



# Engineering insect resistance using plant specialized metabolites

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Plants in nature are protected against insect herbivory by a wide variety of specialized metabolites. Although insect herbivores generally tolerate the defensive metabolites of their preferred host plants, the presence of additional chemical defenses in otherwise closely related plant species can nevertheless provide resistance. This chemical resistance to insect herbivory can be enhanced by genetic engineering to increase the production of endogenous defensive metabolites, modify existing biochemical pathways, or move the biosynthesis of entirely new classes of specialized metabolites into recipient plants. However, current plant genetic engineering strategies are limited by insufficient knowledge of the biosynthetic pathways of plant specialized metabolism, unintended side-effects that result from redirecting plant metabolism, inadequate transgene construction and delivery methods, and requirements for tissue-specific production of defensive metabolites to enhance herbivore resistance.

## Addresses

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## Introduction

The 350,000 extant species of vascular plants [1] are consumed by an estimated 400,000 species of herbivorous insects [2]. Although all plants are attacked by at least some insects, any individual plant species is resistant to the vast majority of insect herbivores. As a result, the world is mostly green. Along with physical traits like leaf toughness, spines, and low digestibility, many of the more than 600,000 known metabolites in plants [3] provide protection against herbivorous insects. Such toxic and deterrent plant metabolites, if harnessed more efficiently

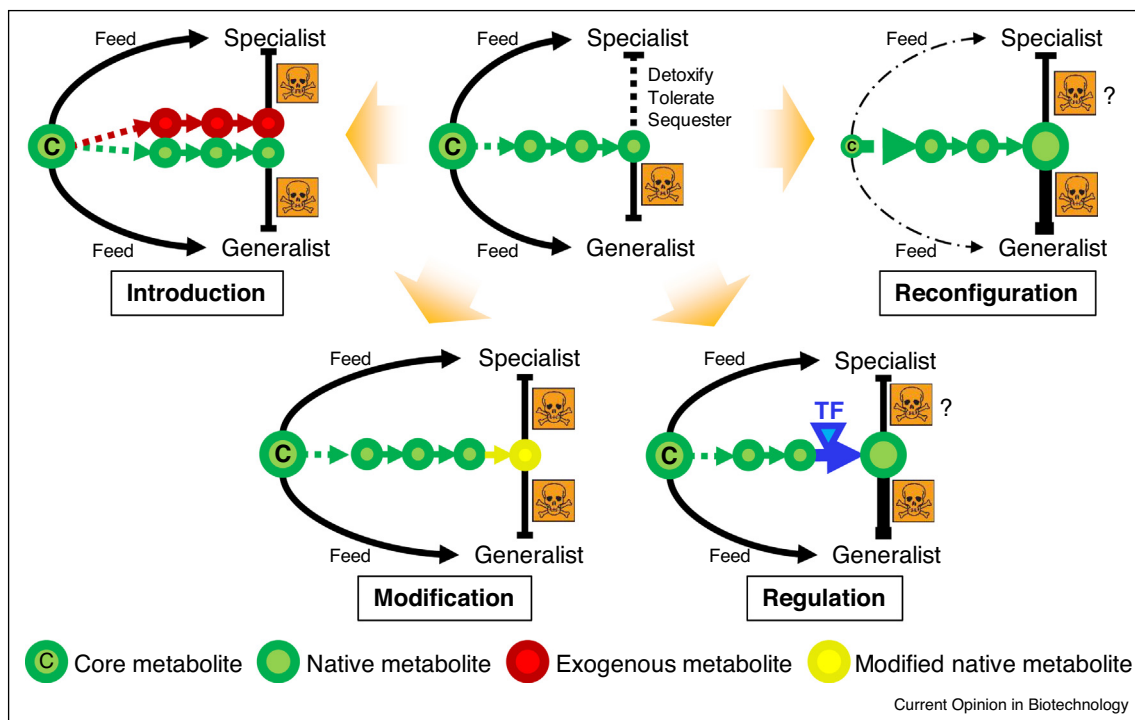
through current biotechnologies, could be used to enhance herbivore resistance in crop plants. In this prospective review, we provide an overview of the current strategies in plant metabolic engineering to increase insect resistance (Figure 1), summarize scientific and technological challenges for this approach, and point to other recent publications that examine the latest progress on addressing each of these challenges in more detail.

## Plant defensive chemicals determine insect host ranges

The efficacy of plant chemical defenses in limiting insect host ranges is most dramatically demonstrated by means of knockout mutations affecting plant biosynthetic pathways and/or insect detoxifying enzymes. Glucosinolates, well-studied defensive compounds that are characteristic of the Brassicaceae [4], can inhibit the growth of insects that are not specialized for feeding on this plant family. For instance, growth of Solanaceae-specialist *Manduca sexta* (tobacco hornworm) larvae is improved by *Arabidopsis thaliana* (*Arabidopsis*) knockout mutations that reduce either glucosinolate biosynthesis [5] or the activity of myrosinases,  $\beta$ -glucosidases that activate glucosinolates to produce isothiocyanates and nitriles [6] (Figure 2). *Bemisia tabaci* (whiteflies) are able to reproduce on *Nicotiana benthamiana* with an acylsugar acyltransferase knocked out, but not on wildtype plants, indicating that acylsugars on the leaf surface are a potent defense against these phloem-feeding insects [7]. On the insect side of the interaction, knockdown of detoxifying enzymes also demonstrates the importance of plant chemical defenses. For instance, *Plutella xylostella* (diamondback moths) can detoxify glucosinolates by means of a sulfatase [8], and reduction of this enzymatic activity by virus-induced gene silencing or CRISPR/Cas9 knockout compromised the performance of *P. xylostella* larvae on *Arabidopsis* [9,10]. Similarly, knockdown of a glucosinolate-detoxifying glutathione *S*-transferase in *B. tabaci* by RNA interference (RNAi) reduced the nymph development rate on *Arabidopsis* [11]. RNAi-mediated knockdown of *Spodoptera frugiperda* (fall armyworm) UDP-glucosyltransferase activity, which detoxifies maize benzoxazinoids by reglycosylation, compromised larval performance on their host plants [12].

Specialized insect herbivores, which tend to tolerate the characteristic defensive metabolites of their preferred host plant species, genera, or families, may nevertheless be deterred by more sporadically distributed chemical defenses. For instance, the well-studied Brassicaceae

Figure 1



Strategies for engineering insect resistance using plant specialized metabolism.

As illustrated in the center panel, all plants produce specialized metabolites from common core metabolic precursors to protect themselves against generalist insect herbivores (indicated by T-shaped arrows). Specialist insect herbivores can circumvent native chemical defenses of their host plants (dashed T-shaped arrows) and thereby benefit from feeding on nutritive core metabolites. Current strategies of engineering insect resistance using specialized metabolism can generally be divided into four categories: **Introduction** [20,22–24,25<sup>\*\*</sup>], whereby completely novel specialized metabolic pathways can be introduced from one species to another across large phylogenetic distances. **Modification** [4,26–32] with genes from closely related species, genotypes, or wild relatives can provide modifying enzymes that act on top of a shared specialized metabolic pathway. **Regulation** [5,37,38,39<sup>\*</sup>,40<sup>\*</sup>] allows targeted activation/de-activation of *cis*-regulatory and *trans*-regulatory elements of transcription factors (TF) to promote upregulation of existing biosynthetic genes (indicated by the bolded arrow) and accumulation of specialized metabolites (indicated by the larger circle at the end) and hence more effective defense. **Reconfiguration** [29,30,43–49] involves manipulation of metabolic gene expression further upstream to divert greater metabolic flux (indicated by the larger green arrow) into existing specialized metabolic pathway to promote accumulation of anti-herbivore specialized metabolites (indicated by the larger circle at the end) while reducing the amount of nutritive core metabolites (indicated by the smaller C-labelled circle) that are available to herbivores.

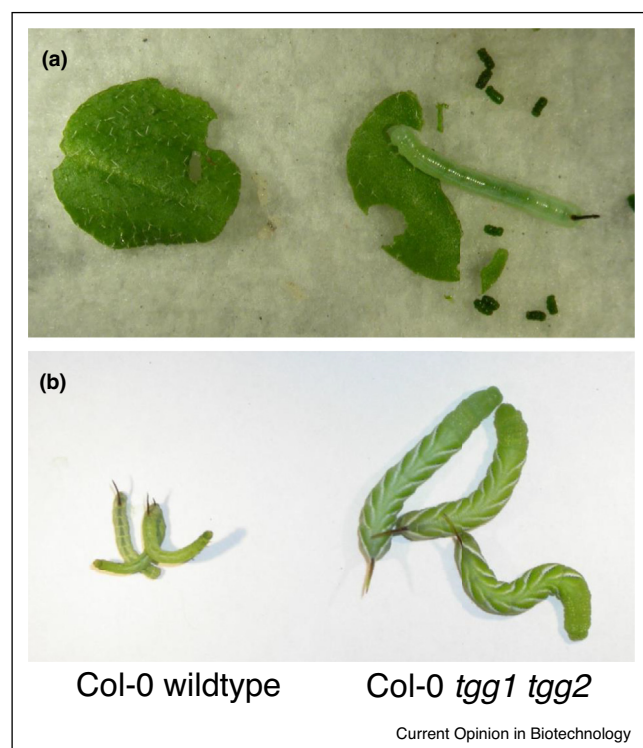
provide excellent examples of plant species that produce additional chemical defenses beyond the canonical glucosinolates that are characteristic of this plant family [4]. G-type (glabrous) *Barbarea vulgaris* (yellow rocket) lineages, which contain more triterpenoid saponins than P-type (pubescent) lineages, are resistant to crucifer-specialist *P. xylostella* [13]. Cardiac glycosides, which are present in the genus *Erysimum* [14<sup>\*</sup>], act as both oviposition and feeding deterrents for Brassicaceae-specialist *Pieris rapae* (white cabbage butterfly) [15,16]. Other examples of non-glucosinolate chemical defenses that are sporadically present in the Brassicaceae and provide protection against herbivory include cucurbitacins in *Iberis umbellata* (candytuft) [17], alliarinoside in *Alliaria petiolata* (garlic mustard) [18], and tropane alkaloids in *Cochlearia officinalis* (scurvygrass) [19]. Although we are not aware of more recently discovered examples, it is likely that a detailed examination of Brassicaceae

chemical diversity would identify additional insect defenses that are limited to particular genera or species within this plant family.

### Biochemical pathway engineering can increase pest resistance

Given the protective effects of plant chemical defenses, it is tempting to improve the existing defensive arsenal of crop plants using transgenic approaches to move biosynthetic pathways from one species into another. However, to date there are very few examples where complete pathways of specialized metabolism were transferred into stable transgenic plants of another species. In an early example of introducing a new biochemical pathway for insect resistance (Figure 1, introduction), transfer of two genes for the biosynthesis of the cyanogenic glucoside dhurrin from *Sorghum bicolor* (sorghum) to *Arabidopsis* increased resistance to *Phyllotreta nemorum* (flea beetles)

Figure 2



*Manduca sexta* (tobacco hornworm) caterpillars **(a)** prefer *tgg1 tgg2* (myrosinase-deficient) leaves over wildtype *Arabidopsis* ecotype Columbia-0 (Col-0) in choice tests, and **(b)** grow more rapidly and are able to reach pupation only on *tgg1 tgg2* mutant plants [6].

[20]. *Nicotiana tabacum* (tobacco) plants that were engineered to produce betalain with genes from *Beta vulgaris* (red beet) had enhanced resistance to *Botrytis cinerea* (grey mold) [21]. A pathway for benzylglucosinolate biosynthesis (Figure 3a) has been engineered in stable transgenic tobacco, though with a low overall yield [22]. *Triticum aestivum* (wheat) plants were genetically engineered to produce (E)- $\beta$ -farnesene, an aphid alarm pheromone, but this did not reduce aphid infestations in the field [23]. Other pathways for the biosynthesis of plant specialized metabolites, for instance saponins from *B. vulgaris* [24] and colchicine from *Colchicum* (fall crocus) [25<sup>••</sup>], have been expressed transiently in *N. benthamiana* using viruses or *Agrobacterium tumefaciens*, but not yet in stable transgenic plants.

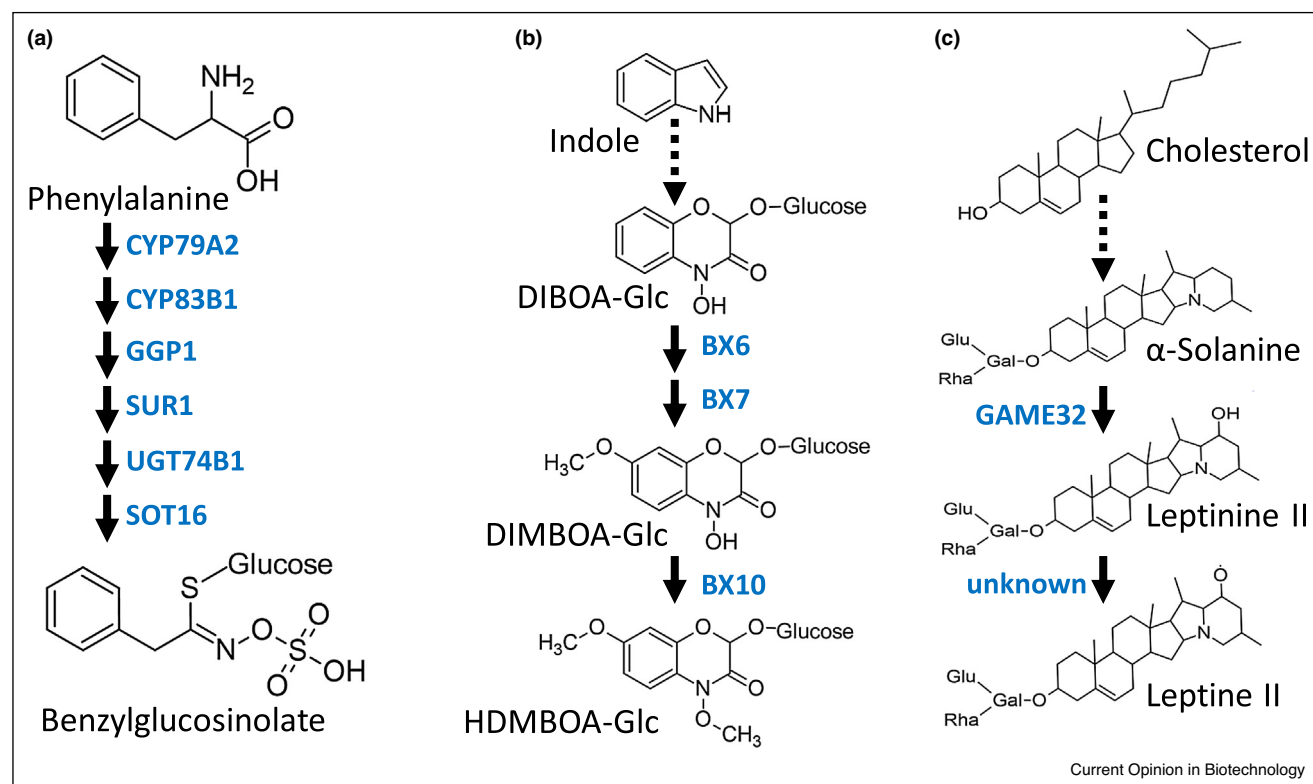
Whereas transfer of new chemical defenses into unrelated species is difficult, a currently more feasible approach is the modification of well-characterized biosynthetic pathways based on natural variation that is present in closely related species. In cases where the core biosynthetic pathway already is present in the target species, fewer exogenous genes need to be transformed and there may be fewer unanticipated changes in other aspects of plant metabolism. If genes are transferred from one food crop to another,

this would involve the biosynthesis of compounds that are already part of the human diet and negative physiological effects from consuming such transgenic plants may be less likely. For example, whereas maize and wheat predominantly accumulate DIMBOA-Glc (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside), HDMBOA-Glc (2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside), and other modified benzoxazinoids, in *Secale cereale* (rye) the benzoxazinoid biosynthetic pathway ends with DIBOA-Glc (2,4-dihydroxy-1,4-benzoxazin-3-one glucoside) [26]. Insect growth is improved on maize *bx6* mutants, which accumulate primarily DIBOA-Glc instead of DIMBOA-Glc and HDMBOA-Glc [27], suggesting that adding maize benzoxazinoid biosynthetic enzymes to rye could enhance pest resistance (Figure 1, modification of an existing biochemical pathway; Figure 3b).

There have been several successes in modifying the glucosinolate content of both *Arabidopsis* and *Brassica* species through transgenic approaches [28]. For instance, accumulation of 2-phenylethylglucosinolate, which is not normally abundant in *Arabidopsis* leaves, can be increased by phenylalanine overproduction (Figure 1, reconfiguration), leading to enhanced *Trichoplusia ni* (cabbage looper) resistance [29,30]. Although there are more than 120 known glucosinolates [4], most of these are not present in *Brassica* crops. As the core glucosinolate biosynthetic pathway already exists in crops like *Brassica oleracea* (cabbage) and *Brassica napus* (rapeseed), it would be feasible to modify existing defensive pathways (Figure 1) by introducing a small number of glucosinolate biosynthetic genes, most likely using genetic engineering approaches, and thereby enhancing herbivore and/or pathogen resistance. In an example of how this might be achieved, expression of glucosinolate biosynthesis genes from *Manihot esculenta* (cassava) in *Arabidopsis* increased resistance to the bacterial pathogens *Erwinia carotovora* and *Pseudomonas syringae* [31].

Solanaceous crops also provide examples of metabolic pathway transfers between related species. Heterologous expression of a sesquiterpene synthase gene from *Solanum habrochaites* in *Solanum lycopersicum* (cultivated tomato) induced the production of a novel insecticidal compound (Figure 1, modification) and increased resistance against whiteflies and spider mites (*Tetranychus urticae* and *Tetranychus evansi*) [32]. Leptines, steroidal glycoalkaloids that act as defenses against *Leptinotarsa decemlineata* (Colorado potato beetle), a devastating pest of potatoes worldwide, are present in *Solanum chacoense*, but not in *Solanum tuberosum* (cultivated potato) [33]. Recently, a 2-oxoglutarate-dependent dioxygenase, the first of two enzymes that would be needed to produce leptines (Figure 3c) was cloned in *S. tuberosum* [34<sup>•</sup>]. If the second, as yet unidentified gene can be cloned in a similar manner, it may be possible to engineer potato plants that are more resistant to Colorado potato beetles. Although

Figure 3



Examples of plant biochemical pathways that could be engineered for insect resistance. **(a)** Six genes for the biosynthesis of benzylglucosinolate were moved from Brassicaceae into tobacco [17]. Higher levels of benzylglucosinolate accumulation might increase resistance against Solanaceae-specialist herbivores feeding on these transgenic plants. **(b)** Transforming *BX6*, *BX7*, and *BX10* genes from maize into rye, which accumulates DIBOA-glucoside, would allow biosynthesis of DIMBOA-glucoside and HDMBOA-glucoside and likely would increase pest tolerance. **(c)** α-Solanin a glycoalkaloid in cultivated potatoes is converted to leptinine II by *GAME32* from *Solanum chacoense* [29]. An as yet unidentified enzyme converts leptinine II to leptine. Production of leptine in cultivated potatoes could increase resistance to Colorado potato beetles.

acylsugars provide effective resistance against insect pests in several wild relatives of cultivated tomatoes, efforts to introgress genes from wild relatives to produce tomatoes that have both elevated insect resistance and good fruit quality have been unsuccessful [35]. However, now that most or perhaps all enzymes of tomato acylsugar biosynthesis have been identified [36], it will be feasible to overexpress this biosynthetic pathway in cultivated tomatoes in a more targeted manner.

### Regulatory networks to enhance plant metabolic resistance

In addition to engineering biosynthetic genes directly, advances in the understanding the genetic regulatory networks of plant specialized metabolism open the possibility of *cis*-regulatory and *trans*-regulatory element engineering (Figure 1, regulation). However, most published examples of this approach are functional genetics studies under laboratory conditions. For instance, overexpression of specific MYB and MYC transcription factors in transgenic *Arabidopsis* can lead to significant alteration of both defensive metabolites and herbivore performance

[5,37,38]. Other recent examples showing upregulation of plant defensive metabolite biosynthesis with transcription factors include transient expression of *ORCA6* transcription factor in *Catharanthus roseus* (Madagascar periwinkle) to increase terpenoid indole alkaloid accumulation [39] and increased accumulation of nicotine in stable transgenic tobacco overexpressing the *NtERF189* transcription factor [40].

It is worth noting that, although insect resistance in plants is commonly associated with specialized metabolism, the dichotomy between plant 'primary' and 'secondary' metabolism is fading. Instead, a more holistic view of plant metabolic networks is required for engineering to the desired plant defense outcomes. Although we are not aware of plant genetic engineering efforts based on such principles, nature has provided ample examples of defense-related interactions that extend beyond specialized metabolites (reviewed in Ref. [41]). For instance, aphids and other phloem-feeders have highly efficient osmoregulatory systems that allow them to cope with their sugar-rich and nitrogen-poor diet [42], and some

even actively manipulate host plant metabolism to increase the nitrogen content in their diet [43–45]. On the plant side, studies with both monocot and dicot species demonstrate that plants relocate nutritive metabolites away from insect feeding sites to hamper herbivore growth and/or promote re-growth after insect infestation, thereby providing increased tolerance of herbivory [46–49]. These studies of natural plant–insect interactions should inspire novel perspectives in rewiring local and systemic plant biochemical environments to promote insect resistance.

### Current limitations for engineering insect resistance in plants

Several biological factors and technical bottlenecks limit plant metabolic engineering to enhance anti-herbivore defenses on a broader scale: (1) Successful metabolic engineering examples are mostly restricted to biosynthetic pathways of a few well-characterized anti-herbivore compounds. This suggests that insufficient knowledge of defensive compounds, biosynthetic pathways, and regulatory mechanisms remains a major limitation in applying metabolic engineering approaches to enhance plant defense. (2) In addition to genetic elucidation of plant biosynthetic pathways, a better understanding of enzyme functions at the cell biology level, such as tissue-specific and subcellular enzyme localization [50–52] and metabolon assembly [53], is also required for the proper engineering of a new metabolic pathway in a heterologous plant species. (3) Plant genetic engineering currently is limited by the host-specificity and genetic load limit of the commonly used *Agrobacterium*-mediated transgene delivery system, as well as technical bottlenecks in tissue culture and other steps required for generation of a stable transgenic plants. Therefore, the successful metabolic engineering approaches described here have involved short pathways and well-studied model plant species. (4) The introduction of a heterologous metabolic pathway can have indirect effects such as depletion or provision of substrates for other metabolic pathways, feedback inhibition, and phytotoxicity that may negatively impact the normal biological processes and economic traits of the engineered plants. For instance, nicotine overproduction in tobacco caused stunting [40<sup>•</sup>], enhanced biosynthesis of the essential amino acid phenylalanine altered glucosinolate accumulation in *Arabidopsis* [29,30], and glucosinolate biosynthesis in transgenic tobacco likely was limited by substrate availability [22]. (5) The volatile legal and legislative landscape, public opinion regarding transgenic plants, and possible flavor changes resulting from new defensive metabolites in food crops could impose non-scientific hurdles to the adoption of metabolically engineered plants.

### Conclusions

Despite of the many challenges ahead, recent scientific and technological advancements have greatly accelerated

progress in removing obstacles from plant metabolic engineering. The ten enzyme-encoding genes required for the biosynthesis of bioactive zealexins in maize were identified in 2020, only nine years after the initial discovery and functional validation of these compounds [54<sup>••</sup>,55<sup>••</sup>]. By contrast, more than 40 years were required to characterize the first five benzoxazinoid biosynthetic genes in maize [56]. Indeed, genetic dissection and metabolic modeling of plant specialized metabolic pathways using a multi-omics platform is currently one of the most active areas of plant research [57]. Meanwhile, development of modular cloning systems, nano-particle-based bio-material delivery systems, and other synthetic biology tools will expand the targets of efficient genetic transformation beyond model species in the near future [57–60]. The integration of accumulating knowledge on the structural bases of post-translational modifications, identification of protein–protein interactions, and precise gene-editing tools give rise to the concept of ‘metabolic editing’, which could result in seamlessly edited *cis*-genic plants with negligible side-effects on normal plant physiology (reviewed in Ref. [61]). With this rapid progress in scientific knowledge and technical tools, engineering plant metabolism for improved insect resistance could become a vital component of a sustainable integrated pest management strategy in agricultural ecosystems.

### Conflict of interest statement

Nothing declared.

### CRedit authorship contribution statement

**Shaoqun Zhou:** Conceptualization, Writing - original draft, Writing - review & editing. **Georg Jander:** Conceptualization, Writing - original draft, Writing - review & editing.

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