

β -Glucuronidase activity in seedlings of the parasitic angiosperm *Cuscuta pentagona*: developmental impact of the β -glucuronidase inhibitor saccharic acid 1,4-lactone

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Abstract. Endogenous plant β -glucuronidase (β -GUS) activity was detected in germinating seeds, seedlings, stems, flowers and haustoria of the parasitic angiosperm *Cuscuta pentagona* Engelm. *In vitro* characterisation of this activity showed it to have an acidic pH optimum, similar to previously characterised plant activities, and a sensitivity to the β -GUS inhibitor saccharic acid 1,4-lactone (SAL). Application of SAL to seeds immediately after chemical scarification resulted in a significant developmental delay and, frequently, in the total arrest of seedling growth. In contrast, application of SAL subsequent to the emergence of the radicle did not produce a significant effect on the development of the seedling. Thus, the distribution of activity and the developmentally contingent potency of SAL in inhibiting growth suggest a role for β -GUS at an early stage of seed germination or seedling growth. Further, the inability of the inhibitor to prevent subsequent shoot elongation suggests that at least some plant growth processes do not require this activity, or that it is required only at minimal levels and is unaffected by the application of SAL.

Additional keywords: germination, glycosyl hydrolase, seedling development.

Introduction

Cuscuta pentagona Engelm, a representative of the genus commonly known as the dodders, is a leafless, rootless, epiphytic parasite. Although apparently derived from the Convolvulaceae (Neyland 2001; Stephanovic and Olmstead 2004) it has a strikingly altered habit comprising a suite of adaptations, including the hallmark of plant parasites, host-penetrating haustoria. Emerging from seed, the dodder seedling uses environmental cues, such as phytochemical emissions (Runyon *et al.* 2006) and light quality (Furuhashi *et al.* 1995; Haidar *et al.* 1997), to seek potential host plants. Through a combination of nutation and directional growth, the seedling twines around any object it encounters. After contacting most plant tissues, including its own, dodder uses secretions to adhere to the target, subsequently breaching the host surface with penetrating haustoria that grow into an association with the host vascular tissues (Lee and Lee 1989; Vaughn 2002, 2003, 2006). It is from the host phloem that the parasite appears to draw most of its fixed carbon and nitrogen (Jeschke *et al.* 1994), and there is evidence for the transport of macromolecules, such as proteins (Haupt *et al.* 2001) and nucleic acids (Roney *et al.* 2007), from the host.

In the process of establishing parasitic connections to its host, dodder uses a battery of hydrolytic enzymes, primarily cell-wall-modifying glycosyl hydrolases (Fry 2004), which have been observed directly through their activities (Nagar *et al.* 1984) or indirectly through their structural consequences during

host-tissue invasion (Vaughn 2003). Further, dodder appears to induce hydrolytic activities within its host (Nagar *et al.* 1984; Albert *et al.* 2004).

Among the several glycosyl hydrolase activities identified in plants (Fry 2004) is β -glucuronidase (β -GUS). β -GUS is an activity familiar to many plant biologists because of the use of the *Escherichia coli* β -GUS gene *uidA* as a reporter for gene expression studies (Jefferson *et al.* 1986, 1987). The occurrence of endogenous plant β -GUS activity was reported more than half a century ago by Levvy (1954). Since then, however, only a handful of papers have addressed the characterisation (Schulz and Weissenbock 1987; Alwen *et al.* 1992; Hodal *et al.* 1992; Shi *et al.* 1995) or the physiological function (Morimoto *et al.* 1998; Sasaki *et al.* 2000) of this activity in plants. Among these reports, a frequent concern was the specific identification and elimination of endogenous β -GUS activities to avoid the misinterpretation of β -GUS reporter gene studies (Alwen *et al.* 1992; Hodal *et al.* 1992). More recently, attention has been given to the physiological role of the endogenous plant β -GUS. A recent study by Sudan *et al.* (2006) used a pharmacological inhibitor of β -GUS, saccharic acid 1,4-lactone (SAL) and demonstrated that its application to seedlings of model plant species, such as *Nicotiana tabacum*, resulted in the arrest of growth and the inhibition of root-hair development.

The anatomy and development of *Cuscuta* spp. provide a distinct contrast to the several model plants examined by

Sudan *et al.* (2006). Embedded in endosperm tissue, the embryo is coiled and lacks cotyledons, possessing only leaf scales. Furthermore, dodder's embryonic root (sometimes termed a radicular appendage) lacks an apical meristem and is ephemeral, briefly storing translocated endosperm resources from the seed and subsequently exporting them to the rapidly elongating shoot, after which this organ senesces (Kuijt 1969). Based on this distinct anatomy, it has been posited that the seeds of *Cuscuta* spp. contain not embryos, but seedlings (Rao and Rama Rao 1990). Because of their general lack of pigmentation and unobscured apical meristem, *Cuscuta* spp. have been proposed as an alternative system for developmental studies that would be difficult in other model plant systems (Deeks *et al.* 1999). Noting that *C. pentagona*'s adopted habit represents a striking departure from that of non-parasitic plants, even in the embryonic stages, we undertook to determine whether the occurrence of β -GUS was comparable to, or distinct from, that noted in other model systems, and to determine, through the use of a pharmacological inhibitor, whether its occurrence was of physiological consequence in growth and development.

Materials and methods

Plant materials

Cuscuta pentagona Engelm was maintained as a glasshouse colony on clonal cuttings of ornamental *Coleus* spp. Seeds were collected from dried fruits and stored at room temperature until use. *C. pentagona* seeds were chemically scarified by soaking for 2 h in concentrated sulfuric acid. Seeds were subsequently washed repeatedly with distilled water until the pH of the seed suspension was neutral. The pigmented outer seed coat was removed by rubbing, leaving the inner, unpigmented layer of the seed coat. Seeds for *in vitro* growth experiments were maintained in flasks of distilled water on an orbital shaker or in multi-well microtiter plates under continuous fluorescent light.

Histochemical detection of glycosyl hydrolase activities

To detect and localise glycosyl hydrolase activities *in situ*, we incubated seeds or tissues in a buffer comprising 50 mmol L⁻¹ citric acid/sodium citrate (adjusted to stated pH), 10 mmol L⁻¹ β -mercaptoethanol, 10 mmol L⁻¹ EDTA, 0.1% Triton X-100 and either 4 mmol L⁻¹ or 1 mmol L⁻¹ of the artificial substrates X-GlucA or X-Gal for the detection of β -glucuronidase or β -galactosidase activities, respectively. For experiments monitoring the impact of pH or inhibitor concentration, tissues were pre-equilibrated for 1 h before the addition of the artificial substrates. Infiltration of tissues was promoted by drawing a vacuum on the sample for 2 min. Tissues were incubated at 37°C for the indicated time and subsequently cleared of pigmentation, if necessary, using a solution comprising 60% EtOH, 10% glycerol and 5% acetic acid in water.

In vitro β -glucuronidase assay

β -GUS activity was quantified by measuring hydrolysis of the artificial substrate 4-methyl-umbelliferyl- β -D-glucuronide (MUG; Gallagher 1992). The pigmented outer seed coats of chemically scarified seeds were removed and the remaining seed mass was homogenised in five volumes (volume to mass) β -GUS extraction buffer that had the same composition as the

buffer used for *in situ* detection. The resulting homogenate was centrifuged for 20 min at 15 000g, 4°C, and the supernatant was retained for *in vitro* assays. All prepared materials were maintained at 4°C or on ice. Aliquots of the protein extract were added to nine volumes of β -GUS buffer with MUG (1 mmol L⁻¹ final reaction concentration). Aliquots (100 μ L) of this reaction were transferred to stop solution (2 mL of 0.2 mol L⁻¹ Na₂CO₃) before and after incubation at 37°C for up to 2 h. Fluorescence at 455 nm from 365 nm excitation, proportional to MU released during the reaction, was measured using a Picofluor 8000-003 fluorometer (Turner Designs, Sunnyvale, CA, USA). The protein concentration of the extracts was measured using Protein Assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Seedling growth studies

Scarified seeds were sorted and inspected under a dissecting microscope to identify and remove damaged seeds. The progression of germinating seedlings through the developmental stages (see results) was monitored at 24-h intervals by non-invasive inspection under a dissecting microscope. Final determination of seedling length was performed by allowing partial desiccation, to the point of turgor loss, which permitted straightened seedlings in a glass dish to be superimposed over a ruler and measured to the nearest millimetre. Experimental treatments of SAL or control media were applied to groups of seedlings in multi-well microtiter plates, such that the seeds or seedlings were completely immersed in the solution (2 mL total volume). Immersed seedlings were placed under a vacuum for 2 min to promote infiltration and were subsequently maintained on an orbital shaker under constant light for the duration of the experiment. Fresh media were applied on a daily basis.

Statistical analysis

Treatment effects on seedling growth and development were examined by repeated-measurement ANOVA (Winer *et al.* 1991). If the interaction between time and treatment was significant a one-way ANOVA, followed by Scheffé's multiple-comparison method (Sokal and Rohlf 1981), was used at each time point to determine any significant differences between treatments.

Results

Growth conditions for seedling experiments

Preliminary studies were performed to optimise the conditions for *in vitro* growth experiments with dodder seeds and seedlings. Chemical scarification for 2 h with subsequent mechanical removal of the outer seed coat resulted in the majority of seedlings emerging within 24–48 h of scarification. The growth of dodder seedlings was measured across a range of media pH values, in the presence of buffers, and in the presence of osmoticum. No difference in growth was observed among seedlings grown *in vitro* at pH values ranging from 4.5 to 7.0; a significant decrease in seedling growth was observed in seedlings grown at pH 4.0; seedling death occurred after 1–2 days at or below pH 3.5 (data not shown). The inclusion of buffer in the medium had the effect of slowing the rate of germination (see subsequent results). Studies examining

the impact of osmoticum and pH on *in vitro* seedling growth showed that seedlings grown in mannitol concentrations up to 100 mmol L⁻¹ did not show reduced rates of elongation relative to control seedlings in water. All subsequent experiments were performed to maximise germination uniformity, to maintain the pH within a nominal range, and to control for buffer and osmotic effects.

Histochemical detection of glycosyl hydrolase activities in dodder seedlings

Germinating dodder seeds and seedlings were incubated with the artificial substrates X-GlucA and X-Gal to observe the patterns of β -GUS and β -galactosidase, respectively. Blue colour developed within hours with each of these substrates and distinct patterns were most easily determined using seedlings that were transferred from the staining solution after 4 to 6 h.

Blue staining indicative of β -GUS activity was evident in swollen but ungerminated seeds from which the outer seed coat had been removed, and in subsequent stages of germination (Fig. 1). The anatomy of the mature seed coat of *Cuscuta* spp. has been characterised as having four distinct layers comprising a pitted epidermis, hypodermis, at least one sclerified palisade layer (varying by species) and multiple layers of crushed parenchyma (Hutchison and Ashton 1979; Lyshede 1984, 1989, 1992; Baskin and Baskin 1998). Chemical scarification of *C. pentagona* seeds resulted in the loosening and frequent shedding of all testa layers to the outside of the sclerified layer, presumably comprising the remnants of the epidermal and hypodermal layers, and subsequently here referred to simply as the 'outer seed coat'. Distinct stained domains were observed within the inner seed coat (Fig. 1A, B). These domains were regularly spaced, although occasionally overlapping domains could be identified (Fig. 1B). The shape and distribution of the stained domains matched the pattern visible through the translucent wall of an unstained seed coat after it had been shed (Fig. 1C). The additive depth of staining at the points of apparent overlap suggested that the staining was occurring in two distinct tissue layers, rather than in a single, irregularly shaped layer. Separation of the inner seed coat from the endosperm mass, in which the coiled embryo is embedded, revealed that the domains of glycosyl hydrolase activity lay just within the sclerified layers of the inner seed coat (Fig. 1D). After removal from the endosperm, these domains assumed sac-like hemispherical shapes, bulging inward and occasionally overlapping. This same pattern was observed in the stained domains of inner seed coats that had been shed by maturing seedlings (not shown). Traces of stain, shaped similarly to the pattern observed from the intact seed, were observed as spots on the freed endosperm mass, indicating that the surface of the endosperm had been in close contact with the stained domains (Fig. 1D). The layers containing these stained domains, by our best estimation, occur within the crushed parenchyma layers of the testa derived from nucellar or integumentary tissues (described by Hutchison and Ashton (1979) and Lyshede (1984) and confirmed by Gehan Jayasuriya and Carol Baskin, pers. comm.). Staining was not observed in unswollen seeds or in the pigmented layers of the outer seed coat (data not shown).

Strong staining, comparable to that seen with short-term staining of emerging seedlings, was not observed in the endosperm tissue proper, although prolonged staining (>24 h) resulted in nearly all tissues acquiring a blue tint. In contrast, the emerging seedling demonstrated a very distinct pattern of staining that varied with the stages of germination and emergence (Fig. 1A). Pre-emergent seeds stained for 4–6 h showed only staining of the seed coat and not of the embryo. However, seeds from which the radicle had emerged showed pronounced staining from the point at which the radicle emerged from the endosperm. Elongation and swelling of the root occurs over the course of a few hours following emergence. The root does not have a root apical meristem or a root cap, and growth of this organ occurs via cell expansion. Seedlings at intermediate stages of this process showed a gradual decrease in the depth of staining (Fig. 1A) that might be attributed to the overall diffusion of activity as the cells expand. Patterns of staining with X-Gal were similar to those observed with X-GlucA, including the distinct patterns within the seed coat and along the emerging radicle (results not shown).

Pre-emergent embryos were excised from swollen seeds to observe the activity-staining patterns independent of the endosperm. Remarkably, these excised embryos, when stained under identical conditions (24 h) to swollen, pre-emergent seeds, showed less staining than embryos that were stained within the seed coat and subsequently removed. While the tip of the radicle retained some staining, overall, less staining was observed under these conditions and staining was observed to occur at irregular locations on the coiled embryo (Fig. 1E). The entire embryo (within the endosperm) stained for activity after 24 h, which suggests that the radicle-localised pattern observed after short-term incubation in emergent seedlings (Fig. 1A) is in part or entirely a consequence of differential substrate infiltration. However, the reduced staining of the excised embryo compared with the embryo stained within the seed (Fig. 1E) may indicate an interaction between the embryo and the endosperm in the regulation of embryonic β -GUS activity. This is, however, in the absence of further evidence, a speculative relationship that will require further inquiry. Prolonged staining of seedlings following the shedding of the seed coat and shoot elongation revealed comparatively low levels of β -GUS activity along the seedling axis relative to the activity observed in germinating seeds and embryos (Fig. 1F). Longitudinal sectioning of the shoot before staining did not reveal additional activity that may have been masked by poor infiltration of substrate (not shown).

β -GUS in other plant organs

β -GUS staining patterns in dodder stem organs were sensitive to the degree of substrate infiltration and the time permitted for staining. Higher degrees of staining were frequently observed at cut surfaces or punctures. The possibility that this staining resulted from wound-induced activity was addressed by freeze-killing stem tissue before wounding and staining, after which the identical pattern was observed. Given these qualifications, we report the most consistently observed patterns of β -GUS staining.

Rapidly growing shoot tips, stems with haustoria and flowers were collected from glasshouse-grown vines and incubated

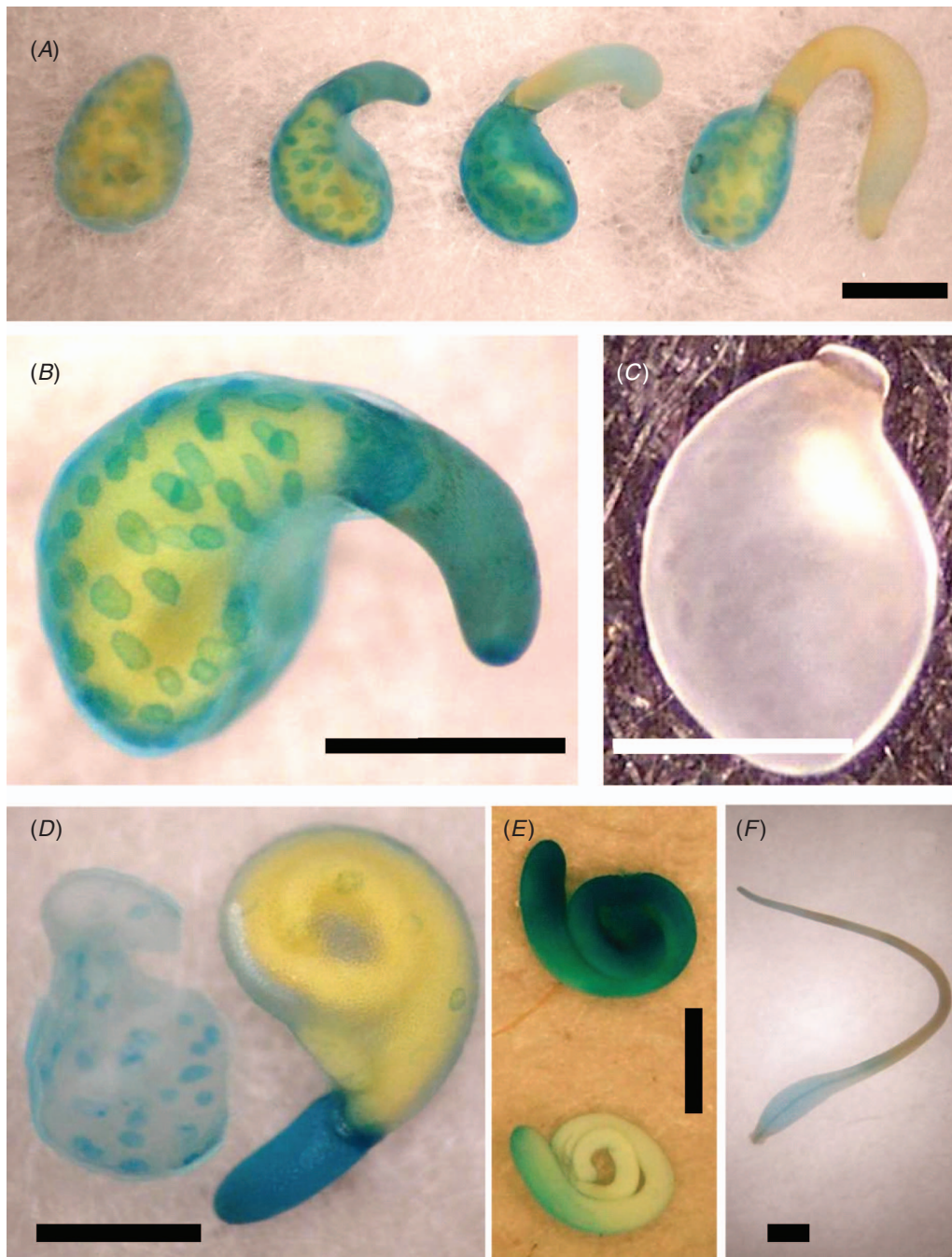


Fig. 1. Occurrence and distribution of β -GUS activity in germinating *Cuscuta pentagona* seeds, embryos and seedlings detected by histochemical activity staining for 4 h (panels A, B and D) or 24 h (panels E and F). (A) Distribution of activity before and during germination and swelling of the radicle. (B) Detail of stained domains as seen through the inner seed coat. Note that some domains overlap. (C) Shed seed coat displaying a distribution of internal structures matching that seen in (B). (D) Stained seed with emerging embryo with seed coat removed after staining. (E) Embryos following prolonged staining within the seed (top) or after removal from the endosperm (bottom). (F) Elongating seedling after prolonged staining. All scale bars are 1 mm.

with X-GlucA (4 mmol L⁻¹) or X-Gal (1 mmol L⁻¹) for 24 h. Growing shoot tips incubated with X-GlucA showed only a faint blue tint distributed throughout the tissue, whereas tissues stained with X-Gal, used to provide a point of comparison and to

control against patterns resulting only from infiltration, showed staining at the margins of developing scale leaves, and only at the most apical positions on the stem (Fig. 2A). Sectioning or wounding of shoot apices occasionally revealed higher levels

of β -galactosidase activity than were detected in intact sections, but neither of these treatments revealed any additional β -GUS activity at the shoot apex (not shown). Staining of longitudinally cut mature stem tissue (Fig. 2B) showed that both β -GUS and β -galactosidase activities occurred throughout these tissues.

Inflorescences stained for β -GUS showed the occurrence of activity in the pistil and stamens, as has been noted in other plant systems (Plegt and Bino 1989; Sudan *et al.* 2006), with particularly intense staining occurring in the anther and stigma (Fig. 2C). A striking pattern of β -GUS staining was observed in portions of stem tissue where haustorial development had been initiated (Fig. 2D) in response to coiling on a plastic target placed in the path of the growing vine. β -GUS staining was seen initially (after 5–6 h, data not shown) only in the centre of the developing haustorial masses, although eventually (by 24 h) the stain was seen to occur throughout the coiled portion of the stem. This staining did not extend to adjacent, non-coiled stem tissue. Patterns resulting from incubation with X-Gal were generally similar to those with X-GlucA, although there was a trend for the staining to occur more locally in the central mass of the haustorium (data not shown). To determine whether GUS staining observed in twining dodder stems is a phenomenon common to twining stems, newly twined stems of glasshouse-grown ivy-leaf morning glory (*Ipomoea hederacea*) were incubated in the presence of X-GlucA. Although staining was observed to occur at locations within the stems and petioles

of *I. hederacea* it did not occur in a pattern comparable to that observed in the coils and haustoria of *C. pentagona* (data not shown).

In vitro and *in situ* characterisation of β -GUS: effects of pH and inhibitor

To determine general characteristics of *C. pentagona* β -GUS, activity was quantified in crude soluble protein extracts of emerging seedlings (including inner seed coat, endosperm and embryo) or in stem tissues. As has been demonstrated previously in other plant systems (Alwen *et al.* 1992; Hodal *et al.* 1992; Sudan *et al.* 2006), the detection of endogenous β -GUS activity is pH sensitive. Maximum *in vitro* activity in seedling extracts was measured at pH 5.3 (Fig. 3A), with measurements at pH 4.3 and 6.3 dropping to less than half of this maximum. Minimal activity (comparable to non-specific background) was measured *in vitro* at pH 3.9 and below, or at 6.9 and above. Experiments using stem tissue showed a similar pH dependence, although the absolute levels of activity were lower (data not shown). Comparison of *in vitro* measurements with *in situ* activity staining patterns in germinating seedlings (Fig. 3B) reveals a similar point of maximum activity in the range of pH 4.8–5.3, although the decrease in staining at pH 6.3 and above is more striking than that seen at pH 3.9 and below. This difference could reflect the different environments of the *in vitro* and *in situ* assays, possibly resulting from a buffering capacity of the intact seed;

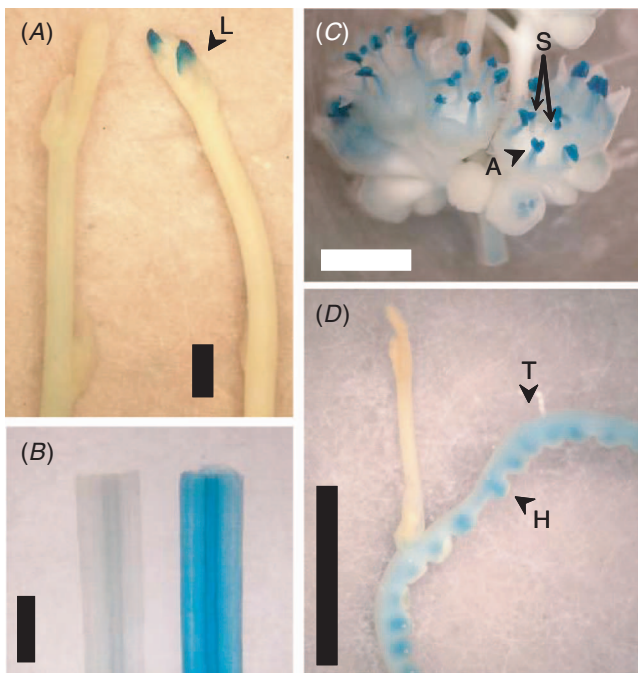


Fig. 2. Distribution of β -glucuronidase (β -GUS) activity in *Cuscuta pentagona* shoot organs. Intact growing shoot tips (A) and paired longitudinal sections of mature, thickened stem (B) stained for β -GUS activity (left) and for β -galactosidase activity (right). (C) Inflorescence stained for β -GUS activity. (D) Twining stem and haustoria stained for β -GUS activity. Each tissue was incubated for 24 h in buffer containing the respective substrate. A, anther; H, haustorium; L, scale leaf; S, stigma; T, twining stem. Scale bars: panels (A) and (B) are 1 mm; panels (C) and (D) are 5 mm.

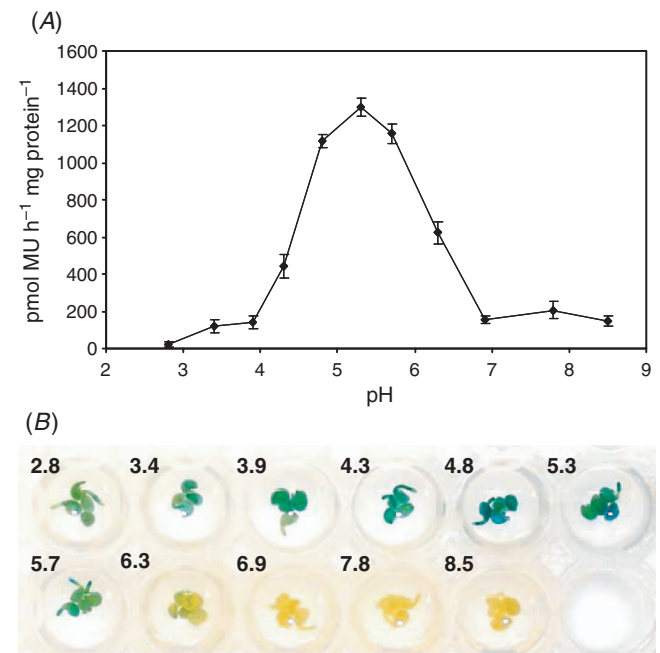


Fig. 3. *In vitro* and *in situ* detection of β -glucuronidase (β -GUS) activity is pH sensitive. (A) *In vitro* quantitation of β -GUS activity in *Cuscuta pentagona* seedling extracts by hydrolysis of 4-methyl-umbelliferyl- β -D-glucuronide (MUG). Each point represents the mean of six replicates; error bars are standard error. Reported data are representative of three experiments. (B) *In situ* detection of β -GUS activity in germinating *C. pentagona* seedlings in the same buffers used for *in vitro* quantitation of activity in panel (A); pH values are indicated next to each sample. Seedlings were stained for 24 h with 4 mmol L⁻¹ X-GlucA.

however, as the germinating seeds were pre-incubated in the staining buffer without substrate in an effort to equilibrate the internal pH environment, the exact basis of this difference is not immediately evident.

To examine the utility of the β -GUS inhibitor SAL as a tool for examining β -GUS function in plant development, we tested its capacity to inhibit *C. pentagona* β -GUS *in vitro* and *in situ*. SAL has previously been demonstrated to inhibit plant β -GUS *in vitro*, with Alwen *et al.* (1992) reporting 50% inhibition with the inclusion of 5 mmol L⁻¹ SAL and up to 75% inhibition with 25 mmol L⁻¹ SAL. Comparable results were reported by Hodal *et al.* (1992). Sudan *et al.* (2006) demonstrated the elimination of *in situ* β -GUS staining in tobacco tissues with the application of 10 mmol L⁻¹ SAL, but did not report measurements of *in vitro* inhibition. In contrast, β -GUS activity in *C. pentagona* seedling extracts was less sensitive to the inclusion of SAL when measured *in vitro*. Inclusion of 1, 10 and 20 mmol L⁻¹ SAL under optimum pH conditions (pH 5.3) resulted in 9, 18 and 21% inhibition, respectively (Fig. 4A). This same trend was observed

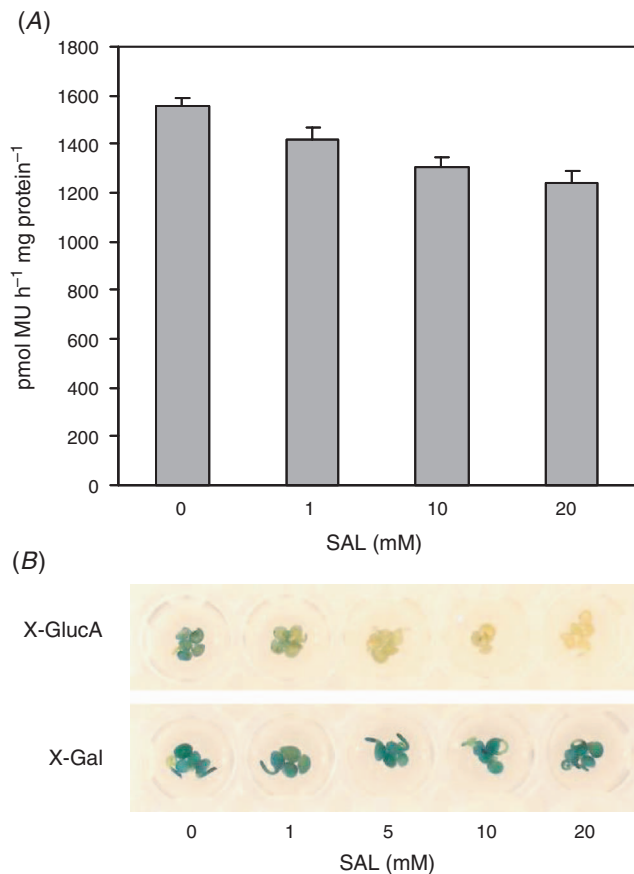


Fig. 4. *In vitro* and *in situ* inhibition of β -glucuronidase (β -GUS) activity by saccharic acid 1,4-lactone (SAL). (A) *In vitro* β -GUS activity. Column height is the mean of six replicates; error bar is standard error. Reported data are representative of three experiments. (B) *In situ* staining for β -GUS (top) and β -galactosidase (bottom) activities with X-GlucA (4 mmol L⁻¹) and X-Gal (1 mmol L⁻¹), respectively, with increasing concentrations of SAL. Germinating seedlings were incubated in buffer (pH 5.3) with substrate for 24 h.

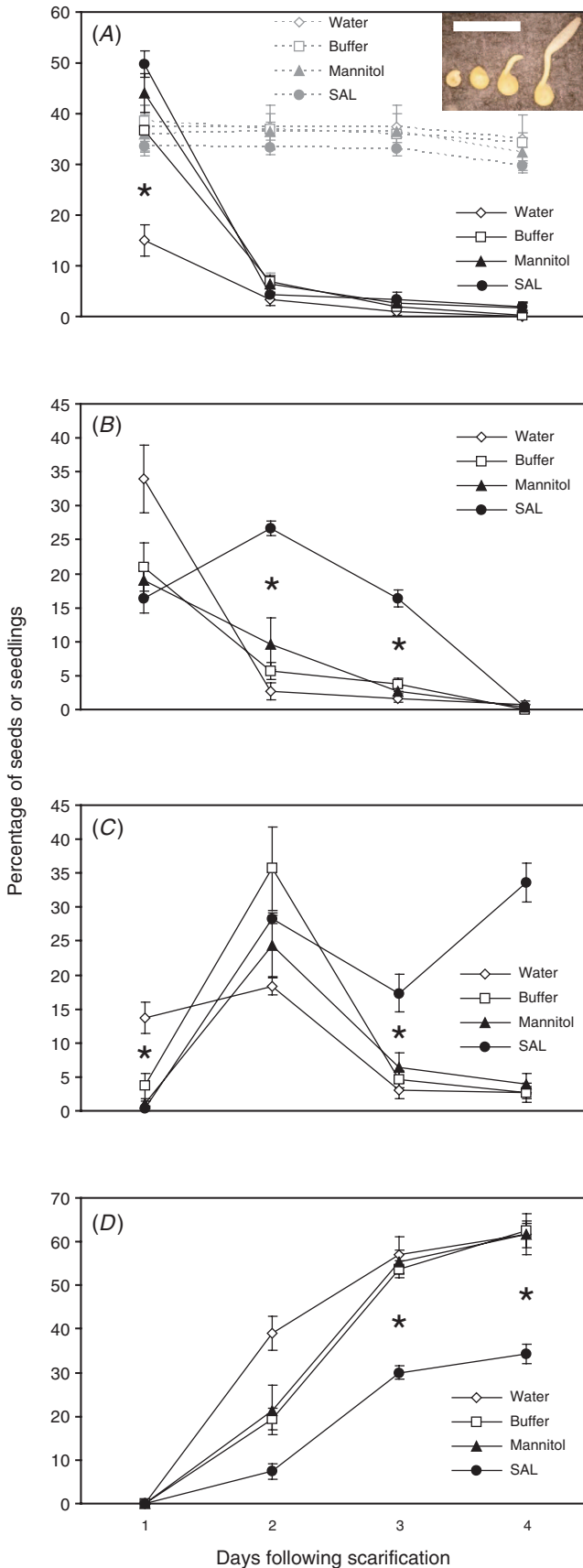
when the assay was performed at a higher pH (6.3), although inhibitor potency was decreased when the assay was performed at pH 4.3 (data not shown). In striking contrast, sensitivity of β -GUS activity to SAL was much more clearly evident when tested *in situ*. Germinating seedlings pre-equilibrated in buffer (pH 5.3) and SAL before the addition of substrate showed a noticeable difference in the depth of staining after 24 h (Fig. 4B). A decrease in staining was noticeable with the inclusion of 1 mmol L⁻¹ SAL; staining was minimal with 5 mmol L⁻¹; and seedlings incubated with 10 or 20 mmol L⁻¹ SAL had essentially no staining. To demonstrate that the effect of SAL on seed enzymatic activities was not non-specific, SAL inhibition of β -galactosidase activity was also examined. No decrease in staining was observed in seedlings incubated with X-Gal across the range of SAL concentrations (Fig. 4B). Based on these observations we concluded provisionally that SAL could be used to examine the function of β -GUS *in situ* and *in vivo*.

Developmentally contingent inhibition of growth by the β -GUS inhibitor SAL

To determine if the occurrence of β -GUS in germinating seedlings reflected a physiological role in germination or seedling growth, we observed seedling development in the presence or absence of SAL. Results of preliminary experiments examining the impact of SAL on seedling growth varied depending on the timing of SAL application, suggesting that sensitivity to SAL varied with developmental stage. Subsequent experiments used closely synchronised populations of seeds or seedlings generated by long (2 h) chemical scarification treatments followed by inspection of seeds under a dissecting microscope.

As previously noted, preliminary work showed that *C. pentagona* seedling growth *in vitro* was sensitive to both pH and to the inclusion of buffer. To control the acidifying effect of SAL, it was necessary to include a buffer in the medium. Sudan *et al.* (2006) used a strongly buffered system (100 mmol L⁻¹ Mes, pH 5.2) for observations of SAL treatment on tobacco seedlings. As a compromise, we used a system of 10 mmol L⁻¹ potassium phosphate buffer (pH 6.5) with 20 mmol L⁻¹ SAL, and we monitored pH throughout the experiment with a pH probe or indicator paper, replacing the medium as needed to maintain a constant pH. Controls for the effects of the buffer and non-specific osmotic effects were also included.

To examine the impact of SAL applied to seeds before emergence, synchronised populations were incubated in distilled water, buffer control (10 mmol L⁻¹ potassium phosphate pH 6.5), osmotic control (buffer plus 20 mmol L⁻¹ mannitol) or SAL (buffer plus 20 mmol L⁻¹ SAL). The progression of seeds through stages of germination and growth (Fig. 5A, inset) was recorded at 24 h intervals for up to 4 days after scarification. Seeds observed immediately after scarification and removal of the outer seed coat could be distinguished from subsequent stages, which had imbibed water, by size and opacity and were termed 'unswollen' in contrast to seeds observed as 'swollen' but not yet showing emergence. Seeds were scored as having an 'emergent radicle' if the radicle protruded beyond the inner seed coat, and seedlings were scored as 'swollen radicle' when the width of the radicle was greater than that of the rest of the



seedling axis. Finally, the seedling was scored as having 'shed seed coat' when the seed coat was released and shoot elongation proceeded.

Among the four treatments, seeds germinated in distilled water proceeded through the early stages of germination most rapidly, with more than one-third passing through the 'unswollen' and 'swollen' stages and proceeding to 'emergent radicle' by the first observation (Fig. 5, compare panels A and B). By contrast, all buffer-containing treatments were delayed relative to the water control, with smaller proportions of the buffer-, mannitol- or SAL-treated seeds reaching the 'emergent radicle' stage within 1 day following scarification. By 2 days after scarification, small proportions of the control treatments (water, buffer and mannitol) remained at the 'emergent radicle' stage because these populations had proceeded to the 'swollen radicle' stage (Fig. 5C), and more than 40% of the water control had reached the final 'shed seed coat' stage, whereupon shoot elongation had begun. By contrast, the frequency of 'emergent radicle' seedlings in the SAL treatment increased significantly relative to the control treatments (Fig. 5B), apparently owing both to delayed emergence and possibly to a decreased rate of procession to subsequent developmental stages. By the third day following scarification, water-, buffer- and mannitol-treated populations showed similar rates of 'shed seed coat' approaching 60% (Fig. 5D), with a majority of the balance remaining as 'unswollen', presumably hardened or inviable seeds (compare with Fig. 5A), while only ~30% of SAL-treated seedlings had reached this stage. By the final observation at 4 days, nearly all germinated seeds of the water-, buffer- and mannitol-treated populations had shed seed coats and had significant shoot elongation, while the frequency of 'shed seed coat' was approximately half among the SAL-treated seedlings (Fig. 5D) and a greater proportion only achieved 'swollen radicle' by this time (compare with Fig. 5C).

Although a significant proportion of SAL-treated seedlings progressed through the series of developmental stages defined above, these seedlings differed noticeably from control (water-, buffer- and mannitol-treated) seedlings. Seedlings were scored as having a swollen radicle on the basis of whether the width of the radicle appeared to be wider than the rest of the seedling

Fig. 5. Germination and growth of saccharic acid 1,4-lactone (SAL)-treated pre-emergent seeds. The developmental progress of scarified seeds was monitored in water (open diamond), 50 mmol L⁻¹ phosphate buffer, pH 6.8 (open square), phosphate buffer with 20 mmol L⁻¹ mannitol (closed triangle) or phosphate buffer with 20 mmol L⁻¹ SAL (closed circle). (A) Unswollen seed (upper trend, dashed) and swollen seed (lower trend, solid line). Seedling stages (inset) used to monitor the progression of germination and growth (left to right): unswollen seed, swollen seed, emergent radicle, swollen radicle. Scale bar is 5 mm. (B) Frequency of seedlings at the emergent radicle stage. (C) Frequency of seedlings at the swollen radicle stage. (D) Frequency of seedlings that had shed the seed coat and progressed to shoot elongation. Each point is the mean proportion of six populations of 50 seeds each. Error bar is standard error. Reported data are representative of three experiments. Statistically significant differences in frequencies are indicated for water-treated compared with buffer-, mannitol- or SAL-treated seedlings (day 1, panels A and C) and for SAL-treated seedlings compared with water-, buffer- and mannitol-treated seedlings (days 2–4, panels B–D); **P* = 0.01.

axis. However, the radicles of SAL-treated seedlings were often less swollen and they tended not to exhibit the characteristic curvature frequently observed in emerging dodder seedlings (Fig. 6A). Seedlings with this appearance were prone to arrest at this stage, failing subsequently to shed the seed coat and proceed to shoot elongation. These tissues remained turgid through the course of the experiment, suggesting that the arrest was not due to death of the plant tissue. Efforts to rescue 'arrested' seedlings

in this state, by transferring them to SAL-free medium, were not successful (data not shown).

SAL-treated seedlings that managed to shed the seed coat and proceed with shoot elongation were significantly shorter than control seedlings. Fig. 6B shows the mean maximum length achieved by all SAL-treated seedlings that had shed seed coats in comparison to estimated means calculated from randomly selected seedlings of the three control treatments. The reduced mean shoot length of SAL-treated seedlings is likely to result in part from a delay in arriving at the shoot elongation stage. However, it should be pointed out that a small fraction of SAL-treated seedlings had shed their seed coats by 2 and 3 days (contemporaneous with significant fractions of buffer- and mannitol-treated seedlings), but very few of these ultimately achieved a shoot length comparable to the mean length achieved by control seedlings. This could indicate a persisting effect from the early SAL treatment rather than a difference resulting simply from delayed development.

In contrast to the developmental impact observed when SAL was applied to pre-emergent seeds, the effect of SAL application during or shortly after emergence was minimal. Treatment of uniformly germinated seeds – from which the radicle was seen to emerge, but that had not yet achieved swelling of the radicle – were treated with the same media used in the pre-emergence experiment and incubated for an additional 4 days with daily exchange of medium. Comparison of the mean length of these seedling populations (Fig. 7) showed little difference between SAL-treated seedlings and water- and buffer-grown controls, and only a small difference between these and buffer-containing controls, although a statistically significant difference was observed between SAL-treated and mannitol-treated seedlings at this stage. Collectively, these results suggest a physiological role for SAL-inhibitable β -GUS activity at the early stages of seed germination and seedling development, the significance of which is reduced or absent after the seedling has emerged from the seed.

To test whether the application of SAL to rapidly growing stems could inhibit haustoria formation, 10 cm stem-tip cuttings were permitted to take up experimental media (100 mmol L^{-1} mannitol control or 100 mmol L^{-1} SAL in water) through the cut end of the stem. After a 24 h pre-incubation, stem apices were affixed to plastic sticks with adhesive tape 2–3 cm from the growing tip. Both mannitol- and SAL-treated stems were able to coil around the target and initiate haustorial development within the following 24 h, showing that the SAL treatment, thus applied, was not able to prevent formation of haustoria (20 observations, data not shown).

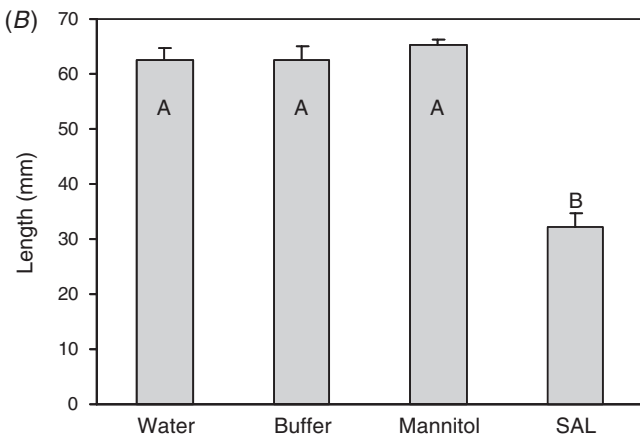
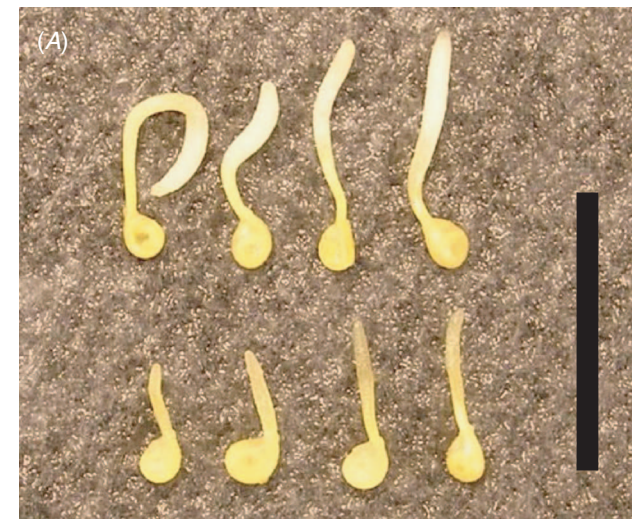


Fig. 6. Developmental impact of early saccharic acid 1,4-lactone (SAL) treatment on *Cuscuta pentagona* seedlings. (A) Seedlings grown in buffer with 20 mmol L^{-1} mannitol (top) and seedlings grown in buffer with 20 mmol L^{-1} SAL. Scale bar is 10 mm. (B) Seedling length after 4 days growth in water (control), 50 mmol L^{-1} phosphate buffer, pH 6.8 (buffer), buffer with 20 mmol L^{-1} mannitol (mannitol) or buffer with 20 mmol L^{-1} SAL (SAL). Column height reports the average of six population means. Water, buffer and mannitol treatment population means were estimated by randomly selecting 10 seedlings from among all seedlings that had shed seed coats. SAL-treated population means were calculated by measuring all seedlings that had shed seed coats in these populations (typically 15–20 seedlings from each population of 50 seeds). Error bars are standard error. One-way ANOVA results: $P < 0.0001$, $F_{3,20}=53.5$. Columns sharing the same label (A or B) do not differ significantly. Reported data are representative of three experiments.

Discussion

Histochemical activity staining for endogenous plant β -GUS revealed abundant activity in germinating seeds and seedlings of *C. pentagona* (Fig. 1). Staining occurred in distinct regions of the testa, the endosperm tissue, and to the embryonic plant itself. The striking spotted pattern observed in the germinating seed suggests specific domains of activity. The anatomy of dodder seeds and seedlings has been examined in detail by Hutchison and Ashton (1979) and Lyshede (1984, 1989, 1992). Staining

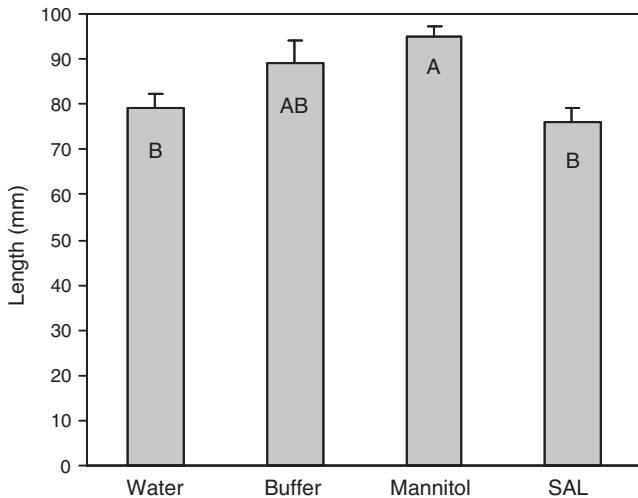


Fig. 7. Developmental impact of post-emergence saccharic acid 1,4-lactone (SAL) treatment on *Cuscuta pentagona* seedlings. Treatment media were of the same composition as that reported in Figs 5 and 6 and were applied after the radicle had emerged from the inner seed coat. Column height is the average of six populations, each with 10 seedlings, after 4 days growth post-application; error bar is the standard error. One-way ANOVA results: $P = 0.0033$, $F_{3,20} = 6.38$. Columns sharing the same label (A or B) do not differ significantly. Reported data are representative of three experiments.

indicative of β -GUS activity occurred within what is described as the 'crushed parenchyma' just below the sclerified palisade layer. While faint staining developed within the endosperm with time, the activity observed in the emerging radicle was much more pronounced and showed a developmental regularity, fading as the cells of the radicle elongated and swelled. By comparison, β -GUS activity in the elongated seedling shoot was minimal. The apparent localisation of β -GUS activity to the radicle should be regarded critically because prolonged staining of intact, pre-emergent seeds revealed staining of the entire embryo, although the degree of this staining was lessened when the embryo was first excised from the endosperm. Significant activity was also observed in stem tissues, particularly in twining stems and in developing haustoria, and in anthers and stigmas (Fig. 2), as has previously been observed (Plegt and Bino 1989; Sudan *et al.* 2006). Remarkably, very little activity was indicated in the shoot tips, where activity might be posited to occur if β -GUS plays a significant role in cell division and growth processes. The apparent increase in activity suggested by the staining patterns at sites of haustorial initiation may indicate a role for β -GUS in the establishment of parasitism, as has been proposed for several other glycosyl hydrolases (Nagar *et al.* 1984). Alternatively, it may be a general consequence of cell proliferation at these sites, without specific association with parasitism.

In vitro characterisation of β -GUS activity from seedling extracts showed that *C. pentagona* β -GUS activity is similar to previously characterised plant β -GUS in having a maximum activity at an acidic pH (Alwen *et al.* 1992; Hodal *et al.* 1992; Sudan *et al.* 2006), which contrasts with the neutral pH optimum of the *uidA*-encoded reporter β -GUS (Hodal *et al.* 1992).

C. pentagona β -GUS was inhibited by SAL, a general inhibitor of β -GUS activity (Karunairatnam and Levvy 1949), although the apparent degree of inhibition was more pronounced when demonstrated by *in situ* activity staining than by *in vitro* assay (Fig. 4), and staining with the artificial substrate X-Gal in the presence of SAL demonstrated that the effect of the inhibitor was not non-specific. SAL has been used in studies of plant β -GUS function and has typically been used at concentrations ranging from 1 to 30 mmol L⁻¹ (Alwen *et al.* 1992; Hodal *et al.* 1992; Morimoto *et al.* 1998; Sudan *et al.* 2006). A potential role for β -GUS in the early stages of *C. pentagona* seed germination or seedling development, during which β -GUS activity is pronounced, is suggested by the observation that immediate application of SAL to scarified seeds resulted in a significant slowing in the rates of seedling emergence (Fig. 5) and in the overall growth of the emerged seedling (Fig. 6). In striking contrast, application of the inhibitor at any stage following the emergence of the radicle resulted in no significant reduction in growth or change in development relative to water-grown control seedlings (Fig. 7). This observation is consistent with the general reduction of β -GUS staining in seedlings observed following emergence from the seed coat. Thus, a physiological role may be posited for β -GUS during the early events of germination and seedling development. The impact of SAL on seedling morphology, as shown in Fig. 6, might be attributed to a role for β -GUS in cell elongation processes. However, for this to be the case, the affected processes must be early acting because SAL application at the time of emergence and before the onset of radicle swelling does not yield this altered morphology. Further, it appears that, at least in this instance, subsequent cell growth and elongation may occur without the participation of β -GUS. Even so, as β -GUS inhibition *in vitro* was incomplete at the same concentrations used *in planta*, a possible role of β -GUS across a wider range of circumstances than indicated in these experiments cannot be entirely dismissed. The failure of SAL to prevent the formation of haustoria in cut stem tips might indicate that β -GUS activity occurring at these sites serves a function that is not critical to haustorial development. However, a role for β -GUS in haustoria formation cannot be entirely ruled out because the efficiency of SAL uptake and transport by the stem or, consequently, its concentration at the target site is not known.

Evidence of the critical role of sugar metabolism in seedling development has recently been provided by Bonfig *et al.* (2007). Developmental arrest and subsequent death of *Arabidopsis* seedlings expressing a transgene-encoded proteinaceous invertase inhibitor led the authors to suggest a key role for invertases in the partitioning of seedling resources. By contrast, the impact of altered glucuronic acid metabolism could be due to its role in cell regulatory processes rather than a disruption of core metabolic functions. Wen *et al.* (2004) proposed a role for β -GUS in the regulation of mitosis based on two observations. First, transformation of alfalfa, pea and *Arabidopsis* with a transgene targeting expression of the bacterial β -GUS *uidA* gene to meristems resulted in a lethal phenotype. Second, treatment of root tips with 10–20 mmol L⁻¹ SAL increased the number of border cells generated by the root apical meristem, while higher levels of SAL (100 mmol L⁻¹) were

lethal to seedlings. SAL-mediated disruption of cell division has been reported in cell suspension cultures (Hodal *et al.* 1992) and treatment of tobacco seedlings with 10 mmol L⁻¹ SAL in a heavily buffered medium suppressed seedling root growth and root hair development (Sudan *et al.* 2006).

Wen *et al.* (2004) proposed that UDP-glucuronyltransferase (UGT) and β -GUS act coordinately to control progression through the cell cycle through the reversible glycosylation, and consequent inactivation, of a mitosis-repressing factor. A subsequent report by Woo *et al.* (2007) showed that three β -GUS-like genes, with significant similarity to the functionally characterised β -GUS cDNA from *Scutellaria baicalensis* (Sasaki *et al.* 2000), occur within the *Arabidopsis* genome: AtGUS1 (At5 g07830), AtGUS2 (At5 g61250) and AtGUS3 (At5 g34940). Consistent with the proposed model for coordinate function of β -GUS and UGT during cell division, both UGT and β -GUS transcripts were found to occur at sites of cell division in *Arabidopsis* (Woo *et al.* 2007). The predicted β -GUS proteins encoded by the three loci showed significant similarity to the 'family 79' glycosyl hydrolases, including the mammalian family of heparanase β -glucuronidases distinct from the 'family 2' β -glucuronidases to which the *E. coli uidA* gene product belongs. Analysis of the N-terminal domains of the predicted β -GUS proteins suggested that AtGUS2 and AtGUS3 were membrane associated, whereas AtGUS1 was secreted. This prediction is consistent with a report by Bayer *et al.* (2006) that identified At5 g07830 (AtGUS1) as occurring within the *Arabidopsis* cell wall proteome.

To determine unequivocally the physiological function of plant β -GUS activities, it will be necessary to identify endogenous substrates and to assign them to specific genes and their encoded activities. To date, two endogenous plant compounds have been identified as substrates for hydrolysis by plant β -GUS. Schulz and Weissenbock (1987) reported the partial purification of β -GUS from rye (*Secale cereale*) leaves, with specificity for luteolin-triglucuronide, a flavonoid glycoside. Morimoto *et al.* (1998) demonstrated the participation of a SAL-inhibitable β -GUS activity in the hydrolysis of baicalein 7-*O*- β -D-glucuronide from *S. baicalensis*, the same plant in which plant β -GUS activity had first been demonstrated nearly half a century earlier by Levvy (1954). The cDNA subsequently reported by Sasaki *et al.* (2000) encodes an enzyme capable of baicalein 7-*O*- β -D-glucuronide hydrolysis. In this system, the aglycone flavonoid baicalein is subsequently oxidised to 6,7-dehydrobaicalein by H₂O₂-consuming peroxidases in an elicitor-mediated stress response (Morimoto *et al.* 1998). Speculating on the nature of SAL-mediated inhibition of root growth and root hair development, Sudan *et al.* (2006) proposed a role for plant β -GUS in the metabolism of cell wall polysaccharides. The acidic pH optimum of the enzyme could be indicative of its function in the relatively acidic apoplastic environment. Ultimately, confirmation of this hypothesis will require the identification of specific cell wall components that are hydrolysed by plant β -GUS. The potency of SAL as an inhibitor of *Cuscuta* seedling growth, only when applied at the earliest stages following scarification, may point to a function in the metabolism or modification of cell components in the seed, possibly in endosperm metabolism, in which case it may be informative to compare

the seed coat remnants shed from control and SAL-inhibited seedlings.

Sudan *et al.* (2006) demonstrated an induction of GUS activity following the application of plant growth regulators, including gibberellic acid (GA). Growing shoot tips of *Cuscuta* may be cultured experimentally and have been shown to be influenced by the inclusion of GA in the culture medium (Maheshwari *et al.* 1980). Although the application of SAL to cut stem tips did not result in a measurable inhibition of shoot growth or haustorial development, it will be of interest to determine whether the inclusion of SAL in culture medium might modify the effects of growth regulators.

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