

Dear Editor:

Thanks very much for your comments and suggestions concerning our manuscript (ID: cimb-4022955) entitled "Uncovering the Molecular Response of Oregano (*Origanum vulgare* L.) to $^{12}\text{C}^{6+}$ Heavy-Ion Irradiation through Transcriptomic and Metabolomic Analyses". Based on the comments we received, we have made careful modifications to the original manuscript and the revised text in manuscript was marked with blue characters to highlight the changes. Point-by-point responses to the two nice reviewers are listed below this letter.

Reviewer 2 comments:

The manuscript entitled "Uncovering the Molecular Response of Oregano (*Origanum vulgare* L.) to $^{12}\text{C}^{6+}$ Heavy-Ion Irradiation through Transcriptomic and Metabolomic Analyses" represents comprehensive study that elucidates the molecular mechanisms underlying metabolic reprogramming induced by heavy-ion irradiation. Heavy-ion beams are accepted as a novel powerful mutagen because they are able to induce mutations with high frequency at a relatively low dose at which virtually all plants survive, and they induce a broad spectrum of phenotypes without affecting other plant characteristics. This research is one step further that leads to the future metabolic engineering and precision breeding. Great idea! The authors engaged several techniques which provided clear results. The work is well described, and the results are placed in an appropriate context. My comments below are intended to strengthen this study.

1. Were these two, NZ1 and NZ2, the only phenotypes you have observed throw-out generations?

Response: We sincerely appreciate the reviewer's valuable question, which prompts us to clarify the scope of phenotypic variations observed in our study.

In fact, NZ1 and NZ2 are not the only mutant phenotypes identified during the multi-generational observation. During the screening process across generations, we initially obtained a total of 18 mutant lines with distinct phenotypic variations after $^{12}\text{C}^{6+}$ heavy-ion irradiation. These variations covered multiple traits, including plant architecture (e.g., dwarfism, bushy growth), leaf characteristics (e.g., leaf color variation from green to light green, leaf shape changes such as lanceolate to ovate, and leaf thickness differences), and growth cycle (e.g., early flowering and late maturity). However, NZ1 and NZ2 were selected as the focus of this study for the following key reasons: First, they exhibited the most significant and stable differences in both morphological traits (plant height, leaf morphology, biomass) and metabolic profiles (especially the accumulation of target bioactive compounds such as carvacrol and thymol) compared to the wild type (WT). Second, preliminary genetic stability tests showed that their core traits (e.g., terpenoid metabolite composition and key morphological features) maintained consistent expression across two consecutive generations, indicating potential heritability. Third,

transcriptomic analysis revealed that these two lines had the most enriched DEGs in terpenoid biosynthesis pathways, which aligned with our research objective of unraveling the molecular mechanisms underlying metabolic reprogramming induced by heavy-ion irradiation.

The other mutant lines either showed unstable phenotypic expression (e.g., trait reversal in the next generation), insignificant changes in target metabolites, or severe growth defects (e.g., low fertility, poor adaptability), making them unsuitable for in-depth multi-omics analysis. We believe that focusing on NZ1 and NZ2, which have clear phenotypic characteristics and metabolic advantages, can more effectively address the core scientific questions of this study. We will supplement the information on the initial screening of mutant lines in the revised manuscript to provide a more comprehensive overview of the phenotypic variation spectrum induced by irradiation.

2. Do you think that selected traits are stable since you observed them for only 2 generations and we should bear in mind that it takes up to M4 to M8 generations for a mutant trait to become stable and reliably inherited after CIB mutagenesis? Can you state that you have homozygous lines?

Response: We highly agree with the reviewer's insightful comment. The reviewer's reminder about the time required for mutant trait stabilization (M4 to M8 generations) after heavy-ion beam mutagenesis is scientifically rigorous and fully aligns with the general principles of plant mutagenesis breeding. We sincerely appreciate this critical perspective, which helps us improve the rigor of our research conclusions.

In response to the concerns about trait stability and homozygosity, we would like to provide the following explanations: First, regarding trait stability: In our study, the observation of NZ1 and NZ2 across two generations (M2 and M3) showed that their key traits—including morphological indicators (plant height, leaf length/width ratio, biomass accumulation) and metabolic traits (content of 62 differentially accumulated volatile compounds, especially carvacrol and thymol)—exhibited no significant fluctuations between generations. This preliminary stability suggests that the mutations induced by $^{12}\text{C}^{6+}$ heavy-ion irradiation may have occurred in key functional genes related to these traits, laying a foundation for stable inheritance. However, we fully acknowledge that two generations of observation are insufficient to confirm long-term genetic stability, as the reviewer correctly pointed out that mutant traits typically require M4 to M8 generations to achieve reliable heritability. At present, we have continued to propagate NZ1 and NZ2 to the M4 generation, and ongoing phenotypic and metabolic monitoring shows that their core traits remain consistent with the M2 and M3 generations. We will supplement the M4 generation data in the revised manuscript to provide stronger evidence for trait stability. Second, regarding homozygosity: We currently cannot definitively state that NZ1 and NZ2 are fully homozygous lines. Heavy-ion beam mutagenesis can induce both homozygous and heterozygous mutations in

the genome. Given that the study has progressed to the M3 generation, it is plausible that some loci related to the observed traits have achieved homozygosity, while other non-target loci may still be heterozygous. To verify homozygosity, we have initiated genotyping work for key candidate genes (e.g., *TPS3*, *TPS6A*, *TPS6C*, *CYP71D178*) identified in the transcriptomic analysis. Preliminary sequencing results show that the coding regions of these candidate genes in NZ1 and NZ2 have consistent genotypes across multiple individuals of the same line, suggesting that these key loci may have been homozygous. However, comprehensive genome-wide homozygosity verification (e.g., whole-genome sequencing or SSR marker analysis) is still ongoing.

In summary, we fully recognize the reviewer's concern about trait stability and homozygosity. The current data indicate preliminary stability of the selected traits in NZ1 and NZ2, but long-term multi-generational observation (up to M4-M8) is still needed to confirm reliable inheritance. While key candidate gene loci may be homozygous, comprehensive homozygosity verification is ongoing. In the revised manuscript, we will clearly state the limitations of the current generation of materials and supplement the latest M4 generation phenotypic data and preliminary candidate gene genotyping results. We plan to continue propagating these lines to the M5-M6 generation to further verify trait stability and homozygosity, which will provide a more solid foundation for future metabolic engineering and breeding applications.

3. Could you please rephrase the sentence in the Abstract “we identified key enzyme-encoding genes, such as GPPS, GGPPS, DXS, and HMGR, involved in the terpenoid backbone biosynthesis pathway, as well as critical candidate genes (*TPS3*, *TPS6A*, *TPS6C*, *CYP71D178*, *CYP71D181*, and *CYP71D10B*) that may regulate the biosynthesis of carvacrol and thymol.” - those are well known key genes involved in terpenoid biosynthesis and since you do not have genome sequencing results to be sure where did mutagenesis occurred (are there point mutations in the coding regions of these genes for instance) we could assumed that altered expression of these genes could be also a consequences of mutagenesis itself (stress response) or a description of regulatory networks due to random mutations.

Response: We thank the reviewer for this valuable comment. We have rephrased the text to more accurately reflect that changes in metabolite levels result from alterations in the transcriptional expression patterns of these genes, rather than necessarily being direct mutagenic targets. The revised wording emphasises their regulatory correlation, rather than a causal mutagenic relationship.

4. Line 428 – there is a repetition "These divergent"

Response: We sincerely apologize for the repetition statement and have corrected in revised

manuscript.

Thanks again for your kind help. I look forward to hearing from you.

Sincerely,

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