



RESEARCH ARTICLE

Molecular screening of various apple genotypes against woolly apple aphid

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Received: 13 June 2026; Accepted: 30 July 2026; Available online: Version 1.0: 10 September 2026

Cite this article: Pawar ST, Sajad M, Akhtar AK, Abu M, Abdul RM, Shivani, Khalid ZM. Molecular screening of various apple genotypes against woolly apple aphid. Plant Science Today (Early Access). <https://doi.org/10.14719/pst.16057>

Abstract

The study evaluated 15 apple genotypes (Mitch Gala, Red Jonaprince, King Fuji, Golden Delicious Reinders, Gala Redlum, Fuji Zehn Aztec, Pinova, Red Braeburn, Devil Gala, Red Chief, Elstar, Crab apple, M-7, M-9 T337 and MM-106) for resistance to woolly apple aphid (WAA). Field samples were collected from experimental orchards at Sher-e-Kashmir University of Agricultural Sciences and Technology, Shalimar, Jammu and Kashmir, India. Molecular marker analysis was conducted at the transcriptomics K-Lab, Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences and Technology, Shalimar, Jammu and Kashmir, India. The WAA is a serious pest that causing significant yield and quality losses in apple orchards. This study aimed to evaluate the presence of molecular markers associated with WAA resistance genes (*Er1*, *Er2* and *Er3*) in 15 apple (*Malus domestica* Borkh.) genotypes. The results indicated that several apple cultivars exhibited differential responses to WAA infestation. Polymerase chain reaction (PCR) was used to screen apple cultivars for 8 molecular markers (NZsc_C20, NZsc_GS327, NZsc_O05, NZms_EB145764, NZsn_O05, NZms_EB106753, NZsc_E01, NZsc_A01) associated with the main WAA resistance genes (*Er1*, *Er2* and *Er3*). The NZms_EB145764 marker is connected to the *Er2* resistance gene and detected in the 12 cultivars: Mitch Gala, Red Jonaprince, King Fuji, Golden Delicious Reinders, Gala Redlum, Fuji Zehn Aztec, Pinova, Red Braeburn, Devil Gala, Elstar, Crab apple and M-9 T337. The NZsc_O05 marker is connected to the *Er1* resistance gene and is found in the following cultivars: Mitch Gala, Golden Delicious Reinders, fuji Zehn Aztec, Pinova, Red Braeburn, Devil Gala, Red Chief, Elstar, Crab apple, M-7 and M-9 T337. The NZsc_A01 marker is connected to the *Er3* resistance gene and is found in the 11 cultivars: Mitch Gala, Red Jonaprince, King Fuji, Golden Delicious Reinders, Gala Redlum, Pinova, Red Braeburn, Devil Gala, Red Chief, Elstar and Crab apple. The NZsn_O05 marker is connected to the *Er1/Er3* resistance gene and is found in only one cultivar: Crab apple.

Keywords: gene markers; molecular screening; resistance; susceptibility; woolly apple aphid

Introduction

Apple is widely recognised as one of the most important temperate fruit crops grown worldwide. Its commercial importance stems not only from its widespread cultivation and economic value but also from its nutritional and health promoting properties. Apples are rich in dietary fibre, antioxidants and flavonoids, making them a vital component of a balanced diet (1).

Beyond their appealing flavour and versatility, apples are considered a nutritional powerhouse due to their high content of phytochemicals such as quercetin, catechin, phloridzin and chlorogenic acid. These compounds act as potent antioxidants, contributing to the prevention of oxidative stress and offering protective effects against chronic diseases (2). The presence of such bioactive molecules not only enhances the fruit's dietary value but also underscores its role in functional nutrition and human health.

Consequently, apple cultivation holds dual significance economic and nutritional making it a cornerstone of temperate horticulture worldwide.

The woolly apple aphid (WAA), *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae) is a widespread quarantine pest of apples. Aphids can cause fruit distortion, stunted shoot growth, decreased tree vigour, decreased apple output and even tree death when they colonise branches, roots, bark and injury sites (3). The WAA outbreaks in orchards vary with temperature during the growing season (April to late October) (4).

Woolly apple aphid, native to North America, is a significant economic pest in apple-producing regions and is commonly found on nursery supplies (5). It is believed that imported nursery apple stocks were brought to India, with the first documented instance of apple trees being affected occurring in 1889 in Coonoor, South India,

where an outbreak was discovered damaging the trees. In 1920, it became a major pest of apples in Kashmir following the introduction of nursery stocks from China (6).

Pesticides are commonly employed to suppress WAA (*Eriosoma lanigerum*) infestations in commercial orchards. However, their effectiveness is often limited due to the unique biology and behaviour of the pest. Woolly apple aphids tend to conceal themselves within bark crevices, cracks and protected sites on the tree, which reduces direct exposure to chemical sprays and allows populations to persist despite repeated applications. In addition, the aphids produce copious waxy secretions that envelop their bodies, forming a protective barrier against insecticides and environmental stressors (7). This wax covering not only shields them from chemical penetration but also complicates mechanical removal, thereby enhancing their survival. As a result, reliance on pesticides alone has proven inadequate for long-term management, highlighting the need for alternative strategies such as host plant resistance and molecular screening of apple genotypes to identify durable sources of resistance.

Molecular studies have been instrumental in unravelling the genetic basis of aphid resistance in apple. Quantitative trait loci (QTLs) associated with resistance have been identified, providing valuable insights into the inheritance of resistance traits. Several molecular markers have been successfully linked to QTLs conferring resistance against different aphid species, including the pink apple aphid (*Dysaphis plantaginea*), the leaf curling aphid (*Dysaphis devecta*) and the green apple aphid (*Aphis pomi*) (8). These findings highlight the genetic diversity within *Malus* germplasm and the potential to exploit resistant cultivars in breeding programmes.

Recent advancements have further refined this approach, leading to the development of molecular markers closely associated with resistance genes against WAA (*Eriosoma lanigerum*). Notably, three major resistance genes *Er1*, *Er2* and *Er3* have been identified and linked to molecular markers, enabling marker-assisted selection in breeding (9). The availability of such markers accelerates the screening of diverse apple genotypes, facilitates the introgression of resistance into elite cultivars and enhances the efficiency of breeding programs aimed at durable pest resistance. Collectively, these molecular tools represent a significant step toward sustainable management of WAA through genetic resistance.

Breeding initiatives aim to provide rootstocks that are resistant to biotic stress and promote sustainable agriculture by minimising the use of pesticides (10). The "Northern Spy" cultivar was previously used as an apple rootstock due to its resistance to WAA (11). Apple breeding programs try to combine the *Er1* gene with other minor genes that provide resistance to *E. lanigerum* (12, 13). Gene pyramiding involves developing many resistance genes against pests or diseases to boost their longevity. Using DNA techniques like microsatellites and SSR markers to map genes linked to *E. lanigerum* resistance can help apple breeding programs identify promising seedlings with the desired gene shortly after germination. Several marker systems such as simple sequence repeats (SSRs), amplified fragment length polymorphisms (AFLPs), restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNAs (RAPDs) have been employed in genetic linkage mapping to identify markers associated with important genes and traits (14–17). Among these, SSR markers were preferred in the present study because they are highly reproducible, co-dominant and abundant in plant genomes, offering greater polymorphism and

resolution compared to dominant markers like RAPDs and AFLPs. Unlike RFLPs, SSRs require less DNA and are more amenable to high-throughput analysis, making them particularly suitable for constructing reliable linkage maps and for marker-assisted selection.

East Malling Institute collaborated with John Innes Institute to create the MM series and Merton Immune of resistant apple rootstocks to WAA (18, 19). MM106 and MM111 are still the most common resistant rootstocks worldwide (20).

To verify the presence of molecular markers associated with the *Er1*, *Er2* and *Er3* resistance genes in the selected apple cultivars, a comprehensive molecular analysis was undertaken. This screening involved the use of specific DNA markers linked to these resistance loci, enabling precise detection of resistance alleles within the genotypes. By employing marker-assisted techniques, the study aimed to distinguish resistant cultivars from susceptible ones, thereby providing valuable insights into the genetic architecture of WAA resistance. Such molecular characterisation not only facilitates the identification of resistant germplasm but also strengthens breeding programs by accelerating the introgression of durable resistance traits into elite apple varieties.

Materials and Methods

The study evaluated 15 apple genotypes (Mitch Gala, Red Jonaprince, King Fuji, Golden Delicious Reinders, Gala Redlum, Fuji Zehn Aztec, Pinova, Red Braeburn, Devil Gala, Red Chief, Elstar, Crab apple, M-7, M-9 T337 and MM-106) for resistance to WAA. Field samples were collected from experimental orchards at Sher-e-Kashmir University of Agricultural Sciences and Technology, Shalimar, Jammu and Kashmir, India. Molecular marker analysis was conducted at the transcriptomics K-Lab, Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences and Technology, Shalimar, Jammu and Kashmir, India. The molecular analysis was used to assess variation among the genotypes and determine their resistance status to woolly apple aphid. The identified resistant genotypes can be used as potential sources for breeding programmes aimed at developing WAA-resistant apple cultivars.

Healthy leaf tissues were collected from all 15 apple genotypes (Mitch Gala, Red Jonaprince, King Fuji, Golden Delicious Reinders, Gala Redlum, Fuji Zehn Aztec, Pinova, Red Braeburn, Devil Gala, Red Chief, Elstar, Crab apple, M-7, M-9 T337 and MM-106) and stored at -20°C until use. The tissues were ground to a fine powder in liquid nitrogen using a sterile mortar and pestle. Template DNA was extracted following a modified CTAB protocol (21). For each genotype, 0.5 g of fine leaf powder was suspended in 2mL of DNA extraction buffer containing 0.22M EDTA (pH8), 0.8M NaCl, 0.14M sorbitol, 0.2M Tris-HCl (pH8), 0.8 % CTAB, 0.5 % SDS and 0.5 % β -mercaptoethanol. The suspension was vigorously mixed with approximately 500 μ L of chloroform and incubated at 65°C for 40min before centrifugation.

The supernatant was carefully extracted and DNA was precipitated with an equal volume (500 μ L) of isopropanol. The DNA pellet was collected by centrifugation and washed with 70 % ethanol to remove impurities. Finally, the pellet was air-dried and dissolved in 100 μ L of TE buffer (10mM Tris-HCl, 1mM EDTA and pH8). DNA concentration and purity were determined using UV spectrophotometry at 260nm and 280nm. Molecular markers linked to *Er1*, *Er2* and *Er3* resistance genes were amplified using specific primers reported by previous researchers, as enlisted in the Table 1 (9).

A total volume of 25 µL was used for each PCR amplification cycle. About 300 ng of template DNA, 12.5 µL of 2x PCR master mix solution, 5 pmol of each primer and distilled water were added to the PCR reaction mixture to complete the volume. The eight molecular markers (NZsc_C20, NZsc_GS327, NZsc_O05, NZms_EB145764, NZsn_O05, NZms_EB106753, NZsc_E01, NZsc_A01) were amplified using an extension time of 2 min and an annealing temperature of 60 °C. The remaining markers were amplified using a touch down PCR cycling regime, where the annealing temperature was lowered from 65 to 60 °C in steps of 0.5 °C per cycle.

The PCR products were separated and analysed using agarose gel electrophoresis on 1–1.5 % agarose gels stained with ethidium bromide. The gels were prepared by dissolving agarose in 1x TAE buffer, followed by heating and cooling before the addition of ethidium bromide to visualise DNA fragments under UV light. Electrophoresis was carried out at a constant voltage until clear band separation was achieved. Results were scored based on the presence or absence of specific amplicons, thereby indicating whether the resistance-linked molecular markers were successfully amplified.

Results and Discussion

The study evaluated 15 apple genotypes (Mitch Gala, Red Jonaprince, King Fuji, Golden Delicious Reinders, Gala Redlum, Fuji Zehn Aztec, Pinova, Red Braeburn, Devil Gala, Red Chief, Elstar, Crab apple, M-7, M-9 T337 and MM-106) for resistance to woolly apple aphid. These genes have laid the groundwork for the development of cultivars with long-term resistance to WAA infestations. In the current study, the apple cultivars under consideration underwent molecular screening to determine the existence of resistance-associated markers. Eight different genetic markers connected to the *Er1*, *Er2* and *Er3* loci were amplified and examined using PCR (Table 2). The amplification profiles obtained with these markers are

presented in Fig. 1 and the resulting banding patterns enabled the differentiation of resistant and susceptible genotypes. The presence of multiple resistance markers in certain apple cultivars indicates that these genotypes diverse resistance loci which act additively or synergistically to confer enhanced protection against WAA. In contrast, cultivars with few or no markers lack these loci, rendering them highly susceptible. Marker-based assessment improves breeding operations by making it easier to identify resistant cultivars and accelerate the introduction of resistance genes into elite apple cultivars.

The NZsc_C20 marker is linked to *Er1* resistant gene and found in only two cultivars: Red Jonaprince and M-9 T337. NZsc_GS327 marker is linked to *Er1* resistance gene and is present in Mitch Gala, King Fuji, Gala Redlum, Elstar, M-9 T337 cultivars. The NZsc_O05 marker is connected to the *Er1* resistance gene and is found in the following cultivars: Mitch Gala, Golden Delicious Reinders, Fuji Zehn Aztec, Pinova, Red Braeburn, Devil Gala, Red Chief, Elstar, Crab apple, M-7 and M-9 T337. The NZms_EB145764 marker is connected to the *Er2* resistance gene and is found in the 12 cultivars: Mitch Gala, Red Jonaprince, King Fuji, Golden Delicious Reinders, Gala Redlum, Fuji Zehn Aztec, Pinova, Red Braeburn, Devil Gala, Elstar, Crab apple and M-9 T337.

The NZsn_O05 marker is connected to the *Er1/Er3* resistance gene and is found in only one cultivar: Crab apple. The NZms_EB106753 marker is connected to the *Er1/Er3* resistance gene and is found in the 10 cultivars: Mitch Gala, Red Jonaprince, Golden Delicious Reinders, Fuji Zehn Aztec, Pinova, Devil Gala, Elstar, Crab apple, M-7 and MM-106. The NZsc_E01 marker is connected to the *Er3* resistance gene and is found in the 8 cultivars: Red Jonaprince, King Fuji, Golden Delicious Reinders, Pinova, Red Braeburn, Elstar, M-7 and M-9 T337. The NZsc_A01 marker is connected to the *Er3* resistance gene and is found in the 11 cultivars: Mitch Gala, Red Jonaprince, King Fuji, Golden Delicious Reinders, Gala Redlum, Pinova, Red Braeburn, Devil Gala, Red Chief, Elstar and Crab apple.

Table 1. Molecular markers and specific primers linked to *Er1*, *Er2* and *Er3* resistance genes

Name of marker	Woolly apple resistance gene	Primer sequence 5' → 3'		Product size (bp)
		Forward	Reverse	
NZsc_C20	<i>Er1</i>	TCTCTAACTCAATAACTCCCAAGAC	ACTTCGCCACCATTATCACTCCTGA	2000
NZsc_GS327	<i>Er1</i>	GCCAAGCTTCAATGTCGGAGTAGAT	CAAGCTTCCCCTAAGGCTATTGCCA	1600
NZsc_O05	<i>Er1</i>	CCCAGTCACTAACATAATTGGCACA	CCCAGTCACTGGCAAGAGAAATTAC	1700
NZms_EB145764	<i>Er2</i>	TTCCAGCGATCCAAAACAAT	GCTCAGGAACACCTCGTTCT	198
NZsn_O05	<i>Er1/Er3</i>	AACGTCAATGTCAATAT	CCCAGTCACTGGCAAGAGAAATTAC	880
NZms_EB106753	<i>Er1/Er3</i>	TCTGAGGCTCCCAAGTCC	TAGGAGCAGAAGAGGTGACG	175
NZsc_E01	<i>Er3</i>	CCCAAGGTCCGAACACAAATGAGAG	CCCAAGGTCCAAACTATCCCGAAG	1350
NZsc_A01	<i>Er3</i>	CAGGCCCTTCAGCAAGAGGTGTCT	CAGGCCCTTCACTACTAATAAGAAC	1250

Table 2. Gene markers associated with woolly apple aphid (WAA) resistance

Gene markers associated with WAA resistance									
SN	Cultivars	NZsc_C20	NZsc_GS327	NZsc_O05	NZms_EB145764	NZsn_O05	NZms_EB106753	NZsc_E01	NZsc_A01
1	Mitch Gala	-	+	+	+	-	+	-	+
2	Red Jonaprince	+	-	-	+	-	+	+	+
3	King Fuji	-	+	-	+	-	-	+	+
4	Golden Delicious Reinders	-	-	+	+	-	+	+	+
5	Gala Redlum	-	+	-	+	-	-	-	+
6	fuji Zehn Aztec	-	-	+	+	-	+	-	-
7	Pinova	-	-	+	+	-	+	+	+
8	Red Braeburn	-	-	+	+	-	-	+	+
9	Devil Gala	-	-	+	+	-	+	-	+
10	Red chief	-	-	+	-	-	-	-	+
11	Elstar	-	+	+	+	-	+	+	+
12	Crab apple	-	-	+	+	+	+	-	+
13	M-7	-	-	+	-	-	+	+	-
14	M-9 T337	+	+	+	+	-	-	+	-
15	MM-106	-	-	-	-	-	+	-	-

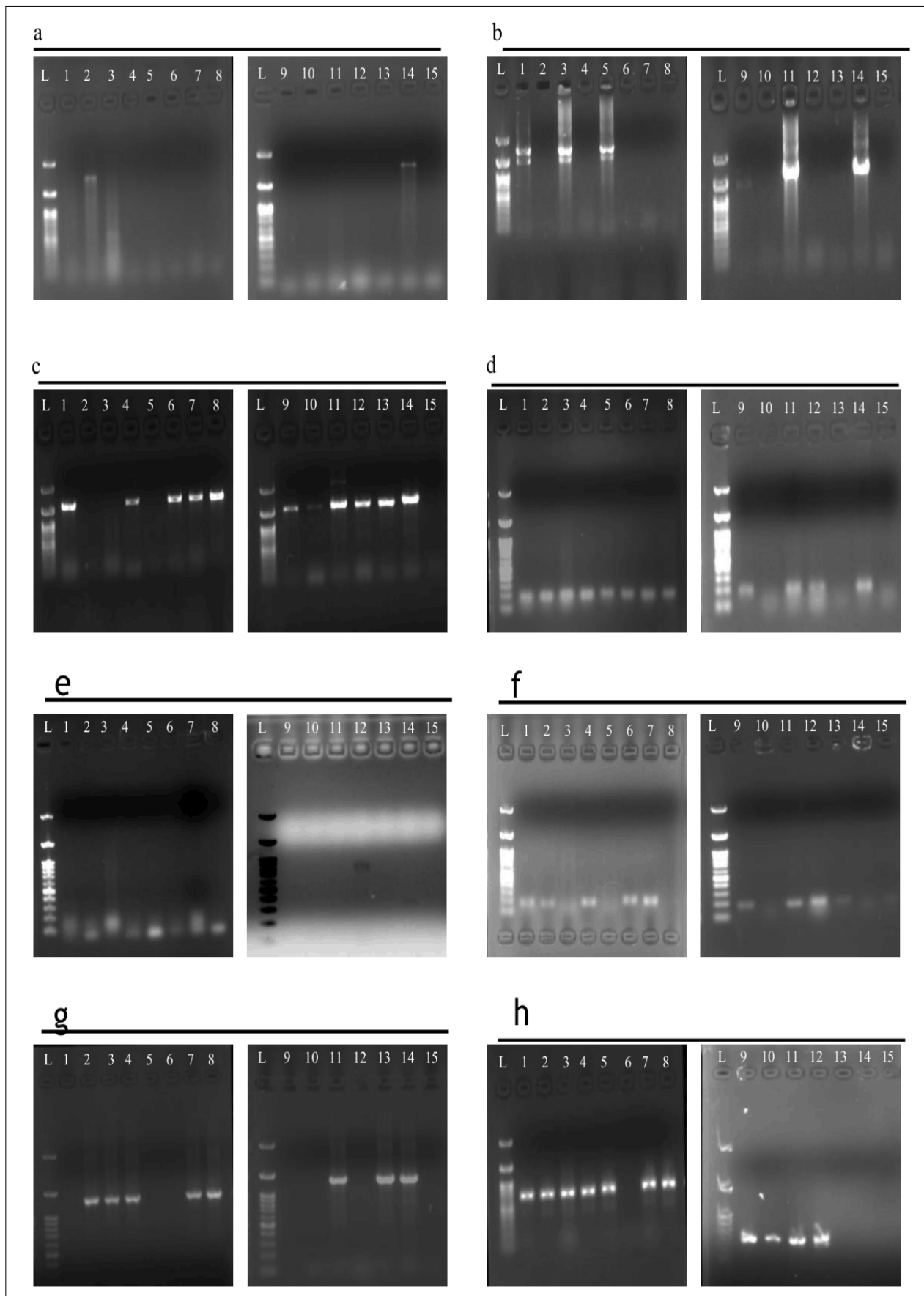


Fig. 1. Polymerase chain reaction (PCR) amplification of the target gene (*Er1*, *Er2* and *Er3*) using various primers on different genotypes (Mitch Gala; Red Jonaprince; King Fuji; Golden Delicious Reinders; Gala Redlum; Fuji Zehn Aztec; Pinova; Red Braeburn; Devil Gala; Red chief; Elstar; Crab apple; M-7; M-9 T337; MM-106) of the apple growing in SKUAST-K, Shalimar to identify the resistance against Woolly apple aphids (a) PCR amplification using primer NZsc_C20; (b) PCR amplification using primer NZsc_GS327; (c) PCR amplification using primer NZsc_O05; (d) PCR amplification using primer NZms_EB145764; (e) PCR amplification using primer NZsn_O05; (f) PCR amplification using primer NZms_EB106753; (g) PCR amplification using primer NZsc_E01; (h) PCR amplification using primer NZsc_A01; L:ladder; (1) Mitch Gala; (2) Red Jonaprince; (3) King Fuji; (4) Golden Delicious Reinders; (5) Gala Redlum; (6) Fuji Zehn Aztec; (7) Pinova; (8) Red Braeburn; (9) Devil Gala; (10) Red chief; (11) Elstar; (12) Crab apple; (13) M-7; (14) M-9 T337; (15) MM-106.

A previous study found that three major WAA resistance genes (*Er1*, *Er2* and *Er3*) have been utilised in apple breeding. The study identified three resistant cultivars ('Stark Gold', 'Early Gold', 'Argi Gala'), two moderately resistant ('Evanee', 'Black Stayman'), six moderately susceptible ('Vista Bella', 'Jonagold', 'Royal Gala', 'Jersey Mac', 'Granny Spur Type', 'Summerred') and five susceptible ('Jonathan', 'Nagava 6', 'Florina', 'Red Chief', 'Gold Iralis'). 'Royal Gala' is a well-known susceptible cultivar. The markers NZms_EB106753 (linked to *Er1/Er3*) and NZsc_E01 (*Er3*) were present in all cultivars, while NZsn_O05 was absent. The NZsc_A01 (*Er3*) occurred in all except 'Fuji 6', 'Jonagold' and 'Granny Spur Type' (22). Previous work showed that *Er3*-mediated resistance in 'Aotea' was overcome by WAA, highlighting its distinction from *Er1* and *Er2* resistance (23). According to previous studies, the WAA resistant genotypes "Northern Spy" and "Robusta 5" have *Er1* and *Er2* resistant genes, respectively (23, 24). A previous study found that NZsn_O05 and NZsc_E01 markers were therefore more effective in identifying resistant plants. Similarly, NZms_EB145764, NZms_EB106753 and NZsc_A01 showed monomorphic bands in both susceptible and tolerant plants but NZsc_C20, NZsc_GS327 and NZsc_O05 (connected to *Er1*) showed no PCR product (25). The study showed that the responses to WAA varied significantly among apple varieties. Resistant cultivars carried *Er* genes that prevented aphid colonisation, but susceptible cultivars allowed for ongoing infestation. The study verified incompatibility as a reliable sign of resistance, emphasising its importance in breeding operations. Resistant rootstocks thus provide good resistance to WAA (26).

Conclusion

Polymerase chain reaction (PCR) screening was employed to evaluate eight molecular markers (NZsc_C20, NZsc_GS327, NZsc_O05, NZms_EB145764, NZsn_O05, NZms_EB106753, NZsc_E01 and NZsc_A01) associated with the three major woolly apple aphid (WAA) resistance genes (*Er1*, *Er2* and *Er3*). Among these, the marker NZms_EB145764, linked to the *Er2* resistance gene, was successfully amplified in 12 cultivars, indicating the widespread occurrence of this resistance allele across the tested germplasm. Similarly, the markers NZsc_O05 and NZsc_A01 were detected in 11 cultivars, each demonstrating their potential utility in resistance screening.

The successful amplification of these markers across multiple cultivars demonstrates their usefulness in identifying resistant and potential resistant genotypes. These findings highlight the significance of molecular markers in resistance breeding, since they provide dependable tools for marker-assisted selection and accelerate up the introduction of lasting resistance genes into elite apple cultivars. Overall, the findings show that molecular screening is a viable technique for discovering resistant cultivars and strengthening breeding programmes aiming at sustainable management of WAA.

Acknowledgements

The authors gratefully acknowledge the financial support provided through the CSIR fellowship, which was instrumental in the successful completion of this study. The authors also thank Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir (SKUAST-K), Srinagar, for providing the research facilities and institutional support necessary to conduct this research.

Authors' contributions

PST performed DNA isolation, PCR amplification, molecular screening and initial data collection and contributed to drafting the manuscript. SM conceived and designed the study and supervised the experimental work. AM maintained the plant material and supported data validation. ARM provided the field map of apple genotypes and leaf samples. S contributed to the literature review, data organisation and manuscript editing. KZM and AAK guided the molecular methodology, supervised the overall research framework, interpreted the data and finalised the manuscript for submission. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors utilised ChatGPT to assist in reviewing and refining the English translation. These tools were employed solely to ensure that the language was presented in proper academic English. Following their use, the authors carefully reviewed and edited the content as necessary and take full responsibility for the accuracy and integrity of the publication.

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Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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