RESEARCH LETTER

A gene determining a new member of the SARP family contributes to transcription of genes for the synthesis of the angucycline polyketide auricin in Streptomyces aureofaciens CCM 3239

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Abstract

Three regulators, Aur1P, Aur1R and a SARP-family Aur1PR3, have been previously found to control expression of the aur1 cluster for the angucycline antibiotic auricin in Streptomyces aureofaciens CCM 3239. Here, we describe an additional regulatory gene, aur1PR4, encoding a homologue from the SARP-family regulators. Its role in auricin regulation was confirmed by its disruption that dramatically affected auricin production. However, transcription from the aur1Ap promoter, directing expression of 22 auricin biosynthetic genes, was not substantially affected in the Δaur1PR4 mutant. A new promoter, sa13p, directing transcription of four putative auricin tailoring genes, was found to be dependent on aur1PR4. Moreover, analysis of the sa13p promoter region revealed the presence of three heptamer repeat sequences corresponding to putative SARP-binding sites. Expression of aur1PR4 is directed by a single promoter, aur1PR4p, which is induced after entry into stationary phase. Transcription from aur1PR4p was absent in a S. aureofaciens Δaur1P mutant strain, and Aur1P was shown to bind specifically to the aur1PR4p promoter. These results indicate a complex network of regulation of the auricin gene cluster. Both Aur1P and Aur1PR3 are involved in regulation of the core aur1A-U biosynthetic genes, and Aur1PR4 in regulation of putative auricin tailoring genes.

Introduction

The Gram-positive soil bacteria of the genus Streptomyces are one of the most important groups of microorganisms, which produce bioactive secondary metabolites, including many known antibiotics. The genes required for antibiotic biosynthesis are generally clustered and they are coregulated by pathway-specific transcriptional activators that are located in these clusters. The expression of these regulatory genes is controlled by complex regulatory mechanisms that determine the onset of antibiotic production (Bibb, 2005; Martin & Liras, 2010). Pathway-specific transcriptional activators have been classified into different families. One of the most prominent of them is the Streptomyces antibiotic regulatory protein (SARP) family. The members of this family are activators characterized by an N-terminal OmpR-type winged helix-turn-helix (HTH) DNA-binding domain (Wietzorreck & Bibb, 1997). This family is represented by several antibiotic pathway-specific activators, like ActII-ORF4 and RedD for actinorhodin and undecylprodigiosin in Streptomyces coelicolor (Fernandez-Moreno et al., 1991; Takano et al., 1992), DnrI for daunorubicin in Streptomyces peucetius (Sheldon et al., 2002), CcaR for clavulanic acid and cephamycin in Streptomyces clavuligerus (Santamarta et al., 2011), FdmR1 for fredericamycin in Streptomyces griseus (Chen et al., 2008), and TylS for tylosin in Streptomyces fradiae (Cundliffe, 2008). However, it contains also well-studied pleiotropic regulatory protein AfsR (Tanaka et al., 2007). Studies of the interactions between SARP-family...
activators with their target genes identified heptameric direct repeats (5'-TCGAGXX-3') with 4-bp spacers, located stringently 8 bp upstream of the -10 regions of promoters (Wietzorreck & Bibb, 1997; Arias et al., 1999; Sheldon et al., 2002; Tanaka et al., 2007).

We previously identified a polyketide synthase gene cluster, aur1, responsible for the production of the angucycline antibiotic auricin in Streptomyces aureofaciens CCM 3239 (Novakova et al., 2002). Auricin, which is a unique angucycline polyketide conjugated to an amino deoxyhexose D-fosamine, is produced during a very narrow time period following a short period of several hours following entry into stationary phase. This unusual phenomenon likely arises from a strict, but complex regulation of auricin biosynthesis, involving several predicted transcriptional regulators (Kutas et al., 2013). Three of them have already been characterized, and their role in regulation of auricin biosynthesis is described in the Fig. 6. The aur1P gene, which encodes a protein belonging to a family of 'atypical' response regulators, has been shown to specifically activate expression of the auricin biosynthesis genes by directly binding and activating the aur1Ap promoter, triggering the expression of 22 structural genes (aur1A-aur1U) of the auricin cluster (Novakova et al., 2005, 2011a). The product of the aur1R gene, which encodes a protein homologous to the transcriptional repressors of the TetR family, has been shown to repress expression of the aur1P gene by directly binding the aur1Rp promoter (Novakova et al., 2010a). Another regulatory gene, aur1PR3, encoding a homologue of the SARP family of transcriptional activators, has also been found to be involved in auricin regulation, and it is also under the direct control of Aur1R (Novakova et al., 2011b).

The auricin cluster is located on pSA3239, a large linear plasmid. Although the main part of the auricin-specific genes is clustered, several genes encoding auricin tailoring enzymes are scattered across a rather large aur1-flanking region (Novakova et al., 2013). The aur1 cluster is even interrupted by eight genes encoding proteins with no auricin-specific function, including bpsA, a gene involved in the biosynthesis of the blue pigment indigo-dine (Novakova et al., 2010b). Intriguingly, disrupting of bpsA, by replacing it with the resistance marker gene aac3 (IV), causes increased auricin production. A possible explanation of this increase may be a polar effect of the aac3(IV) gene on the downstream sa9 gene encoding a SARP regulator (Novakova et al., 2010b). In the present study, we describe the characterization of sa9 (aur1PR4). We provide evidence that it is essential for auricin production in S. aureofaciens CCM 3239. We describe the identification of a putative target promoter of this regulator. In addition, we investigate the transcriptional regulation of aur1PR4 and show that the auricin-specific activator Aur1P directly regulates its expression.

Materials and methods

Bacterial strains, plasmids and culture conditions

The S. aureofaciens CCM 3239 wild type was from the Czechoslovak Collection of Microorganisms, Brno, Czech Republic. The S. aureofaciens Δaur1P mutant strain was described in Novakova et al. (2005), the S. aureofaciens Δaur1R mutant strain in Novakova et al. (2010a) and the S. aureofaciens Δaur1PR3 mutant strain in Novakova et al. (2011b). Escherichia coli SURE and a pBluescript II SK- plasmid (Stratagene) were used for E. coli-cloning experiments. Escherichia coli BW25113/pIJ790 was the host for PCR-targeted gene disruption using the plasmid pIJ773 and E. coli ET12567/pUZ8002 as a nonmethylating host for conjugation (Gust et al., 2003). The Streptomyces integrative plasmid pPM927 (Smokvina et al., 1990) was used for complementation studies. The growth of S. aureofaciens CCM 3239 strains was carried out in Bennet medium (Horinouchi et al., 1983) as described in Novakova et al. (2010a). For RNA isolation, spores (5 × 10^8 CFU) of the particular S. aureofaciens CCM 3239 strain being studied were inoculated in 50 mL of liquid Bennet medium and grown at 28 °C to different growth phases. Conditions for E. coli growth and transformation are described by Ausubel et al. (1995).

Disruption of the S. aureofaciens CCM 3239 aur1PR4 gene

The S. aureofaciens CCM 3239 cosmig library (comprising Sau3AI partially digested DNA fragments in the BamHI site of SuperCos-1) is described in Novakova et al. (2010a). Deletion of the whole coding region of the S. aureofaciens CCM 3239 aur1PR4 gene (aside from its start and stop codons) in the cosmid pCosSA19 (Novakova et al., 2010b) was achieved using the PCR-targeted REDIRECT method (Gust et al., 2003). In brief, the apramycin resistance cassette was PCR amplified using the primers SARP5DIR (5'-AATTGGCGGT GTTCTGCT GACGAGGAGTCATCACCCATGTGTAGGCTGGAGCTG CTTC-3') and SARP5REV (5'-CCGATACCGGAGCTCC TTCGTGTCGTGCGCCGGTGCTCAATT CCGGGGATCC GTCGACC -3') and the template plasmid pIJ790. The resulting PCR product was used to electroporate E. coli BW25113/pIJ790 containing cosmid pCosSA19.

The replacement of the coding region in the resulting SuperCosI-derived cosmids pCosSA19-aur1PR4/AmR was verified by restriction mapping. This cosmid was transformed into the nonmethylating E. coli ET12567/ pUZ8002 strain and introduced into S. aureofaciens CCM 3239 by conjugation. Four apramycin-resistant and
kanamycin-sensitive colonies were selected from the conjugation and the double-cross event verified by Southern blot hybridization (data not shown). One representative strain, \textit{S. aureofaciens} \textit{Daur1PR4} was chosen for further study. The plasmid pPM-aur1PR4, used for complementation of the \textit{Daur1PR4} mutation, was prepared by inserting a 1280-bp Acc65I-BsiWI fragment of the cosmid pCosSA19 (containing the \textit{aur1PR4} gene with its promoter) in pPM927 digested with Acc65I.

**RNA isolation and S1-nuclease mapping**

Isolation of total RNA and high-resolution S1 nuclease mapping were performed according to Kormanec (2001). 40-g samples of RNA were hybridized to c. 0.015 pmol of a corresponding DNA probe labelled at one 5′ end with \(\gamma\)-\[^{32}\text{P}\] ATP (c. 10^6 d.p.m. pmol^{-1} of probe). The S1 probes were prepared as follows: probe 1 (for the \textit{aur1PR4p} promoter, Fig. 1a) was prepared by PCR amplification from the cosmid pCosSA19 using the 5′ end-labelled reverse primer SRP5REV (5′-CGTGTG AGCAGCAGTGCC AGC-3′) from the \textit{aur1PR4} coding region and the unlabelled direct primer SRP5DIR (5′-CAACGGTTACTCCCG CGGAGCC-3′) from \textit{aur1PR4} upstream region; probe 2 (for the \textit{sa13p} promoter) was prepared similarly by PCR amplification using 5′ end-labelled primer Sa13S1rev (5′-GCCAGCTCCTGCGCCTTGACC -3′) from the \textit{sa13}-coding region and the unlabelled primer Sa13S1dir (5′- GCTTGTCGTAGACGGGCAGCAGC -3′) from \textit{sa13} upstream region; the probes for the \textit{aur1Ap} promoter and the control \textit{hrdBp2} promoter were described previously (Kormanec & Farkasovsky, 1993; Novakova et al., 2005). The primers were labelled at the 5′ end with \(\gamma\)-\[^{32}\text{P}\] ATP (ICN, 4500 Ci mmol^{-1}) and T4 polynucleotide kinase.

![Fig. 1.](image-url)

(a) Physical map of the \textit{Streptomyces aureofaciens} CCM 3239 plasmid region in the downstream part of the \textit{aur1} cluster (Novakova et al., 2010b). The genes are indicated by arrows. Bent arrows indicate the position of and direction of transcription from the \textit{aur1PR4p} and \textit{sa13p} promoters. The thin lines below the map represent DNA fragments (5′ labelled at the end marked with an asterisk) that were used as probes in the S1-nuclease mapping and binding experiments. (b) Alignment of \textit{Aur1PR4} with the previously characterized \textit{S. aureofaciens} CCM3239 SARP-family regulators \textit{Aur1PR2} and \textit{Aur1PR3} (Novakova et al., 2011b), and the well-characterized SARP activators, \textit{ActII-ORF4} (Fernandez-Moreno et al., 1991) and \textit{DnrI} (Stutzman-Engwall et al., 1992). The predicted secondary structure of the N-terminal DNA-binding domain of the SARP family (Wietzorreck & Bibb, 1997) is shown, indicating the HTH motif. Identical residues are highlighted in black. Similar residues are shaded. The numbers refer to the deposited amino acid sequences in databases. Sequences were retrieved from GenBank and aligned using CLUSTALX.
following the method of Ausubel et al. (1995). The protected DNA fragments were analysed on DNA sequencing gels together with G+A and T+C sequencing ladders derived from the end-labelled fragments (Maxam & Gilbert, 1980).

Electrophoretic mobility-shift assay (EMSA)

A 500-bp DNA fragment (probe 1, Fig. 1a) comprising the aur1PR4 promoter region was prepared by PCR amplification from pCosSA19 using the 5’t end-labelled primer SRP5REV and the unlabelled primer SRP5DIR. EMSA with purified Aur1P protein was performed as described in Novakova et al. (2005). The 32P-labelled DNA fragment (0.4 ng, c. 5000 d.p.m.) was incubated with increasing amounts of the Aur1P protein for 15 min at 30 °C, and protein-bound and free DNA were resolved on a nondenaturing polyacrylamide gel as described by Wietzorreck & Bibb (1997). This secondary structure motif and the DNA-binding domain, which contains two helical segments (α2 and α3) separated by an 11-residue loop (Wietzorreck & Bibb, 1997). This secondary structure motif and the DNA-binding region are also conserved in the protein produced by sa9, leading us to change the name of the gene to aur1PR4.

The aur1PR4 gene is essential for auricin production

In order to confirm the proposed role of the aur1PR4 gene in auricin biosynthesis, the coding region of aur1PR4 (aside from the start and stop codons) was deleted from the chromosome using a PCR-targeting system designed for the disruption of Streptomyces genes and replaced by a resistance cassette (Gust et al., 2003). This mutation did not affect growth and differentiation. The auricin production of this S. aureofaciens Δaur1PR4 mutant was investigated as described previously (Novakova et al., 2010a). The deletion of aur1PR4 had a dramatic effect on auricin production. The inhibition zones corresponding to auricin in the mutant were much smaller than for the wild-type S. aureofaciens CCM 3239 strain for all time points examined (Fig. 2b and c). The quantities of auricin produced were determined by HPLC of the ethyl acetate extracts (Fig. 2d). Deletion of aur1PR4 caused a dramatic, 20-fold decrease in auricin production. To verify that this phenotype was due to the deletion of aur1PR4, the S. aureofaciens aur1PR4 strain was complemented in trans by transformation with the plasmid pFM-aur1PR4, which contained the aur1PR4 gene, including its promoter. The level of auricin produced by the complemented strain was similar to that of the wild-type strain, confirming that the decrease in auricin production by S. aureofaciens Δaur1PR4 is indeed due to aur1PR4 deletion. These results suggest that aur1PR4 has an important role in the positive regulation of auricin biosynthesis.

We previously characterized the aur1Ap promoter that directed expression of 22 structural genes (aur1A-aur1U) of the auricin cluster (Novakova et al., 2005, 2011a).

Dependence of the aur1Ap transcription on aur1PR4 was investigated by S1-nuclease mapping with RNA isolated from S. aureofaciens CCM 3239 wild-type and the aur1PR4-mutant strains cultivated in liquid Bennet medium. In addition, RNA from the aur1PR3-mutant strain with deleted gene encoding previously characterized SARP-regulator Aur1PR3 (Novakova et al., 2011b) was used in this experiment. In contrast to the aur1PR3 mutant, where the level of aur1Ap mRNA was substantially lower (Novakova et al., 2011b), the level of aur1Ap mRNA from all the time points was not substantially affected in the aur1PR4 mutant (Fig. 3a). We investigated expression of additional putative auricin biosynthetic genes flanking to the aur1 cluster and their proposed dependence on aur1PR4 by S1-nuclease mapping. A putative operon of four genes, sa13, sa12, sa11, sa10, encoding proteins with similarity to several polyketide tailoring enzymes (Novakova et al., 2010b) is located just downstream of the aur1PR4 gene (Fig. 1a). S1-nuclease
mapping was performed using a probe 2 (Fig. 1a) and the same RNA samples as for the aur1Ap promoter. As shown in Fig. 3a, an RNA-protected fragment was identified that corresponded to the sa13p promoter with the transcriptional start point (TSP) at a G 50-bp upstream from the ATG initiation codon of sa13 (Fig. 3c). The promoter was inactive during exponential phase (9 h) and was induced at the beginning of stationary phase (12 h) that correlates with onset of auricin production. The level of sa13p mRNA decreased during late stationary phase. No RNA-protected fragment was identified using the tRNA control. The level of sa13p mRNA was dramatically lower in the Δaur1PR4 mutant. However, its level was only partially reduced in the Δaur1PR3 mutant. As an internal control, S1-nuclease mapping was performed with the same RNA samples using a probe fragment specific for the S. aureofaciens hrdBp2 promoter, which is expressed fairly constantly during growth and differentiation (Kormanec & Farkasovsky, 1993). RNA-protected fragments of similar intensities corresponding to the hrdBp2 promoter were identified in all RNA samples (Fig. 3b). As shown in Fig. 3d, the upstream region of the sa13p promoter contained three heptameric direct repeats, which are similar to the consensus sequence of typical SARP-binding sites (5’-TCGAGXX-3’) and located correctly 8 bp upstream of the -10 region. These results indicate that the sa13p promoter is dependent on the aur1PR4 gene, and likely all four genes are under the control of this SARP regulator. In addition, these genes are also partially dependent upon other SARP-regulator Aur1PR3.
Fig. 3. (a) High-resolution S1-nuclease mapping of the TSP of the aur1Ap and sa13p promoters in Streptomyces aureofaciens CCM3239 wild-type (wt), S. aureofaciens aur1PR3-disrupted (Δaur1PR3) and S. aureofaciens aur1PR4-disrupted (Δaur1PR4) strains. RNA was isolated from cultures grown in liquid Bennet medium at the time points indicated, which corresponded to the different growth phases seen in Fig. 2a. Escherichia coli tRNA was used as a control (lane C). (b) Control S1-nuclease mapping experiments with the same RNA samples and a DNA probe for the hrdBp2 promoter (Kormanec & Farkasovsky, 1993). The lanes A and T denote the G+A and T+C sequencing ladders, respectively. Thin horizontal arrows indicate the positions of RNA-protected fragments and thick bent vertical arrows indicate the nucleotide corresponding to TSP. Before assigning the TSP, 1.5 nucleotides were subtracted from the length of the protected fragment to account for the difference in the 3′ ends resulting from S1-nuclease digestion and the chemical sequencing reactions. In every experiment, the same RNA preparations were hybridized in parallel with both probes. All S1-nuclease mapping experiments were performed twice with independent sets of RNA with similar results. (c) The nucleotide sequence of the S. aureofaciens CCM 3239 sa13p promoter region. The inferred protein product is given in the single-letter amino acid code in the second position of each codon. The TSP of the sa13p promoter is indicated by the bent arrow. The putative -10 box of the promoter is in bold and underlined. The heptameric repeat sequences corresponding to putative SARP-binding sites are shaded. The nucleotide sequence shown is a part of the sequence that was deposited in the GenBank, EMBL and DDBJ databases under accession number FJ479754. (d) Comparison of the three heptameric direct repeats of the sa13p promoter with several predicted or proven binding sites for SARP regulators: actVI-ORF1p and actVI-ORF4p are recognized by ActII-ORF4 (Arias et al., 1999); dnrGp and dpsEp by DnrI (Sheldon et al., 2002); fdmM1p, fdmCp, and fdmWp by FdmR1 (Chen et al., 2008); srrZp by SrrY (Suzuki et al., 2010).
Transcriptional analysis of the aur1PR4 gene

To investigate the expression of the aur1PR4 gene, S1-nuclease mapping was performed with RNA isolated from the S. aureofaciens CCM3239 wild-type strain cultivated to various growth stages in liquid Bennet medium. We previously characterized two auricin-specific regulators, an atypical response regulator Aur1P (Novakova et al., 2005) and a TetR-family repressor Aur1R (Novakova et al., 2010a). To investigate whether these regulatory genes have an effect on the transcription of aur1PR4, RNA was also isolated from S. aureofaciens mutant strains Δaur1P and Δaur1R from the same time points as wild-type strain. A single RNA-protected fragment was identified that corresponded to the aur1PR4 promoter with the (TSP) at an A 88-bp upstream from the ATG initiation codon of aur1PR4 (Fig. 4a and c). The promoter had similar time course as the sa13p promoter. The level of aur1PR4p mRNA was not substantially different in the Δaur1R mutant; however, the mRNA level was dramatically lower in the Δaur1P mutant. RNA-protected fragments of similar intensities corresponding to the control aur1PR4p promoter and that of the aur1PR4p promoter (Fig. 6). The presence of correctly timed transcriptional activator into the binding sites for ActII-ORF4, from the well-characterized representatives of this family (Fernandez-Moreno et al., 1991). In addition, domain prediction revealed both SARP-family typical domains, the N-terminal Trans_reg_C DNA binding domain (pfam00486, aa 23–94) and the C-terminal bacterial transcriptional activator domain (pfam03704, aa 103–248). The secondary structure of the N-terminal DNA-binding domain of Aur1PR4 was also very similar to that of the OmpR-like DNA-binding domain of the SARP family, containing a winged HTH region composed of two α-helices (α1 and α2) separated by an 11-residues loop (Fig. 1b).

Deletion of aur1PR4 dramatically reduces auricin production. Therefore, along with Aur1PR3, Aur1PR4 constitutes an additional auricin pathway-specific activator of the SARP family. However, in contrast to aur1PR3, deletion of aur1PR4 does not substantially affect transcription of the biosynthetic promoter aur1Ap, which controls the expression of 22 auricin biosynthetic genes in the aur1 operon (Novakova et al., 2005, 2011a). However, a single promoter, sa13p, located upstream of a putative operon of four genes, sa13, sa12, sa11, sa10 (Fig. 1a) encoding proteins with similarity to several polyketide tailoring enzymes (Novakova et al., 2010b), was found to be dependent on aur1PR4 (Fig. 6). The presence of correctly located three heptameric sequences similar to the SARP consensus sequence (Wietzorreck & Bibb, 1997; Arias et al., 1999; Sheldon et al., 2002; Tanaka et al., 2007) suggested that the promoter is under the control of SARP-family regulator. Therefore, these four genes are likely targets for Aur1PR4. Comparison of these heptameric sequences with several predicted or proven binding sites for SARP regulators (Fig. 3d) revealed the highest similarity to the binding sites for ActII-ORF4 regulator for the
activation of actinorhodin gene cluster in S. coelicolor (Arias et al., 1999). This is consistent with the highest similarity of the Aur1PR4 DNA-binding region with those for ActII-ORF4 (Fig. 1b). All the SARP-binding sites were most conserved in the first four positions of the heptameric sequences, which was previously identified as a TCGA logo for the SARP consensus sequence (Santamarta et al., 2011). However, there are some exceptions from this conservation. Two recently characterized SARP regulators, NanR1 and NanR2, directing biosynthesis
of nanchangmycin in *S. nanchangensis* likely recognize different binding sites with the consensus motif TTAG (N)_6 TT(A/T)AG (Yu *et al.*, 2012).

Interestingly, in contrast to *aur1PR3*, the *aur1PR4* gene is not under the direct control of Aur1R, but is directly regulated by Aur1P. Moreover, *aur1PR4* was induced later during growth than *aur1PR3*. This makes the regulation of auricin even more complex, involving two differently regulated SARP-family regulators (Fig. 6).

A single pathway-specific transcriptional activator is usually responsible for the activation of biosynthetic genes in antibiotic clusters. However, there is an increasing number of cases in which several pathway-specific activators are involved in the regulation of antibiotic clusters. For instance, two SARP-family regulators, TyIS and TyIT, and three additional regulatory proteins, TyIR, TyIU and TyIQ, are involved in the regulation of the macrolide antibiotic tylosin in *S. fradiae* (Cundliffe, 2008). However, only TyIS and TyIR are required for tylosin production. A similar complex regulation has been described for the kinamycin-producing *alp* clusters in *S. ambofaciens*, where five regulatory genes, encoding three SARP homologues and two TetR-family homologues, have been identified, and the *alpV* gene, encoding one of the SARP regulators, appears to be involved in kinamycin regulation (Bunet *et al.*, 2011). Two SARP homologues, SrrY and SrrZ, also appear to be involved in lankamycin regulation in *S. rocveis*; one of them, SsrZ, is directly controlled by the other, SsrY (Suzuki *et al.*, 2010). Two other SARP homologues, NanR1 and NanR2, are required for the transcription of nanchangmycin polyketide genes; the authors suggested that they might form a heterodimer to bind and activate the genes (Yu *et al.*, 2012). The situation of auricin regulation is more complex as outlined in Fig. 6. In addition to the auricin-specific regulator Aur1P, which directly controls expression of the *aur1* core biosynthetic genes *aur1A-aurlU*, two additional SARP-family regulators, *aur1PR3* and *aur1PR4*, have roles in auricin regulation. *aur1PR3* is involved in an indirect activation of the biosynthetic *aur1Ap* promoter, and *aur1PR4* likely directly controls additional transcriptional unit comprising four genes encoding putative auricin tailoring enzymes. A partial dependence of the *sa13p* promoter on *aur1PR3* indicates dependence also on this SARP

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**Fig. 5.** Detection of an Aur1P-DNA complex by EMSA using a 32P-labelled DNA fragment comprising the *aur1PR4p* promoter (probe 1, Fig. 1a) with increasing amounts of purified His-tagged Aur1P (Novakova *et al.*, 2005). Lane 1, a labelled DNA fragment in the absence of protein; lanes 2–6, 0.5, 1, 3, 4 and 4 µg, respectively, of the purified His-tagged Aur1P protein. Addition of 200 ng of the unlabelled *aur1PR4p* promoter DNA fragment was used to demonstrate Aur1P binding specificity (lane 6). The arrows indicate the free DNA fragment and the shifted fragment corresponding to the proposed complex. All binding experiments were performed twice with independent sets of protein samples, giving similar results.

**Fig. 6.** Model of the regulation of auricin biosynthesis. The genes are indicated by arrows; the auricin biosynthetic genes in grey colour and the regulatory genes in black one. Bent arrows indicate the position and direction of promoters. A line ending with an arrow indicates transcriptional activation, whereas one ending with a perpendicular line indicates repression. Indirect activation is indicated by a broken arrow. See text for further details.
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