: Number of leaves above the uppermost ear, P1000: Weight of 1000 kernels, KRE: Number of kernel rows per ear.

Through evaluating the agronomic characteristics and tolerance of 20 maize lines, we found that 16 lines, including PM1, PM3, PM4, PM5, PM6, PM7, PM8, PM9, PM10, PM12, PM14, PM15, PM16, PM17, PM18, and PM19, had higher grain yield than CML165 (2.34 tons ha^-^{1}), good growth, and tolerance, especially at the seedling stage. These lines were used to analyse the presence of the *opaque-2* gene using SSR markers.

3.2. Identifying the presence of the opaque-2 gene among the studied maize lines with simple sequence repeat markers

The results revealed that 10 lines showed DNA bands appearing at approximately 150 bp at the same location as the band of the positive check CML161, indicating they are homozygous recessive (o_2o_2) lines, including PM1, PM5, PM7, PM8, PM10, PM12, PM14, PM15, PM16, and PM17. On the other hand, implementing electrophoresis with primer *phi112* resulted in marking six lines (PM3, PM4, PM6, PM9, PM18, and PM19) with the appearance of a DNA band at the position of about 160 bp corresponding to the band of the negative check Leafy5 (without the *opaque-2* gene), while the others, along with CML161, showed no DNA band, indicating that these lines possess the homozygous recessive *opaque-2* gene (o_2o_2) (Figs. 1A, 1B).

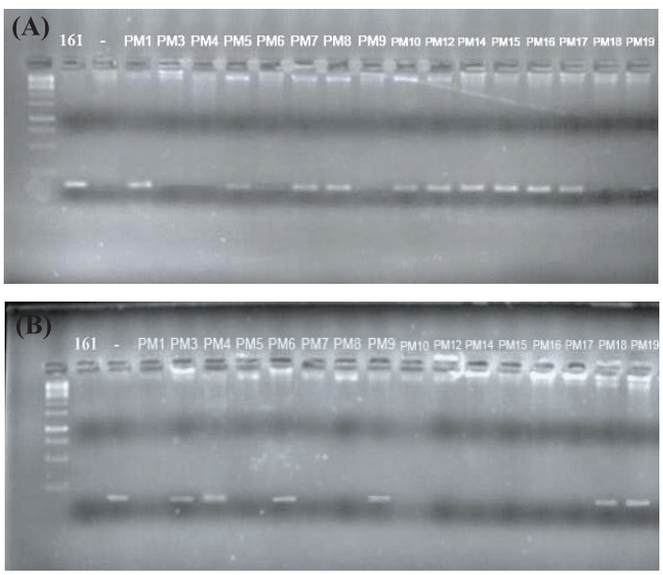

Fig. 1. Identifying the *opaque-2* (o_2o_2) gene of maize lines with simple sequence repeat markers *phi057* (A) and *phi112* (B). Lane 1: 1 kb ladder; Lane 2: CML161 (QPM line); Lane 3: Leafy5 (non-QPM line); Lanes 4, 7, 9, 10, 12, 13, 14, 15, 16, 17: PM1, PM5, PM7, PM8, PM10, PM12, PM14, PM15, PM16, PM17 (o_2o_2 gene); Lanes 5, 6, 8, 11, 18, 19, 20: PM3, PM4, PM6, PM9, PM18, PM19 (O_2O_2 gene).

3.3. Protein content and quality of maize lines

Analysing the protein content of 20 lines revealed diversity from 8.20 g (PM15) to 11.57 g (PM5) in 100 g of maize kernels. The results also indicated that in 100 g of maize grain, *opaque-2* gene lines (PM1, PM5, PM7, PM8, PM10, PM12, PM14, PM15, PM16, PM17) had tryptophan content ranging from 67.1 to 90.8 mg or 0.76 to 0.89% of total protein and lysine from 270.4 to 308.6 mg, equivalent to 2.60-3.30% of total protein. The results are presented in Table 3.

Table 3. Results of analysing protein content and quality of the studied maize lines.

Name of lines	Protein (g/100 g)*	Tryptophan (mg/100 g)*	Tryptophan (% protein)	Lysine (mg/100 g)*	Lysine (% protein)	Quality index
PM1	10.36	79.6	0.77	298.2	2.88	0.77
PM2	10.91					
PM3	10.80					
PM4	9.82					
PM5	11.57	88.5	0.76	300.5	2.60	0.76
PM6	8.43					
PM7	10.00	80.3	0.80	280.7	2.81	0.80
PM8	10.30	84.3	0.82	297.3	2.89	0.82
PM9	9.42					
PM10	9.24	81.7	0.88	280.9	3.04	0.88
PM11	11.00					
PM12	10.10	85.2	0.84	302.0	2.99	0.84
PM13	11.56					
PM14	9.86	82.7	0.84	290.1	2.94	0.84
PM15	8.20	67.1	0.82	270.4	3.30	0.82
PM16	9.80	80.6	0.82	288.7	2.95	0.82
PM17	10.15	90.8	0.89	308.6	3.04	0.89
PM18	10.03					
PM19	10.22					
PM20	11.51					
CML161**	10.8-11.2		0.84-0.88		4.02-4.16	0.84-0.88

*: Analysis results by Centre for Food Quality and Safety Research (CEFORES), Vietnam; **: Analysis results under the project "Research on breeding QPM hybrids with high yield, good tolerance for processing livestock feed".

3.4. Developing leafy maize materials with high protein quality

Based on the results of evaluating the agronomic characteristics and tolerance of the studied lines combined with identifying the *opaque-2* gene with the SSR markers *phi057* and *phi112*, 10 elite lines (PM1, PM5, PM7, PM8, PM10, PM12, PM14, PM15, PM16, PM17) and CML161 were confirmed to carry the recessive *opaque-2* gene (Table 4). These were crossed with the line Leafy5 to develop high-protein quality leafy materials (LM).

Table 4. Summary table of main agronomic traits, resistance and tolerance, protein content, and quality of *opaque-2* gene lines.

Name of lines	LA (leaves)	Corn borer (score)	Sheath blight (score)	Root lodging (score)	Grain yield (tons.ha^{-1})	Protein (g/100 g)	Tryptophan (mg/100 g)	Lysine (mg/100 g)	Quality index
PM1	5-6	1	1	1	2.54	10.36	79.6	298.2	0.77
PM5	5-6	1	1	2	2.89	11.57	88.5	300.5	0.76
PM7	6-7	1	1	1	3.10	10.00	80.3	280.7	0.80
PM8	6-7	1	1	1	3.11	10.30	84.3	297.3	0.82
PM10	5-6	2	1	1	3.26	9.24	81.7	280.9	0.88
PM12	5-6	1	1	1	3.22	10.10	85.2	302.0	0.84
PM14	5-6	1	1	2	3.00	9.86	82.7	290.1	0.84
PM15	5-6	1	1	1	2.47	8.20	67.1	270.4	0.82
PM16	5-6	1	1	2	2.52	9.80	80.6	288.7	0.82
PM17	5-6	1	1	1	3.01	10.15	90.8	308.6	0.89

LA: Number of leaves above the uppermost ear; Score 1: The best; Score 5: The worst.

Table 5. Morphological characteristics and resistance of leafy maize materials.

Name of materials	Origin	PH (cm)	EH (cm)	LA (leaves)	Stay-green (score)	Root lodging (score)	Stalk lodging (score)
LM1	PM1 x Leafy5	242	92	11-15	2	1	1
LM2	PM5 x Leafy5	260	95	9-12	2	1	1
LM3	PM7 x Leafy5	275	100	15-18	1	1	1
LM4	PM8 x Leafy5	255	85	11-15	2	1	1
LM5	PM10 x Leafy5	285	110	15-16	2	2	1
LM6	PM12 x Leafy5	258	115	12-14	1	1	1
LM7	PM14 x Leafy5	285	100	9-12	2	1	1
LM8	PM15 x Leafy5	290	115	12-13	2	1	1
LM9	PM16 x Leafy5	245	102	8-9	2	1	1
LM10	PM17 x Leafy5	280	120	10-11	1	1	1
LM11	CML161 x Leafy5	275	130	12-13	2	1	1
Check 1	LCH9	216	103	6-7	1	1	1
Check 2	NK7328	225	123	6-7	1	1	1

PH: Plant height; EH: Ear height; LA: Number of leaves above the uppermost ear; Score 1: The best; Score 5: The worst.

The research results showed that LM3, LM6, and LM10 have a higher number of leaves above the uppermost ear with more than eight leaves, good growth, low ear height, good resistance to lodging (score 1), and high stay-green (score 1) (details in Table 5).

Due to LM3 having 15-18 leaves above the ear, leading to a long maturity of more than 130 days, it is not reasonable for practical production. Therefore, individual plants with only 10-12 leaves above the ear of LM6 (PM12 x Leafy5) and LM10 (PM17 x Leafy5) were carefully selected (Figs. 2 and 3) as segregating populations for background selection with SSR markers in identifying the presence of the *opaque-2* gene among leafy individuals for the development of leafy maize lines with high protein quality in the next generations.

Fig. 2. Leafy maize material LM6.

Fig. 3. Leafy maize material LM10.

3.5. Screening opaque endosperm maize kernels on a light table

Following morphological evaluation, the best individual plants of LM6 and LM10 with 10-12 leaves above the uppermost ear were selected as primary materials for researching and developing high-protein quality maize hybrids specialised for green forage and silage. The F_2 seeds of these materials were screened for the opaque endosperm ratio on a light table before planting. As a result, modified kernels with the endosperm of around 50% opaque (score 3) of these leafy maize materials were classified (Fig. 4).

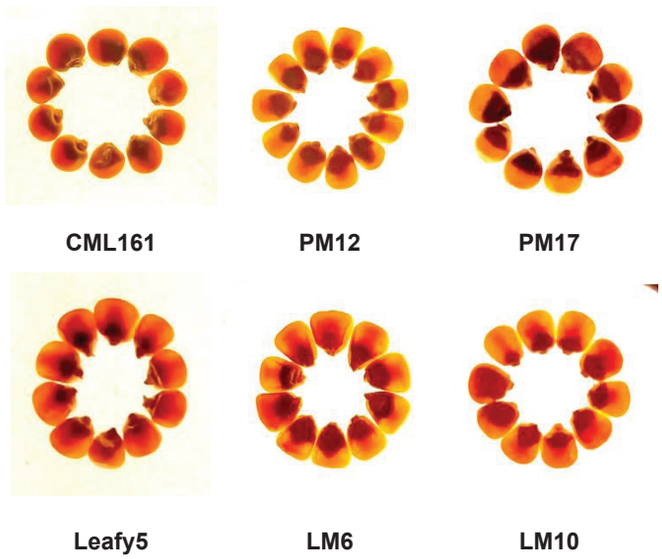

Fig. 4. Opaque endosperm seeds (CML161, PM12, PM17), normal endosperm seeds (Leafy5) and variation in opaque endosperm seeds of LM6 and LM10.

These F_2 seeds were grown to form $F_{2:3}$ populations for background selection with SSR markers to identify the presence of the *opaque-2* gene among leafy individual plants of LM6 and LM10, combining with conventional maize breeding for the development of high-protein quality leafy maize lines for green forage and silage.

4. Discussion

Previous studies have confirmed that the *Leafy1 (Lfy1)* mutant gene, controlling the leafy trait in maize (four or more leaves above the uppermost ear compared to normal maize), is dominant [7] or incompletely dominant [14], and is identified on chromosome 3, near the RFLP marker *bnl7.26* and 2 SSR markers *mmc0001* and *umc1578*. This trait helps increase biomass yield and quality [9, 11, 12], making it a significant direction for maize breeding for green forage and silage, especially in North America [6, 9].

In addition to high whole-plant yield, forage maize must also be of high quality. Therefore, it is essential to use qualified maize germplasms such as mutant genes *bm* (brown midrib), *wx, floury-2*, etc. However, applying these genes in practice has not met expectations due to low average productivity, poor agronomic characteristics, high susceptibility to pests and diseases, poor resistance to lodging, and variable quality [9]. Scientists have demonstrated that the *opaque2* (o_2) is a recessive gene that improves protein quality in maize by increasing lysine and tryptophan content in the kernel endosperm compared to normal maize [16, 22, 32], and it has been used in QPM breeding programs [19, 29, 33].

Based on the scientific foundation and genetics of these traits, studies on developing high-protein quality leafy maize materials for forage breeding were carried out. In any hybrid maize breeding program, inbred lines are considered the foundation. Therefore, evaluating agronomic traits and tolerance is crucial to selecting the best lines suitable for high-protein quality forage breeding. In Spring 2022, due to low temperatures (18-20°C) during the germination and seedling stages, the maturity of lines, including checks CML161 and CML165, was prolonged to 121 days (PM20). Among grain-yield components of the lines, the range of the average weight of 1,000 kernels is more significant than that of the number of kernel rows per ear. This can be explained by the fact that the number of kernel rows per ear is a heritable character [34] and is mainly inherited by additive effects [35]. Although most of the lines have higher grain yield than the check CML165, tolerance to diseases and pests, and resistance to lodging (score 1-2), 16 lines (PM1, PM3, PM4, PM5, PM6, PM7, PM8, PM9, PM10, PM12, PM14, PM15, PM16, PM17, PM18, and PM19) may be more appropriate as parental lines in breeding maize hybrids specialised for green forage and silage.

In breeding high-protein quality maize hybrids, it is fundamental to develop maize lines carrying the *opaque-2* (o_2) gene mutation that regulates an increase in lysine and tryptophan content in the kernel endosperm compared to normal maize [16, 19, 22]. Phenotyping combined with applying molecular markers to confirm the presence of the o_2o_2 gene has been successfully used in many QPM maize breeding programs [26, 36]. SSR markers (*phi057, phi112*, and *umc1066*) have been applied to identify individuals carrying homozygous recessive (o_2o_2) and heterozygous (O_2o_2) genes [28, 37]. The SSR marker *phi112* is dominant

in distinguishing QPM from normal maize [34], because the o_2o_2 genotype is homozygous recessive, so both dominant and co-dominant markers must give the same results. In contrast, *phi057* is a co-dominant marker [28], linked more closely to the *opaque-2* gene than *phi112*, and can detect homozygous dominant (O_2O_2), heterozygous (O_2o_2), and homozygous recessive (o_2o_2) individuals. The presence of a DNA band in the amplified PCR product at the position corresponding to the positive control determines the presence of the *opaque-2* gene, whereas the absence of this DNA band indicates normal maize. Therefore, these SSR markers used in this study help find (o_2o_2) homozygous recessive gene lines accurately and effectively. The analysis of results showed that DNA bands of 10 lines (PM1, PM5, PM7, PM8, PM10, PM12, PM14, PM15, PM16, PM17) appeared at approximately 150 bp as CML161 with the primer *phi057*, while others of 6 lines (PM3, PM4, PM6, PM9, PM18, and PM19) had the position at around 160 bp with the primer *phi112*. This result is similar to previous studies on the application of *phi057* and *phi112* markers in QPM maize breeding [28, 33, 38].

Besides identifying the *opaque-2* gene, to develop quality protein maize, the analysis of tryptophan and lysine content to verify protein quality must be done. As a result, the tryptophan content of the *opaque-2* gene-carrying lines is twice as high, while the lysine content is 1.5 times higher than in normal maize. A reduction in lysine content during selection is also observed in QPM breeding programs [39], and the wide range of variation in lysine content depends on germplasm. In contrast, the tryptophan content in total corn kernel protein is less variable, with 0.5% on average for conventional maize and 0.8% for QPM [18, 19]. Thus, the tryptophan content is used as the quality index (QI) in QPM maize breeding [19, 29, 40]. Remarkably, identifying PM7, PM8, PM10, PM12, PM14, PM15, PM16, and PM17 with QI above the minimum of 0.80 initially indicates that these are QPM lines. However, the analysis results also showed that PM5 has high levels of tryptophan and lysine, with 88.5 and 300.5 mg respectively in 100 g kernel grain samples. However, due to the high total protein content of 11.57 g, the quality index (QI=0.76) is low, leading to the conclusion that the correlation between protein content and quality, and between total protein content and QI, is negative, similar to previous research results [19].

Through phenotyping, genotyping, and analysing the protein quality of 20 inbred lines, we found that PM7, PM8, PM10, PM12, and PM17 are the most promising lines with high protein quality. Our studies focused on developing new high-protein quality leafy materials by crossing homozygous recessive gene o_2o_2-carrying lines with the leafy line (Leafy5). By evaluating the morphological characteristics and resistance of these leafy materials, the results showed that individual plants with eight leaves above the primary ear in all materials were successfully developed. However, not all plants in a given material have a higher number of leaves above the ear, probably because the *Leafy1* gene (*Lfy1*) regulating this trait is an incompletely dominant gene and is mainly inherited from the genetic background [14]. Among the well-phenotyped leafy materials, LM3, with 15-18 leaves above the ear, leading to long maturity (more than 130 days), is unsuitable for production in Vietnam [6]. Therefore, individuals of LM6 and LM10 were selected with 10-12 leaves above the primary ear, good agronomic characteristics, and adaptability to the ecological conditions in Vietnam. They were developed as segregating populations for identifying *opaque-2* gene-carrying individual plants by applying SSR markers. Combined with screening opaque endosperm maize kernels on a light table, kernels with about 50% opacity should be chosen before planting these materials. As a result, both genotyping and phenotyping help select high-protein quality individual plants more precisely and efficiently [19]. This study provides early results in maize breeding for green forage and silage and paves the way for further study of high-protein quality leafy lines in the future.

5. Conclusions

To confirm whether an inbred line is QPM, it is necessary to both genotype the polymorphism on the *opaque-2* gene and analyse protein quality using the quality index. Among the 20 studied maize lines, PM7, PM8, PM10, PM12, PM14, PM15, PM16, and PM17, which carry the o_2o_2 gene and have a QI greater than 0.80, are considered QPM lines. By crossing *opaque-2* gene-carrying lines and CML161 with the leafy line, 11 leafy maize materials (LM) were successfully developed. Among them, only LM6 (PM12 x Leafy5) and LM10 (PM17 x Leafy5) with 10-12 leaves above the uppermost ear, good agronomic traits, especially resistance to lodging (score 1), high stay-green (score 1), and adaptability to the tropical climate, were selected for future development of high-protein quality leafy maize lines.

CRediT author statement

Chi Thanh Nguyen: Data collection, Methodology, Formal analysis, Writing, Editing; Xuan Thang Nguyen: Reviewing, Methodology, Formal analysis, Writing, Editing; Viet Long Nguyen: Formal analysis, Writing, Editing; Thi Kim Chung Nguyen: Data collection, Methodology; Thi Bich Thao Doan: Methodology, Formal analysis, Writing.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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