Management of Genetic Biodiversity by Plant Breeding and Sustainable Agricultural Technologies

NARDI Fundulea

MOLECULAR ANALYSIS FOR DETECTING GENETIC VARIABILITY AT THE NAM-A1 LOCUS IN A COLLECTION OF WHEAT VARIETIES, OLD POPULATIONS, LINES AND WILD SPECIES

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ABSTRACT - The determinant key to market potential and economic value for the novel wheat varieties is wheat grain quality, a characteristic that influences both nutritional content and food processing quality. The NAC transcription factor, encoded by the "No Apical Meristem" (NAM) gene, accelerates senescence by promoting the remobilization of nutrients from leaf tissues to growing grains. Hexaploid wheat contains five NAM genes, consisting in two homeologs (on chromosomes 6A and 6D) and three paralogs (on chromosomes 2A, 2B, and 2D). Among these, NAM-A1 (6A) functions is similarly to NAM-B1 (Gpc-B1 - *Triticum turgidum L.* subsp. *dicoccoides*), which positively affects the baking quality and nutritional value of cereals. This study aimed to detect allelic variants at the NAM-A1 locus using the KASP technique with two molecular markers related to SNPs 1 and 2. Analysis of 102 varieties and old populations highlighted the predominant presence of the "T" allele for SNP-1 and "A" allele for SNP-2, resulting in the haplotypes NAM-A1a, NAM-A1c and NAM-A1d along with some heterozygous haplotypes. Analysis of 42 winter wheat lines revealed the presence "T" allele for SNP 1 and "del" allele for SNP 2 resulting in haplotypes: NAM-A1a, NAM-A1c and NAM-A1d. Analysis of the 44 wild species revealed the presence of the "C" allele for SNP-1 and the "A" allele for SNP-2, resulting in the haplotypes NAM-A1a and NAM-A1c, along with some heterozygous haplotypes. To further elucidate the role of the NAM-A1, NAM-B1 and NAM-D1 genes in wheat, as well to identify the most favorable allelic combinations specific to each growing environment, additional research is necessary to better understand their functions and their interactions with the environment.

INTRODUCTION - The improvement of wheat genotypes with the aim of increasing the efficiency of nitrogen use is necessary to obtain superior grain production worldwide. In wheat, remobilization of previously absorbed nitrogen at anthesis accounts for most of the nitrogen accumulated in the grain. The No Apical Meristem (NAM) gene at the Gpc-B1 locus (chromosome arm 6BS), identified in both hexaploid wheat (*Triticum aestivum* L.) and tetraploid wheat (*Triticum turgidum* L. ssp *durum),* encodes a known NAC transcription factor for accelerating senescence and increasing the remobilization of nutrients from tissues, thus contributing to increasing the protein concentration of wheat grains. Cultivated wheat presents in most cases the non-functional allele of the NAM-B1 gene, therefore its physiological characterization began after the introgression of the chromosomal segment from the species *Triticum turgidum* L. subsp. *dicoccoides* carrying the functional allele. Hexaploid wheat also has five other NAM genes, three paralogous (2A, 2B and 2D) and two homeologs (6A and 6D) of which NAM-A1 (6A), gene which has a similar function to NAM-B1, with beneficial effects on the nutritional quality of cereals and on the baking properties (Avni et al., 2014). The highlighted polymorphism at the level of the NAM-A1 gene is due to the identification of two SNPs. The first SNP (SNP-1) was identified in the NAC domain of the gene and is determined by the C/T inversion. This SNP causes the substitution of the amino acid alanine with the amino acid valine in the protein sequence. The second SNP is determined by a insertion/deletion (IN/DEL) A/del resulting in a truncated protein. These two SNPs determine a genetic variability embodied by four haplotypes (NAM-A1a, NAM-A1b, NAM-A1c and NAM-A1d). NAM-A1b may have arisen as following recombination between haplotypes NAM-A1c and NAM-A1d (Cormier et al., 2015). The aim of this study was to screen a diverse wheat germplasm to determine the genetic variability existing at the level of the NAM-A1 gene with the help of the KASP technique.

MATERIAL AND METHOD - For DNA extraction (both grains and green leaves were used) a NucleoSpin Plant II extraction kit (Macherey-Nagel) and the extraction protocol recommended by the manufacturer were used. The FluOstar Omega (BMG Labtech) was used to quantify the quality and quantity of extracted DNA.
Molecular analysis - the detection of allelic variants at the level of the NAM-A1 locus was carried out with the help of the KASP technique based on the use of fluorescently labeled oligo-extensions. Amplification was performed using a reaction mix containing universal FRET cassettes (FAM, HEX/VIC), passive reference fluorophore (dye) ROX, Taq polymerase, free nucleotides and MgCl2 in an optimized buffer solution (LGC Bioresearch Technologies). The markers used in this technique were represented by two forward primers specific to each allele at the SNP level and a common reverse primer. The forward primers contain at the 5′ end, in addition to the sequence specific to the region of interest, a unique tail that corresponds to one of the universal FRET cassettes: the FAM tail - GAAGGTGACCAAGTTCATGCT and the HEX/VIC tail - GAAGGTCGGAGTCAACGGATT. DNA amplification reactions for the KASP technique were performed with PACE® 2.0 Genotyping Master Mix from 3CR Bioscience.To read and interpret the results of the KASP technique, the FLUOstar Omega plate reader (BMG Labtech) and the KlusterCaller software (LGC Biosearch Technologies) were used. The sequence of the primers used was identified with the help of specialized literature (Cormier et al., 2015).

RESULTS AND DISCUSSION - The two KASP molecular markers related to SNPs 1 and 2 (fig. 1) were used for the analysis of the Nam-A1 locus for wheat varieties and ancient populations (102), winter wheat lines (42) as well as for wild species (44 genotypes). The analyzes revealed that in the wheat germplasm originating from the 102 wheat varieties and old populations (table 1) the "T" allele prevail for SNP-1 (74.5%) and the "A" allele for SNP-2 (65, 6 %), resulting in three haplotypes: NAM-A1a, NAM-A1c and NAM-A1d but also heterozygous forms (table 1, 3). Among the haplotypes, NAM-A1c was the haplotype most present in the material, being highlighted in 44 genotypes (43,13 %), followed by 27 heterozygous forms (26,4%), than the NAM-A1d haplotype (19%) and NAM-A1a (11,76 %) (table 3).
Molecular analysis performed for the 42 winter wheat lines with KASP markers highlighted the predominance of "T" allele for SNP-1 (81%) and the "del" allele at SNP-2 (71%), resulting in 3 haplotypes: NAM-A1a, NAM-A1c and NAM-A1d, but also heterozygous forms (table 2). Among these haplotypes, NAM-A1d was the haplotype most present in the material, being highlighted in 30 wheat lines (71,4%), followed by the NAM-A1a haplotype (14,2 %) and NAM-A1c (9,5 %) (table 4).

Table 1 - KASP analyzes in varieties and old populations ot wheat for NAM-A1 locus

No.	Genotype	Nam-SNP1	Nam-SNP2	Nam-6A	Nr. crt.	Proba	Nam-SNP1	Nam-SNP2	Nam-6A
1	PL1	T	del	d	52	PL52	T	A	c
2	PL2	H	A	H	53	PL53	T	A	c
3	PL3	T	A	c	54	PL54	H	A	H
4	PL4	T	del	d	55	PL55	C	A	a
5	PL5	T	A	c	56	PL56	H	A	H
6	PL6	T	del	d	57	PL57	T	A	c
7	PL7	T	del	d	58	PL58	C	A	a
8	PL8	T	A	c	59	PL59	T	A	c
9	PL9	T	del	d	60	PL60	T	H	H
10	PL10	T	del	d	61	PL61	H	H	H
11	PL11	T	A	c	62	PL62	T	del	d
12	PL12	T	A	c	63	PL63	C	A	a
13	PL13	T	del	d	64	PL64	T	A	c
14	PL14	T	H	H	65	PL65	T	A	c
15	PL15	T	del	d	66	PL66	T	A	c
16	PL16	C	A	a	67	PL67	T	A	c
17	PL17	C	A	a	68	PL68	T	H	H
18	PL18	T	A	c	69	PL69	T	A	c
19	PL19	T	A	c	70	PL70	T	H	H
20	PL20	T	A	c	71	PL71	T	del	d
21	PL21	T	A	c	72	PL72	T	del	d
22	PL22	C	A	a	73	PL73	H	A	H
23	PL23	T	del	d	74	PL74	T	del	d
24	PL24	T	H	H	75	PL75	T	A	c
25	PL25	T	A	c	76	PL76	T	H	H
26	PL26	T	del	d	77	PL77	C	A	a
27	PL27	T	del	d	78	PL78	H	H	H
28	PL28	T	del	d	79	PL79	H	A	H
29	PL29	H	A	H	80	PL80	T	A	c
30	PL30	T	H	H	81	PL81	C	A	a
31	PL31	T	A	c	82	PL82	H	A	H
32	PL32	T	A	c	83	PL83	H	H	H
33	PL33	T	H	H	84	PL84	T	A	c
34	PL34	T	A	c	85	PL85	H	A	H
35	PL35	T	A	c	86	PL86	C	A	a
36	PL36	T	A	c	87	PL87	H	A	H
37	PL37	T	A	c	88	PL88	T	A	c
38	PL38	T	A	c	89	PL89	T	A	c
39	PL39	C	A	a	90	PL90	T	H	H
40	PL40	H	H	H	91	PL91	H	H	H
41	PL41	T	del	d	92	PL92	T	A	c
42	PL42	T	A	c	93	PL93	H	A	H
43	PL43	C	A	a	94	PL94	H	A	H
44	PL44	T	A	c	95	PL95	T	H	H
45	PL45	T	A	c	96	PL96	T	del	d
46	PL46	T	A	c	97	PL97	T	A	c
47	PL47	T	A	c	98	PL98	T	A	c
48	PL48	T	A	c	99	PL99	T	del	d
49	PL49	T	A	c	100	PL100	T	A	c
50	PL50	C	A	a	101	PL101	T	A	c
51	PL51	T	A	c	102	PL102	T	H	H

Table 2 - KASP analyzes in winter wheat lines for NAM-A1 locus

No	Line	Nam-SNP1	Nam-SNP2	Nam-6A
1	GGEN-1b	T	del	A1d
2	GGEN-1c	T	del	A1d
3	GGEN-2	T	del	A1d
4	GGEN-3	T	del	A1d
5	GGEN-4	T	del	A1d
6	GGEN-5	T	del	A1d
7	GGEN-6	T	del	A1d
8	GGEN-7	T	del	A1d
9	GGEN-8	T	del	A1d
10	GGEN-9	T	del	A1d
11	GGEN-10	T	del	A1d
12	GGEN-11	T	del	A1d
13	GGEN-12	C	H	A1a/b
14	GGEN-13	T	del	A1d
15	GGEN-14	T	del	A1d
16	GGEN-15	C	A	A1a
17	GGEN-16	T	del	A1d
18	GGEN-17	C	A	A1a
19	GGEN-18	T	del	A1d
20	GGEN-19	T	del	A1d
21	GGEN-20	T	del	A1d
22	GGEN-21	C	na	na
23	GGEN-22	T	A	A1c
24	GGEN-23	T	A	A1c
25	GGEN-24	T	A	A1c
26	GGEN-25	C	H	A1a/b
27	GGEN-26	T	del	A1d
28	GGEN-27	T	del	A1d
29	GGEN-28	T	del	A1d
30	GGEN-29	T	del	A1d
31	GGEN-31	T	del	A1d
32	GGEN-32	T	del	A1d
33	GGEN-35	T	del	A1d
34	GGEN-36	C	A	A1a
35	GGEN-37	C	H	h
36	GGEN-38	T	del	A1d
37	GGEN-39	T	A	A1c
38	GGEN-40	C	H	A1a/b
39	GGEN-41	T	del	A1d
40	GGEN-42	T	del	A1d
41	GGEN-43	T	del	A1d
42	GGEN-44	T	del	A1d

Figure 1 - Results o KASP analysis for the NAM-A1 locus (varieties and ancient pop.) for 2 SNPs (red = HEX-type allele; blue = FAM-type allele; green = heterozygous; black = control sample no DNA)

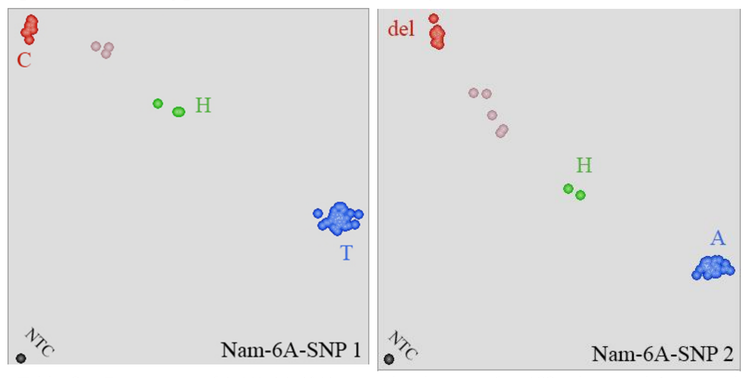

Table 3 - Frequency of NAM-A1 (6A) gene haplotypes for 102 wheat cultivars and old populations

Haplotype	SNP-1	SNP-2	Haplotype frequency (%)
NAM-A1a	C	A	12 (11,76 %)
NAM-A1b	C	Del	0 (0 %)
NAM-A1c	T	A	44 (43,13 %)
NAM-A1d	T	Del	19 (18,6 %)
Heterozygoud	C/T	A/Del	27 (26,4 %)

Table 4 - Frequency of NAM-A1 (6A) gene haplotypes in winter wheat lines

Haplotype	SNP-1	SNP-2	Haplotype frequency (%)
NAM-A1a	C	A	6 (14,28%)
NAM-A1c	T	A	4 (9,52 %)
NAM-A1d	T	Del	30 (71,42 %)
Heterozygous	C/T	A/Del	1 (2,38 %)
Undetermided	C	NA*	1 (2,38)

Table 6 - Frequency of NAM-A1 (6A) gene haplotypes for the 22 wild species

Haplotype	SNP-1	SNP-2	Haplotype frequency (%)
NAM-A1a	C	A	33 (75 %)
NAM-A1b	C	Del	0 (0 %)
NAM-A1c	T	A	9 (20,5 %)
NAM-A1d	T	Del	0 (0 %)
Heterozygous	C/T	A/Del	2 (4,5 %)

Table 5 - Wild-type KASP analyzes for the NAM-A1 locus

No	Genotype	Nam-6A-SNP1	Nam-6A-SNP2	Nam-6A	Nr.crt	Probe	Nam-6A-SNP1	Nam-6A-SNP2	Nam-6A
1	AE SH1	C	A	a	23	DIC 5	C	A	a
2	AE SH10	C	A	a	24	SPH 17	T	A	c
3	AE SH11	C	A	a	25	SPL 1	C	A	a
4	AE SH12	C	A	a	26	SPL 3	C	A	a
5	AE SH13	C	A	a	27	SPT 14	T	A	c
6	AE SH2	C	A	a	28	SPT 16	T	H	H
7	AE SH3	C	A	a	29	TIM 18	C	A	a
8	AE SH4	C	A	a	30	TIM 21	C	A	a
9	AE SH5	C	A	a	31	TIM 25	C	A	a
10	AE SH6	C	A	a	32	TIM 28	C	A	a
11	AE SH7	C	A	a	33	TIM 34	C	A	a
12	AE SH8	C	A	a	34	TIM 39	T	A	c
13	AE SH9	C	A	a	35	TIM 47	C	A	a
14	AMA 11	H	A	H	36	TRG 54	T	A	c
15	AMA 12	T	A	c	37	TRG 55	T	A	c
16	ASP 19	C	A	a	38	TRG 56	T	A	c
17	ASP 21	C	A	a	39	TRG 57	T	A	c
18	ATA 22	C	A	a	40	TRG 58	T	A	c
19	ATA 26	C	A	a	41	TRG 77	C	A	a
20	ATA 29	C	A	a	42	URT 59	C	A	a
21	AVE 30	C	A	a	43	URT 67	C	A	a
22	DIC 4	C	A	a	44	URT 71	C	A	a

The 44 wild species analysis with KASP markers revealed the predominance of the "C" allele (tab 5) for SNP-1 (72.7%) and the "A" allele for SNP-2 (97.7%), resulting in two haplotypes: NAM-A1a and NAM-A1c, along with heterozygous forms. Among the haplotypes, NAM-A1a was most present in the material, being highlighted in 33 wild species (75%), followed by the NAM-A1c haplotype (20,5 %) and 2 heterozygous forms (4,5 %) (table 6).

CONCLUSIONS - a total of 102 wheat varieties and old populations, 42 lines and 44 wild species were analyzed and characterized using KASP molecular markers for allele variations locus NAM-A1.
- The high protein content (GPC) associated with the NAM-A1, haplotype NAM-A1a, was predominantly found only in the 44 wild species, whereas the NAM-A1c haplotype was predominant in the varieties and old populations. In the 42 winter wheat lines, the NAM-A1d haplotype was predominant.
- Pyramiding of the NAM-A1 and NAM-B1 alleles, which are associated with high protein content, into new cultivars can be achieved through marker-assisted selection."

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