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Expression of the *Arabidopsis* histone *H2A-1* gene correlates with susceptibility to *Agrobacterium* transformation

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Summary

Transformation of plant cells by *Agrobacterium tumefaciens* involves both bacterial virulence proteins and host proteins. We have previously shown that the *Arabidopsis thaliana* gene *H2A-1* (*RAT5*), which encodes histone H2A-1, is involved in T-DNA integration into the plant genome. Mutation of *RAT5* results in a severely decreased frequency of transformation, whereas overexpression of *RAT5* enhances the transformation frequency (Mysore et al., 2000b). We show here that the expression pattern of RAT5 correlates with plant root cells most susceptible to transformation. As opposed to a cyclin-GUS fusion gene whose expression is limited to meristematic tissues, the *H2A-1* gene is expressed in many non-dividing cells. Under normal circumstances, the *H2A-1* gene is expressed in the elongation zone of the root, the region that is most susceptible to *Agrobacterium* transformation. In addition, when roots are incubated on medium containing phytohormones, a concomitant shift in *H2A-1* expression and transformation susceptibility to the root base is observed. Inoculation of root segments with a transfer-competent, but not a transformation-deficient *Agrobacterium* strain induces *H2A-1* expression. Furthermore, pre-treatment of *Arabidopsis* root segments with phytohormones both induces *H2A-1* expression and increases the frequency of *Agrobacterium* transformation. Our results suggest that the expression of the *H2A-1* gene is both a marker for, and a predictor of, plant cells most susceptible to *Agrobacterium* transformation.

Keywords: *Agrobacterium*, histone, genetic transformation, phytohormones, *Arabidopsis*.

Introduction

Agrobacterium tumefaciens is a soil-borne phytopathogen that induces crown gall disease on many dicotyledonous plants and some monocots and gymnosperms (Anderson and Moore, 1979; DeCleene and DeLey, 1976; Porter, 1991). The molecular mechanism of tumour production involves the transfer of specific regions (the transferred [T]-DNA) of the tumour inducing (Ti) plasmid from the bacterium to the plant cell. Following T-DNA transport to the plant nucleus, T-DNA integrates into the plant genome and expresses genes that direct the production of auxins and cytokinins, as well as novel low molecular weight metabolites termed opines. Phytohormone overproduction results in the loss of cell cycle regulation with the ensuing production of tumours (recently reviewed in Gelvin, 2000; Tzfira *et al.*, 2000; Zupan *et al.*, 2000).

Research in many laboratories during the past 20 years has resulted in a reasonably detailed understanding of the events that take place within the bacterium during the early stages of tumorigenesis. These events include perception of wound-induced plant phenolic and sugar molecules that act as molecular signals to initiate virulence (*vir*) gene induction, processing of the T-DNA from the Ti-plasmid by the VirD1/VirD2 endonuclease, and export of the VirD2/T-DNA complex, as well as VirE2 and VirF proteins, into the plant cell via the VirB/VirD4 Type IV secretion pathway (Baker *et al.*, 1997; Christie, 1997; Vergunst *et al.*, 2000; Winans *et al.*, 1996; Zupan and Zambryski, 1997). In contrast to our extensive knowledge of these events that occur within the bacterium, we currently have relatively little knowledge of events that occur with respect to the plant cell that ultimately result in stable transformation of the plant. These events include bacterial attachment to the plant cell, T-DNA and Vir protein transfer through the plant cell wall and membrane into the cytoplasm, nuclear targeting of the T-complex, and integration of the T-DNA into the plant genome (Gelvin, 2000).

Our laboratory recently embarked upon a series of projects to identify plant genes involved in the *Agrobacterium*

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transformation process (Gelvin, 2000; Mysore *et al.*, 2000b; Nam *et al.*, 1997, 1999). In the course of these studies, we discovered that a mutation in one of the 13-member family of *Arabidopsis thaliana* histone H2A genes resulted in a *rat* (*r*esistant to *Agrobacterium t*ransformation) phenotype. This mutant, *rat5*, contains a T-DNA insertion in the 3′ untranslated region of the histone *H2A-1* gene, and is deficient in its ability to integrate T-DNA into the plant genome. However, T-DNA transfer to the nucleus occurs at normal frequency (Mysore *et al.*, 2000b). Although roots of the *rat5* mutant could not easily be transformed by *Agrobacterium*, transformation by the flower vacuum infiltration method (Bechtold *et al.*, 1993; Clough and Bent, 1998) was as efficient as that of wild-type plants (Mysore *et al.*, 2000a). This result, as well as the studies by Sangwan's group (Chateau *et al.*, 2000; Sangwan *et al.*, 1992; Villemont *et al.*, 1997) and others (De Kathen and Jacobsen, 1995) showing that specific host cells and stages of the plant cell cycle may be important targets for transformation, prompted us to examine the relationship between histone *H2A-1* gene expression and plant cell transformation by *Agrobacterium*. This relationship may be especially interesting in light of the involvement of histones in the cell cycle, and the synthesis of certain classes of histones in a cell cycle-dependant manner at the beginning of S phase.

We report here a correlation between expression patterns of the histone *H2A-1* gene and *Arabidopsis* root cells that are preferentially transformed by *Agrobacterium*. Exposure of root segments to phytohormones or wounding increases both histone *H2A-1* expression and transformation. These perturbations do not, however, increase the expression of a cyclin promoter in the same way. We propose that histone *H2A-1* gene expression is not strictly linked to the S phase of the mitotic cell cycle, and that expression of this gene is both an early indicator and is predictive of plant cells that are most susceptible to *Agrobacterium* transformation.

Results

Main root elongation zone and the lateral root primordia regions are most susceptible to Agrobacterium *transformation*

Previous *Arabidopsis* root transformation assays involved cutting root segments from axenically grown plants into 3–5 mm long segments, randomizing the segments and inoculating them with *Agrobacterium*, then spreading the randomized segments on medium containing antibiotics to kill the bacteria and to select for transformation (Mysore *et al.*, 2000a, 2000b; Nam *et al.*, 1997, 1998, 1999). Although the protocol of this assay is useful for generating statistically significant data because of the large number of root segments assayed in each experiment, it does not permit localization of specific regions of the root that may be more or less susceptible to transformation. We therefore cut axenic roots into segments, inoculated the segments with *Agrobacterium*, and 'reassembled' the segments on selection medium in the same order that they normally appeared on the plant. In order to identify segments that were most susceptible to transformation, we used a bacterium inoculum concentration of 10^6–10^7 cells ml^{-1}, in contrast to the concentration of 10^9 cells ml^{-1} used in previous experiments. After 1 month, we scored tumour formation on the various segments.

Figure 1 shows that specific regions of the root were hyper-susceptible to transformation. These regions included segments near but basal, to the meristematic zone of the main root tip (the elongation zone), and segments containing lateral root primordia. Thus, the preferential susceptibility of *Arabidopsis* roots to *Agrobacterium* transformation is highly localized to specific areas.

Expression pattern of the histone H2A-1 *gene*

We previously showed that the *Arabidopsis* histone *H2A-1* gene is involved in *Agrobacterium*-mediated transformation of root segments. An *Arabidopsis* mutant, *rat5*, contains a T-DNA insertion in the 3′ untranslated region of this gene. This mutant can support T-DNA nuclear transport, but not T-DNA integration into the chromosome (Mysore *et al.*, 2000b). Histone proteins make up nucleosomes, and are consequently important for regulating gene activity and for establishing chromatin structure. To determine whether a correlation exists between root cells that are most transformable and cells that express the histone *H2A-1* gene involved in T-DNA integration, we investigated the expression patterns of two genes, histone *H2A-1* and a cyclin gene (*cyc1At*) which is important for cell division (Ferreira *et al.*, 1994). We expressed translational fusions of the promoters plus part of the respective open reading frames to GUS in transgenic *Arabidopsis* and examined the pattern of expression of these fusion genes.

Both the histone *H2A-1* and cyclin promoters were expressed in roots, shoots, floral meristems, and lateral root primordia (Figure 2). In keeping with its function in cell division, the expression of the cyclin promoter was limited to meristematic regions of the plant. These include shoot and floral meristematic regions (Figure 2b,d,f), root meristems (Figure 2h,j,n), and lateral root primordial (Figure 2h,l). The expression pattern of the histone *H2A-1* promoter was broader. In addition to the meristematic regions listed above (Figure 2a,c,e,g,i,k,m), the histone *H2A-1* promoter was expressed in the elongation region of the root (Figure 2g,i,m) and in newly emerged young and expanding areas of leaves and stems (Figure 2a,c,e). To verify the histone *H2A-1* promoter activity of our reporter system, we per-

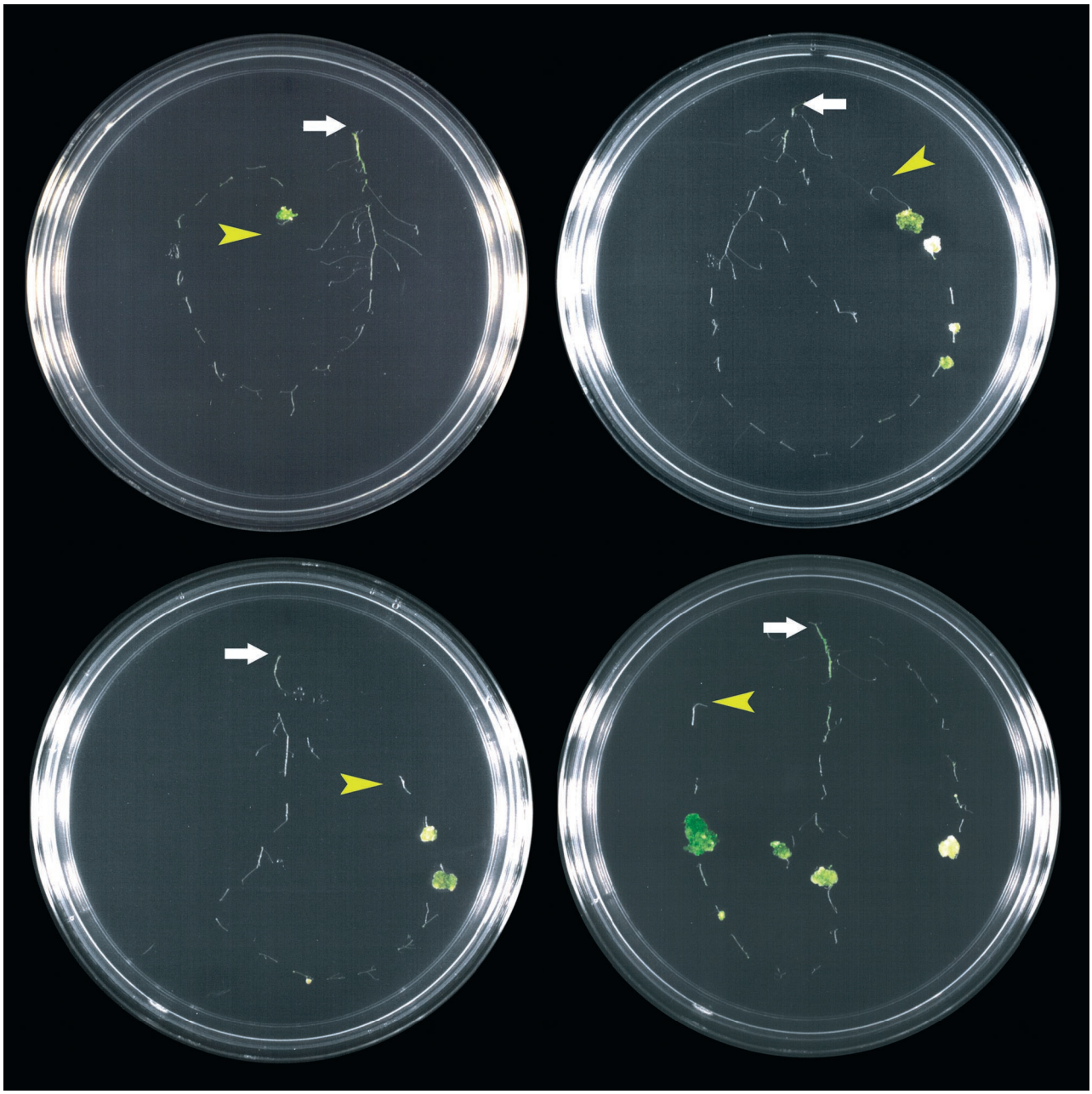
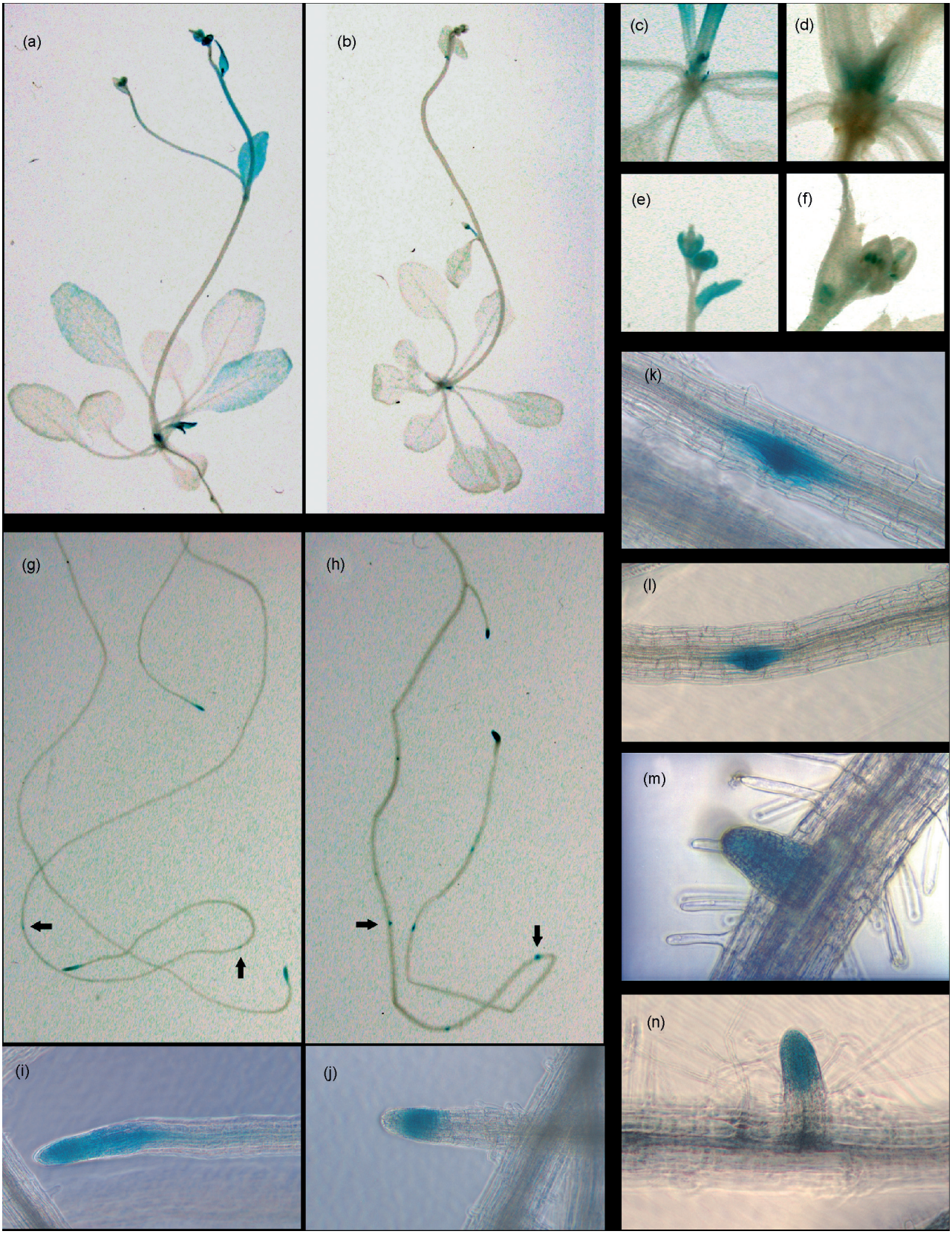


Figure 1. Transformation competence of various regions of *Arabidopsis* roots.
Main roots, from which most lateral roots were eliminated, were cut into segments and infected with *A. tumefaciens* A208. After 2 days, the roots were transferred to MS medium containing timentin (to kill the bacteria) and lacking phytohormones (to select for tumours) and the segments were arrayed as they initially appeared. The plates were incubated for 1 month, then photographed. Arrows indicate the basal root segments nearest the crown of the plant. Arrowheads indicate the most apical segments containing the root apical meristem.

formed whole mount *in situ* hybridization using histone *H2A-1* gene-specific probes. We detected histone *H2A-1* transcripts in the floral bud and margins of newly emerging leaves (Figure 3b), the expanding leaf (Figure 3d), and the root tip, including both the elongation and meristematic zones (Figure 3f). Control sense polarity hybridization probes did not reveal these transcripts (Figure 3a,c,e). These results establish a correlation between our promoter-reporter gene studies and our *in situ* hybridization data, and indicate that GUS activity directed by the histone *H2A-1* promoter-*gusA* gene is representative of histone *H2A-1* gene expression. Our results further show that the expression pattern of the histone *H2A-1* gene in the roots correlates well with the cells in the root elongation zone that are most susceptible to *Agrobacterium* transformation, as depicted in Figure 4.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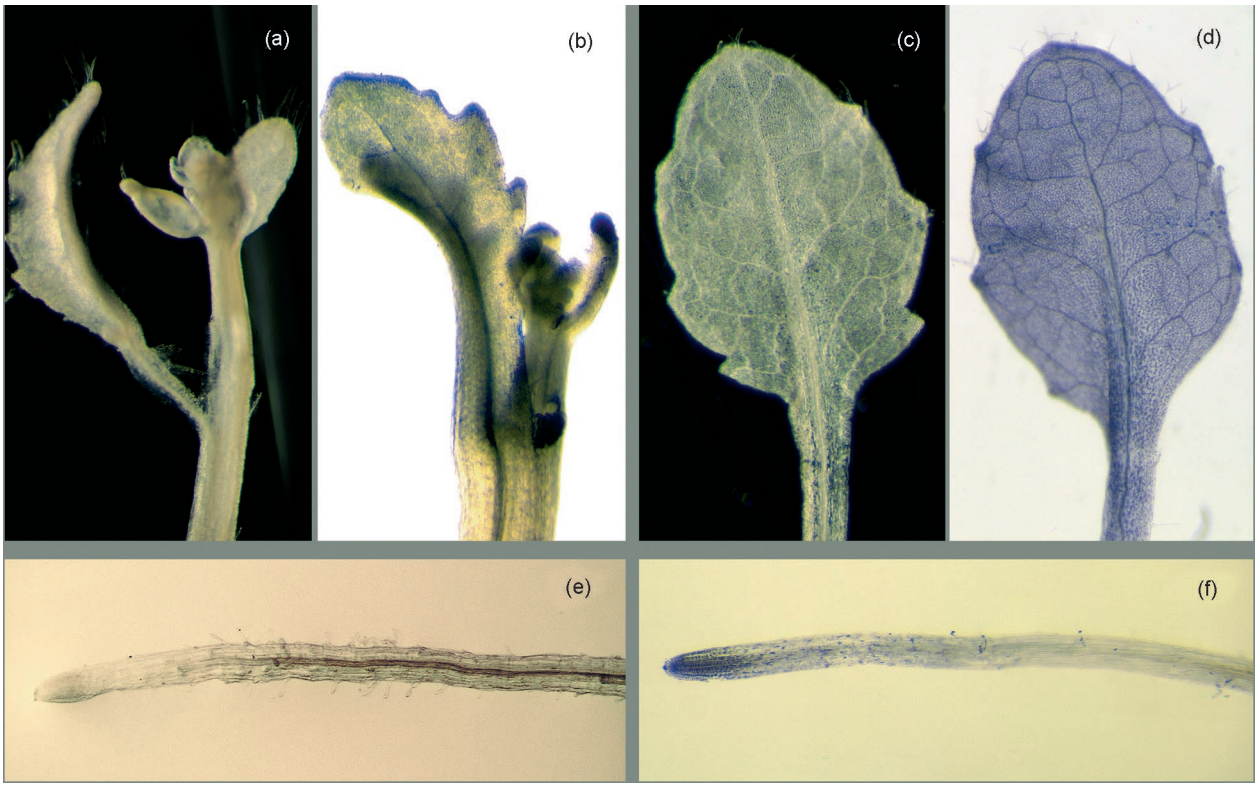


Figure 3. Whole mount *in situ* hybridization with histone *H2A-1*-specific probes.
Whole-mount *in situ* hybridizations were performed with a digoxygenin-labelled (DIG) *H2A-1* gene specific antisense probe (b, d and f) and with a DIG-labelled sense probe (a, c and e). Purple coloured staining regions represent RNA/RNA hybrids detected with anti-DIG Fab fragment conjugated to alkaline phosphatase.

Susceptibility of cells to Agrobacterium transformation correlates with the expression of the histone H2A-1, but not the cyclin cyc1At, promoter in cut root segments

To define further the correlation between cells expressing the histone *H2A-1* promoter and plant cells most susceptible to *Agrobacterium* transformation, we investigated the expression of the histone *H2A-1* promoter in cut root segments similar to those used for tumorigenesis assays. We first determined the expression pattern of the histone *H2A-1* promoter in uninfected root segments of histone *H2A-1::gusA* transgenic plants. For these experiments, we did not include segments derived from the meristem and elongation regions of the roots. Figure 5(a) shows that 2 days after cutting, these segments displayed histone *H2A-1* promoter activity in cells surrounding the vascular tissue, most likely pericycle, near but not at the cut end of the root segment. We infected cut root segments from non-transgenic plants with *A. tumefaciens* At802. This strain, derived from the oncogenic strain A208, additionally contains the T-DNA binary vector pBISN1 (Narasimhulu *et al.*, 1996) which encodes a *gusA*-intron gene under the control of a 'super-promoter' (Ni *et al.*, 1995). Expression of GUS activity directed by this *gusA*-intron gene occurs only in the plant and not in the bacterium (Liu *et al.*, 1992). Figure 5(b) shows that, following infection of cut root segments with *A. tumefaciens* At802, GUS expression appears predominantly in the same region of the root segments that expresses the

Figure 2. Expression of the histone *H2A-1* and cyclin *cyc1At* promoters in transgenic *Arabidopsis* plants.
Transgenic plants containing a *H2A-1::gusA* fusion gene (panels a, c, e, g, i, k and m) or a *cyc1At::gusA* fusion gene (panels b, d, f, h, j, l and n) were grown to maturity and stained with X-gluc.
(a and b) Aerial portions of the plant.
(c and d) Shoot apical meristem region.
(e and f) Floral buds.
(g and h) Whole roots.
(i and j) Root tip region.
(k and l) Lateral root primodia.
(m and n) Young lateral roots.
Note that expression of the cyclin fusion gene is limited to meristematic regions, whereas the histone *H2A-1* fusion gene additionally expresses in non-dividing tissues. Arrows in Panels (g) and (h) indicate lateral root primodia.

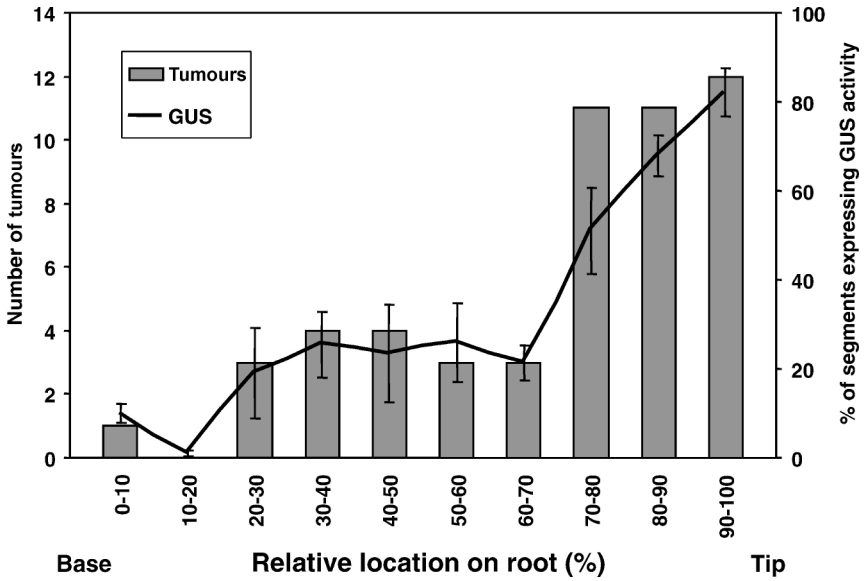


Figure 4. Relationship between histone *H2A-1* promoter expression and tumour formation in *Arabidopsis* root segments.
Segments from main roots of wild-type Ws plants were co-cultivated with *A. tumefaciens* A208 for 2 days. Similarly, segments from the main root of histone *H2A-1*::*gusA* transgenic plants were incubated on MS medium without *Agrobacterium* infection. The infected segments were subsequently transferred to MS medium lacking phytohormones but containing timentin to assay for tumour formation, or the uninfected transgenic segments were incubated in X-gluc to assay for expression of the histone *H2A-1* promoter, respectively. The relative position (0–100%) along the root was noted, and the number of tumours was scored after 1 month. The pattern of GUS expression was determined after staining for 24 h. Thick vertical bars indicate tumorigenesis. Thin lines indicate GUS staining. Note that both tumorigenesis and histone *H2A-1* promoter expression occurred primarily near the tip of the roots. For this figure, a total of 30 Ws and 50 *H2A-1*::*gusA* transgenic plants were used for the assay. The experiment was repeated 3 times on different dates.

histone *H2A-1* promoter. Thus, transformation-competence and histone *H2A-1* promoter activity correlate well in these cut root segments.

We further investigated the correlation between histone *H2A-1* and cyclin *cyc1At* promoter expression in uninfected cut root segments. We incubated root segments on solidified MS medium lacking phytohormones and assayed segments daily for GUS activity. Figure 6 shows that histone *H2A-1* promoter expression first became detectable 2 days after incubation and reached maximal levels after 4 days, following which GUS activity decreased and eventually disappeared after 6 days. Thus, the histone *H2A-1* promoter

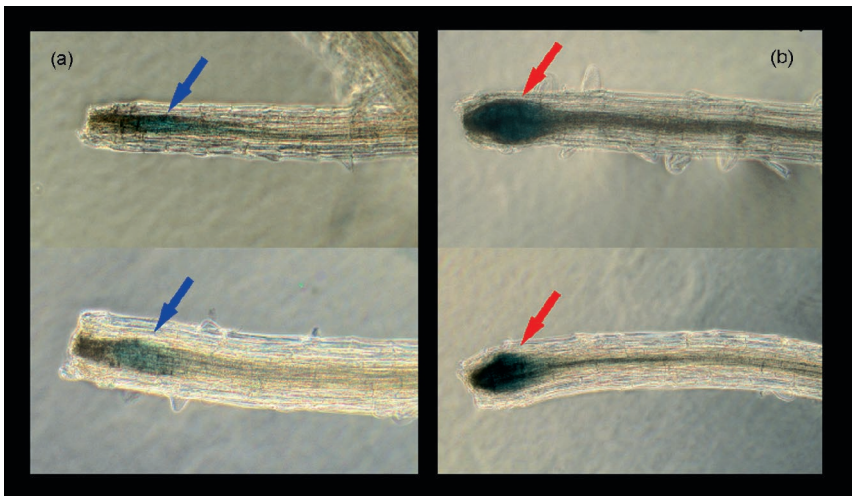


Figure 5. Correlation between histone *H2A-1* promoter expression and *Agrobacterium* transformation in cut root segments.
Root segments were cut from transgenic histone *H2A-1*::*gusA* plants (panel A) or wild-type Ws plants (panel B). Two days after cutting, the segments from the transgenic plant were stained with X-gluc to detect regions of histone *H2A-1* promoter expression. Root segments from wild-type Ws plants were infected with *A. tumefaciens* At802 containing the binary vector pBISN1. Two days after infection, the roots were stained with X-gluc to identify cells transformed by the binary vector. Arrows in Panels (a) and (b) indicate regions of GUS staining representing histone *H2A-1* promoter expression or *Agrobacterium* transformed cells. Note the coincidence of cells expressing the histone *H2A-1* promoter and cells susceptible to *Agrobacterium* transformation.

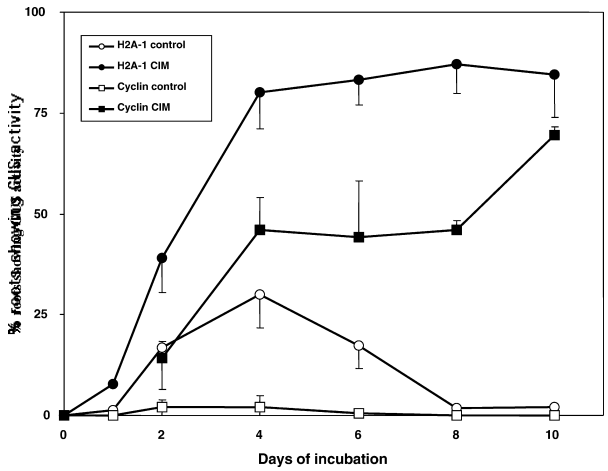


Figure 6. Induction kinetics of the histone *H2A-1* and cyclin *cyc1At* promoters in root segments.
Root segments from the respective transgenic plants were incubated on MS medium lacking phytohormones, or callus inducing medium (CIM) containing phytohormones. After various periods of time, the segments were stained with X-gluc and the percentage of root segments showing GUS activity was determined. ○, histone *H2A-1* MS medium control; ●, histone *H2A-1* CIM; □, cyclin *cyc1At* MS medium control; ■, cyclin *cyc1At* CIM. For each point, at least 100 root segments were tested. Each experiment was repeated seven times.

is induced transiently by the process of cutting the roots into small segments. Under the same conditions, however, cyclin *cyc1At* promoter activity was only weakly detectable in a very few segments. Thus, the transient expression of the histone *H2A-1* promoter in root segments may not be correlated with cell division. This observation further supports our conclusions that expression of this gene may be needed for T-DNA integration for reasons other than a dependency upon actively dividing cells.

Figures 6 and 7 show that when we incubated cut root segments on callus-inducing medium containing the phytohormones auxin and cytokinin, the activity of both the histone *H2A-1* and cyclin *cyc1At* promoters was induced. The expression patterns of induction differed, however, between the two promoters. The histone *H2A-1* promoter initially expressed in cells near the cut ends of the segments (Figure 7a), but after 5 days incubation on hormones this promoter was active throughout the root segment (Figure 7b). Cyclin *cyc1At* promoter activity was initially undetectable (Figure 7c), but was induced predominantly at cut ends of the roots in newly generated callus (Figure 7d). (The appearance of GUS-staining cells in the middle of a few root segments in Figure 7d most likely represent inadvertent wounding of these segments during the experimental procedure). These results further indicate that the expression patterns of the histone *H2A-1* and cyclin *cyc1At* promoters differ, and that the histone *H2A-1* gene is expressed in cells that are not actively dividing.

Incubation of whole roots on medium containing phytohormones alters the pattern of histone H2A-1 *promoter expression and tumorigenesis concomitantly*

When we incubated main roots on callus-inducing medium containing phytohormones, there was a co-ordinate alteration in the pattern of both histone *H2A-1* promoter expression and susceptibility to transformation. The initial pattern of histone *H2A-1* promoter expression in the elongation region of the root tip, shown in Figures 2 and 8a, changed. After 3 days, histone H2A-1 promoter activity was undetectable in either the elongation or meristematic regions of the root (Figure 8a). Six days after incubation, expression reappeared, but at the basal end of the root (Figure 8a). To determine whether the pattern of susceptibility to transformation was also altered, we incubated roots on hormones for 7 days, cut them into segments, and infected the segments with *A. tumefaciens* A208. Segments were subsequently moved to MS medium lacking phytohormones to monitor the development of tumours. Figure 8(b) shows that, in accordance with the altered pattern of histone *H2A-1* promoter activity, most tumours now appeared at the basal region of the root. These results suggest that expression of the histone *H2A-1* promoter could be an early indicator of cells most susceptible to *Agrobacterium* transformation.

Histone H2A-1 *promoter expression is higher in tumorous cells than in non-tumorous cells*

We investigated whether the histone *H2A-1* promoter is preferentially active in tumorous, compared to non-tumorous, root cells. Two weeks after root segments were inoculated with *A. tumefaciens* A208 tumours began to develop on some, but not all, segments. Of the 469 root segments inoculated, 120 (26%) developed tumours, and 96% of these tumours expressed GUS activity resulting from expression of the histone *H2A-1* promoter. Only 4% of the non-tumorous root segments expressed GUS activity. Thus, tumorous root segments preferentially expressed the histone *H2A-1* promoter, whereas root segments lacking tumours preferentially did not express this promoter.

Infection of root segments by transformation-proficient Agrobacterium *cells induces histone* H2A-1 *but not cyclin promoter activity*

To determine whether infection of root segments by *Agrobacterium* affects histone *H2A-1* or cyclin *cyc1At* promoter activity, we infected transgenic root segments with either *A. tumefaciens* A208 (an oncogenic strain) or the near-isogenic strain A136 (a non-oncogenic strain that lacks a Ti-plasmid). After 2 days, we stained root segments from these infections, and non-infected control roots, with X-gluc to determine the expression of the relevant promoters.

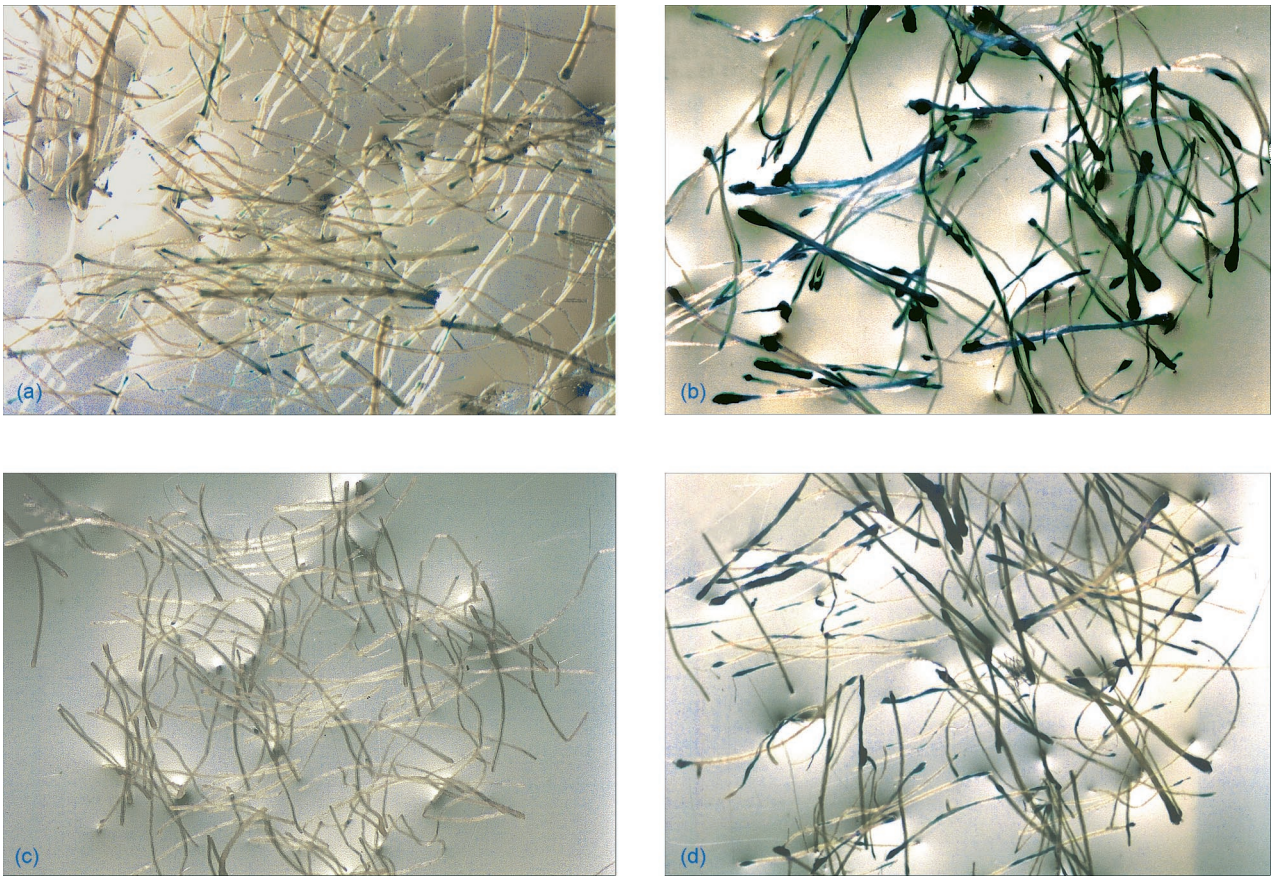
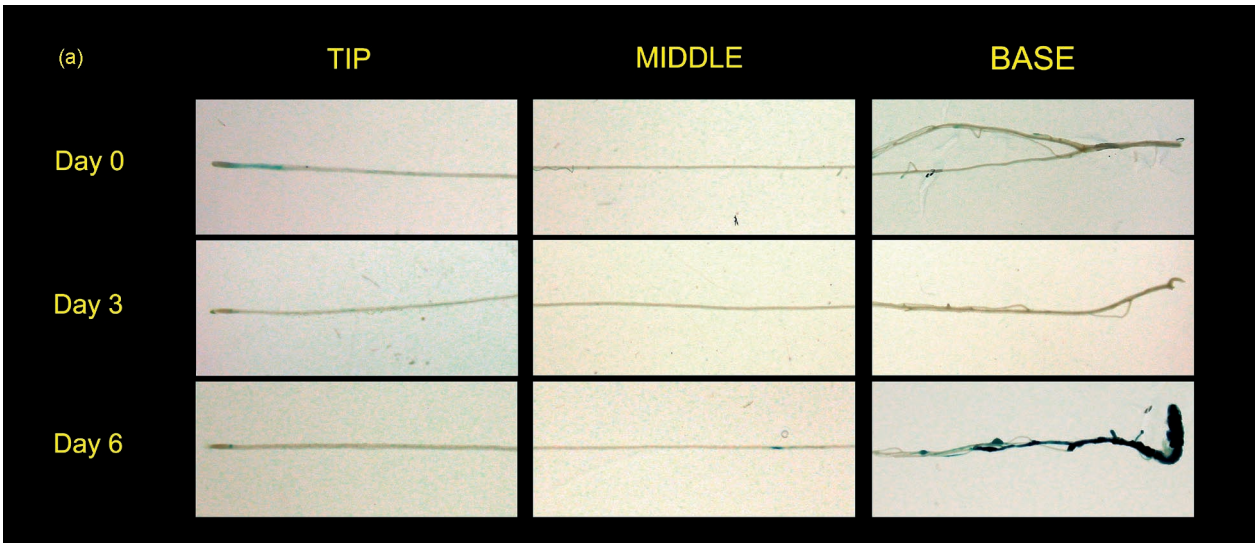


Figure 7. Induction of the histone *H2A-1* and cyclin *cyc1At* promoters in root segments.
Root segments from the respective transgenic plants were stained either after 5 days incubation on MS medium lacking phytohormones (panels a and c) or on callus inducing medium containing phytohormones (panels b and d).
(a and b) Histone *H2A-1*::*gusA* transgenic plants.
(c and d) Cyclin *cyc1At*::*gusA* transgenic plants.
Note that after incubation on callus inducing medium, GUS expression is found throughout the root segments of the *H2A-1*::*gusA* transgenic plants, whereas GUS expression in the root segments of the *cyc1At*::*gusA* transgenic plants is limited to the proliferating callus at the ends of the root segments.

Table 1 shows that histone *H2A-1* and *cyc1At* transgenic roots infected with the non-oncogenic strain *A. tumefaciens* A136 showed a percentage of promoter activity similar to that of uninfected roots. However, roots infected with the oncogenic strain *A. tumefaciens* A208 showed induced activity of the histone *H2A-1*, but not the cyclin *cyc1At*, promoter. These results indicate that the histone *H2A-1* promoter can be induced by T-DNA and/or virulence protein transfer to the plant in a cell division-independent manner.

Pre-incubation of root segments on medium containing phytohormones increases their susceptibility to Agrobacterium *transformation*

Our data shown in Figures 6 and 7 indicate that the histone *H2A-1* gene is induced by phytohormones, and that there is an excellent correlation between the expression of the histone *H2A-1* gene and susceptibility to *Agrobacterium* transformation. Our results depicted in Table 1 also demonstrate that infection of root segments by a transfer-competent *Agrobacterium* strain induces histone *H2A-1* promoter activity. We therefore asked whether pre-incubation of roots on phytohormones would increase their transformation efficiency. To answer this question, we pre-incubated whole roots or cut root segments of *Arabidopsis* ecotype Ws on MS medium (lacking phytohormones) or CIM medium (containing phytohormones) for various periods of time, after which we infected the cut root segments, or cut the whole roots into segments and infected them with *Agrobacterium*. Because this ecotype is highly susceptible to transformation, we diluted the bacteria 100-fold (10^7 cells ml^{-1}) from the inoculum concentration that we used in other experiments. We scored tumour production after 1 month. Figure 9 shows that pre-incubation of either cut root segments or whole roots on MS medium had relatively



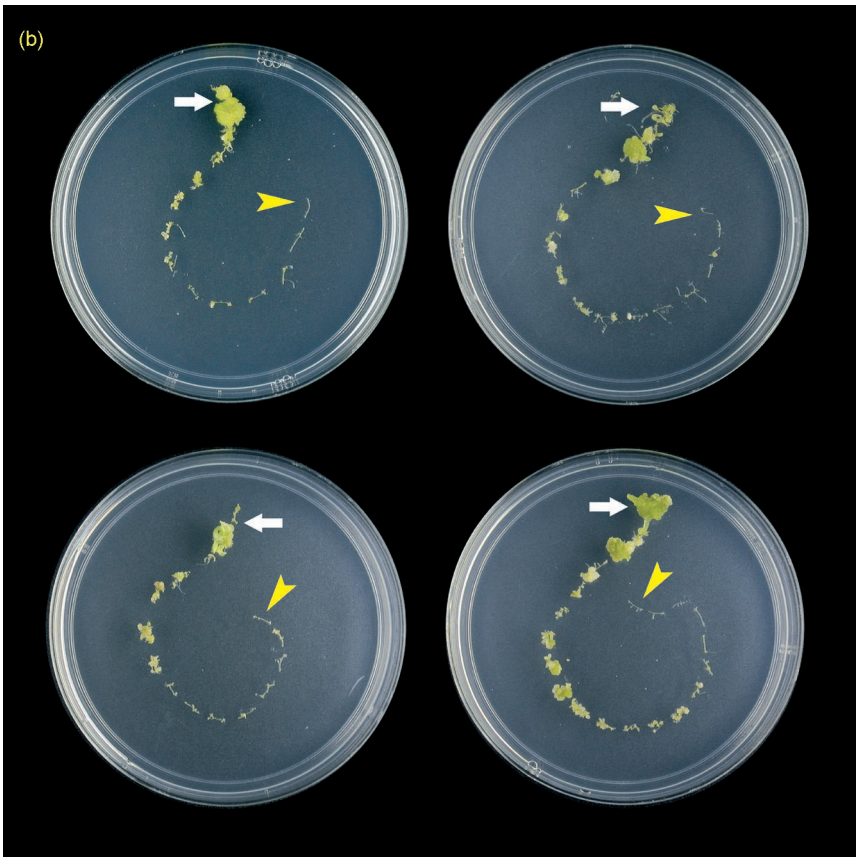


Figure 8. Phytohormone treatment of *Arabidopsis* roots co-ordinately alters the pattern of histone *H2A-1* promoter expression and transformation susceptibility.
(a) Roots of histone *H2A-1*::*gusA* transgenic plants were stained for GUS activity with X-gluc after incubation on callus inducing medium for 0–6 days.
(b) After incubation on callus inducing medium for 6 days, roots were cut into segments and infected with *A. tumefaciens* A208. After 2 days co-cultivation, the segments were arrayed as they initially appeared on MS medium containing timentin (to kill the bacteria) and lacking phytohormones (to select for tumours). Arrows indicate the basal root segments nearest the crown of the plant. Arrowheads indicate the most apical segments containing the apical meristem. Note that with increasing time of incubation on callus inducing medium, the pattern of histone *H2A-1* promoter activity changes from the root tip to the root base, and that there is a co-ordinate alteration in the pattern of transformation susceptibility to segments near the root base.

Table 1 Induction of the histone *H2A-1* and cyclin *cyc1At* promoters by *Agrobacterium* infection

	% of root segments showing GUS expression	
Infection	Histone *H2A-1*	Cyclin *cyc1At*
Uninfected	8.3 ($n=2900$)	0.7 ($n=1183$)
A. tumefaciens A208[a]	31.7 ($n=979$)	1.5 ($n=661$)
A. tumefaciens A136[b]	3.6 ($n=396$)	0.6 ($n=312$)

[a]T-DNA and Vir protein transfer-competent strain.
[b]T-DNA and Vir protein transfer-deficient strain.

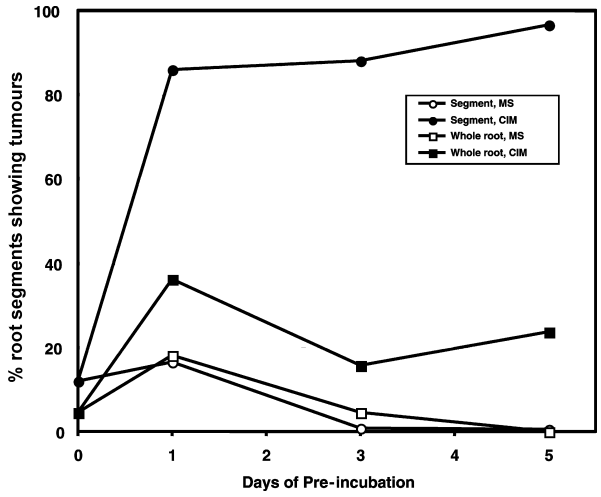


Figure 9. Phytohormone pre-treatment of *Arabidopsis* roots or cut root segments increases the transformation frequency by *Agrobacterium*.
Roots or cut root segments of *Arabidopsis* ecotype Ws were either infected with *A. tumefaciens* A208 directly, or incubated on MS medium lacking phytohormones or callus inducing medium (CIM) containing phytohormones for various periods before inoculation with *Agrobacterium*. Two days after infection, the segments were moved to MS medium lacking phytohormones but containing timentin. Tumorigenesis was scored after 1 month. ○, Root segments MS medium; ●, Root segments CIM; □, Whole roots MS medium; ■, Whole roots CIM. For each point, 70–80 root segments were assayed. The experiment was repeated twice. The experiment shown is representative of the two experiments performed.

little effect on tumour formation. Cut root segments showed only a 1.4-fold increase in transformation after 1 day incubation, and whole roots showed a 3.6-fold increase. Transformation rates dropped with further pre-incubation times. In contrast, 1 day pre-incubation on CIM medium dramatically increased the efficiency of tumorigenesis. After 1 day pre-incubation, the transformation of cut root segments increased approximately 7-fold, and this level of transformation was maintained for 3 and 5 days pre-incubation. Transformation rates also increased approximately 6-fold when whole roots were pre-incubated on CIM. However, this increase in transformation frequency was not maintained for roots pre-incubated for 3 and 5 days. Interestingly, when whole roots were pre-incubated on CIM medium for 5 days, subsequent *Agrobacterium* infection of cut root segments yielded many regenerated plants and atypical teratomas.

Our data indicate that pre-incubation of whole roots, or especially cut root segments, on phytohormones for a short period can enhance transformation by *Agrobacterium*.

Discussion

We have determined that the elongation zone, not the meristem, of *A. thaliana* roots is most susceptible to transformation by *A. tumefaciens*, and that this region contains cells that most highly express the histone *H2A-1* gene. We have further shown that following incubation on phytohormones, roots change their pattern of susceptibility to *Agrobacterium* transformation, and that this pattern correlates with a change in expression pattern of the histone *H2A-1* but not the cyclin *cyc1At* promoter. Furthermore, pre-incubation of *Arabidopsis* roots on phytohormones can substantially increase their susceptibility to *Agrobacterium*-mediated transformation.

Particular plant species (van Wordragen and Dons, 1992) and in some instances cultivars or ecotypes of a given species (Akama *et al.*, 1992; Chateau *et al.*, 2000; Nam *et al.*, 1997) can differ significantly in their transformation competence. In addition, specific tissues or cell types of a plant may display different degrees of transformation competence (Akama *et al.*, 1992; Pawlicki *et al.*, 1992). For example, Geier and Sangwan (1996) showed that in *Kohleria* internode explants, the most highly transformable cells constitute the vascular tissue, including pericycle cells. De Kathen and Jacobsen (1995) demonstrated that de-differentiating cells near the vascular system of epicotyl and cotyledon regions of pea are most susceptible to transformation. Similarly, in *Arabidopsis* ecotype C24 roots, de-differentiating pericycle cells were most transformation competent (Sangwan *et al.*, 1992). These cells were morphologically characterized as small and isodiametric with thin primary walls, multiple small vacuoles, and dense cytoplasm. In this latter example, however, the cells were induced for transformation competence only upon phytohormone pre-treatment of the roots. Our results, using ecotype Ws, indicate that even without phytohormone pre-treatment, cells in the elongation region of the root are competent for transformation. The difference in our results may result from the higher transformation efficiency of ecotype Ws compared to ecotype C24 (Nam *et al.*, 1997).

Data presented in this paper suggest a molecular marker for transformation competence: expression of the histone *H2A-1* gene. In all instances tested, the cells found most competent for *Agrobacterium*-mediated transformation correlated with cells most highly expressing this gene. Thus, in *Arabidopsis* roots, cells surrounding the vascular

tissue in the elongation zone were both most highly transformable and expressed the histone *H2A-1* gene to the greatest extent (Figures 1 and 5). The pattern of histone *H2A-1* expression could be altered by incubation of the roots with phytohormones prior to cutting and infection. Tumorigenesis susceptibility shifted to the basal regions of the root nearer to the crown of the plant, and we noted a parallel shift in histone *H2A-1* expression to this region of the root (Figures 2 and 8).

Research in several laboratories had previously suggested that actively dividing cells were most transformation competent, both to *Agrobacterium*-mediated transformation and to 'naked' DNA uptake (Kartzke *et al.*, 1990; Nagata *et al.*, 1986; Villemont *et al.*, 1997). We were thus surprised to identify the root elongation zone, rather than the root meristem, as the region most highly susceptible to *Agrobacterium* transformation (Figures 1 and 4). Although we detected expression of the histone *H2A-1* gene in the meristematic region of roots, much of the expression also localized to the elongation zone comprised of non-dividing cells. The use of a cyclin *cyc1At* promoter-*gusA* fusion construction clearly differentiated dividing from non-dividing cells. As reported previously (Ferreira *et al.*, 1994), GUS activity directed by this transgene localized to root, shoot and floral meristematic regions, whereas the histone *H2A-1* gene additionally expressed in non-dividing but elongating leaf, stem, and floral tissues (Figures 2 and 3). When cut root segments were treated with phytohormones (Figure 7) or were infected with *Agrobacterium* (Table 1), the expression patterns of the histone *H2A-1* and cyclin *cyc1At* promoters also did not correlate.

There are many reports of a class of 'replacement' histones that are expressed in a non-cell cycle-dependent manner (Chaubet *et al.*, 1992; Huh *et al.*, 1995; Kapros *et al.*, 1992; Meshi *et al.*, 2000; Robertson *et al.*, 1996; Waterborg and Robertson, 1996). In view of its expression in non-dividing cells, histone H2A-1 may possibly be classified as a 'replacement' histone. In addition, the histone H2A-1 protein does not contain the 'SPKK' motif commonly found near the carboxy-terminus of cell cycle-dependent histones (Huh *et al.*, 1995, 1997; Sundas *et al.*, 1993). It is interesting to note that a T-DNA insertion between two closely spaced 'replacement' histone H3 genes (Chaubet *et al.*, 1992; Chaubet-Gigot *et al.*, 2001) also results in the rat phenotype (J. Li, A. Kaiser, A. Kopecky, and S.B. Gelvin, unpublished). We thus speculate that 'replacement' histones may be involved in *Agrobacterium*-mediated transformation, especially in the step of T-DNA integration. However, DNA endoreduplication that allows plant cells to become polyploid takes place in many tissues and cells including leaf epidermal cells, hypocotyls, root hairs, and trichomes (Barlow, 1975; Dosier and Riopel, 1978; Galbraith *et al.*, 1991; Gendreau *et al.*, 1997, 1999; Jacqmard *et al.*, 1999; Melaragno *et al.*, 1993; Joubes and Chevatier, 2000). Although the fundamental reason for endoreduplication is still unclear, many investigators have suggested that an increase in ploidy is related to an increase in nuclear volume or cell size. Because endoreduplication occurs in many cells in which the histone *H2A-1* gene expresses, we cannot rule out the possibility that this gene is expressed in the S phase of cells undergoing the endoreduplication cycle but not in cells undergoing the normal mitotic cell cycle. To determine whether the histone *H2A-1* gene encodes a true 'replacement' histone (i.e. one whose expression is not linked to the S phase of the mitotic cell cycle), more detailed investigations will be required.

We and others have found that treatment of *Arabidopsis* tissues with phytohormones prior to *Agrobacterium* infection significantly enhances transformation. For example, Chateau *et al.* (2000) recently determined that pre-treatment of many *Arabidopsis* ecotypes, and the UV radiation-sensitive mutant *uvh1*, with phytohormones could significantly increase the transformation of *Arabidopsis* petiole segments. Without phytohormone pre-treatment, petioles from many of the ecotypes were highly recalcitrant to transformation. The transformation competence of other species, including *Pinus radiata* (Bergmann and Stomp, 1992), grape (Lowe and Krul, 1991), and *Petunia hybrida* (Villemont *et al.*, 1997) is also increased by treatment with phytohormones. In addition, many *Arabidopsis* transformation protocols using leaves, roots, hypocotyls, and cotyledons include phytohormone pre-treatment before *Agrobacterium* infection (Akama *et al.*, 1992, 1995; Chaudhury and Signer, 1989; Clarke *et al.*, 1992; Lloyd *et al.*, 1986; Mandal *et al.*, 1993; Valvekens *et al.*, 1988). Although *Arabidopsis* ecotype Ws is a relatively easily transformed ecotype (Nam *et al.*, 1997), we have shown that pre-treatment of whole roots and especially cut root segments can increase transformation 6- to 7-fold (Figure 9). Pre-treatment of roots from many highly recalcitrant ecotypes with phytohormones can also substantially increase their susceptibility to transformation (HC Yi and S.B. Gelvin, unpublished). Interestingly, the transformation frequency of roots from many *rat* (*r*esistant to *Agrobacterium t*ransformation) mutants can also be substantially increased by phytohormone pre-treatment (HC Yi and S.B. Gelvin, unpublished). Under these conditions, the cyclin *cyc1At* promoter is not substantially induced. These observations, in conjunction with our previous observations that many *rat* mutants can easily be transformed by flower vacuum infiltration (Mysore *et al.*, 2000a), suggests that the physiological state of various cell types differs with regard to transformation susceptibility, and that this physiological state can be altered by phytohormone treatment in the absence of cell division.

Infection of *Arabidopsis* root segments with a transformation-competent, but not a transformation-deficient, *Agrobacterium* strain increases the percentage of seg-

ments expressing the histone *H2A-1* promoter 6-fold. Under these infection conditions, the percentage of segments expressing the cyclin *cyc1At* promoter increases only slightly (Table 1). These data indicate that transfer of T-DNA, virulence proteins, or both can induce the histone *H2A-1* gene, allowing for more favourable T-DNA integration. They further indicate that the expression patterns of the histone *H2A-1* and cyclin *cyc1At* promoters are not coordinate. We have recently conducted experiments to identify plant genes that are rapidly induced or repressed following *Agrobacterium* infection of tobacco BY-2 suspension culture cells. Interestingly, we have found that all core histone genes, including histone H2A genes, are rapidly induced after infection, and that induction is similarly dependent upon T-DNA and/or virulence protein transfer (Veena and S.B. Gelvin, unpublished). Thus, it appears that in two different experimental systems (*Arabidopsis* roots and tobacco cell suspensions), plant histone genes are induced by infection with transfer-proficient *Agrobacterium* strains. Our previous work showed that the histone *H2A-1* gene is involved in T-DNA integration, and that overexpression of the histone *H2A-1* gene in transgenic *Arabidopsis* increases its susceptibility to transformation (Mysore *et al.*, 2000b). Taken together, these findings establish a central role for histones in the *Agrobacterium* transformation process.

Experimental procedures

Growth and infection conditions

A. tumefaciens was grown in YEP rich or AB minimal medium (Lichtenstein and Draper, 1986) at 30°C. When appropriate, antibiotics were used at the following concentrations (mg l^{-1}): rifampicin, 10; kanamycin, 100 (on solidified medium) or 25 (in liquid medium). *A. thaliana* seeds were surface sterilized and germinated on solidified B5 medium as described by Nam *et al.* (1999). When appropriate, kanamycin was incorporated into the medium at a final concentration of 50 mg l^{-1}. Root segments were cut and infected with *A. tumefaciens* as previously described (Nam *et al.*, 1999). For tumorigenesis assays, we used either *A. tumefaciens* A208 (a tumorigenic strain; Sciaky *et al.*, 1978) or A136 (an avirulent strain; Watson *et al.*, 1975). For transformation assays to express GUS activity in plants, we used *A. tumefaciens* At802 [A208(pBISN1)].

Generation of histone H2A-1::gusA *transgenic plants*

A 650-bp *Hin*dIII fragment containing 620 bp of upstream sequence and the first 30 bp of the histone H2A-1 coding sequence was cloned into the *Hin*dIII site of pBluescript. A *Sma*I-*Xba*I fragment from this resulting plasmid was cloned into the corresponding sites of pBI101.3, generating a translation fusion of the first 10 amino acids of the histone H2A-1 protein with the β-glucuronidase protein. The resulting plasmid, pKM3, was introduced into *A. tumefaciens* GV3101 (Koncz and Schell, 1986) by triparental mating (Ditta *et al.*, 1980) and used to transform *A. thaliana* ecotype Ws by the flower vacuum infiltration method (Bent and Clough, 1998). Transgenic plants were selected on B5 medium containing 50 mg l^{-1} kanamycin.

GUS activity assays

Assays to detect β-glucuronidase (GUS) expression in plant tissues were performed using 5-bromo-4-chloro-3-indolylglucuronide (X-gluc) according to Jefferson *et al.* (1987).

Hybridization probes

A histone *H2A-1* gene-specific probe was designed from the 3′-untranslated (UTR) region of the gene. 145 bp of 3′-UTR sequences were amplified by PCR and subcloned into pBluescript KS+ (Stratagene, La Jolla, CA, USA). The plasmid was digested with *Sma*I and *Eco*RV for *in vitro* transcription to generate sense and antisense RNA probes, respectively. *In vitro* transcription was performed using a RNA transcription kit with digoxigenin-11-uridine-5′-triphosphate as specified by the supplier (Stratagene, La Jolla, CA, USA).

In situ *hybridization*

For whole-mount *in situ* hybridization, *A. thaliana* seedlings were fixed in PBS, 67 mM EGTA, and 1% formaldehyde for 25 min at room temperature and pre-treated as described in the non-radioactive *in situ* hybridization application manual, 2nd edition (Boehringer Mannheim, Indianapolis, IN, USA). *In situ* hybridization was carried out at 37°C using digoxygenin-labelled RNA probes. Anti-digoxigenin antibody coupled to alkaline phosphatase (Boehringer Mannheim) was diluted 1 : 1000 in DIG blocking buffer (Boehringer Mannheim) and absorbed to hybridized seedlings for 1–3 h at 37°C. The chromogenic reaction with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma, St Louis, MO, USA) was used for detection of *H2A-1* RNA signals. A sense probe was used as a control for non-specific reactivity.

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