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ORIGINAL ARTICLE

Evidence from Co-expression Analysis for the Involvement of Amidase and INS in the Tryptophan-Independent Pathway of IAA Synthesis in *Arabidopsis*

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Abstract

The reverse genetic approach has uncovered indole synthase (INS) as the first enzyme in the tryptophan (trp)-independent pathway of IAA synthesis. The importance of INS was reevaluated suggesting it may interact with tryptophan synthase B (TSB) and therefore involved in the trp-dependent pathway. Thus, the main aim of this study was to clarify the route of INS through the analysis of *Arabidopsis* genome. Analysis of the top 2000 co-expression gene lists in general and specific conditions shows that *TSA* is strongly positively co-expressed with *TSB* in general, hormone, and abiotic conditions with mutual ranks of 89, 38, and 180 respectively. Moreover, *TSA* is positively correlated with *TSB* (0.291). However, *INS* was not found in any of these coexpressed gene lists and negatively correlated with *TSB* (−0.046) suggesting unambiguously that these two routes are separately and independently operated. So far, the remaining steps in the INS pathway have remained elusive. Among all enzymes reported to have a role in IAA synthesis, *amidase* was found to strongly positively co-expressed with *INS* in general and light conditions with mutual ranks of 116 and 141 respectively. Additionally, *amidase1* was found to positively correlate with *INS* (0.297) and negatively coexpressed with *TSB* concluding that amidase may exclusively involve in the trp-independent pathway.

Keywords Amidase · Co-expression · Indole synthase · Tryptophan synthase A · Tryptophan synthase B

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Introduction

Auxin is a key signaling phytohormone regulating almost all aspects of plant growth and development from embryogenesis to senescence [1]. Indole-3-acetic acid (IAA), the main naturally occurring auxin in the plant kingdom, has long been proposed to be synthesized from both tryptophan through trp-dependent pathways (Fig. 1) and directly from tryptophan precursors through a trp-independent pathway [2, 3].

Biochemical and genetic experiments have recently revealed the indole pyruvic acid (IPA) pathway as the first complete route of IAA synthesis via the trp-dependent pathway. In this simple two-step pathway, tryptophan aminotransferase (TAA) converts tryptophan into IPA, whereas YUCCA, a flavin-containing monooxygenase, catalyzes the rate-limiting reaction by converting IPA into IAA [4, 5]. Additionally, indole-3-acetaldoxime (IAOx) has been demonstrated as a second trp-dependent pathway [6, 7]. Even though the cytochrome P450 monooxygenase CYP79B2/CYP79B3 has been suggested to produce IAOx from tryptophan, the remaining intermediates and enzymes are still largely unidentified. Furthermore, aldehyde oxidase (AAO), nitrilase (NIT), and amidase convert indole-3-acetaldehyde (IAAld), indole-3-acetonitrile (IAN), and indole-3-acetamide (IAM) to IAA. They all were recognized as naturally occurring enzymes. However, pathways including these enzymes are yet to be characterized [8–11].

Three decades ago, a combination of labeling, genetic, and metabolic approaches led to the proposal that IAA is biosynthesized from tryptophan precursors via the

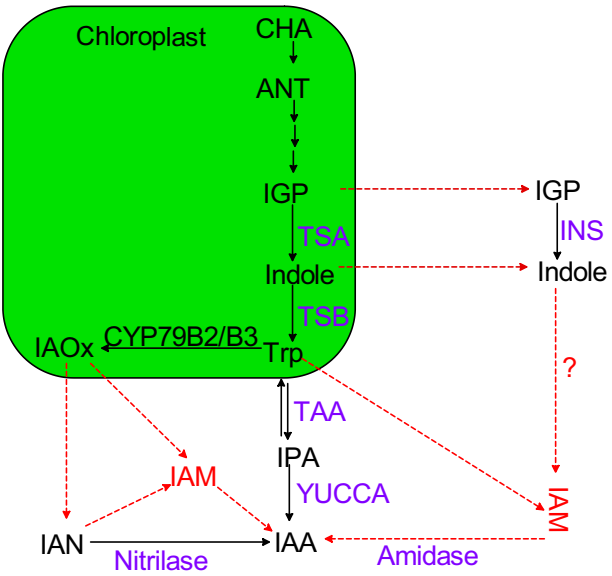

Fig. 1 Potential IAA biosynthesis pathways in *Arabidopsis*. CHA, chorismate; ANT, anthranilate; IGP, indole-3-glycerol phosphate; TSA, tryptophan synthase A; TSB, tryptophan synthase B; Trp, tryptophan; INS, indole synthase; IAOx, indole-3 acetaldoxime; IAN, indole-3-acetonitrile; IAM, indole-3-acetamide; TAA, tryptophan aminotransferase of *Arabidopsis*; IPA, indole-3-pyruvic acid; YUCCA, a Flavin monooxygenase; IAA, indole-3-acetic acid. Enzymes are indicated in blue color, whereas intermediates are in black. Arrows in red colors refer to undefined or unconfirmed enzymes, whereas black colors refer to identified enzymes

trp-independent pathway [12, 13]. This pathway was suggested to be ubiquitous as it was proposed to synthesize IAA in broad taxonomic groups of plants, including *Arabidopsis*, tomato, maize, carrot, *Lemnagibba*, and bean [14–18]. Recently, indole severe sensitive 1 mutant *(iss1)* was described to display trp-independent IAA synthesis as well as related physiological activities including longer petioles, adventitious roots, and leaf narrowing [19]. Six years ago, feeding and genetic approaches were uncovered INS (At4g02610) as the first enzyme in the trp-independent pathway and an essential role for this pathway in early embryogenesis was suggested [20]. The cytosolic INS was demonstrated to regulate hypocotyl length at high temperature in concert with the IPA pathway in *Arabidopsis* based on the observation that *ins-1* mutants are not 100% penetrant and have an additive effect with *wei8* (weakly ethylene insensitive8) mutants. Wang et al. [20] hypothesis has been questioned by Nonhebel bioinformatics work. The possibility for the INS or its product, indole, to interact with TSB and therefore with the trp-dependent pathway was not excluded [21]. The additive effect of *wei8* and *ins* was further used to support this hypothesis. Therefore, the involvement of INS in either trp-dependent or independent pathways was considered in this article. In this pathway, indole-3-glycerol phosphate is converted to indole by the indole synthase in the cytosol [20]. However, enzymes catalyzing the remaining steps have not been identified so far. Therefore, this piece of work was aimed to find out enzymes potentially act with INS in the INS pathway of IAA synthesis. All previously reported genes to have roles in IAA synthesis were investigated in the list of genes most strongly positively co-expressed with *INS*. Additionally, a list of genes negatively co-expressed with *INS* was also studied to shorten the list of genes most probably act with INS in the trp-independent pathway.

Coexpression analysis is a powerful tool that has been used for identifying genes related to a specific metabolic pathway [22]. For instance, this approach has been adopted to identify genes regulating cellulose synthesis and transcription factors involved in the glucosinolate synthesis pathway [23, 24]. In this research, ATTED-II, a web-based tool, was used as a secondary database to analyze publicly available microarray data for the dicot model plant *Arabidopsis thaliana* [25]. Unlike Hirai et al. [24], who used Pearson's correlation coefficient for combinations of *Arabidopsis* genes available in ATTED-II, mutual rank (MR) as a powerful measure was used in the latest release of the database. Co-expressed gene relationships are primarily based on the vast amount of microarray data stored in TAIR as well as NASCArrays [26].

Methods

The co-expression analysis tool (CoExSearch: co-expressed gene list from multiple query genes) available on the ATTED-II (http://atted.jp) database version 9.2 was used to find genes positively and negatively co-expressed with the *Arabidopsis INS* and *TSB* genes. Genes previously reported to have a possible role in IAA synthesis are listed in Table 1. The available top 2000-gene lists positively and 300-gene lists negatively co-expressed with seed genes were studied and analyzed. The four *TSB* genes in *Arabidopsis* (At5g38530, At5g28237, At4g27070, and At5g54810) were uploaded into the CoExSearch tool of ATTED-II. Affy data were only available to *TSB* (At5g38530).

Table 1 Summary of IAA-synthesis genes previously reported having a role in IAA synthesis. *INS*, indole synthase; *TSA*, tryptophan synthase A; *TSB*, tryptophan synthase B; *YUCCA*, a flavin monooxygenase; *TAA*, tryptophan aminotransferase of *Arabidopsis*; *TAR*, tryptophan aminotransferase related; *AAO*, aldehyde oxidase; *NIT*, nitrilase

Gene	Locus number	Gene	Locus number	Gene	Locus number
INS	At4g02610	*AAO2*	At3g43600	*TAR4*	At1g34060
TSA	At3g54640	*AAO3*	At2g27150	*YUCCA1*	At4g32540
TSB1	At5g54810	*AAO4*	At1g04580	*YUCCA2*	At4g13260
TSB2	At4g27070	*NIT1*	At3g44310	*YUCCA3*	At1g04610
TSB3	At5g28237	*NIT2*	At3g44300	*YUCCA4*	At5g11320
TSB4	At5g38530	*NIT3*	At3g44320	*YUCCA5*	At5g43890
Amidase1	At1g08980	*NIT4*	At5g22300	*YUCCA6*	At5g25620
Amidase2	At5g07360	*CYP79B2*	At4g39950	*YUCCA7*	At2g33230
Amidase3	At5g64440	*CYP79B3*	At2g22330	*YUCCA8*	At4g28720
Amidase4	At5g09420	*TAA1*	At1g70560	*YUCCA9*	At1g04180
Amidase5	At4g34880	*TAR1*	At1g23320	*YUCCA10*	At1g48910
Amidase6	At3g25660	*TAR2*	At4g24670	*YUCCA11*	At1g21430
AAO1	At5g20960	*TAR3*	At1g34040		

Results and Discussion

Tryptophan Synthase A but Not Indole Synthase Interacts with Tryptophan Synthase B to Produce Tryptophan

A significant role for the involvement of indole synthase in the trp-independent pathway of IAA synthesis has been quite recently reported. The cytosolic INS was suggested to produce indole from the indole-3-glycerol phosphate [20, 27]. However, this suggestion was challenged by a proposal that INS may interact with TSB to produce tryptophan and therefore with the trp-dependent pathway [21]. In this work and through the implementing co-expression analysis of *Arabidopsis* genome obtained from the ATTED-II secondary database [25], we excluded the possibility of INS's interaction with TSB. Among the 21,000 genes analyzed, the available 2000 gene list correspond to 90% cut-off value positively significantly co-expressed with TSB in general conditions (condition-independent manner) were obtained and studied. Furthermore, co-expressed gene lists obtained from specific samples under certain conditions, including hormone, tissue, biotic, and abiotic stress, were also obtained and investigated. Table 2 shows a short list of all genes related to IAA synthesis significantly positively co-expressed with *TSB* under general and specific conditions.

Indole synthase gene was found neither in the general nor in the specific co-expression data lists, suggesting that this enzyme may not interact with TSB to produce IAA via the trp-dependent pathway. In contrast to *INS*, *TSA* which its product forms a complex with TSB [28], was strongly co-expressed with the query gene with mutual ranks of 38, 98, and 180 in the hormone, general, and abiotic conditions respectively. Interestingly, *INS* was not also found in the top 300 gene list negatively co-expressed with *TSB*, suggesting that trp-dependent and independent pathways may interact to produce IAA in *Arabidopsis*. Another evidence for the involvement of INS in the trp-independent pathway came from the analysis of correlation patterns. Figure 2 shows the correlation of expression patterns for *INS*

Table 2 Summary of IAA-synthesis genes positively co-expressed with *TSB* in the top 2000 genes lists in general and specific conditions. Specific conditions include hormone, tissue, biotic, abiotic, and light. Coexpression index is represented by mutual rank

Gene	Locus	General Condition	Hormone	Tissue	Biotic	Abiotic	Light
Amidas2	At5g07360	1686		200	594	152	
Amidas5	At4g34880			2471			
Amidas6	At3g25660						1419
AAO1	At5g20960	182	645	117	876	412	898
AAO2	At3g43600			328		1456	302
AAO3	At2g27150	307	1220				1431
NIT3	At3g44320	168	992	901		78	1072
NIT4	At5g22300	728	212		1499		
CYP79B2	At4g39950	382	540	1150			
CYP79B3	At2g22330			1816			
YUC5	At5g43890			2017			
TSA	At3g54640	89	38			180	

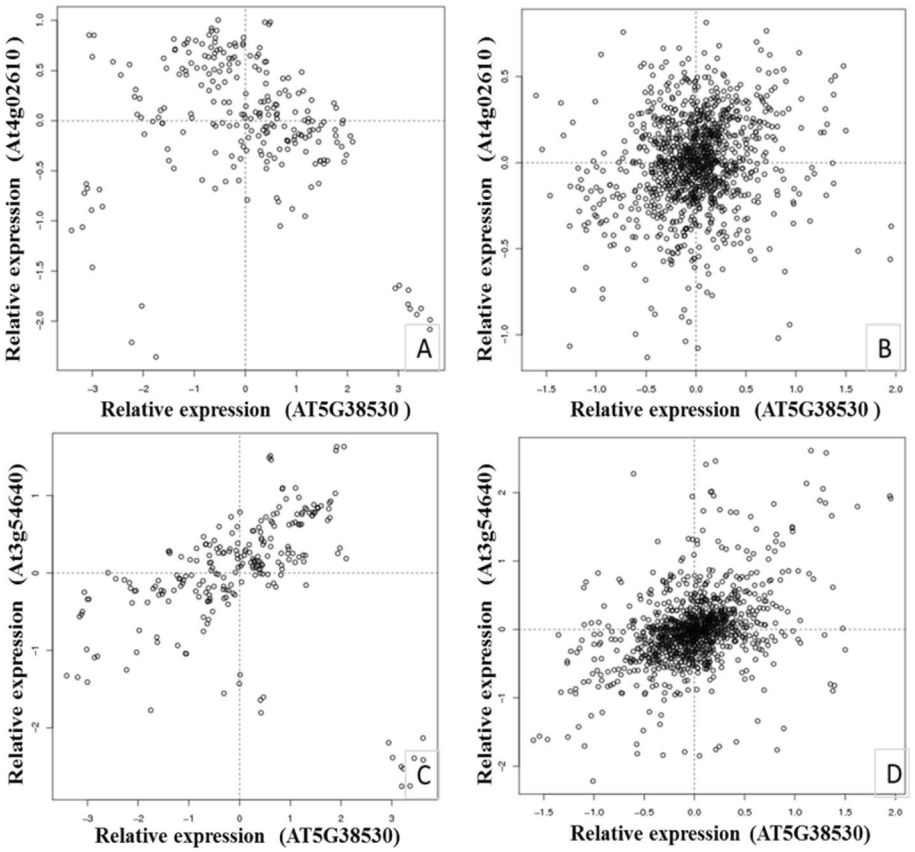

Fig. 2 Correlation of the expression obtained from developmental and vegetative samples for *INS* and *TSB* (**A** and **B**) and for *TSA* and *TSB* (**C** and **D**)

and *TSB* (A and B) as well as *TSA* and *TSB* (C and D) for samples obtained from both developmental and vegetative tissues, respectively. The correlation of expression for INS and TSB is −0.046, whereas for TSA and TSB is 0.291 is further supports the involvement of INS in the trp-independent pathway of IAA synthesis. This suggestion is in agreement with the independent production of indole by TSA and INS suggested in maize [27].

Amidase May Interact with INS in the trp-Independent Pathway

Even though Wang et al. [20] reported a significant role for INS in IAA production through the trp-independent pathway, the remaining reaction(s) in this pathway needs to be identified. Functional identification of gene(s) expected to work in the same pathway of IAA synthesis with INS was also attempted in this study using the publically available co-expression database ATTED-II [25]. Indole synthase was used as a seed gene to search for other possible genes in the tryptophan-independent pathway of IAA synthesis. Among all genes previously reported to be involved in IAA synthesis, *amidase1* (At1g08980) was strongly co-expressed with *INS* in general and light conditions with mutual ranks of 116 and 141 respectively (Table 3). Additionally, *Amidase1* was found to positively correlate with *INS* (0.297) (Fig. 3). This correlation value was similar to that found between *TSA* and *TSB*; their products constitute an enzyme complex involved in the production of trp from indole-3-glycerol phosphate. Taken together and in addition to the observation that this gene was not found to positively co-expressed with *TSB*, suggests that amidase1 may be exclusively involved in the trp-independent pathway of IAA synthesis. Finding *amidase1* in the top 300 gene list negatively co-expressed with *TSB* (MR 20,628) further supports this hypothesis. It concludes that amidase may catalyze the rate-limiting step in this pathway. These findings are in agreement with the observation that Amidase1 is specifically convert IAM to IAA (*K*m 972 μM) [29]. Furthermore, the importance of amidase1 in IAA synthesis has been quite recently suggested by Gao et al. [30].

Amidase5 (At4g34880), an *Arabidopsis* amidase member that functionally not characterized, was co-expressed with *INS* in the general and light conditions with a MR of 1000 and 926 respectively. However, statistical supportability of the co-expression relation for this gene was found to be weak. The same thing was observed for the expression of this gene with *TSβ* in the tissue condition (MR 2471) but again with low statistical supportability suggesting that the gene product may be involved in the production of substance other than IAA. Amidase2 (At5g07360) which was proposed to convert substrate other than indole-3-acetamide (IAM) [31] was co-expressed with *TSβ* in the general, tissue, biotic, and abiotic specific conditions whereas co-expressed with *INS* in the abiotic conditions

Table 3 Summary of genes co-expressed with *INS* (The top 1st 2000 genes). *TAA1*, tryptophan aminotransferase of *Arabidopsis*. Coexpression index is represented by mutual rank

Gene	Locus	General condition	Hormone	Tissue	Biotic	Abiotic	Light
Amidase1	At1g08980	116			1571		141
Amidase2	At5g07360					539	
Amidase5	At4g34880	1000					926
AAO2	At3g43600		1020				
TAA1 (WEI8)	At1g70560	1078					996

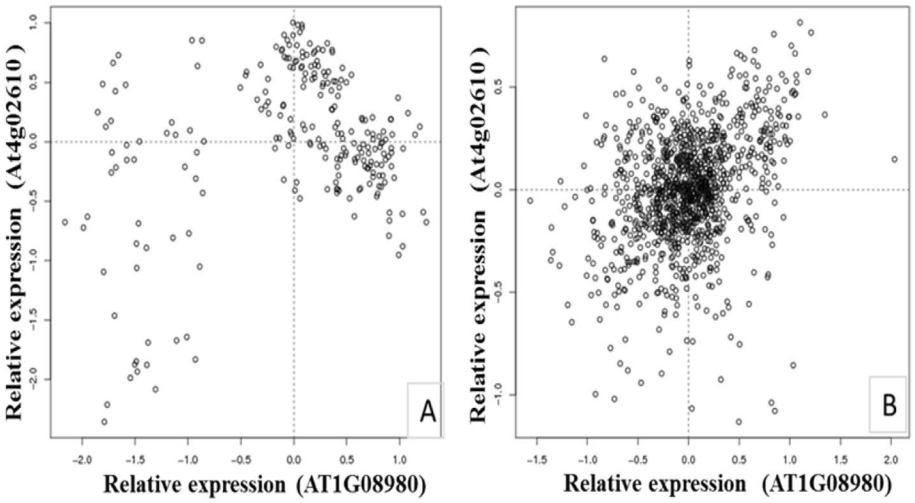

Fig. 3 Correlation of the expression for *INS* and *amidase1* obtained from developmental samples (**A**) and vegetative samples (**B**)

only. Amidase6 (At3g25660), another uncharacterized *Arabidopsis* amidase member, was co-expressed only with *TSβ* in the light condition (MR 1419).

Role of Aldehyde Oxidase and Nitrilase in IAA Synthesis

Condition-dependent and independent co-expression lists were also searched for previously genes reported to be involved in IAA biosynthesis. A group of IAA synthesis genes including *NIT*, *AAO*, *YUC*, and *CYP79B2/B3* is positively co-expressed with *TSB*. The nitrilase isoform (*NIT3)*, which was reported exclusively in Brassicaceae [32, 33], is co-expressed with *TSB* in all but biotic conditions (Table 2). Through the analysis of *NIT2*RNAi lines, it has been shown that the level of IAA is considerably reduced due to non-detected NIT1-3 [34] suggesting that NIT3 may participate in IAA synthesis through the trp-dependent pathway. Additionally, *NIT4* found in monocot and dicot plants is strongly co-expressed with *TSB* in hormone conditions in addition to general and biotic conditions (Table 2). NIT4 is primarily involved in cyanide detoxification [35]. These controversial data need to be clarified for a possible role of NIT4 in IAA synthesis, particularly in the trp-dependent pathway. None of the *NIT* isoforms was found to co-expressed with *INS* suggesting that this enzyme may not have any possible role in IAA production through the INS pathway.

Aldehyde oxidase α and γ are homodimers of AAO1 and AAO2 respectively whereas aldehyde oxidase β is a heterodimer of AAO1 and AAO2. They have shown to have limited preferences for abscisic aldehyde [36]. In this work, co-expression of *INS* was noted only with *AAO2* in the hormone-specific condition, suggesting a possible role for AOγ in IAA biosynthesis through the trp-independent pathway. However, aldehyde oxidase1, 2, and 3 were found to co-express with *TSB*. Aldehyde oxidase1 is co-expressed with *TSB* in all analyzed general and specific conditions, whereas *AAO2* co-expressed with *TSB* in the tissue, abiotic and light conditions with mutual ranks of 328, 1456, and 302, respectively. Therefore, a possible role for these enzymes in IAA synthesis through the trp-dependent pathway is not excluded. Additionally, *AAO3* product which was reported to oxidize

abscisic aldehyde efficiently is co-expressed with *TSB* in general, hormone, and light conditions. This finding may explain the crosstalk between IAA and ABA hormones.

Conclusion

The trp-independent pathway was earlier proposed as a major route of IAA biosynthesis in *Arabidopsis* based on metabolites quantification and feeding experiments in trp biosynthesis mutants. More recent evidence supporting the involvement of the trp-independent pathway in auxin biosynthesis through INS was further illustrated. However, the interaction of INS with TSB to produce trp and, as a result, a possible role for this enzyme in the trp-dependent pathway was also suggested based mainly on bioinformatics studies. Therefore, the main aim of this work was to collect evidence through the implementation of coexpression analysis of the *Arabidopsis* genome for the specific role of INS in auxin biosynthesis. Among the 21,000 genes of the *Arabidopsis* genome available on the ATTED-II (http://atted.jp) database, *TSA* was found to be strongly positively coexpressed with *TSB* in the top 2000 gene lists of general, hormone, and abiotic conditions. Mutual ranks under these conditions were found to be 89, 38, and 180, respectively. Furthermore, *TSA* was found to positively correlate with *TSB* (0.291) in a large group of vegetative and developmental *Arabidopsis* samples. The correlation was clearer in developmental samples comparing to vegetative ones. However, *INS* was co-expressed with *TSB* neither in general nor in specific conditions and was found to negatively correlate with *TSB* (−0.046), suggesting that INS is operated in the trp-independent pathway. Absence of co-expression and correlation between *INS* and *TSB* may suggest that trp-dependent and independent pathways may separately and independently operate to synthesize IAA.

Production of IAA through INS in the trp-independent pathway is unclear in terms of other enzymes involved in this pathway. Therefore, all previously reported enzymes to have a possible role in IAA synthesis were analyzed in this research. Among all these enzymes, amidase was proposed to interact with INS in the trp-independent pathway. Amidase is the only gene found to be strongly positively coexpressed with *INS*. Mutual ranks for coexpression between *INS* and *amidase* (in particular *amidase1*) were 116, and 141 in general and light conditions respectively. Mutual ranks for these two genes were similar to that found between *TSA* and *TSB*, in which their products form an active complex. *INS* and *amidase* were positively correlated with each other. The value of correlation was similar to that found between *TSA* and *TSB*. Taken together and in addition to the observation that *amidase* is negatively correlated with *TSB*, we suggested that amidase1 is solely involved in the trp-independent pathway of IAA synthesis.

Author Contribution All authors contributed to the study conception and design. YMA-Z designed the study and supervised the work. Introduction was written by AA-Z and ARAT. Materials and methods were designed and written by KGF and SMA. The final results, discussion and conclusion were written by YMA-Z and FAA. SP revised and commented the final draft of the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability Not applicable.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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