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Smoke exposure alters endogenous gibberellin and abscisic acid pools and gibberellin sensitivity while eliciting germination in the post-fire annual, *Nicotiana attenuata*

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Abstract

Exposure to smoke is required for the germination of seeds from dormant genotypes of *Nicotiana attenuata*, a post-fire annual of the Great Basin Desert. Germination can be elicited by $GA_{1,3,4,7}$ treatments and inhibited by the GA biosynthesis inhibitor, paclobutrazol (PAC), abscisic acid (ABA) and terpenes leached from unburned litter of the plant's natural habitat. We analysed the endogenous GA and ABA dynamics during the 22 h after imbibition, when smoke-treated dormant seeds commit to germination. Extractable GA_{1+3} pools decreased in all seeds, but the decrease was more dramatic within 2 h of smoke exposure, which was followed by an increase between hours 2 and 4. Extractable ABA pools increased shortly after imbibition and remained stable in control, water-treated seeds, but decreased sharply in smoke-treated seeds. PAC completely inhibited smoke-induced germination when seeds were treated for 12 h after smoke exposure, consistent with the requirement of *de novo* GA synthesis for germination. Smoke treatment in the dark did not result in germination, whereas GA_3 treatment did, a result consistent with phytochrome-mediated GA biosynthesis. Smoke exposure dramatically increased the sensitivity of seeds to exogenous GA_3 treatments in both the light and dark, and light exposure increased this sensitivity an additional tenfold. Taken together, these results suggest that while germination requires endogenous GA synthesis, the effects of smoke treatment increase GA sensitivity, which is correlated with a decrease in endogenous ABA pools.

Keywords: abscisic acid, dormancy, gibberellic acid, germination, *Nicotiana attenuata*, paclobutrazol, smoke

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Introduction

Seed germination and dormancy are under control of the phytohormones, gibberellins (GAs) and abscisic acid (ABA), in a variety of species (Karssen *et al*., 1989; Derkx *et al*., 1994; Koornneef *et al*., 2002; Olszewski *et al*., 2002). In the solanaceous species, tomato, tobacco and *Datura,* GA plays a crucial role in the weakening of endosperm layers, which physically restrain the growing embryo and inhibit germination (Welbaum *et al*., 1998; Wu *et al*., 2001; Sanchez *et al*., 2002). Several hydrolases, such as endo-β-mannanase, expansins and β-1,3-glucanase show enhanced activity before endosperm weakening and are induced by GA (Dahal *et al*., 1997; Chen and Bradford, 2000; Leubner-Metzger and Meins, 2000; Nonogaki *et al*., 2000). Furthermore, GA may stimulate embryo growth, as has been demonstrated in pea and *Brassica napus* (Swain *et al*., 1997; Hays *et al*., 2002). In contrast, ABA acts as a suppressor of germination in both tomato and tobacco seeds by preventing endosperm weakening (Toorop *et al*., 2000; Krock *et al*., 2002), specifically by inhibiting β-1,3-glucanase in tobacco (Leubner-Metzger and Meins, 2000). Furthermore, ABA is known to induce, and may maintain, seed dormancy (Hilhorst and Karssen, 1992; Bewley, 1997) and, accordingly, over- and antisense-expression of an ABA biosynthetic enzyme in *Nicotiana plumbaginifolia* results in high- and low-dormancy phenotypes, respectively (Frey *et al*., 1999).

While the initiation of germination is not thought to result from a *de novo* synthesis of GA, a change of sensitivity of the seed to stored GA is thought to start the process (Karssen and Laçka, 1986; Derkx *et al*., 1994). However, *de novo* GA synthesis is necessary to complete germination, and this is under control of activated phytochromes (Casal and Sánchez, 1998; Yamaguchi *et al*., 1998), the mechanisms of which are rapidly emerging. For example, transcripts of GA20ox and GA3ox, enzymes that contribute to the last steps of GA formation, exhibit a light-modulated increase

during germination in *Arabidopsis*, lettuce and de-etiolated pea seedlings (Kamiya and García-Martínez, 1999; García-Martínez and Gil, 2001).

Fire synchronizes the germination in many species, and different fire-related mechanisms can elicit germination (Brown and van Staden, 1997; Minorsky, 2002). For example, fire-related physical/chemical scarification of seed coats, heat shock and nutritive- and signal-mediated stimulation play a role in some species (Baldwin *et al*., 1994; Egerton-Warburton, 1998). However, several species that exhibit fire- or smoke-elicited germination in laboratory studies do not originate from post-fire habitats, e.g. lettuce, celery and rice (Thomas and van Staden, 1995; van Staden *et al*., 1995; Doherty and Cohn, 2000). In contrast *N. attenuata*, a native annual of the Great Basin Desert, USA, which times its germination with the post-fire environment by responding to different chemical signals, provides an ecologically realistic system for the study of smoke-induced germination (Preston and Baldwin, 1999). Distributed throughout California, Idaho, Nevada, Utah and Arizona, this species produces 90% dormant seeds, occurs for up to three subsequent growing seasons following a fire, and seeds can survive in the soil for at least 150 years, awaiting the next burn. The seeds' thin and water-permeable testa allows them to germinate in response to smoke cue(s) derived from burned biomass (Baldwin *et al*., 1994). Furthermore, seeds regulate dormancy by responding to compounds leached from unburned litter (ABA and four terpenes – bornane-2,5-dione, 1,8-cineole, β-thujaplicin and camphor) from the habitat's dominant vegetation (e.g. juniper, sagebrush and blackbrush) (Krock *et al*., 2002). These negative cues can override the stimulus of smoke in areas adjacent to burns, where intact litter is covering the ground. From this research, it is clear that *N. attenuata* chemically eavesdrops on its environment and times its germination with the post-fire environment by responding to terpenes, ABA and smoke from its environment. The physiological basis of these responses has been examined with inhibitors of hormone synthesis, paclobutrazol (PAC; GA inhibitor) and fluridone (ABA inhibitor), which suppress or induce germination, respectively, and by mimicking the inhibitory effect of leachates from unburned litter on smoke-induced germination with a combination of ABA and the four terpenes (Krock *et al*., 2002).

Here, we extend the physiological analysis of the smoke-elicited germination by quantifying, by gas chromatography/mass spectrometry (GC/MS), the dynamics of endogenous GA and ABA pools in the seeds of a dormant *N. attenuata* genotype immediately after exposure to smoke and water imbibition (and hence committed to germination) and in water-imbibed seeds (which remained dormant). Smoke exposure increased seed sensitivity towards GA, associated with a large, short-term decrease of the GA pool, which was not found in imbibed, non-germinating seeds. Endogenous ABA content decreased in smoke-exposed seeds, but not in water-treated controls. Moreover, smoke and light exposure increased (each by ten times) the efficacy of exogenous GA treatments in enhancing germination, which was consistent with a role for smoke in increasing sensitivity to GA and the *de novo* synthesis of GA by light. With paclobutrazol treatments, we determine the minimum duration of GA biosynthesis for successful germination to be 12 h.

Materials and methods

Germination bioassays

The procedures used have been described at length in Krock *et al*. (2002). Briefly, bioassays were carried out in small closed cups filled with 4 g analytical sand (Merck, Darmstadt, Germany), containing 10 seeds each, and each was replicated three times. The sand was saturated with 0.5 ml water before adding the treatment, and in the case of smoke treatments, seeds were exposed to 1 ml of smoke solution for 1 h before being transferred to the cups. The cups were placed in a climate chamber (Economic DeLuxe 432 L, Snijders Scientific, Tilburg, The Netherlands) under 16 h light and 8 h dark period at 32°C and 28°C, respectively. Seeds were imbibed with 1 ml of water containing different concentrations of: GA_3 and GA_7 (1 mM, 100 µM, 10 µM, 1 µM), (Duchefa, Haarlem, The Netherlands); the GA biosynthesis inhibitor paclobutrazol [(2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)pentane-3-ol] (Riedel-deHaën, Deisenhofen, Germany) at 1 µM and 10 µM; smoke, prepared from liquid smoke (House of Herbs INC., Passaic, New Jersey, USA) diluted in water 1/300 (v/v); or only water. For germination in the dark, seeds were treated under reduced light (<0.001 µmol m^{-2} s^{-1}), and seed cups were wrapped in aluminium foil before being placed in a climate chamber. Smoke-treated seeds from a *N. attenuata* genotype (collected near Flagstaff, Arizona, USA) that remain dormant in the dark, but germinate after very short (less than 30 s) exposure to light, served as a control for light exposure. Since determining whether dark-grown seeds had germinated required microscopic examination under light of sufficient intensity to interrupt the dark period, all examined seed cups were removed from the experiment. Hence, for these experiments, a full replicated set of seed cups was included for each examination time in the experiment. Percentage germination data from each

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cup were arcsin transformed before treatment comparisons were made using the *t*-test (StatView, Version 5.0, SAS, Cary, North Carolina, USA).

Extraction and quantification of GA and ABA

Seeds (0.500 g, approx. 3500 seeds) from a dormant genotype collected near Flagstaff, Arizona in 1996 and subsequently selfed under glasshouse conditions for four generations, were treated with either 20 ml of tap water (control) or a 1:300 liquid smoke:water dilution in a 50 ml Erlenmayer flask and stored in a climate chamber for 2 h under light from 60 W fluorescent bulbs (Philips, USA) at 32°C. Each sample and control treatment was replicated three times. Seeds and treatment solutions were transferred to 145 mm diameter Petri dishes, previously filled with 50 g analytical sand (Merck, Darmstadt, Germany). Water (30 ml) was added and the dishes were stored in a growth chamber (York International, Mannheim, Germany) for a total exposure of 16 h of light followed by an 8 h dark period to simulate one diurnal cycle. Treatments were terminated 2, 4, 12 or 22 h after imbibition by flash-freezing seeds in liquid N_2. Thawed seeds were separated from the 50 g sand and immediately ground for 2 min with a pestle and mortar, which was filled with 2 g sand and 4 ml acetonitrile (ACN) (Roth, Karlsruhe, Germany). Non-imbibed dry seeds (0 h) were also sampled. Ground seeds were transferred into flasks containing 150 ml ACN. Flasks were covered with aluminium foil and placed on a shaking table (Certomat BS-1, Braun, Melsungen, Germany) for 14 h at 160 rpm and 20°C. Afterwards, the ACN volume was reduced to 40 ml in a rotary evaporator at 160 mbar and 50°C, and the sample/ACN mixture was centrifuged (Avanti J-25, Beckman, Palo Alto, California, USA) in 50 ml tubes (Falcon tubes, Becton-Dickinson, Heidelberg, Germany) for 45 min at 12,000 rpm. The ACN supernatant was evaporated to 3 ml in a rotary evaporator at 160 mbar and 50°C, and 10 ml water was added to facilitate the removal of the remaining ACN during rotoevaporation. The residual 10 ml aqueous phase was stored at 4°C until 15 ml of methanol/water ($MeOH/H_2O$) (15/85, v/v) were added. The acidity of all methanol solutions and water used for the following solid-phase extraction (SPE) was adjusted to pH 3 with acetic acid (Merck, Darmstadt, Germany). Internal standards of 100 ng 17,17-D_2 GA_1, GA_3, GA_4 and GA_7 (Professor Lewis Mander, The ANU, Acton ACT 0200, Australia) and 100 ng D_4 ABA (Dr R. Baraldi, Istituto di Ecofisiologica delle Piante Arboree da Frutto, CNR, Bologna, Italy) in 100 µl water were added to each sample, for detection and quantification of the native hormones by comparison of retention times and peak areas of significant masses.

A 6 ml LC-18 cartridge with 1 g filling in a vacuum manifold (Supelco, Deisenhofen, Germany) was used for extractions. The cartridge was conditioned by sequential elution with 20 ml $MeOH/H_2O$ (50/30; v/v) and 20 ml water, respectively. The sample containing GA and ABA internal standards was loaded on the cartridge, and washed with 20 ml water and 20 ml $MeOH/H_2O$ (15/85; v/v). GA_1, GA_3 and ABA were eluted with 50 ml $MeOH/H_2O$ (40/60; v/v). GA_4 and GA_7 were eluted with 50 ml $MeOH/H_2O$ (70/30; v/v) (Fig. 1). The eluates were reduced to 1.5 ml with a rotary evaporator at 50°C and 150 mbar for the MeOH fraction and then 28 mbar for the H_2O fraction. Residues were transferred to 2 ml crimp vials and dried at 45°C and low pressure (Concentrator 5301, Eppendorf, Hamburg, Germany). Freshly prepared diazomethane (300 µl) was added to the sample vials containing the dried residue of the 40% MeOH SPE-fraction, after which the vials were sealed and placed in a heating block (Techne Dri-Block DB2D, Labtech Int., Burkhardtsdorf, Germany) for 2 h at 40°C in a fume hood to methylate free acids. The ether was removed by evaporation with a concentrator. The residue was dissolved in 80 µl toluene and transferred into the 200 µl glass insert of a crimp vial. For quantification of ABA, 15 µl of each sample were injected into a GC/MS system (Agilent GC/MS-System 6890/5973 Series with Auto Sampler and PTV, Agilent, Waldbronn, Germany) under the following conditions: large volume injection, 5 × 3 µl; injector temperature, –20°C for 3 min, ramped to 250°C at 700°C/min; carrier gas, He; constant flow, 1.0 ml min^{-1}; oven temperature programme, initial 80°C, 4 min hold, 40°C min^{-1} to 200°C, 7.5°C min^{-1} to 260°C, 10 min hold, 100°C min^{-1} to 300°C, 4.6 min hold; total run time, 30 min; column, DB-5MS, length 30 m, ID 0.25 mm, film thickness 0.25 µm (J&W Scientific, Fisher Scientific, Nidderau, Germany). Ion fragments of ABA were quantified by single ion monitoring (SIM) at m/z 190 for native and at m/z 194 for deuterated isomers. The second derivatization step for trimethylsilylation of GA_3 and GA_1 was carried out by adding 15 µl MSTFA [*N*-methyl-*N*-trimethylsylil-triflouroacetamide] (Macherey-Nagel, Düren, Germany) to the remaining 65 µl of sample in 200 µl glass inserts. Crimped vials were placed in a heating block for 15 min at 60°C. Samples were again injected with the same GC method and column, as described above, and quantified with SIM at m/z 506 for native GA_1, m/z 504 for native GA_3, m/z 508 for deuterated GA_1 and m/z 506 for deuterated GA_3. For detection of GA_4 and GA_7, dried residues of the 70% MeOH SPE fraction were derivatized and detected as described above for GA_1 and GA_3. SIM detection was performed at m/z 418, 390 and 386 for native GA_4, m/z 416, 384 and 356 for native GA_7, m/z 420, 392

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and 388 for deuterated GA_4 and m/z 418, 386 and 358 for deuterated GA_7. Quantities of native GA were corrected for the amount of native GA occurring in the deuterated standards, as determined by separate analysis, and were expressed as ng g^{-1} dry seed. Quantities were compared with *t*-tests (StatView, Version 5.0, SAS, Cary, North Carolina, USA). Figure 1 summarizes the extraction and quantification method.

Results

Dynamics of endogenous GA and ABA pools

ABA, GA_1 and GA_3 were detected in every seed sample, but not GA_4 and GA_7 (Fig. 2). The detection limits of the analytical procedures for GA_1 and GA_3 were 1 ng and 5 ng for GA_4 and GA_7 in each 500 mg sample of dry seeds. It should be noted that only free hormones were extracted and detected with the methods used.

In the genotype of seeds used in this study, 90% of the seeds germinated (defined by endosperm rupture by the radicle) 3 d after exposure to smoke, while water-treated seeds showed no germination. Endogenous GA and ABA were measured (in three independent replicates per harvest and treatment) at 0 h (dry seeds), and at 2, 4, 12 and 22 h after both smoke and water treatments, because previous experiments had demonstrated that seeds are committed to germination within 24 h of smoke exposure. In every sample, the amount of GA_1 was clearly lower than that of GA_3, and was detected at 1:10 amount ratios at every harvest, except at the 0 h harvest, when it was detected at 1:6. In smoke-treated seeds, a large short-term decrease of GA_3 was observed after 2 h, when it declined from 440 ng g^{-1} dry seeds to 80 ng g^{-1} seeds ($P < 0.0001$, $t = 20.48$). The extractable pool subsequently increased to 200 ng g^{-1} seeds between 2 and 4 h ($P < 0.001$, $t = 13.33$) and returned to 80 ng g^{-1} seeds at the 22 h harvest (Fig. 2A). GA_1 extractable pools exhibited a similar pattern of changes over time (Fig. 2B). In water-treated seeds, GA_3 content declined steadily to 100 ng g^{-1} seeds by 22 h, after a short-term decrease in the first 2 h from 440 ng g^{-1} seeds to 280 ng g^{-1} seeds ($P < 0.05$, $t = 5.734$). This decrease was not observed in GA_1 extractable pools ($P > 0.05$). In water-treated seeds, neither GA increased between 2 and 4 h after imbibition.

The ABA content of water-treated seeds (Fig. 2C) increased dramatically in the first 2 h after imbibition, from 30 ng g^{-1} seeds (0 h) to 93 ng g^{-1} seeds ($P < 0.05$, $t = 4.39$). After 4 h, extractable ABA concentrations stabilized at 57 ng g^{-1} seeds, for the 22 h duration. In smoke-treated seeds, the ABA content also increased during the first 2 h, from 30 ng g^{-1} seeds to 56 ng g^{-1} seeds ($P > 0.2$, $t = 1.557$), but less strongly compared to water-treated seeds. In marked contrast to what was observed in the water-treated seeds, extractable ABA pools steadily decreased through the 22 h harvest, to 6 ng g^{-1}, which were significantly lower values ($P < 0.01$, $t = 5.365$) than those found in water-treated seeds at 22 h (53 ng).

Combined effects of light on smoke- and GA-treatments

Seeds were treated with GA_3 (at 1 mM, 100 µM, 10 µM and 1 µM) alone, or combined with smoke, and with or without light exposure (Fig. 3), to examine the interactive effects of smoke, light and GA on

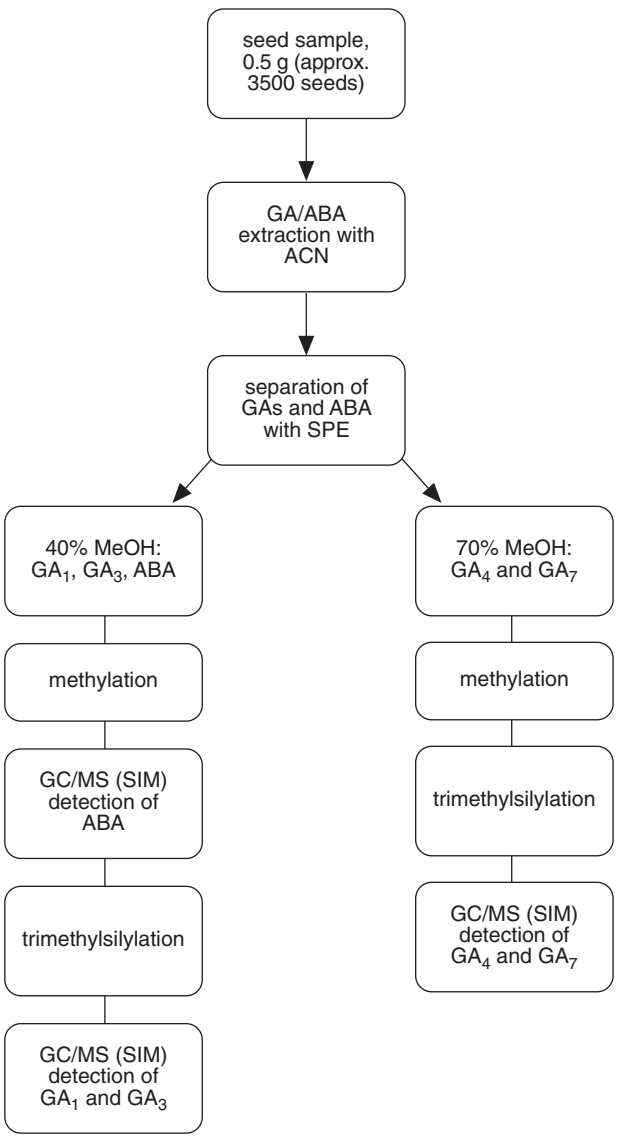

Figure 1. Flow-chart of extraction method, derivatization and detection of GAs and ABA in a seed sample of *Nicotiana attenuata*.

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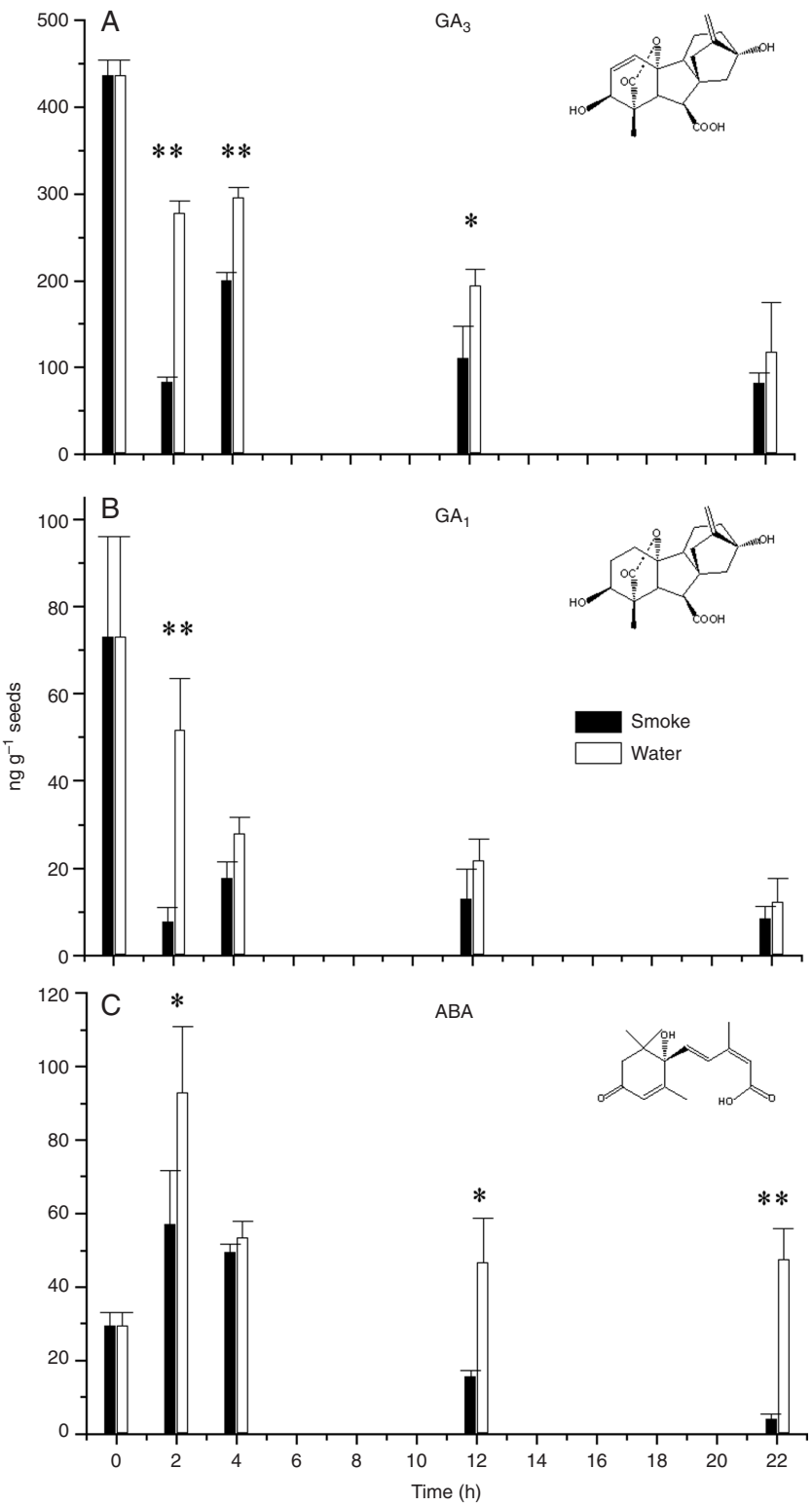

Figure 2. Endogenous amounts (mean + SEM of three replicates) of GA_1, GA_3 and ABA (ng g^{-1} dry seed) during 22 h after smoke and water treatments in *Nicotiana attenuata* seeds, dormant genotype Arizona (0 h = dry seeds). Asterisks indicate a significant difference between treatments at one harvest time (*, $P < 0.05$; **, $P < 0.01$).

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germination. Smoke treatment of seeds in the dark did not result in germination, but smoke significantly enhanced GA-elicited germination at 100 µM GA_3 and at 10 µM GA_3 treatments, in comparison to the germination of seeds treated only with GA_3 ($P < 0.01$, $t = 6.26$ for percentage germination at day 3 with GA_3 at 100 µM with and without smoke; $P < 0.001$, $t = 12.57$ for percentage germination at day 5 with GA_3 at 10 µM with and without smoke). Seeds treated with smoke and 1 µM GA_3 in the dark did not germinate (data not shown). Smoke treatments showed the same germination-enhancing effects when GA-treated seeds were exposed to light as was found in the dark, although, at one order of magnitude lower concentrations of applied GA, compared to the dark. For example, seeds treated with smoke and 1 µM GA_3 did not germinate when grown in the dark (data not shown), but did so in the light (Fig. 3).

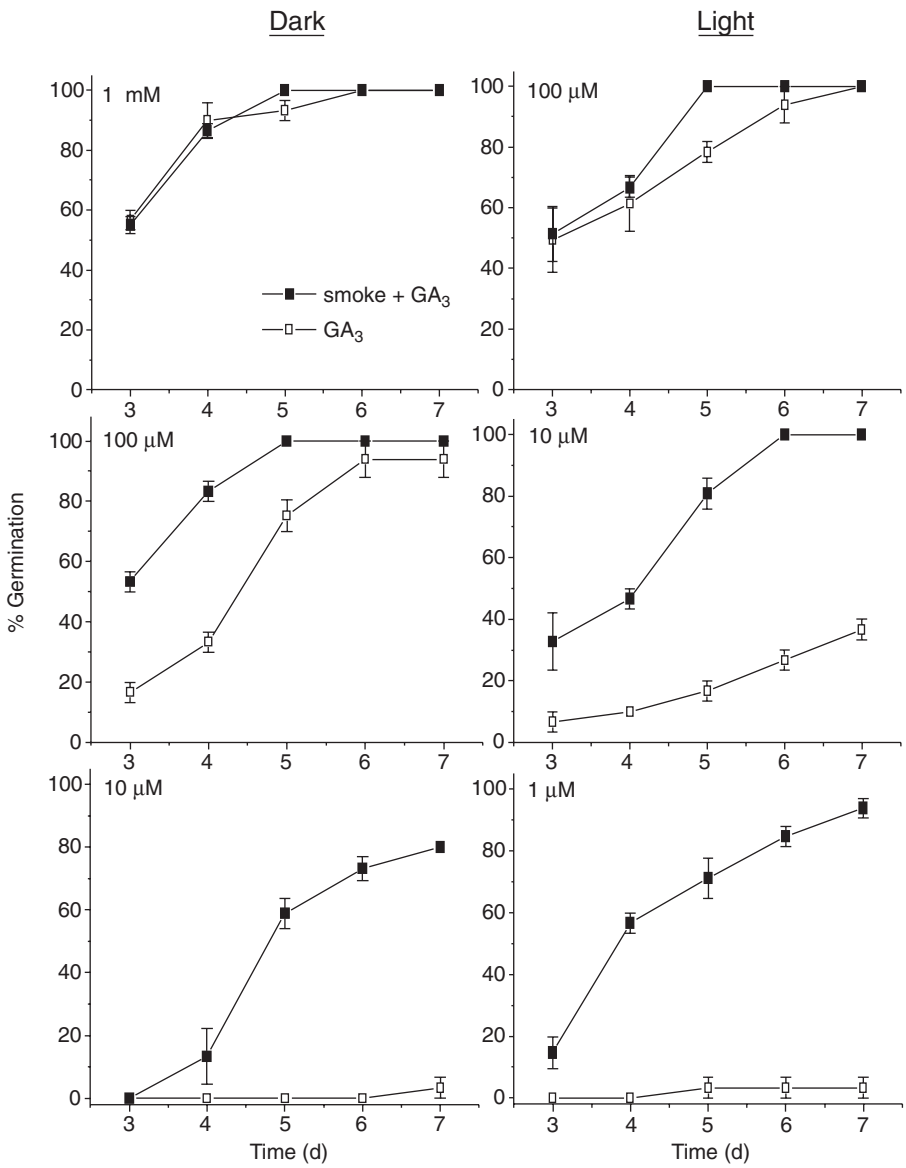

Figure 3. Mean (± SEM) percentage germination (endosperm rupture by radicle) of ten *Nicotiana attenuata* seeds, in three replicate germination cups per observation, after treatment with smoke (smoke/water 1:300, v/v) and/or GA_3 at different concentrations (1 mM, 100 µM, 10 µM, 1 µM) in the dark (left) and under light (right). For all dark-grown seeds, seed cups were discarded after each observation to avoid the confounding effects of light exposure during observations, while light-exposed seed cups were recounted throughout the experiment. The genotype of *N. attenuata* used here shows slightly delayed germination compared to the genotype used in other experiments.

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Paclobutrazol treatment

The GA biosynthesis inhibitor PAC [which is thought to interrupt the formation of GAs by inhibiting the oxidation of *ent*-kaurene to *ent*-kaurenoic acid; Rademacher (2000)] completely inhibited germination at 10 μM when applied up to 12 h after smoke treatment (Fig. 4). At 1 μM PAC, the duration of inhibition was shorter, with a quarter of seeds germinating after 6 d, when PAC was applied 12 h after smoke treatment. This inhibitory effect was probably not due to toxicity, because all PAC-treated seeds germinated within 2 d after a subsequent 1 mM GA_3 treatment (data not shown).

Discussion

Exposure to smoke increases sensitivity to GA, indicating that smoke cues facilitate GA signalling (Fig. 3). A rapid decrease in GA contents is consistent with the utilization of previously stored GAs in the initiation of germination, rather than the *de novo* synthesis of GAs (Karssen and Laçka, 1986; Derkx *et al*., 1994) (Fig. 2). Smoke exposure increased the sensitivity of dark-grown seeds to exogenous GA tenfold [as was also found in lettuce by van Staden *et al*. (1995)], and this smoke-enhanced sensitivity was again increased tenfold when seeds were exposed to light, which, most likely, is caused by *de novo* synthesis of GA. While the underlying mechanisms mediated by the (unknown) smoke cues remain to be

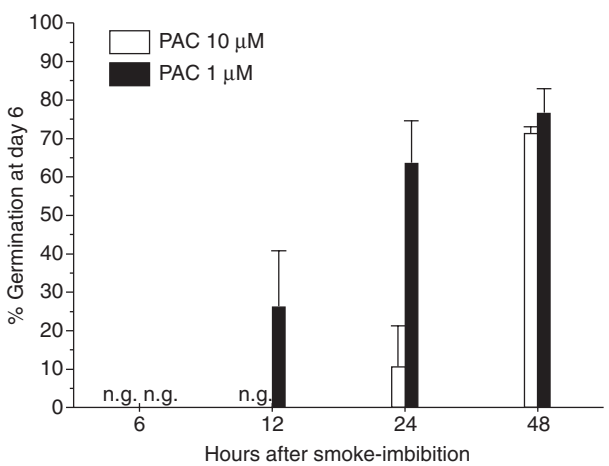

Figure 4. Mean (± SEM) percentage germination (endosperm rupture by radicle) of ten smoke- and GA biosynthesis inhibitor paclobutrazol (PAC)-treated *Nicotiana attenuata* seeds, in three replicate germination cups per observation, 6 d after smoke treatment. Smoke was applied at 0 h, PAC was applied at 0 (no germination, not shown), 6, 12, 24 and 48 h after smoke treatment; germination was detected after 6 d. n.g., no germination detected. All seeds treated only with smoke had germinated by day 6.

discovered (Minorsky, 2002), these observations are consistent with the growing literature on phytochrome-mediated GA synthesis during germination. Furthermore, after-ripening-mediated release of photodormancy of *Nicotiana tabacum* seeds is correlated with a reduced requirement for GA; however, whether this is due to increased sensitivity or due to increased endogenous GA remains unknown (Leubner-Metzger, 2002).

Imbibition results in, first, a rapid decrease in extractable GA pools during the first 2 h, which suggests that a sufficient amount of GA deactivating enzymes (such as GA2ox for GA_1) (Hedden and Phillips, 2000) are present in the seed before the start of germination. The decrease is followed by a rapid GA increase in smoke-treated seeds between 2 and 4 h, which may be light induced. Phytochromes have been shown to induce *ga4*, a gene that encodes GA3ox and contributes to the last step of GA biosynthesis (García-Martínez and Gil, 2001; Yamaguchi and Kamiya, 2002). Transcripts for GA_4 in *Arabidopsis* and lettuce increase within 1 h after red light treatment (Toyomasu *et al*., 1998; Yamaguchi *et al*., 1998), providing a mechanism for the phytochrome mediation of the rapid increase in GA resulting from *de novo* synthesis. The tenfold enhanced germination under light (Fig. 3) also suggests a phytochrome-mediated increase in *de novo* GA synthesis. A similar phytochrome-dependent mechanism was found by Gardner *et al.* (2001) for smoke-induced germination of lettuce, which could be reversed by far-red light exposure. However, lettuce seeds also germinated after smoke treatment in the dark, indicating a phytochrome-independent mechanism, which was not observed in *N. attenuata*. Moreover, in contrast to *N. attenuata*, lettuce seeds contain no active GAs until they are induced by light or smoke (Gardner *et al*., 2001).

The results of the PAC treatments are consistent with the hypothesis that *de novo* GA synthesis is necessary for germination, here within 12 h of smoke exposure, even though GA content decreased during this time (Figs 2, 4). The decrease in GA pools over 22 h before endosperm rupture likely results from GA catabolism being slightly higher than the light-induced synthesis. However, the results of the PAC treatments should be interpreted with caution, because PAC treatment may result in effects other than the inhibition of GA biosynthesis; for example, the reduction of ethylene formation in pineapple, and of brassinosteroids and flavonoids that require the PAC-inhibited cytochrome P_{450}-type monoxygenases (Min and Bartholomew, 1996; Rademacher, 2000). However, subsequent application of GA_3 after smoke and PAC treatment to non-germinated seeds resulted in 100% germination after 2 d, indicating that GA was the limiting factor for germination.

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Of the bioactive GAs, only GA_1 and GA_3, but not GA_4 or GA_7, were found in *N. attenuata* seeds. This is consistent with the finding that the early 13-hydroxylation pathway, which leads to GA_1 and GA_3, is the most important pathway of GA synthesis in tobacco (Vidal *et al.*, 2001). In other experiments (data not shown), we found exogenously applied GA_4 and GA_7 to induce germination 100 times more effectively than GA_1 and GA_3 applications (all at pH 3.00), a result also reported for lettuce and tomato seeds (van Staden *et al.*, 1995), and to different degrees from *N. tabacum* (Hashimoto and Yamaki, 1959; Leubner-Metzger *et al.*, 1996). Since GA_1 and GA_3 differ from GA_4 and GA_7 by one additional hydroxyl group, the differences in activity may reflect differences in uptake due to differences in polarity.

GA content in water-treated seeds also exhibited a dynamic consistent with GA signalling, but in these seeds germination did not occur. Different mechanisms may prevent germination in these water-imbibed seeds. First, the sensitivity towards GA may still be reduced, and/or GA effects may be suppressed by the increased ABA, which, in turn, may result from activation of previously stored conjugates of ABA, as reported in *Fagus sylvatica* (Le Page-Degivry *et al.*, 1997). Specifically, the inhibition of transcription of a β-1,3-glucanase in tobacco (Leubner-Metzger and Meins, 2000) may contribute to the maintenance of dormancy of these water-imbibed seeds. The significant decrease in ABA content in smoke-treated seeds of *N. attenuata* is consistent with findings in GA-treated germinating *N. plumbaginifolia* seeds (Grappin *et al.*, 2000). This suggests that reduction of ABA is necessary to initiate germination and, in particular, to initiate endosperm weakening, and is consistent with previous results with germinating fluridone-treated *N. attenuata* seeds. Furthermore, reduced embryo sensitivity to ABA may also play a role, as has been shown in yellow cedar seeds (Schmitz *et al.*, 2000). However, ABA alone is not sufficient to completely inhibit germination of *N. attenuata,* because the endosperm swells and breaks through the testa (due to the expansion of the embryo) in ABA- and smoke-treated seeds (Krock *et al.*, 2002). This indicates that ABA primarily inhibits at least a part of endosperm weakening and that other regulators suppress embryo growth. A recently described putative transcription factor of the DELLA family, RGL2, which is a negative regulator of GA, may be an interesting candidate for future work. In *Arabidopsis* this factor is up-regulated after imbibition in both dormant and germinating seeds, and down-regulated by GA during germination, while it remains elevated in dormant seeds (Lee *et al.*, 2002). Moreover, *rgl2* was able to suppress the GA-deficient non-germinating *ga1–3* phenotype (Lee *et al.*, 2002).

The natural regulation of germination and dormancy of *N. attenuata* is a complex network of responses to different environmental cues. Seeds in nature not only promote dormancy by producing ABA, but are exposed to ABA leached from leaves of their competitors and, additionally, respond to terpenes to enforce secondary dormancy, even when smoke is present (Krock *et al.*, 2002). These terpenes inhibit germination without eliciting increases in endogenous ABA contents in the seeds, indicating an ABA-independent mechanism underlying inhibition. Terpene treatment might suppress embryo growth, because the endosperm of terpene-exposed seeds does not erupt through the testa after smoke treatment. Whether terpenes directly affect GA signalling, in particular by blocking GA receptors, remains unknown.

In summary, the amounts of GA and ABA in *N. attenuata* seeds have been shown to exert a powerful influence on seed development after imbibition. Increased ABA is associated with prolonged dormancy and declines rapidly after smoke exposure. Smoke-treated seeds differ from water-treated seeds in their GA dynamics in the initial stages of germination, declining at first but then increasing for a short period. Smoke-induced germination of *N. attenuata* seeds is associated with three different events in the ABA/GA dynamics of a seed. The first is most likely a rapid change of GA sensitivity induced by smoke. The second is a continuous production of GA, which is required for at least 12 h to complete germination. The third is a large reduction of ABA content. The maintenance of dormancy after imbibition, on the other hand, requires increased ABA, and also requires an additional unknown mechanism that may function at a transcriptional level, according to terpene treatments. However, the fact that hormonal changes associated with germination and dormancy occur during the first 2 h after treatments, may indicate that these processes are not primarily regulated on a transcriptional level. Our results correspond to the revised hormone-balance hypothesis for seed dormancy proposed by Karssen and Laçka (1986), which states that ABA and GA act at different times and sites during seed development. Here, ABA maintains dormancy after imbibition and GA promotes germination. A decline in ABA content, decreased sensitivity to ABA and increased sensitivity to GA are involved in loss of dormancy in many species (Hilhorst, 1995; Li and Foley, 1997; Debeaujon and Koornneef, 2000; Grappin *et al.*, 2000), which seem to occur in a very short time in 'smoke-dormant' *N. attenuata* seeds.

How the different environmental cues act chemically, and whether they activate GA receptors directly, are involved in the initiation of ABA decreases and increases, or if they activate phytochrome signalling, remain open questions.

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However, it is clear that the two dueting phytohormones, GA and ABA, mediate the ability of the seed to react quickly to its environment and recognize accurately environmental signals that portend ecological conditions favourable for successful germination and growth.

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