Cell Type–Specific Chromatin Decondensation of a Metabolic Gene Cluster in Oats

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Transcription-related chromatin decondensation has been studied in mammals for clusters of structurally and/or functionally related genes that are coordinately regulated (e.g., the homeobox locus in mice and the major histocompatibility complex locus in humans). Plant genes have generally been considered to be randomly distributed throughout the genome, although several examples of metabolic gene clusters for synthesis of plant defense compounds have recently been discovered. Clustering provides for genetic linkage of genes that together confer a selective advantage and may also facilitate coordinate regulation of gene expression by enabling localized changes in chromatin structure. Here, we use cytological methods to investigate components of a metabolic gene cluster for synthesis of developmentally regulated defense compounds (avenacins) in diploid oat (Avena strigosa). Our experiments reveal that expression of the avenacin gene cluster is associated with cell type–specific chromatin decondensation, providing new insights into regulation of gene clusters in plants. Importantly, chromatin decondensation could be visualized not only at the large-scale level but down to the single gene level. We further show that the avenacin and sterol pathways are likely to be inversely regulated at the level of transcription.

INTRODUCTION

In mammalian systems, transcription-related chromatin decondensation has been observed in a number of artificial gene clusters, in highly transcribed chromosomal regions and for clusters of functionally related genes that are coordinately regulated during development or between different cell types, such as the Hoxb locus (Tumbar et al., 1999; Tsukamoto et al., 2000; Volpi et al., 2000; Williams et al., 2002; Chambery and Bickmore, 2004; Janicki et al., 2004; Chambery et al., 2005; Sproul et al., 2005; Morey et al., 2007). In plants, genes are normally considered to be randomly distributed throughout the genome, with the obvious exception of groups of physically linked genes that share sequence homology and that have arisen through tandem gene duplication (e.g., gene-for-gene type disease resistance genes) (Friedman and Baker, 2007). Recently, however, several examples of clusters of genes for synthesis of plant defense compounds have been found (Frey et al., 1997; Qi et al., 2004; Wilderman et al., 2004; Shimura et al., 2007; Field and Osbourn, 2008; Swaminathan et al., 2009). The identification of groups of genes that do not share sequence relatedness and that contribute to a common pathway raises some intriguing questions about the evolutionary pressures responsible for such self-organization (Amoutzias and Van de Peer, 2008). Clustering will facilitate the inheritance of beneficial combinations of genes that together confer a selective advantage (Gierl and Frey, 2001; Qi et al., 2004; Wong and Wolfe, 2005; Field and Osbourn, 2008), while disruption of such metabolic gene clusters may lead not only to loss of ability to synthesize a beneficial pathway end product but also to accumulation of toxic pathway intermediates (Field and Osbourn, 2008; Mylona et al., 2008). Physical clustering may also facilitate coordinate regulation of expression of pathway genes by enabling localized changes in chromatin structure (Sproul et al., 2005; Osbourn and Field, 2009).

Our investigations of the role of natural products in plant defense have led us to identify an operon-like gene cluster for the synthesis of antimicrobial terpenes (avenacins) in diploid oat (Avena strigosa) (Papadopoulou et al., 1999; Qi et al., 2004). These defense compounds accumulate in the roots, where they confer broad-spectrum resistance to attack by soil-borne pathogens (Papadopoulou et al., 1999). The ability to produce avenacins is restricted to the genus Avena and has evolved since the divergence of oats from other cereals and grasses. To date, we have cloned a total of five genes in this cluster and have defined a further two loci that show complete genetic cosegregation with these five genes (Papadopoulou et al., 1999; Haralampidis et al., 2001; Qi et al., 2004, 2006; Mugford et al., 2009). The expression of the avenacin biosynthesis genes is tightly regulated and is restricted to the epidermal cells of the root meristem, which is the site of accumulation of avenacins (Haralampidis et al., 2001; Qi et al., 2006; Mugford et al., 2009). Here, we have selected the avenacin biosynthetic genes Sad1 and Sad2 for cytological investigation of chromatin
RESULTS

Sad1 and Sad2 Are Cotranscribed in Root Epidermis Cells and Are Transcriptionally Silent in the Cortex

Avenacin A-1 is the major UV fluorescent compound present in oat roots and accumulates in the epidermal cells of the root tips, which are the site of synthesis (Hostettmann and Marston, 1995; Haralampidis et al., 2001; Qi et al., 2006; Mugford et al., 2009) (Figures 1A and 1B). We used mRNA fluorescence in situ hybridization (FISH) to examine the expression of the Sad1 and Sad2 genes in root cells. All experiments were performed on wax-embedded root sections of 3-d-old seedlings of A. strigosa. Sad1 and Sad2 were simultaneously transcriptionally active in the nuclei of over 80% of root tip epidermal cells (Figure 1C, Table 1). Sense probes for these genes did not give signals, indicating that the signals that we observed were derived from mRNA and not DNA (see Supplemental Figure 1 online). Sad1 was additionally expressed in ~50% of the cells of the outermost cortex layer, referred to here as the subepidermis (Figures 1A and 1C), while Sad2 transcripts were seldom detected in this cell layer. Sad1 and Sad2 transcripts were not observed in other cell types within the root (see Supplemental Figure 1 online). There were no obvious differences between G1 and G2 nuclei in coexpression of Sad1 and Sad2 in root tip epidermal cells (Figure 1C), implying that expression of Sad1 and Sad2 is not cell cycle dependent.

Cytoplasmic proteins and nuclear-encoded plastid and mitochondrial proteins are generally translated on free ribosomes in the cytoplasm, while integral membrane proteins and those that are secreted are translated on ribosomes bound to the ER. Sad1 and Sad2 are not predicted to be integral membrane proteins, but based on structural predictions and by analogy with their sterol biosynthetic counterparts, both are likely to be membrane associated (Haralampidis et al., 2001; Qi et al., 2004, 2006). In addition to the nascent transcripts that we detected in the nuclei (Figure 1), cytoplasmic transcripts derived from Sad1 and Sad2 were readily detectable in the root tip epidermal cells (Figure 2). Cytoplasmic Sad1 transcript was also evident at lower levels in the cytoplasm of the cells of the subepidermis (see Supplemental Figure 2 online). The spatial pattern of the Sad2 transcript resembled a typical reticulate endoplasmic reticulum (ER) distribution in the cytoplasm. By contrast, the Sad1 transcript was localized in foci that may represent branched strands of a membranous system, possibly specialized subdomains of the ER. These experiments indicate that the patterns of localization of the Sad1 and Sad2 mRNAs in the cytoplasm are distinct and rarely overlap, suggesting that the translation of these two mRNA species into proteins is unlikely to be spatially coordinated. A few examples of targeting of transcripts to specific parts of the cytoplasm in plant cells have been reported, including transcripts for expansins and seed storage proteins (Choi et al., 2000; Okita and Choi, 2002; Crofts et al., 2004; Washida et al., 2004). Avenacins accumulate in the cell vacuole (Mylona et al., 2008), but the route by which pathway intermediates are transported from the ER to this final destination is...
unknown. It is possible that this process could be mediated via zip-coded regions of the ER (Grotewold and Davies, 2008).

The Genomic Region Spanning Sad1 and Sad2 Is Decondensed in the Root Epidermis

We then used DNA FISH to determine whether expression of genes within the avenacin gene cluster is accompanied by chromatin decondensation. Previously, we constructed and sequenced a BAC contig spanning the Sad1/Sad2 region and showed that these two genes lie within 58 kb of each other in the A. strigosa genome (Figure 3A) (Qi et al., 2004, 2006). We generated probes for the Sad1 and Sad2 genes (15.2 and 5.8 kb, respectively) (Figure 3A). Using these probes, we were able to detect the Sad1 and Sad2 loci in nuclei of the epidermal, subepidermal, and cortical cells of A. strigosa root tips by DNA FISH (Figures 3B and 3C). The hybridization signal for Sad1 in the nuclei of epidermal cells frequently comprised more than one fluorescent focus. In several cases, this was also true for Sad2. However, in the subepidermal and cortical cells, each locus was almost always visualized as a discrete focus. The nuclei that we examined in these experiments were all G1 nuclei (see Methods) and so these multiple foci are not attributable to signals derived from sister chromatids. The multiple foci that we have observed for Sad1 (and to a lesser extent Sad2) in the nuclei of the root tip epidermal cells are therefore likely to reflect decondensation of the respective loci. Beaded signals have also been seen in much larger, transcriptionally active chromosome regions spanning groups of major histocompatibility complex and other genes in mammalian cells (Volpi et al., 2000; Müller et al., 2004).

We then examined the length of the region encompassing Sad1 and Sad2 in the nuclei of the epidermal, subepidermal, and cortical cells. To do this, we measured a path consisting of the shortest distance in three dimensions traversing the intensity maxima of all fluorescent foci, starting with all Sad1 foci (green) and ending with all Sad2 foci (red), as shown by Figure 3C and the accompanying path outlines (Figure 3D). These data are summarized in Figure 3E. We calculated a mean length for the whole region of 0.97 μm for epidermis nuclei and of 0.41 and 0.42 μm for subepidermis and cortex nuclei, respectively. The length differences between epidermis and subepidermis/cortex are significant (P = 0.0001 for both, two-tailed Mann-Whitney U-test) and correspond to a chromatin compaction of 28-fold for the epidermis and 66- and 65-fold for subepidermis and cortical cells, compared with naked B-DNA (Watson and Crick, 1953). The length differences between subepidermal and cortical cells are not significant (P = 0.7, two-tailed Mann-Whitney U-test). Thus, the region shown in Figure 3A is more extended in nuclei of cells expressing both Sad1 and Sad2 (the epidermal cells) than in those cells that do not express these genes (the cortical cells) or in the subepidermal cell layer where Sad1 is expressed more sporadically and at lower levels. This suggests that coordinate expression of Sad1 and Sad2 in the root epidermal cells may be associated with chromatin decondensation. However, lower-level expression of Sad1 in the subepidermal cell layer is not associated with significant chromatin decondensation as assessed using these methods. In order to distinguish the contributions of the two genes to chromatin decondensation from that made by the intergenic sequence, we compared the distances between the closest Sad1 and Sad2 foci for each chromosome that we examined, as an estimated lower bound for the intergenic distance. The mean lengths obtained were 0.47 μm for epidermis nuclei and 0.31 and 0.34 μm for subepidermal and cortex nuclei, respectively. These differences between epidermis and subepidermis/cortex nuclei were significant (P = 0.03 for the difference between epidermis and cortex and P = 0.02 for the difference between epidermis and subepidermis, two-tailed Mann-Whitney U-test). There was no significant difference in the length of the intergenic regions when the values for subepidermis and cortex were compared (P = 0.9, two-tailed Mann-Whitney U-test). Our strategy for defining the paths that we have measured is based on the assumption that the closest foci that we observe for each gene are nearest neighbors in terms of the linear organization of the gene and that the Sad1 and Sad2 foci that are closest to each other represent the shortest intergenic distance. Clearly, this need not always be the case, and so we may have underestimated the degree of chromatin decondensation for both of these parameters.

Sad1 Is Decondensed in the Root Epidermis

Next, we examined decondensation at the single gene level. First, we performed DNA FISH experiments using the

![Figure 2](image-url)
same 15.2-kb *Sad1* probe as in our previous experiments (Figures 3A and 4A) and analyzed the number of fluorescent foci in G1 and G2 nuclei of cells in the epidermis, subepidermis, and cortex of the root tips (Figures 4B and 4C) by three-dimensional optical sectioning through the nuclei. There were more fluorescent foci for *Sad1* in the nuclei of epidermal cells when compared with the nuclei of subepidermal and cortical cells, consistent with decondensation of *Sad1* in the nuclei of the epidermal cells. These differences were significant for both G1 and G2 nuclei (G1, \( P = 0.001 \); G2, \( P = 0.002 \), for epidermis compared with subepidermis; G1, \( P = 0.000004 \); G2, \( P = 0.00002 \) for epidermis compared with cortex; Figure 4C). There also appeared to be more *Sad1* foci in the subepidermis compared with the cortex, although these differences were significant for G2 nuclei (\( P = 0.03 \)) but not for G1 nuclei (\( P = 0.2 \)). These results are consistent with our expression data (Figure 1C, Table 1), which indicate that *Sad1* is expressed in the nuclei of root epidermal cells and to a lesser extent in nuclei of the subepidermal cells but not in the cortex. *Sad1* shows an intermediate degree of decondensation when G2 nuclei of the cortex, subepidermis, and epidermis are compared (Figure 4), although cell cycle–related differences in *Sad1* expression were not observed (Figure 1).

We then labeled the 5' and 3' parts of the *Sad1* locus with different fluorochromes (Figure 5). In this case, we did not
differentiate between G1 and G2 nuclei. We restricted our analysis to those loci that showed one green and one red focus (and so represented either G1 nuclei or overlapping chromatids in G2 nuclei) or that showed two red and two green foci that could be identified as belonging to two different chromatids (Figures 5B and 5C). We then measured the distances in three dimensions between the intensity maxima of the two halves for nuclei of root epidermal and cortical cells (Figure 5D). Based on these measurements, the mean length of Sad1 was calculated to be 0.35 µm in the epidermis and 0.23 µm in the cortex. This corresponds to a chromatin compaction of 15-fold in the epidermis and 22-fold in the cortex compared with naked B-DNA (Watson and Crick, 1953). These length differences are highly significant (P = 0.0004).

CS1, the Sad1 Homolog in Sterol Biosynthesis, Is Expressed in the Root Cortex but Not in the Epidermis of the Root Tip

Sad1 is likely to have been recruited from primary sterol metabolism by duplication of the cycloartenol synthase gene CS1, followed by acquisition of new function (Haralampidis et al., 2001; Qi et al., 2004). Sterols are essential for plant growth and development, and the genes for sterol biosynthesis are normally expressed throughout the plant (Ohyama et al., 2009). Previously, we cloned and characterized the A. strigosa CS1 gene and showed by RNA gel blot analysis that this gene (which shares 42% nucleotide sequence identity with Sad1) is expressed in the roots, stems, leaves, and flowers, as expected (Haralampidis

Figure 4. The Sad1 Gene Locus Is Decondensed in Nuclei of Root Tip Epidermal Cells as Shown Using a 15.2-kb Probe Spanning Sad1.

(A) Diagram showing the 15.2-kb probe spanning the Sad1 gene locus used for DNA in situ hybridization (coding region in light green).

(B) Representative examples of Sad1 loci in different cell types. The images are overlays of several optical sections, section spacing 0.2 µm. Blue, chromatin stained with DAPI. Bar = 5 µm.

(C) Distributions of numbers of fluorescent foci in G1 and G2 nuclei in the three different cell types.
et al., 2001; Qi et al., 2004). Here, we have performed mRNA in situ hybridization using alkaline phosphatase signal amplification to examine the spatial expression pattern of CS1 at higher resolution. Surprisingly, we found that CS1 transcripts were not detectable in the root tip. While Sad1 transcripts were detected primarily in the epidermal cells of the meristematic zone of the root tips of 3-d-old A. strigosa seedlings (and more weakly in the subepidermal cell layer and in parts of the columella), CS1 transcripts were only evident in the cortical cells behind this region (Figure 6). This suggests that the two pathways are likely to be inversely regulated at the level of transcription. Inverse coordinate regulation of the two pathways may be important because the two pathways compete for a common substrate (2,3-oxidosqualene) (Chappell, 2002). It could also represent a self-protection mechanism, since amphipathic triterpene glycosides, such as avenacins, exert their toxic effects on cells by complexing with sterols, causing membrane permeabilization (Morrissey and Osbourn, 1999). A cycloartenol

**Figure 5.** Decondensation of the Sad1 Gene Locus Can Be Visualized Using Probes for the 5' and 3' Ends of the Gene. (A) Diagram showing the probes for the 5' and 3' ends of the Sad1 gene used for DNA in situ hybridization. The two probes split the locus in half. The parts of the coding region encompassed by each probe are shown in lighter shades of green or red. (B) G2 nuclei in epidermal and cortical root cells each showing two Sad1 loci: 5' probe in green and 3' probe in red. Bar = 5 μm. (C) Detailed views of individual gene loci (with the framed loci shown in (B) on the left of each set of panels). Bar = 1 μm. The images shown in (B) and (C) are overlays of several optical sections, section spacing 0.2 μm. Blue, chromatin stained with DAPI. (D) Length distributions of the Sad1 locus in nuclei of epidermal and cortical cells. The length of each locus was determined by measuring the distance in three dimensions between the intensity maxima of the two halves.

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The higher-order structure of chromatin is still a matter of considerable debate. There is good evidence that nucleosomes are organized into 10-nm fibers, visualized as beads on a string.
under the electron microscope, which would have a compaction of sevenfold (Table 2) (Olins and Olins, 1974; Oudet et al., 1975; Goodrich and Tweedie, 2002). Thus, our data for Sad1 suggest that on average this gene is more compact than a 10-nm fiber, although it is likely that decondensation varies along the gene and may be greater where the polymerases are bound. There is very little data so far available about the condensation status of active genes. The Balbiani rings (giant chromosomal puffs) of the Dipteran midge Chironomus have been shown by electron microscopy to have a compaction of 3.6, well below that of the 10-nm fiber (Daneholt et al., 1982), while the rRNA genes in pea (Pisum sativum) show a greater degree of compaction (8- to 11-fold), which corresponds well with that of the 10-nm fiber (Gonzalez-Melendi et al., 2001) (Table 2). The Chironomus and pea genes studied in these investigations are very highly transcribed and have many polymerases attached, which may be expected to lead to a higher degree of decondensation. Most active genes are likely to have only one or two polymerases attached, so our value of 15 for compaction of active Sad1 seems reasonable.

The proposed levels of structural organization above the 10-nm fiber are highly controversial. A well-publicized secondary structure of chromatin is the 30-nm fiber, which is 40- to 50-fold more condensed than naked B-DNA (Goodrich and Tweedie, 2002). Such 30-nm fibers have been observed in vitro (Robinson et al., 2006), but as yet there is no clear evidence for these in vivo or, indeed, for more condensed higher-order structures (Branco and Pombo, 2007; Fakan and van Driel, 2007). Our mean chromatin compaction values indicate that in both the epidermis and the cortex, Sad1 is packaged into a structure that has a higher packing order than the 10-nm fiber and a lower one than...
the 30-nm fiber. For the Sad1/Sad2 region with its repeat-rich intergenic region (Qi et al., 2006), the packing order increases but is still less than the 30-nm fiber level in the epidermis, while it is above this level in the cortex (Table 2).

Our data provide cytological evidence to link cell type–specific chromatin decondensation with expression of the avenacin gene cluster in nuclei of oat root epidermal cells, providing new insights into regulation of gene clusters in plants. It has been suggested that opening up domains of secondary chromatin structure may place genes in a transcriptionally permissive environment, where their expression can then be triggered by transcription factors (Sproul et al., 2005). Further experiments are required to establish the cause and effect of this phenomenon and to identify the factors required for pathway regulation. The most satisfactory way to achieve this will be through characterization of regulatory mutants. We have isolated a resource of 100 avenacin-deficient mutants of A. strigosa (Papadopoulou et al., 1999; Qi et al., 2006; Mugford et al., 2009). Analysis of this mutant collection is expected to lead to the identification of loci that are required for regulation of expression of the gene cluster. These may include genes for pathway-specific transcription factors and also for regulation at the chromatin level.

METHODS

Plant Material

The diploid oat (Avena strigosa) accession number S75 (from the Institute of Grasslands and Environmental Research, Aberystwyth, Wales, UK) was used for all experiments.

RNA and DNA in Situ Hybridization

Oat seeds were imbibed in water for 24 h at 4°C and germinated on wet filter paper for 3 d at 22°C. Root tips were fixed in 4% (w/v) formaldehyde in PBS and embedded in wax and longitundinal sections (12 μm) prepared. Probes for in situ hybridization were prepared by in vitro transcription (for RNA probes) or by nick translation (for DNA probes) and labeled with either digoxigenin-11-UTP (Roche) or dinitrophenol-11-UTP (Perkin-Elmer) (see Supplemental Methods online for further details of probes, hybridization, and immunodetection methods).

Image Acquisition, Analysis, and Measurements

RNA in situ experiments with enzymatic detection were analyzed with a Nikon Eclipse 800 microscope using a ×10 objective with a numerical aperture of 0.3. Images were taken on a Pixera Pro 600 digital camera. For the FISH experiment, root sections were analyzed using a ×60 objective (numerical aperture 1.4, oil) on a Nikon Eclipse 600 microscope equipped with a Hamamatsu Orca ER cooled CCD digital camera, a motorized stage, and a z-focus drive. Further details concerning filters, correction of chromatic aberration, deconvolution of raw data, differentiation between G1 and G2 nuclei, length measurements, and preparation of images are given in Supplemental Methods online.

Accession Numbers

Sequence data for the genes referred to in this article can be found in the GenBank/EMBL data libraries under accession numbers DQ680849 (Sad1 and Sad2) and AY618693 (CS1).

Supplemental Data

The following materials are available in the online version of this article:

Supplemental Figure 1. Control RNA in Situ Hybridization Experiments for Sad1 and Sad2.

Supplemental Figure 2. Transcripts of Sad1 (red) and Sad2 (green) in Oat Root Tips.

Supplemental Methods.

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Table 2. DNA Compaction

| DNA          | Size (kb) | Untranscribed Length | Transcribed Length (μm) | kb/μm | Compaction | Reference               |
|--------------|-----------|-----------------------|-------------------------|-------|------------|-------------------------|
| B-DNA       | –         | –                     | 2.9                     | 1.0   | Watson and Crick (1953) |
| 10-nm fiber | –         | –                     | 20.3                    | 7.0   | Woodcock and Dimitrov (2001) |
| 30-nm fiber | –         | –                     | 116–145                 | 40–50 | Woodcock and Dimitrov (2001) |
| Hox1b-Hox3   | 90        | 0.1                   | 900                     | 310   | Chambery and Bickmore (2004) |
|              |           | 0.9                   | 100                     | 34    |                         |
| MHCII        | 375       | 1.2                   | 312                     | 107   | Müller et al. (2004)    |
| Sad1-Sad2    | 79        | 0.42 (cortex)         | 178                     | 65    | This study             |
|              |           | 0.97 (epidermis)      | 81                      | 28    |                         |
| Sad1         | 15        | 0.23 (cortex)         | 65                      | 22    | This study             |
|              |           | 0.35 (epidermis)      | 43                      | 15    |                         |
| Pea rDNA (transcribed) | 6.6  | 0.2-0.3               | 22–33                   | 7.6–11.4 | Gonzalez-Melendi et al. (2001) |
| Chironomus BR2 (transcribed) | 37  | 3.5                   | 10.4                    | 3.6   | Daneholt et al. (1982)  |
| Wheat glutenin array (20 copies) | 180 | 2.17                  | 83                      | 29    | Wegel et al. (2005)     |
|              |           | 13.66                 | 13                      | 4.5   |                         |
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