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Molecular, Biochemical and Physiological Characterization of Gibberellin Biosynthesis and Catabolism Genes from *Nerium oleander*

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Abstract

Genomic and cDNA clones encoding two gibberellin (GA) 20-oxidases (named *NoGA20ox1* and *-2*) and three GA 2-oxidases (*NoGA2ox1*, *-2*, and *-3*) have been isolated and characterized from *Nerium oleander* L. (oleander), a plant of horticultural importance in the Mediterranean region. *NoGA2ox2* and *-3* transcripts were abundant in expanding leaves and flower buds, whereas *NoGA20ox1*, *-2* and *NoGA2ox1* transcripts were barely detected by Northern blot analysis in the vegetative and reproductive tissues analyzed. The expression of *NoGA20ox2*, but not that of *NoGA20ox1*, was subject to negative feedback regulation by active GAs. In contrast, the expression of *NoGA2ox2*, but not that of *NoGA2ox1* and *-3*, was subject to positive feedforward regulation by active GAs. Multiple transcripts with different 3′ untranslated regions, due to alternative polyadenylation sites, have been identified for each of the isolated genes except *NoGA2ox1*. Enzyme assays confirmed that NoGA20ox1 and -2 were involved in GA biosynthesis, catalyzing the conversion of GA_{12} to GA_9 and GA_{53} to GA_{20}, and that NoGA2ox1, -2, and -3 were involved in GA catabolism, converting GA_1 to GA_8, GA_{20} to GA_{29}, and GA_9 to GA_{51}. NoGA2ox3 catalyzed a further oxidation leading to GA_{29}- and GA_{51}-catabolite. Ectopic expression of *NoGA2ox3* in tobacco reduced the level of active GA_1 in transgenic plants, and independent transgenic lines displayed a range of dwarf phenotypes correlated with the level of transgene expression. These results define the physiological context of the control of GA metabolism in oleander, and they provide molecular tools for the biotechnological modification of GA metabolism in this species.

Key words: Gibberellin; Tobacco transgenic; Dwarf; GA 2-oxidase; GA 20-oxidase; *Nerium oleander*.

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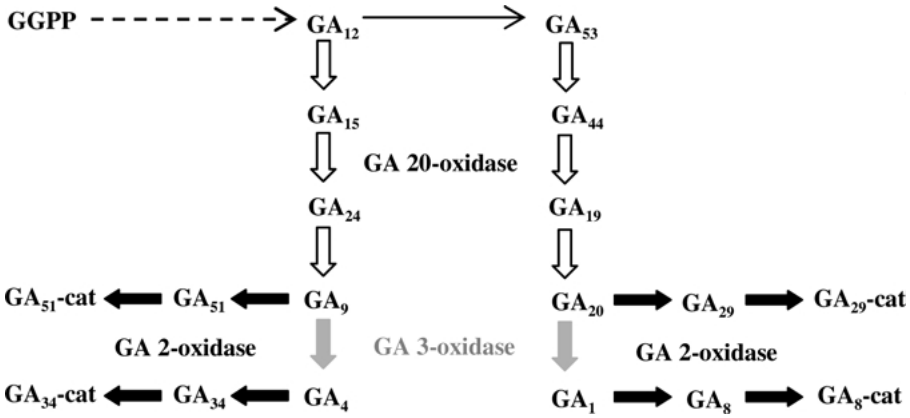


Figure 1. Giberellin metabolism pathway. White arrows represent steps catalyzed by GA 20-oxidases; gray, by GA 3-oxidase; and black thick arrows, by GA 2-oxidases. Broken arrows indicate steps catalyzed by more than one enzyme.

Introduction

Gibberellins (GAs) are growth substances that regulate different physiological processes including germination, stem elongation, and fruit-set and development (García-Martínez and Hedden 1997). For example, it has been shown that shoot length and content of active GAs in many species are positively correlated (Reid and Howell 1995). There are several pathways of GA biosynthesis, depending on the species and tissue considered (Sponsel 1995), and many of the genes encoding GA biosynthetic and catabolic enzymes have been cloned (Hedden and Kamiya 1997; Hedden and Phillips 2000; Olszewski and others 2002). In many species the main pathway of biosynthesis involves early-13-hydroxylation, in which the precursor GA_{53} is metabolized to the active GA_1 by two enzymes acting consecutively, GA 20-oxidase and GA 3-oxidase (Figure 1). Giberellin 20-oxidase is a multifunctional enzyme that converts GA_{53} to GA_{20} (and/or GA_{12} to GA_9 in the parallel non-early-13-hydroxylation pathway), regulating the GA biosynthetic flux (Hedden and Kamiya 1997). Giberellin 2-oxidases introduce a 2β-hydroxyl group in C_{19}-GAs, such as GA_1, GA_4, GA_9, and GA_{20} (Lester and others 1999; Martin and others 1999; Thomas and others 1999), although a few GA 2-oxidases appear to use C_{20}-GAs as substrates, such as AtGA2ox7 and -8 from *Arabidopsis* (Schomburg and others 2003). With respect to the activity of these enzymes, some GA 2-oxidases are multifunctional enzymes that catalyze a further oxidation at C-2 producing GA-catabolites (Thomas and others 1999; Lee and Zeevaart 2002). Both GA 3-oxidase and GA 2-oxidase may therefore directly regulate the levels of the active GAs GA_1 and GA_4 and their immediate precursors, GA_{20} and GA_9 (for reviews, see Hedden and Phillips 2000; Yamaguchi and Kamiya 2000). Transcript levels of biosynthetic genes encoding GA 20-oxidases and GA 3-oxidases are generally subjected to feedback regulation (Hedden and Kamiya 1997; Cowling and others 1998), whereas the expression of catabolic genes encoding GA 2-oxidases has been reported to be under feed-forward regulation (Thomas and others 1999) by active GAs.

Nerium oleander L. is a tropical evergreen shrub distributed throughout the world, commonly known as oleander. Oleander plants are used for ornamental purposes in Mediterranean regions because they are highly tolerant to drought and because of their beautiful white to red flowers. An inconvenience for the commercial use of this species as ornamentals is that they are considered to be too tall. Current horticultural practice involves the use of inhibitors of GA biosynthesis (for example, paclobutrazol, PCB) to reduce the height of oleander potted plants to prevent lodging and improve commercial characteristics (Banon and others 2001).

The isolation and characterization of genes involved in GA biosynthesis and catabolism in oleander is a prerequisite to understanding the regulation of GA metabolism in this species that may provide useful information for genetic manipulation of active GA levels. This could permit the reduction of shoot length of these plants and improve their commercial potential. As the first investigation of GA metabolism in oleander, in this report we describe the isolation and characterization of cDNA and genomic clones of genes belonging to GA 20-oxidase and GA 2-oxidase gene families (*NoGA20ox1* and *–2*, *NoGA2ox1*, *-2*, and *-3*), and the analysis of transgenic tobacco expressing the catabolic enzyme NoGA2ox3, as an attempt to provide the tools for a biotechnological strategy that may permit the reduction of the height of plants by reducing GA levels. The results obtained suggest unique and shared aspects of GA biology between

oleander and other studied species (for example, *Arabidopsis*), and provide the tools for biotechnological manipulation of GA-related developmental processes in this important ornamental plant.

Materials and Methods

Plant Material and Treatments

Plants of *Nerium oleander* L. var. *splendens giganteum*, dark pink (purchased from Asociación Profesional de Flores, Plantas y Afines, Comunidad Valenciana, Spain), were grown in vermiculite:peat (1:1) mixture in the greenhouse.

Apical shoots (apex plus the two first expanding leaves) from oleander were collected from: (1) plants irrigated with 10^{-6} M paclobutrazol (Duchefa, Haarlem, Netherlands) for 45 days, to isolate *NoGA20ox1* and *-2* cDNA clones, and (2) plants treated daily with 1 μg GA_3 per shoot apex in 0.1% Tween 80 aqueous solution, for 5 days, to isolate *NoGA2ox1, -2*, and *-3* cDNA clones. Other oleander material such as apical shoots, elongating and fully elongated internodes, expanding and fully expanded leaves, and roots were collected for transcript and DNA analysis.

Nicotiana tabacum cv. *xanthi* seeds were surface sterilized, plated in Petri dishes with germinating medium (GM) (Murashige and Skoog [MS] salts [Murashige and Skoog 1962] [Duchefa], 1% w/v sucrose, and 0.8% w/v agar [Pronadisa, Alcobendas, Spain]), and shoot apices were transferred into vessels with rooting medium (RM) (MS salts plus Gamborg B5 vitamins [Duchefa], 2% w/v sucrose, 0.8% w/v agar, and 0.1 mg l^{-1} IAA). The cultures were maintained in a growth chamber at 26° ± 2°C under a 16-h photoperiod (photon fluence rate of 120 μmol m^{-2} s^{-1} provided by 58 W fluorescent tubes, Grolux, Sylvania), and leaf sections (1 cm × 1 cm), isolated from about 3-week-old plants, were used as explants in transformation experiments.

Seeds of control (untransformed) and transgenic tobacco plants were germinated and grown in Petri dishes containing solidified sterile GM and different concentrations of GA_3 (10^{-5} M or 10^{-6} M) (Fluka, Alcobendas, Madrid, Spain) when that was necessary. Adult tobacco plants were treated with GA_3 by spraying the entire plant surface with a 10^{-5} M GA_3 solution containing 0.1% v/v Tween 80, and/or by watering the plants with nutrient solution containing 10^{-5} M GA_3. The material was frozen in liquid N_2 immediately after collection, and stored at –70°C until use.

Nucleic Acid Isolation

Poly(A^+) RNA was isolated from homogenized, frozen tissues using the proteinase K extraction method followed by phenol-chloroform extraction (Bartels and Thompson 1983), and oligo(dT) cellulose column chromatography. DNA was isolated from young leaves according to the method of Dellaporta and others (1983).

Isolating NoGA20ox1, -2, NoGA2ox1, -2, and -3 cDNA, and Genomic Clones

A detailed table including the sequences of all the primers used in this work is available upon request. *NoGA20ox1, -2, NoGA2ox1, -2*, and *-3* partial cDNA clones were isolated by reverse transcription polymerase chain reaction (RT-PCR) using degenerated primers. The PCR amplifications of single-strand cDNA, synthesized from poly(A^+) RNA with a ''First-strand cDNA Synthesis Kit'' (Amersham International, Bucks., UK), were performed using the Expand™ High Fidelity PCR system (Boehringer Mannheim, Germany). *NoGA20ox1* and *-2* clones were isolated using the degenerated primers N1/N6 and *NoGA2ox1, -2*, and *-3* clones were isolated by RT-PCR followed by nested-PCR using the degenerate primers F2/R3 and F3/R1, respectively. The PCR reactions occurred in 25 μl reaction mixtures containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, 10 pmol of each primer, and 1 unit of Taq DNA polymerase (Amersham International, Bucks., UK). Reaction mixtures were heated at 94°C for 5 min and then subjected to the following thermocycling conditions: 5 × (94°C 1 min, 40°C 3 min, 72°C 2 min) + 35 × (94°C 1 min, 50°C 1 min, 72°C 2 min) + 72°C 10 min. The PCR products were analyzed by 1% (w/v) agarose gel electrophoresis, and bands of interest were cloned into the pGEM-T easy vector (Promega, Madison, Wisconsin, USA). Positive clones were isolated and sequenced. The same procedure was used to isolate an *actin* cDNA partial clone from oleander (*NoActin*), using ACT-F and ACT-R as degenerated primers.

5′ rapid amplification of c-DNA ends (5′-RACE) for all the cDNA clones from oleander were performed using the ''Marathon cDNA Amplification'' and ''SMART™ RACE cDNA Amplification'' kits (Clontech) according the manufacturer's instructions. 3′-RACE studies were performed by RT with a primer that had attached a known sequence followed by a PCR amplifying the 3′-ends of the clones. The gene-specific primers used in 5′- and 3′-RACE were AS64/AS88 and AS432/AS380 for *NoGA20ox1*; BS372/

BS180 and BS65/BA178 for *NoGA20ox2*; XR0/XR1/XR2 and XF1/XF2 for *NoGA2ox1*; YR0/YR1/YR2 and YF1/YF2 for *NoGA2ox2*; and ZR1/ZR2 and ZF1/ZF2 for *NoGA2ox3*. Full-length cDNA clones were amplified by RT-PCR using the specific sense/antisense primers AF0/AR0, BF0/BR0, XXF0/XXR0, YYF0/YYR0, and ZZF0/ZZR0 for *NoGA20ox1, -2* and *NoGA2ox1, -2*, and *-3*, respectively. The same specific sense/antisense primers were employed in the isolation of the genomic clones, but with genomic DNA as the template. Thermocycling conditions were: 94°C 5 min + 5 × (94°C 1 min, 55°C 2 min, 72°C 2 min) + 30 × (94°C 1 min, 55°C 1 min, 72°C 2 min) + 72°C 10 min.

Nucleotide sequences were determined by the dideoxy-nucleotide chain-termination method using an automated sequencing system (ABI1337, Applied Biosystems). Multiple sequence alignments were performed using CLUSTALW-MP interactive Multiple Sequence at EBI (http://www.ebi.ac.uk/clustalw/index.html) and plotted using the GeneDoc, Multiple Sequence Alignment Editor & Shading Utility, version 2.2.000 (Nicholas and others 1997). The phylogenetic tree was drawn using PhyloDraw (Choi and others 2000). The intron positions were determined by aligning the genomic sequences and their corresponding cDNA sequences using the ''Spidey'' program (http://www.ncbi.nlm.nih.gov/spidey/index.html). Intron position for *Arabidopsis* and rice genes were obtained from <http://atensembl.arabidopsis.info/> and <http://www.gramene.org/> respectively. Protein molecular mass (PMM) and isoelectric point (pI) were calculated using http://us.expasy.org/cgi-bin/pi_tool (Table 1).

Transcript Analysis

Northern blots were prepared by electrophoresis of 2 μg poly(A^+) RNA (or total RNA isolated as described by Bugos and others 1995), in formaldehyde agarose gels, transferred with 20× SSC to Nytran 0.45 nylon filters (Schleicher and Schuell, Dassel, Germany) and cross-linked with UV Strata linker 800 (Stratagem, La Jolla, California, USA). After 1 hour of prehybridization, filter hybridization was carried out overnight at 42°C in a solution containing 50% formamide, 5× SSC, 5× Denhardt's, 0.1% SDS, 0.1 mg/ml denatured salmon sperm DNA, and random-primed ^{32}P-labeled, full-length cDNA probes. Filters were washed (10 min per wash three times at room temperature in 2× SSC and 0.1% sodium dodecyl sulfate (SDS) solution followed by washes at 37°C, 42°C, and 65°C in 0.1× SSC and 0.1% SDS solution. Blots were stripped and re-hybridized with *NoActin* as loading control.

The abundances of the *NoGA20ox2* multiple transcripts were analyzed by RT-PCR. Single-strand cDNA carrying extra nucleotides in 5′(AP2 sequence, included in the end of the oligo-dT primer used for reverse transcription) was used as the template, and BF3/AP2 were used as primers. The AP2 sequence (present in all cDNA molecules) served as the nonspecific reverse primer, and BF3 (located in the coding region, 10 nt upstream from the stop codon) served as the specific upstream primer. Thermocycling conditions were: 94°C 5 min + 5 cycles at [94°C 1 min, 52°C 3 min, 72°C 2 min] + 15 cycles at [94°C 1 min, 45°C 1 min 45 s, 72°C 2 min] + 72°C 10 min. The three amplification products (253, 277, and 357 bp) were separated by electrophoresis in a 2.5% (m/v) agarose gel, transferred to a membrane, and hybridized with the longest transcript amplification product (357 nt) as the probe.

Heterologous Expression of NoGA20ox1, -2, NoGA2ox1, -2, and -3 in *E. coli*

Full-length cDNA clones of *NoGA20ox1, -2* and *NoGA2ox1, -2*, and *-3* were subcloned in pTrcHis expression vector and expressed in *Eschericia coli* TOP10 cells (Xpress Kit; Invitrogen, San Diego, California, USA). Aliquots of cell lysate supernatants were incubated as described previously (García-Martínez and others 1997), using 15,000 dpm of [^{14}C]GA_{12} (55 Ci/mol) or [^{14}C]GA_{53} (34 Ci/mol) as substrates of NoGA20ox1 and -2 and 15,000 dpm of [^{14}C]GA_1 (34 Ci/mol), [^{14}C]GA_9 (55 Ci/mol), and [^{14}C]GA_{20} (55 Ci/mol) as substrates of NoGA2ox1, -2, and -3 (obtained from Prof. L. Mander, Australian National University, Canberra, Australia). Supernatants of reaction media were analyzed by high-performance liquid chromatography (HPLC), using a 4 μm C_{18} Novapak column (150 mm long by 3.9 mm i.d.) and a linear gradient of 10% to 100% methanol (containing 50 ml acetic acid l^{-1}) over 40 min at 1 ml min^{-1}. The radioactive products were identified by gas chromatography-selected ion monitoring.

Heterologous Expression of NoGA2ox3 in Tobacco Plants

The full-length *NoGA2ox3* cDNA clone with a 9-bp 5′-untranslated sequence and a 72-bp 3′-untranslated sequence, was amplified by PCR using the primers ZZF0/ZZR0 with *Bam*HI sites, introduced in the *Bam*HI site of pBluescript SK^+ and reintroduced into the *Bam*HI site of the pBin19-JIT60 plasmid. The vector, pBin19-JIT60-*NoGA2ox3*, was introduced into *Agrobacterium tumefaciens* strain LBA4404

Table 1. Full-length cDNA Clone Features of NoGA20ox1, -2 and NoGA2ox1, -2, and -3

	NoGA20ox1	NoGA20ox2	NoGA2ox1	NoGA2ox2	NoGA2ox3
ORF (aa)	376	359	339	332	334
PMM (kD)	42.7	41.4	38.1	37.4	37.6
pI	5.97	7.25	6.64	7.01	6.04

ORF: open reading frame (represented in amino acids [aa]; PMM, protein molecular mass; pI, isoelectric point.

(Hoekema and others 1983) for transformation of *Nicotiana tabacum*. Tobacco leaf sections were infected, and transgenic tobacco plants were regenerated according to the method of Vidal and others (2001). Non-infected explants were cultured as a control. Independent transgenic lines were selected in GM medium containing 125 mg l^{-1} kanamycin. The T_1 generations were self-pollinated to obtain the T_2 generations. The lines showing a 3:1 segregation (resistant:sensitive) were selected to obtain the homozygous T_3 plants.

Quantification and Identification of GAs

Giberellins in the apical leaves (the five youngest leaves) were extracted, purified, and quantified by GC-MS as described by Rodrigo and others (1997). The samples were spiked at the time of extraction with [^{3}H]GA_{20} and [^{3}H]GA_9 (from Prof. L. Mander, Australian National University, Canberra, Australia) to check recoveries during the purification procedure, and with [17-$^2H_2$]GA_1, [17-$^2H_2$]GA_4, [17-$^2H_2$]GA_8, [17-$^2H_2$]GA_9, [17-$^2H_2$]GA_{19}, [17-$^2H_2$] GA_{20} ,and [17-$^2H_2$]GA_{29} (purchased from Prof. L. Mander) as internal standards for quantification. The HPLC fractions were methylated and trimethylsilylated before analysis by GC-MS in a Hewlett Packard 5890 gas chromatograph coupled to a 5971A Mass Selective Detector and quantified as described previously (Rodrigo and others 1997).

DNA sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY594289 to *NoGA20ox1*, AY594290 to *NoGA20ox2*, AY594291 to *NoGA2ox1*, AY594292 to *NoGA2ox2*, AY588978 to *NoGA2ox3*, and AY594293 to *NoActin*, a partial clone of *Actin* from oleander.

Results

Isolating GA 20- and GA 2-Oxidase Gene Families from *Nerium oleander*

GA 20- and 2-oxidase cDNA clones were isolated from apical shoots of *Nerium oleander* (oleander) by RT-PCR using poly(A)$^+$ RNA. We manipulated the growth of oleander to aid isolation of these genes. Treatments with paclobutrazol (PCB, a GA biosynthesis inhibitor) reduced plant height, whereas treatment with the active GA_3 increased stem elongation (Figure 2). In addition, it has previously been shown that transcript levels of GA 20- and 2-oxidase genes are generally subjected to negative feedback or positive feedforward regulation, respectively, by active GA (Hedden and cKamiya 1997; Cowling and others 1998; Thomas and others 1999). Therefore, to facilitate the isolation of cDNAs corresponding to transcripts of these genes, plants were treated with PCB to enrich for GA 20-oxidases or GA_3 to enrich for GA 2-oxidases before material collection. Partial cDNA clones were isolated by RT-PCR using degenerated primers, and subsequently full-length clones were obtained by RACE-PCR (see *Materials and Methods*). Two full-length cDNA clones encoding putative GA 20-oxidases, named *NoGA20ox1* and *NoGA20ox2*, and three cDNA clones encoding putative GA 2-oxidases, designated *NoGA2ox1, NoGA2ox2*, and *NoGA2ox3*, were obtained using this enrichment procedure. The amino acid sequences encoded by the isolated genes (Table 1) share several conserved regions with other dioxygenases, such as putative 2-oxoglutarate-binding sites (Arg and Ser) and iron binding sites (His, Asp, and His) as indicated in Figure 3a.

The genomic structures of *NoGA20ox1, -2* and *NoGA2ox1, -2*, and *-3* genes were determined spanning the entire coding regions of each gene. Comparison of genomic and cDNA sequences revealed that all gene structures consisted of three exons and two introns (Figure 3b). Alignment among all GA 20- and 2-oxidase gene sequences from oleander, *Arabidopsis*, and rice showed that intron positions were conserved in both gene families in different species (Xu and others 1995; Spielmeyer and others 2002) (Figure 3a).

NoGA20ox1, -2 and NoGA2ox1, -2, and -3 amino acid sequences were compared with those of other GA 2-oxoglutarate-dependent dioxygenases involved in GA metabolism (including GA 3-oxidases)

Figure 2. Effect of GAs in *Nerium oleander* 60-day-old seedlings. GA_3 10^{-6} M supplemented in the medium produced elongated hypocotyls and long internodes, whereas PBC 10^{-6} M reduced hypocotyl length and produced short internodes compared with the control (untreated plants).

from dicotyledonous species. The phylogenetic tree based on these alignments showed three main groups (Figure 4), a first group for GA 20-oxidases, where NoGA20ox2 and -1 were included, a second group for GA 3-oxidases, and a third group for GA 2-oxidases (which use C_{19}-GAs as substrates) where NoGA2ox1, -2, and -3 grouped. AtGA2ox7 and –8, described as GA 2-oxidases that use C_{20}-GAs as substrates (Schomburg and others 2003), were clearly separated from the rest of GA 2-oxidases, which use C_{19}-GAs as substrates.

Expression Patterns of NoGA20ox1 and -2 and NoGA2ox1, -2, and -3 Transcripts

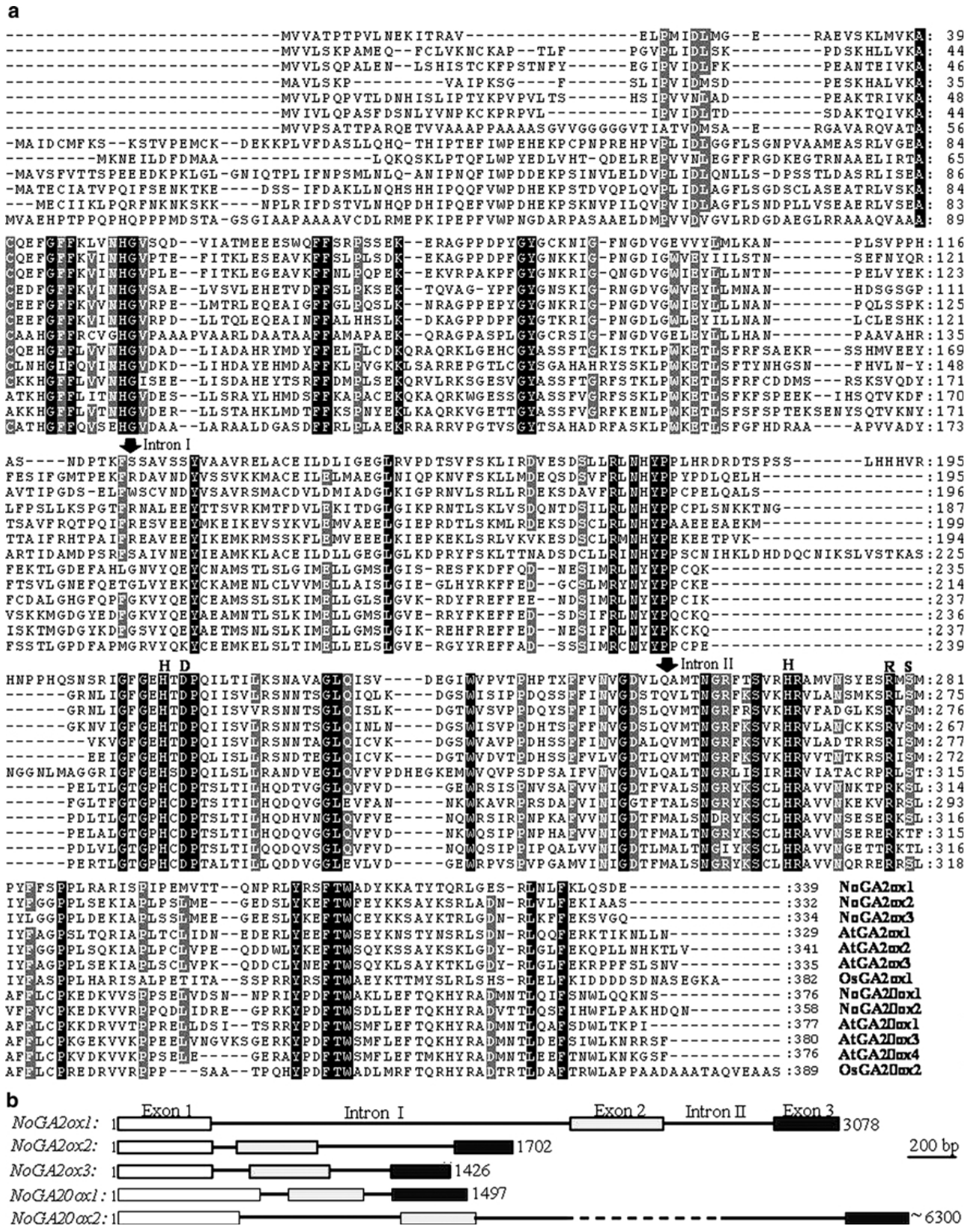
The expression patterns of *NoGA20ox1, -2* and *NoGA2ox1, -2*, and *-3* genes were studied by RNA gel-blot analysis using poly(A)$^+$ RNA isolated from several reproductive and vegetative organs of oleander (expanding leaves, apical shoots, and immature floral buds). *NoGA20ox1*, *-2* and *NoGA2ox1* transcripts were undetectable in these tissues (data not shown). *NoGA2ox2* and *NoGA2ox3* transcripts were abundant in expanding leaves (EL) and floral buds (FB) but were expressed at lower levels in apical shoots (AS) (Figure 5a). Other experiments showed that in roots or internodes, transcripts from all the genes analyzed were not detected (data not shown).

Many plant species exhibit negative feedback regulation of transcripts of GA 20-oxidase and positive feedforward regulation of GA 2-oxidase by active GAs (Hedden and Phillips 2000). We examined whether *NoGA20ox1, -2* and *NoGA2ox1, -2*, and *-3* also exhibited this mechanism of GA homeostasis by determining whether transcript levels in apical shoots were altered by GA_3 or PCB treatments. *NoGA20ox1* transcripts were not detected in control plants, even after PCB application (data not shown). Expression of *NoGA20ox2* (expressed at very low levels in untreated shoots), was induced by PCB, but reduced by GA_3 treatment (Figure 5b), indicating that *NoGA20ox2* expression is subjected to negative feedback regulation by active GAs.

Transcript levels of *NoGA2ox3* did not change in untreated (control), GA_3-, or PCB-treated plants, whereas GA_3 treatment increased *NoGA2ox2* transcripts levels (Figure 5c). *NoGA2ox1* transcripts were not detected, even in GA_3-treated plants (data not shown). These results suggest that only *NoGA2ox2*, out of the three GA 2-oxidase genes isolated from oleander, is subject to feedforward regulation by GAs.

Transcripts from *NoGA20ox1, -2* and *NoGA2ox2* and *-3* genes exhibited multi-polyadenylation sites. Sequencing 3′ ends of eight independent cDNA clones from each gene (for *NoGA2ox1*, only two identical cDNA clones were isolated) revealed the existence of transcripts of different length due to attachment of the poly(A)$^+$ tail at different sites in the 3′-untranslated region. Transcripts corresponding to each gene differed only in their 3′ untranslated region length, maintaining identical coding regions and therefore producing identical proteins. Five different transcripts from *NoGA20ox1* were identified: three from *NoGA20ox2*, three from *NoGA2ox2*, and four from *NoGA2ox3* (Figure 5e).

To define the relative abundance and regulation of each transcript population for one example gene, the number of different polyadenylated *NoGA20ox2* transcripts in response to GA_3 and PCB treatments was estimated using an RT-PCR–based approach. A similar pattern of three bands (253, 277, 357 bp), corresponding to the three *NoGA20ox2* transcripts previously described, was obtained in all the cases (Figure 5d). All three species of transcripts increased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Intron I
Intron II
H D
H
R S
NoGA2ox1
NoGA2ox2
NoGA2ox3
AtGA2ox1
AtGA2ox2
AtGA2ox3
OsGA2ox1
NoGA20ox1
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b
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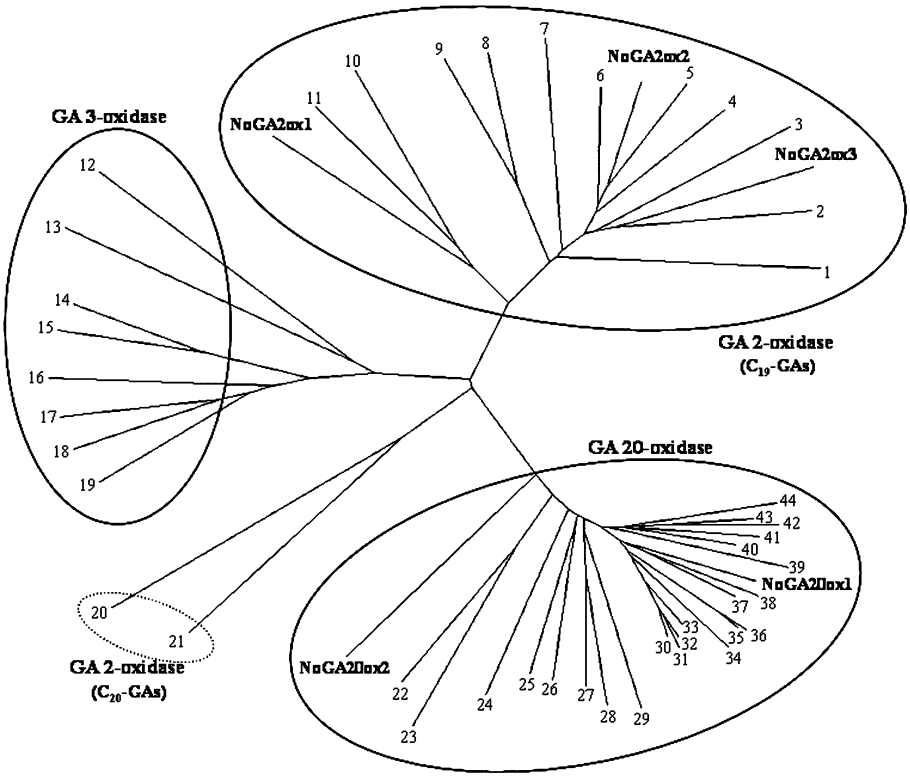

Figure 4. Phylogenetic tree based on the comparison of the deduced amino acid sequences of GA 20-oxidases, GA 2-oxidases (that use C_{20}-GAs or C_{19}-GAs as substrates), and GA 3-oxidases from different dicotyledonous species. This unrooted phylogenetic tree was created using ClustalW and plotted with PhyloDraw. Accession numbers are in brackets and numbers in bold represent the sequences in the tree: **1**, AtGA2ox1 (AJ132435), **2**, PcGA2ox1 (AJ132438), **3**, SoGA2ox1 (AF506281), **4**, PsGA2ox1 (AF100954), **5**, LsGA2ox1 (AB031206), **6**, CmGA2ox (AJ302041), **7**, LsGA2ox2 (AB031207), **8**, AtGA2ox2 (AJ132436), **9**, AtGA2ox3 (AJ132437), **10**, PsGA2ox2 (AF100955), **11**, SoGA2ox2 (AF506282), **12**, CmGA2,3ox (U63650), **13**, LsGA3ox3 (AB031203), **14**, AtGA3ox1 (L37126), **15**, AtGA3ox2 (T51691), **16**, PsGA3ox1 (AF007766), **17**, LsGA3ox1 (AB012205), **18**, LsGA3ox2 (AB012206), **19**, NtGA3ox1 (AB032198), **20**, AtGA2ox7 (AC079284), **21**, AtGA2ox8 (AL021960), **22**, ClGA20ox1, (AF074709), **23**, CmGA20ox (X73314), **24**, PvGA20ox3 (U70532), **25**, NtGA20ox2 (Ntc-16, AB016084), **26**, SoGA20ox1 (U33330), **27**, AtGA20ox2 (X83380), **28**, AtGA20ox1 (X83379), **29**, AtGA20ox3 (X83381), **30,** LeGA20ox1 (AF049898), **31**, StGA20ox1 (AJ291453), **32**, SdGA20ox1 (AJ252088), **33**, Ntc-12 (AB012856), **34**, LeGA20ox3 (AF049900), **35**, LeGA20ox2 (AF049899), **36**, StGA20ox2 (AJ291454), **37**, LsGA20ox1 (AB012203), **38**, LsGA20ox2 (AB012204), **39**, ap20ox (AB037114), **40**, CcGA20ox1 (AJ250187), **41**, PttGA20ox1 (AJ001326), **42**, PsGA20ox1 (U70471), **43**, PvGA20ox1 (U70530), **44**, PvGA20ox2 (U70531).

in response to PCB and decreased in response to GA_3 compared to untreated material (Figure 5d). Moreover, relative intensities between the three bands were the same in treated and untreated plants, indicating that relative transcript abundance was also maintained.

Functional Analysis of NoGA20ox1 and -2 and NoGA2ox1, -2, and -3 *In Vitro*

To demonstrate that the cDNA clones *NoGA20ox1* and *-2* encode functional GA 20-oxidases and that *NoGA2ox1, -2,* and *-3* encode functional GA 2-oxid-

Figure 3. Analysis of *NoGA20ox1, -2, NoGA2ox1, -2,* and *–3* sequences. **a.** Alignment of the deduced amino acid sequences of NoGA20ox1, -2 and NoGA2ox1, -2, and -3 with other GA 20- and GA 2-oxidases, including AtGA20ox1, -3, -4 (At4g25420, At5g07200, At1g60980), OsGA20ox2 (GR:0060842), AtGA2ox1, -2, -3 (At1g78440, At1g30040, At2g34555), and OsGA2ox1 (GRMG00000169901), from *Arabidopsis* and rice. Similarities are represented in gray to black boxes. The conserved residues H-D-H, that are supposed to bind Fe^{2+}, and the residues R-S, that putatively bind 2-oxoglutarate are indicated on the top of the sequence. Black arrows show intron positions, conserved in all the sequences. **b.** Representation at scale of gene structure for *NoGA2ox1, -2, -3* and *NoGA20ox1* and *-2*. Three exons (boxes white to black) and two introns (black line) of different lengths were detected in all the genes. The scale represents a fragment of 200 bp.

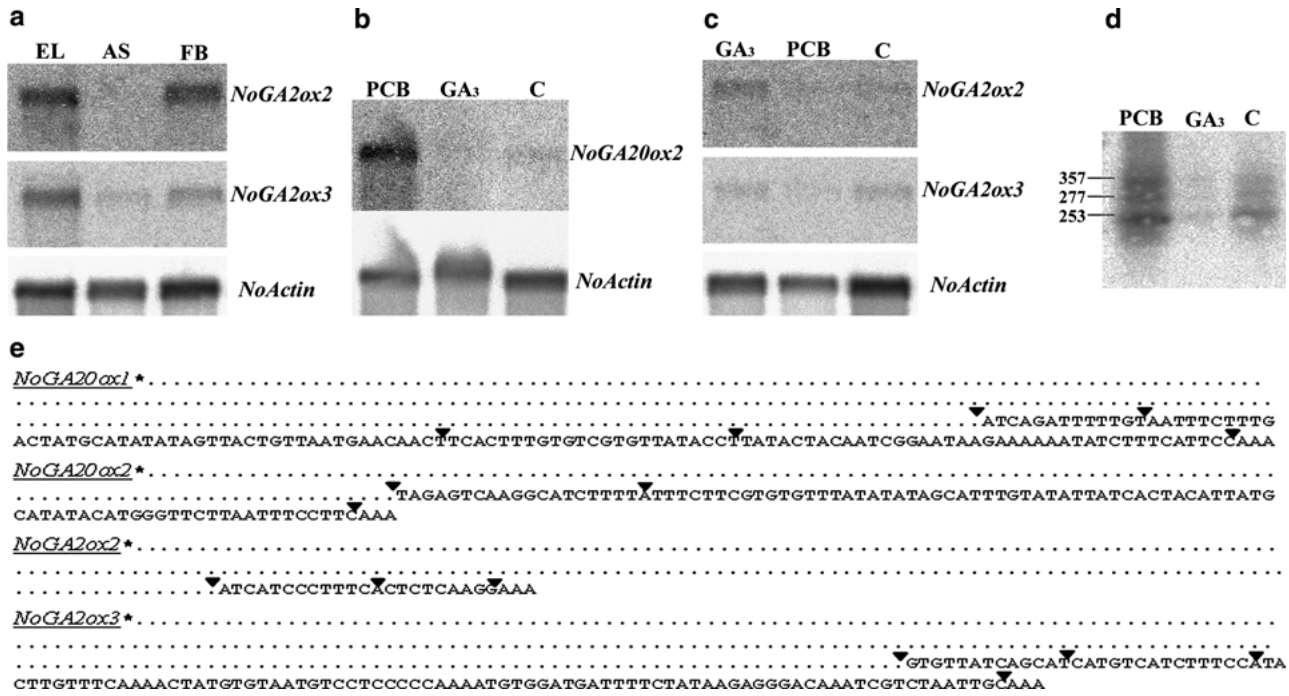

Figure 5. Transcript analysis of *NoGA2ox2, -3 and NoGA20ox1* and *-2* genes in oleander. Northern blot analyses (**a–c**) were carried out using 2 μg poly(A)$^+$, and expression of *NoActin* was used as a control for RNA loading. **a.** Transcript levels of *NoGA2ox2* and *-3* in expanding leaves (EL), apical shoots (AS), and floral buds (FB). **b, c.** Effect of exogenous addition of GA_3 and PCB on expression of *NoGA20ox2* and *NoGA2ox2* and *-3* in apical shoots. Controls (C) are untreated plants. **d.** RT-PCR showing the individual *NoGA20ox2* transcript populations and the effect of PCB and GA_3 treatment on them. cDNA from apical shoots was amplified by PCR, and products were separated on a 2.5 % (w/v) agarose gel, transferred to a nylon membrane and hybridized with a radioactive DNA probe corresponding to the 357 pb transcript. **e.** Representation of the 3′ untranslated region from *NoGA20ox1, -2, NoGA2ox2* and *-3* genes. An asterisk indicates the stop codon position; dots indicate the 3′ untranslated region identical in all the transcripts from each gene; and black triangles indicate the sites of poly(A$^+$) tail attachment in different transcripts isolated from each gene.

ases, their coding regions were expressed as fusion proteins in *E. coli*, and soluble protein extracts were used to assay GA 20-oxidase and GA 2-oxidase activities using several radioactive GAs as substrates. The reaction products were separated by reverse-phase HPLC and detected by radioactive scintillation counting. Retention times (Rt) of the products were compared with those of standard ^{14}C-labeled GAs for tentative identification.

Both NoGA20ox1 and -2 recombinant proteins converted [^{14}C]GA_{12} and [^{14}C]GA_{53} to products with Rt corresponding to [^{14}C]GA_9 and [^{14}C]GA_{20}, respectively (Figures 6a–c and 6d–f), suggesting that *NoGA20ox1* and *-2* encode GA 20-oxidases. Despite the relatively large fraction of substrates unmetabolized in the incubations with NoGA20ox2 (Figures 6c and 6f), intermediate products of GA 20-oxidase such as GA_{15}, GA_{24}, GA_{44}, and GA_{19} were not detected.

The three NoGA2ox1, -2 and -3 recombinant proteins converted [^{14}C]GA_1, [^{14}C]GA_9, and [^{14}C]GA_{20} to products with Rt corresponding to [^{14}C] GA_8, [^{14}C]GA_{51}, and [^{14}C]GA_{29}, respectively (Figures 6g–j, 6k–n and 6o–r). These products were identified by gas chromatography–mass spectroscopy (GC-MS; data not shown), thus demonstrating that *NoGA2ox1, -2*, and *-3* encode GA 2-oxidases. In the case of NoGA2ox3, a second HPLC product (peak) was also found in incubations with [^{14}C]GA_9 and [^{14}C]GA_{20}, with Rt corresponding to a GA_{51}-catabolite and GA_{29}-catabolite, respectively (Figures 6n and 6r). No conversion of GA_{12} or GA_{53} (C_{20}-GAs) was detected in incubations with NoGA2ox1 or NoGA2ox3 (data not shown). Thus, it appears that NoGA2ox1 and NoGA2ox3 use specifically C_{19}-GAs as substrate and not C_{20}-GAs.

Functional Analysis of NoGA2ox3 *In Vivo*

To analyze the potential to modify GA levels as strategy to reduce plant height, we expressed the catabolic gene *NoGA2ox3* in tobacco plants. Tobacco leaf explants were infected with *A. tumefaciens* carrying pBin19-JIT60-*35S::NoGA2ox3*, and a total of 25 T_1 putative transgenic plants resistant to kanamycin

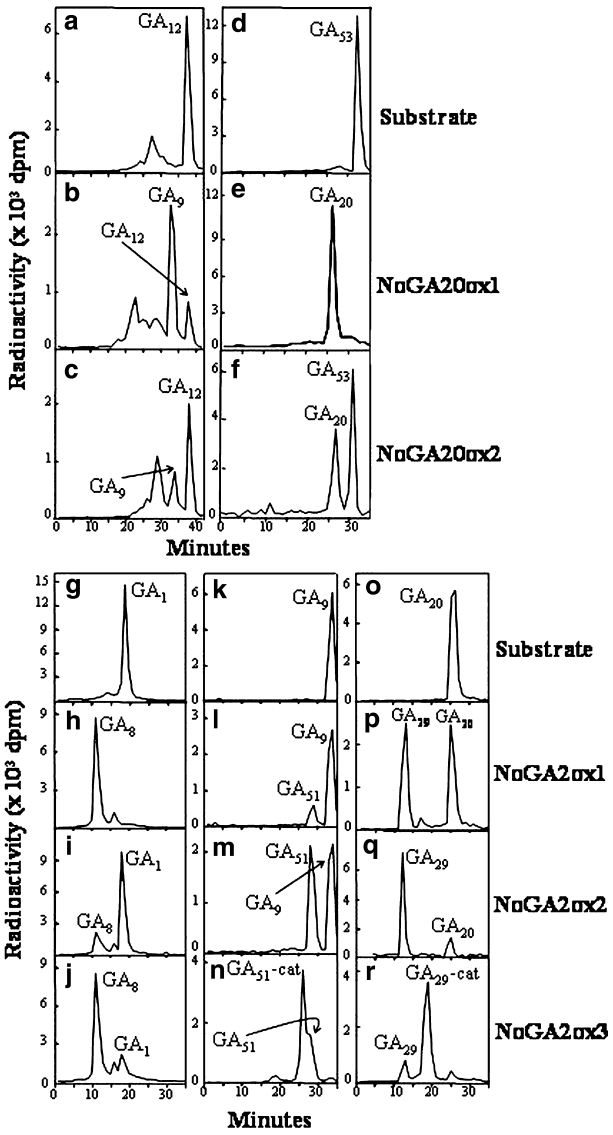

Figure 6. Radiochromatograms showing separation by HPLC of ^{14}C-labeled GA substrates and products after incubation with recombinant NoGA20ox1, -2 and NoGA2ox1, -2, and -3 proteins. Identities of the peaks are indicated. Substrates [^{14}C]GA_{12} (**a–c**) and [^{14}C] GA_{53} (**d–f**) were incubated with the recombinant NoGA20ox1 and -2 proteins. Substrates [^{14}C]GA_1 (**g–j**), [^{14}C]GA_9 (**k–n**) and [^{14}C]GA_{20} (**o–r**) were incubated with NoGA2ox1, -2, and -3, and products, except GA-catabolites (GA-cat), were identified by gas chromatography–mass spectroscopy. Equal amounts of bacterial lysates (85 µl) containing the expressed proteins were used in the incubations.

were obtained. Transformants carrying the transgene (tested by Northern blot analysis) were selected and exhibited different phenotypes, ranging from almost wild-type, to semi-dwarf (approximately half the height of control plants; Figure 7a) and dwarf plants (compact rosettes with no visible internodes; Figure 7b). Hemizygous T_2 plants of the dwarf line L-41 and homozygous T_3 populations of dwarf L-40.9 and semi-dwarf L-45.3 lines were obtained and phenotypically analyzed.

Northern blot analysis of total RNA isolated from expanding leaves of L-40.9, L-41, and L-45.3 plants demonstrated that there was a higher accumulation of transcripts corresponding to the transgene in dwarf (L-40.9 and L-41) lines than in semi-dwarf (L-45.3) plants (Figure 7c), suggesting a correlation between the level of accumulation of transgene mRNA and reduction of plant height.

We have shown that, *in vitro*, NoGA2ox3, catalyzes the inactivation of the bioactive GA_1 as well as its immediate precursors, GA_{20} and GA_9 (Figures 6j, 6n, and 6r). We tested the influence of the ectopic expression of this enzyme in tobacco on endogenous GAs by analyzing the GA content of transgenic plants expressing *35S::NoGA2ox3*. In semi-dwarf L-45.3 plants, the amounts of GA_1, GA_9, GA_{19}, and GA_{20} were dramatically reduced (Table 2). In dwarf L-40.9 and L-41 plants, the amount of GA_1 was reduced to trace levels (data not shown). It was not possible to quantify GA_4, GA_8, and GA_{29} in extracts of any of these plants because they were below detection limits.

The phenotypes conferred by the transgene were studied during development. Seeds of control (non-transformed plants) and transgenic L-40.9, L-41, and L-45.3 lines were imbibed on GM media for 10 days, and their germination potential was assayed (Figure 8a). L-41 seeds showed a reduction in germination, whereas all L-40.9 seeds germinated, although with a clear delay in germination time (Figure 8b), and L-45.3 seed germination was similar to that of control seeds. Application of GA_3 (10^{-5} M) to the medium partially rescued the low germination of L-41 seeds (Figure 8a) and completely rescued the delay in germination time found in L-40.9 seeds (data not shown).

Hypocotyl length was reduced in dwarf transgenic plants; for example 10-day-old L-41 seedlings showed a large reduction in hypocotyl length when compared to control seedlings. A weaker reduction was detected in L-40.9 seedlings, and no reduction in hypocotyl length was detected in L-45.3 seedlings (a semi-dwarf line) (Figure 8c). GA_3 application rescued the dwarf hypocotyl phenotypes of L-41 and L-40.9 seedlings (data not shown).

The height of adult transgenic plants expressing *NoGA2ox3* was reduced with increasing severity in semi-dwarf (L-45.3) and dwarf (L-41) phenotypes (Figure 8d).

The number of internodes developed before flowering in 6-month-old plants of the semi-dwarf

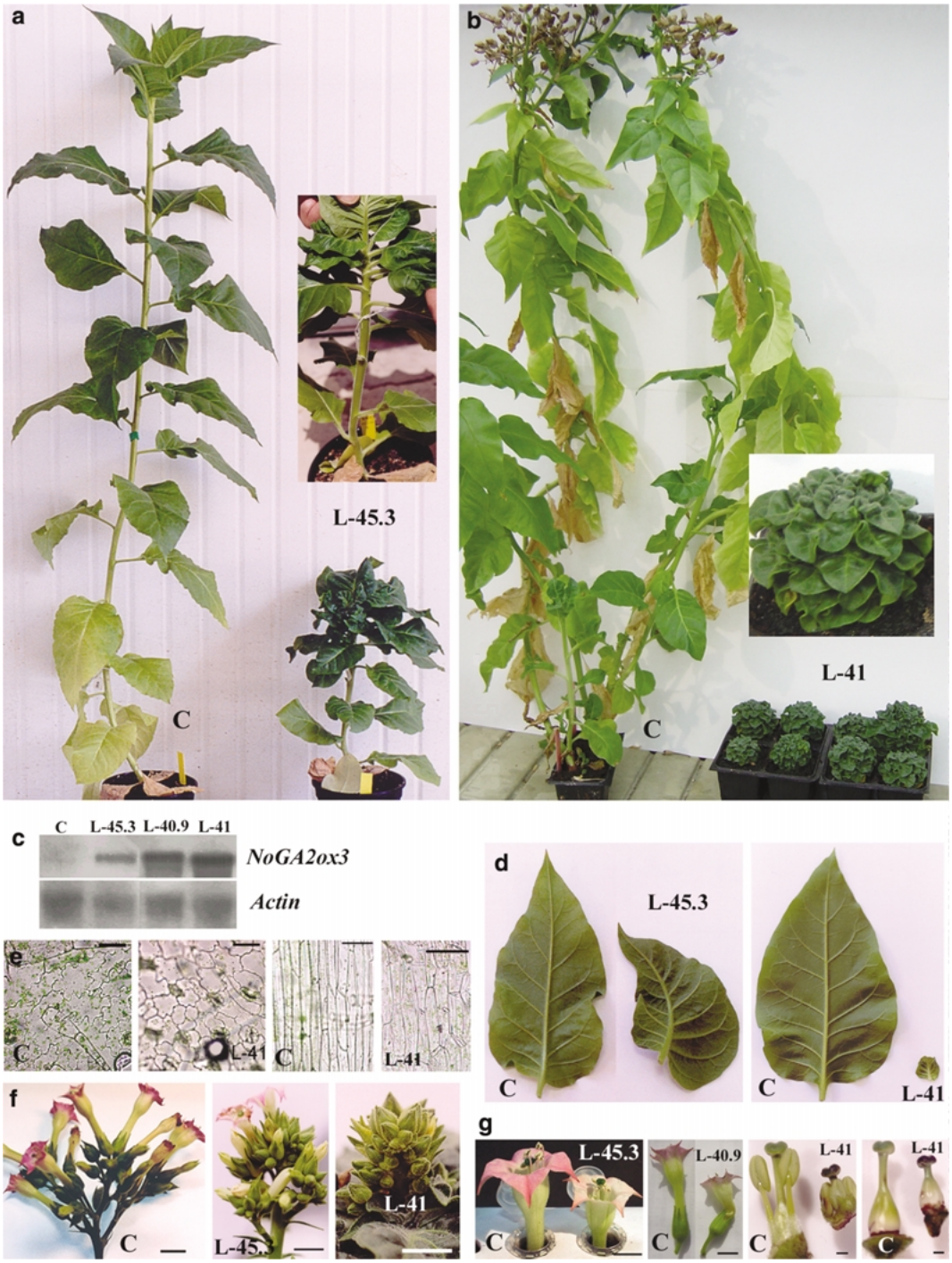

Figure 7. Ectopic expression of *NoGA2ox3* reduces stem growth and produces morphological alterations in leaves and flowers in *Nicotiana tabacum*. **a, b.** Reduction in stem elongation was detected in semi-dwarf (L-45.3) and dwarf (L-41) transgenic plants when compared with control (C, untransformated) plants; insets show a close-up of the transgenic plants. **c.** Northern blot analysis, using total RNA (20 μg) from untransformed (C), L-45.3, L-40.9, and L-41 plants and using the full-length *NoGA2ox3* cDNA clone as the probe, indicated higher transgene expression in dwarf L-41 plants than in the semi-dwarf line L-45.3. Expression of *Actin* was used as a control for RNA loading. **d.** Leaves in transgenic plants were smaller and more dark-green than those of control plants. **e.** Epidermal cells in the abaxial leaf blade (left) and in the central leaf vein (right) were smaller in dwarf L-41 plants than in control plants; scale bars from left to right: 50, 50, 100, 100 μm. **f.** Inflorescences from semi-dwarf L-45.3 and dwarf L-41 plants were compacted when compared with control plants (scale bar, 1 cm). L-41 plants produced flowers uniquely after spraying with GA_3 10^{-5} M. **g.** Flowers from transgenic plants were smaller and pale (left) and, in the case of the L-41 flowers, short filaments and small ovaries were developed (right); scale bars from left to right: 1 cm, 1 cm, 1 mm, 1 mm.

Table 2. Gibberellin Content (ng/g fresh weight [FW]) of Control (untransformed) and Semi-dwarf L-45.3 Tobacco Plants Expressing *35S::NoGA2ox3*

Line	GA_1	GA_9	GA_{19}	GA_{20}
Control	0.96	0.10	3.25	2.3
L-45.3	Trace	Trace	Trace	0.07

GA: giberellin; Trace: < 0.05 ng/g FW.

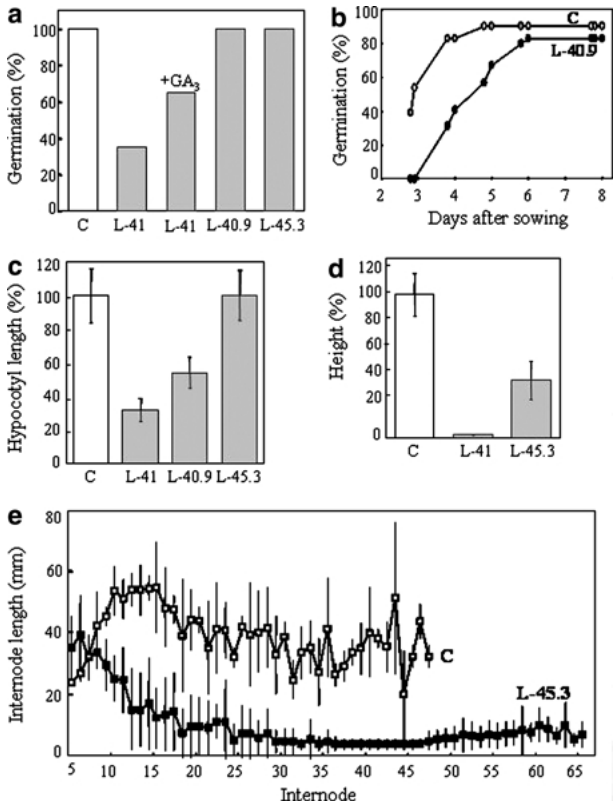

Figure 8. Analysis of germination, seedling development, and internode length of transgenic tobacco plants. **a.** Percentage of seeds germinated in GM medium 10 days after sowing (n = 100). L-41 line showed low germination, partially rescued by application of GA3 (10^{-5} M) to the media, when compared with control (C, untransformed) seeds. **b.** Germination-time curves showing a delay in germination in L-40.9 seeds. **c.** Short hypocotyls were detected in dwarf L-41 and L-40.9 lines but not in the semi-dwarf L-45.3 plants when compared with control plants. **d.** The height of adult transgenic plants (6-months-old) was reduced severely. **e.** Internode lengths of 6-month-old L-45.3 plants (black square) and control plants (white square); n = 9; bars mean standard deviation.

L-45.3 line was higher than in untransformed plants (Figure 8e). The phenotype of semi-dwarf plants was similar to that of control plants until approximately the 8th internode (counted from the base); subsequently the developing stem of L-45.3 plants showed a GA-deficient phenotype of shorter internodes (Figures 7a and 8e). In contrast, in dwarf transgenic plants all internodes were extremely short, including those developed at early developmental stages. In the case of the dwarf line L-41, rosettes were very compact (Figure 7b), and values of average internode length in 1-year-old plants were estimated by dividing the length of the segment of the stem carrying nonsenescent leaves by the number of leaves present on it. This gave average internode length values of 0.4 ± 0.1 mm (n = 10) for L-41 plants versus 25.6 ± 3.5 mm (n = 3) for control plants, indicating that L-41 plants developed 32.1 ± 9.6 leaves per centimeter of stem versus 0.4 ± 0.1 leaves per centimeter of stem developed in untransformed plants, demonstrating that the reduction of internode length was the cause of their extremely reduced height.

Leaves of transgenic plants had wrinkled surfaces, and were smaller and more dark-green than those of untransformed tobacco plants. Leaves of semi-dwarf L-45.3 plants were also twisted, due to unequal growth of both sides of the blade. Leaves from dwarf plants L-40.9 and L-41 also showed a dramatic size reduction (Figure 7d). The morphology of cells in the leaves of dwarf L-41 plants was analyzed to determine whether leaf growth inhibition was caused by a reduction of cell division and/or cell elongation. Epidermal cells of the blade were smaller and lacked the characteristic shape of control plants. Epidermal cells of the central nerve in the leaf of dwarf L-41 plants were shorter than those of untransformed plants (Figure 7e). Therefore, the reduction of leaf size in dwarf L-41 plants was due to inhibition of both cell elongation and expansion.

GA_3 is a bioactive form of GA that cannot be metabolized by GA 2-oxidases (Nakayama and others 1990). Thus, application of GA_3 should rescue dwarfism induced by expression of *NoGA2ox3.* After application of GA3 (10^{-5} M) via irrigation to semi-dwarf L-45.3 plants, a significant reversion of dwarfism was observed (data not shown), with number and morphology of leaves similar to those

of wild-type plants. In the case of dwarf L-40.9 and L-41 plants, however, even simultaneous applications of GA_3 to the leaves (spraying) and to the roots (via irrigation) only partially rescued the dwarf phenotype. In all the cases, when GA_3 application was discontinued, the transgenic plant growth reverted to the dwarf phenotype.

Semi-dwarf transgenic L-45.3 plants flowered later than untransformed plants and produced more vegetative leaves (62 ± 6 versus 45 ± 6 leaves). The application of GA_3 via irrigation rescued the late-flowering phenotype, indicating that the decreased level of endogenous GA was responsible for flowering delay. Dwarf L-40.9 plants rarely flowered, and usually remained in a vegetative stage even after 10 months of culture, in contrast with untransformed plants, which flowered before 6 months. Dwarf L-41 plants did not flower and remained vegetative for more than 1 year. Continuous GA_3 application via irrigation and spraying rescued flowering in L-40.9, but very poor rescue was obtained for L-41 plants.

The number of flowers in transgenic plants expressing *NoGA2ox3* was also reduced, being more severe in dwarf L-40.9 than in semi-dwarf L-45.3 plants (data not shown). The inflorescences of transgenic plants were more compact than those of untransformed plants (Figure 7f). This effect was particularly conspicuous in the case of L-41 dwarf plants, where the inflorescences, obtained only after spraying with GA_3, were extremely compact, and most flower buds reached senescence at an early developmental stage. Flower abortion was prevented in dwarf L-40.9 plants by GA_3 application. However, in the case of dwarf L-41 plants, even after intensive GA_3 applications (spraying plus irrigation) only a few fertile flowers producing fruits and seeds were obtained.

Flower development was altered in transgenic plants (Figure 7g). In semi-dwarf L-45.3 plants, flowers showed a reduction in size, corolla pigmentation, and stamen length compared to untransformed flowers, although flowers were fertile and produced fruits and seeds. Flowers of L-40.9 dwarf plants also exhibited bent corollas, and fertility was reduced because pollen was non-viable, and/or ovule development was altered (fertilization of these flowers with wild-type pollen frequently failed to produce fruits and seeds). In the case of dwarf L-41 plants (induced to flower with GA_3) the most severe morphological alterations were reduction of stamen filament length and of carpel size (ovary and style), whereas anther size was relatively unaltered compared with untransformed plants (Figure 7g).

Discussion

Two cDNA clones encoding GA 20-oxidases (*NoGA20ox1* and *-2*) and three cDNA clones encoding GA 2-oxidases (*NoGA2ox1, -2*, and *-3*) were isolated from apical shoots of *Nerium oleander* plants.

Comparative analysis of amino acid sequences of NoGA20ox1, -2 and of NoGA2ox1, -2 and -3 proteins showed great similarity with previously characterized GA 20- and 2-oxidases respectively, from other species and also revealed conservation of the amino acid residues proposed to bind 2-oxoglutarate and Fe^{2+} at the catalytic site in GA 2-oxoglutarate-dependent dioxygenases (Valegard and others 1998; Figure 3a). Analysis of the structures of genes indicated conservation of intron position in all cases in comparison with orthologous genes from *Arabidopsis* and rice (Figure 3).

Northern blot analysis showed the presence of *NoGA2ox2* and *NoGA2ox3* transcripts in expanding leaves and flowers; *NoGA2ox3* transcripts were also detected in apical shoots (Figure 5a). In contrast, transcripts of the other GA 2-oxidase, *NoGA2ox1*, were below detection levels in all tissues analyzed. These results are similar to the spatial expression pattern described for GA 2-oxidases from *Arabidopsis* (Thomas and others 1999). For the GA 20-oxidase, *NoGA20ox1* transcripts were not detected in any of the tissues investigated, whereas low levels of *NoGA20ox2* transcripts were detected in the apical shoot (Figure 5b).

The biosynthesis and catabolism of physiologically active GAs has been shown to be subjected to negative feedback and positive feedforward regulation, respectively. Both mechanisms regulate transcript accumulation of GA biosynthetic genes (GA 20- and GA 3-oxidase) and GA catabolic genes (GA 2-oxidase) (Hedden and Phillips 2000). *NoGA20ox2* transcript levels were reduced by GA_3, and increased by PCB treatment (Figure 5b), indicating that *NoGA20ox2* expression is subjected to negative feedback regulation by active GAs in oleander. This is in agreement with the regulation described for the GA 20-oxidases *OsGA20ox1* from rice (Toyomasu and others 1997), *PttGA20ox1* from hybrid aspen (Eriksson and Moritz 2002) and *CcGA20ox1* from *Carrizo citrange* (Vidal and others 2003). However, *NoGA20ox1* transcripts were not detected by Northern blot analysis, even after treatment with PCB, suggesting a very low expression, under detection levels. However, the isolation of this gene by RT-PCR demonstrates that the gene is expressed, albeit below the level of detection by Northern blot analysis, rejecting

the possibility that it is a pseudogene. *NoGA2ox2* transcript levels were increased by GA_3 and decreased by PCB treatments (Figure 5c), indicating a feedforward regulation of expression by active GAs. However, no change in transcript abundance was detected after GA_3 or PCB treatment for *NoGA2ox1* or *NoGA2ox3* genes (Figure 5c). Therefore, not all GA 2-oxidases isolated from oleander appear to be subject to positive feedforward regulation. Similar results have been found in rice, where *OsGA2ox2* expression is regulated by a feedforward mechanism (Sakai and others 2003) but not that of *OsGA2ox1* (Sakamoto and others 2001). Differential regulation by GA of members of the oleander GA 2-oxidase gene family may indicate that these genes are expressed in different cell types of the tissues studied, or that their expression in the same cells is differentially regulated by the corresponding promoter elements. Further analysis of this differential expression will be required to determine the underlying mechanism(s). The expression of a large number of genes has been reported to be post-transcriptionally regulated by specific sequences in the corresponding mRNAs, usually in the 3′ untranslated region (UTR), although the mechanism of regulation has been proposed in only a very few cases (de Moor and others 2005). Transcripts with alternative polyadenylation sites are common in eukaryote mRNA. Studies of human transcripts, where extensive cDNA data are now available, show that at least 28% of the mRNAs displayed two or more polyadenylation sites (Beaudoing and others 2000). Multiple polyadenylation, described in plant transcripts (Dean and others 1986), has very rarely been associated with a functional or regulatory role. One such case is the cell-wall invertase gene (*Incw1*) of maize (Cheng and others 1999), which produces two transcripts differing in 150 nt length at the 3′ UTR, that are differentially accumulated in the presence of metabolizable sugars. We have shown that genes encoding GA 20-oxidases (*NoGA20ox1* and *-2*) and GA 2-oxidases (*NoGA2ox2* and *NoGA2ox3*) from oleander produce transcripts with different 3′ untranslated regions due to the use of different polyadenylation sites (Figure 5e).

The phenomenon of multiple transcripts has been documented for GA 20-oxidases. Eriksson and Moritz (2002) reported the presence of multiple *PttGA20ox1* transcripts in hybrid aspen, and we identified two transcript types with polyadenylation sites separated by 126 nucleotides in *CcGA20ox1* (AJ250187) from *Carrizo citrange* (unpublished work). There is also evidence of multiple transcripts for these gene families in other species, such as *Arabidopsis* (*AtGA20ox2*, UniGene Cluster At.28053). However, the phenomenon of multiple transcripts has not been described previously for GA 2-oxidases.

In the case of the three *NoGA20ox2* transcripts detected in this work with different polyadenylation sites (Figure 5e), their levels were not differentially regulated in response to variation of active GA content (PCB and GA_3 treatments) (Figure 5d). Therefore, multi-site polyadenylation does not appear to be involved in the feedback GA regulated expression, at least in the case of *NoGA20ox2* transcripts. This does not exclude, however, a role in response to other regulatory and developmental signals. More work is necessary to clarify a possible biological role for multiple polyadenylated transcripts in genes involved in GA biosynthesis and catabolism.

The proteins encoded by *NoGA20ox1* and *-2* catalyzed the conversion of GA_{12} to GA_9 and GA_{53} to GA_{20} (Figures 6a–f), and proteins encoded by *NoGA2ox1*, *-2* and *-3* converted GA_1 to GA_8, GA_9 to GA_{51}, and GA_{20} to GA_{29} (Figures 6g–r). NoGA2ox3 also catalyzed further oxidations at C-2 producing GA_{51}- and GA_{29}-catabolites (Figures 6n and 6r), suggesting that NoGA2ox3 is a multifunctional enzyme, similar to PcGA2ox1 from runner bean, AtGA2ox2 and-3 from *Arabidopsis* (Thomas and others 1999), PsGA2ox1 from pea (Lester and others 1999; Martin and others 1999), OsGA2ox3 from rice (Sakai and others 2003), and SoGA2ox1 from spinach (Lee and Zeevaart 2002). Multifunctional activity was not detected for the proteins NoGA2ox1 or -2, in agreement with results obtained for AtGA2ox1 from *Arabidopsis* (Thomas and others 1999), PsGA2ox2 from pea (Lester and others 1999), SoGA2ox2 from spinach (Lee and Zeevaart 2002), and OsGA2ox1 from rice (Sakamoto and others 2001). This implies that GA 2-oxidase families may contain enzymes with or without multifunctional catalytic activity. GA 2-oxidases can differ not only in their catalytic activity but also in their substrate preference, as has been described for GA 2-oxidases that oxidize C_{20}-GAs (GA_{53} and GA_{12}) instead of C_{19}-GAs (Schomburg and others 2003). SoGA2ox1 from spinach can use both C_{20}-GA (GA_{53}) and the typical C_{19}-GAs as substrates (Lee and Zeevaart 2002). The ability of NoGA2ox1 and NoGA2ox3 to metabolize C_{20}-GAs (GA_{53} and GA_{12}) was tested by *in vitro* assays, but no substrate transformation was detected (date not shown). These results suggest that NoGA2ox1 and NoGA2ox3 share substrate specificity (C_{19}-GAs) with the majority of GA 2-oxidases isolated up to now, including AtGA2ox1, -2, and -3 proteins described in *Arabidopsis* (Thomas and others 1999).

The expression of *NoGA2ox3* under the control of the CaMV 35S promoter generated tobacco plants displaying a range of dwarf phenotypes, from nearly wild-type to severely dwarf compact rosettes, that correlated to transgene expression (Figures 7a–c).

It was found that tobacco plants expressing *NoGA2ox3* had reduced or trace levels of GA_1, as well as reduced levels of GA_1 precursors (GA_{19} and GA_{20}) (Table 2). In addition to GA_1 (the active GA in the early-13-hydroxylation pathway), GA_4 (in the non-13-hydroxylation pathway, Figure 1) may also be important in the control of tobacco stature, at least under certain conditions (Vidal and others 2001). In this work, although it was not possible to quantify GA_4 in transgenic plants, GA_4 was probably also reduced because the levels of GA_9, its immediate precursor, were reduced to trace levels. Surprisingly, no accumulation of GA 2-oxidation products (mainly GA_8, GA_{51}, and GA_{29}) was detected. This may be due to the multifunctional activity of the NoGA2ox3 enzyme, because it has been found that *in vitro* NoGA2ox3 can catalyze further oxidation of GA_8, GA_{51}, and GA_{29} to the corresponding GA-catabolites (Figure 6n and 6r).

Exogenous application of GA_3, a GA described as resistant to catabolism by GA 2-oxidases (Nakayama and others 1990), rescued the height reduction of semi-dwarf L-45.3 plants, and dwarfism was restored when treatment was discontinued. The reversion to normal growth by exogenous application of GA_3 provides a potential method for horticultural manipulation of transgenic plants expressing the GA 2-oxidase gene. Landscape managers can choose to accelerate early growth of transgenic plants via GA_3 application, thereby allowing the growth rate to be reduced only after the plants have reached a desired size. In the case of the dwarf L-40.9 and L-41 lines, which exhibited a severe dwarfism, restoration of the phenotype with GA_3 treatment was partial, even after intensive GA_3 treatment via irrigation plus spraying. The fact that the restoration of the dwarfism in these lines is directly proportional to the GA_3 dosage (spraying GA_3 on the plants showed restoration that was improved with the further addition of GA_3 via irrigation) suggests that GA_3 was not able to fully restore growth in dwarf L-40.9 and L-41 plants because of its inability to reach target tissues at the right concentration. This could be due to low uptake as a consequence of the morphological alterations detected in these severely dwarfed plants. The small and wrinkled leaves, with a high density of trichomes per unit of surface (as a result of smaller epidermal cells) might reduce the GA_3 uptake when sprayed, and the reduced root development in these lines could reduce GA_3 uptake when applied via irrigation. All these results on restoration of dwarfism by GA_3 in semi-dwarf and dwarf transgenic plants strongly support the idea that dwarfism was due to a deficiency of bioactive GAs.

Transgenic tobacco plants displayed the main phenotypic features previously described in dwarf and semi-dwarf GA-deficient mutants, including reduced germination potential, short internodes, dark green leaves, delayed flowering, and deficiencies in flower development (Ross and others 1997).

Germination and seedling development were modified in the dwarf lines L-41 and L-40.9 but not in the semi-dwarf L-45.3 line (Figure 8a). In *Arabidopsis*, only the most severe GA-deficient mutants (for example, *ga1-3* and *ga2-1*) are deficient in germination (Koornneef and van der Veen 1980). Hypocotyl length reduction in transgenic dwarf lines (Figure 8c) was similar to that observed in *Arabidopsis* plants overexpressing the *AtGA2ox7* and *AtGA2ox8* genes (Schomburg and others 2003).

Dwarfism in tobacco transgenics was associated with reduced internode length, which is the most easily observed phenotypic trait associated with GA deficiency in growing plants (Hedden and Proebsting 1999). Surprisingly, L-45.3 semi-dwarf plants produced internodes similar to wild-type plants during early developmental stages (up to the eight internode, approximately), and short internodes at later stages (Figures 7a and 8e). This ''delayed dwarfism'' was a general feature in the transgenic lines, with the semi-dwarf phenotype obtained in this work observed in five independent lines (including L-45.3). Because transgene expression could be detected at early developmental stages by Northern analysis (data not shown), delayed dwarfism did not appear to be a result of lack of *NoGA2ox3* expression.

Small dark-green leaves and a reduction in epidermal cell size were associated with ectopic *NoGA2ox3* expression (Figure 7d–e), similar to the effect observed in stems of tobacco transgenic plants with reduced GA levels caused by overexpression of a dominant-negative form of a bZip transcriptional activator, which regulates GA_1 levels (Fukazawa and others 2000). However, the reduction of cell size observed in dwarf L-41 plants was not sufficient to explain the strong reduction of leaf size in those plants, implying that cell divisions may also be reduced in the transgenic plants.

Ectopic expression of *NoGA2ox3* delayed flower induction in transgenic plants by increasing the number of vegetative nodes. This delay was proportional to dwarfism severity and was ovecome by GA_3 treatment. Semi-dwarf L-45.3 plants had 38%

more nodes than wild-type plants, whereas dwarf L-41 plants did not flower unless they were treated with GA_3. This phenotype agrees with results obtained by constitutive overexpression of other GA 2-oxidases in species like rice (*OsGA2ox1*; Sakamoto and others 2003) and *Arabidopsis* (*AtGA2ox7* and *AtGA2ox8*; Schomburg and others 2003). In *Arabidopsis*, severe reduction of endogenous GA levels delays flowering in long days and prevents flowering in short days (Wilson and others 1992). This feature may be of special interest for ornamental plants requiring small size but prolonged vegetative life.

The morphology of reproductive organs was also altered in transgenic plants (Figure 7f–g). This effect was proportional to the severity of the dwarf phenotype because flowers from semi-dwarf plants were just slightly smaller than flowers from wild-type plants, whereas flowers from dwarf plants were also paler, bent, with shorter peduncles and sterile pollen, and often did not attain full development. These effects are not surprising because it is known that GAs are required for corolla development (Weiss 2000), and GA deficiency may lead to male sterility (Goto and Pharis 1999).

There are no published reports of genetic transformation of oleander, and therefore at present it is only possible to use heterologous systems (such as reported here with tobacco) to assay the function of oleander genes. If transformation becomes feasible, future studies will be able to dissect the function of the GA metabolism genes studied here directly in oleander.

In this work we have characterized for the first time GA metabolic genes from oleander and have shown that aspects of the control of their expression are conserved with other species. We have also identified multiple transcripts associated with these genes that appear more prevalent in oleander than in other studied species. The enzyme encoded by *NoGA2ox3* was shown to reduce active GA content and induce dwarfism very efficiently in tobacco, therefore providing the tools for possible biotechnological modification of GA levels in oleander as an important target for Mediterranean horticulture.

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