University of Nebraska - Lincoln

DigitalCommons@University of Nebraska - Lincoln

Biochemistry -- Faculty Publications

Biochemistry, Department of

2-2004

Tissue-specific expression and developmental regulation of cytochrome b561 genes in *Arabidopsis thaliana* and *Raphanus sativus*

Wim Verelst

University of Antwerp, verelst@mpiz-koeln.mpg.de

Jyoti Kapila

University of Antwerp

Janice De Almeida Engler

University of Gent

Julie M. Stone

University of Nebraska-Lincoln, jstone2@unl.edu

Roland Caubergs

University of Antwerp

See next page for additional authors

Follow this and additional works at: <https://digitalcommons.unl.edu/biochemfacpub>

Part of the [Biochemistry, Biophysics, and Structural Biology Commons](#)

Verelst, Wim; Kapila, Jyoti; Engler, Janice De Almeida; Stone, Julie M.; Caubergs, Roland; and Asard, Han, "Tissue-specific expression and developmental regulation of cytochrome b561 genes in *Arabidopsis thaliana* and *Raphanus sativus*" (2004). *Biochemistry -- Faculty Publications*. 90.

<https://digitalcommons.unl.edu/biochemfacpub/90>

This Article is brought to you for free and open access by the Biochemistry, Department of at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Biochemistry -- Faculty Publications by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Authors

Wim Verelst, Jyoti Kapila, Janice De Almeida Engler, Julie M. Stone, Roland Caubergs, and Han Asard

Tissue-specific expression and developmental regulation of cytochrome *b561* genes in *Arabidopsis thaliana* and *Raphanus sativus*

Wim Verelst,¹ Jyoti Kapila,¹ Janice De Almeida Engler,² Julie M. Stone,³
Roland Caubergs,¹ and Han Asard³

1. Laboratory of Plant Physiology, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium

2. Laboratory of Plant Genetics, University of Gent-VIB, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

3. Department of Biochemistry, Plant Science Initiative, Beadle Center, University of Nebraska – Lincoln, Lincoln, NE 68588-0664, USA

Corresponding author – Wim Verelst. Present address: Max Planck Institute for Plant Breeding Research, Department of Molecular Plant Genetics, Carl-von-Linné-Weg 10, D-50829 Cologne, Germany; e-mail verelst@mpiz-koeln.mpg.de

Abstract

Ascorbate (Asc) is an essential molecule in many aspects of development and stress responses in plants and animals. Cytochromes *b561* (cyts *b561*) are tightly coupled to Asc homeostasis. These proteins are found in mammalian tissues, where they are involved in the regeneration of Asc, serving the synthesis of catecholamine neurotransmitters, and in intestinal iron reduction. Plant genomes encode homologous membrane-associated, Asc-reducible cyts *b561*. The expression of these proteins in plants, however, has so far not been studied. We have now examined the expression of two *Arabidopsis thaliana* cyt *b561*-encoding genes – *Artb561-1* and *Artb561-2* – using relative-quantitative RT-PCR and in situ hybridization (ISH) techniques. The genes show overlapping and distinct tissue- and organ-specific expression patterns. Transcripts of both genes are found in leaf epidermal cells, and expression seems to correlate with leaf maturation and cessation of cell elongation. Both genes are also expressed in the epidermal cell layer of stems and roots in the L1 layer of the shoot apex, in the vascular system of leaves, stems and roots, and in the root pericycle. In addition, *Artb561-1* is expressed in the root cap, whereas *Artb561-2* mRNA is found in the epidermis of lateral roots, in the root meristem, and in unfertilized ovules. These observations provide important information for the elucidation of the physiological function of cyts *b561* in plants.

Abbreviations: Asc – ascorbate; cyt(s) *b561* – cytochrome(s) *b561*; DHA – dehydro ascorbate; ISH – in situ hybridization; MDHA – monodehydro ascorbate

Introduction

Ascorbate (Asc) plays a prominent role in a large number of cellular processes, as a major component in maintaining redox homeostasis. Apart from its function as an antioxidant, it acts as a cofactor for several metabolic enzymes (Arrigoni and De Tullio 2000). As a consequence of these processes, the pool of reduced Asc molecules becomes depleted. The hydrophilic nature of Asc prevents it from diffusing freely through biological membranes. Therefore, subcellular compartments that cannot synthesize Asc require mechanisms for replenishing reduced Asc molecules. This can be achieved by either *trans*-membrane transport of the molecule itself (Horemans et al. 2000a, 2000b), by *trans*-membrane transport of electrons, using oxidized Asc (dehydro ascorbate, DHA) or monodehydro ascorbate

(MDHA) as electron acceptors (Asard et al. 1992, Horemans et al. 1994), or by a combination of both mechanisms. One protein known to replenish reduced Asc in a mammalian subcellular compartment is the chromaffin granule cytochrome *b561* (cyt *b561*). This protein is responsible for the transfer of electrons into specialized chromaffin granule vesicles, where catecholamine neurotransmitters are synthesized by an Asc-dependent enzyme (Wakefield et al. 1986, Njus et al. 1987).

A family of membrane proteins with sequence homology to the chromaffin granule cyt *b561*, likely to play an important role in plant cellular Asc metabolism, has only recently been identified (Asard et al. 2001). The conservation of structural features between the putative plant and animal cyt *b561* protein sequences is striking (Asard et al. 2001). The overall amino acid identity between human

chromaffin granule cyt *b*561 and the proteins encoded by the *A. thaliana* *Artb561-1* and *Artb561-2* genes is 36% and 35%, respectively. Conserved features include six predicted transmembrane helices, four His residues likely to be involved in heme ligation, and a predicted binding site for MDHA (Asard et al. 2001). A putative binding site for Asc (Okuyama et al. 1998) is not as well conserved among plant cyts *b*561 (Verelst and Asard 2003). At least one member of this family of plant proteins is plasma membrane localized and has been extensively characterized at the biochemical level (Asard et al. 1998, 2000, 2001).

The bovine adrenal chromaffin cyt *b*561 has been purified (Perin et al. 1988, Tsubaki et al. 1997), and recent attempts to purify the corresponding protein from plants have been met with some success (Trost et al. 2000, Asard et al. 2001, Bérczi et al. 2001). All available information on plant cyt *b*561 has been obtained through biochemical studies and sequence comparisons with the animal system. Although most biochemical work has been performed on purified plasma membranes of bean hypocotyls (*Phaseolus vulgaris* L.), the presence of cyt *b*561 has been reported in a variety of organs from a large number of vascular plants (Asard et al. 1998, 2001). However, the physiological function of the protein in plants remains unknown. The mammalian chromaffin cyt *b*561 shuttles electrons across the chromaffin granule membrane, from cytosolic Asc to intravesicular MDHA, thereby replenishing the pool of reduced Asc molecules (Wakefield et al. 1986, Njus et al. 1987, 2001). In vitro studies on bean hypocotyl plasma membrane vesicles have shown that the plant cyt *b*561 is also capable of transmembrane electron transport from Asc to MDHA and ferricyanide (Asard et al. 1992, Horemans et al. 1994). These experiments indicate that plant plasma membrane cyt *b*561 could shuttle electrons between intracellular Asc and an extracellular acceptor, similar to the mode of action of the chromaffin cyt *b*561 (Asard et al. 1998). Therefore, the plasma membrane cyt *b*561 in plants may be capable of regenerating Asc in the apoplast using electrons from cytosolic Asc. It may thus be involved in the physiological actions of Asc in the extracellular space (Asard et al. 2001).

Cyt *b*561 research has advanced by the recent identification of additional cyt *b*561 homologues in animals. A ferric reductase in the plasma membrane of duodenal brush border cells was found to be highly homologous to the chromaffin granule cyt *b*561 (McKie et al. 2001). The presence of multiple cyts *b*561 supports the hypothesis that cyts *b*561 may occur in different subcellular locations in different tissues, carrying out specialized functions.

Based on similarities to the animal cyt *b*561 protein sequences, a number of putative cyt *b*561-encoding genes have been identified in dicots and monocots (Asard et al. 2001, Verelst and Asard 2003). As in mammals, more than one cyt *b*561 homologue is present in most plant species where cyt *b*561 genes have been identified. The occurrence of different isoforms of the cyt *b*561 protein in plants also points to potentially different functions for these proteins in various tissues. Moreover, there is evidence for plant

cyts *b*561 to reside in multiple subcellular compartments, as indicated by sucrose gradient centrifugation analyses of cauliflower, bean and mung bean membranes (Asard et al. 1987, Scagliarini et al. 1998).

Using relative-quantitative RT-PCR, we studied the expression patterns of two putative *Arabidopsis thaliana* cyt *b*561 encoding genes (*Artb561-1* and *Artb561-2*, TAIR accession numbers At4g25570 and At5g38630, respectively) at the organ level in *A. thaliana*. In addition, in situ hybridization analyses (ISH) were used to examine the expression patterns of both genes in more detail, at the tissue level in *A. thaliana* and *Raphanus sativus* (radish) sections. While all previous studies on cyt *b*561 in plants yielded information on biochemical properties at the membrane level, these experiments are the first to provide information at the whole plant level. These results may contribute to our understanding of the physiological functions of cyt *b*561 in plants, by promoting the formulation of working hypotheses that can be the basis for future studies.

Materials and methods

Plant material and growth conditions

A. thaliana L. cv. Columbia Col-0 and *R. sativus* L. seeds (bought from a local market) were surface sterilized and sown on K1 germination medium (Valvekens et al. 1988). Petri dishes were placed under growth chamber conditions (60% humidity, 22°C, 80 $\mu\text{E m}^{-2} \text{s}^{-1}$) with a light regime of 16 h of light and 8 h of darkness. *A. thaliana* seedlings were collected 7 days after germination, and radish roots and shoot apices 12 days after germination. *A. thaliana* plants were also grown in soil, under the same growth conditions as sterile-grown plants, and after 4–6 weeks flowers and siliques were collected from these plants for ISH.

A. thaliana roots used for RNA isolation were grown in liquid cultures following the procedure of the Meyerowitz Laboratory (<http://www.caltech.edu/meyerowitz>), and collected 20 days after germination.

Probe preparation

Using EST clones 170N15T7 (accession number R65413) and ATTS1779 (accession number Z26702) as templates, the ESTs of *Artb561-1* and *Artb561-2*, respectively, were amplified by PCR, using SP6 and T7 primers. The PCR products of, respectively, 927 and 678 bp were purified by gel extraction and used as templates for in vitro transcription, using an SP6/T7 Transcription Kit (Roche Diagnostics, Mannheim, Germany) and [α - ^{35}S]-UTP (Amersham, Roosendaal, the Netherlands). Sense and antisense radiolabeled riboprobes of both genes were synthesized and hydrolyzed to an average length of 300 nucleotides as described by de Almeida Engler et al. (2001), and column-purified using 'Bio-Spin 30 Chromatography Columns' (Bio-Rad, Hercules, CA, USA). The quality and length of the transcripts

was checked on a denaturing agarose gel (de Almeida Engler et al. 2001).

In situ hybridization (ISH)

ISH was essentially performed as described by de Almeida Engler et al. (2001). Plant material was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0, and subsequently embedded in paraffin as described by de Almeida Engler et al. (2001). *A. thaliana* and *R. sativus* tissue sections were hybridized with sense or antisense ^{35}S -UTP labeled RNA probes of the *Artb561-1* and *Artb561-2* genes. For each slide 15×10^6 cpm of [α - ^{35}S]-UTP-labeled RNA-probe were used for hybridization. Exposure times varied. Images were taken with a digital Axiocam (Zeiss) under standard dark and bright field optics.

Relative-quantitative RT-PCR

Total RNA was isolated from 100 to 300 mg frozen *A. thaliana* tissues (seedlings, leaves, flowers, roots) using Tri-Pure Isolation Reagent (Roche Diagnostics, Mannheim, Germany), following the manufacturer's instructions. The concentration of total RNA was quantified spectrophotometrically at 260 nm. About 3 μg of this RNA was used for cDNA synthesis with a 'First Strand cDNA Synthesis Kit' (Fermentas, Vilnius, Lithuania). RT-PCR conditions were optimized for both *Artb561-1* and *Artb561-2* genes, using 150 ng of cDNA and gene-specific primers overlapping intron regions. The manufacturer's instructions were followed, and care was taken that the PCR amplification reactions were stopped in the linear range, to allow reliable quantification of the amplicons. For quantification, Universal 18S rRNA primers and competimers (QuantumRNA 18S Internal Standards; Ambion, Austin, TX, USA) were used in a ratio of one to nine, as determined in trial experiments (according to the kit's manual). RT-PCR bands were visualized in agarose gels using ethidium bromide. After scanning, band intensities were quantified using OptiQuant Image Analysis Software (Packard, Meriden, CT, USA).

Results

Relative-quantitative RT-PCR analyses

Relative-quantitative RT-PCR was used to examine the expression of *Artb561-1* and *Artb561-2* mRNA in different *A. thaliana* organs. We tested the expression of both genes in young seedlings (11 days after germination (DAG)), in roots (from liquid cultures, 20 DAG), young and mature (fully expanded) leaves (both from plants at 25, 30, 47 and 65 DAG), flower buds, unfertilized flowers and fertilized flowers (from plants 65 DAG). At least three independent samples were tested for each organ type. Levels of 18S rRNA were used as an internal standard. The amplification

of this abundant transcript was suppressed using Ambion's competimer technology (Ambion), so that the amount of 18S amplicon in RT-PCR was of the same order of magnitude as that of the *Artb561-1* and *Artb561-2* amplicons. Expression levels were expressed relative to that of 18S rRNA. To compare the mRNA levels of the *cytb561* genes in different organs, the average amount of *cytb561* mRNA in seedlings was arbitrarily set to 1, and the levels in other organs were expressed relative to this value (Figure 1). It was not possible to make an absolute quantitative comparison between the two genes studied in these experiments, since the RT-PCR method is only relative-quantitative, and different amounts of PCR cycles were used for optimal amplification of both transcripts.

For both genes the mRNA levels in young leaves were comparable to those in seedlings (1.09 ± 0.48 and 0.82 ± 0.30 , respectively). In mature leaves the levels were slightly higher than in seedlings, but statistically not significantly different (1.54 ± 0.27 and 1.32 ± 0.39 , respectively). Plant age (DAG) did not affect the results for leaves, because similar values were obtained for leaves of comparable developmental stages collected from plants 25, 30, 47 and 65 DAG (data not shown).

In flower buds and unfertilized flowers, the *Artb561-1* transcript levels (0.28 ± 0.06 and 0.27 ± 0.05) were significantly lower than in seedlings, whereas the levels of *Artb561-2* mRNA in these samples were comparable to those in seedlings (1.19 ± 0.21 in flower buds and 1.32 ± 0.16 in unfertilized flowers). In fertilized flowers that were starting to form seeds, levels of both genes were very low: the *Artb561-1* and *Artb561-2* transcript levels were 0.08 ± 0.02 and 0.43 ± 0.13 , respectively. For both genes the reduction in expression in fertilized flowers was about 70%, when compared to flower buds or unfertilized flowers. The expression in flowers thus suggested developmental regulation of both genes. There was also a pronounced organ-specificity, especially apparent in roots as compared to other organs. In roots, the expression levels of both genes were significantly higher than in seedlings (3.50 ± 0.36 and 5.78 ± 0.76). However, we can not fully exclude an effect of growth conditions (liquid culture versus soil-grown plants) on the *Artb561-1* and -2 expression levels in roots. Our data suggest a differential expression of these two genes.

In situ hybridization

ISH was used to investigate the expression patterns of the two putative cyt b561 encoding genes in tissue sections of *A. thaliana* and its closely related species *R. sativus* (radish). The *A. thaliana* and *R. sativus* genomes are very similar (Martinez et al. 1992), and radish tissues have the advantage of being considerably larger than *A. thaliana*, which allows more refined analysis of the expression patterns. Control hybridization with sense probes gave no significant background signal, and therefore only results obtained with antisense probes are presented here.

Figure 1. Relative mRNA expression levels of two *A. thaliana* genes encoding cyts *b561*, *Artb561-1* (dashed bars) and *Artb561-2* (dotted bars) in *A. thaliana* organs, as determined by relative-quantitative RT-PCR analysis. Amounts of *cytb561* mRNA are expressed relative to 18S rRNA levels (ratio *cytb561*/18S rRNA). The expression level in seedlings is chosen as reference, and levels from all other organs are expressed relative to the former. Values represent average expression levels relative to seedlings, $\pm$ se ($n = 3-10$). The age of the plants used for RNA extraction was 11 DAG for seedlings, 25, 30, 47 and 65 DAG for both young and mature (fully expanded) leaves, 65 DAG for flower buds and flowers, and 20 DAG for roots. For leaves, averages of samples from plants of all different ages are shown. The level of significance for samples in which the mRNA level is significantly different from that in seedlings, as found in a *t*-test, is marked as (*) for $\alpha = 0.01$ and (**) for $\alpha = 0.001$.

In the vegetative aerial parts of radish and *A. thaliana* seedlings, both genes *Artb561-1* and -2 had identical expression patterns. In the shoot apical meristem of radish, *Artb561-1* and -2 were expressed in the L1 layer of the shoot apex, in the epidermis of leaf primordia and young leaves, and in vascular bundles (Figures 2A & 2B, respectively). Interestingly, a gradient of expression was observed in the epidermis of maturing leaves, with high levels at the base and diminishing towards the leaf tip. This points to developmental control of *Artb561-1* and -2 expression in that tissue. A detailed transverse section through a maturing radish leaf confirmed high-level *Artb561-1* expression in vascular bundles, with a weaker expression in the epidermis (Figure 2C). In stems, both genes were expressed in the vascular cylinder and also weakly in the epidermis (Figure 2D). The observations made in radish sections were confirmed in *A. thaliana* tissues. Figures 2E & 2F show the expression of *Artb561-1* and -2, respectively, in *A. thaliana* seedlings. The epidermal patterns were visible, although sections from radish gave us better results. The expression in the L1 layer of the shoot apical meristem was apparent for *Artb561-2* in *A. thaliana* (Figure 2F), but not for *Artb561-1*, as the shoot apex was not present in this section. However, comparison to radish strongly suggested that *Artb561-1* was also expressed in the L1 layer of the *A. thaliana* shoot apex.

In radish roots, the expression patterns of both genes varied depending on the developmental stage of the tissues, comparable to the situation in leaves. More mature tissues, such as the differentiation zone, showed a strong hybridization signal of the *Artb561-1* probe in the pericy-

cle (Figure 2G), and to a lesser extent in the vascular tissue and in the epidermis. In a younger region of the root, the elongation zone, transcripts corresponding to *Artb561-1* were similarly detected in the pericycle, vascular tissue and epidermis, and expression was also observed in the cortex (Figure 2H). Similar localizations were obtained for *Artb561-2* in the elongation and differentiation zones of roots (data not shown). Figure 2I shows a cross section through the differentiation zone of an *A. thaliana* root, in which *Artb561-1* was expressed in the pericycle and in the epidermis, but not in the cortex. Again, this was consistent with the observations made in radish.

Whereas all observations presented thus far showed identical expression patterns for both genes, there were also some striking differences, namely in lateral roots, the root meristematic region and flowers. Radish lateral roots showed weak or no expression of the *Artb561-1* gene (Figure 2J), whereas *Artb561-2* mRNA could be detected strongly in the epidermal cell layer (Figure 2K). These differences could also be observed in *A. thaliana* lateral roots (Figure 2L for *Artb561-2*). This not only confirmed that the probes used in these experiments were hybridizing with two different genes in radish, it also suggested that these genes were most likely the *Artb561-1* and -2 orthologues.

At the root tip, *Artb561-1* was strongly expressed in the lateral part of the root cap and in the epidermis, whereas no expression could be seen in the meristematic tissue (Figure 2M). In contrast, *Artb561-2* was strongly expressed in the cortical region of the root tip, which mainly consists of elongating cells, but not in the root cap (Figure 2N). Strong expression of *Artb561-2* in the root meristematic

Figure 2. In situ hybridization with *Arth561-1* and *Arth561-2* antisense probes in *R. sativus* (A, B, C, D, G, H, J, K, M, N, O) and *A. thaliana* (E, F, I, L, P, Q) tissue sections, detected by autoradiography. For most pictures both dark and bright field images are shown (the latter with prime). The hybridization signal is seen as white dots in dark field and as black dots in bright field optics. (A) *Arth561-1* expression in a radish shoot apex showing hybridization signal in the epidermis of leaf primordia (LP), young leaves (YL), maturing leaves (ML), and in the L1 layer of the shoot apical meristem (SAM); (B) *Arth561-2* expression in a radish shoot apex showing a similar but weaker signal in the epidermis as in (A); (C) Transverse section of a radish leaf, showing *Arth561-1* transcripts in the epidermal cell layer (Ep) and in the vascular bundles (VB), but not in the mesophyll (Me); (D) Transverse section through a radish stem, showing *Arth561-2* expression in the vascular cylinder; (E) and (F) Expression of *Arth561-1* and *Arth561-2*, respectively, in the leaf epidermis and L1 layer of *A. thaliana* seedlings; (G) Transverse section through the differentiation zone of a radish root hybridized with the *Arth561-1* antisense probe, showing signal in the pericycle cell layer (Pe); X = xylem, Ep = epidermis; (H) Transverse section through the elongation zone of a radish root, showing *Arth561-1* expression in pericycle, vascular cylinder, epidermis and cortex; a similar pattern was observed for *Arth561-2* (data not shown); (I) *Arth561-1* expression in the pericycle of an *A. thaliana* root, confirming the pattern seen in radish; (J) Longitudinal section of an emerging radish lateral root showing *Arth561-1* expression in the pericycle, but not in the lateral root; (K) Similar view for *Arth561-2*, showing expression in the epidermis of the emerging lateral root; (L) *A. thaliana* root with emerging lateral roots, expressing *Arth561-2* (LRM: lateral root meristem); (M) Transverse section through a radish root meristem hybridized with *Arth561-1*, showing expression in the lateral part of the root cap, and in the epidermis; (N) Transverse section through the root elongation zone, where *Arth561-2* is expressed in all expanding tissues, but primarily in the cortex; (O) Longitudinal section through a radish root meristem, showing expression of *Arth561-2* in the meristem; (P) Ovary of an *A. thaliana* flower, expressing *Arth561-2* in unfertilized ovules. Arrows point to examples of unfertilized ovules; (Q) Mature embryo of *A. thaliana* with *Arth561-2* expression in the root tip and mainly in the tips of the cotyledons. Bars in A and B = 200 μ m; in C, D, E, F, G, H, J, K, N, O, P and Q = 100 μ m; in I, L and M = 50 μ m.

tissue could be easily observed in a longitudinal section (Figure 2O).

In *A. thaliana* flowers, no clear hybridization signal could be detected for the *Arthb561-1* gene (data not shown). In contrast, *Arthb561-2* was highly expressed in unfertilized ovules, whereas the signal in other parts of flowers was insignificant (Figure 2P). In heart-shaped embryos, a homogenous weak expression was observed for both genes (data not shown), whereas in mature *A. thaliana* embryos, both *Arthb561-1* and *Arthb561-2* were expressed primarily in the epidermis, cotyledon tips, and root tip (Figure 2Q for *Arthb561-2*).

Discussion

Despite the importance of Asc in various aspects of plant development and stress responses, many of the key players regulating Asc homeostasis are unknown (Pastori and Foyer 2002). Components known to support Asc recycling in animals and plants include the chromaffin granule cyt *b561*, thioredoxins, protein disulphide isomerase, and various Asc and DHA transporters (Njus et al. 1987, Wells et al. 1990, May et al. 1997, Rumsey and Levine 1998, Horemans et al. 2000a, 2000b, Patel et al. 2001).

In this paper, we present expression analyses of two *A. thaliana* genes encoding cyt *b561* proteins (*Arthb561-1* and *Arthb561-2*), potentially involved in Asc recycling in plants. While the expression patterns of these two genes were similar in most organs, there were notable differences, suggesting that they support distinct physiological functions in plants. RT-PCR was used to assess expression at the organ level (Figure 1), after attempts to detect *Arthb561-1* transcripts by Northern blot using total RNA isolated from *A. thaliana* tissues had failed. Perhaps this was due to expression in a very restricted group of cells, which was supported by the *in situ* hybridization experiments. ISH demonstrated that these genes are expressed in the shoot apex, in leaves, stems, roots and inflorescences. However, expression was restricted to certain tissues: the epidermis, vascular tissue, pericycle, root cortex, root meristem and ovules.

In addition to this observed tissue-specific expression, the genes were developmentally regulated. Transcripts of both genes were detected in the epidermal cell layer of mature embryos (Figure 2Q). This expression pattern continued throughout seedling development, with highest levels of epidermal expression in leaf primordia, emerging leaves and root meristems. The L1 layer of the shoot apical meristem, which gives rise to the epidermis, strongly expressed *Arthb561-1* and *Arthb561-2* (Figures 2A & 2B). In young leaves, transcripts were observed throughout the entire epidermis, whereas in maturing leaves a gradient of expression was seen, with the expression diminishing towards the tip (Figure 2A). This is consistent with acropetal cell differentiation in leaves (Van Lijsebettens and Clarke 1998). Similar observations were made in the root epidermis, where expression decreased with increasing distance from the root tip (for example, expression was lower in the differentiation zone than in the elongation zone; Fig-

ures 2G & 2H, respectively). Therefore, in epidermal cells of both leaves and roots, expression levels of both genes decreased upon cell differentiation.

Developmental regulation of *Arthb561-1* and *Arthb561-2* was also observed in flowers. RT-PCR analysis showed a dramatic reduction in expression levels following fertilization (Figure 1). This was consistent with the ISH results showing that *Arthb561-2* had a particularly high expression in unfertilized ovules compared with fertilized ovules (Figure 2P). The hybridization signal in other floral parts was insignificant, suggesting that the observed decrease in *Arthb561-2* mRNA following fertilization may have been solely accounted for by a decrease in ovule-specific expression of this gene.

Although the overall expression patterns of these two genes were similar, there were also some important differences. At the root tip, *Arthb561-1* transcripts were mainly present in the epidermis and lateral root cap (Figure 2M), whereas *Arthb561-2* mRNA was detected throughout the root meristem (Figures 2N & 2O). Emerging lateral roots did not express *Arthb561-1* (Figure 2J), whereas they highly expressed *Arthb561-2*, primarily in the epidermis (Figure 2K). This differential expression indicates that the two genes analyzed may support different physiological functions, and/or have a different subcellular localization. This would resemble the situation in mammals, where two cyts *b561* are reported to have a different subcellular localization and possibly a different function: in chromaffin tissue, a cyt *b561* is present in the membrane of an intracellular granule (Wakefield et al. 1986), while in the duodenal brush border another cyt *b561* protein is situated in the plasma membrane (McKie et al. 2001).

It should be noted that we only report on the expression patterns of two cyt *b561* encoding genes in plants, but that two more homologous genes have been identified (*Arthb561-3* and *-4*, TAIR accession numbers At1g14730 and At1g26100, respectively). The expression patterns of these other cyts *b561* are not known. We are currently investigating the subcellular localization and expression patterns of all known *A. thaliana* cyts *b561*, to elucidate the roles of these proteins in plant physiology.

Acknowledgements – The authors kindly thank Prof. Gilbert Engler (RUG, Ghent, Belgium) for valuable advice, and Ruth De Groodt and Marleen Brunain (RUG, Ghent, Belgium) for technical assistance in the ISH procedures, Rebecca Verbanck (RUG, Ghent, Belgium) for art work, Prof. Yves Guisez, Dr. Nele Horemans, Dr. Marcel Jansen and Dr. Kris Visserberg (UA, Antwerp, Belgium) for interesting discussions and for critically reading the manuscript, and Jan Neefs (UA, Antwerp, Belgium) for taking care of soil-grown *Arabidopsis* plants. This research was supported by grants from the Fund for Scientific Research (FWO) and the University of Antwerp; W.V. is Research Assistant of the Fund for Scientific Research, Flanders (Belgium) (FWO). A contribution of the University of Nebraska Agricultural Research Division, Lincoln, NE 68583, USA. Journal Series no. 13822. The published version of this paper was edited for *Physiologia Plantarum* by C. H. Foyer.

References

- de Almeida Engler J, De Groodt R, Van Montagu M, Engler G (2001) *In situ* hybridization to mRNA of *Arabidopsis* tissue sections. *Meth Enzymol* 23: 325–334
- Arrigoni O, De Tullio MC (2000) The role of ascorbic acid in cell metabolism: between gene-directed functions and unpredictable chemical reactions. *Plant Physiol* 157: 481–488
- Asard H, Caubergs RJ, Renders D, DeGreef JA (1987) Duroquinonone-stimulated NADH oxidase and *b*-type cytochromes in the plasma membrane of cauliflower and mung beans. *Plant Sci* 53: 109–119
- Asard H, Horemans N, Caubergs RJ (1992) Transmembrane electron transport in ascorbate-loaded plasma membrane vesicles from higher plants involves a *b*-type cytochrome. *FEBS Lett* 306: 143–146
- Asard H, Horemans N, Preger V, Trost P (1998) Plasma membrane *b*-type cytochromes. In: Asard H, Bérczi A, Caubergs RJ (eds.) *Plasma Membrane Redox Systems and Their Role in Biological Stress and Disease*. Kluwer, Dordrecht, The Netherlands, pp 1–31
- Asard H, Kapila J, Verelst W, Bérczi A (2001) Higher-plant plasma membrane cytochrome *b*₅₆₁: A protein in search of a function. *Protoplasma* 217: 77–93
- Asard H, Terol-Alcayde J, Preger V, Del-Favero J, Verelst W, Sparla F, Pérez-Alonso M, Trost P (2000) *Arabidopsis thaliana* sequence analysis confirms the presence of cyt *b*-561 in plants: Evidence for a novel protein family. *Plant Physiol Biochem* 38: 905–912
- Bérczi A, Lüthje S, Asard H (2001) *b*-Type cytochromes in plasma membranes of *Phaseolus vulgaris* hypocotyls, *Arabidopsis thaliana* and *Zea mays* roots. *Protoplasma* 217: 50–55
- Horemans N, Asard H, Caubergs RJ (1994) The role of ascorbate free radical as an electron acceptor to cytochrome *b*-mediated trans-plasma membrane electron transport in higher plants. *Plant Physiol* 104: 1455–1458
- Horemans N, Foyer C, Asard H (2000a) Transport and action of ascorbate at the plant plasma membrane. *Trends Plant Sci* 5: 263–267
- Horemans N, Foyer C, Potters G, Asard H (2000b) Ascorbate function and associated transport systems. *Plant Physiol Biochem* 38: 531–540
- Martinez MC, Jørgensen J-E, Lawton MA, Lamb CJ, Doerner PW (1992) Spatial pattern of *cdc2* expression in relation to meristem activity and cell proliferation during plant development. *Proc Natl Acad Sci USA* 89: 7360–7364
- May J, Mendioretta S, Hill KE, Burk RF (1997) Reduction of dehydroascorbate to ascorbate by the selenoenzyme thioredoxin reductase. *J Biol Chem* 272: 22607–22610
- McKie AT, Barrow D, Latunde-Dada GO, Rolfs A, Sager G, Mudaly E, Mudaly M, Richardson C, Barlow D, Bomford A, Peters TJ, Raja KB, Shirali S, Hediger MA, Farzaneh F, Simpson RJ (2001) An iron-regulated ferric reductase associated with the absorption of dietary iron. *Science* 291: 1755–1759
- Njus D, Kelley PM, Harnadek GJ, Pacquing YV (1987) Mechanism of ascorbic acid regeneration mediated by cytochrome *b*₅₆₁. *Ann NY Acad Sci* 493: 108–119
- Njus D, Wigle M, Kelley PM, Kipp BH, Schlegel HB (2001) Mechanism of ascorbic acid oxidation by cytochrome *b*₅₆₁. *Biochemistry* 40: 11905–11911
- Okuyama E, Yamamoto R, Ichikawa Y, Tsubaki M (1998) Structural basis for the electron transfer across the chromaffin vesicle membranes catalyzed by cytochrome *b*₅₆₁: Analyses of DNA nucleotide sequences and visible absorption spectra. *Biochim Biophys Acta* 1383: 269–278
- Pastori GM, Foyer CH (2002) Common components, networks, and pathways of cross-tolerance to stress. The central role of 'Redox' and abscisic acid-mediated controls. *Plant Physiol* 129: 460–468
- Patel M, McIntosh L, Bliss T, Ho D, Sapolsky R (2001) Interactions among ascorbate, dehydroascorbate and glucose transport in cultured hippocampal neurons and glia. *Brain Res* 916: 127–135
- Perin MS, Fried VA, Slaughter CA, Südhof TC (1988) The structure of cytochrome *b*₅₆₁, a secretory vesicle-specific electron transport protein. *EMBO J* 7: 2697–2703
- Rumsey SC, Levine M (1998) Absorption, transport, and disposition of ascorbic acid in humans. *J Nutr Biochem* 9: 116–130
- Scagliarini S, Rotino L, Bäurle I, Pupillo P, Asard H, Trost P (1998) Purification of the ascorbate reducible *b*-type cytochrome of the plasma membrane of etiolated bean seedlings. *Protoplasma* 205: 66–73
- Trost P, Bérczi A, Sparla F, Sponza G, Marzadori B, Asard H, Pupillo P (2000) Purification of cytochrome *b*-561 from bean hypocotyl plasma membrane: evidence for the presence of two heme centres. *Biochim Biophys Acta* 1468: 1–5
- Tsubaki M, Nakayama M, Okuyama E, Ichikawa Y, Hori H (1997) Existence of two heme B centers in cytochrome *b*₅₆₁ from bovine adrenal chromaffin vesicles as revealed by a new purification procedure and EPR spectroscopy. *J Biol Chem* 272: 23206–23210
- Valvekens D, Van Montagu M, Van Lijsebettens M (1988) *Agrobacterium tumefaciens* mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc Natl Acad Sci USA* 85: 5536–5540
- Van Lijsebettens M, Clarke J (1998) Leaf development in *Arabidopsis*. *Plant Physiol Biochem* 36: 47–60
- Verelst W, Asard H (2003) A phylogenetic study of cytochrome *b*₅₆₁ proteins. *Genome Biol* 4: R38
- Wakefield LM, Cass AEG, Radda GK (1986) Functional coupling between enzymes of the chromaffin granule membrane. *J Biol Chem* 261: 9739–9745
- Wells WW, Xu DP, Yang YF, Rocque PA (1990) Mammalian thioltransferase (glutaredoxin) and protein disulfide isomerase have dehydroascorbate reductase activity. *J Biol Chem* 265: 15361–15364