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Thermoinhibition Uncovers a Role for Strigolactones in Arabidopsis Seed Germination

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Strigolactones are host factors that stimulate seed germination of parasitic plant species such as *Striga* and *Orobanche*. This hormone is also important in shoot branching architecture and photomorphogenic development. Strigolactone biosynthetic and signaling mutants in model systems, unlike parasitic plants, only show seed germination phenotypes under limited growth condition. To understand the roles of strigolactones in seed germination, it is necessary to develop a tractable experimental system using model plants such as Arabidopsis. Here, we report that thermoinhibition, which involves exposing seeds to high temperatures, uncovers a clear role for strigolactones in promoting Arabidopsis seed germination. Both strigolactone biosynthetic and signaling mutants showed increased sensitivity to seed thermoinhibition. The synthetic strigolactone GR24 rescued germination of thermoinbibited biosynthetic mutant seeds but not a signaling mutant. Hormone analysis revealed that strigolactones alleviate thermoinhibition by modulating levels of the two plant hormones, GA and ABA. We also showed that GR24 was able to counteract secondary dormancy in Arabidopsis ecotype Columbia (Col) and Cape Verde island (Cvi). Systematic hormone analysis of germinating *Striga helmonthica* seeds suggested a common mechanism between the parasitic and non-parasitic seeds with respect to how hormones regulate germination. Thus, our simple assay system using Arabidopsis thermoinhibition allows comparisons to determine similarities and differences between parasitic plants and model experimental systems for the use of strigolactones.

Keywords: Arabidopsis • Germination • GR24 • *Striga hermonthica* • Strigolactones • Thermoinhibition.

Abbreviations: ABA, abscisic acid; CK, cytokinin; Col, Columbia; Cvi, Cape Verde islands; DMSO, dimethyl sulfoxide; Flu, fluridone; GA, gibberellin; GA3ox2, gibberellin 3-oxidase2; iP, N^6-(Δ^2-isopentenyl)adenine; JA, jasmonic acid; JA-Ile, jasmonoyl-isoleucine; MAX, MORE AXILLARY BRANCHES; NCED, 9-*cis*-epoxycarotenoid dioxygenase; PAC, paclobutrazol; SA, salicylic acid; SL, strigolactone; TDZ, thidiazuron; TI_{50}, 50% inhibition of seed germination by thermoinhibition; tZ, *trans*-zeatin.

Introduction

The establishment of seed dormancy and subsequent germination, like all developmental processes in plants, are heavily influenced by hormones (for a review, see Kucera et al. 2005). Moreover, it appears that extensive interaction between various hormones is required to determine the optimal developmental state for seed germination in various environments. Perhaps not surprisingly, different plant species seem to have different hormone requirements, which most probably represent the different environments to which these species have evolved (Finch-Savage and Leubner-Metzger 2006). This has made the assembly of a hormone interaction map for seed dormancy and germination challenging (Nambara and Marion-Poll 2005). However, even with these species differences, a simplified model for the roles of major hormones in seed germination has begun to emerge. Clearly, abscisic acid (ABA) is required for the establishment of seed dormancy and inhibits germination. Gibberellins (GAs), on the other hand, counteract ABA responses and are considered to be a promoting factor in seed germination (Yamaguchi et al. 2007, Seo et al. 2009). Ethylene appears to be able to counteract many of the negative functions of ABA during germination (Ghassemian et al. 2000). Finally, cytokinins (CKs) also appear to act positively on germination, possibly by stimulating ethylene synthesis (Lieberman 1979).

Much of this germination model of hormone action is based on molecular studies using model experimental systems. Therefore, an understanding of how hormones regulate seed dormancy and germination in a context of evolutionary and environmental constraints needs to be translated to tractable experimental systems such as Arabidopsis. This need is

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nowhere more urgent than in the field of parasitic plant infestations of the species *Striga* and *Orobanche*. *Striga*, for example, has contaminated >40% of Africa's arable savanna regions, leading to crop and economic losses close to US$13 billion every year (Joel et al. 2007). One key for parasitic plants to complete infections successfully is the sensing of a suitable host plant (Yoder 2001, Xie et al. 2010). Normally parasitic plant seeds remain dormant in the soil. Once a crop is planted, the parasite germinates and infects the roots of the host. The germination cue for parasite seeds is a small group of structurally related compounds called strigolactones (SLs) that are exuded from the host's roots. The need for SLs for parasitic seed germination most probably means that these species have different hormonal requirements from those of autotrophic plant species. Nevertheless, how host SLs stimulate *Striga* germination is a critical target of biotechnology, although it has been difficult to develop it into an experimental system due to obligate requirement for host plants to complete its lifecycle.

In contrast, SLs have functions in apical shoot branching in a variety of non-parasitic plant species including Arabidopsis, and hence it has been possible to identify SL biosynthetic and signaling mutants (for a review, see Dun et al. 2009). However, none of these mutants shows obvious seed germination defects under normal growth conditions (Nelson et al. 2011). A simple explanation for the differences between parasitic plants and other species may be that parasitic plants have had a unique evolutionary trajectory that has resulted in the obligate requirement of SLs for their seed germination. Alternatively, it is possible that SLs do have promotive roles in seed germination in a broader collection of plant species, but these roles cannot be easily seen under normal growth conditions optimal for germination (Tsuchiya and McCourt 2009). If this is the case, modifying the environment to produce suboptimal conditions may be able to reveal the germination phenotypes of these mutants. Support for this possibility comes from the observation that some SL-defective Arabidopsis mutants show seed germination defects under limited growth conditions while a synthetic SL, GR24, stimulates Arabidopsis seed germination in these environments (Shen et al. 2007, Nelson et al. 2009, Tsuchiya et al. 2010).

Temperature is a main seasonal cue for seed germination. For example, germination of winter annual seeds, such as Arabidopsis, is suppressed by high temperature during the summer, which enables the seeds to germinate in the season appropriate for their growth (Baskin and Baskin 1998). High temperature conditions can also be mimicked under laboratory conditions to cause thermoinhibition of Arabidopsis seeds, which is dependent on various hormones (Tamura et al. 2006, Toh et al. 2008). For example, exposing seeds to inhibitory temperatures causes the accumulation of ABA and decreases GA levels. With this in mind, we examined if a simple experimental system using temperature could be developed to sensitize the effects of SL function during Arabidopsis germination. We initially limited ourselves to the ecotype Columbia (Col) because this is the most widely used ecotype in molecular and genetic analyses, and then we extended the study to ecotype Cape Verde islands (Cvi) for secondary dormancy. We found that thermoinhibition was able to uncover a clear positive role for SLs in Arabidopsis germination, which was achieved by reducing the ABA to GA ratio in seeds. We next directly assayed hormone levels in *Striga hermonthica* seed to see if SLs had a similar effect on ABA and GA hormone levels and to determine what other hormone changes may occur. We found that SL addition does decrease the ABA/GA ratio and also found that SLs strongly increased CK levels in germinating *Striga* seeds. Increases in CKs normally occur during the imbibition of Arabidopsis seeds and have been correlated with the germination rate (Preston et al. 2009, Yano et al. 2009). It therefore appears that *Striga* seeds may share similar hormonal changes with Arabidopsis under certain conditions during germination. Our work offers the simple experimental condition, thermoinhibition, for assaying SL functions in seeds of non-parasitic systems such as Arabidopsis, and data gleaned from this system may be applicable to parasitic seed biology.

Results

Strigolactones are involved in thermoinhibition of Arabidopsis seeds

A promotive role for SLs in germination is difficult to see under standard laboratory growth conditions because the most commonly used Arabidopsis ecotype, Col, does not have deep seed dormancy (Shen et al. 2007, Tsuchiya et al. 2010, Nelson et al. 2011). Germination of Col seeds, however, decreases at temperatures above optimal (Tamura et al. 2006, Toh et al. 2008). Practically, temperature can be finely tuned and is relatively easy to standardize between experiments. Therefore, we thought that optimizing this condition might make the levels of SLs a rate-limiting factor that could then be easily assayed at the whole plant level. In our growth condition, wild-type seeds germinated well at 24°C, but showed a progressive inhibition of germination as the temperature was raised, resulting in complete suppression at 32–34°C (**Fig. 1B**), which is comparable with the findings of previous reports (Toh et al. 2008). In contrast, application of the synthetic SL, GR24, clearly shifted the thermoinhibition curve in the 30–32°C range (**Fig. 1B**). Consistent with this, GR24 also showed a dose response with respect to suppressing thermoinhibition (**Fig. 1C**). GR24 was almost as efficient as fluridone (Flu), an ABA synthesis inhibitor, or GA_3. This is somewhat surprising since these two hormones are the major factors in the establishment and breaking of Arabidopsis seed dormancy under optimal growth conditions.

We next tested the physiological function of SL biosynthesis and signaling genes on thermoinhibition by analyzing the germination efficiencies of various *max* (*more axillary branches*) mutant seeds under different temperatures. While SL

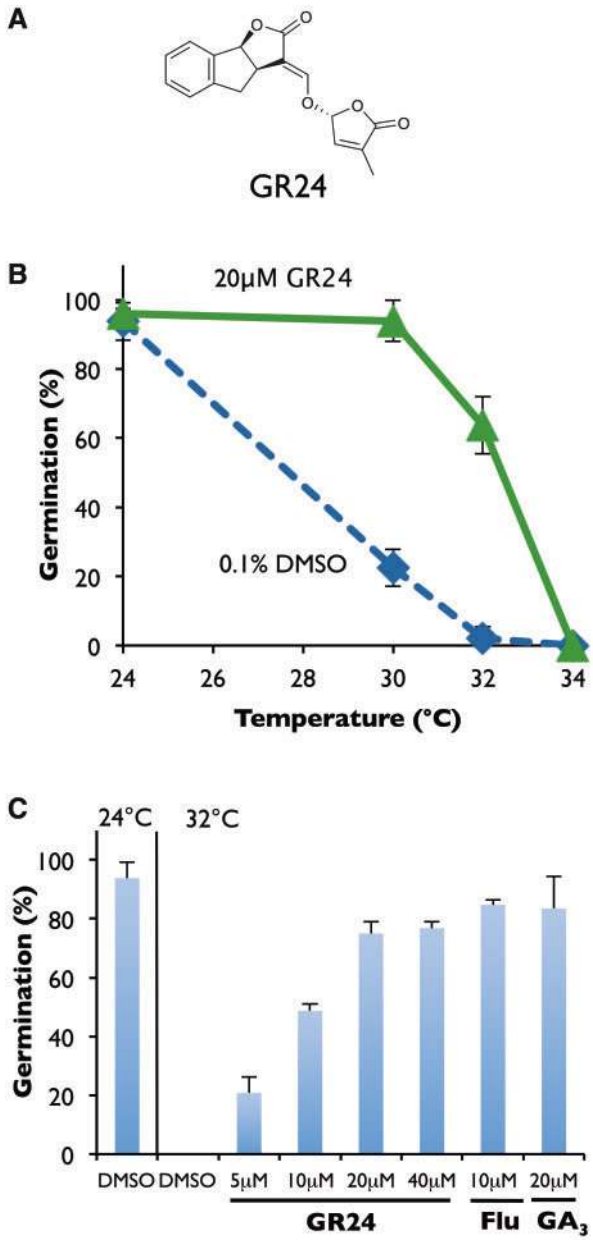

Fig. 1 GR24 alleviates seed thermoinhibition in Arabidopsis. (A) Chemical structure of synthetic strigolactone GR24. (B) Effect of GR24 on seed thermoinhibition in wild-type Arabidopsis (Col). The *y*-axis indicates the percentage germination in the presence of 20 μM GR24 (green triangle and solid line) or an equivalent amount of DMSO (0.1%, blue square dotted line) at the temperature indicated by the *x*-axis. Germination was counted 7 d after imbibition. (C) Dose effect of GR24 on thermoinhibition. The *y*-axis indicates the percentage germination in DMSO control (0.1%), GR24 (5–40 μM), Flu (10 μM) or GA_3 (20 μM) at the temperatures indicated at the top of graph. Error bars represent the SD ($n = 3$). Germination assays were performed using three independent batches.

biosynthetic *max3-9* seeds showed no significant differences from wild-type seeds, another biosynthetic mutant *max1-1* and the SL signaling mutant *max2-1* were more sensitive to thermoinhibition compared with wild-type seeds (**Fig. 2A**). As expected, GR24 application alleviated thermoinhibition in the biosynthetic mutant but not in the signaling mutant. A closer inspection of thermoinhibition in these mutants using the parameter of 50% inhibition of seed germination by thermoinhibition (TI_{50}) showed that *max1-1* and *max2-1* are 3°C more sensitive to temperature than wild-type seeds (**Fig. 2B**). Again exogenous GR24 increased the TI_{50} of the wild type, *max3-9* and *max1-1*, but not *max2-1*, indicating that the temperature hypersensitivity was SL dependent. Together, these data indicated that wild-type Col seeds require SLs to overcome thermoinhibition and, consequently, that various temperatures can be used as experimental conditions to follow the role of SLs in Arabidopsis seed germination.

Strigolactones function at, or upstream of, ABA and GA in seed germination

To investigate the relationship between SLs, ABA and GA, we exposed SL synthesis and signaling mutant seeds to either ABA or GA biosynthesis inhibitors to determine if decreasing either of these hormone levels influenced SL-dependent seed thermosensitivity. The application of the ABA biosynthesis inhibitor Flu to *max1* and *max2* seeds alleviated their thermal hypersensitivity (**Fig. 2C**). Flu, however, is an inhibitor of phytoene desaturase, an enzyme for carotenoid biosynthesis. Because both ABA and SLs are products of the carotenoid biosynthetic pathway it would be expected that Flu application decreases both ABA and SL levels. However, since reducing SL levels should increase thermoinhibition in seeds, the rescue of thermoinhibition by Flu was most probably due to reduced ABA levels. Moreover, that Flu rescued seed thermoinhibition of the SL-insensitive *max2-1* further suggests that ABA acts at, or downstream of, SL function to regulate seed thermoinhibition.

When we applied the GA biosynthesis inhibitor paclobutrazol (PAC) to *max* mutant seeds we found that thermoinhibition was not rescued by GR24 application (**Fig. 2C**). This result indicated that the GR24 rescue of thermoinhibited SL mutant seeds requires GA synthesis. Previous studies showed that thermoresistance of the ABA biosynthetic mutant *aba2-2* was suppressed by PAC, suggesting that the accumulation of ABA is upstream of GA effects on Arabidopsis seed thermoinhibition (Toh et al. 2008). Consistent with this, we also found that PAC strongly suppressed seed germination of *aba2-2* at high temperatures; however, this PAC suppression was not rescued by GR24 application (**Fig. 2D**). Together, these inhibitor experiments suggest that SLs function at, or upstream of, ABA, which in turn down-regulates GA with respect to thermoinhibition of seed germination.

GR24 decreases ABA levels and increases GA levels during the alleviation of thermoinhibition.

Our hormone biosynthesis inhibitor experiments suggest that SLs influence the ABA/GA ratio in seed thermoinhibition. The simplest explanation is that application of GR24 suppresses

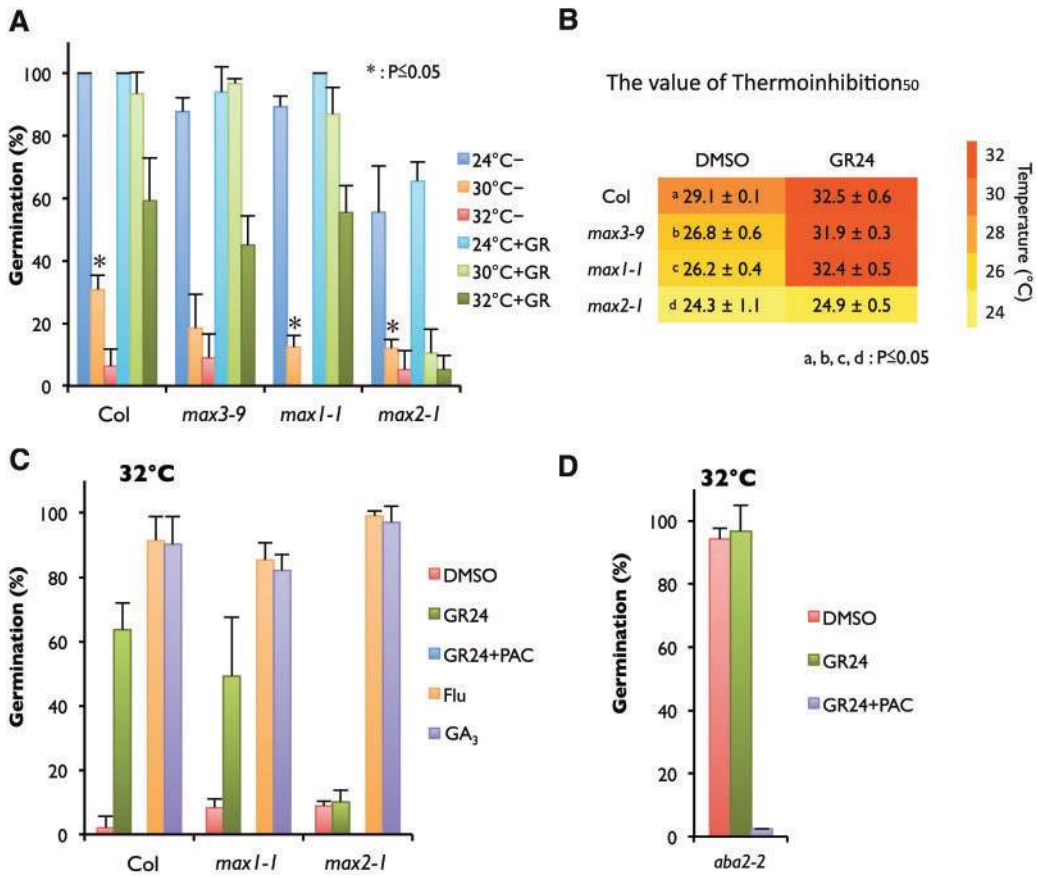

Fig. 2 Effect of thermoinhibition on SL biosynthetic and SL signaling mutants. (A) Thermoinhibition of Col and *max* (*more axillary branches*) mutant (*max1-1*, *max2-1* and *max3-9*) seed germinations in the presence (+GR) or absence (−GR) of 20 μM GR24. (B) A heat-map representation of the thermoinhibition$_{50}$ (TI_{50}) value on Col and *max* mutants in the presence or absence of 20 μM GR24. The numbers within the box represent the temperatures at which half maximal germination (±SD) was observed (see the Materials and Methods for details). An indicator for colors in the heat-map is show to the right. (C) Effect of the GA biosynthesis inhibitor pacrobutrazol (PAC), the ABA biosynthesis inhibitor fluridone (Flu) and GA_3 on thermoinhibition of Col and *max* mutants. DMSO control (0.1%), GR24 (20 μM), GR24 with PAC (20 μM each), Flu (10 μM) or GA_3 (20 μM) was applied to Col and *max* seeds at 32°C. Germination assays were performed using three independent batches. (D) Effect of GR24 (20 μM) and PAC (20 μM) on germination of the ABA biosynthetic mutant *aba2-2*. All error bars represent the SD ($n = 3$).

ABA accumulation or enhances GA accumulation during the alleviation of thermoinhibition. To test these possibilities, we first measured endogenous ABA levels at 24 h after the start of a thermoinhibition treatment in the presence or absence of GR24 (**Fig. 3A**). As observed in previous studies, wild-type seeds showed increased levels of ABA at 32°C compared with those at 24°C. In contrast, in the presence of GR24, ABA levels at 32°C were as low as those observed at 24°C. When we measured ABA levels in *max1-1* seeds under the same conditions, ABA levels were again lower in the mutant seeds treated with GR24 compared with those in untreated seeds. On the other hand, the presence of GR24 did not reduce the levels of ABA in *max2-1* seeds exposed to 32°C. 9-*cis*-Epoxycarotenoid dioxygenase9 (NCED9), one of the key enzymes for ABA biosynthesis in seeds, has been shown to play a major role in thermoinduced ABA accumulation (Argyris et al. 2008, Toh et al. 2008). Application of GR24 suppressed *NCED9* transcript accumulation to levels similar to those seen at 24°C (**Fig. 3B**).

GA_4 is considered a key bioactive GA in seed germination. In wild-type seeds the endogenous GA_4 levels in imbibed seeds were significantly lower at 32°C than at 24°C (Toh et al. 2008; **Fig. 3C**). However, GR24 increased the endogenous GA_4 levels in both wild-type and *max1-1* seeds at 32°C. In contrast, *1* GA_4 levels in *max 2-1* did not respond to GR24, which suggests that SLs can increase GA accumulation through *MAX2*. Repression of gibberellin 3-oxidase2 (GA3ox2), a key seed enzyme in the production of bioactive GA, is critical for thermoinhibition (Toh et al. 2008). Consistent with this, transcript levels of *GA3ox2* were significantly decreased at 32°C compared with its levels at optimal temperature (**Fig. 3D**). However, GR24 application did not profoundly increase *GA3ox2* transcript levels, which suggests that the GA dependence of GR24 action is due to activation of other steps in the GA biosynthetic pathway, repression of GA catabolism, or both. Together with experiments using biosynthesis inhibitors, it appears that GR24 can influence the ABA/GA ratio in thermoinhibited seeds by

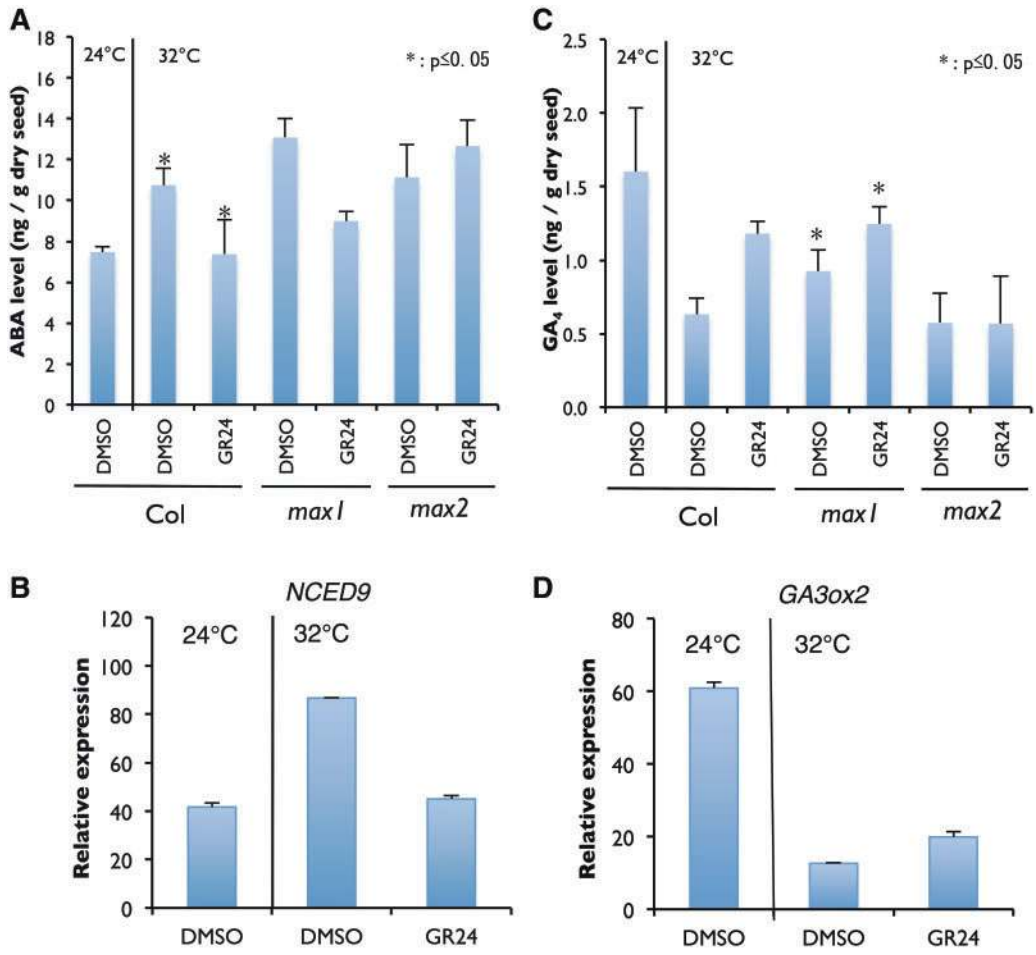

Fig. 3 ABA and GA levels are affected by GR24 during thermoinhibition. (A) Effects of thermoinhibition and GR24 on endogenous ABA levels in imbibed seeds. Col and *max* mutant seeds were imbibed at 24 or 32°C in 0.1% DMSO or 20 μM GR24 for 24 h. ABA levels were quantified by liquid chromatography/tandem mass spectroscopy (LC-MS/MS). Similar results were obtained from two independent batches. Representative results are shown. (B) Effects of GR24 on expression of *9-cis epoxycarotenoid dioxygenase9* (*NCED9*) in imbibed seeds. Real-time PCR analysis was carried out using total RNA from after-ripened seeds imbibed for 24 h at 24 or 32°C in the presence or absence of 20 μM GR24. The *y*-axis represents relative expression of *NCED9 against 18S rRNA*. (C) Effects of thermoinhibition and GR24 on endogenous GA_4 levels in imbibed seeds under the same condition as in A. (D) Effects of GR24 on *GA3ox2* transcript levels in imbibed seeds under the same condition as in B. Similar results were obtained from two independent experiments using different seed batches. Data from one of the replicates are shown. All error bars represent the SD ($n = 3$).

both decreasing ABA synthesis and stimulating GA accumulation and thus alleviate thermoinhibition of seed germination.

Effect of strigolactones on secondary dormancy

Secondary dormancy is the ability of seeds to re-establish the dormant state in response to an unfavorable environment. For example, prolonged exposure of seeds at high temperature, limited light conditions or osmotic stress reduces the ability to germinate even after the environmental conditions become suitable for germination (Khan and Karssen 1980, Cadman et al. 2006, Leymarie et al. 2008). In a series of thermoinhibition experiments, we noticed that germination of Col seeds at 24°C was inhibited when seeds were pre-imbibed at 34°C for 7 d (**Fig. 4A**). However, application of GR24 under the same conditions increased seed germination dose dependently (**Fig. 4B**). In contrast, germination of *max2* in the same condition was completely suppressed regardless of GR24 application (**Fig. 4A**), indicating that SLs counteracted thermoinduced secondary dormancy.

To clarify further the effects of SLs on secondary dormancy, we used another Arabidopsis ecotype, Cvi, that has clearer secondary dormancy compared with the weakly dormant Col. Germination of Cvi seeds is not only light dependent, but is also inhibited by prolonged incubation in the dark due to secondary dormancy (Cadman et al. 2006). We found that GR24 also reversed dark-induced secondary dormancy in Cvi seeds (**Fig. 4C**). This reversal of secondary dormancy by GR24 was also observed when Cvi seeds were exposed to GR24 only after the

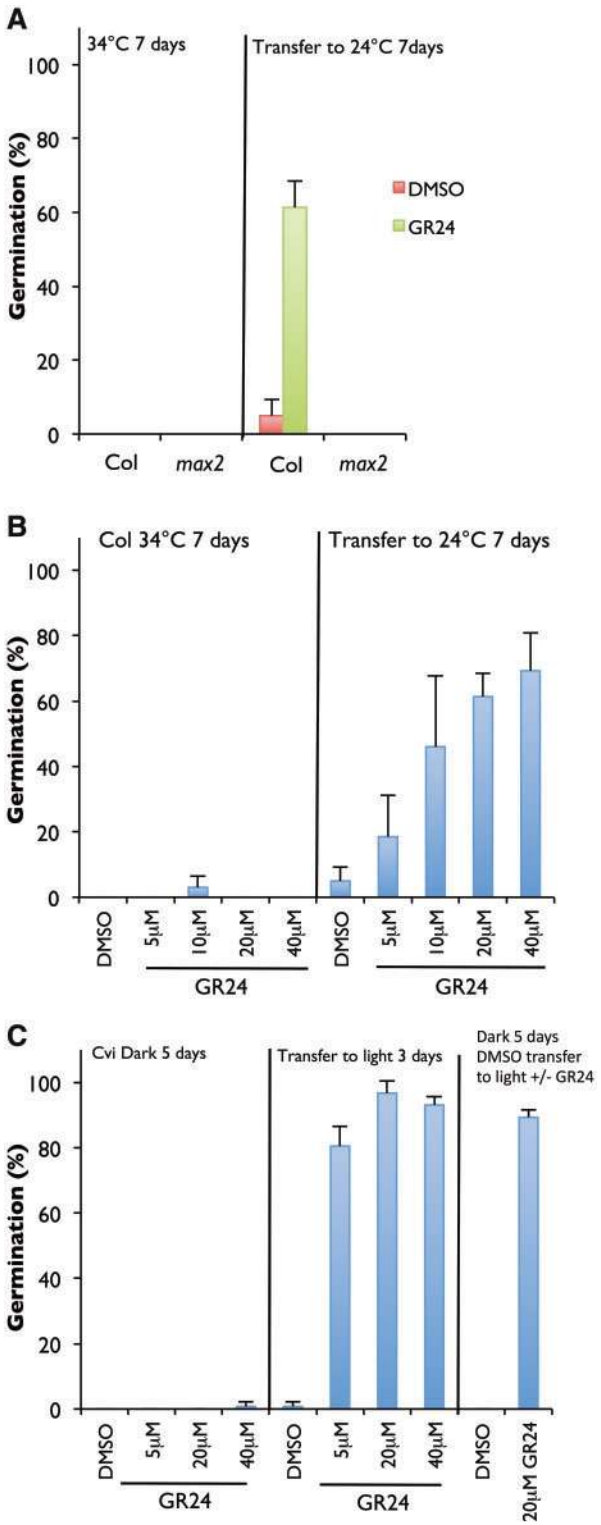

Fig. 4 GR24 counteracts secondary dormancy. (A) Thermoinduced secondary dormancy in Col seeds. To induce secondary dormancy in after-ripened Col and *max2-1* mutants, seeds were incubated with or without 20 μM GR24 for 7 d at 34°C and germination was counted (left). Then the plates were transferred to 24°C for 7 d and germination was counted again (right). (B) Dose effect of GR24 on thermoinduced secondary dormancy in Col. (C) Dark-induced secondary dormancy in Cvi. After-ripened Cvi seeds were incubated in the dark at 24°C for 5 d to induce secondary dormancy on DMSO or GR24 (left), then transferred to light conditions and incubated at 24°C for a further 3 d (center). The same experiments were performed except for adding GR24 after the induction of secondary dormancy (right). Error bars represent the SD ($n = 3$). Germination assays were performed using three independent batches.

induction of secondary dormancy (**Fig. 4C**). Thus, the positive role of SLs on germination can be extended to breaking secondary dormancy in Arabidopsis.

Hormone analysis of *Striga hermonthica* seeds.

The use of thermoinhibition of Arabidopsis seeds clearly indicated that SLs influence the levels of other hormones. This suggests that any of the SL hormonal relationships seen in our Arabidopsis thermoinhibition system may be transferable to parasitic plant seed germination. Previous studies using *S. helmonthica* seeds showed that both CK and ethylene can stimulate germination in the absence of SLs (Yoshikawa et al. 1978, Logan and Stewart, 1991, Babiker et al. 1993, Babiker et al. 1994, Sugimoto et al. 2003). Studies on *Orobanche* showed that ABA levels decrease during seed conditioning (Chae et al. 2004). However, the relationship between SLs and ABA/GA levels in parasitic seeds is not clear because a systematic analysis of hormones has not been performed for their germination. Therefore, we performed profiling of hormone levels in *S. hermonthica* seeds imbibed in the presence or absence of GR24 for 24 h (**Fig. 5A**). Under these conditions, GR24 stimulated 50% of the seeds to germinate as assayed by root protrusion after 24 h imbibition (**Supplementary Fig. S1**). Our system detected ABA, GA_4, GA_1, *trans*-zeatin (tZ), N^6-(Δ^2-isopentenyl)adenine (iP), IAA, jasmonic acid (JA), jasmonoyl-isoleucine (JA-Ile) and salicylic acid (SA), while we were not able to detect GA_4 and JA in *S. helmonthica* seeds. As was observed with thermoinhibited Arabidopsis seeds, ABA increased after rehydration, which was suppressed by GR24. Also, GA_1 increased with GR24 treatment. IAA levels remained high in GR24 compared with those in the dimethylsulfoxide (DMSO) control, whilst JA-Ile decreased after transfer to either DMSO or GR24. SA did not show significant differences among all treatments. Intriguingly, GR24 strongly induced tZ accumulation.

Although GR24 increased the ABA/GA ratio in germinating *S. helmonthica* seeds, application of either GA or Flu is not sufficient to stimulate germination of several parasitic plant species (Takeuchi et al. 1995, Chae et al. 2004). We also found that GA or Flu alone did not stimulate *S. helmonthica* germination (**Fig. 5B**). However, simultaneous application of GA and Flu did stimulate germination to a level comparable with that by GR24 (**Fig. 5B**). Consistent with strong induction of CK by GR24, studies of parasitic seed germination showed that CKs are sufficient to germinate seeds (Yoshikawa et al. 1978, Babiker et al. 1993). We also found that tZ and the

synthetic CK thidiazuron (TDZ) stimulated *Striga* seeds, while seedling morphology was also affected (**Fig. 5B, C**). Taken together, hormonal interaction of SLs, GA, ABA and CK appeared to be involved in *S. helmonthica* seed germination.

Discussion

In this study, we found that thermoinhibition of Arabidopsis seeds uncovered a promotive role for SLs on seed germination. A number of studies have previously shown that a consequence of exposing Arabidopsis and lettuce seeds to high temperatures is to increase the ABA/GA ratio, which in turn reduces germination (Yoshioka et al. 1998, Gonai et al. 2004, Argyris et al. 2008, Toh et al. 2008). It appears that genetically reducing seed SL levels increases the ABA/GA ratio in seeds, resulting in an enhanced response to thermoinhibition, whilst SL application reduces the ABA/GA ratio, which alleviates seed thermoinhibition. These results posit a simple working model in which SLs act upstream of these key seed hormones to inhibit ABA synthesis and stimulate GA accumulation (**Fig. 6A**). Presently, it is unclear how SLs influence seed ABA/GA levels, but it appears in the case of ABA that transcription of key biosynthetic enzymes such as NCED9 is repressed by GR24. Certainly with the molecular tools available in Arabidopsis it should be possible eventually to understand how this hormone controls ABA and GA levels in seeds. In a broader sense, the information gleaned from Arabidopsis may allow further insights to be obtained into how SL functions in less tractable experimental plants such as *Striga* and *Orobanche*. For example, GA synthesis is necessary for GR24 to alleviate thermoinhibition of Arabidopsis, and GA is also necessary for germination of conditioned *Orobanche ramosa* seeds in the presence of GR24 (Zehar et al. 2002, Toh et al. 2008; **Fig. 2C, D**). However, although GA was sufficient to rescue our Arabidopsis seed thermoinhibition assay it is not sufficient to induce germination in parasitic plants (Takeuchi et al. 1995, Chae et al. 2004; **Fig. 5B**). These differences indicate that there will be nuances between Arabidopsis and parasitic plants seeds with respect to its interactions with ABA, GA and SLs.

With respect to which hormones are influenced by SLs during parasitic seed germination, we took a systematic approach to measuring the level of a variety of hormones during *S. hermonthica* seed germination to determine if any similarities exist between this parasitic plant species and Arabidopsis. As observed with thermoinhibition in Arabidopsis seeds, SL application to *S. helmonthica* decreased the ABA levels and increased GA accumulation in germinating seeds (**Fig. 5A**). Interestingly, GA could induce *S. helmonthica* seed germination when ABA biosynthesis was inhibited at the same time (**Fig. 5B**). This may suggest that antagonistic interaction between ABA and GA is compromised in this parasitic plant species (**Fig. 6**). Possibly, a function of SLs is to reduce the ABA level and to increase the GA level at the same time may partially account for their germination-stimulating activity. SL application also unexpectedly enhanced CK levels (**Fig. 5A**). Simultaneous application of the CK kinetin and GA has been shown to alleviate lettuce seed thermoinhibition (Saini et al. 1986). After-ripened Arabidopsis seeds accumulate higher levels of iP after imbibition, which correlates with the germination rate (Preston et al. 2009, Yano et al. 2009). Interestingly, ethylene also alleviates thermoinhibition in lettuce seeds, and inhibiting ethylene synthesis cancels the suppressive effects of kinetin on thermoinhibition (Abeles 1986, Saini et al. 1986, Khan and Prusinski 1989). Similar experiments on parasitic plant seeds involving ethylene and CKs show that these hormones can also stimulate parasitic plant seed germination without SL treatment, which suggests that they may work downstream of SLs (Yoshikawa et al. 1978, Logan and Stewart, 1991, Babiker et al. 1993; Babiker et al. 1994, Sugimoto et al. 2003; **Fig. 6B**). In Arabidopsis, CKs stimulate ethylene production, and ethylene has a strong promotive effect on seed germination (Beaudoin et al. 2000, Ghassemian et al. 2000, Ogawa et al. 2003, Siriwitayawan et al. 2003). Possibly, the promotive effect of CK on parasitic plant germination and seed thermotolerance is through increased ethylene production (**Fig. 6**). Although we demonstrated that CKs stimulated *S. hermonthica* germination, the roots look developmentally compromised, suggesting that other factors are required for normal radicle development (**Fig. 6B**). Our hormone analyses were performed at the stage where 50% of *S. helmonthica* seeds were germinated. Therefore, to reach any conclusion on the relationship between causes and effects, it is necessary to extend the hormone analysis to early time points and to examine effects of hormone biosynthesis inhibitors when exogenous SLs were applied. Nevertheless, considering the large difference between Arabidopsis and *Striga* in terms of strategy of survival, the hormonal interaction maps of non-parasitic seed thermoinhibition and *Striga* seed germination appear unexpectedly similar (**Fig. 6**). The information obtained from our work will be used to address more specific questions in the future. For example, *Striga* and *Orobanche* are extremely sensitive to SLs with concentrations as low as 100 pM, while our thermoinhibition assay showed that Arabidopsis required the micromolar range (Wigchert et al. 1999). The species difference might be related to their evolutionary constraints and thus to the dependence on hormonal interaction.

Our results of thermoinhibition in Col and secondary dormancy in Cvi seeds have interesting implications with respect to germination of *Striga* seeds. *Striga* seeds stay dormant in soil even when the physical environmental conditions are ideal for germination, which is akin to secondary dormancy in non-parasitic plants. Since SLs appear to break secondary dormancy in Arabidopsis, SLs probably play a similar role in parasitic plants. Hence, the ability of *Striga* to stay dormant in the absence of a host may possibly reflect the evolution of a secondary dormancy that requires SLs to be broken.

In summary, our work demonstrated that simple assays of thermoinhibition and secondary dormancy in the model plant

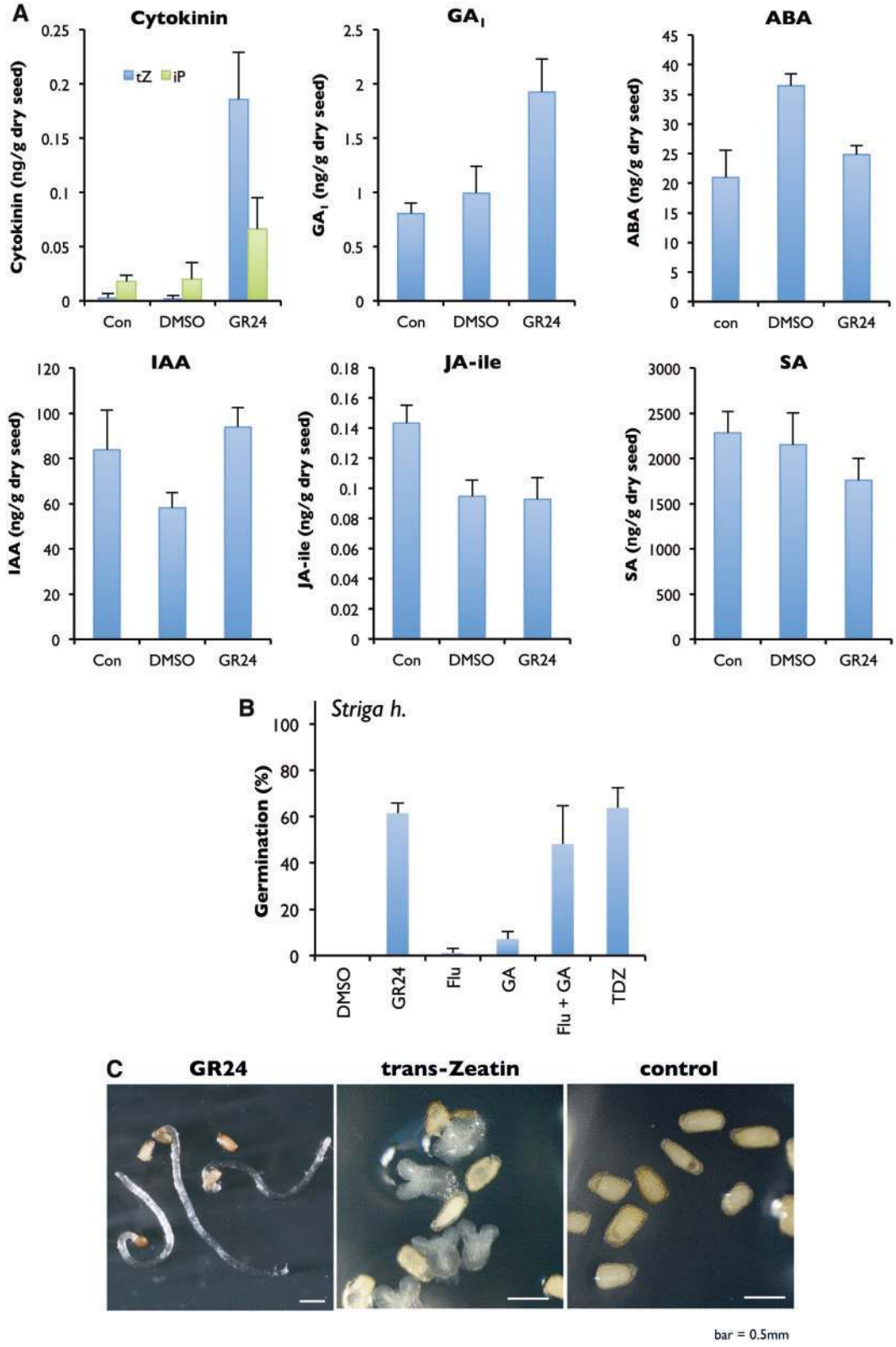

Fig. 5 Systematic hormone analysis of *Striga hermonthica* seeds. (A) Hormone levels in pre-conditioned (stratified) *S. helmonthica* seeds (Con), treated with 0.03% DMSO for 24 h or with 0.1 μM GR24 for 24 h. Error bars represent the SD ($n = 3$). See the Materials and Methods for details. (B) Induction of *Striga* seed germination by Flu, GA and the CK thidiazuron (TDZ). Conditioned *S. helmonthica* seeds were exposed to DMSO, 0.1 μM GR24, 10 μM Flu, 20 μM GA, 10 μM Flu + 20 μM GA, or 20 μM TDZ. (C) Representative picture of germinating *S. hermontica* seeds on DMSO (Control), 0.1 μM GR24 or 25 μM *trans*-zeatin. A bar represents 0.5 mm.

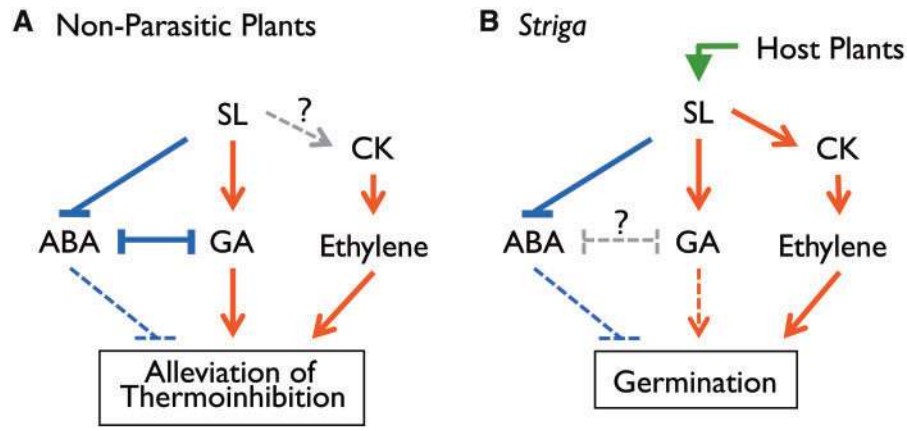

Fig. 6 A working model for the role of SLs in the alleviation of thermoinhibition in non-parasitic plants and on the stimulation of *Striga* seed germination. (A) Hormonal interactions in non-parasitic seed thermoinhibition. In Arabidopsis seeds SLs alleviate thermoinhibition by decreasing the ABA level and increasing the GA level. With GA, CK is able to alleviate thermoinhibition in lettuce seeds by increasing the ethylene level. A negative arrow from ABA to alleviation of thermoinhibition (blue dashed line) is possible. Hormonal interaction of SLs and CK is presently unknown. (B) Hormonal interactions in *Striga* seed germination. Host-exudate SLs increase GA levels and decrease ABA levels, which is similar to Arabidopsis thermoinhibition (A). GA is necessary, but not sufficient, to induce *Striga* germination. SLs also increase CK levels. A simple scheme of SLs > CK > ethylene can explain why these three hormones are sufficient to stimulate *Striga* seed germination. A negative arrow from ABA to germination is possible. Interaction between ABA and GA is presently unknown.

Arabidopsis could be used to uncover a positive role for SLs in seed germination. These experimental systems allow dissection of the mechanisms underlying how SLs influence other key hormones involved in seed dormancy and germination. Moreover, as we demonstrated, systematic assessment of hormone levels in *Striga* seeds will allow for comparisons to be made with Arabidopsis.

Materials and Methods

Plant material

Except for dark-induced secondary dormancy, Arabidopsis (*Arabidopsis thaliana*) ecotype Col was used as the wild type in this study. SL mutant alleles used were all Col background (*max1-1*, *max2-1* and *max3-9*). Seeds were harvested and stored under constant conditions (24°C in the dark) for after-ripening. Cvi seeds were after-ripened for 2 years. To obtain different seed batches, plants were grown in pots placed in separate trays. *Striga hermonthica* (Del.) Benth. seeds used in this study were collected from infested sorghum from Wad Medani, Sudan in 2006.

Thermoinhibition assay

Seeds were harvested and stored for about 6 months. Thirty seeds were imbibed in 400 µl of distilled water in a 24-well plate (Sarstedt, Inc.). Plates were incubated at constant temperature under continuous fluorescent light (12 µmol m^{-2} s^{-1}) for 7 d to count germinations. Working stocks of GR24, Flu (Sigma-Aldrich Co.), GA_3 (Sigma-Aldrich Co.) and PAC (ZENECA Ag Products Inc.) were dissolved in DMSO. The final concentration of DMSO was ≤0.1%, and there was no effect on thermoinhibition by DMSO alone. Germination was scored as radicle protrusion. All germination tests were conducted in at least two independent seed batches with three replicates in each. A representative result is presented in the figures. To obtain the TI_{50} value, seed germination was counted for three replicates at 24, 30 and 32°C, respectively. For each temperature point, the germination value (%) was randomly taken from one of triplicate samples and fit with a sigmoid curve using the Solver program in Microsoft Excel. The TI_{50} was calculated as a temperature value of inflection points on the sigmoid curve. This procedure was repeated 10 times and the mean ± SD is presented. Note that the TI_{50} represents the temperature which inhibits half of maximal germination (usually germination at 24°C). A heat-map was created with Microsoft Excel.

Secondary dormancy assay

For thermoinduced secondary dormancy in Col, 30 seeds were incubated in 400 µl of distilled water in a 24-well plate, incubated with 0.1% DMSO or 20 µM GR24 at 34°C for 7 d under continuous light and then the plates were transferred to 24°C for 7 d under continuous light to count germination. Cvi seeds were incubated on 0.8% agar containing GR24 or DMSO. Murashige and Skoog salt was not included in this assay. The seeds were initially incubated in the dark for 5 d to induce secondary dormancy, then transferred to light conditions and incubated for another 3 d to count germination. The result was obtained from triplicate experiments.

Striga seed pre-conditioning

A 30 mg aliquot of *S. hermonthica* seeds was surface-sterilized and sown on a 2.5 cm glass microfiber filter (Whatman GF/B).

Seed conditioning was conducted by placing the discs into a sterile Petri dish, sealing it with parafilm and incubating at 30°C in the dark for 11 d. Discs were dried briefly, which did not affect the germination efficiency. Then the discs were transferred to two layers of filter paper on a new Petri dish and treated with 0.03% DMSO or 0.1 μM GR24 for 24 h at 24°C. Seeds were frozen in liquid N_2 and stored at −80°C until use.

Hormone analysis

For hormone analysis in Arabidopsis, 30 mg of seeds were surface-sterilized and sown on two layers of filter paper wetted with 3 ml of the designated solution in a 60 mm Petri dish. The Petri dish was sealed with scotch tape and incubated at the designated temperature in the light for 24 h after the start of imbibition. Extraction and purification of ABA, GA_4 (from Arabidopsis and *S. helmonthica*), IAA, tZ, iP, SA, JA and JA-Ile (from *S. helmonthica*) were performed by solid-phase extraction as described previously (Dobrev and Kamínek 2002, Yano et al. 2009). Hormone levels were analyzed by a Triple Quad LC-MS/MS G6400 series (Agilent Technologies). The following are stable isotope-labeled compounds used as internal standards in this study: D_6-ABA (Icon Isotopes); D_2-GA_4, D_2-GA_1, D_5-tZ and D_6-iP (Olchemim); D_2-IAA and D_6-SA (Sigma-Aldrich); D_2-JA and [$^{13}C_6$]JA-Ile (Tokyo Kasei); and synthetic [$^{13}C_6$]JA-Ile (Jikumaru et al. 2004). MS/MS transitions for quantifications are as follows: (*m*/*z*): ABA (263/153), D_6-ABA (269/159), GA_1 (347/273), D_2-GA_1 (349/275), GA_4 (331/ 257), D_2-GA_4 (333/259), IAA (174/130), D_2-IAA (176/132), iP (204/136), D_6-iP (210/137), tZ (220/136), D_5-tZ (225/136, 137), JA-Ile (328/136) and [$^{13}C_6$]JA-Ile (322/130). As for the identification of hormones in *Striga*, MS/MS transitions for conformations are also analyzed on the following signals: (*m*/*z*): ABA (263/219), D_6-ABA (269/225), GA_1 (347/259), D_2-GA_1 (349/261), GA_4 (331/225), D_2-GA_4 (333/227), IAA (174/128), D_2-IAA (176/130), iP (204/148) and D_6-iP (210/148). Hormone analysis in Arabidopsis was done in at least two independent seed batches with three replicates for each. A representative result is presented but all results were consistent.

Quantitative reverse transcription–PCR (QRT–PCR)

Total RNA was extracted using an RNAqueous kit with Plant RNA Isolation Aid (Ambion). First-strand cDNA was synthesized from 1 μg of RNA using a QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. Quantitative PCRs were performed using the PowerSYBR Green PCR Master mix following the manufacturer's instructions on the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). cDNA concentrations were normalized to a standard of *18S rRNA* transcript levels. The gene-specific primers for *NCED9*, *GA3ox2* and *18S rRNA* were as described previously (Ogawa et al. 2003, Seo et al. 2004). Genomic DNA was used for the standard curve. For each sample, the mean value from triplicate real-time PCRs was adapted to calculate the transcript abundance, and the mean values were plotted with the SDs. For biological replicates, experiments were performed at least twice using different seed batches, and similar results were obtained.

Supplementary data

Supplementary data are available at PCP online.

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