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Seasonal cardenolide production and *Dop5βr* gene expression in natural populations of *Digitalis obscura*

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This study is dedicated to Prof. H. Ulrich Seitz (Tübingen)

Abstract

Productivity variations and seasonal fluctuations of cardenolides have been studied in 10 natural populations of *Digitalis obscura* distributed in three bioclimatic belts. Main cardenolides in *D. obscura* plants are those of the series A and such predominance (ca. 80–85%) over the series B metabolites is independent of the population studied or the degree of maturity of the leaves. Primary glycosides represent ca. 50–60% of total cardenolides; this percentage did not vary among populations or with the leaf age but increased in summer and decreased in winter. A correlation analysis between plant biomass and cardenolide content showed a positive relationship of these parameters, which, according to the bioclimatic distribution of the populations, suggests that certain environmental conditions may cause marked decreases in plant biomass together with a reduction in productivity. Cardenolide contents changed in the timecourse of the four seasons as a multiple response to distinct plant and/or environmental factors. The lowest production was recorded in May, followed by a fast cardenolide accumulation in summer, a decreasing phase in autumn, and a stationary phase in winter. We also analysed the seasonal expression of the gene encoding the progesterone 5β-reductase, enzyme producing the required 5β-configured intermediaries of cardenolides. A fragment of the isolated partial genomic sequence was used as a probe for Northern analysis to study the seasonal gene expression in selected populations. The expression pattern showed increasing levels from February to July and a further reduction in autumn, although harmful climatic conditions seems to induce overexpression of this gene.

Keywords: *Digitalis obscura*; Scrophulariaceae; Secondary metabolism; Seasonal variability in natural environment; Cardenolides; *Dop5βr* gene; Progesterone 5β-reductase

1. Introduction

Plants produce a wide variety of secondary metabolites, which are indispensable for the survival of plants since many of these natural products have important ecological functions such as resistance against diseases and herbivores (Hartmann, 1996). Besides this, plant secondary metabolism is the source for many fine chemicals of commercial importance.

One group of natural products of major interest in the pharmaceutical industry is cardiac glycosides from *Digitalis* species. Treatment with these compounds is still the only safe inotropic drug for oral use that improves haemodynamics in patients with compromised cardiac function (Schwinger et al., 2003). *Digitalis* cardiac glycosides possess a basic skeleton, a steroid genin, namely digitoxigenin (series A), gitoxigenin (series B) or digoxigenin (series C).

Levels of plant carbon-based secondary compounds are partly under genetic control and determined in part by environmental conditions (Koricheva et al., 1998); therefore, in order to maximise the production of a specific natural product, it will be necessary to understand the various factors that control and influence its biosynthesis.

In the case of *Digitalis* plants, previous studies have reported that cardenolide biosynthesis is basically dependent on morphological differentiation (Eisenbeiß et al., 1999) and genotype (Gavidia et al., 1996; Nebauer

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et al., 1999), although numerous environmental factors may determine, in a greater or lesser degree, plant productivity. Thus, it is known the influence that mineral nutrients (Gavidia and Pérez-Bermúdez, 1997; Roca-Pérez et al., 2004), CO_2 and water stress (Stuhlfauth et al., 1987), and light conditions (Brugidou et al., 1988) exert on cardenolide accumulation. Moreover, other studies on different cardiac glycoside-producing species showed that the production of these compounds seems to be related to plant biomass (Kosinski, 1996) or defence responses against damage caused by feeding of herbivores (Malcolm and Zalucki, 1996).

Our work deals with *Digitalis obscura* L., a perennial bush with a wide distribution in basophilic shrubland in the Iberian Peninsula. The study includes plants from 10 natural populations, distributed in three bioclimatic belts, in order to estimate the variability of cardenolide production in wild populations, and to study the seasonal fluctuations of these secondary compounds under natural environmental conditions. Besides this, we also analyse the seasonal expression of the gene encoding the progesterone 5β-reductase. This enzyme has been proposed to have a key function in the cardenolide biosynthetic pathway (Gärtner et al., 1990), producing the required 5β-configured intermediary products leading to the different genins.

2. Results and discussion

2.1. Cardenolide analysis and production variability in D. obscura wild populations

Qualitative and quantitative HPLC analyses show that the cardiac glycosides produced by leaves of *D. obscura* largely belong to series A and B cardenolides (digitoxigenin and gitoxigenin derivatives, respectively). The main metabolites in young and mature leaves were lanatoside A (38–59%) and digitoxin (31–51%), while lanatoside B and gitoxigenin presented lower concentrations (3–9%) in all samples. These products were accompanied by very low contents or traces of different glycosides or genins such as evatromonoside, gitoxin, or digitoxigenin.

According to these data, it seems clear that the predominant cardenolides in *D. obscura* plants (Fig. 1) are those of the series A, which is in agreement with previous results reported for this species (Lichius, 1991; Gavidia and Pérez-Bermúdez, 1997). Our results also demonstrate that the predominance of the series A cardenolides (ca. 80–85%) over those of the series B is independent of the natural population studied or the degree of maturity of the leaves (Fig. 1(a) and (b)). All these accumulated data on cardenolides from *D. obscura* are in apparent contradiction with those found by Nebauer et al. (1999) who observed metabolic variations in this species, namely predominance of cardenolides of the series A or B, depending on the locations where the plants were collected.

On the other hand, Kreis and May (1990) reported that *Digitalis* cardenolides are accumulated in the leaves as primary glycosides. In the present study we have found that primary glycosides represent ca. 50–60% of total cardenolides (Fig. 2). Our results also demonstrated that the ratio between primary and secondary glycosides did not markedly vary among populations or with the age of the analysed leaves (Fig. 2(a) and (b)). In addition, we have performed correlation analyses among the different compounds found in *D. obscura*, detecting only positive significant relationships (data not shown). Such direct correlations reflect simultaneous variations in the content of the different cardenolides, and is similar to that found between lanatoside C and total cardenolides in *Digitalis lanata*

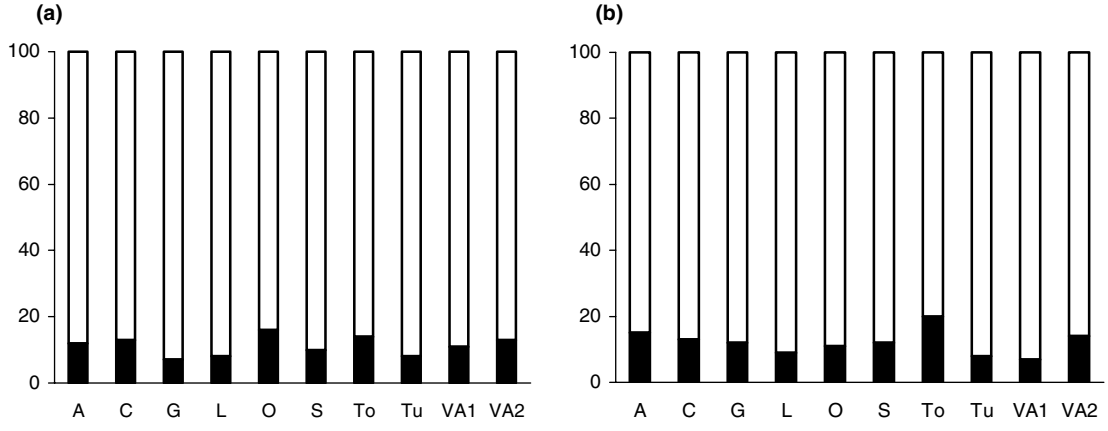

Fig. 1. Percentages of series A (□) and series B (■) cardenolides in young (a) and mature (b) leaves of *Digitalis obscura* plants from different natural populations. A, Ayora; C, Camporrobles; G, Garbí; L, Llanorel; O, Olocau; S, Sinarcas; To, Toro; Tu, Tuéjar; VA1, Vall d'Alcalá 1; VA2, Vall d'Alcalá 2.

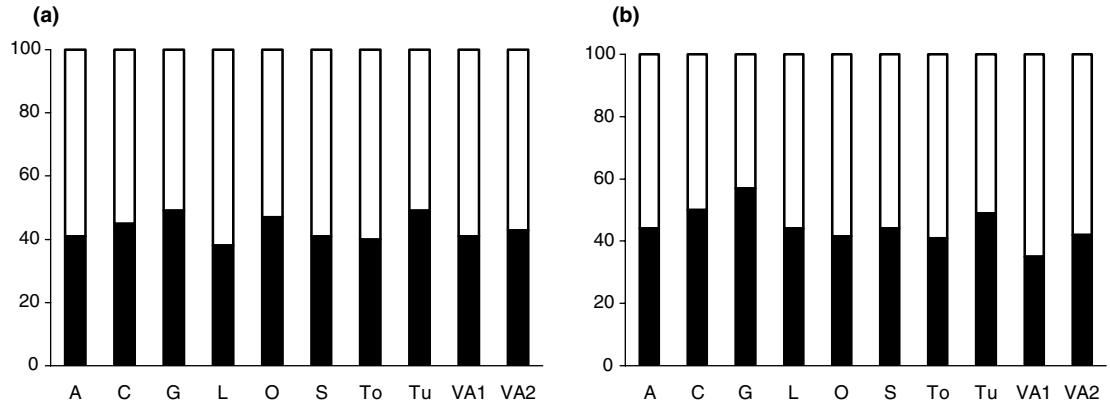

Fig. 2. Percentages of primary (□) and secondary (■) glycosides in young (a) and mature (b) leaves of *Digitalis obscura* plants from different natural populations. A, Ayora; C, Camporrobles; G, Garbí; L, Llanorel; O, Olocau; S, Sinarcas; To, Toro; Tu, Tuéjar; VA1, Vall d'Alcalá 1; VA2, Vall d'Alcalá 2.

plants at two different stages of growth (Castro Braga et al., 1997).

Total cardenolide contents determined in the populations studied (Table 1) are within the range previously reported for this species by Gavidia et al. (1996). Moreover, we have detected variations in cardenolide production among populations, which is of great interest for the selection and multiplication of specimens with a higher capacity to biosynthesise cardiac glycosides. Thus, the plants from the most productive population (Ayora, with a mean content of 2257 $\mu g\,g^{-1}$ dry wt.) presented cardenolide contents significantly higher than those from Camporrobles, Olocau and Toro, which presented mean values lower than 1000 $\mu g\,g^{-1}$ dry wt. (Table 1). In a preliminary unpublished study, we analysed single plants within populations and estimated that the percentage of variation among populations was much higher than that observed within populations and related to single plant differences (ca. 72% versus 28%, respectively). In contrast, in a similar experiment with this species, Nebauer et al. (1999) found that cardenolide variations were mainly attributable to single plant differences (50.6%), and claimed that such results could be related to the similar bioclimatic conditions of the sampled populations.

Table 1
Total cardenolide contents ($\mu g\,g^{-1}$ dry wt.) in young and mature leaves of *Digitalis obscura* plants from different populations

	Leaf type		Total mean
	Young	Mature	
Ayora	2238	2277	2257a
Camporrobles	842	1085	964b
Garbí	1199	1300	1249ab
Llanorel	1468	1615	1541ab
Olocau	851	939	895b
Sinarcas	1009	1215	1112b
Toro	667	741	704b
Tuéjar	1350	1443	1396ab
Vall d'Alcalà-1	1335	1497	1419ab
Vall d'Alcalà-2	1290	1517	1403ab

Data are means of four sampling periods. Values followed by the same letter are not significantly different ($p < 0.05$) according to Tukey's test.

It is well-known that secondary metabolism activity is related to environmental conditions and recent investigations in wild populations demonstrate it for different natural products (De Santos Galíndez et al., 2000; Covelo and Gallardo, 2001). In regard with cardenolides, it has been reported that mild environmental conditions, plant population density, and high metabolite accumulation were positively correlated (Kosinski, 1996). A similar relationship (biomass/productivity) was suggested by ourselves in a previous work on *D. obscura* (Roca-Pérez et al., 2002). Now, we have performed a correlation analysis between plant biomass and cardenolide production using the data from the ten populations evaluated. Biomass was determined in July, when plant development is maximal (see Roca-Pérez et al. (2002) for details concerning the allometric equation for calculating biomass of *D. obscura* plants). Our analysis showed a positive significant correlation between plant biomass (g dry wt./plant) and cardenolide content ($\mu g\,g^{-1}$ dry wt.) in leaves: $y = 24.5x + 1153$, where y is cardenolide content and x is biomass; ($r = 0.718$, $p < 0.05$). According to the bioclimatic distribution of the selected *D. obscura* populations, this result confirms that certain environmental conditions, namely those of the supramediterranean belt, may cause marked decreases in plant biomass together with a reduction in the cardenolide content. On the contrary, well-developed specimens usually presented a higher ability to biosynthesise these metabolites. As discussed below, light and thermic conditions seem to be key factors for cardenolide production.

2.2. Seasonal variations of cardenolides

Changes in the cardenolide contents of *D. obscura* leaves in the timecourse of the four seasons are displayed in Fig. 3. The results presented show a similar pattern of seasonal variation of total cardenolides in young and mature leaves. A comparison of the profiles of both types of leaves showed that the lowest cardenolide productions were recorded in May. This was followed by a fast cardenolide accumulation phase, and the maximum productions were reached in July. Cardenolides decreased past the summer season, this represents a decreasing phase corresponding to the autumn. In winter, during a stationary phase, cardenolides remained practically unchanged with respect to the preceding sampling period (Fig. 3).

These results are quite similar to those reported by Brugidou et al. (1988) in *D. lanata* plants grown in controlled conditions and natural environment. These authors found that the variability in cardenolide production of *D. lanata*, expressed as digoxin contents, was related with the seasonal variations of light intensity, photoperiod and thermoperiod.

Recent works have reported seasonal variations of distinct secondary metabolites such as taxanes from *Taxus baccata* (Veselá et al., 1999), harpagosides in *Scrophularia scorodonia* (De Santos Galíndez et al., 2000), or phenolics from *Quercus robur* (Covelo and Gallardo, 2001) and *Betula pubescens* (Riipi et al., 2002). Increasing and decreasing phases of productivity varied with the species studied, but the influence of plant status or environmental factors on productivity were not discussed in most of these works.

Young and mature leaves of *D. obscura* showed similar cardenolide contents in the different sampling dates (Fig. 3). The exception was the very low value found in young leaves, that in May presented a cardenolide content of 445 $\mu g\,g^{-1}$ dry wt. versus 1139 $\mu g\,g^{-1}$ dry wt. of the mature leaves (Fig. 3). This low accumulation in young immature leaves, which were still growing during springtime, is basically explained considering that cardenolide production increases in parallel with morphological differentiation and leaf maturation (Weiler and Zenk, 1976; Eisenbeiß et al., 1999).

The marked increase observed in July may be a multiple response to distinct plant and/or environmental factors. At this time the young leaves were completely developed, reached the same degree of differentiation that mature leaves, and consequently the cardenolide yields of young and old leaves became similar (Fig. 3). Therefore, this increasing phase seems to be necessarily determined, as discussed above, by the environmental conditions typical of the summer: high temperature and light intensity, and extended photoperiod.

Besides this environmental regulation, a parallel and concomitant phenomenon may explain the high productivity observed in the summer. This explanation is based on the growth differentiation balance (GDB) hypothesis as proposed by Herms and Mattson (1992) who postulated that the synthesis of carbon-rich secondary metabolites is dependent on the availability of photosynthates. The leaves of *D. obscura* are low in cardenolides in May when the growth rate of the plant becomes maximal and, therefore, demands large amounts of carbon. In July, with higher photosynthetic rates and lower plant development rates, most part of carbon is funnelled into secondary metabolism and, then, cardenolide biosynthesis and accumulation increase. Finally, we suggest that a third factor could be involved in this seasonal increase of cardenolides. It is uncertain what adaptative advantage may represent

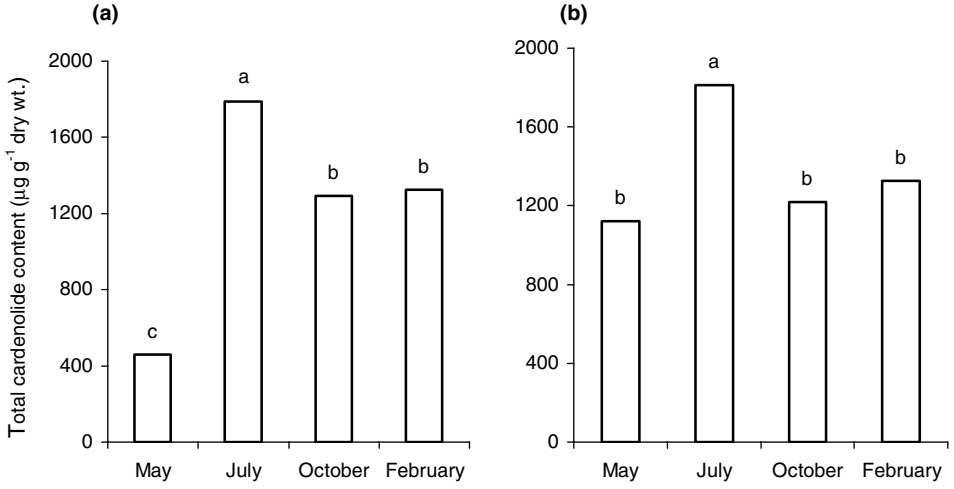

Fig. 3. Seasonal variation of total cardenolide contents in young (a) and mature (b) leaves of *Digitalis obscura* plants. Each value represents the mean of ten natural populations. Within each graph, bars with the same letter are not significantly different ($p < 0.05$) according to Tukey's test.

cardenolides for *Digitalis* plants, but it has been suggested the role of triterpenes against herbivore feeding (Harborne, 1982; Malcolm and Zalucki, 1996). Then, it cannot be ruled out that besides constitutive cardenolides an induced cardenolide biosynthesis occurs as a defensive response against the multitude of insects interacting with *D. obscura* plants during late spring and summer.

Past the summer cardenolides decreased, according to the regulation imposed by the environmental conditions of the new season, and finally the content of these metabolites remained stationary during the winter (Fig. 3). In this respect, Brugidou et al. (1988) pointed out photoperiodism and a more accented daily thermoperiodism of late summer and early autumn as the main factors involved in the decrease of digoxin rate in *D. lanata* plants. Nevertheless, the fact that cardenolides have been found in different sink organs and move around phloem sap (Vogel and Luckner, 1981; Rothe et al., 1999) suggests that catabolism and translocation may also provoke a decrease in the leaf cardenolide contents during the autumn. For instance, it has been reported that both phenomena, translocation (MacLeod and Pridham, 1966) and catabolism (Riipi et al., 2002), are involved in the reduction of phenolics in leaves of different species.

We have also tried to determine whether *D. obscura* cardenolides changed qualitatively in the timecourse of the four seasons. The percentages of series A cardenolides and primary glycosides obtained for each season and natural population are summarised in Table 2. As discussed above, series A cardenolides is the major group in *D. obscura* leaves, independently of the population studied or the degree of maturity of the leaves (Fig. 1(a) and (b)), and results showed in Table 2 extend this predominance along the year, since seasonal changes were not found. In the present study we have also observed that primary glycosides represent ca. 50–60% of total cardenolides and such percentage did not markedly vary among populations or with the age of the leaves (Fig. 2(a) and (b)), although we have found that the ratio between primary and secondary glycosides was altered along the year (Table 2). Thus, in all populations, the percentage of primary glycosides was higher in July (67–83%) and May (49–72%) than in October (32–54%) and February (23–51%). These results suggest that cardenolide glucosylation or deglucosylation by glucosyltransferases or glucohydrolases (Kreis and May, 1990; Eisenbeiß et al., 1999), respectively, is affected by environmental conditions. The activities of anabolic or catabolic enzymes could be the signals for cardenolide accumulation in the leaves during spring and summer, as primary glycosides in the vacuole, or for cardenolide degradation and transport during autumn and winter.

2.3. Seasonal expression of the gene *Dop5βr* encoding the progesterone 5β-reductase

We have previously isolated the *p5βr* gene from *Digitalis purpurea* (Gavidia et al., in preparation). In addition, primers based on the sequence of (*Dpp5βr* were used to isolate a partial genomic sequence of this gene in *D. obscura* (*Dop5βr*). This sequence contains one intron and resulted in a DNA fragment of 1278 bp corresponding to amino-acid 4–392 (Fig. 4), being highly similar to the *Dpp5βr*. Their nucleotide sequences exhibit 97.16% identity and their amino-acid sequences show a 96.94% identity.

Comparison analysis revealed that the *Dop5βr* nucleotide sequence present the higher percentages of identity with four plant clones from *Arabidopsis thaliana* (accession numbers At4g24220 and CAA68126), *Populus tremuloides* (AAO63776) and *Oryza sativa* (BAC20032). Their functions are unknown except for the protein from *Populus* which is presumably involved in vascular development.

A *Hin*dIII/*Eco*47I fragment of the *Dop5βr* clone (697 bp) was used as a probe for Northern analysis in order to study the seasonal expression of the gene in plants from the populations of Ayora and Toro. These populations were selected since their plants showed the maximum and minimum productivity of cardenolides. Hybridisation

Table 2
Seasonal variations of series A cardenolides and primary glycosides in leaves of *Digitalis obscura* plants from 10 natural populations

	Series A cardenolides (%)				Primary glycosides (%)			
	May	July	October	February	May	July	October	February
Ayora	88	88	87	81	72	75	44	40
Camporrobles	92	90	81	85	52	80	40	38
Garbí	84	92	90	91	61	67	32	23
Llanorel	94	92	87	94	71	74	38	51
Olocau	88	88	83	86	65	74	45	36
Sinarcas	90	89	88	90	62	83	40	41
Toro	79	88	92	74	66	77	49	45
Tuéjar	93	89	94	93	49	77	37	30
Vall d'Alcalà-1	88	93	91	92	70	79	54	46
Vall d'Alcalà-2	78	92	87	87	68	77	50	33

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                M  S  W  W  W  A  G  A  I  G  A  A  K
  1 TAGCTGAAAAAATGAGCTGGTGGTGGGCTGGAGCGATCGGCGCTGCAAAGgtaagttaac

 61 atttatcgcataattgaaacaatcctagctatttatgtgtaatctgactggtaattggtt
          K  K  L  E  E  D  D  A  P  P  K  H  S  S  V  A  L  I
121 gaaatagAAAAAGTTGGAAGAAGATGACGCACCGCCAAAGCATTCGAGCGTGGCGTTGAT
      V  G  V  T  G  I  I  G  N  S  L  A  E  I  L  P  L  A  D  T
181 AGTTGGGGTAACCGGAATCATCGGCAACAGCCTGGCGGAGATCCTGCCACTGGCCGACAC
      P  G  G  P  W  K  V  Y  G  V  A  R  R  T  R  P  A  W  H  E
241 CCCCGGCGGTCCGTGGAAGGTATACGGCGTCGCCCGCCGCACCAGACCCGCCTGGCATGA
      D  N  P  I  N  Y  V  Q  C  D  I  S  D  P  D  D  S  Q  A  K
301 GGATAATCCGATCAATTACGTCCAGTGCGACATATCCGATCCAGATGACTCCCAAGCCAA
      L  S  P  L  T  D  V  T  H  V  F  Y  V  T  W  A  N  R  S  T
361 GCTGTCACCTCTGACTGATGTTACCCACGTGTTCTACGTTACCTGGGCTAATCGATCCAC
      E  Q  E  N  C  E  A  N  S  K  M  F  R  N  V  L  D  A  V  I
421 CGAACAAGAAAACTGTGAAGCCAATAGCAAAATGTTCAGGAACGTGCTTGATGCAGTTAT
      P  N  C  P  N  L  K  H  I  S  L  Q  T  G  R  K  H  Y  M  G
481 CCCTAATTGCCCCAATTTGAAACACATCTCATTGCAGACTGGGAGGAAGCATTACATGGG
      P  F  E  S  Y  G  K  I  E  S  H  D  P  P  Y  T  E  D  L  P
541 ACCATTTGAATCGTACGGGAAAATAGAATCCCATGATCCACCCTACACTGAGGATTTGCC
      R  L  K  Y  M  N  F  Y  Y  D  L  E  D  I  M  L  K  E  V  E
601 CAGGTTGAAGTACATGAACTTTTACTATGATTTAGAGGATATTATGCTTAAGGAGGTGGA
      K  K  E  G  L  T  W  S  V  H  R  P  G  N  I  F  G  F  S  P
661 GAAGAAGGAGGGTTTGACTTGGTCGGTTCATCGCCCAGGGAATATATTCGGGTTTTCTCC
      Y  S  M  M  N  L  V  G  T  L  C  V  Y  A  A  I  C  K  H  E
721 ATATAGTATGATGAATTTGGTGGGTACCCTTTGTGTTTATGCAGCTATTTGCAAACACGA
      G  K  V  L  R  F  T  G  C  K  A  A  W  D  G  Y  S  D  C  S
781 GGGAAAGGTTTTGAGGTTTACTGGTTGTAAGGCTGCGTGGGATGGGTACTCGGATTGCTC
      D  A  D  L  I  A  E  H  H  I  W  A  A  V  D  P  Y  A  K  N
841 TGATGCGGATTTGATAGCGGAGCATCATATTTGGGCTGCAGTGGATCCTTATGCAAAAAA
      E  A  F  N  V  S  N  G  D  V  F  K  W  K  H  F  W  K  V  L
901 TGAGGCCTTTAATGTGAGTAATGGAGATGTGTTTAAATGGAAGCATTTTTGGAAGGTGTT
      A  E  Q  F  G  V  E  C  G  E  Y  E  E  G  E  D  L  K  L  Q
961 GGCGGAGCAGTTTGGGGTAGAGTGTGGAGAGTATGAAGAAGGGGAGGATTTGAAATTGCA
      D  L  M  K  G  K  E  P  V  W  E  E  I  V  R  G  N  G  L  T
1021 GGATTTAATGAAGGGGAAGGAGCCGGTTTGGGAGGAAATCGTGAGGGGGAATGGATTGAC
      P  T  K  L  K  D  V  G  I  W  W  F  G  D  V  I  L  G  N  E
1081 ACCTACGAAACTGAAGGATGTTGGGATTTGGTGGTTTGGTGATGTTATACTTGGGAATGA
      C  F  L  D  S  M  N  K  S  K  E  H  G  F  L  G  F  R  N  S
1141 GTGTTTCCTGGATAGTATGAACAAGAGCAAGGAGCATGGCTTTTTGGGATTTAGGAACTC
      K  N  A  F  I  S  W  I  D  K  A  K  A  Y  K  I  V  P  *
1201 CAAGAATGCGTTCATTTCTTGGATTGACAAGGCAAAAGCTTACAAGATTGTTCCTTGACA
1261 TGGTTTCTCTTCAGTTTGTCTTTGGTAATAATGTGTTAGTTCACCAACTGTGTGGTGT
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Fig. 4. Nucleotide sequence of the *Dop5βr* gene and the deduced amino-acid sequence. The intron sequence is in lower case letters. Sequence available from the EMBL database under the accession number AJ555127.

analysis of total RNA detected a single mRNA band (as a representative example see Fig. 5(a) and (b)). These mRNAs were quantified using a radioanalytical imaging system and results depicted in Fig. 5(c).

The profile of the seasonal expression of *Dop5βr* mRNA in plants from Ayora showed increasing levels from February to July, when the maximum was reached, and a further progressive reduction in autumn until the minimum levels detected in the winter samples. A similar pattern of seasonal expression was found in plants from Toro, but in this population *Dop5βr* transcripts were expressed at lower levels than in Ayora plants. Surprisingly, however, this seasonal pattern was altered in leaves from Toro collected in February, which presented the maximum expression of the gene (Fig. 5(a)). Thus, *Dop5βr* transcript level in these winter samples (16.6 mol of mRNA) almost doubled that measured in the corresponding samples from Ayora, which presented the lowest seasonal level of this population (Fig. 5(c)).

This result could be explained considering that Toro is a population located in the supramediterranean belt where very low temperatures are reached during the winter. It is widely accepted that plant protection is a major function of natural products, then is not surprising that under harmful climatic conditions some genes would be specifically overexpressed in *D. obscura* as a response indispensable for the survival of the plants. This could be the case of the gene encoding progesterone 5β-reductase. Moreover, we have some evidence that *Dop5βr* is not the only gene overexpressed in response to freezing. Interestingly, the leaves of the plants from Toro are dark-red coloured in winter. This chromatic response reflects the stress provoked by low temperature, which is an environmental factor known to induce anthocyanin biosynthesis for plant protection (Holton and Cornish, 1995; Pietrini et al., 2002). We determined the anthocyanin content in *D. obscura* leaves and the results showed that these metabolites increased in winter

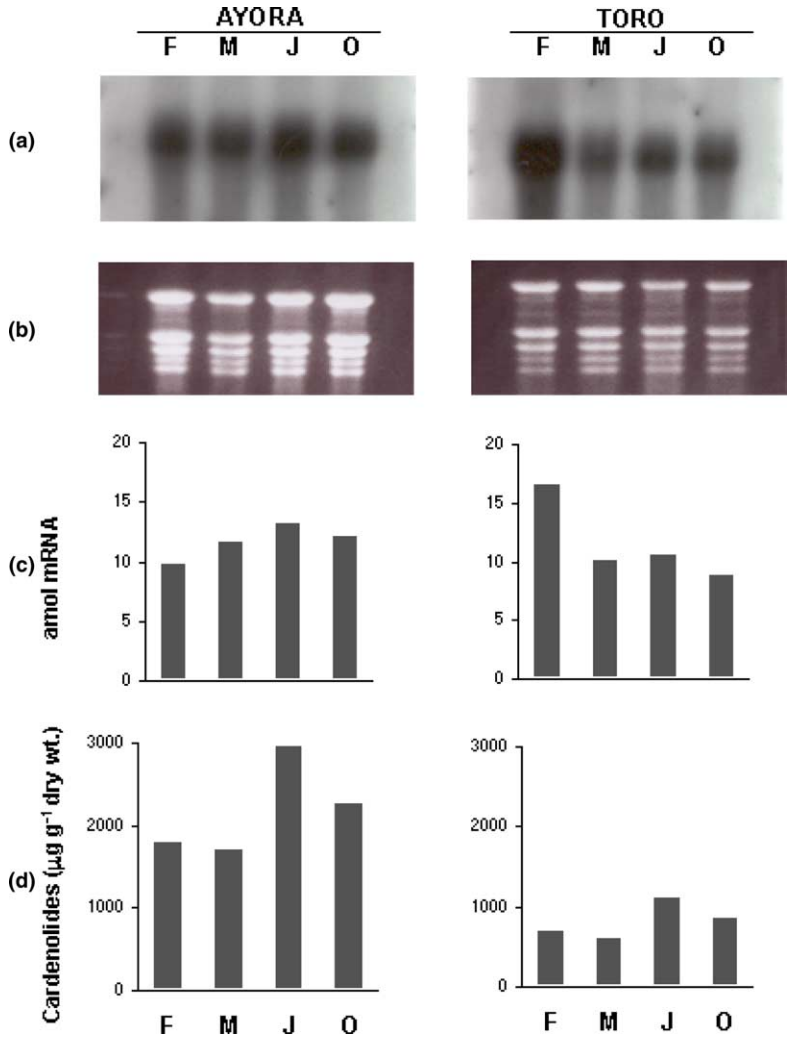

Fig. 5. Seasonal expression of the *Dop5βr* gene in leaves of *Digitalis obscura* plants from Ayora and Toro populations collected in February (F), May (M), July (J) and October (O): (a) RNA gel blot analysis of *Dop5βr* transcript levels; (b) ethidium bromide staining of RNA used for RNA gel blots in (a); (c) quantification of the *Dop5βr* mRNAs using a radioanalytical imaging system; (d) quantification of cardenolides. Values in (c) and (d) are mean of three different analyses with at least two replicates each.

within a range of 22–36% in respect of the other data collection (data not shown).

Finally, we have evaluated the possible relation between the rate of cardenolide biosynthesis and the expression levels of the gene *Dop5βr*. Although the mentioned freezing-induced overexpression of *Dop5βr* was unrelated to cardenolide production, our results basically showed parallel increases of *Dop5βr* transcripts and total cardenolides in the populations studied. Thus, a higher cardenolide yielding of Ayora plants is accompanied by a higher constitutive expression level of *Dop5βr*, and concomitant highest values of both parameters are found in the samples collected in summer from both, Ayora and Toro populations (Fig. 5(c) and (d)).

We have isolated the gene encoding progesterone 5β-reductase, which represents the only knowledge on the molecular genetics of cardenolide biosynthesis. This protein is a key enzyme producing the first stereospecific cardenolide precursor (5β-pregnane-3,20-dione), which requires at least four further enzymatic transformations to form the genins and then distinct cardiac glycosides, therefore more information concerning the enzymes and genes involved in the route is necessary. Currently we are studying the transcription level of *p5βr* gene under different stress conditions and how cardenolide production is affected.

3. Experimental

3.1. Cardenolide analysis

3.1.1. Study area and sampling

The study was conducted in 10 natural populations of *Digitalis obscura* L. (Scrophulariaceae) located in the

Valencian Community (Spain): Ayora, Camporrobles, Garbí, Llanorel, Olocau, Sinarcas, Toro, Tuéjar, Vall d'Alcalà-1 and Vall d'Alcalà-2. Bioclimatically, these populations belong to the thermo- (Garbí, Llanorel, Olocau, Vall d'Alcalà), meso- (Ayora, Tuéjar) or supramediterranean belts (Camporrobles, Sinarcas, Toro).

The collection of leaves for cardenolide extraction took place in May, July, October 2000 and February 2001. Ten plants were selected within each population and, for each date, the experimental samples consisted of 40 young leaves (the first four underneath the apical bud, still growing leaves) or 40 mature leaves (the first four above the clearly senescent leaves) collected separately. The samples were processed immediately.

3.1.2. Cardenolide extraction

The leaves were surface cleaned with distilled water, dried at 50 °C for 72 h and pulverised. Solidphase extractions of cardenolides were performed as described by Wiegrebe and Wichtl (1993) with some modifications. 50 mg of the dried leaf powder were treated with 12 ml of 70% MeOH; 1 ml of β-methyldigoxin (0.5 mg ml^{-1}) was added as internal standard. This solution was incubated in a boiling water bath for 10 min and then rapidly cooled to room temperature. After addition of 2 ml of a lead acetate solution (15%), mixing and precipitation, 2 ml of monosodium phosphate (4%) were added. After complete precipitation, the extract was diluted with distilled water up to 24 ml and centrifuged for 5 min at 3300 *g*. Supernatant was passed through a pre-treated LiChrolut column (Merck) and, after washing with water, cardenolides were eluted with 2 ml MeOH. Finally, the extracts were filtered through 0.22 μm acetate filters.

3.1.3. HPLC determinations

Cardenolide separation and analysis were carried out by HPLC as described by Gavidia and Pérez-Bermúdez (1997). The extracts were analysed in a Merck-Hitachi LaChrom chromatograph (L-7400 UV detector, L-7100 pumps, L-7200 auto-sampler) coupled to a 20 μl injector (Rheodyne 7725). Cardenolides were separated at 30 °C on a LiChroCART 250-4 column packed with LiChrospher 100 RP-18 (5 μm) from Merck, and detected at 230 nm under a flow rate of 1 ml min^{-1}. A gradient elution of H_2O (A) and CH_3CN (B) was employed: initial = 20% B; 35 min = 32% B; 45 min = 40% B; 55 min = 50% B; 59 min = 55% B; 65 min = 60% B; 70 min = 20% B. The identities and amounts of the cardenolides were checked by co-chromatography with commercial available standards (Sigma). Four replicates were analysed per each experimental sample. Final data are presented as μg of cardenolides g^{-1} dry wt.

3.1.4. Statistical analysis

All the variables analysed were first tested for data normality and variance homogeneity by using Shapiro-Wilk's test or Leven's test, respectively. To study the influence of sampling date or the variability among plant populations, data on cardenolide accumulation were subjected to a one-way ANOVA. When appropriate, means were compared with Tukey's test. To establish relationships between the different cardenolide types, we used Pearson's correlation. All analyses were undertaken with the SPSS 9.0 program.

3.2. Experiments for *Dop5βr* expression

3.2.1. Plant material

For this experiment, the samples were collected in February, May, July and October 2002 from the populations of Ayora, and Toro, which presented the maximum and minimum productivity of cardenolides, respectively. Three plants of similar biomass were selected within each population and leaves from the intermediate region of the shoots were collected for each individual plant. Two separate groups of leaves were used; one was collected and processed as previously described for cardenolide determinations, the other group was used for molecular studies. In this latter experiment, and in order to avoid external contamination, the leaves were thoroughly rinsed with distilled water and surface cleaned before collection. Then, the leaves were cut and immediately immersed in liquid nitrogen to prevent physiological alterations due to wounding. These samples were stored at −80 °C until use.

3.2.2. DNA extraction and PCR amplification of *Dop5βr*

Total genomic DNA from *D. obscura* leaves was isolated according to Dellaporta et al. (1983), with an additional polyethylene glycol precipitation step as proposed by Del Castillo Agudo et al. (1995) for this species. The primers for amplification by the polymerase chain reaction (PCR) of *Dop5βr* sequence were 5′-TAGCTGAAAAAATGAGCTGG and 5′-ACACCAC ACAGTTGGTGAAC, which were designed according to the *Dpp 5βr* sequence. Genomic DNA (100 ng) was used as a template for 25 cycles of: 95 °C/30 s, 52 °C/45 s, 74 °C/90 s. The reaction mixture contained 100 μM of each primer, 100 M dNTPs, 0.5 units of Taq DNA polymerase and reaction buffer according to Ecogen. PCR reactions were carried out in a Perkin–Elmer 9600 thermocycler. The amplification product with the expected length, ca. 1400 bp, was ligated into a pGEM-T Easy vector (Promega). The resultant clone was named *Dop5βr*.

3.2.3. DNA sequencing

Sequencing was carried out by the dideoxy-chain termination method with a DNA sequencing kit (PE

Biosystems) on an ABI 310 Genetic Analyser (Perkin–Elmer). Complete nucleotide sequences were determined for both strands and analysed by the DNASTAR program package (Lasergene) and CLUSTALW.

3.2.4. RNA isolation and Northern blot analysis

Total RNA was extracted from *D. obscura* leaves according to the procedure described by Salzman et al. (1999) with some modifications: (a) the extraction buffer used was that reported by Steimle et al. (1994) supplemented with 100 mg of soluble polyvinylpyrrolidone (PVP 40) and 200 μl 2-mercaptoethanol per 10 ml buffer; (b) prior to the first precipitation with absolute EtOH and 5 M NaCl, proposed in the original protocol, two purification steps were introduced: precipitation with 0.7 ml NaOAc (3 M pH 5.2), and the treatment of the supernatant with insoluble polyvinylpolypyrrolidone (100 mg PVPP/g tissue); each purification step was followed by incubation on ice (30 min) and centrifugation (13,000 *g*, 10 min at 4 °C).

Northern analyses, using 20 μg of total RNA, were carried out as previously described by Gavidia et al. (2002). A quantitative measure of transcript abundance was obtained using a radioanalytical imaging system (InstantImager 2024, Packard Instruments). Radioactivity was directly measured on the gel blots, and the attomoles of mRNA were determined as proposed by Gómez-Gómez and Carrasco (1998). Autoradiography of the membranes was obtained on XOMAT AR films (Kodak) using an intensifying screen at −80 °C.

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