BIOINFORMATICAL AND EXPERIMENTAL APPROACHES OF STRIGOLACTONES RECEPTORS

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Abstract. Strigolactones (SLs) are plant hormones with significant roles in plant growth, development and environmental interactions. SLs were first discovered to stimulate the germination of parasitic plants such as *Striga* and *Orobanche*, but they have now been revealed to regulate a variety of physiological processes in plants. Since their detection as germination stimulants, SLs have received a lot of attention for their several activities in controlling shoot branching, stress responses and symbiotic interactions with beneficial microorganisms. This review examines recent bioinformatics approaches to evaluating SLs and their receptors. By thoroughly exploring the significance of SLs in plant biology, this article highlights the potential for interdisciplinary research to fully use SLs in agriculture and other applications.

Key words: strigolactone, phytohormones, receptor, ligand, bioinformatics.

INTRODUCTION

Strigolactones (SLs) are a new class of phytohormones that serve important functions in plant physiology and development, gaining significant attention over the past few years [60]. These compounds act as both endogenous and exogenous signaling molecules, impacting a variety of processes including shoot branching, abiotic stress response, senescence, chlorophyll synthesis, and signaling pathways for host identification by arbuscular mycorrhizal fungi and parasitic plants [3, 18, 22, 38, 46]. The first identified SL, strigol, was isolated in 1966 from the root exudates of *Gossypium hirsutum* L. (cotton), a non-host for *Striga* species, and was found to act as a germination stimulant for *Striga lutea* Lour. (witchweed) [51]. Neither of their use is highly developed. Plant hormone agonists have been shown to be important in both basic and practical research [20]. Important discoveries were represented by the discovery of SL biosynthesis intermediate carlactone (CL) and

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the recognition of proteins, such as D14 in rice, involved in SL perception and signaling pathways [40].

SL receptors, which are vital for acquiring biological signals, initiate the cellular response, triggering a series of molecular events that influence many physiological processes in plants. This signaling cascade regulates gene expression, resulting in a variety of physiological activities. Plants boost their fitness and adaptability by modifying their growth and development in response to environmental factors. Understanding these receptors is critical for understanding plant hormone signaling pathways and increasing agricultural productivity [5, 11, 17, 27].

STRIGOLACTONE OVERVIEW

Based on the structure, there are two main groups of natural SLs: canonical and non-canonical [54]. Canonical SLs are the most thoroughly investigated and well-studied type of SL. They feature a conserved structure composed of an ABC ring system (tricyclic lactone part) connected to a D-ring (butenolide) via an enol-ether bridge, with the natural SLs maintaining a 2'R configuration at this link. The differences between the B-ring and C-ring configurations produce two separate SL families: strigol-like and orobanchol-like [3, 10, 36, 49]. Both families can undergo numerous chemical transformations, including methylation, epoxidation, and hydroxylation, which impact the AB fragment [1]. The most well-known canonical SLs are: strigol, 5-deoxystrigol, orobanchol, orobanchyl acetate, sorgomol [54]. Non-canonical SLs lack the conventional ABC-ring structure but maintain the important enol-ether bridge and D-ring fragment, which can be coupled to a variety of configurations [3, 51]. Some examples of non-canonical types are carlactone, lotuslactone, zealactone, and heliolactone [22]. Research on the structure-activity relationship (SAR) of SLs has shown that the D-ring fragment and the enol-ether bridge are essential for their biological activities. Additionally, modifications such as the addition of a hydroxyl group to the A-ring or B-ring reduce the stability of SLs, specifically promoting the germination of *Striga* species but not *Orobanche* species [54]. Plant roots are the primary location of SL biosynthesis, but only trace amounts of SLs are released into the rhizosphere (10-7-10-15 M) [23].

Currently, a large variety of branching mutants have assisted researchers in comprehending SL production and signaling pathways. Significant examples include more axillary growth mutants (max) in *Arabidopsis thaliana* (L.) Heynh., dwarf (d) or high-tillering dwarf (htd) mutants in rice, decreased apical dominance mutants (dad) in *Petunia* × *hybrida* hort. ex E. Vilm., and ramosus mutants (rms) in *Pisum sativum* L. *Arabidopsis*'s max3 and max4, rice d10 and d17, and pea rms1 and rms5 are deficient in carotenoid cleavage dioxygenases CCD7 and CCD8 [14, 29, 32, 39]. CCD8 then processes this intermediate to produce carlactone. The cytochrome P450 enzyme MAX1 further converts carlactone into active SLs [53], which have major implications in controlling plant development and interactions with the environment [26].

The development of synthetic SL analogs for commercial use is actively being pursued [13]. This is an important step forward in SL research because these synthetic analogs have the same bioactive core as natural SLs, although with slightly lower activity but generally superior stability [23]. Synthetic SLs are useful tools for understanding the biological activities of these hormones in plants and developing novel agricultural tactics to eliminate parasitic weeds and improve crop growth. They can affect plant growth and development, control parasitic weed germination, and enhance the effectiveness of beneficial symbiotic connections between plants and soil bacteria [2, 37, 42]. Among the most popular synthetic SL analogs were GR24, Triton-X-100, Nijmegen-1, GR5, GR7, and 2-MN [22]. The complete synthesis of the ABC rings, followed by bonding the functional side chains and the D-ring it is occasionally yield-limited. Based on the discovery of the bioactiphore in SLs, the D-ring, which is necessary for activity, chemical synthesis of SL analogues is more realistic and practical [30, 60]. A simpler version, which is being studied to observe if it is more efficient, is SL mimics. These are molecules lacking the ABC rings but keeping the D-ring attached to a suitable substituent at C-5. The word "mimic" stems from the discovery that these compounds replicate SL action. Because of their simpler structures in contrast to natural and synthetic equivalents of SLs, they can be considered viable alternatives for agricultural uses. There are two types of SL mimics obtainable. At C-5, the initial contains a substituted phenyloxy group. One of the most active is para-bromo-phenyloxy butenolide. *Striga hermonthica* (Delile) Benth. seeds have poor germination activity; however, they are quite effective as branching inhibitors. The substituent in the phenoxy ring has been changed, and the most powerful structure (para-bromo) for branching inhibition has been discovered (tiller bud outgrowth in rice). In terms of branching inhibition, this SL mimic is as effective as GR24. The second group described almost simultaneously, contains an aroyloxy group at C-5. These SL mimics have a low germination activity for *S. hermonthica* seeds but a high germination activity for *Orobanche cernua* Loefl. seeds [30, 60]. Simple ketones like 1-tetralone and 1-indanone can be transformed into highly active SL mimics in a few steps of synthesis. Keto enols like dimedone and hydroxycoumarin require only one step to produce an active SL derivative [58].

In recent years, the signaling pathway of SLs has been an active area of research and experiments are still being done to fully understand these aspects. Structural biologists continue to face significant challenges in capturing the entire SL molecule inside the receptor. The discovery of an SL receptor protein that additionally serves as an active catalytic enzyme marks a significant advance toward comprehending SL perception [22]. SLs are recognized by dual-functional receptor/hydrolase proteins with poor substrate turnover, which adds to the difficulty of determining how the signal is transduced at the molecular level [10]. The binding of SLs with a protein receptor is the first step in the bioprocesses mediated by SLs

[59], with the receptors discovered through research of SL-insensitive mutants, the rice dwarf 14 (d14) and petunia deceased apical dominance 2 (dad2) [42].

STRUCTURE AND MECHANISM OF ACTION OF STRIGOLACTONE RECEPTORS

The SL receptor is an α/β hydrolase in petunia (DAD2, Decreased Apical Dominance 2), DWARF14 in *Arabidopsis* (AtD14) and rice (OsD14), and RAMOSUS3 in pea (RMS3) with a very sluggish but vitally necessary enzymatic activity towards its hormone substrate [24]. The α/β hydrolases are a major family of enzymes that exist in all living species. Moreover, the α/β hydrolase fold acts as a fundamental framework for ligand receptors and phytohormones that include SL, karrikin, and gibberellin. D14 and KAI2 are members of the α/β hydrolase family with four helices creating a lid and a base built by beta strands and the rest of the helices. The Ser-Asp-His catalytic triad is functionally active and is located in the back of the ligand-binding pocket [22, 33, 52].

SL receptors are non-canonical, irreversibly binding SLs and producing a covalently linked intermediate molecule (CLIM) attached to the histidine in the catalytic triad [15, 52]. In Arabidopsis, the D14 receptor's catalytic triad (S97-H247-D218) breaks SL molecules into ABC-ring and D-ring products [25]. SL binding sends signals to leucine-rich repeat F-box proteins (MAX2 in *Arabidopsis* and D3 in rice), which connect to an SCF complex. This causes the ubiquitination and destruction of transcriptional repressors such as SMXLs in *Arabidopsis* and D53 in rice [18]. The pathway, including the interaction between MAX2 and D14, is preserved in various species [12]. In *Arabidopsis* plants, the MAX2 protein degrades BES1, a transcriptional regulator that positively regulates brassinosteroid signaling. SLs and D14 both contribute to BES1 deterioration. Not all SL reactions modify gene expression; for example, after 10 minutes of treatment, SLs deplete the auxin transporter PIN1 from the plasma membrane of xylem parenchyma cells [42].

D14-TYPE RECEPTOR

D14 is a 318 amino acid protein produced by the Dwarf14 gene. In rice, the phenotype provided by its known mutant allele is similar to that of SL-deficient mutants. Because exogenous SLs are unable to fix this condition, D14 has a role in SL perception rather than synthesis [21]. This receptor interacts with MAX2 to initiate the polyubiquitination and degradation of D53-type proteins in the SMXL family after SL perception [50, 55] and is crucial for rice shoot branching [57].

A D14-type receptor lacking SL is in the open state. The SL is hydrolyzed after binding, releasing the ABC rings. Hydrolysis occurs via a nucleophilic interaction by the S96 amino acid of the catalytic triad, resulting in the formation of an ABC ring. A covalent link is then formed between the C5 moiety of the D ring and H247, resulting in a D-ring intermediate. The enzymatic dissociation of the D-ring is a

critical step in the action mechanism of SLs. The generated hydroxy butenolide causes conformational changes in the receptor pocket, triggering a chain reaction in signal transmission [31]. It is hypothesized that D14 signaling partners are selected during this step, promoting the receptor's transition to a closed state [41].

Since all of these biological effects of SLs are related to agricultural productivity, accurate control of SL signaling might represent a novel approach to crop production enhancement. DWARF and heavily branching structures are attractive qualities for increasing biomass, particularly in well-fertilized areas [3, 28].

A study led by Hu *et al.* [25] identified two putative SL genes in the *Saccharum spontaneum* species, SsD14a and SSD14b. Using Yeast-two-hybrid assays, they indicated that only SsD14a interacts with downstream signaling partners AtMAX2 and AtSMXL7/SsSMXL7. The increased expression of SsD14a, but not SsD14b, reversed the *Arabidopsis* d14-1 mutant's enhanced branching phenotype, showing that SsD14a operates similarly to the documented SL receptor, AtD14. The crystal structure of the N-terminal truncated SsD14a was determined, revealing an architecture identical to AtD14 and OsD14 in the open state, which is compatible with its capability to inhibit branching process in plants. The interaction between SsD14a and SMXL proteins is dependent on the R310 amino acid residue in the 10th alpha helix. Once the equivalent residue in SsD14b (P304) was substituted with arginine (P304R), SsD14b's ability to interact with SMXL proteins was restored, reversing the *Arabidopsis* d14-1 max2-3 double mutant phenotype [25].

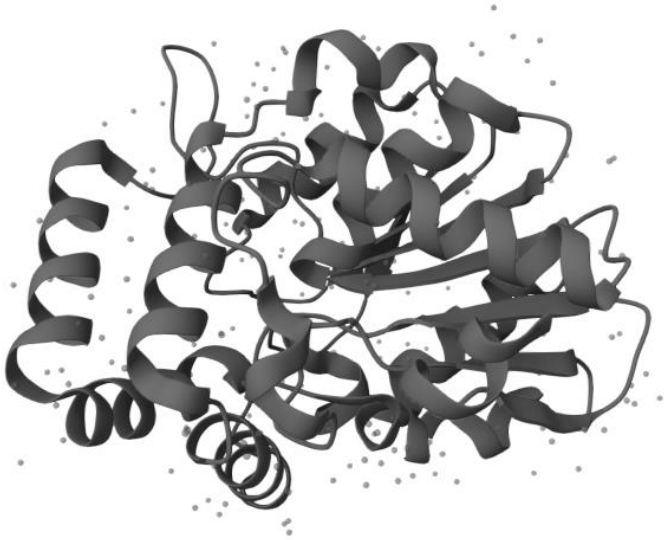

Fig. 2. Crystal structure of rice DWARF14 (D14), PDB ID: 4IH9, original after [56].

HYPOSENSITIVE TO LIGHT/KARRIKIN INSENSITIVE2 (HTL/KAI2)

HTL is a similar α/β hydrolase gene that has been demonstrated to play a function in *Arabidopsis* germination. HTL homologs may play comparable roles in parasitic plant species because *Striga* demands SLs to germinate. On the opposite, *Arabidopsis* HTL binds karrikin. Karrikin is a smoke-derived germination stimulant. *Striga* is responsive to SL picomolar concentrations and can not germinate in response to karrikin. Additionally, *Striga* can discriminate between hosts by recognizing distinct strigolactone combinations. It is challenging to figure out the functional involvement of *Striga* HTL/KAI2 homologs (ShHTLs) in SL perception whereas *S. hermonthica* is an obligate and outcrossing hemiparasite [46].

While SL receptors (*S. hermonthica* hyposensitive to light [ShHTL]) have been found, determining their functions has been challenging because these parasites cannot be simply cultivated in the laboratory, because seed susceptibility to SL demands precise temperatures and humidity, achieved in the field at the start of a new rainy season. Furthermore, most *Striga* species are obligatory outcrossers that are not transformable and thus not accessible to genetic research. As a result, in manageable genetic systems like *Arabidopsis*, activities are mainly assigned by expressing *Striga* SL receptors [4].

ShHTL4-ShHTL9 receptors confer SL sensitivity to *Arabidopsis* seeds, with ShHTL7 providing SL hypersensitivity to *Striga* germination levels. These observations, in addition to the conclusion that activating ShHTL7 is significant for *Striga* germination, suggest that this receptor is important for germination [4].

ShHTL6 and 7 are likely to be responsible for seed germination in *S. hermonthica*, as suggested by sensitivity to SL in transgenic *Arabidopsis* HTL-deficient mutants expressing ShHTLs and the affinity of different SLs for recombinant ShHTLs. Due to the structural diversity of SL receptors across and within species, sensitivity to a range of SL agonists varies in concentration and bioactivity [44].

In *S. hermonthica*, ShHTL7 was found to be the most active SL receptor [52]. Soporidine blocks the ShHTL7 receptor, preventing seed germination in *Striga* [45]. The ShHTL7 perceiving method consists of ShHTL7 binding to SL then occurs the hydrolyzing of SL into CLIM. CLIM is a D-ring-derived intermediate. The receptor protein conformation changes and interacts with ShMAX2/MAX2-based SCF complex and SMAX1 to induce SL signal transduction to regulate seed germination [34].

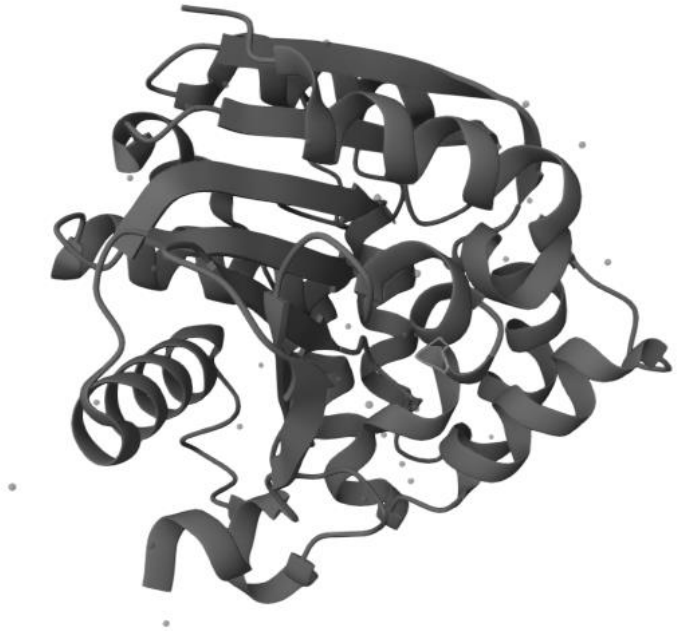

Fig. 2. Strigolactone receptor in Striga ShHTL7, PDB ID: 7WA8, original after [49].

Sobecks *et al.* [43] explores the molecular mechanisms behind the enhanced SL sensitivity of the Var64 mutant of the *A. thaliana* KAI2 receptor compared to the wild-type. Using long-timescale molecular dynamics simulations and Markov state models, the researchers found that the Var64 mutant (with mutations W153L, F157T, and G190T) has a higher affinity for binding GR24 synthetic analog, with the binding free energy getting about 2 kcal/mol higher compared to the wild-type receptor. Thus, the mutant receptor has a significantly bigger binding pocket volume in comparison with the wild-type receptor, allowing the ligand to attach to it easier. Furthermore, the mutant enhances binding kinetics, increasing the flux from unbound to bound states by a factor of ten. These modifications in thermodynamics and kinetics contribute to the higher SL sensitivity revealed in the Var64 mutant as opposed to the wild-type KAI2.

BIOINFORMATICS STRATEGIES FOR ANALYZING STRIGOLACTONES AND THEIR RECEPTORS

Bioinformatics of natural and synthetic compounds [6, 7] can give us important information about their structural features, biological activities, or pharmacological features [47].

Bioinformatics research of SL receptors provides valuable information into the functioning of these small molecules. Conducting such bioinformatic analyses is desirable both for understanding the roles of strigolactones in agriculture, plant growth, and the symbiotic relationship they mediate between parasitic plants and the plants that secrete them [8, 25, 48], as well as for their potential role in biomedicine, a subject currently under active study [16, 35].

In 2020, Yoshimura *et al.* [55] announced DL1, a powerful small molecule inhibitor that targets D14 receptors in plants. DL1 was discovered through a chemical search for D14 inhibitors, which used the fluorescent turn-on strigolactone analog yoshimulactone green (YLG) as a mediator. In rice and *Arabidopsis*, DL1 showed significant efficiency at sub-micromolar levels, inhibiting the expression of SL production genes while encouraging shoot branching. Unlike traditional breeding or genetic manipulation, DL1 provides a chemical-based method for variable management of plant architecture without requiring permanent genetic changes. Furthermore, DL1 shows promise for improving plant-Arbuscular Mycorrhizal (AM) fungal relationships. A SAR investigation led to the synthesis of DL1 analogs with increased potency. Per the published crystal structure of D14 in association with synthetic SL GR24, a docking model of the D14-DL1 complex was developed. D14's binding pocket was generated by eliminating GR24 from the D14-GR24 complex. To validate this kind of binding, several substituents on DL1 were replaced with hydrogen atoms, and the inhibitory effects of the DL1 derivatives were quantified using IC50 values for YLG hydrolysis by AtD14. The observations support the docking hypothesis in which the DL1 ethyl indole moiety is housed close to the D14 catalytic site. The substitution of bromoadamantane over bromonaphthalene significantly increased D14 inhibitory effects. The bromonaphthalene derivative, DL1b, had the best IC50 value (IC50 = 0.29 mM) compared to other D1 analogs. DL1b has a superior efficacy for in vivo branching induction and ought to serve as the lead chemical in the development of a first-in-class regulator of plant growth.

β-lactones act as irrevocable antagonists for SL receptors. The mentioned compounds suppress the plant (AtD14) and parasitic weed (ShHTL7) receptors. The IC50 values range from 0.16–7.9 to 0.74–77 μM, depending on side-chain changes at the third and fourth locations in the lactone ring. Tolfenamic acid and other N-phenylanthranilic acid derivatives with low micromolar binding affinity DAD2 inhibitors have been researched throughout the years as analytical tools have advanced. The acid connects to the DAD2 internal cavity and interacts with highly conserved residues in SL receptors, as structural investigations demonstrate. A subsequent SAR analysis of 138 N-phenylanthranilic acid closely related compounds using the Differential Scanning Fluorimetry (DSF) test on DAD2,

OsD14, and AtD14 revealed that one molecule, 2-(2'-methyl-3'-nitroanilino)benzoic acid (MNAB), has a higher affinity for all three receptors than tolfenamic acid [24]. Among the antagonists of SLs is 2-methoxy-1-naphthaldehyde (2-MN). 2-MN inhibits the interaction of D14 with D53 to rescue SL-suppressed rice tillering buds. 2-MN also prevents the germination of Striga seed [10].

While the SL binding pocket is 25 amino acids in length which means it represents 12% of total protein length, it can contain substitutions that change ligand selectivity among paralogs or orthologs. Eight of these residues (H26, G27, 29, H96, S97, F175, D218, H247 in AtD14) are constant. These residues' conservation throughout the D14/KAI2 family is believed to preserve receptor functionality and ligand accessibility and this is demonstrated by G28D mutation in pea, which led to the severe general instability of RMS3 (G27D=AtD14) [22].

Arellano-Saab *et al.* [5] analyzed dormirazine (DOZ), a new antagonist of SL perception in Striga, using a hybrid strategy that integrated phenotypic screening and target-specific structural data. The work focused on the key receptor ShHTL7 and differentiated it from previous approaches that targeted nonparasitic SL receptors before investigating Striga receptors. While focusing on ShHTL7, DOZ blocked several receptors due to the inherent promiscuity of SL receptors. The researchers revealed that RG6-derived compounds attach to certain amino acids in ShHTL7's lid domain that are not targeted by the natural SL molecule. Increasing the flexibility of SL receptors, especially through lid amino acids, was critical for interactions with downstream signaling partners. The RG6-ShHTL7 complex preserved the receptor in an inactive, open state. Triton X-100, when attached to the lid domain of ShHTL7, considerably inhibited Striga germination, highlighting the possibility of novel SL receptor antagonists. Molecular dynamics simulations provided new perspectives on protein-ligand interactions that were similar to experimental results. The study discovered new interactions between ligands and ShHTL7 residues, which might improve the inhibition found with DOZ and related drugs. While the binding techniques of RG6, RG6-6, and DOZ were similar, minor variations in the amino acids they contacted were crucial to their inhibitory activity. They examined a series of SL antagonists, RG1-RG7, using *Arabidopsis*, which encodes a ShHTL-like α/β hydrolase. Structural examination revealed that RG6 fit into the ShHTL7-binding pocket, antagonizing SL by limiting access to the pocket. This compound proved selectivity for sensitive SL receptors and was optimized by computational drug design. Scanning the CORE and ChemBridge libraries revealed 21 lead nonhydrolyzable compounds with RG6-like structures. These antagonists were docked with ShHTL7 and tested phenotypically, diminishing rac-GR24's capacity to germinate thermoinhibited seeds. RG6-6 is a highly effective SL inhibitor, composed of piperazine moieties that bind to ShHTL7 and limit the flexibility of the αE loop. These results underline the importance of targeting specific areas of SL receptors when generating effective antagonists.

Another study conducted by Fiorilli *et al.* [19] discovered a structural homolog of plant D14 in the pathogenic fungus *Cryphonectria parasitica* (CpD14). They confirmed CpD14's potential to bind and hydrolyze natural SL stereoisomers using computational modeling and biological analysis. Knockout mutants of CpD14 showed lower susceptibility to SL treatment, indicating that CpD14 is involved in SL responses in this fungus. The workflow was based on molecular homology modeling and docking simulations to predict the binding of SLs to CpD14. These predictions were tested using biochemical experiments, including electrospray ionization mass spectrometry (ESI-MS), which showed CpD14 binding and hydrolytic activity on SLs. The research results suggested that CpD14 is essential for the fungal response to SLs, underlining the multifunctional value of SLs in plant-AM fungi symbiotic relationships. The study's major findings include CpD14's ability to bind and hydrolyze SL stereoisomers, which highlights its function in SL signaling. Knockout mutants missing CpD14 revealed lower sensitivity to SLs, confirming the receptor's role in fungal response mechanisms.

CONCLUSIONS

The study of SLs provides many possibilities to improve the knowledge of plant physiology and agricultural practices. Important achievements in SL biosynthesis, receptor biology, and synthetic analog development highlight their potential for improving crop yields and combating invasive plants. Researchers are discovering more about SL receptors' roles in plant development and signaling cascades as they study their structure and functions. Furthermore, bioinformatics approaches present useful tools for evaluating SL-mediated physiological responses. Moving forward, interdisciplinary research will be mandatory to characterize the entire potential of SLs in agriculture and beyond.

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