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Multiple Gibberellin Receptors Contribute to Phenotypic Stability under Changing Environments

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The pleiotropic and complex gibberellin (GA) response relies on targeted proteolysis of DELLA proteins mediated by a GA-activated GIBBERELLIN-INSENSITIVE DWARF1 (GID1) receptor. The tomato (*Solanum lycopersicum*) genome encodes for a single DELLA protein, PROCERA (PRO), and three receptors, SlGID1a (GID1a), GID1b1, and GID1b2, that may guide specific GA responses. In this work, clustered regularly interspaced short palindromic repeats (CRISPR) /CRISPR associated protein 9–derived *gid1* mutants were generated and their effect on GA responses was studied. The *gid1* triple mutant was extremely dwarf and fully insensitive to GA. Under optimal growth conditions, the three receptors function redundantly and the single *gid1* mutants exhibited very mild phenotypic changes. Among the three receptors, GID1a had the strongest effects on germination and growth. Yeast two-hybrid assays suggested that GID1a has the highest affinity to PRO. Analysis of lines with a single active receptor demonstrated a unique role for GID1a in protracted response to GA that was saturated only at high doses. When the *gid1* mutants were grown in the field under ambient changing environments, they showed phenotypic instability, the high redundancy was lost, and *gid1a* exhibited dwarfism that was strongly exacerbated by the loss of another *GID1b* receptor gene. These results suggest that multiple GA receptors contribute to phenotypic stability under environmental extremes.

INTRODUCTION

The plant hormone gibberellin (GA) regulates various developmental processes, including seed germination, cell and shoot elongation, leaf expansion, transition to flowering, flower growth, and fruit development (Davière and Achard, 2013). The nuclear DELLA proteins inhibit GA responses (Locascio et al., 2013). GA binding to the soluble GIBBERELLIN-INSENSITIVE DWARF1 (GID1) receptor increases the affinity of the latter to DELLA, leading to the formation of a GID1-GA-DELLA complex. This facilitates the interaction of DELLA with an Skp, Cullin, F-box (SCF) E3 ubiquitin ligase complex, via GID2/SLEEPY1 (SLY1) F-box proteins. The SCF^{SLY1} complex then polyubiquitinates DELLA, targeting it for degradation by the 26S proteasome (Sasaki et al., 2003; Dill et al., 2004; Griffiths et al., 2006; Harberd et al., 2009; Hauvermale et al., 2012). DELLA degradation releases DELLA-interacting transcription factors, leading to transcriptional reprogramming and activation of GA responses.

GID1s can be divided into several evolutionarily conserved groups, with monocots expressing one group and eudicots expressing two: A type and B type (Yoshida et al., 2018). GID1 is a soluble protein showing sequence similarity to carboxylesterase (CXE) enzymes that hydrolyze short-chain fatty-acid esters; however, it lacks CXE activity (Ueguchi-Tanaka et al., 2007; Yoshida et al., 2018). GID1 was first identified in rice (*Oryza sativa*); the *gid1-1* rice mutant is dwarf and insensitive to GA (Ueguchi-Tanaka et al., 2005). Although rice has a single *GID1* gene, Arabidopsis (*Arabidopsis thaliana*) has three homologs named *GID1a*, *GID1b*, and *GID1c* (Griffiths et al., 2006; Nakajima et al., 2006). GID1a and GID1c belong to the A type group and GID1b to B type (Yoshida et al., 2018). Because the triple *gid1a gid1b gid1c* mutant in Arabidopsis is extremely dwarf and completely insensitive to GA, it was concluded that GID1 proteins are the only GA receptors in this species (Griffiths et al., 2006; Iuchi et al., 2007; Willige et al., 2007). GID1 contains two main motifs: the CXE domain that functions as a GA binding "pocket" and the N-terminal extension (N-Ex) domain. When GA binds to GID1, the N-Ex closes the GA binding pocket and then binds DELLA (Murase et al., 2008). Yamamoto et al. (2010) showed that Arabidopsis GID1b interacts with DELLA in a GA-independent manner. They also showed that although rice GID1 requires GA for interaction with DELLA, a mutation at the loop region (P99S) enables GA-independent interaction and responses.

The role of GAs in tomato (*Solanum lycopersicum*) plant development has been studied for many years. GA biosynthetic mutants, such as *gib-1*, *gib-2*, and *gib-3* (GA-deficient mutants), as well as the GA signaling mutant *procera* (*pro*), were identified and characterized (Bensen and Zeevaart, 1990; Koornneef et al., 1990; Martí et al., 2007; Carrera et al., 2012; Livne et al., 2015). *PROCERA* (*PRO*) codes for the single tomato DELLA protein and loss-of-function *pro* alleles were identified (Van Tuinen et al., 1999; Livne et al., 2015) and found to cause significant stem elongation, late flowering, parthenocarpic fruit development, and increased transpiration (Livne et al., 2015; Nir et al., 2017). Tomato plants overexpressing the gain-of-function, stable DELLA mutant gene

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IN A NUTSHELL

Background: Gibberellin (GA) is a growth-promoting phytohormone that regulates numerous developmental processes throughout the plant life cycle, including seed germination, stem elongation, leaf expansion, flowering, fruit development, and more. The DELLA proteins inhibit all these GA responses. When GA binds to its receptor GID1, they interact with DELLA and stimulate DELLA degradation, leading to the activation of GA responses. All dicot plants have multiple GA receptors, raising the question of whether different receptors regulate different developmental responses. Tomato has three GA receptors and one DELLA protein, making tomato an ideal system to study this question. Using genome editing technology (CRISPR/Cas9), we created *gid1* single, double and triple mutants in tomato and studied the role of each receptor in plant development and GA responses.

Question: We wanted to know if the different tomato GA receptors have unique roles in the regulation of plant development and if the existence of several receptors provides some evolutionary benefit to the plant.

Findings: When the single and double mutant plants were grown under optimal controlled growth conditions, they were almost normal and very similar to the wild type, suggesting that each receptor can fully replace the others (redundancy). However, when grown in the field, under changing environments, all mutant lines, but not the wild type, exhibited a high degree of phenotypic variability, including semi-dwarfism and even extreme dwarfism. Thus, while under optimal controlled growth conditions, one active GA receptor is sufficient to support normal growth, in the "real world", under a changing environment, all three are needed.

Next steps: We would like to find the environmental factor that causes instability in *gid1* mutants, to reveal the mechanism of this instability and to understand how three receptors with overlapping functions provide growth stability under environmental extremes.

proΔ17, were dwarf and exhibited reduced transpiration (Nir et al., 2017). A recent study identified three putative *SlGID1* (GID1) GA-receptor genes in tomato, but mutants were not found yet (Gazara et al., 2018; Shinozaki et al., 2018; Yoshida et al., 2018).

Arabidopsis has three GID1s, but rice and barley only one, raising the question of the necessity for multiple receptors. Mutant analyses in Arabidopsis revealed high redundancy, with differences in the contribution of each GID1 to GA responses (Griffiths et al., 2006). Although *gid1a*, *gid1b*, and *gid1c* single mutants and the *gid1b gid1c* double mutant exhibited normal development, *gid1a gid1c* and *gid1a gid1b* double mutants displayed clear developmental defects, including reduced inflorescence-stem length and lower fertility. This suggests that in Arabidopsis, GID1a has the most significant contribution to GA responses, which has been attributed to its high expression level (Griffiths et al., 2006). Althouh it has been speculated that different Arabidopsis GID1s bind specific DELLAs, analyses in yeast (*Saccharomyces cerevisiae*) suggested that all GID1s can interact with all five DELLA proteins (Griffiths et al., 2006; Nakajima et al., 2006). By contrast, Gallego-Giraldo et al. (2014) showed that during Arabidopsis fruit development, each GID1 variant binds specific DELLAs, dictating a unique role. The fact that Arabidopsis has three GID1s and five DELLAs makes it difficult to dissect the specific activities of each receptor. Tomato has three putative receptors (Shinozaki et al., 2018) but only one DELLA. Thus, tomato presents an ideal system for studying the specific and overlapping roles of the different SlGID1s (GID1s), and the importance of multiple GA receptors in the overall GA activity and plant development.

Here, we assessed the role of the tomato GID1 receptors in GA responses throughout tomato plant life cycle. The results show high redundancy in the activities of the three receptors in GA sensing and signaling under optimal controlled growth conditions. However, when grown in the field under changing environments, the redundancy was lost and all mutants showed phenotypic variability with increased dwarfism, suggesting that redundancy in GA sensing contributes to phenotypic stability under extreme environments.

RESULTS

The Three Tomato GID1s Are Functional GA Receptors

The tomato genome contains three putative *GID1* genes (Shinozaki et al., 2018); one belongs to the type A group, and two to type B. We named them *GID1a* (Solyc01g098390), *GID1b1* (Solyc09g074270), and *GID1b2* (Solyc06g008870; Supplemental Figure 1; Supplemental Data Set). To test whether the putative GID1s function as GA receptors, we heterologously expressed them in the semi-dwarf Arabidopsis *gid1a gid1c* mutant (Griffiths et al., 2006). All three *GID1s* restored normal growth in the transgenic mutant (Figure 1A), suggesting that all are functional GA receptors. Following GA binding, GID1 interacts with DELLA to initiate GA responses (Hauvermale et al., 2012). We then tested the interaction of the tomato GID1s with the tomato DELLA protein PRO, in a yeast two-hybrid assay. In the presence of GA_3, all three GID1s interacted with PRO (Figure 1B).

We next generated clustered regularly interspaced short palindromic repeats (CRISPR) /CRISPR associated protein 9 (Cas9)-derived *gid1* mutants. The mutations were analyzed by PCR and sequenced (Supplemental Figures 2A and 2B), and two independent mutant lines were identified for *GID1a*, four for *GID1b1*, and three for *GID1b2*. Homozygous mutants were obtained for each line, and the Cas9 construct was segregated out by backcrossing to M82. One homozygous line for each gene was selected for further study: *gid1a*-1-4 had a 1-bp insertion, causing a frame-shift and premature stop-codon; *gid1b1-12* had a 672-bp deletion; and *gid1b2*-4-2 had a 132-bp deletion (Supplemental

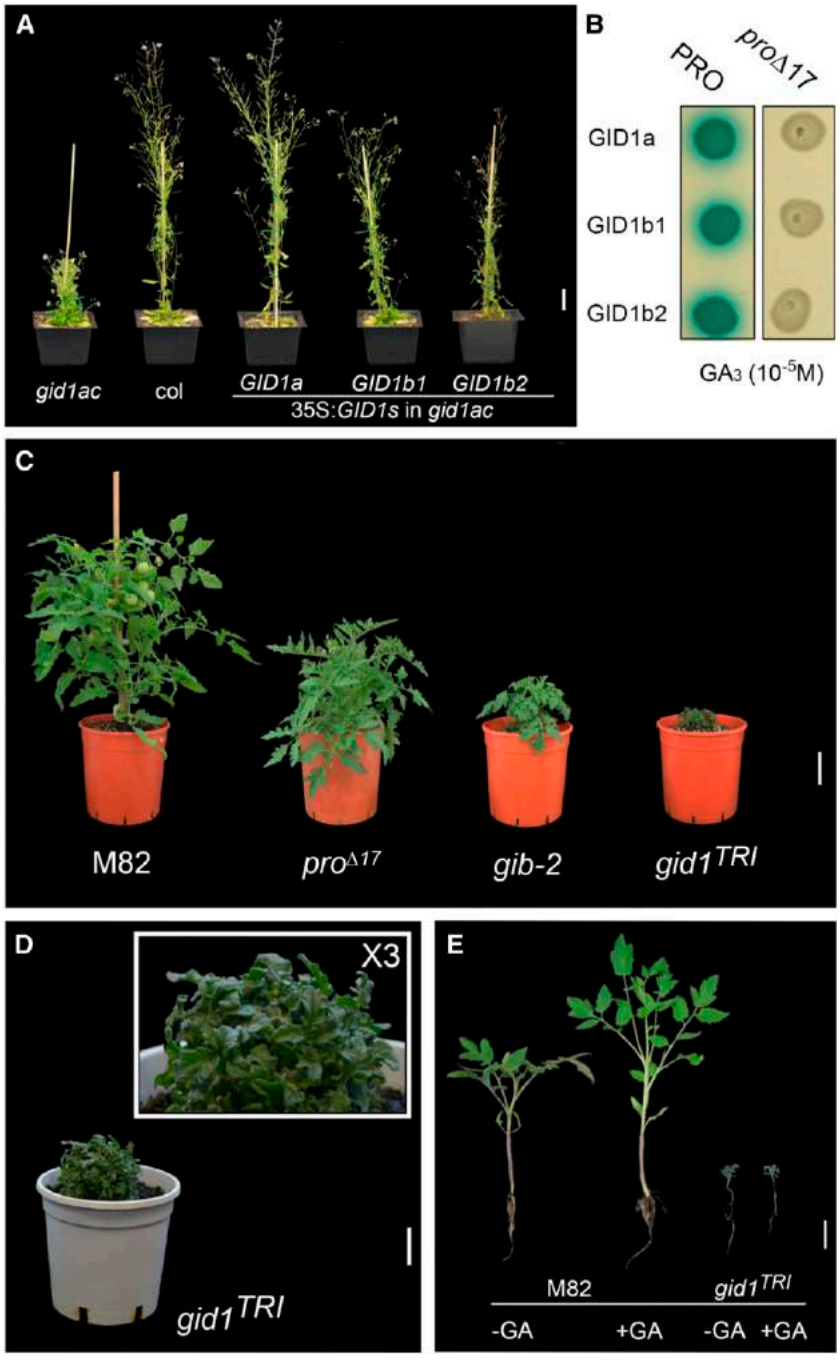

Figure 1. The Tomato GID1.

(A) Complementation of the Arabidopsis *gid1a gid1c* (*gid1ac*) mutant with the tomato*GID1* genes. The three *GID1* genes were heterologously expressed under the regulation of the 35S promoter in the Arabidopsis *gid1a gid1c* mutant (in Col-0 background). Bar = 3 cm.
(B) The interaction between PRO and the three GID1s in the presence of 10^{-5}M GA_3 in yeast two-hybrid assays. proΔ17 was used as a negative control.
(C) Two-month-old representative M82, stable DELLA (*proΔ17*)-overexpressing M82, GA-deficient mutant *gib-2* and *gid1* triple mutant (*gid1*^{TRI}). Bar = 5 cm.
(D) Sixteen-month-old *gid1*^{TRI} plant. Bar = 2 cm.
(E) The response of M82 and *gid1*^{TRI} to repeated application of GA_3 (100μM). Bar = 3 cm.

Figure 2A). We then generated double and triple (*gid1*^{TRI}) mutants by crosses. Homozygous *gid1*^{TRI} seeds did not germinate even after scarification. For germination, the seed coat had to be removed, and the embryos rescued and placed on Murashige and Skoog (MS) medium. *gid1*^{TRI} plants exhibited very slow growth, extreme dwarfism, and very small and dark-green leaves (Figures 1C and 1D). Flowers of *gid1*^{TRI} were very small, did not open, their style did not elongate, and their carpels slowly degenerated (Figure 2E). As a result, *gid1*^{TRI} plants were sterile. Some old *gid*^{TRI} plants (more than 12 months old) produced extremely small parthenocarpic fruits. Although the lifespan of M82 plants is approximately five months, the first generated *gid1*^{TRI} plants were more than 16 months old (Figure 1D).

In comparison with plants overexpressing the DELLA gain-of-function mutant gene *proΔ17* (Nir et al., 2017) and the GA-deficient mutant *gib-2* (Koornneef et al., 1990), *gid1*^{TRI} dwarfism was much stronger (Figure 1C). To test if *gid1*^{TRI} is fully insensitive to GA, we treated M82 and *gid1*^{TRI} repeatedly with 100 μM GA_3. The GA treatment had no effect on *gid1*^{TRI} development (Figure 1E). As expected, the steady state level of *GA20ox1*, whose expression is suppressed by GA as a result of the negative feedback regulation (Livne et al., 2015), was six times higher in *gid1*^{TRI} than in M82 (Supplemental Figure 3). Although GA application to M82 strongly suppressed *GA20ox1* expression, it had no effect in *gid1*^{TRI}, further demonstrating the insensitivity of *gid1*^{TRI} to GA and suggesting that *gid1*^{TRI} is null in all three receptor genes.

High Redundancy in the Regulation of Plant Growth by GID1s

To study specific and overlapping activities of the different GID1s, we examined GA-regulated developmental responses in the single and the double mutants. All of the single mutants seeds and the double mutant *gid1a gid1b1* and *gid1b1 gid1b2* seeds germinated normally, similar to wild type M82 seeds (Supplemental Figure 4A). However, *gid1a gid1b2* seeds showed reduced and delayed germination (Figure 2A). We then examined epicotyl elongation and leaf development in the different mutants. Whereas *gid1b1* and *gid1b2* plants did not show obvious phenotypic changes, *gid1a* stems were slightly shorter (Figures 2B and 2C). Epicotyl length of *gid1b1 gid1b2* plants was similar to M82, but their leaves were smaller with normal leaflets (Figures 2B to 2D). *gid1a gid1b1* plants were semi-dwarf and had long leaves but dark-green leaflets (Figures 2B to 2D; Supplemental Figure 4B). *gid1a gid1b2* had the strongest phenotype; they were semi-dwarf with smaller and darker, green leaves (Figures 2B to 2D; Supplemental Figure 4B). Taken together, *GID1a* had the strongest effect on germination, stem elongation, and leaflet growth and color.

We previously showed that increased GA activity in *pro* delayed flowering (Livne et al., 2015). Flowering time (measured as leaves to first inflorescence) was slightly delayed in *gid1a gid1b2* only (Supplemental Figure 4C). These suggest that both increased and decreased GA activity delay flowering time in tomato. Flower size was affected only in *gid1b1,* with an additive effect to *gid1b2* in the double mutant *gid1b1 gid1b2* (Figure 2E; Supplemental Figure 4D). No developmental defect in the flowers of the strongest double mutant *gid1a gid1b2* were identified, suggesting that GID1b1 is the most prominent GID1 acting in GA-regulated flower organ growth. We also examined root development of all single and double mutants grown hydroponically. Primary-root length was reduced in *gid1a,* with an additive effect to *gid1b2* in the double mutant *gid1b1 gid1b2* (Supplemental Figures 4E and 4F).

Finally, the effect of the single, double, and triple mutants on the steady state expression of *GA20ox1* and *GA3ox1* was examined. The expression of *GA20ox1* and *GA3ox1* was upregulated in *gid1*^{TRI} only (Figures 2F and 2G), suggesting a high redundancy in the regulation of the feedback response by the three receptors.

GA-Dependent and Independent Interactions between GID1s and PRO

To examine whether the relative contributions of the different GID1s is determined by their expression levels, we analyzed their expression in various tissues in M82 plants. In imbibed seeds, roots, and flowers, *GID1b1* exhibited the highest expression, whereas in elongating stems and young leaves, *GID1a* and *GID1b1* showed similar expression levels (Figure 3A). *GID1b2* exhibited the lowest expression in all tested tissues. The relatively low expression of *GID1a* in stems, seeds, and roots cannot explain its prominent role in stem and root elongation and germination. We therefore examined whether the different GID1s display different affinities to PRO. To this end, we tested the interaction between the three GID1s and PRO in yeast in the presence of different GA_3 concentrations. GID1a interacted with PRO in the absence of GA; addition of the hormone had no effect on the intensity of the interaction (Figure 3B). GID1b2 and GID1b1 interacted with PRO only when the GA_3 concentration exceeded 10^{-8} M and 10^{-7} M, respectively. These results suggest that GID1a has the highest and GID1b1 the lowest affinity for PRO.

To determine whether the GA-independent interaction between GID1a and PRO occurs in planta and induces spontaneous GA responses, we followed stem elongation in double mutants treated with the GA biosynthesis inhibitor paclobutrazol (Pac). We reasoned that in each double mutant combination a single GID1 is active: for example, in *gid1b1 gid1b2* only GID1a is active. Double mutant seedlings were treated repeatedly, three times a week, for 5 weeks, with 10 mg/l Pac before stem elongation was monitored for 2 weeks. All three double mutants showed strong suppression of stem elongation, and the inhibition of *gid1b1 gid1b2* (active GID1a) growth was similar to that of *gid1a gid1b2* (active GID1b1; Figure 3C). Because it was shown previously that GA-induced stem elongation in tomato is PRO dependent (Livne et al., 2015), these results suggest that GID1a, similar to GID1b1 and GID1b2, depends on GA to promote PRO degradation and stem elongation.

We also tested if *GID1a* promotes growth independently of GA when expressed in Arabidopsis. Arabidopsis Col and *gid1a gid1c* mutants expressing the tomato *GID1a*, *GID1b1*, or *GID1b2* (Figure 1A) were treated repeatedly with 1mg/l Pac for 3 weeks, and then rosette diameter was measured. Pac had a similar inhibiting effect on the growth of all lines (Figure 3D). These results suggest that either the GA-independent interaction between GID1a and PRO does not occur in planta or that GID1a and PRO interact in planta but the GID1a-PRO complex cannot bind the F-box SLY1 without GA. Similar to Arabidopsis and rice, tomato

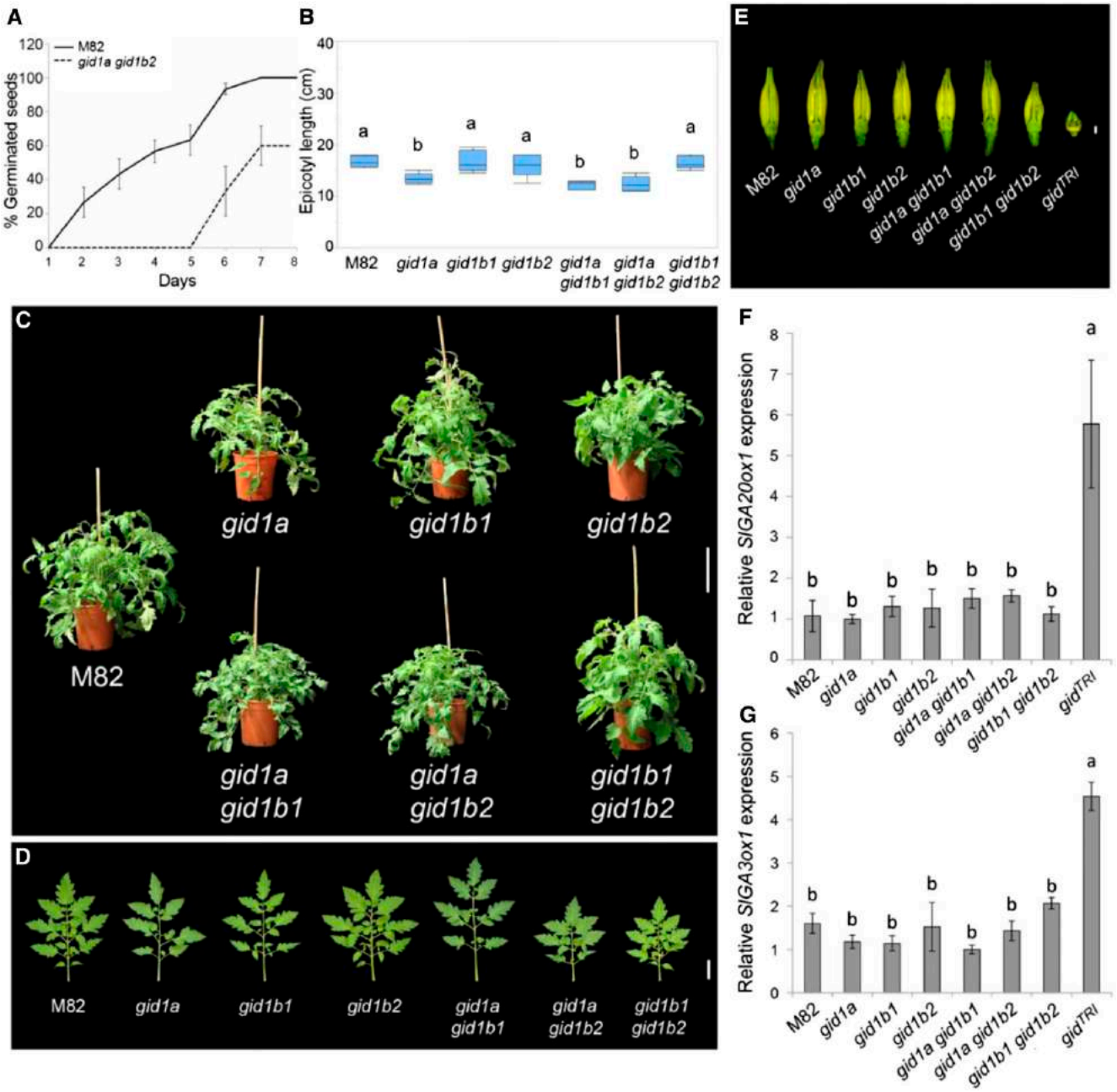

Figure 2. Phenotypic Characterization of the *gid1* Single and Double Mutants.

(A) Germination of M82 and *gid1a gid1b2* seeds. Values (percentage of germinated seeds) are means of three replicates (plates) each containing 30 seeds ±SE.

(B) Epicotyl length of control M82 and single and double *gid1* mutants. Data are graphically presented as whisker and box plots. Statistical significance was tested with Student's *t* test, ($n = 12$, $P < 0.05$). Each set of letters above the columns represents significant differences.

(C) Representative seven-week-old single and double *gid1* mutant plants. Bar = 3 cm.

(D) Representative single and double *gid1* leaves (leaf number 4 from the apex down). Bar = 5 cm.

(E) Representative single and double *gid1* flowers. Bar = 2 mm.

(F) and **(G)** Steady state level of *GA20ox1* **(F)** and *GA3ox1* **(G)** expression in single, double, and triple (*gid1*^{TRI}) *gid1* mutants. Values (gene to *ACTIN*) are means of four biological replicates ±SE. Each set of letters above the columns represents significant differences (Student's *t* test, $P < 0.05$).

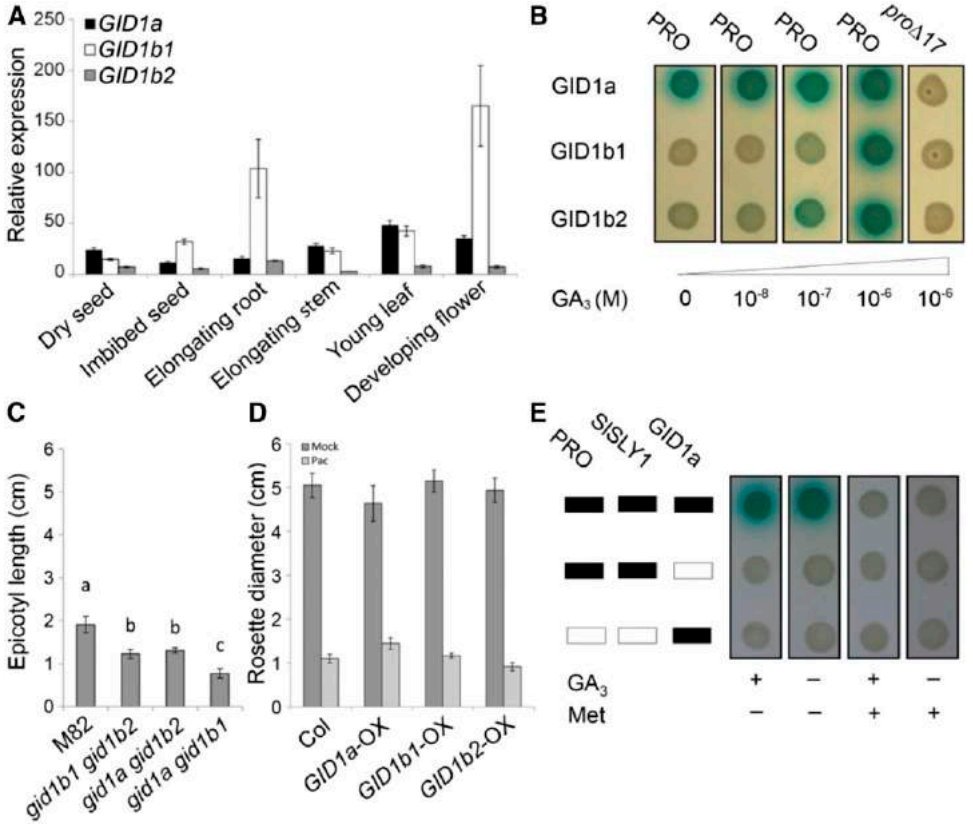

Figure 3. Molecular Analyses of GID1s.

(A) RT-qPCR analysis of *GID1a*, *GID1b1*, and *GID1b2* expression in various tissues. Values (normalized to *ACTIN*) are means of four biological replicates ±SE.

(B) The interaction between PRO and the three GID1s and the effect of GA_3 concentration (10^{-8}–10^{-6} M) on this interaction in yeast two-hybrid assay. proΔ17 was used as a negative control.

(C) Seedlings of the three double mutant lines (each with one active receptor as indicated) were treated repeatedly, for 5 weeks, with 10 mg/l Pac, and then stem elongation was measured for 2 weeks. Values (cm) are means of 18 biological replicates ±SE. Each set of letters above the columns represents significant differences (Student's *t* test, $P < 0.05$).

(D) Transgenic Arabidopsis *gid1a gid1c* mutant expressing the tomato *GID1a*, *GID1b1*, or *GID1b2* and Col seedlings were treated repeatedly with 1 mg/l Pac for 3 weeks, and then rosette diameter was measured. Values (cm) are means of 10 biological replicates ±SE. Each set of letters above the columns represents significant differences (Tukey–Kramer HSD, $P < 0.05$).

(E) GID1a-PRO interaction with SlSLY1 and the effect of GA_3 in yeast three-hybrid assay. SlSLY1 fused to GAL4 BD and PRO were expressed in yeast together with GID1a fused to GAL4 AD. The addition of Met to the growth medium (+Met) suppressed PRO expression. Cells were grown with or without 10^{-5} M GA_3. β-Galactosidase activity (the interaction of all three proteins) was visualized by X-Gal staining.

has a single SLY1 (encoded by *SlSLY1*). We tested the interaction between GID1a, PRO, and SlSLY1 in yeast, in the presence or absence of GA_3 (10^{-5} M). A yeast three-hybrid assay showed that SlSLY1 interacts with GID1a-PRO independently of GA (Figure 3E), suggesting that GA-independent interaction between GID1a, PRO, and SLY occurs in yeast but probably not in planta.

The Unique Role of GID1a in the Regulation of Plant Growth in Response to High GA Levels

Because each double mutant has a single active receptor, we used them to study the contributions of each GID1 to GA-induced stem elongation. Seedlings of the double mutants were treated for 10 d with Pac and then with increasing concentrations of GA_3. All three double mutants responded similarly to GA_3 doses up to 1 μM (Figure 4A). However, although in *gid1b1 gid1b2* (active GID1a) and M82 the elongation response increased further with higher GA_3 concentrations up to 100 μM, *gid1a gid1b2* (active GID1b1) and *gid1a gid1b1* (active GID1b2) exhibited a very mild elongation response to GA_3 concentrations above 1 μM (Figures 4A and 4B). In addition to stem elongation, other phenotypes were affected by the high GA_3 doses in *gid1b1 gid1b2* but not in the other two double mutants, including leaf color and form (simpler leaves with smoother margins in M82 and *gid1b1 gid1b2*; Figure 4B). These results imply that GID1a is the GA receptor that most contributes to the response of tomato stem to high GA doses. It should be noted, however, that the high GA_3 doses used in this experiment are not physiologically relevant. To further explore this

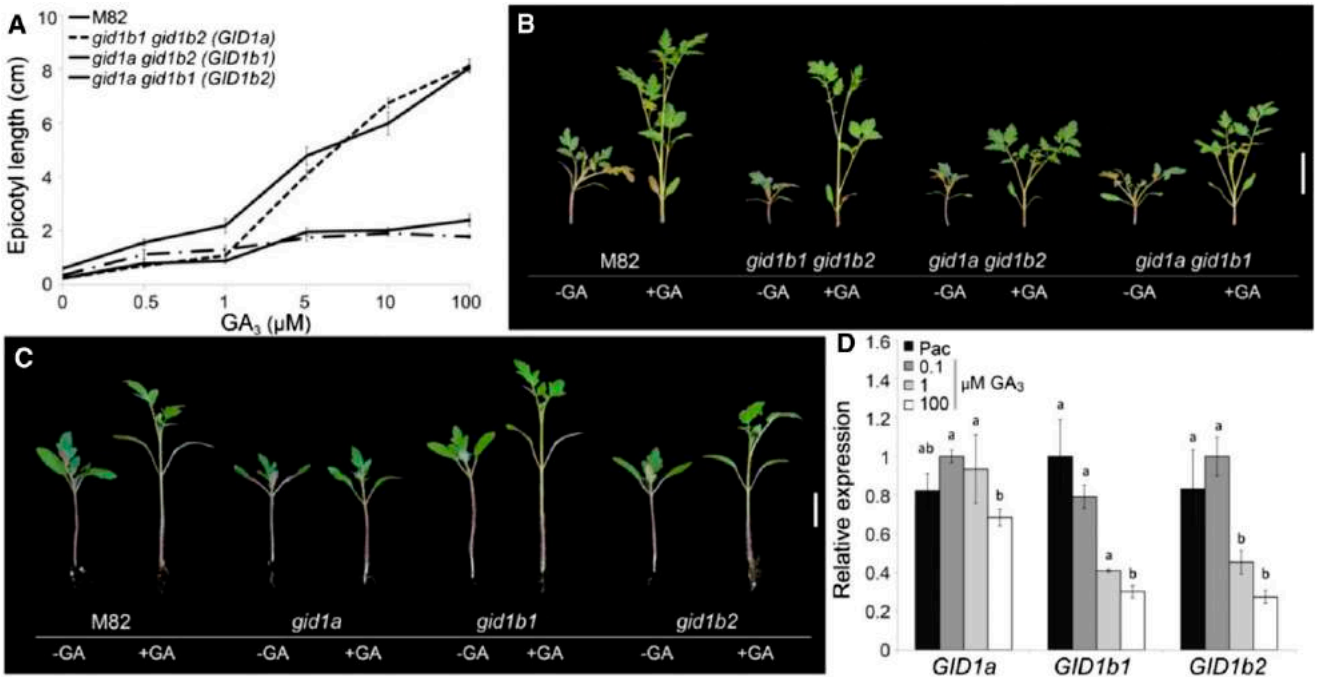

Figure 4. GID1a Is Responsible for Stem Elongation in Response to High GA Doses.

(A) Epicotyl elongation in response to GA_3 treatment. Seedlings of the double mutants with two leaves were treated for 10 d with Pac, followed by single treatment with different concentrations of GA_3 (0.5 μM–100 μM). Then 7 d later, stem elongation was measured. The active GID1 is indicated in parenthesis for each double mutant. Values are means of eight replicates ±SE.

(B) Representative plants of M82 and the three double *gid1* mutants treated for 10 d with Pac, followed by single treatment with 100 μM GA_3. Then 7 d later, pictures of the treated plants were taken. Bar = 4 cm.

(C) The effect of GA_3 treatment (100 μM) on stem elongation in the different single mutants. Representative seedlings of M82 and the three single *gid1* mutants are presented. Bar = 2 cm.

(D) RT-qPCR analysis of *GID1a*, *GID1b1*, and *GID1b2* expression in response to GA treatment. M82 seedlings were treated for 4 d with Pac and then with different concentrations of GA_3 (0.1 to 100 μM); and 3 h later, leaves were taken for the analysis of *GID1a*, *GID1b1*, and *GID1b2* expression. Values (normalized to *ACTIN*) are means of four biological replicates ±SE. The highest value for each gene was set to 1.

phenomenon, we examined shoot elongation responses in the single mutant plants to a single treatment with high GA_3 concentrations (100 μM). Although *gid1b1* and *gid1b2* exhibited a strong elongation response, similar to M82, GA_3-induced elongation was strongly suppressed in *gid1a* (Figure 4C; Supplemental Figure 5A); similar results were obtained with GA_4 (Supplemental Figure 5B). Following repeat treatments (every 3 d with 100μM GA_3), GA-induced elongation was strongly suppressed in *gid1a*, but not in *gid1b1* or *gid1b2* (Supplemental Figure 5C). Similar responses were observed with another *gid1a* allele (Supplemental Figure 5D).

In Arabidopsis, *GID1* expression is repressed by GA (Middleton et al., 2012), but the severity of the repression differs among the three *GID1* genes (Griffiths et al., 2006). We tested if the three tomato *GID1*s differ in their feedback response to GA treatment. M82 seedlings were treated for 4 d with Pac and then with different concentrations of GA_3 (0.1 to 100 μM), and 3 h later, leaves were taken for the analysis of *GID1a*, *GID1b1*, and *GID1b2* expression. *GID1b1* and *GID1b2* expression was reduced by 1 μM GA_3, and the inhibition effect increased with higher GA concentrations (Figure 4D). By contrast, *GID1a* expression was repressed only by 100 μM GA_3, and the level of inhibition was significantly lower: although *GID1b1* and *GID1b2* were inhibited by approximately 75%, *GID1a* was inhibited only by approximately 30% following the application of 100 μM GA_3. We also examined the expression of the active *GID1* genes in each of the respective double mutants, following treatments with 100 μM GA_3, and found similar results (Supplemental Figure 5E). A similar feedback response was also found in elongating stems (Supplemental Figure 5F). These results suggest that the strong inhibition of *GID1b1* and *GID1b2* by high GA doses may reduce their contribution to GA-induced stem elongation.

*gid1*s Exhibit Phenotypic Instability under Semi-Controlled, Nonoptimal Growth Conditions

GA activity promotes transpiration due to increased stomatal conductance (Nir et al., 2017). To examine the contribution of each receptor to whole-plant transpiration, M82 and the double *gid1* mutants were grown in a partially controlled greenhouse (natural light and day-length and temperature ranging from 22°C to 32°C) in pots on an array of load cells (lysimeters), which simultaneously followed plant weight change and provided information on biomass gain and transpiration (Nir et al., 2017). Whole-plant daily

transpiration and weight gain in M82 were significantly higher than in all double mutants (Figure 5A; Supplemental Figure 6). However, the mutant lines exhibited relatively high phenotypic variability, with few *gid1a gid1b2* plants showing much stronger dwarfism than when grown under optimal growth conditions (Figure 5B). We evaluated the degree of variability in plant weight, using the parameter of coefficient of variation (CV- SD/mean; Fisher et al., 2017). The variability in M82 plant weight was much lower than that of all double mutants (Figure 5C). Among the three receptors, GID1a had the strongest effect on phenotypic stability, and among the three double mutants, *gid1b1 gid1b2* (with an active GID1a) exhibited the lowest variability. These results show that the stable phenotype of the *gid1* mutants observed under optimal growth conditions is partially lost under less optimal growth conditions.

The Loss of Redundancy under Changing Environments

We next tested the development of all single and double mutant *gid1* lines under an ambient changing environment. When grown in the soil in a greenhouse (Supplemental Figure 7A), under ambient light and temperature conditions (temperature ranging from 20°C to 40°C), the different lines exhibited increased dwarfism compared with their counterparts grown under controlled optimal growth condition. More specifically, *gid1a* and *gid1a gid1b2* exhibited approximately 50% and 65% reduction in stem length (compared with wild type M82) under ambient growth conditions and only 15% and 20% stem length reduction under optimal growth conditions, respectively (Figures 6A and 6B versus Figure 2B; Supplemental Figure 8A). Moreover, all mutant lines exhibited a high degree of phenotypic variability (Figures 6B and 6C; Supplemental Figure 7B), which was significantly higher than that of M82. Phenotypic variability (based on epicotyl length) of *gid1a* was 3-fold and that of *gid1a gid1b2* was 4-fold higher than that of M82 (Figure 6C). PCR and sequencing were performed to confirm the genotype of individual plants showing strong and mild dwarfism (Supplemental Figure 9). Because *gid1a gid1b2* exhibited the strongest dwarfism and the highest phenotypic instability (Figures 6B and 6C), it was grown next to M82 under optimal controlled growth conditions to compare their phenotypic stability. As expected *gid1a gid1b2* was only semi-dwarf under these conditions (Supplemental Figure 8A) and exhibited low and similar phenotypic variability as compared with M82 plants (Figure 6D). We collected seeds from field-grown semi-dwarf and dwarf *gid1a gid1b2* fruits (after selfing) and grew the plants under controlled optimal conditions. All plants were semi-dwarf and exhibited low phenotypic variability, similar to M82 plants (Supplemental Figures 8B and 8C).

Under optimal growth conditions, the expression of the GA-biosynthesis genes *GA20ox1* and *GA3ox1* was affected only in the triple *gid1*^{TRI} and not in the single and double mutants (Figures 2F and 2G). To test if the high redundancy in the regulation of the feedback response is lost under a changing environment, *GA20ox1* and *GA3ox1* expression was then analyzed in field-grown M82 and semi-dwarf and dwarf *gid1a gid1b2* plants. *GA20ox1* and *GA3ox1* expression was increased in *gid1a gid1b2*, and the levels of their expression were in line with the severity of dwarfism (Figures 7A and 7B). Previously, we showed that PRO promotes the expression of the abscisic acid-regulated gene RESPONSIVE TO ABSCISIC ACID18 (SlRAB18; Nir et al., 2017). We tested the effect of *gid1a gid1b2* on the expression of *SlRAB18* under the different growth conditions. Although the expression of *SlRAB18* was similar in *gid1a gid1b2* and M82 under controlled optimal growth conditions (Figure 7C), under ambient conditions it was much higher in the dwarf *gid1a gid1b2* (Figure 7D), suggesting

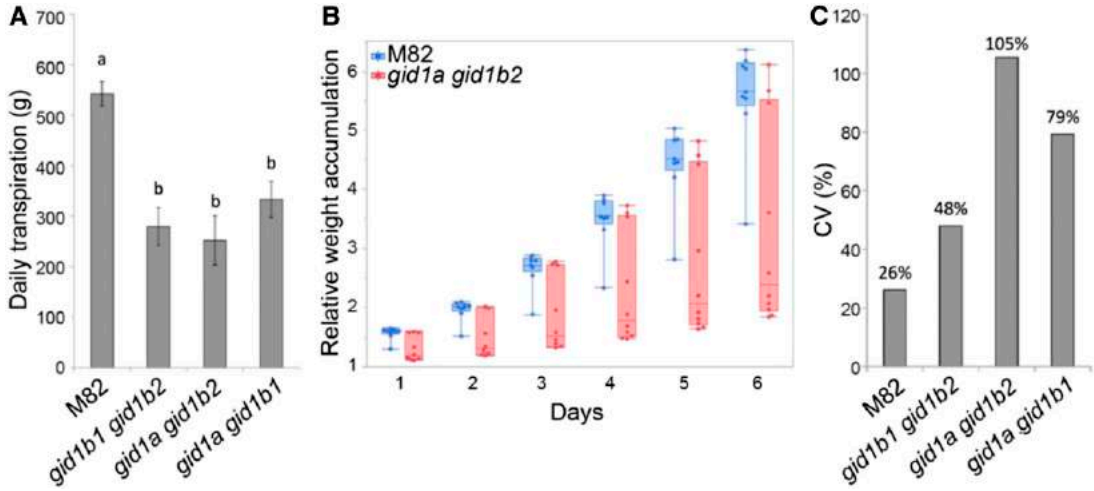

Figure 5. *gid1* Mutants Exhibit Phenotypic Instability Under Semi-Controlled, Nonoptimal Growth Conditions.

(A) Plants were placed on lysimeters in semi-controlled greenhouse. Pot (pot + soil + plant) weight was measured every 3 min. Values (daily transpiration in g) are means of thirteen plants ±SE. Each set of letters above the columns represents significant differences (Student's *t* test, $P < 0.05$).

(B) Relative weight gain in M82 and *gid1a gid1b2* mutants grown as in A during 6 d. Total weight, including plant, pot, and soil, was taken each pre-dawn, immediately after irrigation to saturation and drainage. The weight difference (delta) from previous pre-dawn measurement is the accumulated plant weight during a single day. Data are graphically presented as whisker and box plots.

(C) Coefficient of variation (CV - SD/mean) of plant weight taken at d 6 (see **[B]**).

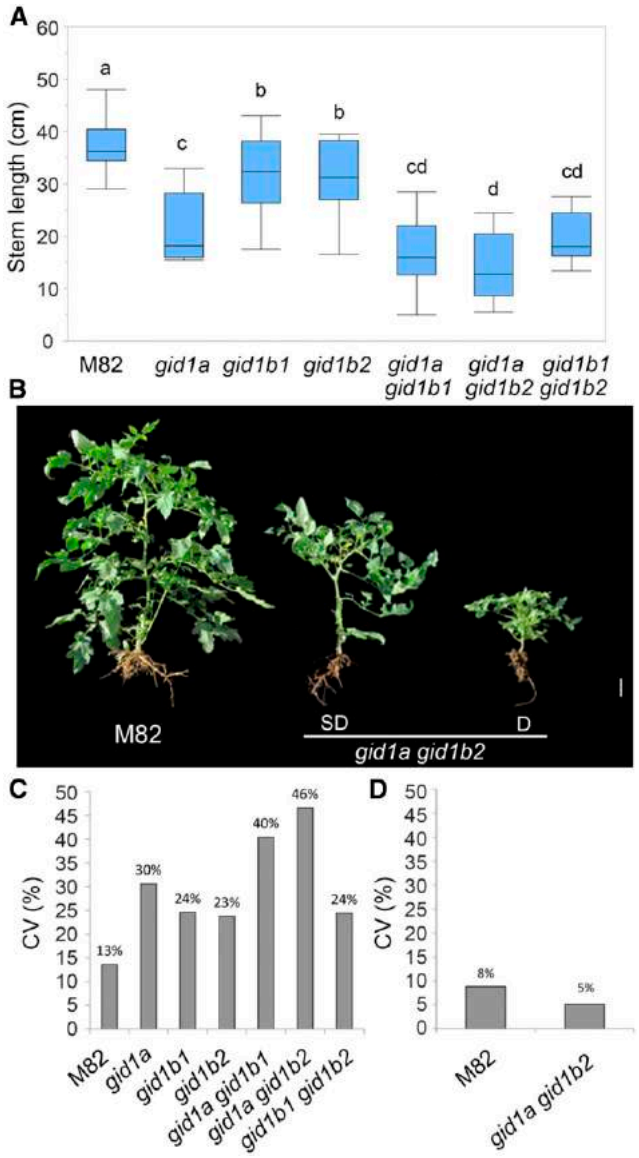

Figure 6. The High Redundancy Between GID1s Is Lost Under Changing Environments.

All *gid1* single and double mutants were grown in the soil in a greenhouse under ambient light and temperature conditions.

(A) Epicotyl length. Data are graphically presented as whisker and box plots. Statistical significance was tested with Student's *t* test, ($n = 12$, $P < 0.05$). Each set of letters above the columns represents significant differences.

(B) Representative M82 and *gid1a gid1b2* semi-dwarf (SD) and dwarf **(D)** plants grown under ambient conditions. Bar = 3 cm.

(C) Coefficient of variation (% CV) of epicotyl length of plants grown under ambient conditions. Values are means of four replicates (each the mean length of three plants) grown in randomized block design ± SE. Percentages are presented above columns.

(D) CV of epicotyl length of M82 and *gid1a gid1b2* plants grown under optimal growth conditions. Values are means of nine plants ± SE. Percentages are presented above columns.

that under these conditions GA activity is reduced and PRO accumulates. Taken together, these results suggest that the dwarfism of *gid1a gid1b2* in the field was caused by loss of redundancy in GID1 activity and the suppression of GA responses.

We then tested if the loss of redundancy and dwarfism of *gid1a gid1b2* and *gid1a gid1b1* was caused by reduced expression of the remaining active receptor. The expressions of *GID1b1* in dwarf *gid1a gid1b2* and of *GID1b2* in dwarf *gid1a gid1b1* were similar to their expression in M82 under controlled optimal growth conditions and in the field (Figures 7C and 7D; Supplemental Figures 8D to 8F). Moreover, we did not find correlations between dwarfism to changes in the expression levels of the remaining active receptor in the double mutants in the field (Supplemental Figures 8E and 8F); whereas *GID1b1* expression was lower, that of *GID1b2* was higher. Thus, reduced expression of the remaining active receptor is probably not the cause for the loss of redundancy under ambient growth conditions.

DISCUSSION

Most eudicot plants express multiple GA GID1 receptors (Yoshida et al., 2018), whereas some monocots express only one, raising questions regarding the possibility of GA responses driven by specific receptors and of evolutionary benefits of multiple receptors. Because tomato has three GID1s but only one DELLA, we used this system to study specific and overlapping roles of the different receptors, and the importance of multiple GA receptors to the overall GA activity and plant development under different growth conditions.

The three tomato GID1s were expressed in all examined tissues and exhibited overlapping activity. However, their expression levels did not correlate with their relative contribution to GA-regulated processes. Only in developing flowers was *GID1b1* expression highest and its effect on flower organ growth the strongest. In all other tested developmental and physiological responses, GID1a played the dominant regulatory role. *GID1a*, however, did not show the highest expression, and in most tissues, it exhibited similar or lower levels of expression than the less active receptor, *GID1b1*. Yeast two-hybrid assays suggested that GID1a has the highest affinity to the DELLA protein PRO. It also showed that the affinity of GID1b2 to PRO was higher than that of GID1b1. Thus, the affinity of the three tomato GA receptors to DELLA may determine their relative contributions to most GA-regulated developmental processes.

GA application or complete loss of DELLA activity has a dramatic effect on stem elongation in tomato, suggesting that, in tomato, GA activity is not saturated under normal growth conditions (Livne et al., 2015). Our results suggest that only GID1a drives the strong response to high GA doses in tomato. The growth-promoting effects of activated GID1b1 and GID1b2 were rather weak, and the corresponding double mutants (*gid1a gid1b1* and *gid1a gid1b2*) exhibited a very mild elongation response to exogenous GA treatments. Thus, in this specific response, the three receptors did not show overlapping activity. The unique role of GID1a may be the result of differences in the expression levels of the different receptors following GA application. *GID1b1* and *GID1b2* expression was strongly suppressed by GA, due to feedback inhibition (Middleton et al., 2012), whereas the expression of *GID1a*

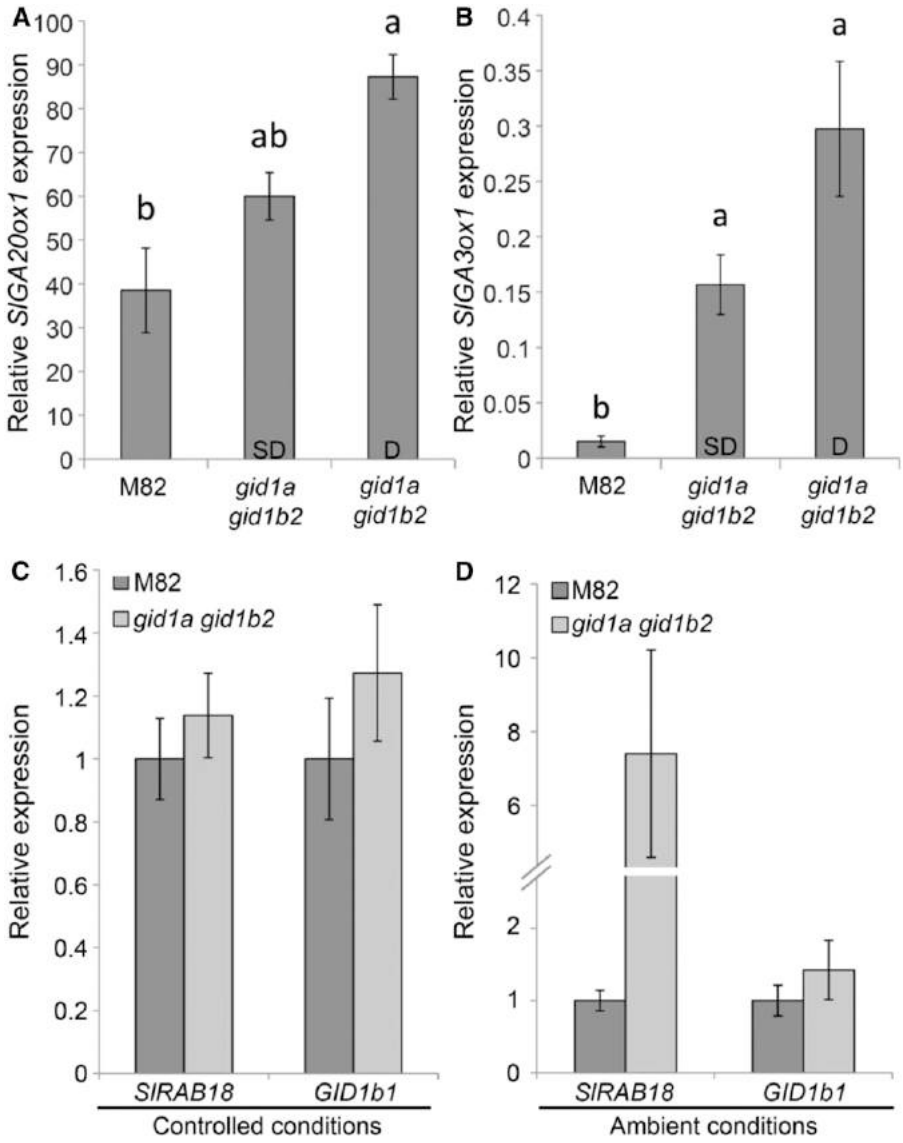

Figure 7. Loss of Redundancy in GA Sensing Under Ambient Growth Conditions.

(A) and **(B)** RT-qPCR analysis of *SlGA20ox1* **(A)** and *SlGA3ox1* **(B)** expression in M82 and semi-dwarf (SD) and dwarf **(D)** *gid1a gid1b2* grown under ambient growth conditions. Values (normalized to *ACTIN*) are means of four biological replicates ± SE. Letters above the columns represent significant differences between respective treatments (Student's *t* test, $P < 0.05$).

(C) and **(D)** RT-qPCR analysis of *SlRAB18* and *GID1b1* expression in M82 and *gid1a gid1b2* plants (in fully expanded young leaves) grown under optimal growth conditions **(C)** or ambient conditions in the field **(D)**. Values (normalized to *ACTIN*) are means of four biological replicates ± SE. Letters above the columns represent significant differences between respective treatments (Student's *t* test, $P < 0.05$). Expression values for all analyzed genes in M82 were set to 1.

was much less affected. This, together with the higher affinity of GID1a to PRO, may underlie the strong response to the hormone and subsequent effect on stem elongation, as well as on other GA-related developmental responses, such as leaf color and form. Whether this unique activity of GID1a has a true biological role is not yet clear. GA levels increase in response to specific environmental conditions to induce rapid and strong stem elongation (e.g., shade-avoidance response; Yang and Li, 2017). GID1a may be the only receptor mediating these types of responses.

The type A GID1 (GID1a) exhibited GA-independent interaction with PRO in yeast. Similar results were shown recently by Shinozaki et al. (2018). In Arabidopsis, type B GID1 (GID1b) exhibits GA-independent interaction with DELLA (Griffiths et al., 2006), suggesting that this GID1 unique characteristic evolved independently in different species (Yoshida et al., 2018). Yamamoto et al. (2010) speculated that GA-independent interactions between specific GID1s and DELLAs are enabled by a unique conformation of GID1 in which the N-Ex domain is

partially closed even in the absence of GA. Our yeast three-hybrid assay showed that the GID1a-PRO complex interacts with the F-box protein SlSLY without GA, suggesting that GID1a can induce PRO degradation and GA responses in the absence of GA. However, continuous Pac treatment (GA-limited conditions) had a similar dwarfing effect on all *gid1* double mutants (each containing a different active GID1), suggesting that similar to GID1b1 and GID1b2, GID1a depends on GA for its activity. It is possible that the high affinity between GID1a and PRO, together with the high expression levels, allowed for spontaneous interaction in yeast even though they do not interact in planta without the hormone.

The three tomato GA receptors, GID1a, GID1b1, and GID1b2, exhibited extensive overlapping activity in the regulation of germination, growth, transition to flowering, flower development, and gene expression. Redundancy, caused by gene duplication, is very common in flowering plants (Veitia, 2005), rendering them resistant to mutation (Abley et al., 2016). Under optimal, controlled growth conditions, mutation in a single *GID1* had almost no effect on most tested GA-regulated developmental processes. However, when grown in the field and exposed to ambient changing environment, the *gid1* mutants showed phenotypic instability and loss of redundancy. Among the three receptors, the loss of GID1a had the strongest effect. This loss of redundancy in *gid1a gid1b2* plants was evident by the strong dwarfism, the activation of the feedback response (upregulation of *GA20ox1* and *GA3ox1* expression), and the upregulation of the DELLA-induced gene *SlRAB18*. All these phenotypes were not found in *gid1a gid1b2* under controlled, optimal growth conditions. Changes in the expression of the remaining active GID1s in the *gid1* single and double mutants, under ambient conditions, are probably not the cause for the loss of redundancy and instability, because they did not correlate with plant phenotype.

The mechanism buffering phenotypes against environmental influences is not fully clear; several factors have been proposed, including redundancy (Abley et al., 2016). Our results support this hypothesis; under optimal growth conditions, the activity of a single receptor was sufficient to maintain rather normal growth, but under a changing environment all three receptors were needed for stable and normal development. GA biosynthesis is affected by environmental cues, such as light, temperature, water availability, and salinity (Yamaguchi, 2008; Colebrook et al., 2014; Wang et al., 2019). Thus, under ambient conditions, extreme changes in the environment may lead to strong fluctuations in GA levels. It is possible that the overlapping activity of the three tomato GID1 receptors is needed to buffer these changes in GA level and that this buffering effect allows stable and normal growth under a changing environment.

METHODS

Plant Materials, Growth Conditions, and Hormone Treatments

Tomato *Solanum lycopersicum* cv M82 (sp^-/sp^-) plants were used throughout this study. The CRISPR-Cas9 *gid1* mutants were generated in the M82 background. The *gib-2* mutant originally in 'Money maker' was backcrossed three times to M82. All plants were grown in a growth room set to a photoperiod of 12/12-h night/days, light intensity (cool-white bulbs) of ~250 μmol m^{-2} s^{-1}, and 25°C. In other experiments, plants were grown in a greenhouse under natural day-length conditions, light intensity of 700 to 1000 μmol m^{-2} s^{-1} and 18-29°C. For growth under ambient conditions, plants were grown in the soil in a greenhouse under natural day-length conditions, with light intensity of ~500-1200 μmol m^{-2} s^{-1} and 20-40°C. For root analysis, seedlings were grown hydroponically in Hoagland nutrient solution (pH 6.5), in a growth room, under the above-described conditions. Arabidopsis (*Arabidopsis thaliana*) *gid1a gid1c* mutant in the Columbia (Col-0) background and wild type Col-0 plants were used in this study. The plants were grown in a growth room, under a controlled temperature (22°C) and long (16-h light/8-h dark) day conditions.

GA_3, GA_4 (Sigma-Aldrich), and Pac were applied to plants by spraying.

Seed Germination Assays

After surface sterilization of the tomato seeds, ~100 seeds were sown on 0.5× MS plates. Seeds were germinated in the dark at 23°C, for 3 d and then transferred to the light~100 μmol m^{-2} s^{-1} at the same temperature. To assess seed germination, radicle emergence was scored each day after sowing.

Molecular Cloning and Arabidopsis Transformation

Solyc01g098390 (*GID1a*), Solyc09g074270 (*GID1b1*), and Solyc06g008870 (*GID1b2*) coding sequences in pENTR were inserted into the Gateway-compatible pGWB6 vector (Bensmihen et al., 2004). The pGWB6 vector drives expression of the recombined gene under control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. The pGWB6 constructs were transferred into *Agrobacterium tumefaciens* GV3101 by electroporation and used to transform *gid1ac* Arabidopsis (Columbia) mutants by the floral dip method. T1 transgenic seeds were selected based on their resistance to hygromycin.

CRISPR/Cas9 Mutagenesis, Tomato Transformation, and Selection of Mutant Alleles

Two single-guide RNAs (sgRNAs; Supplemental Table 1) were designed for each gene, using the CRISPR-P tool (http://cbi.hzau.edu.cn/crispr). Vectors were assembled using the Golden Gate cloning system, as described by Weber et al. (2011). Final binary vectors, pAGM4723, were introduced into *A. tumefaciens* strain GV3101 by electroporation. The constructs were transferred into M82 cotyledons using transformation and regeneration methods described by McCormick (1991). Kanamycin-resistant T0 plants were grown, and independent transgenic lines were selected and self-pollinated to generate homozygous transgenic lines. The genomic DNA of each plant was extracted and genotyped by PCR for the presence of the Cas9 construct. The CRISPR/Cas9-positive lines were further genotyped for mutations using a forward primer to the upstream sequence of the sgRNA1 target and a reverse primer to the downstream of the sgRNA2 target sequence. The target genes in all mutant lines were sequenced. Several homozygous and heterozygous lines were identified, and at least two independent mutant lines for each *SlGID1* gene were selected for further analysis. The Cas9 construct was segregated out by crosses with M82.

RNA Extraction and cDNA Synthesis

Total RNA was isolated from various tissues: seeds, roots, young leaves, elongating stems, flower bud, and all flower organs. Frozen tissues were ground and resuspended in guanidine HCl, and then phenol/chloroform was added. Samples were mixed by vortexing for 30 s, and after 30 min at 4°C the samples were centrifuged at 4°C for 45 min. Ethanol (100% v/v) and 1M acetic acid were then added, and the samples were mixed and stored

overnight at −80°C. NaAc (3M) was added, and samples were washed with cold 70% (v/v) ethanol. cDNA was then synthesized using SuperScript II reverse transcriptase (18064014; Invitrogen) and 3 μg of total RNA, according to the manufacturer's instructions. The RNeasy Micro Kit (QIAGEN) was used, according to manufacturer's instructions, to extract RNA from roots, seeds, and flower organs.

RT-Quantitative PCR Analysis

RT-qPCR analysis was performed using an Absolute Blue qPCR SYBR Green ROX Mix (AB-4162/B) kit (Thermo Fisher Scientific). Reactions were performed using a Rotor-Gene 6000 cycler (Corbett Research). A standard curve was obtained using dilutions of the cDNA sample. The expression was quantified using Corbett Research Rotor-Gene software. Three independent technical repeats were performed for each sample. Relative expression was calculated by dividing the expression level of the examined gene by that of *ACTIN*. Gene to *ACTIN* ratios were then averaged. All primer sequences are presented in Supplemental Table 2.

Yeast Two- and Three-Hybrid Assays

GID1a, *GID1b1*, and *GID1b2* coding regions were fused to GAL4 DNA binding domain (BD) in pBD-GAL4 (Clontech) by PCR amplification, with primers bearing EcoRI and SalI restriction sites. Following restriction digests, the *SlGID1* products were ligated into pBD-GAL4 at the EcoRI and SalI sites. The coding sequence of PRO and *proΔ17* were fused to the transcriptional activation domain (AD) in pACT (Clontech) at the BamHI and XhoI sites, which enables expression of proteins containing a GAL4 AD, by PCR amplification of fragments flanked with EcoRI and SalI restriction sites. All enzymes used were acquired from New England Biolabs. Plasmids were then transformed into *Escherichia coli* (DH5α) by heat shock, and the protein coding regions were sequence-verified. Each pBD GAL-GID1 was individually transformed into yeast (*Saccharomyces cerevisiae*) strain Y190 that contained the pACT vector sub-cloned with either PRO or *proΔ17*. Yeast transformants were selected for the presence of plasmids by growth on synthetic dextrose (SD) agar plates lacking Leu and Trp (LT), and examined for PRO interactions by using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining to monitor β-galactosidase reporter gene expression levels. Individual clones were spotted onto glass, round SD–LT plates containing GA_3 (0 to 10^{-5} μM). After incubation at 28°C, for 2 d, colonies were chloroform lysed and stained to estimate β-galactosidase accumulation. The plate assay was repeated three times.

For yeast three-hybrid assay, *PRO* and *SlSLY1* coding regions were cloned into pBridge (Clontech) at PfIMI and NotI (PRO) and EcoRI and SalI (SlSLY1) sites. The cDNAs in pBridge allowed the expression of two proteins: SlSLY1 fused to GAL4 BD, and PRO. *PRO* expression was driven by a Met-suppressed promoter (suppressed by 2 mM Met). The coding sequence of *GID1a* was cloned into pACT (Clontech) at EcoRI and NcoI sites to express GID1a fused to the GAL4 AD. The pBridge (containing *PRO* and *SlSLY1*) and pACT (containing *GID1a*) were transformed into yeast strain Y190. Yeast transformants were selected for the presence of the plasmids on SD agar plates lacking LT, and examined for GID1a-PRO-SlSLY1 interaction using X-Gal staining to monitor β-galactosidase reporter gene expression levels. Individual clones were spotted onto SD (-LT) plates containing combinations of GA_3 (10^{-5} μM) and/or Met. In the presence of Met, PRO is not expressed. After incubation at 28°C, for 2 d, colonies were chloroform lysed and stained to visualize β-galactosidase accumulation.

Seed Scarification and Embryo Rescue

Seeds were placed on MS plates (without sugar). After 24-48 h, the seed coats were cut with a sharp knife under a binocular microscope, the outer layer of the seed was peeled off, and then a small scar was made at the root tip side of the seed coat. The scarified seeds were placed on MS plates. The full embryo rescue was achieved by peeling off the outer seed layer and then gently making a horizontal cut on the seed coat, peeling away the entire seed coat and exposing the embryo. The naked embryos were placed on MS plates for 2 to 3 d and then planted in the soil.

Soil Plant Analysis Development

The intensity of the green color (greenness) of the leaves was measured using a soil plant analysis development-502 chlorophyll meter (Minolta Camera Co.).

Whole-Plant Transpiration Measurements

Whole-plant transpiration rates were determined using an array of lysimeters placed in the greenhouse (Plantarry 3.0 system; Plant-DiTech) in the "iCORE Center for Functional Phenotyping" (http://departments.agri.huji.ac.il/plantscience/icore.phpon), as described in detail by Halperin et al. (2017). Briefly, plants in 4L pots were grown under semi-controlled temperature conditions (20–32°C d and 18–24°C night), natural day-length, and light intensity of ~1000 μmol m^{-2} s^{-1}. Each pot was placed on a temperature-compensated load cell with digital output (Vishay Tedea-Huntleigh) and sealed to prevent evaporation from the surface of the growth medium. The weight output of the load cells was monitored every 3 min. Daily plant transpiration (weight loss between predawn and sunset) was calculated from the weight difference between the two data points.

Statistical Analysis

All data were analyzed with JMP Pro 14 software. One-way analysis of variance (compared with means), each pair Student's *t*, or all pairs of Tukey's Honestly Significant Difference test were used. Different letters represent differences at a significance level of $P < 0.05$.

Accession Numbers

Sequence data from this article can be found in the Sol Genomics Network (https://solgenomics.net/) under the following accession numbers: *SlACTIN*, Solyc11g005330; *SlGA20ox1*, Solyc03g006880.2.1; *SlGA3ox1*, Solyc03g119910; *GID1a*, Solyc01g098390; *GID1b1*, Solyc09g074270; *GID1b2*, Solyc06g008870; *PRO*, Solyc11g011260; *SlRAB18*, Solyc02g084850; *SlSLY1,* Solyc04g078390.

Supplemental Data

Supplemental Figure 1. Molecular phylogenetic analysis of GID1s.

Supplemental Figure 2. Sequence of *gid1* mutant alleles.

Supplemental Figure 3. *gid1*^{TRI} is resistant to GA.

Supplemental Figure 4. Characterization of the *gid1* mutants.

Supplemental Figure 5. The response of the different *gid1* mutants to GA treatments.

Supplemental Figure 6. Relative weight gain in M82 and *gid1* double mutants.

Supplemental Figure 7. *gid1* mutants showed increased dwarfism and phenotypic instability under changing environmental conditions.

Supplemental Figure 8. *GID1* expression and *gid1* growth under controlled and ambient growth conditions.

Supplemental Figure 9. Genotyping of *gid1* mutants grown in the field under ambient conditions.

Supplemental Table 1. RNA-guides used in this study.

Supplemental Table 2. Primers used in this study.

Supplemental Data Set. Text file of the alignment used for the phylogenetic analysis shown in Supplemental Figure 1.

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AUTHOR CONTRIBUTIONS

N.I.-E. and D.W. designed the research plan, analyzed data, and wrote the article; N.I.-E., U.R., H.S., S.B, S.L., and D.M. performed the research.

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