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Title: A potential role for endogenous microflora in dormancy release, cytokinin metabolism and the response to fluridone in *Lolium rigidum* seeds

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Running title: Involvement of seed bacteria in mediating dormancy

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ABSTRACT

- *Background and aims* Dormancy in *Lolium rigidum* seeds can be alleviated by warm stratification in the dark or by application of fluridone, an inhibitor of plant ABA biosynthesis via phytoene desaturase. However, germination and absolute ABA concentration are not particularly strongly correlated. The aim of this study was to determine if cytokinins of both plant and bacterial origin are involved in mediating dormancy status and in the response to fluridone.
- *Methods* Seeds with normal or greatly decreased (by dry heat pre-treatment) bacterial populations were stratified in the light or dark and in the presence or absence of fluridone in order to modify their dormancy status. Germination was assessed and seed cytokinin concentration and composition was measured in embryo-containing or embryo-free seed portions.
- *Key results* Seeds lacking bacteria were no longer able to lose dormancy in the dark unless supplied with exogenous gibberellin or fluridone. Although these seeds showed a dramatic switch from active cytokinin free bases to *O*-glucosylated storage forms, the concentrations of individual cytokinin species were only weakly correlated to dormancy status. However, cytokinins of apparently bacterial origin were affected by fluridone and light treatment of the seeds.
- *Conclusions* It is probable that resident microflora contribute to dormancy status in *L. rigidum* seeds via a complex interaction between hormones of both plant and bacterial origin. This interaction needs to be taken into account in studies on endogenous seed hormones or the response of seeds to plant growth regulators.

Key words: bacteria, cytokinins, fluridone, germination, *Lolium rigidum*, microflora, seed dormancy

INTRODUCTION

Lolium rigidum (annual ryegrass) is used as a pasture plant but is also a major weed in many regions of the world, particularly those with Mediterranean-type climates (Kloot, 1983). Like many annual weeds, one reason for the persistence of *L. rigidum* is the dormancy of its seeds at shedding, which allows germination to be staggered over a period of months and ensures that at least part of the population encounters conditions favourable for seedling establishment (Batlla & Benech-Arnold, 2007). Dormancy in mature, imbibed *L. rigidum* seeds is released by incubation in warm, dark conditions (known as dark-stratification), a process which is inhibited by the green and blue wavelengths of light (Goggin *et al.,* 2008). During dark-stratification, seed sensitivity to abscisic acid (ABA), a major hormonal mediator of seed dormancy, is decreased (Goggin *et al.,* 2009). This allows the seeds to subsequently respond to the germination stimuli of light and alternating temperature (Steadman 2004).

ABA is synthesised in plants via oxidative cleavage of carotenoids (Nambara & Marion-Poll, 2005). Although the key regulatory step of ABA biosynthesis is the cleavage of violaxanthin and neoxanthin (C_{40}) to xanthoxin (C_{15}) by the enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED) (Han *et al.,* 2004), ABA biosynthesis can be prevented by inhibiting upstream steps of carotenoid synthesis. In the plastid membranes, phytoene desaturase (PDS), in concert with ξ-carotene desaturase, converts the colourless carotenoid precursor 15-*cis*-phytoene to the red-coloured all-*trans* lycopene (Farré *et al.,* 2010). Herbicides such as fluridone (1-methyl-3-phenyl-5-[3-trifluoromethylphenyl]-4-[1*H*]-pyridinone), diflufenican (*N*-[2,4-difluorophenyl]-2-[3-trifluoromethylphenoxy]-3-pyridine-carboxamide) and norflurazon (4-chloro-5-methylamino-2-[3-trifluoromethylphenyl]-3-[2*H*]-pyridazinone) bind non-competitively to PDS and prevent

this conversion, leading to an accumulation of phytoene and a lack of coloured carotenoids (Chamovitz *et al.,* 1993).

The role of ABA in seed dormancy and the inhibition of germination has been widely studied using fluridone to inhibit carotenoid biosynthesis, thereby decreasing endogenous ABA concentrations, in both developing and mature imbibed seeds (e.g. Grappin *et al.,* 2000; Feurtado *et al.,* 2007 and references therein; Martínez-Andújar *et al.,* 2011). However, in seeds of *Sorghum bicolor* (Benech-Arnold *et al.,* 1999), *Orobanche minor* (Chae *et al.,* 2004) and *L. rigidum* (Goggin *et al.,* 2009), fluridone had the expected stimulatory effect on germination, but with no significant alteration in seed ABA concentration. Additionally, changes in dormancy in *L. rigidum* seeds appear to be associated with changes in seed sensitivity to ABA (and the germination-stimulating gibberellins), rather than to absolute ABA concentrations (Goggin *et al.,* 2009; 2010). Therefore, it is possible that fluridone also acts on other pathways regulating seed dormancy/germination.

Until recently, cytokinins (CK), phytohormones synthesised by plants and microorganisms, have been neglected in studies on seed dormancy and germination. The major, biologically active CK are isopentenyladenine (iP) and its hydroxylated derivatives *cis*-zeatin (cZ) (Gajdošová *et al.,* 2011) and *trans*-zeatin (tZ), but conjugation to a range of substrates results in a complex CK modification profile in many organisms (Frébort *et al.,* 2011; Spíchal 2012). Plants contain predominantly sugar conjugates of cZ and tZ (Kuroha *et al.,* 2009), whilst free iP, cZ and tZ, and their methylthiolated derivatives, are found in bacteria (Pertry *et al.,* 2009). Wang *et al.* (2011) demonstrated that there is an antagonistic interaction between ABA- and CK-regulated

transcription factors during seed germination in *Arabidopsis*. Repeated selection of *L. rigidum* seeds for constitutively very low and high dormancy levels resulted in changes in the balance between the CK free base cZ and its less-active riboside derivative in the mature seeds (Goggin *et al.*, 2010); and active regulation of CK composition has been observed in germinating seeds of lucerne, oat and maize (Stirk *et al.*, 2012*b*).

Cytokinin free bases have been shown to stimulate seed germination (e.g. Pence *et al*., 2006 and references therein), and it is possible that endogenous bacteria living within seed tissues are a major source of free CK. For example, treatment of seeds with cultures of the seed-transmitted bacteria that seem to colonise all tissues of higher and lower plants (pink-pigmented facultative methylotrophs) resulted in a stimulation of germination in a range of species (Holland & Polacco, 1994); however, there is conflicting evidence as to whether the effect was due to bacterial CK production (Koenig *et al*., 2002; Lee *et al*., 2006). The aim of the current study was to determine if seed microflora play a role in the response of *L. rigidum* seeds to dormancy-modifying treatments such as fluridone (releases dormancy) and blue light (inhibits dormancy release), and whether this involves changes in CK metabolism. The dormancy-releasing efficacy of two other compounds inhibiting ABA biosynthesis, diflufenican and naproxen ([*S*]-6-methoxy-α-methyl-2-naphthalene acetic acid, an inhibitor of NCED: Matusova *et al.*, 2005), was also compared with that of fluridone.

MATERIALS AND METHODS

Chemicals

Chemicals were purchased from Sigma-Aldrich (Sydney, Australia) unless otherwise specified.

Seed material and dry heat pre-treatment

Dormant *Lolium rigidum* seeds collected in 2007 from a wild population infesting a wheat field in the Western Australian grain belt were used for all germination tests and metabolite analyses. This population was characterised in Goggin *et al.* (2009); briefly, the seeds do not germinate under optimal germination conditions (25/15°C with a 12 h photoperiod of cool white fluorescent light at 90 μmol m^{-2} s^{-1} over 400 – 700 nm), but require 21 d dark-stratification at 20°C to release dormancy and enable them to then respond to germination stimuli. Stratification under blue, green or white light inhibits dormancy release. The moisture content of the mature seeds was 7% (w/w), which is low enough for them not to be adversely affected by a mild heat treatment.

In order to determine if endogenous seed microflora play a role in mediating seed dormancy or in the response to fluridone, the bacterial population in dry seeds was greatly reduced by heating in a 50°C oven for 48 h (Holland & Polacco, 1992). To confirm the success of the heat treatment, subsamples of 10 heated and unheated seeds were homogenised in 1 ml sterile water in a sterile mortar and pestle and the homogenates streaked onto non-selective LB medium (Bertani, 1951) solidified with 1.5% (w/v) agar and containing 30 μg ml^{-1} cycloheximide to inhibit fungal growth (Ryu *et al*., 2006). Plates were incubated at 28°C for 3 d, and three independent experiments were performed on different batches of heated and unheated seeds.

Germination in the presence of putative ABA biosynthesis inhibitors or gibberellin

Unheated seeds were sown on 1% (w/v) agar containing 50 μM fluridone, diflufenican or naproxen. A control treatment of 0.1% (v/v) dimethylsulphoxide, equivalent to the amount of solvent added with the test compounds, was also included (Goggin *et al.*, 2009). Seeds were then incubated under one of three stratification conditions for 21 d: non-stratified seeds were placed directly into optimal germination conditions; dark-stratified seeds were incubated at 20°C in the dark; and light-stratified seeds were incubated under blue (465 nm) LED light at 20 μmol m^{-2} s^{-1} (see Goggin *et al.*, 2008 for further details) at 20°C. All seeds were then transferred to optimal germination conditions for a further 7 d. At the end of this time, visible germination, defined as protrusion of ≥1 mm of radicle from the seed coat, was counted, with dead or empty seeds (i.e. those collapsing under gentle pressure at the conclusion of the experiment) being excluded from calculations. Each treatment consisted of four independent replicates of 50 seeds and in all cases, dead or empty seeds accounted for ≤3% of the total number of seeds.

Similar experiments were performed to investigate the effect of 50 μM fluridone or 10 μM gibberellin A_4 (GA_4) on both unheated and heat-treated seeds.

Effect of fluridone on seed hormones

Unheated and heat-treated seeds were stratified at 20°C for 21 d in the dark or under blue light in the presence or absence of 50 μM fluridone and then used for hormone analysis. ABA and CK measurements were performed on seeds that had been dissected into embryo-containing (top third) and embryo-less (bottom two-thirds) segments, using *c*. 200 seeds per sample with four replicates per treatment. Seeds were dissected on ice, snap-frozen in liquid nitrogen and freeze-

dried. ABA and CK were extracted and analysed by liquid chromatography-mass spectrometry according to Ross *et al.* (2004) and Quesnelle and Emery (2007), respectively.

Statistical analyses

Measurements of germination and metabolites were analysed by one- and two-factor ANOVA at the 5% level of significance. Differences between means were assessed using the least significant difference test. A random forest analysis (Liaw and Wiener, 2002) was performed to assess the contribution of ABA and each CK species to seed germination under all treatments, using the 'randomForest' package in R v3.0.2 (R Core Team 2013). The default settings in R were used except that the number of trees was increased to 10 000; the percent increase in mean squared error was used as the importance measure.

RESULTS

Heat-treatment of Lolium rigidum *seeds reduces their bacterial population*

LB plates streaked with homogenates from dry *L. rigidum* seeds heated at 50°C for 48 h had very low bacterial growth compared to the unheated-seed controls **[Supplementary Data Fig. S1].** It should be noted that although *Lolium* seeds are also often infected with fungal endophytes (Gundel *et al.,* 2006), the above heat treatment is unlikely to have affected the fungal population: Welty *et al.* (1987) demonstrated that dry heating of *L. perenne* seeds at 60°C for 18 weeks only decreased the viable endophyte population by 50%, and that a treatment of 50°C for 48 h would decrease endophytes by only one or two percentage points. In any case, fungal endophytes in *Lolium* have little impact on seed dormancy or germination (Gundel *et al.,* 2006).

Unheated and heat-treated seeds respond differently to stratification treatments

Confirming previous results (Goggin *et al.,* 2009), dark-stratification of unheated seeds resulted in release of dormancy whilst blue light-stratified seeds remained dormant, and fluridone stimulated germination under all stratification conditions (Fig. 1A). GA_4 caused a higher relative stimulation of germination in non-stratified (4-fold relative to the control) and light-stratified (2-fold) seeds compared to seeds stratified in the dark (1.2-fold stimulation) (Fig. 1A).

In contrast, heat-treated, dark-stratified seeds were unable to lose dormancy in the dark, although the basal germination level (i.e. of heat-treated, non-stratified control seeds) was not affected by the heat treatment (Fig. 1A). Fluridone and GA_4 were able to restore germination in heat-treated, dark-stratified seeds and also stimulated germination in the non-stratified and light-stratified heat-treated seeds (Fig. 1A).

Although fluridone had the expected 2- to 3-fold stimulatory effect on the germination of unheated seeds, the other putative ABA synthesis inhibitors did not. Diflufenican had no effect on non-stratified seeds and a slight stimulatory effect on dark- or light-stratified seeds, whilst naproxen had no effect on non- or dark-stratified seeds and inhibited germination of light-stratified seeds (Fig. 1B).

Fluridone affects ABA concentration in the embryo, regardless of heat treatment

Heat treatment had no effect on ABA concentration in the embryo-containing portion of stratified seeds, but 50 μM fluridone caused a 2- to 4-fold decrease under all conditions (Fig. 2A). In the embryo-less seed portions, neither fluridone nor heat treatment had an effect on ABA

concentration, except that in unheated, dark-stratified control seeds, endosperm ABA was 2-fold higher than in (1) the corresponding heated seeds and (2) the unheated, dark-stratified seeds that had been treated with fluridone (Fig. 2B). Similar to previous results (Goggin *et al.*, 2009), there was not a particularly strong relationship between germination and ABA concentration in the embryo-containing seed portion (Fig. 3A), with the linear correlation coefficient (r^2) being 0.4. Overall, as calculated by the random forest analysis, ABA and CK concentration explained 62% of the variation in germination. Ranking of the importance measures (percent increase in mean squared error) for each measured hormone showed that ABA had more influence over germination than any of the CK species **[Supplementary Data Fig. S2]**.

As naproxen unexpectedly inhibited germination in light-stratified seeds (Fig. 1B), ABA was also measured in unheated seeds that were dark- or light-stratified in the presence or absence of 50 μM naproxen. In this case, the inhibition of germination in light-stratified seeds by naproxen corresponded to a 3.5-fold increase in ABA in the embryo-containing portion of the seeds (Fig. 3B).

Changes in seed CK composition due to heating

The CK concentration in dry (unheated) seeds was around 3-fold higher than in the unheated stratified seeds (Table 1) and the predominant species were dihydrozeatin (DZ) and its *O*-glucosylated riboside (DZROG) **[Supplementary Data Table S1]**. Heating of seeds before imbibition did not affect total imbibed CK concentration in the embryo-containing portion of the seeds but caused a 2-fold decrease in the embryo-less portion, and greatly changed CK composition in both tissues (Table 1). Figure 4 shows the fold-changes in CK concentrations in

the embryo-containing and embryo-less portions of stratified seeds relative to the concentrations in the corresponding portions of unheated, dark-stratified control seeds. The free bases *trans*-zeatin (tZ) and isopentenyladenine (iP) were rendered undetectable by heat treatment, as was the nucleotide of DZ (DZNT) (Fig. 4) and the *O*-glucosides of DZ and DZR, whilst DZ itself was dramatically decreased but still detectable **[Supplementary Data Table S1]**. *cis*-Zeatin nucleotide (cZNT) was undetectable in the embryo-containing tissue of heat-treated seeds, and decreased >2-fold relative to unheated seeds in the embryo-less portion (Fig. 4). The riboside and nucleotide of tZ (tZR and tZNT, respectively) were undetectable in dark-stratified seeds following the heating pre-treatment, but their concentration was increased ≥3-fold in the corresponding blue light-stratified seeds (Fig. 4). A similar pattern was seen for the *O*-glucosylated riboside of tZ (tZROG), but only in the embryo-less portion of the seeds, and a light-mediated >2-fold increase of the riboside of iP (iPR) was also observed in all tissue from heat-treated seeds (Fig. 4). Heat treatment caused an increase in levels of methylthiol-zeatin (MeSZ) in the embryo-containing tissue, especially under blue light; in contrast, heating resulted in a decrease in methylthiol-iP (MeSiP) to below detectable levels (Fig. 4). Most remarkably, heat treatment caused a massive increase in the *O*-glucosylated riboside of *cis*-zeatin (cZROG), ranging from 5-fold in embryo-less portions of blue light-stratified seeds to 54-fold in the embryo-containing portions of dark-stratified seeds (Fig. 4). The absolute concentrations of these and the other CK species detected in dry and stratified seeds are given in **Supplementary Data Table S1**.

Changes in seed CK composition due to blue light and/or fluridone

The effects of fluridone and dark- or light-stratification upon seed CK composition were not as marked as those of the heat treatment, and varied widely between CK species and seed tissue type **[Supplementary Data Table S1]**. Only those which showed a ≥2-fold change in response to the light or fluridone treatment (either in unheated seeds, heat-treated seeds, or both) were considered to be potentially biologically significant and are discussed here. In addition to the light-induced changes in heat-treated seeds described above, blue light caused a decrease in the concentration of DZNT in the embryo-containing portions of unheated seeds, whilst fluridone caused an increase in the same tissue, but only in the dark (Fig. 4). Although the random forest analysis showed a weak overall correlation between CK concentration and germination, DZNT was the CK that had the greatest association with germination **[Supplementary Data Fig. S2]**. In the embryo-less portion of the seeds, fluridone prevented light-induced increases in the concentration of iPNT (heat-treated seeds only) and of MeSiP (unheated seeds only) (Fig. 4). Fluridone and blue light also interacted to cause a decrease in MeSZ concentration in the embryo-less portion of unheated seeds (Fig. 4).

DISCUSSION

Seed bacteria may help to mediate dormancy status

The ability of *Lolium rigidum* seeds to lose dormancy via dark-stratification was greatly impaired in the near-absence of bacteria, but in contrast to previous studies (e.g. Holland & Polacco, 1994), the basal (i.e. non-stratified) level of seed germination itself was not inhibited. The fact that exogenous gibberellin A_4 restored dormancy release in heat-treated, dark-stratified seeds suggests that gibberellin synthesis by the seed bacteria contributes to this process. Previous

attempts at measuring gibberellins in *L. rigidum* seed parts have proved unsuccessful due to their extremely low concentrations (Goggin *et al.*, 2009), so this hypothesis remains to be confirmed.

Seed ABA concentration was largely unchanged by the heating pre-treatment, so the bacterial contribution to seed ABA metabolism under the conditions of the study was likely to have been negligible. So far, there is contradictory evidence, from only a few studies, as to whether bacteria are capable of synthesising ABA at all (reviewed in Hartung, 2010). In contrast, CK composition was dramatically altered by heating, suggesting that bacteria play a major role in seed CK metabolism. There was, in this case, no strong link between seed germination ability and endogenous CK concentration or composition, similar to the findings of Stirk *et al.* (2012*a*) in *Tagetes minuta* achenes and Long *et al.* (2012) in pea seeds. However, the fact that fluridone and blue light, so effective in determining seed dormancy status, also modify certain components of the *L. rigidum* seed CK pool suggests that seed CKs (of both plant and bacterial origin) could indirectly influence dormancy and germination.

Heat treatment has the greatest effect on seed cytokinins

Dry heating of the seeds before imbibition had a much more substantial effect on the imbibed seed CK pool than light and/or fluridone, causing a decrease in some CK species to undetectable levels. This strongly suggests that these CK species, including DZNT (which disappears upon heating and was the CK which had the greatest association with germination in the random forest analysis), are maintained in imbibed seeds by bacterial metabolism, which would have been almost absent in the heat-treated seeds. The fact that the undetectable CKs also include tZ, iP and MeSiP supports this hypothesis. Bacteria tend to synthesise free bases such as tZ and iP (Morris

et al., 1993), whereas plants are capable of synthesising and accumulating *O*-glucosylated derivatives (a common reversible storage form of CK) (Kuroha *et al.,* 2009). Additionally, MeSiP has so far only been characterised in bacteria (e.g. Lexa *et al.,* 2003; Omer *et al.,* 2004; von Schwartzenberg *et al.,* 2007; Pertry *et al.,* 2009).

The dramatic increase in cZROG in heat-treated seeds, particularly in the embryo-containing portions where most of the living plant tissue is found (Fig. 4), might indicate that plant cZROG accumulation in imbibed seeds is repressed by high levels of bacterial tZ and/or that seed-synthesised cZROG is only rapidly converted to active forms of CK when bacterial activity is present in the seed. The higher concentration of MeSZ (which is potentially an early intermediate in the tRNA-derived cZ biosynthetic pathway) in the heat-treated seeds could also indicate that, in seeds with a greatly depleted bacterial population, the high level of cZROG is synthesised from tRNA rather than *de novo* (Spíchal, 2012).

Interestingly, the switch from tZ (active) to cZROG (storage) as the dominant CK species in imbibed *L. rigidum* seeds following a bacteria-decreasing treatment parallels an earlier study on the ‘green island’-causing fungus *Colletotrichum graminicola* in maize leaves. Infection of senescing leaves caused a 50% decrease in the major CK present in uninfected tissue, cZOG (storage), and a concomitant increase in cZR and cZNT (precursors of active cZ) (Behr *et al.,* 2012). There is, thus, increasing evidence that plants and the microorganisms that inhabit their tissues are intricately linked in terms of hormone metabolism. As cited in Links *et al.* (2014), a more detailed knowledge of plant interactions with their microflora could be beneficial in terms

of improving the growth and disease resistance of agricultural crops; or to help control weeds such as *L. rigidum*.

A possible role for blue light and fluridone in mediating CK metabolism

The CK pool in heat-treated seeds was more responsive to blue light than in unheated seeds, suggesting that at least some aspects of plant CK metabolism are mediated by light. This is illustrated by the fact that precursor and storage forms of tZ, which were absent (along with free tZ itself) in the embryo-containing portions of heat-treated, dark-stratified seeds, were present at higher concentrations in heat-treated, light-stratified seeds than in their unheated counterparts (Fig 4; **Supplementary Data Table S1**). This difference was further increased by treatment with fluridone. Therefore, plant synthesis of *trans*-CK is apparently light-dependent and can be stimulated by fluridone via an unknown (direct or indirect) mechanism. Concentrations of iPR were not affected by fluridone but were increased by light in both tissues of the heat-treated seeds, suggesting that plant synthesis of iP-type CK is also stimulated by light. There is limited information as to the effect of light on CK homeostasis in green organisms. Cytokinin concentration and composition in a shade-adapted prairie plant was responsive to light intensity and red/far-red ratio (Kurepin *et al.,* 2012), and CK synthesis (particularly cZNT) was stimulated in *Chlorella* cells during exposure to white light (Stirk *et al.,* 2011). Plant responses elicited by both blue light and CK were found to occur via either independent (e.g. photomorphogenesis) or intersecting (e.g. anthocyanin synthesis) signalling pathways, but the effect of blue light on CK metabolism itself has not been investigated so far (Vandenbussche *et al.,* 2007).

MeSiP levels in the embryo-less portion of unheated seeds were increased 3-fold by stratification in the light (Fig. 4) and the presence of fluridone prevented this increase. However, this interesting response is unlikely to be directly important in determining germination, at least in *L. rigidum*, as indicated by the random forest analysis **[Supplementary Data Fig. S2]**. The genes encoding iP methylthiolation activity in bacteria are yet to be characterised (Frébort *et al.*, 2011), and it is unknown whether fluridone directly affects this activity, or whether inhibition of MeSiP synthesis is an indirect result of the effect of fluridone on other pathways.

Differential effects of putative ABA biosynthesis inhibitors on seed germination

It appears from the CK analysis that fluridone has only subtle effects on seed CK metabolism, and thus its precise mode of action in breaking seed dormancy in *L. rigidum* remains obscure. The efficacy of fluridone in breaking *L. rigidum* seed dormancy was far greater than that of two other inhibitors of ABA biosynthesis, diflufenican (also an inhibitor of PDS) and naproxen (which theoretically inhibits the rate-limiting step of ABA biosynthesis), which had little to no stimulatory effect on germination: naproxen actually inhibited germination of light-stratified seeds whilst dramatically and unexpectedly increasing the seed ABA concentration. Although the results of pharmacological studies must always be interpreted with caution, the different effects of the two PDS inhibitors, fluridone and diflufenican, on seed dormancy suggests that inhibition of PDS activity may not be the only mode of action of fluridone in breaking dormancy, especially since ABA concentration (decreased by inhibition of PDS) is not strongly correlated to germination in some grass species (Benech-Arnold *et al.*, 2003; Gianinetti & Vernieri 2007; Goggin *et al.*, 2009 and current study). The unexpected inhibitory activity of naproxen towards germination could be due to the fact that it is an inhibitor of human

cyclooxygenase (McGettigan & Henry, 2000) and thus might also interfere with the activity of the structurally-similar 70 kDa subunit of plant α-dioxygenase (Sanz *et al.,* 1998), which plays a role in fatty acid α-oxidation and is highly active during seed germination (Meisner *et al.,* 2009).

In summary, seed microflora may contribute to the mediation of dormancy in imbibed *L. rigidum* seeds. This adds another level of complexity to the process of seed dormancy release and its inhibition in this species. The understanding that is gradually emerging suggests that: (1) sensitivity to ABA is more important than absolute ABA concentration (Goggin *et al.,* 2009 and current study), although ABA concentration is a greater determinant of dormancy than CK concentration; (2) sensitivity to gibberellins increases in the dark (Goggin *et al.,* 2010) and bacterial gibberellin synthesis could potentially contribute to dark-mediated dormancy release (although this requires confirmation); (3) bacterially-derived CK species, with DZNT as a possible prime candidate, interact with the other plant growth regulators in mediating dormancy, and their metabolism can be affected by light and fluridone. A detailed study of the mode of action of fluridone in regulating CK metabolism would likely yield important information on the interaction between different signal transduction pathways.

SUPPLEMENTARY DATA

Supplementary data consist of: Fig. S1: the effect of dry heat treatment on endogenous bacteria in *Lolium rigidum* seeds, shown by streaking seed homogenate onto non-selective growth medium under sterile conditions; Fig. S2: the output from the random forest analysis showing the relative importance of ABA and CK to germination; and Table S1: concentrations of all CK species detected in unheated and heat-treated *L. rigidum* seeds (separated into embryo-containing

and embryo-less portions) after stratification in the dark or light and in the presence or absence of fluridone.

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FIGURE CAPTIONS

Fig. 1**.** Effect of dry heat pre-treatment (50°C for 48 h) and plant growth regulators on *Lolium rigidum* seed germination. (A) Germination of unheated and heat-treated seeds when imbibed on agar containing no plant growth regulators (Control), 50 μM fluridone (+F) or 10 μM GA_4 (+GA_4), either directly under germination conditions of 25/15°C with a 12 h photoperiod (Non-stratified) or incubated for 21 d in the dark (Dark-stratified) or under blue light (Light-stratified) at 20°C before being transferred to germination conditions. (B) Germination of unheated seeds when imbibed on agar containing no ABA synthesis inhibitors (Control), 50 μM fluridone (+F), 50 μM diflufenican (+DFF) or 50 μM naproxen (+NAP) and stratified as in (A). Values are means ± s.e. (n = 4), with each replicate consisting of 50 seeds. Different letters above bars denote significant (P<0.05) differences between means.

Fig. 2. Effect of fluridone on *Lolium rigidum* seed ABA concentration. ABA concentrations were measured in unheated and heat-treated seeds that were stratified for 21 d in the dark or under blue light at 20°C in the presence (+F) or absence (-F) of 50 μM fluridone. Embryo-containing (A) and embryo-less (B) seed portions were analysed separately. Values are means ± s.e. (n = 4), with each replicate consisting of 200 seeds. Different letters above bars denote significant (P<0.05) differences between means.

Fig. 3. Correlation between hormone concentration and seed germination. (A) The ABA concentration data (individual replicates) from Fig. 2A was plotted against seed germination under the corresponding treatment (shown in Fig. 1A). (B) ABA concentrations were measured in the embryo-containing portions of unheated seeds that were stratified in the dark or light in the presence (+NAP) or absence (-NAP) of 50 μM naproxen. Values in (B) are means ± s.e. (n = 4), with each replicate consisting of 200 seeds. Different letters above bars denote significant (P<0.05) differences between means. Numbers within the bars denote the seed germination under each stratification treatment as shown in Fig. 1B.

Fig. 4. Changes in *Lolium rigidum* seed cytokinin (CK) concentrations due to dry seed heating and stratification. Concentrations of *trans*-zeatin (tZ), isopentenyl adenine (iP), dihydrozeatin nucleotide (DZNT), *cis*-zeatin nucleotide (cZNT), *trans*-zeatin riboside (tZR), *trans*-zeatin nucleotide (tZNT), *O*-glucosylated tZR (tZROG), iP riboside (iPR), methylthiol-zeatin (MeSZ), methylthiol-iP (MeSiP), *O*-glucosylated *cis*-zeatin riboside (cZROG) and iP nucleotide (iPNT) were measured in unheated and heat-treated seeds that were imbibed for 21 d in the dark (D) or under blue light (L) at 20°C in the presence (+F) or absence (-F) of 50 μM fluridone. Embryo-

containing and embryo-less seed portions were analysed separately. CK concentrations in the seed tissues are expressed relative to the concentration in the unheated D–F tissues (n = 4 replicates of 200 seeds; s.e. bars shown). Different letters above bars denote significant (P<0.05) differences between the absolute mean CK concentrations as given in **Supplementary Data Table S1**; bars marked with upper-case letters were analysed separately from those marked with lower-case letters.

Table 1. *Changes in imbibed* Lolium rigidum *seed cytokinin composition in response to heat pre-treatment*

	CK in unheated dry seeds (pmol g^{-1} dwt)		CK in stratified seeds (pmol g^{-1} dwt)			
	+emb	**-emb**	**Unheated +emb**	**Heated +emb**	**Unheated -emb**	**Heated -emb**
Bases	210 ± 9^{b}	227 ± 17^{a}	70 ± 3^{c}	4.9 ± 0.6^{d}	70 ± 3^{c}	4.6 ± 0.3^{d}
Ribosides	70 ± 2^{a}	43 ± 1^{b}	6.7 ± 0.3^{d}	8.2 ± 1.8^{d}	6.9 ± 0.4^{d}	11 ± 1^{c}
NT	53 ± 5^{a}	31 ± 1^{b}	33 ± 2^{b}	10 ± 2^{d}	20 ± 1^{c}	7.3 ± 1.8^{d}
MeS	17 ± 2^{bc}	19 ± 0^{bc}	19 ± 1^{c}	49 ± 6^{a}	24 ± 3^{bc}	31 ± 3^{b}
***O*G**	190 ± 12^{a}	100 ± 9^{b}	38 ± 2^{c}	101 ± 13^{b}	14 ± 1^{d}	11 ± 1^{d}
Total	541 ± 22^{a}	422 ± 26^{b}	165 ± 7^{c}	172 ± 8^{c}	134 ± 6^{d}	65 ± 4^{e}

Seeds (heated or unheated) were stratified at 20°C for 21 d before being separated into embryo-containing (+emb) and embryo-less (-emb) seed portions, and cytokinins (CK) measured in each portion. Data for the different stratification treatments (dark or light, presence or absence of 50 μM fluridone) were combined; values are means ± s.e. (n = 4 replicates of 200 seeds) and significant differences ($P<0.05$) between treatments are signified by different letters across rows. NT, nucleotides; MeS, methylthiols; *O*G, *O*-glucosides.