Functional characterization of amyrin synthase involved in ursolic acid biosynthesis in Catharanthus roseus leaf epidermis

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Abstract: Catharanthus roseus accumulates high levels of the pentacyclic triterpene, ursolic acid, as a component of its wax exudate on the leaf surface. Bioinformatic analyses of transcripts derived from the leaf epidermis provide evidence for the specialized role of this tissue in the biosynthesis of this triterpene that is then secreted to the leaf surface where it accumulates with other components of the leaf exudate including the monoterpenoid indole alkaloid, catharanthine. Cloning and functional expression in yeast of a triterpene synthase derived from this tissue showed it to be predominantly an amyrin synthase (CrAS), since the amyrin to amyrin reaction products accumulated in a 5:1 ratio. Expression analysis of CrAS showed that triterpene biosynthesis occurs predominantly in the youngest leaf tissues and in the earliest stages of seedling development. Further studies using laser capture microdissection to harvest RNA from epidermis, mesophyll, idioblasts, laticifers and vasculature of leaves showed the leaf epidermis to be the preferred sites of CrAS expression and provides conclusive evidence for the involvement of this tissue in biosynthesis ursolic acid in Catharanthus roseus.

Cover Letter

Dear Norman:

We are happy to submit the following manuscript by Yu et al entitled 'The mevalonic and ursolic acid pathways are preferentially expressed in Catharanthus roseus leaf epidermis.' for possible publication in Phytochemistry. We hope that if acceptable the study be included in the special issue that will be issued in commemoration to Professor Meinhardt Zenk who passed away a few months ago.

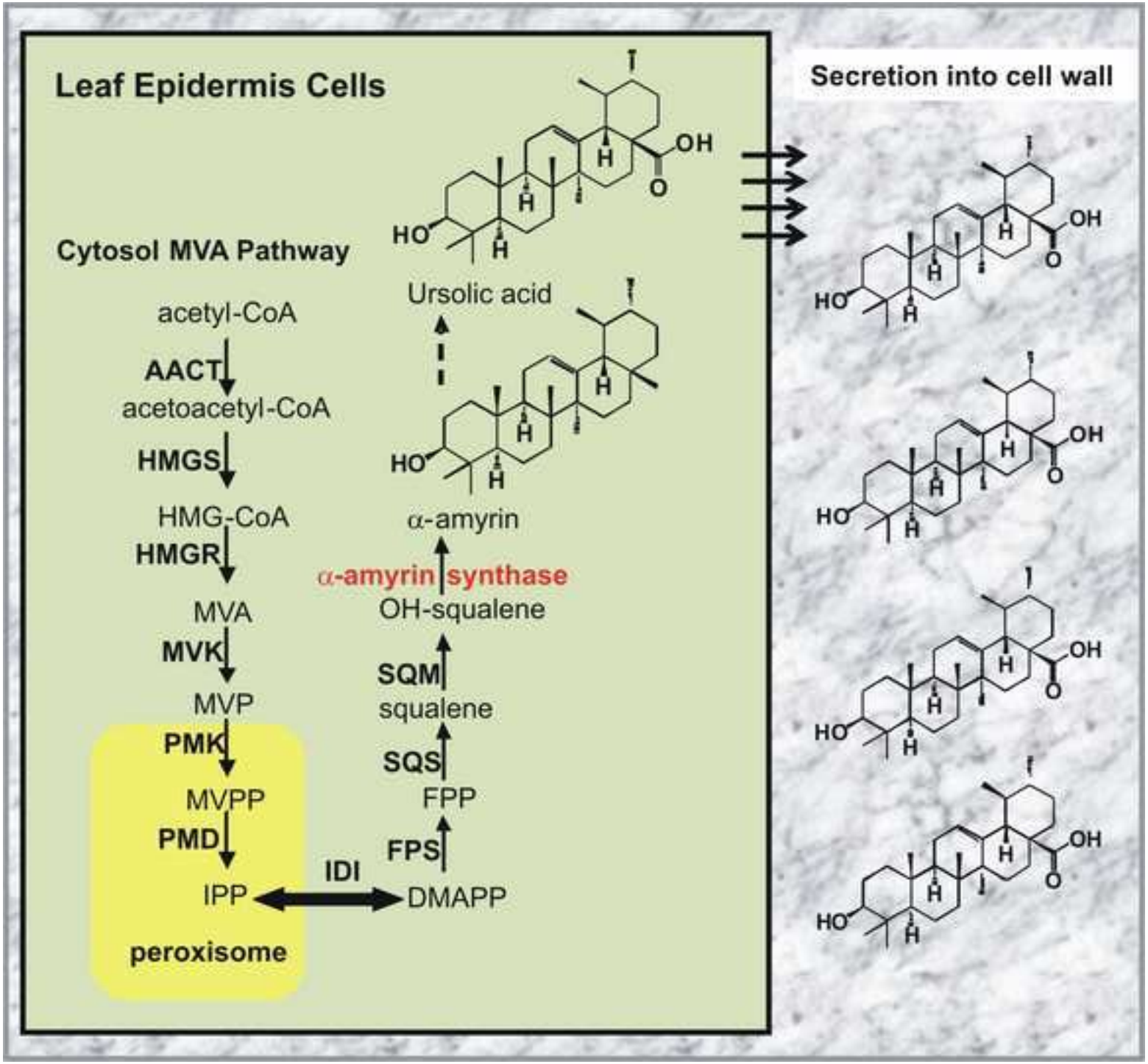
Thank you for handling this manuscript for us and we look forward to hearing from you once the reviews have been performed.

Sincerely,

Vincenzo De Luca

Brock University

*Graphical Abstract



Highlights:

- Molecular and biochemical characterization Catharanthus amyrin synthase (CrAS).
- Expression of CrAS in yeast produces α- and β-amyrin in a 5:1 ratio.
- The leaf epidermis is specialized by preferential expression of the mevalonic and triterpene biosynthesis pathways leading to ursolic acid accumulation on the leaf surface.
- CrAS gene expression is correlated with young leaves and with early stages of seedling development when the majority of ursolic acid accumulates

The mevalonic and ursolic acid pathways are preferentially expressed in Catharanthus roseus leaf epidermis.

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Abstract

Catharanthus roseus accumulates high levels of the pentacyclic triterpene, ursolic acid, as a component of its wax exudate on the leaf surface. Bioinformatic analyses of transcripts derived from the leaf epidermis provide evidence for the specialized role of this tissue in the biosynthesis of this triterpene that is then secreted to the leaf surface where it accumulates with other components of the leaf exudate including the monoterpenoid indole alkaloid, catharanthine. Cloning and functional expression in yeast of a triterpene synthase derived from this tissue showed it to be predominantly an α amyrin synthase (*CrAS*), since the α amyrin to β amyrin reaction products accumulated in a 5:1 ratio. Expression analysis of *CrAS* showed that triterpene biosynthesis occurs predominantly in the youngest leaf tissues and in the earliest stages of seedling development. Further studies using laser capture microdissection to harvest RNA from epidermis, mesophyll, idioblasts, laticifers and vasculature of leaves showed the leaf epidermis to be the preferred sites of *CrAS* expression and provides conclusive evidence for the involvement of this tissue in biosynthesis ursolic acid in *Catharanthus roseus*.

Introduction:

The Madagascar periwinkle (*Catharanthus roseus*) is well known as the only commercial source of the anticancer dimeric monoterpenoid indole alkaloids (MIAs), vinblastine and vincristine, that have been used as chemotherapy agents since the 1970's. While this plant accumulates low levels of these dimers, they do accumulate more significant amounts of their precursor monomers, catharanthine and vindoline. Recent studies have provided extensive evidence that the assembly of MIAs in Catharanthus takes place in separate cell types. The Internal Phloem Associated Parenchyma (IPAP) cells appear to assemble part of the monoterpene skeleton (10 hydroxygeraniol) (Beurlat et al., 2004) and an undetermined intermediate appears to be transported by an unknown mechanism to the leaf epidermis where at least the last 2 steps in this pathway are expressed to assemble secologanin (Irmler et al. 2000; Murata et al., 2008). The leaf epidermis localized tryptophan decarboxylase also converts L-tryptophan into tryptamine that is combined with secologanin through the enzyme, strictosidine synthase to form strictosidine, the central intermediate of MIA biosynthesis (St Pierre et al., 1999), that can be converted to several hundred MIAs found in Catharanthus (van Der Heijden et al., 2004) and to several thousand MIAs characterized in many other plant species (Szabo, 2008). Random sequencing of a cDNA library derived from Catharanthus leaf epidermis enriched RNA provided strong evidence that most of MIA pathway genes were preferentially expressed in this tissue (Murata et al., 2008). Additional studies have now established that while biosynthesis of catharanthine and vindoline occurs in young developing leaves, catharanthine accumulates in leaf wax exudates, while vindoline is found within the leaf explaining in part by the localization of the terminal reactions in

vindoline biosynthesis to leaf mesophyll idioblasts and laticifers (Roepke et al., 2010). The spatial separations of vindoline from catharanthine explain why Catharanthus accumulates such low levels of dimeric anticancer drugs.

The thousands of biologically active triterpenes found in plants are derived from (*3S*)-oxidosqualene to generate over 80 different carbon skeletons (Ebizuka et al., 2003) via the activity of different oxidosqualene cyclase (OSC) enzymes that carry out the carbocation rearrangements responsible for this biological diversity. For example, *Catharanthus roseus* accumulates 2.5 % of their leaf dry weights as the α-amyrin-derived ursane-type triterpene, ursolic acid (Usia et al., 2005). Sequencing of the leaf epidermis-enriched transcripts also identified of the mevalonic acid pathway (Figure 1) as well as a new OSC gene with high amino acid sequence identity to amyrin synthases described in other plant species and metabolite analysis showed that ursolic acid was also secreted exclusively to the cuticular wax layer (Murata et al., 2008). Interstingly 2 steps (PMK and MVD) in the MVA pathway were localized to the peroxisomes (Figure 1) of Catharanthus (Simkin et al., 2011), confirming previous suggestions for compartmentation of this pathway in Arabidopsis (Sapir-Mir et al., 2008) and mammalian cells (Kovacs et al., 2007). The present study describes the functional expression of the *Catharanthus roseus* amyrin synthase (*CrAS*) in *Saccharomyces cerevisiae* and documents the highly regulated leaf epidermis specific expression of this gene during plant growth and development.

Experimental Procedures:

Cultivation of *Catharanthus roseus*

The *Catharanthus roseus* (L.) G. Don, Little Delicata plants used in this study were cultivated in the greenhouse (Photoperiod 16h: 8h/ light: dark at 30 °C). For seedling developmental studies *Catharanthus* seeds were immersed in 70% ethanol for 1 min, and then thoroughly rinsed with sterile distilled water for two times. After sterilization seeds were incubated in water in darkness for 24 h. After rinsing again with sterile distilled water, 20 to 30 seeds per Petri dish were plated on wet filter papers, where they germinated and grew in the greenhouse (Photoperiod 16h: 8h/ light: dark at 30 °C) for up to 12 days and aliquots of 20 seedlings were harvested everyday for gene expression analysis.

Isolation and cloning of *Catharanthus roseus* amyrin synthase

Young leaves from the 1st leaf pair (1g) were harvested, immediately placed in liquid nitrogen, and ground thoroughly with a mortar and pestle. Total RNA was extracted by using TRIzol reagent (Invitrogen) and used for cDNA synthesis by AMV Reverse Transcriptase (Promega) following standard protocols (Murata et al, 2005) to generate cDNAs used directly as PCR templates. A degenerate sense primer (5'-ATGTGGA**R**GCT**R**AAG**R**T**W**GC-3', **R=** G or A, **W**= A or T) was designed according to the highly conserved 5'-end amino acid regions of known Amyrin synthases. The anti-sense primer (5'-CTACAAGAAAACTTCTTGGATTTTTAC-3') was designed according to known partial sequence from leaf epidermal-enriched cDNA library (Murata et al., 2008). PCR was carried out with *pfu* DNA polymerase (Fermentas) and the following cycling condition: 5 min at 95 °C (30 S at 95 °C, 30 S at 52 °C, 3 min at

72 °C)×35 cycles. After gel purification and A-tailing by Taq DNA polymerase (Fermentas) of PCR product, the DNA of desired length was cloned into the pGEM-T easy vector (Promega) and transformed to XL-10 Gold Ultra-competent cells. The full-length sequence of *Catharanthus* amyrin synthase (*CrAs*) was obtained after sequencing of the plasmid DNA.

Functional Expression of CrAS and analysis of Amyrins in *Saccharomyces cerevisiae*

The full-length coding sequence of the *CrAS* was PCR amplified using sense primer (5'-TAT**GAGCT**CATGTGGAAGCTAAAGATAGC-3') which contains a Sac1 restriction site and anti-sense primer (5'-ATA**GGATCC**CTACAAAGCTTTGGTTGGCC-3') which contains BamH1 restriction site. The PCR product was digested with Sac1/BamH1 and cloned into the Sac1/BamH1 sites of the yeast expression vector pSCW231 under control of *ADH1* promoter and *CYCI* terminator to create pSCW231-*CrAS*.

The yeast strain MKP-0 (*MATa canl-100 ade2-1 lys2-1 ura3-52 leu2-3, 112 his3-D200 trpl-D901*) was transformed with pSCW231, pDM067 which contains *S. vaccaria β-Amyrin synthase* (Meesapyodsuk et al., 2007) and pSCW231-*CrAS* by the lithium acetate method, and transformants were selected on SD medium minus tryptophan (SD/-Trp). The recombinant yeast cells were grown on 50 ml liquid SD/-Trp medium at 28 °C until stationary phase. MKP-0 yeast containing pSCW231 and pDM067 were used as controls. For analysis of yeast products, the cells were harvested by centrifugation at 5000 rpm for 10 min, and then saponified with 2 ml 20% KOH/methanol at 80 °C for 1 h. After extraction with 2 ml hexane and 2 ml water, the hexane phase was dried and the residue was dissolved in 100 ul of BSA (Aldrich)/pyridine (1:1). An internal standard of 10 μg of cholesterol in 100ul CH_2Cl_2 was added to each sample and were dissolved in 20 μl

BSTFA/TMCS/pyridine (45:5:50) (Sigma) for analysis. GC-MS analysis was carried out as in Meesapyodsuk et al., 2007, except that the temperature program was from 225 to 325 at 5 degrees per minute. A standard mixture of cholesterol, α-amyrin and β-amyrin (1:1:1) (Sigma) was used for response factor calibration.

Extraction and analysis of triterpenes from Catharanthus leaves

Fresh individual leaf pairs representing different ages and developmental stages of growth were harvested and placed in 5 ml chloroform for one hour in order to harvest leaf surface triterpenes and chloroform extracts were dried by vacuum centrifugation in a SPD SpeedVac (Fisher Scientific) dryer. The chloroform stripped leaves were air-dried in a fume hood for 60 min, pulverized with a mortar and pestle in the presence of liquid nitrogen and the powder was extracted with 5 ml methanol using a homogenizer. The extracts were centrifuged and the collected supernatant that was dried by vacuum centrifugation. The dried chloroform and methanol extracts were dissolved in methanol (200µl), filtered through a (0.22 mm) PALL filter (VWR Canada) and analysed by Ultra Performance Liquid Chromatography-Mass Spectroscopy (UPLC-MS) using an Acquity^{TM} C18 column with particle size of 1.7 mm and column dimensions of 1.0×50 mm. The solvent systems to resolve triterpenes used A (H_2O) and B (Acetonitrile) to form a linear gradient using the following conditions: 0-0.6 min 99 % A, 1 % B at 0.3 mL/min, 0.6-5 min 99 % A and 1 % B at 0.4 mL/min, 5-8 min gradient to 1 % A and 99 % B at 0.4 mL/min, 8.00 min to 8.50 min gradient to 99 % A and 1% B at 0.4 mL/min and 8.50 min to 10 min 99 % A and 1 % B at 0.3 mL/min (Murata et al, 2008).

Sequence alignment and Phylogenetic Analysis

The protein sequence of CrAS (GenBank JQ027033) was aligned with (47) full-length functionally characterized amyrin synthases from other plant species -using ClustalW 2 (http://align.genome.jp/). The *CrAS* nucleotide sequence was submitted to DNA distance neighbour-joining phylogenetic analysis using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and visualized with Phylodraw (http://pearl.cs.pusan.ac.kr/phylodraw/).

Results and Discussion

Phylogenetic analysis associates *Catharanthus roseus* amyrin synthase (CrAS) with known α/β amyrin synthases.

A single candidate amyrin synthase gene encoding an incomplete open reading frame appeared to be represented 19 times (Figure 1) within the Catharanthus leaf epidermis enriched library (Murata et al., 2008). The full length clone (*CrAS*) obtained as described in Materials and Methods was submitted to phylogenetic analysis with 47 putative (some functionally characterized) amyrin synthases (Figure S1). The 762 amino acid open reading frame of CrAS showed 86 % amino acid sequence identity (Figure S1, S2) to a 763 amino acid mixed function oxidosqualene oxidase (OEA) from olive (*Olea europaea*) which produced a mixture of α and β amyrin (Figure 2) as well as small amounts of ψ-taraxasterol and butyrospermol when expressed in yeast (Samara et al., 2007). In contrast CrAS showed only 60 % amino acid sequence identity (Figure S1, S2) to a 760 amino acid *Saponaria vaccaria* β amyrin synthase (SvBS) that produces β amyrin exclusively when expressed in yeast (Figure 2) (Meesapyodsuk et al., 2007). The results provided clues that CrAS might in fact be the α amyrin synthase (Figure 2) involved in production of the direct precursor of ursolic acid that accumulates on the surface of Catharanthus leaves ((Murata et al., 2008). CrAS also contains the amino acid motif DCTAE, thought to form part of the active site that binds oxidosqualene (Abe et al., 1993), a number of QW motifs shown to be shared by the OSC superfamily (Poralla et al., 1994 and the MWCYCR motif that specifies the formation of *β*-amyrin in *Panax ginseng* (Kushiro et al., 2000).

CrAS possesses α amyrin synthase activity when expressed in yeast.

Functional expression of CrAS produced a yeast strain that accumulated α amyrin as a major product (Figure 3a) as well as small amounts of β amyrin when compared to the control strain that does not accumulate either triterpene (Figure 3b) or to yeast expressing SvBS that only accumulates small amounts of these two products (Figure 3c). When three biological replicates were analyzed (Fig 3 inset), the CrAS line accumulated up to 5 times more α amyrin (102.6±18.9 μg) than the β (21.93±4.2 μg) form and together this line accumulated up to 14 times more α/β amyrin than the β amyrin (8.56±1.45 μg) accumulating in SvBS line. This preferred production of α amyrin by CrAS was also observed when apple *MdOSC1* (Brendolise et al., 2011) was expressed in the yeast, *Pichia methanolica*. Remarkably the apple gene aligns more closely to the multifunctional triterpene synthase *KcMS*, and to the lupeol synthase *BgLUS,* while it is not closely related to *CrAS* compared to olive *OEA*, showing only 54 % amino acid sequence identity over 758 amino acids (Supplementary Figure 1). Amino acid sequence comparisons conducted with apple MdOSC1 were used to suggest that substitution of F for W in the ^{256}MFCYCR^{261} motif might be partly responsible for the increased α amyrin product specificity observed (Brendolise et al., 2011). However the presence of W in the ^{256}MWCYCR^{261} motif of CrAS suggests that creation of α amyrin product specificity involves changes to other amino acids on the protein backbone. The similar biochemical properties of CrAS and MdOSC1 also show that creation of α amyrin product specificity could have occurred more than once after divergence of this multifunctional triterpene synthase family.

***CrAS* is preferentially expressed in leaf epidermis, in younger leaves and during the earlier stages of seedling development in *Catharanthus roseus*.**

RNA obtained from the epidermis, mesophyl, idioblast, laticifer and vasculature of leaves by laser capture microdissection was converted to cDNA (Murata and De Luca, 2005; Murata et al., 2006) and each tissue type was probed for expression of *CrAS*. While whole leaves and leaf epidermis were the preferred sites of *CrAS* expression (Figure 4a), the mesophyll, idioblast, laticifer and vasculature cells were not. In contrast actin was expressed to similar levels in all cell types studied. These results corroborate earlier studies suggesting that the leaf epidermis is enriched in the mevalonic acid and ursolic acid pathways (Murata et al., 2008).

Leaves of different ages were extracted for total RNA (Murata et al., 2005) and the expression profile of *CrAS* with age was determined by real time RT-PCR. The results showed that the youngest leaves appear to be most active in ursolic acid biosynthesis and this activity declined with leaf age (Figure 4b). The expression profile of *CrAs* also corresponded closely with the accumulation profile of ursolic acid on the leaf surface where maximum levels were achieved in leaf pair 3 and beyond with levels varying between 0.7 to 1 mg/leaf pair (Figure 4c). When ursolic acid levels per cm^2 of leaf surface in leaves of different ages were compared, the highest levels were found in the youngest leaves and they were diminished rapidly with leaf age and leaf expansion, reflecting the importance of very young tissues in the biosynthesis and secretion of this triterpene in *Catharanthus roseus* (Figure 4d). Together these data suggest that ursolic acid biosynthesis and accumulation follows a very similar developmental program to that of MIA biosynthesis (Murata et al., 2008).

The preferential expression of *CrAs* in young Catharanthus leaves prompted investigations to determine when this gene would be expressed during seedling germination and development (Figure 5). Analyses of whole seedlings showed that triterpene biosynthesis is activated at very early stages of germination with relative expression of *CrAs* at day 1 being already 7 times higher than the day 12 time point and this expression increased until day 5 after which transcript levels decreased continually (Figure 5). The early expression of triterpene biosynthesis is in contrast to MIA biosynthesis that appear to peak over a period of 72 to 96 hr from day 4 to day 7 of seedling development (De Luca, 2011). These results provide novel insights concerning the possible timing differences between triterpene and MIA biosynthesis that might be further investigated using developing seedlings as a model system.

Conclusions:

Catharanthus roseus accumulates up to 2.5 % of its dry weight as the ursane type triterpene, ursolic acid (Usia et al., 2007) as a component of the wax exudates on the leaf surface (Murata et al., 2008). Remarkably, catharanthine, a major MIA of Catharanthus also accumulates almost exclusively in this wax exudate (Roepke et al., 2010). The present study provides chemical, biochemical and molecular evidence that biosynthesis of ursolic takes place in the leaf epidermis by confirming the predominantly α amyrin synthase activity of recombinant CrAs and by demonstrating the preferred expression of this gene in younger leaves and seedlings and in the leaf epidermis rather than in other tissue types isolated by laser capture microdissection (Murata and De Luca, 2005).

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Legends to Figures:

Figure 1. The leaf epidermis is enriched in the mevalonic acid and ursolic acid pathways, but not in the 2-*C*-methyl-D-erythritol 4-phosphate /1-deoxy- D- xylulose 5-phosphate pathway (MEP/DOXP pathway). Bioinformatic analysis of a leaf epidermis enriched RNA sequence database (Murata et al, 2008) showed the frequency with which each step in the mevalonic and ursolic acid biosynthetic pathways were expressed and this is compared with the frequency with which these genes were expressed in young whole Catharanthus leaves using a database containing 653,830 sequences obtained by 454 Pyrosequencing (Roche Diagnostics). The following abbreviations are used for different pathway enzymes: **AACT** (acetoacetyl-CoA synthetase). **HMGS** (HMG-CoA synthase); **HMGR** (HMG-Coa reductase); **MVK** (Mevalonate kinase), **PMK** (phosphomevalonate kinase), **PMD** (mevalonate 5-pyrophosphate decarboxylase); **IDI** (isopenenyll-pyrophosphate isomerase) **FPS** (farnesyl-pyrophosphate synthase); **SQS** (squalene synthase); **SQM** (squalene monooxygenase); **AS** (α amyrin synthase); **DXS** (1-deoxy-D-xylulose 5-phosphate synthase); **DXR** (1-deoxy-d-xylulose 5-phosphate reductoisomerase); **CMS** (4-diphosphocytidyl-2-C-methyl-D-erythritol synthase); CMK (4-diphosphocytidyl-2-C-methyl-D-erythritol kinase); **MCS** (2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase); **HDS** (4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase) **IDS** (HMB-PP reductase)

Figure 2. Biosynthesis of ursolic and oleanolic acids from oxidosqualene involves α/β amyrin synthases that create the ursane and/or oleanane skeletons.

Figure 3. Analysis by GC-MS of the relative accumulation of α- and β-amyrin produced in *S. cerevisiae* strains expressing recombinant CrAS and SvBS. a) MS profile of CrAS expressing yeast extracts. b) Control MS profile of vector without any amyrin synthase expressing yeast extracts. c) MS profile of SvBS expressing yeast extracts. d) MS profile of α- and β-amyrin standards together with the internal cholesterol standard. Figure 3 (insert). Relative ratio of α- and β-amyrin accumulating in CrAS (white bars) and SvBS expressing yeast strains (black bars) when α-amyrin is 1. The error bars represent standard errors from three biological replicates.

Figure 4. Preferential expression of *CrAS* in Catharanthus leaf epidermis, in younger leaf tissues and in younger stages of seedling development is correlated with the timing ursolic acid accumulation of the leaf surface. a) Laser capture microdissection was used to harvest whole leaves (W), leaf epidermis (E), leaf mesophyll (M), leaf idioblast (I), laticifer (L) and leaf vascular (V) cells for RNA isolation and for performing RT-PCR using probes for *CrAs* and *CrActin*. b) Quantitative RT PCR analysis of expression levels of CrAS was performed with extracts from leaves of different developmental ages (leaf pairs 1, 2, 3, 4 and 5). c) Accumulation of ursolic acid (μg/leaf pair) in leaves of different ages (leaf pairs 1, 2, 3, 4, 5, 6, 7, 8 and 9). d) Accumulation of ursolic acid (μg/cm^2 of leaf surface) in leaves of different ages (leaf pairs 1, 2, 3, 4, 5, 6, 7, 8 and 9). The error bars in b, c and d represent standard errors from three biological replicates.

Figure 5. Expression of *CrAS* during seed germination and seedling development. Quantitative RT PCR analysis of expression levels of CrAS was performed with extracts

of seedlings from day 1 to 12 of development. The error bars represent standard errors from three biological replicates.

Figure 1

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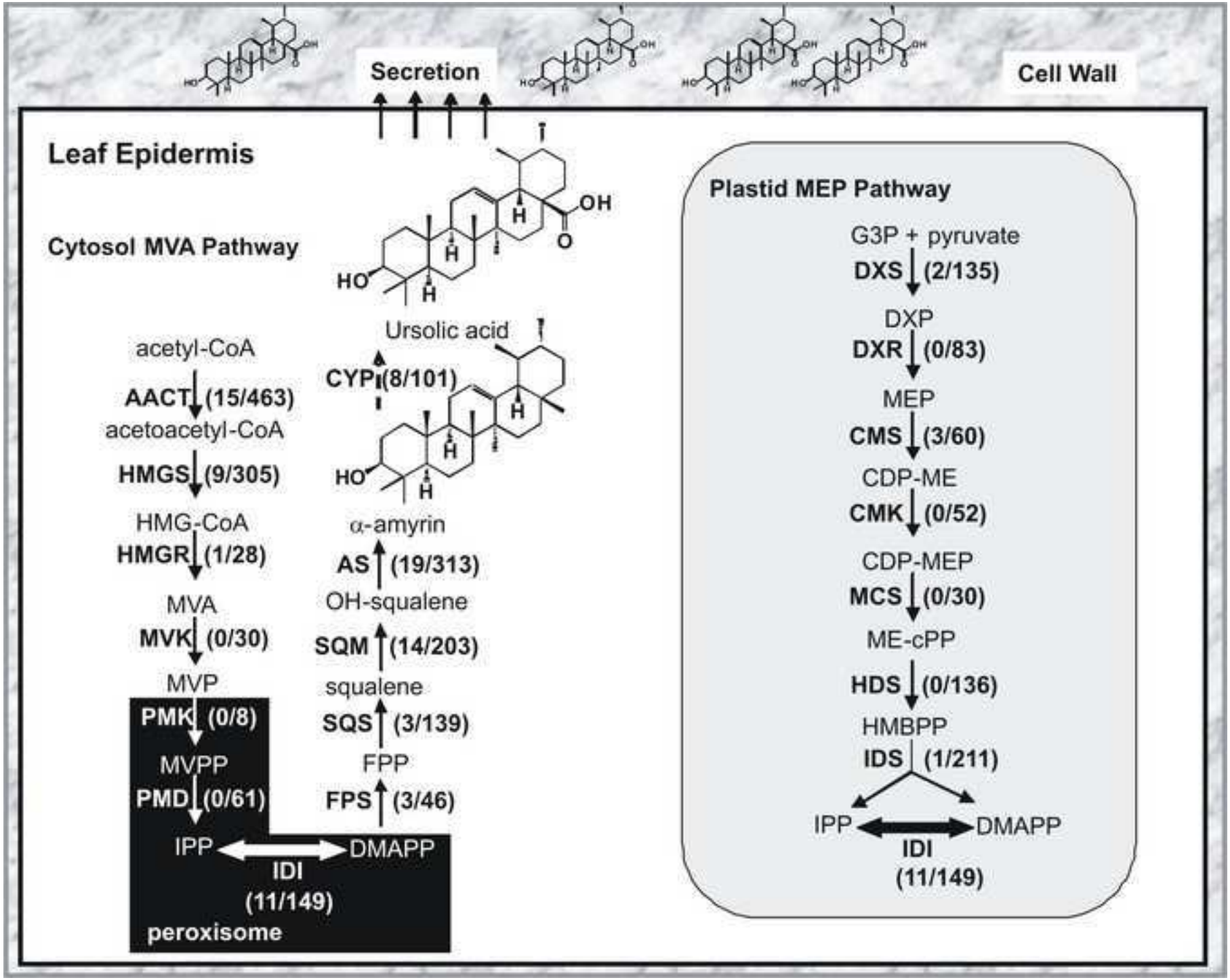

Figure 2

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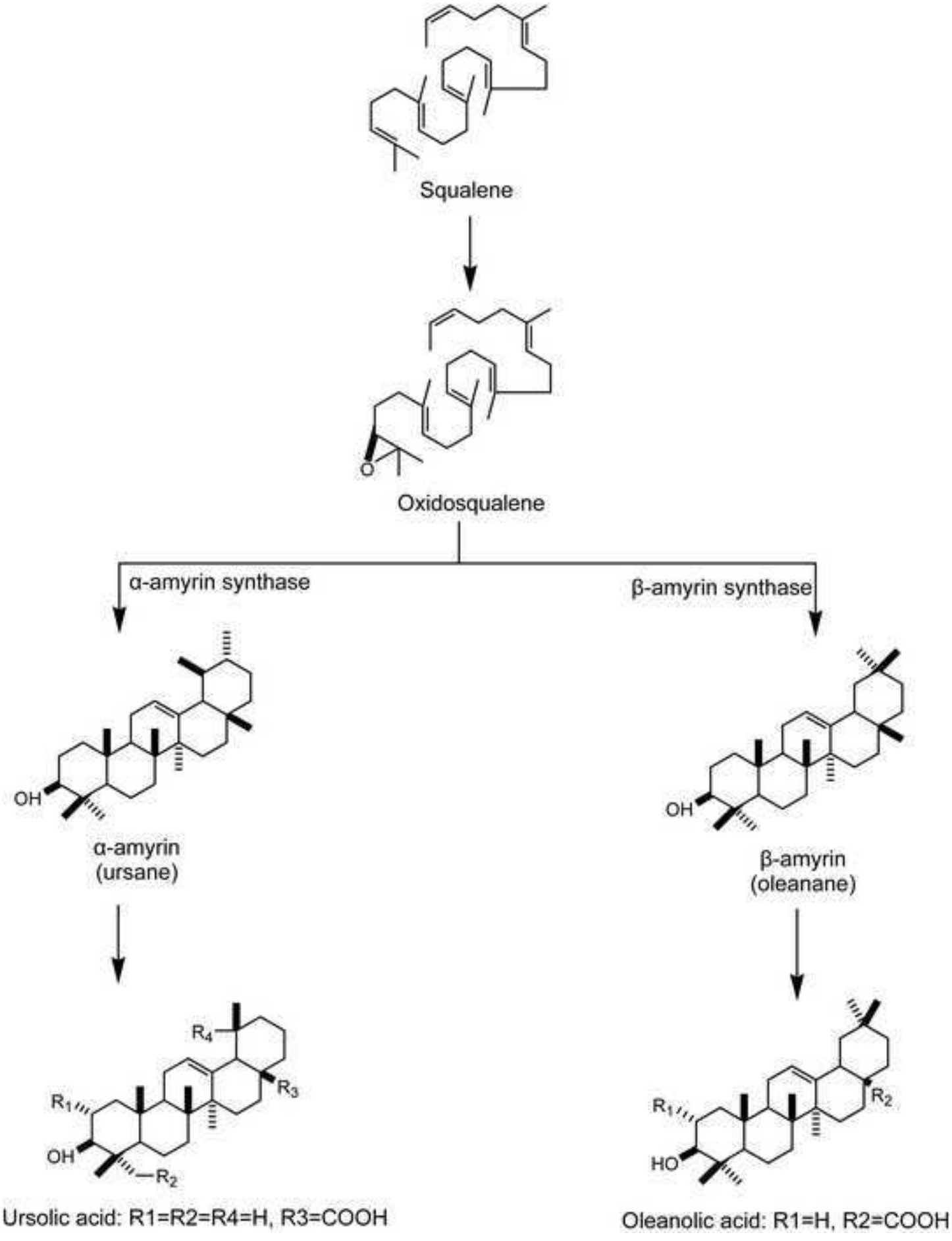

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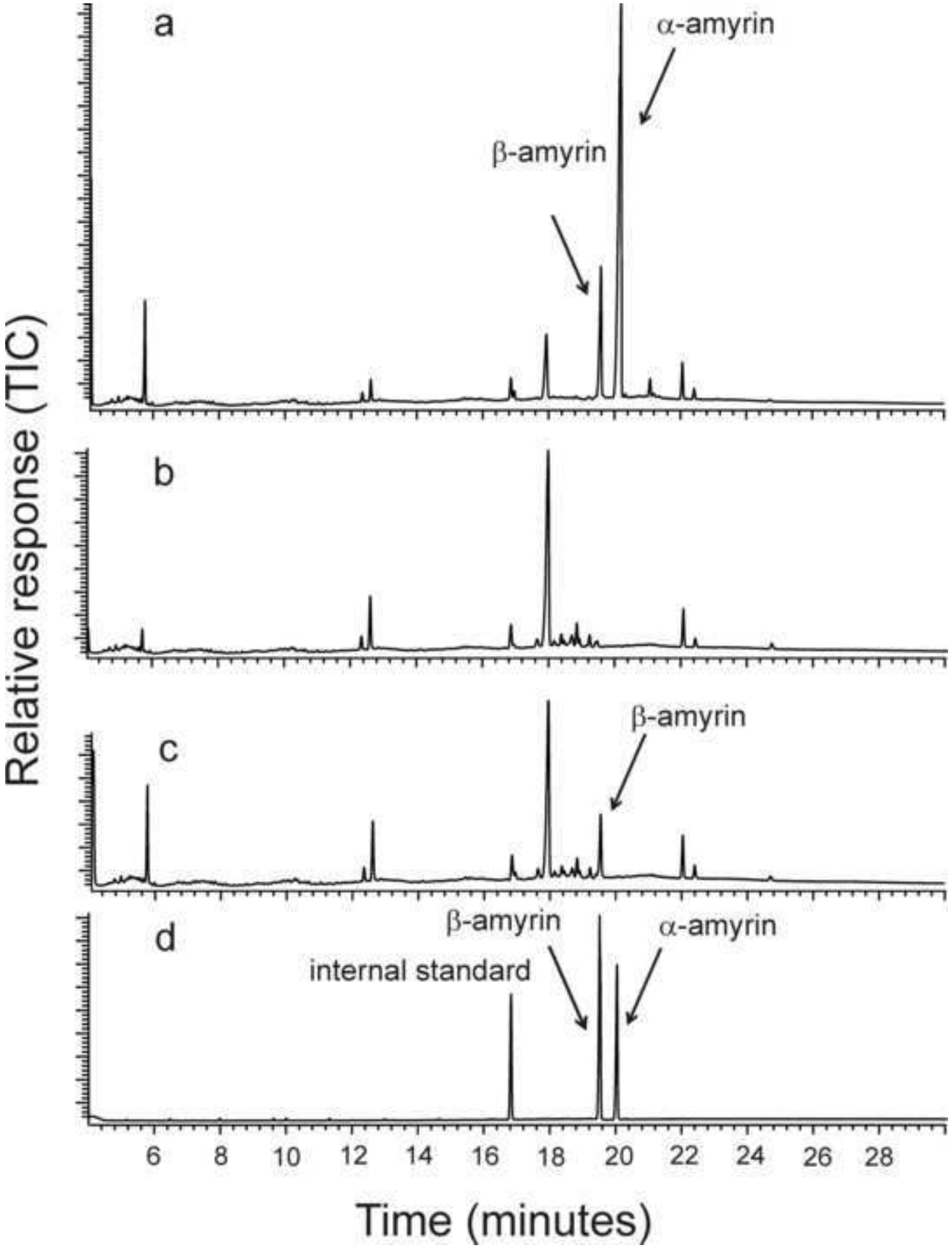

Figure 3 inset
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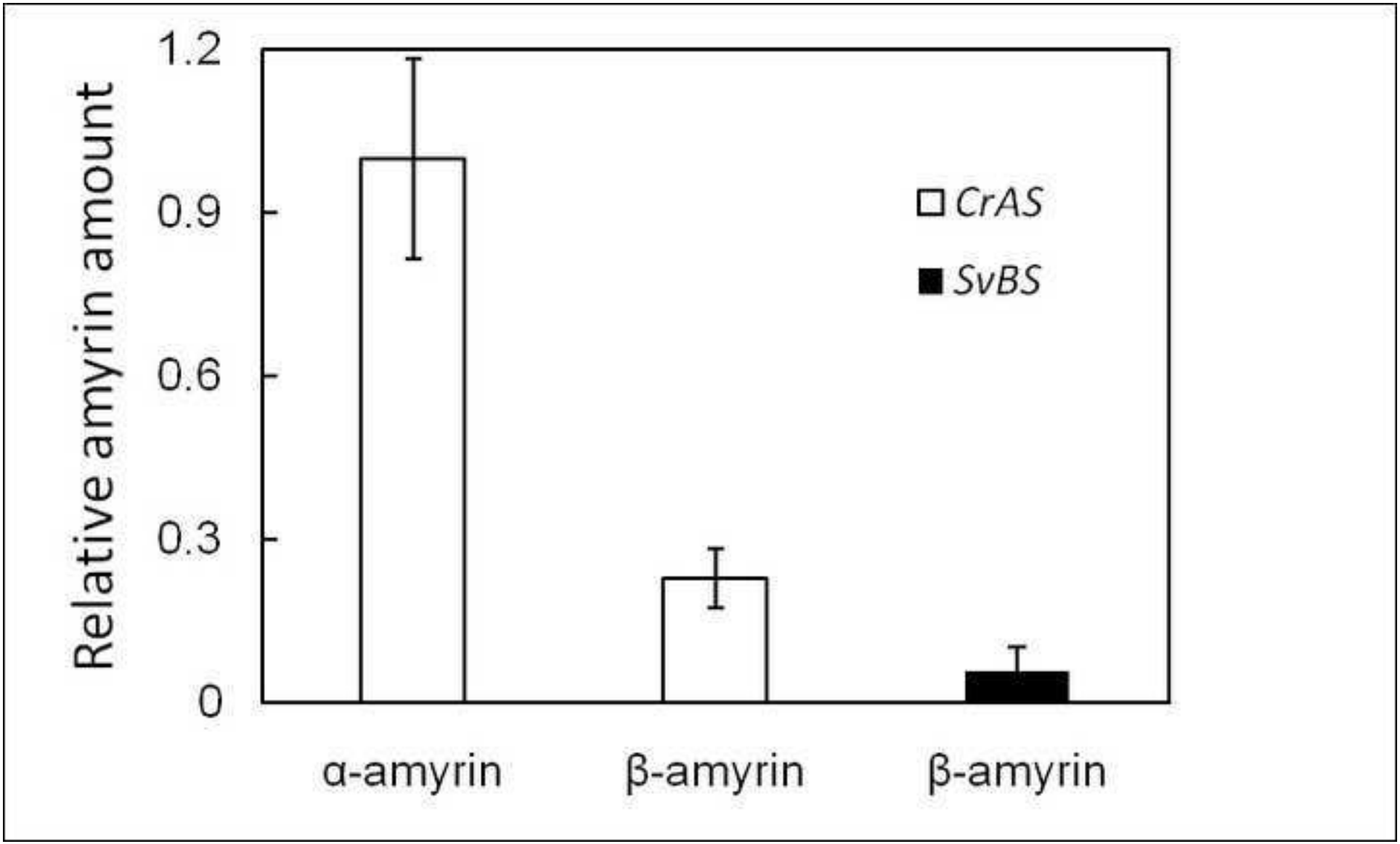

Figure 4a

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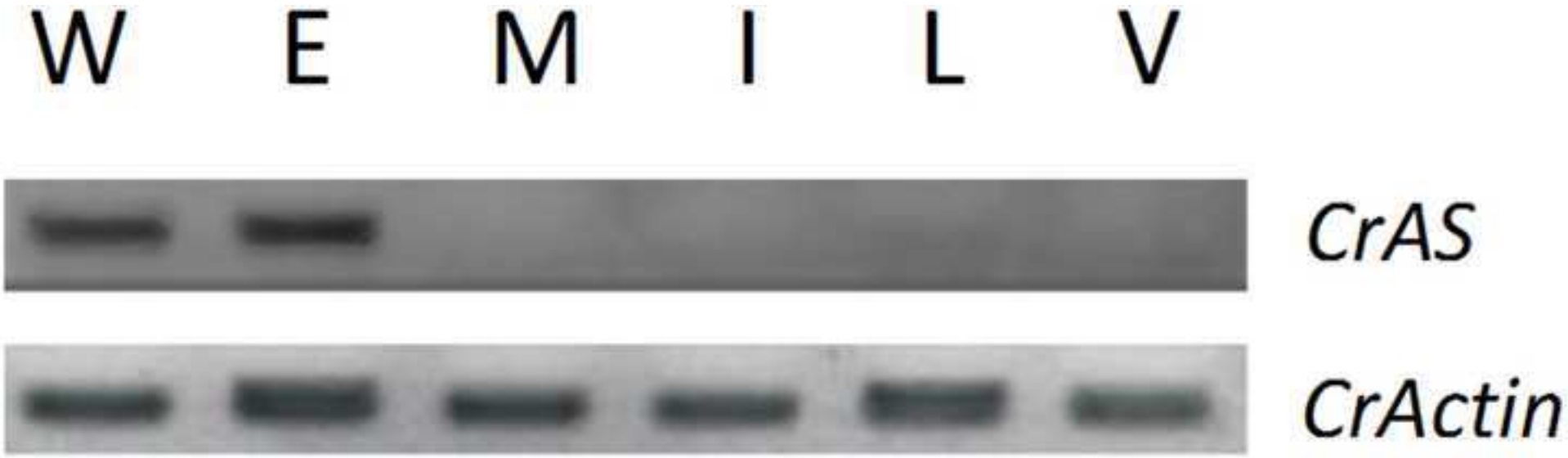

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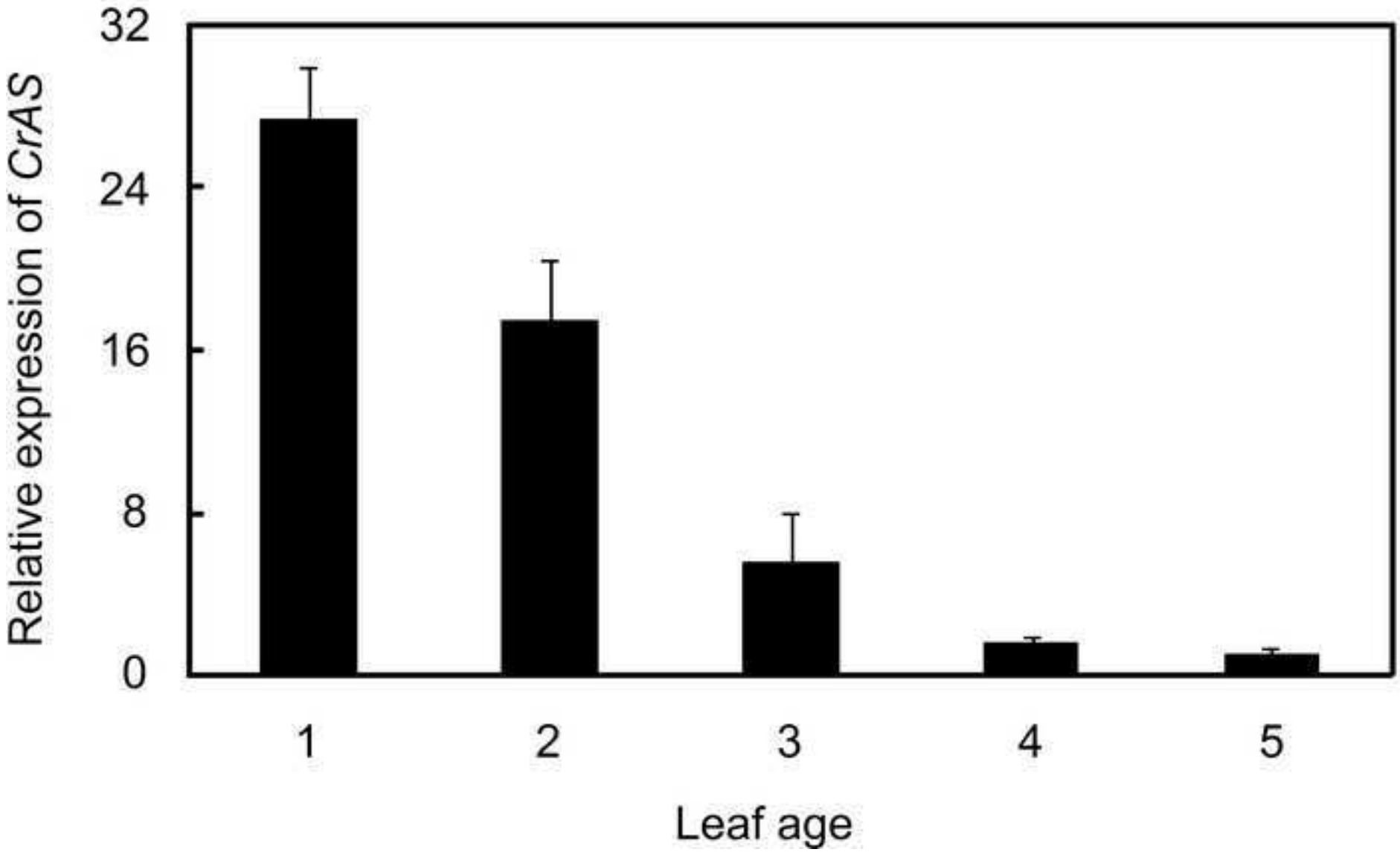

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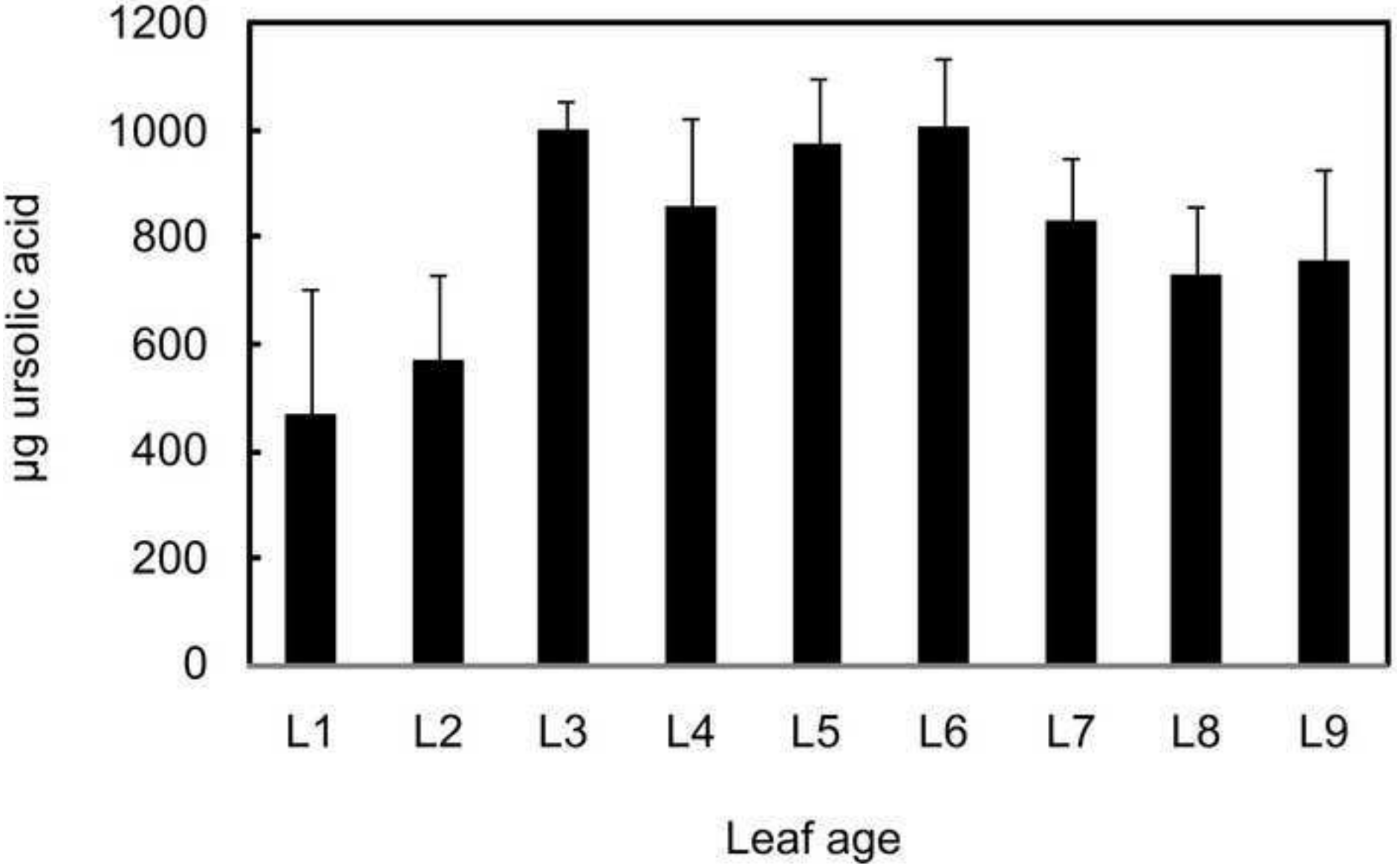

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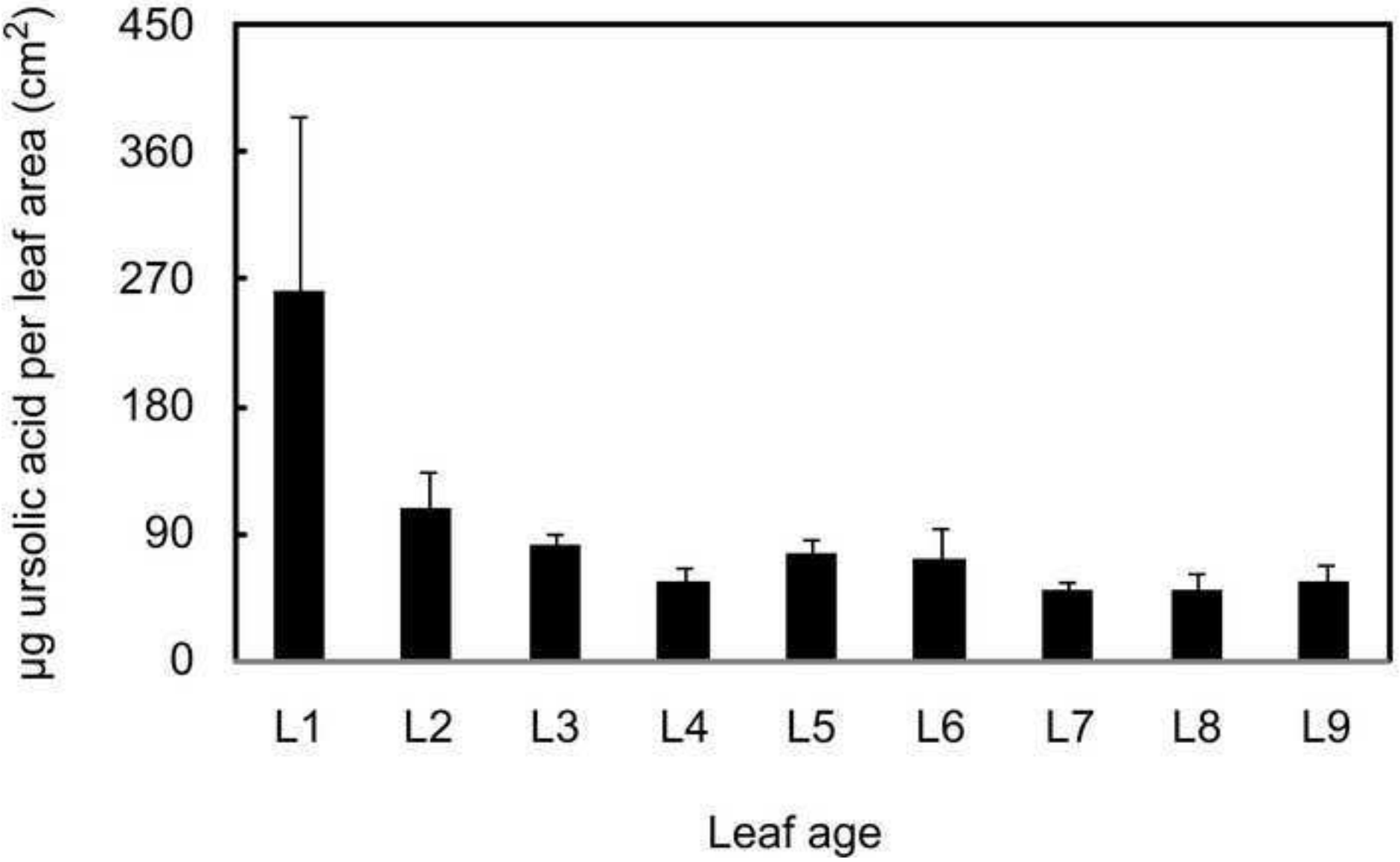

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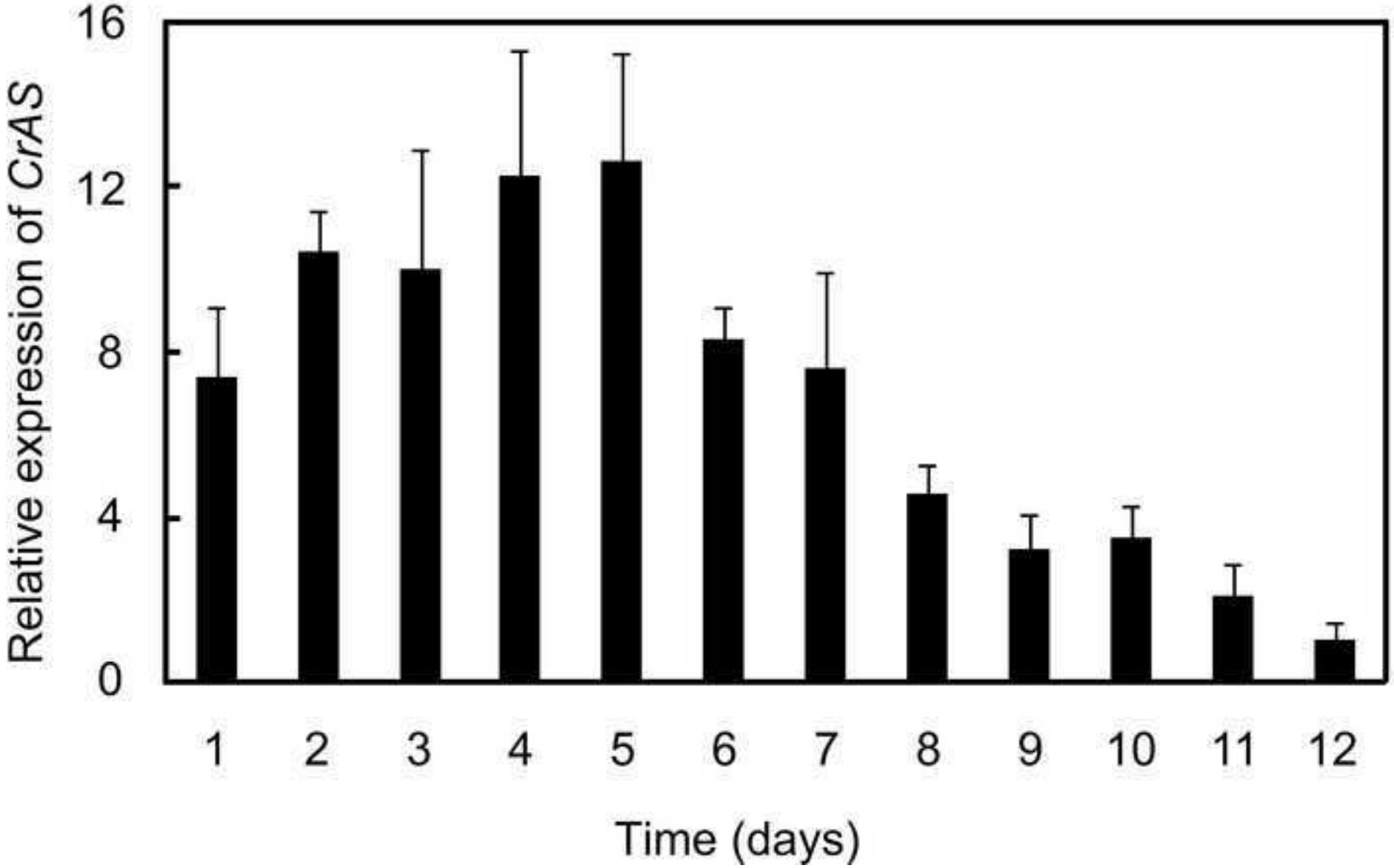

Supplementary Figure 1

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Supplementary Figure 2

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Supplementary Figure legend

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