Research Article

Identification of cytoplasm type and nuclear Ms gene in onion cultivars[1]

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ABSTRACT

Open-pollinated onion cultivars predominate in the southern region of Brazil, due to their higher adaptability to local climatic conditions, unlike commercial hybrids, which have shown a lower adaptability. Cytoplasmic male sterility (CMS) systems are employed to develop hybrid onion cultivars. Two molecular markers, 5'cob and orfA501, were used to differentiate the cytoplasm type, and the AcSKP1 marker to identify the nuclear male fertility-restoring locus (Ms). A total of 1,126 plants from the most common onion cultivars grown in southern Brazil, including Bola Precoce®, Crioula®, Valessul®, Mega®, Joia® and Princesa do Sul®, were analyzed using all the three markers. An extremely rare occurrence of the S cytoplasm was observed among the cultivars, being detected in only 1.8 % of the samples, while the T cytoplasm was the most prevalent, accounting for 56.3 % of the samples. Among the 1,126 plants analyzed, only three exhibited the S cytoplasm and were recessive for the Ms-locus (Smsms). Additionally, 49 plants with the N-cytoplasm (as per the Engelke's classification) and recessive for the nuclear genotype (Nmsms) were identified, 45 of which were pollen producers. The male-fertility restoration occurred in 22.2 % of the crosses between Tmsms male-sterile plants and male-fertile N cytoplasmic-msms plants (as per the Engelke's classification).

KEYWORDS: *Allium cepa* L., cytoplasmic male sterility, onion breeding.

RESUMO

Identificação do tipo de citoplasma e do gene nuclear Ms em cultivares de cebola

O uso de cultivares de polinização livre de cebola predomina na região Sul do Brasil, devido à maior adaptabilidade às condições climáticas locais, enquanto híbridos comerciais têm demonstrado baixa adaptabilidade. A macho-esterilidade citoplasmática (CMS) é utilizada para o desenvolvimento de cultivares híbridas de cebola. Foram utilizados dois marcadores moleculares, 5'cob e orfA501, para diferenciar o tipo de citoplasma, e o marcador AcSKP1 para identificar o gene nuclear Ms responsável pela manutenção ou restauração da macho-fertilidade em cebola. Um total de 1.126 plantas de cultivares de cebola mais comuns no Sul do Brasil, incluindo Bola Precoce®, Crioula®, Valessul®, Mega®, Joia® e Princesa do Sul®, foram analisadas utilizando-se os três marcadores. A ocorrência do citoplasma S mostrou-se extremamente rara nas cultivares analisadas, sendo detectado em somente 1,8 % das amostras, enquanto o citoplasma T foi o mais frequente, sendo detectado em 56,3 % das amostras. Das 1.126 plantas analisadas, apenas três possuíam o citoplasma tipo S e eram recessivas para o locus Ms (Smsms). Também foram identificadas 49 plantas com citoplasma N (de acordo com a classificação de Engelke) e recessivas para o gene nuclear (Nmsms), das quais 45 eram produtoras de pólen. A restauração da macho-fertilidade ocorreu em 22,2 % dos cruzamentos entre plantas macho-estéreis Tmsms e plantas macho-férteis Nmsms (de acordo com a classificação de Engelke).

PALAVRAS-CHAVE: *Allium cepa* L., macho-esterilidade citoplasmática, melhoramento de cebola.

INTRODUCTION

As with most modern crops, the use of hybrid onion (*Allium cepa* L.) has enabled higher yields. However, despite Brazil being a significant global producer, there is still a widespread preference for open-pollinated cultivars, particularly in the southern region of Brazil, which is the primary production area, with approximately 30,000 hectares under cultivation (IBGE 2024). The reluctance to adopt

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onion hybrids in southern Brazil stems from the fact that no commercial cultivar has met the farmers' expectations, primarily due to poor adaptability to the region's climate.

Hybrid onion seeds are produced using sources of cytoplasmic male sterility (CMS). The initial source of onion CMS was reported by Jones & Clarke (1943), who described CMS as being controlled by the interaction of male-sterile cytoplasm (S) with a homozygous recessive genotype at the nuclear male-fertility restoration (Ms) locus (Smsms). The male-sterile plant, referred to as "A" (Smsms), can be propagated by crossing it with a maintainer inbred line, "B", which possesses normal cytoplasm (N) and is homozygous recessive for the nuclear Ms gene (N-msms). All seeds resulting from the cross between plants "A" will be male-sterile, while seeds from the inbred line "B" will be male-fertile (Havey 2004).

At least two additional male-sterile cytoplasms, T and R, have been identified in onion (Berninger 1965, Havey & Kim 2021). According to Schweisguth (1973), interactions among three nuclear loci control the restoration of male fertility in plants with T cytoplasm. Recently, Havey & Kim (2021) identified the R cytoplasm. Comparative analyses of mitochondrial markers in N, S and R cytoplasms revealed that orf725 is a common CMS-associated gene and is responsible for male sterility in both CMS-S and CMS-R cytotypes (Kim et al. 2016 and 2019, Tsujimura et al. 2019).

Since the first male-sterile inbred lines were released in 1952, the S cytoplasm has been widely used in the United States to produce hybrid onion seeds (Goldman et al. 2000). After analyzing the cytoplasm of commercial and control inbred onion lines, Havey & Kim (2021) found that both S and R cytoplasms are frequently used, whereas the T cytoplasm is rarely employed for commercial hybrid seed production.

Several polymorphisms in chloroplast (cp) and mitochondrial (mt) DNA have been identified in the N and S cytoplasms of onion. The 5'cob marker, developed by Sato (1998), was based on mitochondrial DNA variations, as were orfA501 (Engelke et al. 2003) and orf725 (Kim et al. 2009). According to Havey & Kim (2021), although Kim et al. (2019) referred to the T cytoplasm in their study, it was later determined to be a different cytoplasm, now recognized as R cytoplasm. The genuine T cytoplasm could not be distinguished from the N cytoplasm by orf725, as both produce an 833 bp amplicon. Table 1 provides a summary of the markers used for cytoplasm differentiation.

By using only the 5'cob and OrfA501 markers, it is not possible to distinguish N from R cytoplasms. Therefore, it is feasible to classify plants as S, T or "N-like" (sensu Engelke et al. 2003), indicating that some plants are S and T cytotypes, while others belong to the (R + N) group (Havey & Kim 2021).

Huo et al. (2015) developed the codominant marker AcSKP1, which correlates with genotypes at the male-fertility restorer nuclear locus (Ms). This marker has shown partial success when applied to Brazilian germplasm (Ferreira & Santos 2018).

This study aimed to analyze cytoplasm types in 1,710 plants from six widely cultivated, open-pollinated onion cultivars, in southern Brazil, using the 5'cob and orfA501 markers, as well as the nuclear male-fertility restorer locus (Ms) using the AcSKP1 marker, in order to select plants for the development of male-sterile (A) and male-fertile maintainer (B) lines to develop hybrids better adapted to southern Brazil.

MATERIAL AND METHODS

Field trials, including crosses, self-pollination and seed production, were conducted at the Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina (Epagri), in Ituporanga, while the molecular analyses were carried out at the Epagri of Itajaí, both in the Santa Catarina state, Brazil.

In July 2021, following natural vernalization to induce flowering, 288 bulbs from six open-pollinated onion cultivars (Bola Precoce®, Crioula®, Valessul®, Mega®, Joia® and Princesa do Sul®) were planted. After the emergence of the first leaves,

Table 1. Polymorphic markers in mitochondrial (mt) DNAs distinguishing onion cytoplasms based on the results of Sato (1998), Engelke et al. (2003) and Havey & Kim (2021). Adapted from Havey & Kim (2021).

Cytoplasm	5'Cob		OrfA501	Orf725	
	180 bp	414 bp	473 bp	628 bp	833 bp
N	+	-	-	-	+
T	+	-	+	-	+
S	+	+	+	+	-
R	+	-	-	+	+

Normal (N) male-fertile cytoplasm and male-sterile cytoplasms S (Jones & Clarke 1943), T (Berninger 1965) and R (Havey & Kim 2021). + indicates amplification; - indicates no amplification. The amplicon sizes are in base pairs (bp).

DNA was extracted using the Doyle & Doyle (1990) protocol, and its concentration was measured using NanoDrop®. The extracted DNA was diluted to a concentration of 20 ng μL^{-1} for use in PCR reactions. Table 2 lists all primers used in this study.

The molecular markers used to distinguish the onion cytoplasms were 5'cob from Sato (1998) and orfA501 from Engelke et al. (2003), which differentiate the S, T and "N sensu Engelke et al. (2003)" cytoplasms. The alleles at the nuclear male-fertility restorer locus (Ms) were identified using the codominant AcSKP1 marker, developed by Huo et al. (2015). Amplicons were separated using electrophoresis in 1.2 % agarose gel stained with 1 mg mL^{-1} of ethidium bromide. A 100 bp ladder was used as the molecular weight standard.

The onion cytoplasms were classified following Engelke et al. (2003) and the reclassification proposed by Havey & Kim (2021): i) "N-sensu Engelke et al. (2003)" - amplification of only the 180-bp fragment by the 5'cob marker; ii) T cytoplasm - amplification of the 180-bp fragment by 5'cob and the 473-bp fragment by orfA501; iii) S cytoplasm - amplification of the 180-bp and 414-bp fragments by the 5'cob-marker, and the 473-bp fragment by orfA501 (Table 1).

The evaluation of the amplicons at the restorer nuclear locus considered the following: a 898 bp fragment indicated the dominant homozygote (MsMs), both 898 bp and 628 bp fragments indicated the heterozygote (Msms), and a 628 bp fragment indicated the recessive homozygote (msms).

A phenotypic evaluation of the inflorescences from plants identified as S-msms and T-msms was conducted to assess the pollen production. The results of the sample amplifications were obtained by October 2021, before the anthesis, to facilitate the targeted crosses. Later in 2021, the plants identified as Smsms were crossed with plants identified as N sensu Engelke et al. (2003) msms. The aim of this cross was to evaluate the accuracy of the markers in identifying both male-sterile (Smsms) and maintainer (Nmsms) plants. Additionally, 18 plants identified as Tmsms, which showed no visible pollen production, were also crossed with N sensu Engelke et al. (2003) msms plants.

In 2023, the offspring of the test crosses were analyzed: Smsms x N sensu Engelke et al. (2003) msms and Tmsms x N sensu Engelke et al. (2003) msms. The pollen production of 40 plants from the crossbred offspring was manually and visually assessed. Additionally, ten plants from each cross were individually isolated in cages (one plant per cage) and fly pupae were introduced twice weekly to facilitate self-pollination.

RESULTS AND DISCUSSION

Among the 1,728 DNA samples extracted, 1,710 were successfully amplified with the 5'cob and orfA501 markers for cytoplasm identification. A total of 1,126 samples were amplified with the AcSKP1 marker for the Ms locus. Of all the samples, 716 plants (41.9 %) had "N sensu Engelke et al. (2003)" cytoplasm, 963 plants (56.3 %) had sterile T cytoplasm and only 31 plants (1.8 %) had sterile S cytoplasm (Table 3). Therefore, the sterile S cytoplasm was exceptionally rare among these Brazilian populations, while the sterile T cytoplasm was the most prevalent. According to Havey & Kim (2021), the T cytoplasm is rarely used in the development of commercial cultivars in the United States. Khrustaleva et al. (2023) reported that the S cytoplasm is the most common source of CMS used by Russian breeders, with the R cytoplasm being the

Table 2. PCR markers used for the determination of cytoplasm types according to Sato (1998) and Engelke et al. (2003), and Ms locus in onion according to Huo et al. (2015).

Primer names		Reference	Sequence	Amplicon size (bp)
Multiplex	FU898	Huo et al. (2015)	GCAATACACAGCTTCTAGCTGAATT	898
	FD898		AACACACACACAGAGTGAGAAATTTTATATA	
	SU628		TCTGTGTGTGTGTGTAATTTCTCTG	628
	SD628		CGGAAGATTAATATTTTGCGTATACAT	
OrfA501	Primer1	Engelke et al. (2003)	ATGGCTCGCCTTGAAAGAGAGC	473
	Primer2		CCAAGCATTTG GCGCTGAC	
5'cob	S-specific	Sato (1998)	GTCCAGTTCCTATAGAACCTATCACT	414
	N-specific		TCTAGATGTCGCATCAGTGGAATCC	180
	Common		CTTTTCTATGGTGACAACTCCTCTT	

Table 3. Cytoplasm type analysis in six onion cultivars grown in Brazil, using the 5'cob and orfA501 markers.

Cultivar	Cytoplasm type (%)			Number of plants
	S	T	N sensu Engelke	
Bola precoce®	0 (0.0 %)	173 (60.1 %)	115 (39.9 %)	288
Crioula®	2 (0.7 %)	157 (54.7 %	128 (44.6 %)	287
Valessul®	10 (3.5 %)	153 (53.1 %)	125 (43.4 %)	288
Joia®	7 (2.6 %)	208 (76.8 %)	56 (20.7 %)	271
Mega®	7 (2.4 %)	140 (48.6 %)	141 (49.0 %)	288
Princesa do Sul®	5 (1.7 %)	132 (45.8 %)	151 (52.4 %)	288
Total	31	963	716	1,710
Overall (%)	1.8	56.3	41.9	

rarest, while the T cytoplasm was found in 20.5 % of the analyzed breeding lines. Similarly, Khar et al. (2022) observed a predominance of normal (N) cytoplasm and the homozygous recessive genotype (msms) at the Ms locus in Indian onions.

Ferreira et al. (2017), using the 5'cob and orfA501 markers, examined only seven plants from 28 Brazilian onion cultivars and found frequencies of 47, 28 and 25 %, respectively for "N sensu Engelke et al. (2003)", S and T cytoplasms. Ragassi et al. (2012) analyzed 66 samples from cultivars in a bulk of ten plants using the same markers and identified frequencies of 18.2, 56 and 25.8 %, respectively for the "N sensu Engelke et al. (2003)", S and T cytoplasms. Santos et al. (2008) also evaluated the cytoplasm type in two open-pollinated onion populations from northeastern Brazil: in one population they found T and "N sensu Engelke et al. (2003)" cytoplasms, while the other population, derived from a cross using a single male-sterile plant as the female parent, had only the S cytoplasm. The significant differences in cytoplasm frequencies between these studies may result from the small number of samples analyzed by Ferreira et al. (2017) and the bulk analysis approach used by Ragassi et al. (2012). Open-pollinated cultivars exhibit a considerable intrapopulation variation, and a limited sample size can lead to biased results. Additionally, the present study focused on commercial cultivars primarily used in southern Brazil, what may explain the differences in cytoplasm frequencies, when compared to the northeastern cultivars evaluated by Ferreira et al. (2017) and Ragassi et al. (2012).

Table 3 provides the analysis of cytoplasm types in six Brazilian onion cultivars using the 5'cob and orfA501 markers. Analyzing the cytoplasmic markers for each cultivar revealed that Bola Precoce® had no plants with S cytoplasm, 173 plants (60.07 %) had T cytoplasm and 115 plants (39.93 %) "N sensu Engelke et al. (2003)" cytoplasm. The Crioula® cultivar had only two plants (0.7 %) with S cytoplasm, 157 plants (54.7 %) with T cytoplasm and 128 plants with "N sensu Engelke et al. (2003)" cytoplasm. These findings contradict those of Ragassi et al. (2012), who reported that Bola Precoce® had only T cytoplasm and Crioula® only S cytoplasm. This discrepancy likely arises from the analysis of a single DNA bulk and the small sample size used in their study. The results of the current study are more consistent with the origins of these cultivars, which are open-pollinated and derived from mass selection of the Baia onion or natural crosses involving Baia (Yokoyama et al. 1984, Gandin et al. 1986, Barbieri & Medeiros 2007). It is unlikely that two open-pollinated cultivars would differ entirely in cytoplasm type or exhibit only one type within their populations.

Among the cultivars and accessions analyzed by Ragassi et al. (2012) is Rijnsburger, a cultivar that is possibly the source of the sterile R-type cytoplasm (Havey & Kim 2021). According to the data evaluated by these authors, the DNA bulk of ten plants from the Rijnsburger accession, analyzed with the 5'cob and orfA501 markers, indicated the presence of N cytoplasm ["N sensu Engelke et al. (2003)"]. To some extent, these data support the information presented by Havey & Kim (2021) and suggest that, within the group of plants identified as "N sensu Engelke et al. (2003)", there may be plants with N and/or R cytoplasm. However, to fully clarify this issue, it would be necessary to evaluate all plants identified as "N sensu Engelke et al. (2003)" in our study with the orf725 marker. Ferreira et al. (2017) evaluated DNA samples from seven plants of the Bola Precoce® cultivar using the 5'cob, orfA501 and orf725 markers, and the analysis with the first two markers indicated only plants with T cytoplasm, in

the seven plants analyzed. However, amplifications with orf725 revealed that six out of seven plants contained R cytoplasm (presence of both 833 and 628 bp fragments), as per the results of Havey & Kim (2021), while only one plant showed the presence of N or T cytoplasm (presence of only one 833 bp fragment). The analyses with the 5'cob and orfA501 markers differ from the results of this study, where it was observed that 60 % of the plants of this cultivar had T cytoplasm. This discrepancy may be attributed to the small sample size in the study by Ferreira et al. (2017). However, when considering the combined results with the orf725 marker, the results of Ferreira et al. (2017) conflict and diverge completely among the markers used by the authors, according to the amplification patterns of the Cob5', orfA501 and orf725 markers obtained by Havey & Kim (2021).

In the present study, Valessul® had ten plants (3.47 %) with S cytoplasm, 153 plants (53.13 %) with T cytoplasm and 125 plants (43.4 %) with "N sensu Engelke et al. (2003)" cytoplasm. This cultivar was developed through several cycles of mass selection from a segregated population resulting from a cross between Bola Precoce® and Crioula® (Alves et al. 2017). The Joia®, Mega® and Princesa do Sul® cultivars are even more recent and likely derived from the Valessul® through mass selection or crossings, as they share a significant phenotypic similarity, including cycle length and bulb characteristics. The Joia® cultivar had seven plants (2.58 %) with S cytoplasm, 208 plants (76.75 %) with T cytoplasm and 56 plants (20.66 %) with "N sensu Engelke et al. (2003)" cytoplasm, the lowest frequency of "N sensu Engelke et al. (2003)" cytoplasm among the evaluated cultivars (Table 3). The Mega® cultivar had seven plants (2.43 %) with S cytoplasm, 140 plants (48.61 %) with T cytoplasm and 141 (48.96 %) with "N sensu Engelke et al. (2003)" cytoplasm. Princesa do Sul® had five plants (1.74 %) with S cytoplasm, 132 plants (45.83 %) with T cytoplasm and 151 plants (52.43 %) with "N sensu Engelke et al. (2003)" cytoplasm, the highest frequency.

Only Ferreira et al. (2017) analyzed the restorer nuclear locus (Ms) in a southern Brazilian onion cultivar (Bola Precoce®), using the AcSKP1 marker, identifying four homozygous-dominant plants (MSMS) and three heterozygous plants (MSms) among the seven plants evaluated. Ferreira & Santos (2018) also reported a partial success using this marker in some accessions from the onion germplasm bank. Santos et al. (2010) visually estimated a frequency of 2.0 % male-sterile plants in an open-pollinated cultivar from northeastern Brazil, where the cytoplasm was of the T type.

In this study, based on the AcSKP1 marker, 603 plants (53.55 %) had a homozygous-dominant genotype (MsMs), 432 plants (38.37 %) were heterozygous (Msms) and 91 individuals (8.08 %) were homozygous recessive (msms). Thus, the frequency of dominant alleles at the Ms locus was 72.74 %, and the recessive allele frequency was 27.26 % (Table 4).

Three Smsms plants did not produce pollen and 45 out of 49 plants with "N sensu Engelke et al. (2003)" msms produced pollen. Four of the non-pollen producing plants likely have R cytoplasm (R msms) rather than N, as N msms plants should be male-fertile. Of the 45 male-fertile plants, three were crossed with Smsms plants, 18 with Tmsms plants and 24 were self-pollinated. Among the offspring from the S-msms x "N sensu Engelke et al. (2003)" msms crosses, 40 plants produced no pollen, confirming that the pollinators were maintainers (Nmsms). Additionally, 18 Tmsms plants that did

Table 4. Number and percentage of each genotype according to the AcSKP1 marker and the frequency of recessive and dominant alleles of the MS gene locus in each cultivar and in the total number of analyzed plants.

Cultivar	Genotypes according to AcSKP1 (%)			Total	Allele frequency	
	MSMS	MSms	msms		MS	ms
Bola Precoce®	104 (51.0 %)	85 (41.7 %)	15 (7.4 %)	204	71.8 %	28.2 %
Crioula®	129 (44.8 %)	129 (44.8 %)	30 (10.4 %)	288	67.2 %	32.8 %
Valessul®	110 (51.6 %)	86 (40.4 %)	17 (8.0 %)	213	71.8 %	28.2 %
Joia®	68 (64.8 %)	28 (26.7 %)	9 (8.6 %)	105	78.1 %	21.9 %
Mega®	95 (62.1 %)	48 (31.4 %)	10 (6.5 %)	153	77.8 %	22.2 %
Princesa do Sul®	97 (59.5 %)	56 (34.4 %)	10 (6.1 %)	163	76.7 %	23.3 %
Total	603	432	91	1,126	Overall allele frequency	
Overall genotypes (%)	53.6	38.4	8	-	72.7	27.3

not produce pollen were crossed with 18 "N sensu Engelke et al. (2003)" msms plants. The analysis of pollen production in the F1 populations revealed that male fertility was restored in four populations (22.2 %). In the remaining 14 populations, male sterility persisted, and no seeds were produced after self-pollination, confirming their sterility. These finding align with those of Schweisguth (1973) and Havey (2000), what suggests that different loci or alleles control the restoration of male fertility in S and T cytoplasms.

In Brazil, onions are cultivated across a broad latitudinal range, from near the equator (5ºS) to southern regions at 34ºS (IBGE 2024). The availability of both T and S cytoplasmic types with the same or similar phenotypes in southern Brazil suggests that the T cytoplasm can be a more suitable source of male sterility for hybrid onion production, due to its wide prevalence. Considering the most cultivated cultivars in the southern region of Brazil, it was observed that the frequency of cytoplasm types and the distribution of alleles at the MS locus are similar among the cultivars. The S cytoplasm is extremely rare among the main onion cultivars used in southern Brazil; however, its presence is detectable and can be used for hybrid development.

CONCLUSIONS

1. The molecular markers 5'cob, orfA501 and AcSKP1 are valuable tools in onion breeding programs for identifying N "sensu Engelke et al. (2003)" msms, Smsm and Tmsms plants, which can be used to develop inbred lines;
2. The male-sterile T cytoplasm is predominant in open-pollinated onion cultivars in southern Brazil, facilitating the production of male-sterile lines from locally adapted cultivars. In contrast, the sterile S cytoplasm is extremely rare (1.8 %) in these populations;
3. Based on the AcSKP1 marker, the dominant allele frequency at the Ms locus in these populations is 72.74 %, while the recessive allele frequency is 27.26 %.

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