Zucchini TINY4 gene regulates plant development through brassinosteroid signalling pathway

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ABSTRACT. Brassinosteroids (BRs) are steroid plant hormones necessary for the regulation of physiological processes essential for plant growth and survival. The analysis of mutants altered in these responses has provided insight into the genes involved in the BR signalling pathway, allowing molecular components essential for the perception and transmission of this hormonal signal to be identified.[1] The characterization of a collection of the EMS mutants of the *Cucurbita pepo* morphotype *zucchini* allowed us to select five mutants that show alterations in vegetative development, which we have called *tiny* (*tin1/5*) due to their small plant size. In this work, by combining whole-genome sequencing and the mapping of molecular markers of codominant inheritance, it has been possible to identify the first known mutant allele of the zucchini *TINY4* gene, homologous to the *SERK* (*SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE*) gene of *Arabidopsis thaliana*. *SERK* encodes a protein kinase with leucine-rich repeats (LRR-RLK) located in the plasma membrane, which together with two other LRR-RLK proteins, namely BRASSINOSTEROID-INSENSITIVE1 (BRI1) and BRI1-ASSOCIATED KINASE1 (BAK1), form a complex that can be used for the perception and signalling of brassinosteroid. The *bri1* mutant is characterized by short petioles and inward-curved leaves. The *serk* mutation enhances these phenotypic traits, and thus the *bri1 serk* double mutant shows reduced petiole length, a small rosette size, and an excessive leaf curl.[2] The *zucchini tin4* mutant exhibits severe compaction of the vegetative organs of the plant, caused by the reduced petiole size of leaves and stems, a phenotype that resembles the *Arabidopsis bri1 serk* double mutant. To confirm that the *tin4* mutation is responsible for the mutant phenotype, new loss-of-function alleles of this gene are being generated using CRISPR-Cas9 gene editing technology. These results will contribute to the functional genomics of this species and provide further insight into the functionality of the *TIN4* gene in the brassinosteroid perception pathway and thus in the vegetative morphogenesis of *zucchini*.

Phenotypic alterations in *tin1/5* mutants

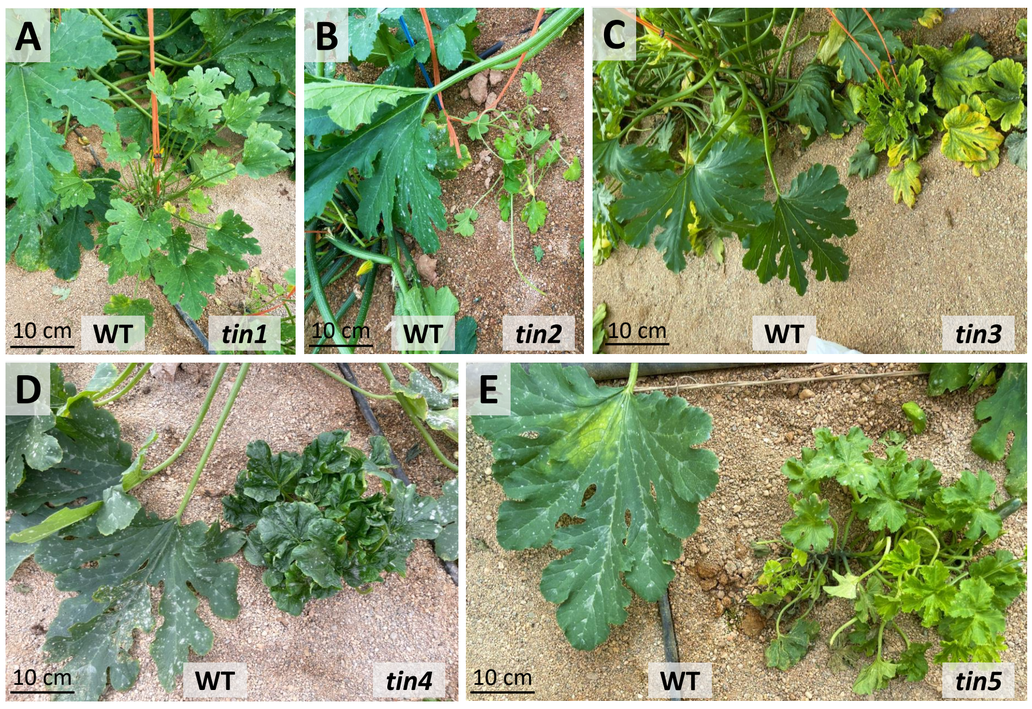


Figure 1. *tin1/5* mutants show a significant reduction in leaf and stem size. Genetic analysis of segregating progenies indicated that the *tiny* mutant phenotype is inherited in each family as monogenic and recessive. The results of chi-square analysis were: *tin1* mutant (**A**) $\chi2 = 0.074$, $P = 0.785$; *tin2* mutant (**B**) $\chi2 = 3.333$, $P = 0.067$; *tin3* mutant (**C**) $\chi2 = 3.100$, $P = 0.078$; *tin4* mutant (**D**) $\chi2 = 0.439$, $P = 0.508$; *tin5* mutant (**E**) $\chi2 = 0.000$, $P = 1.000$.

Molecular characterization of the *TINY4* gene

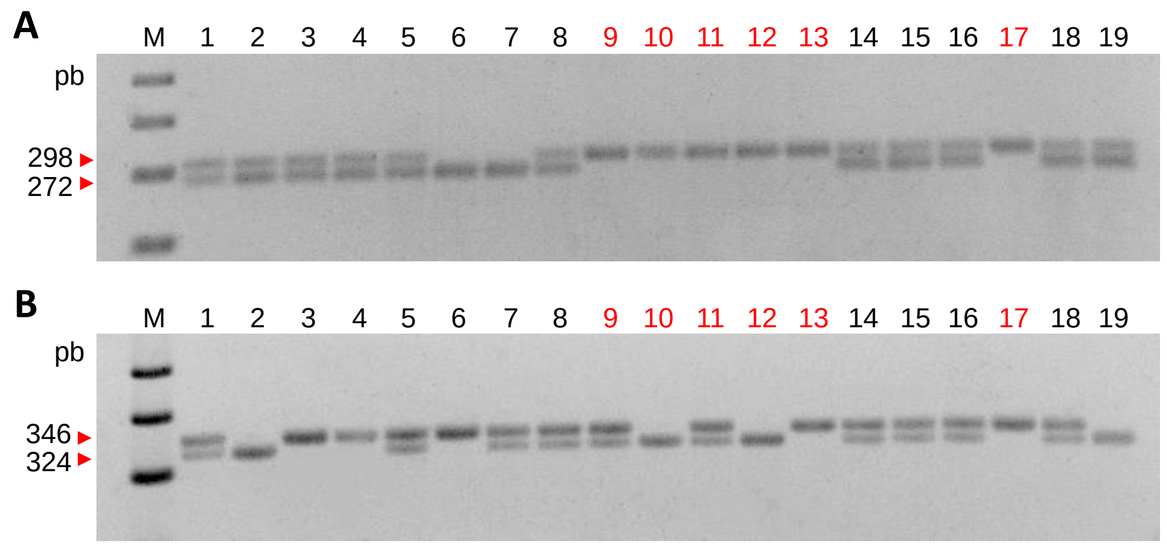
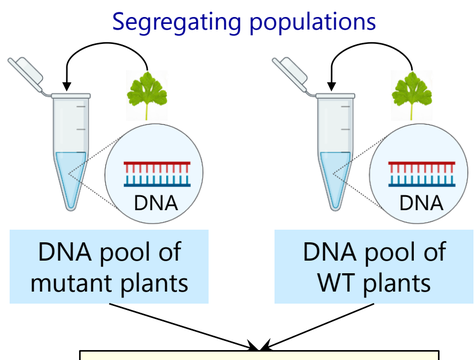


Figure 3. A. Cosegregation analysis of the *tin4* mutant phenotype and the SNV identified in a gene encoding a protein involved in brassinosteroid perception. Digestion of the specific marker for that gene from all mutant phenotype plants (red numbers) showed the restriction fragment expected from plants homozygous for the mutation (298 bp). Genotypes corresponding to plants homozygous for the normal allele (272 bp) as well as heterozygous plants were observed in the normal phenotype plants (black numbers). **B.** As a negative control it is shown the absence of cosegregation between the *tin4* mutant phenotype and the SNV identified in a gene, *Cp4.1LG12g00460*, located on another chromosome.

Identification of *tin1/5* mutations



Mutant	SNV
tin1	19
tin3	74
tin4	40
tin5	39

→

Mutant	SNV
tin1	5
tin3	14
tin4	**2**
tin5	5

Functional impact on gene models

Mutants of *Arabidopsis*

Figure 2. DNA was extracted from plants of segregating populations and two pools of equimolar amounts of DNA were generated by grouping the samples of each family according to their phenotype. Whole-genome sequencing and subsequent bioinformatic analysis of these DNAs allowed the identification of mutant-specific mutations or variants (SNVs). Priority was given to the analysis of SNVs located in genes for which homologous *Arabidopsis* genes with mutant phenotypes similar to the *tiny* mutants have been described and which should have a negative functional impact on the corresponding protein.

Functional analysis of the *TINY4* gene

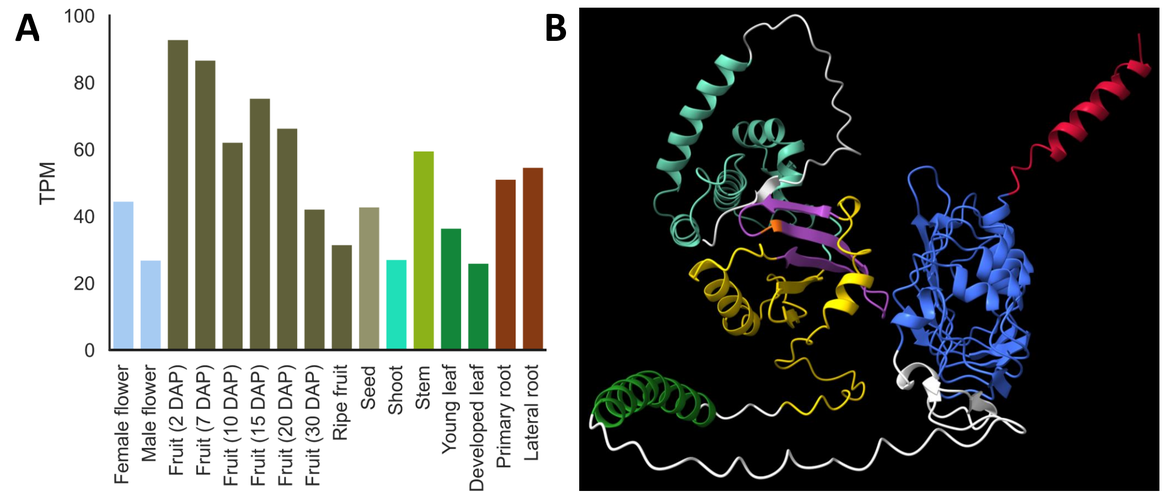


Figure 4. A. Expression profile of the *TINY4* gene in different *zucchini* tissues. The vertical axis indicates the number of transcripts per million (TPM) in each sample. The gene has ubiquitous expression, consistent with the mutant phenotype and shows a maximum of expression in fruits 2 days after pollination (DAP). **B.** Predicted structure of the TINY4 protein. The leucine-rich repeats (LRR) domain is shown in blue, the transmembrane domain in green, the phosphorylase kinase domain in yellow, and the phosphotransferase domain in cyan. The signal peptide is shown in red, the ATP binding site in purple. The identified mutation (Gly310→Asp310) introduces a negative charge at the ATP binding site repelling the binding of this cofactor.

Referencias. [1] Clouse, S. D. (2011). Brassinosteroids. *The Arabidopsis Book/American Society Of Plant Biologists*, *9*. [2] Karlova, R., Boeren, S., Russinova, E., Aker, J., Vervoort, J., & de Vries, S. (2006). The *Arabidopsis* somatic embryogenesis receptor-like kinase1 protein complex includes brassinosteroid-insensitive1. *The Plant Cell*, *18*(3), 626-638. **Acknowledgments.** This work has been funded by the Spanish Ministry of Science and Innovation (project CUCURVAR RTC2019-007247-2).