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Molecular fingerprinting and genetic diversity screening across plant species using conserved RCAR domain-based markers

Molekulárne profilovanie a skríning genetickej variability rastlinných druhov pomocou markera konzervovanej RCAR domény

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ABSTRACT

The RCAR (Regulatory Component of ABA Receptor) domain, a conserved region associated with abscisic acid (ABA) signalling, plays a significant role in plant stress responses and developmental processes. In this study, a newly developed DNA marker technique based on the conserved RCAR domain was used to assess genetic diversity among 25 plant species and 20 tomato (*Solanum lycopersicum* L.) cultivars. PCR amplification using degenerate primers amplified reproducible fragments across all samples. Genetic similarity analysis, based on Jaccard coefficients, revealed high homogeneity among tomato cultivars (>85%), while other species exhibited broader genetic variation (30–80%). UPGMA clustering and AMOVA confirmed clear genetic differentiation between tomato and other plant species, with 40.32% of variation attributed to interspecies differences. These results demonstrate the utility of RCAR domain-based markers in evaluating interspecific and intraspecific genetic relationships, providing a foundation for future studies on the functional roles of RCAR in plant adaptation and signalling.

Keywords: DNA markers, genetic diversity, tomato cultivars, interspecific variation

ABSTRAKT

RCAR doména (regulačná zložka receptora ABA), konzervovaná oblasť spojená so signalizáciou kyseliny abscisovej (ABA), hrá významnú úlohu v stresových reakciách rastlín a vývinových procesoch. V tejto štúdii bola použitá novo vytvorená technika DNA markerov založená na konzervovanej doméne RCAR na posúdenie genetickej diverzity a vzťahov medzi 25 rastlinnými druhmi vrátane liečivých rastlín, stromov a zeleniny a 20 odrodami rajčiakov (*Solanum lycopersicum* L.). PCR amplifikácia s použitím degenerovaných primerov úspešne produkovala amplikóny v rozpätí 200 – 500 bp vo všetkých analyzovaných druhoch. Analýza genetickej podobnosti založená na Jaccardových koeficientoch odhalila vysokú homogenitu medzi kultivarmi rajčiakov (> 85 %), zatiaľ čo ostatné rastlinné vykazovali širšiu genetickú variabilitu (30 – 80 %). Klastrovanie UPGMA a AMOVA podporili genetické odlišnosti medzi rajčiakmi a inými rastlinnými druhmi, pričom 72 % variácií bolo vplyvom medzidruhových rozdielov. Tieto výsledky demonštrujú užitočnosť markerov založených na doméne RCAR pri hodnotení medzidruhových a vnútrodruhových genetických vzťahov, čo poskytuje základ pre budúce štúdie funkčných úloh RCAR v adaptácii a signalizácii rastlín.

Kľúčové slová: DNA markéry, genetická diverzita, odrody rajčiakov, interšpecifická variabilita

INTRODUCTION

Lacking mobility, plants rely on intricate immune responses, including both constitutive and inducible defenses (Li et al., 2020). Abscisic acid (ABA) is a key phytohormone regulating both development and stress tolerance, particularly under drought, salinity, and extreme temperatures. It functions by controlling stomatal closure, ion balance, and activating stress-responsive genes (Zhu et al., 2016; Zhao et al., 2017).

The PYR/PYL/RCAR receptor family is central to ABA signaling (Li et al., 2024). First identified in *Arabidopsis thaliana* (L.) Heynh. (Park et al., 2009), these receptors are now widely recognized as primary ABA sensors (Wang et al., 2013), forming the largest known group of plant hormone receptors (Dupeux et al., 2011). Located in the cytosol, they perceive ABA and initiate downstream signalling (Ma et al., 2009), triggering adaptive responses like stomatal closure and osmotic adjustment (Chen et al., 2020; Akhter et al., 2021; Yang et al., 2019a). The RCAR domain (Regulatory Component of ABA Receptor), a conserved region within these receptors, plays a crucial role in ABA perception and interaction with negative regulators like PP2Cs (Cutler et al., 2010; Li et al., 2024). Its conservation across diverse plant taxa suggests functional stability, while minor sequence variations may reflect adaptive evolution (Komatsu et al., 2020).

The RCAR domain is a conserved structural motif within the Bet v 1 protein superfamily, which plays a critical role in ligand binding and signal transduction in both plants and allergens (Kofler et al., 2012; Fernandes et al., 2013). RCAR domain in Bet v 1 homologs functions as an ABA receptor in plants, mediating stress and defense responses (Müller et al., 2014) and possesses a high degree of conservation (Ma et al., 2009).

Conserved domains of plant DNA are an important source of DNA-based markers. Many of them were developed as universal in their use, such as P450-based Analogue Markers (PBA; Rana et al., 2005), Conserved DNA Derived Polymorphism (CDDP; Collard and Mackill, 2009), and Tubulin-Based Polymorphism (TBP; Bardini et al., 2004). These markers have been proven to be effective in the DNA polymorphism analysis of different plant species. Fingerprints generated by the polymorphism of cytochrome P450 genes could be used for map construction, as it was reported for *Solanum tuberosum* L. (Yamanaka et al., 2005), interspecies polymorphism determination (Moravčíková et al., 2024) or for intraspecies characterization of the endangered medicinal plant *Rhazya stricta* Decne. (Gilani et al., 2013). Conserved DNA-derived polymorphism was used in the analysis of genetic diversity of *Pistacia vera* L. genotypes to define a conservation strategy (Aouadi et al., 2019), in the genetic characterization of micropropagated rose plants (Dessoki et al., 2020) or for the population assessment of *Musa* spp. (Igwe et al., 2021). Tubulin-based polymorphism is reported as a versatile tool for plant genotyping and was successfully used for intraspecific variability analysis of *Phaseolus vulgaris* L. (Braglia et al., 2020) or in the genomic profiling of *Vitis vinifera* L. (Gavazzi et al., 2016). Conserved-domain DNA markers represent an important class of functional molecular markers in plant genetics. They provide reproducible and informative multilocus fingerprints linked to biologically conserved genomic regions. Among these systems, SCoT and CDDP markers frequently exhibit high polymorphism information content (PIC) and strong discriminatory power across diverse plant species (Hamidi et al, 2014).

DNA-based markers are still considered to constitute effective and useful tools available in the research of plant variability, especially those that are polymorphic among individuals or populations (Amiteye, 2021).

This study employed a conserved domain-based primer targeting the RCAR region to examine genetic variation within tomato cultivars (intraspecific) and among various plant species (interspecific), including herbs, fruits, vegetables, medicinal, and forest plants. The aim was to assess RCAR domain conservation and variability and the potential for phylogenetic and molecular marker applications.

MATERIAL AND METHODS

Plant material and DNA extraction

Samples of 25 different plant species (including medicinal plants, vegetables, trees, and fruit species) and 20 tomato cultivars were collected from *in situ* or commercial retail chains, respectively, in the Slovak Republic (Table 1). Mature fruits were collected for tomato varieties, and leaves of young plants were collected for other species.

Table 1. Plant species and tomato varieties used in the study

Plant species	Variety* of *Lycopersicum esculentum*
Achillea millefolium L.	Aramis
Alnus glutinosa (L.) *Gaertn.*	Brixtol
Betula pendula Roth.	Cluster Goliath
Betula pubescens Ehrh.	Cluster Mato
Capsicum annuum L.	Lino Rosso
Daucus carota L.	Medulienka
Fragaria ananassa Duchesne ex Rozier.	Nema Giant
Glechoma hederacea L.	Nema Rock
Helichrysum italicum (Roth) G. Don	Prunus
Hypericum perforatum L.	Roma
Chelidonium majus L.	Strombolino
Lavandula angustifolia Mill.	Stupice
Levisticum officinale W.D.J. Koch	Sunstream cherry
Melissa officinalis L.	Viva Italia
Origanum majorana L.	Tramezzino
Plantago lanceolata L.	Triple Red
Picea abies (L.) H. Karst.	Ty King
Salix alba L.	UG Red
Salvia rosmarinus Spenn.	Ugx
Symphytum officinale L.	Yellow pear
Trifolium pratense L.	-
Thymus vulgaris L.	-
Urtica dioica L.	-
Vaccinium corymbosum L.	-
Verbena officinalis L.	-

* The origin of tomato varieties was in Spain except for Medulienka and Roma that originated in Slovakia

Total genomic DNA was extracted from plant material (leaves or mature fruit of tomato varieties) using the Qiagen DNA Plant Kit following the manufacturer's instructions. Quality and concentration were assessed using Nanodrop spectrophotometry (A260/A280 and A260/A230 ratios), and DNA was quantified using the formula: DNA (µg/mL) = [A260 × 50] × dilution factor.

Primer design and PCR optimization

Degenerate primers targeting the plant RCAR conserved domain (Jain and Kumar, 2015) and annealing full degeneracy of arg-cys-ala-arg (Forward: 5′-AGRTGYGCNAGR-3′, Reverse: 5′-RGANCGYGTRGA-3′) were designed to amplify fingerprinting profiles across diverse plant species. Degenerate primers were designed based on conserved amino acid sequences of RCAR proteins using standard IUPAC nucleotide codes.

PCR conditions were optimized using mixed tomato DNA at a concentration of 50 ng/µL, with tested annealing temperatures ranging from 35.5 °C to 44.7 °C (data not shown). Expected amplicon sizes based on *in silico* prediction ranged between 200–500 bp, and these were used as positive controls in amplification.

PCR and Gel Electrophoresis

PCR was performed in 20 µL reactions containing 10 µL Robust® Green Master Mix, 2 µL DNA template (100 ng/µL), 1 µL of each primer, and 7 µL nuclease-free water using the SureCycler 8800. Thermal cycling conditions were initial denaturation at 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 42 °C for 30 s, and 72 °C for 1 min; followed by a final extension at 72 °C for 10 min. To calculate the size of the amplified DNA fragments, 5 µL of 100 bp DNA ladders were used as molecular weight markers. Amplification products were separated on 2% agarose gels stained with GelRed and visualized under UV light.

Data analysis

The genetic diversity and relationships among the plant species were analyzed in R software using multiple computational techniques. A binary matrix was constructed based on the presence (1) or absence (0) of

bands observed in gel electrophoresis. The Jaccard similarity coefficient was calculated to assess genetic similarity (Jaccard, 1908), and a distance matrix was used to generate a heatmap to visualise relationships. Hierarchical clustering using the unweighted pair group method with arithmetic mean (UPGMA) was performed to generate a dendrogram. Principal Component Analysis (PCA) was conducted to identify components contributing to genetic variation. Analysis of Molecular Variance (AMOVA) was carried out to partition genetic variance within and among groups. Finally, a genetic network image was generated to illustrate interconnections among species. These analyses were executed using R packages such as "pheatmap," "stats," "adegenet," and "vegan" (R Core Team, 2020).

RESULTS AND DISCUSSION

Different parts of plant genomes can be used as effective DNA markers. Here, a new type of marker for Bet v 1-homologous sequences is reported across different plant species. PCR amplification using primers targeting the conserved Regulatory Component of ABA Receptor (RCAR) domain produced clear and reproducible bands across all tested plant species, ranging from 200 to 500 bp. The optimized annealing temperature of 42 °C yielded the most consistent amplification, effectively balancing specificity with product yield (Figure 1).

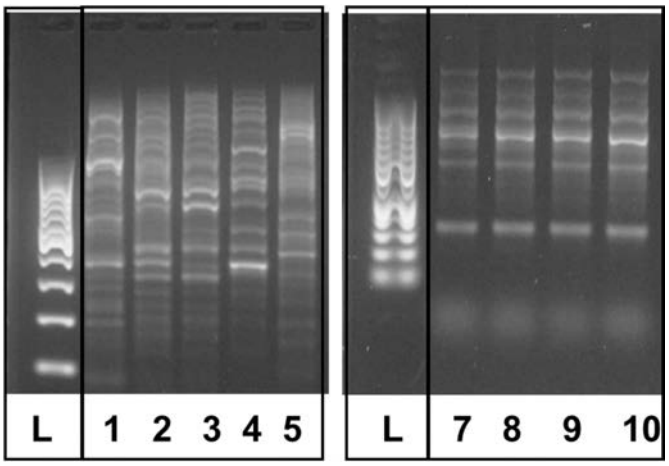


Figure 1. Generated RCAR amplicons in the set of variable plant species (1 - *Verbena officinalis* L., 2 - *Betula pendula* Roth., 3 - *Betula pubescens* Ehrh., 4 - *Alnus glutinosa* (L.) Gaertn., 5 - *Capsicum annuum* L.) and tomato varieties (7 – Cliste Goliath, 8 – Cluster Mato, 9 – Medulienka, 10 – Tramezzino)

A heatmap was constructed to visualize the genetic similarity between plant species and tomato accessions. The heatmap revealed distinct clusters of similarity, with tomato species forming a coherent cluster and other plant species showing variable relationships. The clustering patterns suggested significant genetic diversity both within and between groups. This visualization provided an initial overview of genetic relationships that guided subsequent analyses. A heatmap based on Jaccard similarity coefficients (Figure 2) revealed two major clusters. Tomato cultivars formed a tightly knit cluster with high genetic similarity (Jaccard values > 0.8), indicating low polymorphism among domesticated varieties.

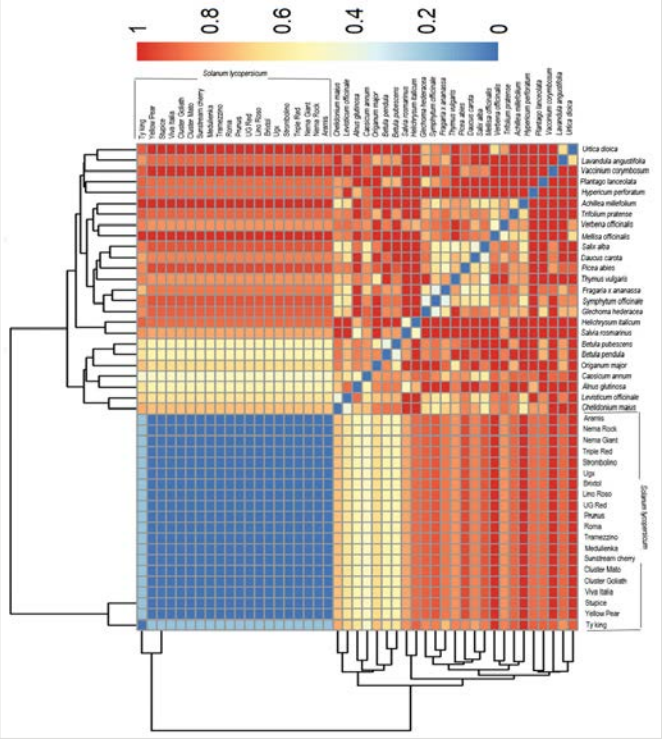


Figure 2. Heatmap of pairwise genetic similarity (Jaccard Index) among tomato varieties and other plant species

In contrast, non-tomato plant species showed broader diversity, with Jaccard values ranging from 0.2 to 0.6. Low similarity values (0.0-0.2) between tomato and other species underscore significant interspecific divergence. These results highlight the utility of the RCAR marker not only for assessing conservation but also for distinguishing genetic variation across and within species groups.

The UPGMA dendrogram (Figure 3) supported the heatmap results, with tomato cultivars clustering together in a compact group, while non-tomato plants were distributed among several subclusters. This pattern emphasizes the higher intraspecific similarity within tomatoes and greater genetic variability among other species.

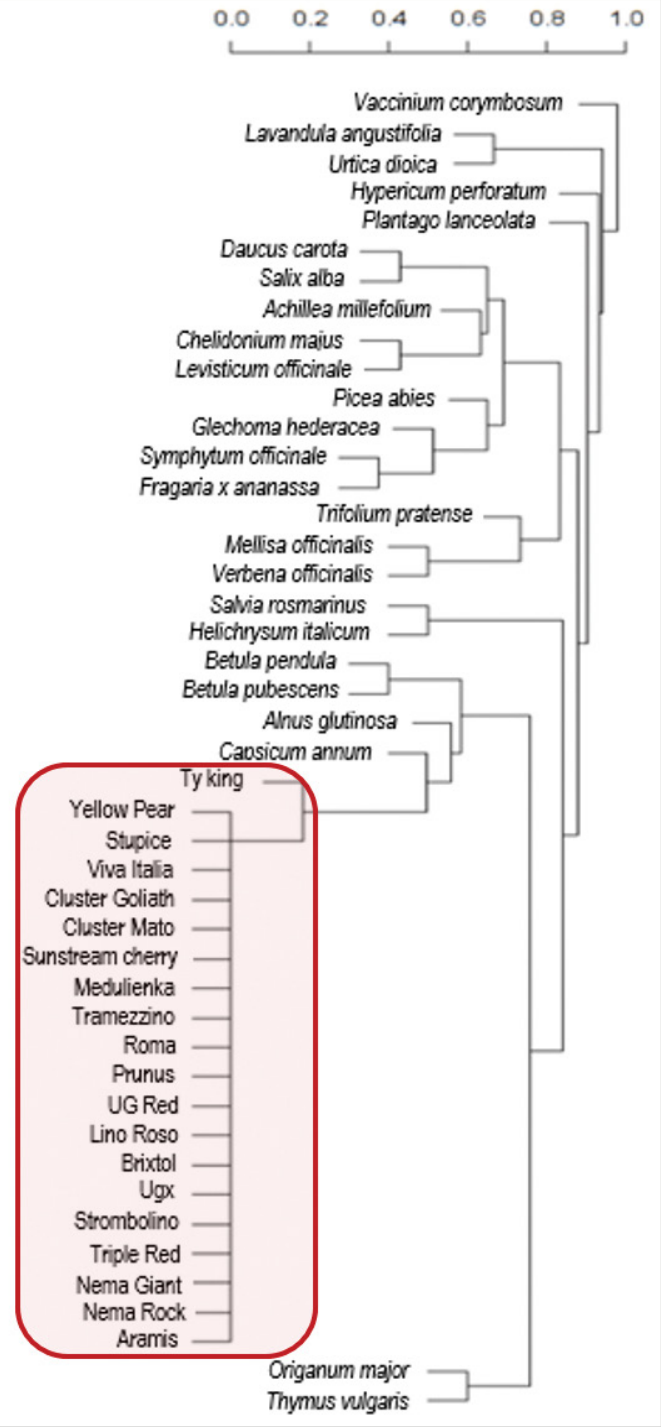


Figure 3. UPGMA dendrogram illustrating hierarchical clustering of analysed accessions (tomato varieties are in the red rectangle)

Analysis of Molecular Variance (AMOVA) indicated that 40.32% of the total genetic variation was attributed to intergroup differences (tomato vs. non-tomato), while 59.68% resulted from within-group variation (Table 2). This relatively high within-group variation-particularly among non-tomato plants-demonstrates the RCAR marker's capacity to reveal meaningful genetic differences even within phylogenetically diverse plant sets.

The average Polymorphism Information Content (PIC) was 0.332, indicating moderate polymorphism across the RCAR domain among tested plant species. The Effective Multiplex Ratio (EMR) of 19 and Marker Index (MI) of 6.308 further highlight the efficiency and informativeness of the marker system. These results support the utility of the RCAR domain as a reliable marker for both intra- and interspecific genetic studies (Table 3).

DNA markers provide an effective source of genomic information that can target different parts of plant genomes. Both markers of coding as well as non-coding regions are widely applied in fingerprinting (Hlavačková et al., 2016; Vivodík et al., 2019; Pipan et al., 2021; Baroudi et al., 2023; Kostova et al., 2024). Compared with anonymous markers such as RAPD or AFLP, conserved-domain markers are often more reproducible and biologically informative because they target functional genomic regions. Their informativeness ranges from moderate to very high (Hamidi et al., 2014). Here, PCR amplification produced clear and reproducible bands in all of analyzed plant species, ranging from 200 to 500 bp. These findings confirm the conserved nature of the RCAR domain across a broad phylogenetic range, including both tomato cultivars and various non-tomato plant species such as herbs, fruit trees, and forest species. This strong conservation of the RCAR domain is in line with earlier reports emphasizing the evolutionary stability and functional importance of ABA receptor families across plant taxa (Komatsu et al., 2020; Cutler et al., 2010). Studies by Szostkiewicz et al. (2010) and Park et al. (2009) also support the view that RCAR domains are widely retained due to their central role in abscisic acid signalling and stress responses.

Table 2. AMOVA results for genetic variation between and within the analysed plant groups

Source of Variation	Degrees of Freedom (Df)	Sum of Squares (SS)	Mean Square (MS)	Variance Components (Σ)	Percentage of Variation (%)
Between Samples	1	77.031	77.031	3.250	40.32%
Within Samples	43	206.880	4.811	4.811	59.68%
Total	44	283.911	6.453	8.061	100.00%

The RCAR domain provides a large hydrophobic binding pocket that accommodates small molecules, allowing conformational flexibility essential for both receptor and allergenic functions (Seutter von Loetzen et al., 2014). PYR/PYL/RCAR receptors were reported to have an essential role in the abscisic acid-dependent responses of plants to different stimuli (Fidler et al., 2022).

Tomato cultivars formed a tightly-knit cluster with high genetic similarity (Jaccard values > 0.8), indicating low polymorphism among domesticated varieties in the RCAR domain. This is consistent with previous findings that domesticated tomato lines exhibit a narrow genetic base due to intensive breeding and selection pressures (Lin et al., 2014; Blanca et al., 2015). All the other analysed plant species resulted in polymorphic RCAR-based fingerprints with very distinctive patterns where no taxonomic background was visible, which is a limitation of the RCAR-based markers for possible intravariability screening. These findings are supported by previous studies indicating that ABA receptor gene families, though structurally conserved, can exhibit considerable variation in sequence motifs among distant taxa, contributing to functional diversification (Yang et al., 2019b; Li et al., 2024). Phylogenetic analyses indicate that Bet v 1 homologs and RCAR domains share a common ancestral fold related to small-molecule transporters (Radauer et al., 2008), what provide a background of the universal use of the DNA markers based on this conserved domain. Compared with ISSR or SCoT markers, here reported RCAR based od CDDP markers often show slightly lower or comparable informativeness, but they have the advantage of targeting functional genomic regions associated with stress responses and development (Bidyananda et al., 2024).

Table 3. Efficiency coefficients of the RCAR Domain Marker System

Marker System	PIC	EMR	MI
RCAR Domain-Based	0.332	19	6.308

PIC – polymorphic information content; EMR – effective multiplex ratio; MI – marker index.

DNA markers based on the conserved parts of genes encoding plant allergens were previously successfully applied for Bet v 1, profilin and LTP allergens by our group (Žiarovská and Urbanová, 2022; Čerteková et al., 2023; Žiarovská et al., 2023; Hovaňáková et al., 2024). The efficiency coefficient reported for these techniques corresponds to our findings for the RCAR technique reported here. BBAP method, which is another marker with the background of Bet v 1 homologuous sequences in plant genomes, was characterized by PIC to be 0.5 for *Arachis hypogaea* L genotypes. PIC values for other coding sequence DNA markers, such as Conserved DNA-Derived Polymorphism (CDDP) markers in plants, vary depending on the species, primer design, and genetic diversity of the analyzed population. In most studies, CDDP markers showed moderate to high informativeness, usually with PIC values between 0.28 and 0.84 (Bidyananda et al., 2024; Maeruf et al., 2024). This is in concordance with the coding-based RCAR marker reported here. RCAR marker reported here belongs to the conservative domain of PR protein families and specifically to the Bet v 1 group. The group of Bet v 1-based fingerprinting was proved to be effective in distinguishing common ragweed genotypes (Klongová et al., 2025) or apple cultivars (Kučerová et al., 2024). A similar distinguished pattern of fingerprints of *Lamiaceae* species could be summarized for this study and a previously reported study of BBAP markers used for *Lamiaceae* herbs (Urbanová et al., 2024). In both of

those studies performed analysis, common thyme and oregano were clustered in a more similar branch of the dendrogram than common sage. The allergen-based marker techniques provide polymorphic potential in the fingerprinting of plants in this study (Urbanová et al., 2024). In legumes, profilin-based fingerprinting was confirmed as a polymorphic profile-generating fingerprint technique (Žiarovská et al., 2023; Kováčik et al., 2024).

In the cross-group comparison of analysed species, the tomato varieties show limited genetic similarity to other plant species, as indicated by low Jaccard Index values (0–0.2) in inter-group comparisons. This finding aligns with the evolutionary divergence between the Solanaceae family and the diverse families represented by the other plant species, but here, this new type of allergen-based DNA marker was proven to be effective in plant fingerprinting.

CONCLUSION

The successful amplification and genetic analysis of the conserved RCAR domain across diverse plant species, including tomatoes, demonstrates its potential as a valuable molecular marker for studying genetic relationships. The high genetic similarity among tomato cultivars contrasts with the broader diversity observed in other plants, reflecting their evolutionary divergence. The combination of simplicity, low cost, cross-species applicability, and functional relevance of this marker makes it valuable for plant breeding, germplasm characterization, phylogenetics, and molecular diversity studies.

In the future, integrating expression analysis of RCAR-linked genes under different stress conditions could provide deeper insights into their functional roles, especially in relation to stress-responsive genes like PR10. Expanding the study to include transcriptomic or proteomic approaches may further unravel the molecular mechanisms governed by conserved domains like RCAR in plant adaptation and defense.

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