A phylogenetic study of cytochrome b561 proteins
Wim Verelst* and Han Asard†

Addresses: *Laboratory of Plant Physiology, Department of Biology, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium. †Department of Biochemistry, University of Nebraska-Lincoln, Lincoln, NE 68588-0664, USA.

Correspondence: Wim Verelst. E-mail: wverelst@ruca.ua.ac.be.

© 2003 Verelst and Asard; licensee BioMed Central Ltd. This is an Open Access article: verbatim copying and redistribution of this article are permitted in all media for any purpose, provided this notice is preserved along with the article’s original URL.

Abstract

**Background:** As an antioxidant and cofactor to numerous metabolic enzymes, ascorbate has an essential role in plants and animals. Cytochromes b561 constitute a class of intrinsic membrane proteins involved in ascorbate regeneration. Despite their importance in ascorbate metabolism, no evolutionary analysis has been presented so far on this newly described protein family.

**Results:** Cytochromes b561 have been identified in a large number of phylogenetically distant species, but are absent in fungi and prokaryotes. Most species contain three or four cytochrome b561 paralogous proteins, and the encoding genes usually have four or five exons. At the protein level, sequence similarities are rather low between cytochromes b561 within a single species (34-45% identity), and among phylogenetically distant species (around 30% identity). However, particular structural features characterizing this protein family are well conserved in members from all species investigated. These features comprise six transmembrane helices, four strictly conserved histidine residues, probably coordinating the two heme molecules, and putative ascorbate and monodehydro-ascorbate (MDHA) substrate-binding sites. Analysis of plant cytochromes b561 shows a separation between those from monocotyledonous and dicotyledonous species in a phylogenetic tree.

**Conclusions:** All cytochromes b561 have probably evolved from a common ancestral protein before the separation of plants and animals. Their phyletic distribution mirrors the use of ascorbate as primary antioxidant, indicating their role in ascorbate homeostasis and antioxidative defense. In plants, the differentiation into four cytochrome b561 isoforms probably occurred before the separation between monocots and dicots.

Background

Ascorbate (vitamin C) is generally known for its detoxification of damaging reactive oxygen species during aerobic metabolism and under stress conditions [1]. Through the modulation of levels of reactive oxygen species, ascorbate is implicated in the control of cell expansion, cell division and programmed cell death [2]. As a cofactor to numerous iron- and copper-containing oxygenases, ascorbate is also involved in the biosynthesis of essential molecules, such as the plant hormones ethylene and gibberellic acid, cell-wall glycoproteins and antimicrobial agents [3,4]. The recent unraveling of the ascorbate biosynthetic pathway in plants [5,6] has
resulted in a renewed interest in this molecule, and in the recognition that much remains to be learned about its regulation and metabolism.

While our knowledge of ascorbate biosynthesis and catabolism is rapidly expanding, little is known about the mechanisms by which ascorbate is regenerated throughout the plant cell. Cytochromes b561 constitute a newly identified class of membrane proteins possibly implicated in replenishing ascorbate pools in plant cells, essential to maintaining the physiological functions of this important molecule [7].

Cytochromes b561 are intrinsic membrane proteins containing two heme molecules, and reducible by ascorbate [8,9]. They have been suggested to function as electron transporters, shuttling electrons across membranes from ascorbate to an acceptor molecule. The one-electron oxidation product of ascorbate, monodehydro-ascorbate (MDHA) has been shown, at least in vitro, to function as an electron acceptor for mammalian and plant cytochromes b561 [8,10,11]. The cytochrome b561-catalyzed reduction of MDHA results in the regeneration of the fully reduced ascorbate molecule.

The presence of cytochromes b561 in plants was first demonstrated on the basis of their biochemical properties. Ascorbate-reducible cytochromes with a wavelength maximum near 561 nm and a typically high redox potential (E° around +140 mV) were found in purified plasma membrane fractions from various species [12,13]. The availability of the primary sequence of the cytochrome b561 from bovine adrenal gland chromaffin cells [14,15] has recently resulted in the identification of homologous sequences in plants [7,16]. Putative cytochrome b561-encoding genes have now been identified in nearly all organisms for which considerable genomic sequence information is available, including invertebrates (insects, nematodes, platyhelminths, tunicates), vertebrates (mammals, amphibians) and plants (both monocots, dicots and gymnosperms) (this paper and [7,17]). The presence of cytochromes b561 in a wide variety of species indicates the general importance of this class of proteins in eukaryotic cell physiology.

Members of the cytochrome b561 protein family are characterized by a number of structural features, likely to play an essential part in their function [7,17,18]. They are highly hydrophobic proteins with six transmembrane helices, four conserved His residues, possibly coordinating two heme molecules, and predicted substrate-binding sites for ascorbate and monodehydro-ascorbate (MDHA). The occurrence of cytochromes b561 in species phylogenetically remote as nematodes and mammals, tunicates and insects, or amphibians and plants, is intriguing. The strict conservation of essential structural features suggests that the mode of action and physiological function of these proteins may be very similar. We present here a further analysis of the similarities and differences between cytochromes b561 throughout the animal and plant kingdoms, at the level of genomic organization and protein structure. Also, we examine the phylogenetic relations between all cytochrome b561 proteins identified so far, to allow hypotheses to be made on the evolution of these ubiquitous proteins.

Results
Analysis of cytochrome b561-encoding genes

Upon the completion of the Arabidopsis thaliana genome project in December 2000, it became possible to identify all genes encoding cytochromes b561 in a plant. Four putative b561 genes were identified in Arabidopsis thaliana (for Arabidopsis thaliana cyt b561) [7]. Genomic sequences possibly encoding cytochromes b561 were also identified in Oryza sativa and Craterostigma plantagineum. Most putative b561-encoding sequences from other plant species, however, are so far only represented by expressed sequence tags (ESTs).

Genomic sequences for cytochromes b561 are also known for a number of animal species, including Drosophila melanogaster, Caenorhabditis elegans, Homo sapiens (human) and Mus musculus (mouse). The genomes of human and mouse each encode three cytochromes b561: one involved in ascorbate regeneration inside chromaffin granules (Hosb561-1, Mumb561-1), one present in the duodenum (Hosb561-2, Mumb561-2) that may function as a ferric reductase in the plasma membrane of duodenal mucosa, and a third that seems to have a ubiquitous distribution in mammalian tissues, but whose physiological function is not known. This is tentatively referred to as 'ubiquitous cytochrome b561' (Hosb561-3, Mumb561-3) (H.A., unpublished work).

All the A. thaliana sequences have a similar organization, with four exons of comparable lengths (Arthb561-1 is shown in Figure 1). The intron-exon structure of cytochrome b561 genes identified in O. sativa and C. plantagineum is similar (Figure 1). Mammalian genes coding for the duodenal cytochrome b561 isofrom also have a similar organisation as their plant homologues, with four exons and three introns (Figure 1). Interestingly, however, genes encoding the two other mammalian b561 cytochromes have five exons (Figure 1). Cytochrome b561 genes with a different intron-exon structure within one species are also present in C. elegans. One of the C. elegans cytochrome b561 proteins (F55H2.5) is encoded by a gene (Caeb561-1) consisting of only two exons and one intron (Figure 1), while the two other genes in this nematode (F39G3.4 and F39G3.5; Caeb561-2 and -3 respectively) each have four exons. Similarly, two homologous genes have been identified in the fruit fly D. melanogaster (CG1275 and CG8776). The first has two different splicing variants, each with four exons, and the second gene has either five or six exons, also depending on splicing.
The presence of five exons in the mammalian chromaffin granule cytochrome b561 has been considered to support a five transmembrane helix structure for these proteins [19]. However, the occurrence of cytochrome b561-encoding genes with very different gene structures indicates that this one-helix-one-exon model is probably not valid for all members of this protein family. Moreover, secondary-structure prediction routines almost invariably indicate the presence of six transmembrane helices for each of the identified putative cytochrome b561 genes. The correlation between predicted transmembrane structures and intron-exon structure is presented in Figure 2 for three different A. thaliana genes (Arthb561-1, Arthb561-2, Arthb561-4), a homolog from rice (Orsb561) and the human chromaffin cytochrome b561.
This analysis indicates that individual transmembrane helices may be encoded by different exons. For example, the predicted transmembrane helices 4 in Artb561-2, helix 6 from Artb561-4 and helix 4 in Hosb561-1 are each encoded by exons 3 and 4 (Figure 2). In rice, transmembrane helix 5 is partially encoded by exon 2 and exon 3. A similar situation, in which single transmembrane helices are encoded by more than one exon, was observed for the plasma membrane ATPase (BT002855) and an Arabidopsis thaliana sodium channel (AY113938) (data not shown). The number of full transmembrane helices encoded by individual exons also varies among the genes. In the different cytochrome b561-encoding genes in Arabidopsis thaliana, the first exon respectively encodes one, two or three helices (Figure 2).

Alternative splicing of cytochrome b561-encoding genes is observed in D. melanogaster. Different transcripts are found for each of the two putative genes (CG1275 and CG8776). The first gene has two transcripts (Drmb561-1 and -2), each encoded by four exons. Drmb561-1 is only different from Drmb561-2 in that it has 86 extra amino acids at the amino terminus. This extra region (predicted to be cytoplasmic) is absent in all other cytochrome b561 proteins identified thus far. The second gene from D. melanogaster has three different transcripts, encoded by five (Drmb561-3) or six (Drmb561-4 and -5) exons. Again, the only difference is seen at the amino terminus of the encoded proteins. Drmb561-5 is nearly identical to Drmb561-3, with only a difference in 20 amino-terminal amino acids. The sequence of the Drmb561-4 protein corresponds to that of the other two proteins, except for the lack of 80 amino acids at the amino terminus. At the protein level, Drmb561-4 thus proves to be the shortest isoform, lacking the first transmembrane helix. All six helices are present in the other two isoforms.

Comparison of putative cytochrome b561 proteins

The only cytochrome b561 protein that has so far been purified and sequenced, is the bovine chromaffin granule cytochrome b561 [14,15]. On the basis of sequence similarity to this protein, a large number of putative cytochrome b561 protein sequences have been identified from EST sequences obtained from a large variety of species [7]. The similarity between sequences from different organisms is usually not high at the DNA level. However, at the protein level well-conserved features are apparent. In Figure 3 a selection of cytochrome b561 protein sequences from phylogenetically diverse species is aligned. As reported by [17], and as apparent from Figure 1, the conservation in the first and sixth...
transmembrane helix of the cytochromes b561 is limited to the overall hydrophobicity. This observation suggests that these helices may be primarily involved in protein folding and stability, rather than in the catalytic activity.

In addition to the conservation of the predicted transmembrane structures, four His residues suggested to bind the two heme groups are strictly conserved in all cytochrome b561 homologs. Furthermore, predicted binding sites for MDHA and ascorbate - as suggested by [18] - are conserved. A potential MDHA-binding motif - xYSLHSWxGx - with x being a hydrophobic amino acid in most proteins, is highly conserved. In the Anopheles gambiae (malaria mosquito) b561 protein, Angb561, the Ser is conservatively replaced by Thr. The Tyr in the consensus sequence is not conserved in the proteins from C. elegans - Caeb561-1, -2 and -3 - nor in Artb561-3, but it is present in all Artb561-3 orthologs identified in other plant species (data not shown).

The suggested ascorbate-binding site (ALLVYRVFR in the mammalian chromaffin cytochrome b561 [18]) is almost perfectly conserved in all mammalian proteins, but the degree of conservation is much lower in plants (Figure 3). The first three or four amino acids of this consensus sequence are generally hydrophobic, and the Tyr is found in all proteins except Artb561-4, in which it is replaced by His. The triplet codons for Tyr and His differ by only one nucleotide, suggesting that a single point mutation may be responsible for this substitution. The His at this position is found in all Artb561-4 orthologs from other plant species (data not shown), and therefore seems a consistent substitution in this isoform of plant cytochromes b561. The Arg from this motif is conservatively replaced by Lys in all available plant sequences. To the best of our knowledge the sequence requirement for ascorbate and MDHA binding has not been experimentally confirmed, and so it is not possible to evaluate whether the observed conservation is sufficient for the functional interaction with the ascorbate-MDHA redox couple.

In addition to the conserved structural features mentioned above, all cytochromes b561 show strict conservation of five Gly, two Pro, one Lys and one Glu (green in Figure 3). A number of aromatic residues are also well conserved (yellow in Figure 3). These have been suggested to take part in the electron transfer between the two heme molecules, possibly by electron tunneling [17].

**Phylogeny of cytochromes b561**

When all available cytochrome b561 protein sequences - including those derived from ESTs - are aligned with Clustal W [20], and subsequently organized in a phylogenetic tree (Figure 4), two main clusters containing the plant and animal cytochromes b561 can be discriminated. However, the position of the Artb561-3 orthologs in the plant cluster is not supported by high bootstrap values (less than 80%).

A closer examination of the plant group reveals the clustering of sequences from Lycopersicon esculentum (tomato, Lyeb561), L. hirsutum (Lyhb561), Solanum tuberosum (potato, Stob561), C. plantagineum (resurrection plant, Crpb561), Beta vulgaris (beet, Bevb561-1), Populus tremula (poplar, Putb561), Medicago truncatula (barrel medic, Metb561-1), Oryza sativa (rice, Orsb561), Zea mays (maize, Zemb561), Hordeum vulgare (barley, Hovb561-1), Sorghum bicolor (sorghum, Sobb561), Triticum aestivum (wheat, Trab561-1) and T. monococcum (Trmb561) with the Artb561-1 protein, suggesting that these proteins are orthologs of Artb561-1 from A. thaliana. The localization of a cytochrome b561 from Pinus taeda (lobolly pine, Pitb561) within this cluster is not supported by a bootstrap value above 80% and should be interpreted with caution. Similarly, sequences from T. aestivum (Trab561-2), H. vulgare (Hovb561-2), M. truncatula (Metb561-2), Glycine max (soybean, Glmb561-2), Zinnia elegans (zinnia, Zieb561) and B. vulgaris (Bevb561-2) are clustered with Artb561-2. Orthologs of Artb561-3 have been identified in Mesembryanthemum crystallinum (ice plant, Mecb561-3), M. truncatula (Metb561-3) and Gossypium hirsutum (cotton, Gohb561), and Artb561-4 orthologs in M. truncatula (Metb561-4) and B. vulgaris (Bevb561-4).

Most of the plant cytochrome b561 sequences so far have been obtained from dicotyledonous plants, but for Artb561-1 and -2 some orthologs from monocotyledons are available. These form separate ‘sub-clusters’ within the clusters of Artb561-1 and -2 respectively, whereas an Artb561-1 ortholog from the animal group, all known chromaffin granule cytochromes b561 - from H. sapiens (human, Hosb561-1), M. musculus (mouse, Mumb561-1), Sus scrofa (pig, Susb561), Bos taurus (bovine, Bothb561) and Ovis aries (sheep, Ovab561) are clustered together with the homolog from Xenopus laevis (African clawed frog, Xeb561). The duodenal cytochromes b561 from humans, mouse and rat (Hosb561-2, Mumb561-2 and Ramb561-2) form a separate group, as do the chromaffin granule cytochromes b561 and the third type of mammalian cytochrome b561 (ubiquitous cytochrome b561 from humans and mouse, Hosb561-3 and Mumb561-3). The position of the cytochrome b561 protein from Ciona intestinalis (Ciib561; sea squirt, a tunicate) is uncertain, as the bootstrap value is below 80%. The cytochrome b561 homologs identified in C. elegans and the flatworm Dugesia japonica could not reliably be associated with any of the clusters.

The outcome for the phylogenetic tree was essentially the same when T-Coffee [21] or POA [22] software packages were used, which are in some cases considered more reliable than Clustal W [23]. Similarly, using only the central cytochrome b561 ‘core domain’ as defined by Ponting [24], instead of the full sequence, the clustering of cytochrome b561 homologs is
Figure 3 (see legend on next page)
identical to that presented in Figure 4, and the overall appearance of the resulting tree is not altered (Figure 5).

To examine the selective pressure on cytochrome b561-encoding genes after various gene duplication events, a Li93 analysis was performed using the DAMBE software package [25,26], based on pairwise comparisons. The amount of synonymous substitutions (Ks in Table 1) is apparently saturated when comparing Artb561-3 to the three other paralogous genes from A. thaliana, indicating that Artb561-3 may represent an early gene duplication event, and has accumulated a large number of ‘silent’ substitutions. Nonsynonymous substitutions (that is, codon alterations leading to amino acid replacements, Ka in Table 1) for these genes range from 0.49 to 0.75 (substitutions/position), which is much lower than the Ks values. Artb561-2 and -4 have the lowest Ks (0.47), suggesting that they originated from a more recent gene duplication in A. thaliana. Comparable values are found for the orthologs of Artb561-2 and -4 in B. vulgaris and M. truncatula, indicating that this last gene duplication had occurred before the radiation within the dicotyledons (data not shown). In mouse, Ks values are equally high (and probably saturated) for the genes encoding the three cytochrome b561 isoforms. Ks values are also comparable to those of the different plant isoforms (Table 1).

Comparison of cytochromes b561 from dicotyledonous species to their orthologs from monocotyledons reveals that Ks and Ka values are generally both around 0.3 to 0.5. Knowing that both clades diversified 130 million years ago [27], the average rate of synonymous and nonsynonymous substitutions is calculated to be around 3 × 10^{-9} substitutions per position per year (Table 1). This is comparable to the synonymous evolution rate of other nuclear-encoded plant genes (5-30 × 10^{-9} substitutions/position/year [28]). Among dicotyledons (using estimated divergence times for different families, derived from Figure 2 in [29]) the average synonymous substitution rate is higher (12.63 ± 2.41 × 10^{-9} substitutions/position/year), but the nonsynonymous rate is very similar (2.38 ± 1.18 × 10^{-9} substitutions/position/year). For the resurrection plant (C. plantagineum), however, which has adapted to life in an extremely dry environment, a particularly high nonsynonymous substitution rate was found as compared to the ortholog from potato (13.25 × 10^{-9} substitutions/position/year), whereas the synonymous rate was the same as for the orthologs in other dicots (Table 1).

Ks and Ka were also calculated for animal cytochrome b561 genes. Comparing the gene from the tunicate C. intestinalis (Ciih561), which branched off the vertebrate lineage around 540 million years ago [30], with the chromaffin granule cytochrome b561 genes from vertebrates (X. laevis, H. sapiens and M. musculus) gives a Ks of around 0.65 and a Ka of 0.8. This is remarkably low, considering the large phyletic distance between tunicates and vertebrates, and implies synonymous and nonsynonymous substitution rates of 1-1.5 × 10^{-9} substitutions/position/year.

The Gu99 likelihood ratio test in the DIVERGE software package [31] was used to examine whether proteins from different branches in the phylogenetic tree have different functional constraints, and whether they may have functionally diverged. The Theta ML value, indicative for the level of functional divergence between proteins [31], is relatively low (0.213) according to [32], when monocotyledous and dicotyledonous Artb561-1 orthologs are compared. This suggests that these orthologs are likely to have similar biochemical functions. The Gu99 likelihood assay also indicated that a functional adaptation may have occurred at only five amino acids between these orthologs, supporting their functional similarity. A similar analysis of Artb561-1 and -2 orthologs yielded a theta ML value of 0.533, and 22 residues that may have functionally adapted. These numbers are still rather low [32]. However, four of these residues (A, I, S and P) are situated in the predicted ascorbate-binding site (AI-XYKSLP in Artb561-1). A similar analysis for the mammalian cyts b561 (chromaffin cyts b561 versus the cluster with the other two mammalian cyts b561) resulted in a very low theta ML of 0.031, and no residues likely to have an altered function.

**Discussion**

At the gene level, conservation among cytochrome b561-encoding genes is not very high [7]. The genomic organization usually comprises four or five exons in plants and mammals (Figure 1). In addition to genes with four or five exons, intron-exons contain homologous genes with only two exons (C. elegans F55H2.5), or with six exons (D. melanogaster). Splice variants are observed for D. melanogaster CG1275 and CG8776. This restricted splicing results in a cytochrome b561 protein with an additional amino-terminal domain (Drmb561-1), and in a protein that lacks the first of the six
transmembrane helices (Drmb561-4), commonly present in plant and mammalian cytochromes b561. The physiological implication of these altered cytochrome b561 structures is unclear. Interestingly, the alternative splice products have in each case retained the cytochrome b561 ‘core structure’ as defined by Ponting [24], that is, four transmembrane helices containing the conserved heme-ligating His residues and the predicted substrate-binding sites.

Despite the availability of extensive genomic sequence information from fungi and prokaryotes, the presence of cytochrome b561-like sequences is restricted to animals and
plants. Some fungal species have been demonstrated to synthesize the ascorbate analogs D-arabo-ascorbate (*Penicillium*) or erythro-ascorbate (*Candida albicans, Saccharomyces cerevisiae*) instead of ascorbate [33,34]. However, these compounds occur at very low concentrations, and they probably have only limited importance - if any - as antioxidants [34]. Ascorbate is apparently completely absent in prokaryotes [33]. The absence of cytochromes b561 in fungi and prokaryotes may therefore be related to the absence of L-ascorbate as a major antioxidant.

Our phylogenetic analysis supports the hypothesis that all cytochrome b561 proteins have probably evolved from a single protein, present in the common ancestor of plants and animals. This conclusion is supported by the perfect conservation of the 'core structural features' in cytochrome b561.
proteins in a range of phylogenetically very distinct species (Figure 3). It should be noted that the conservation of the putative ascorbate-binding sites is high in the mammalian proteins, but considerably less in the plant sequences (Figure 3). Although an ascorbate-reducible cytochrome b561 has been demonstrated in several plant species [12,35], this theoretically leaves open the possibility that other substrates may function as electron donors to the plant cytochromes b561. The Gu99 likelihood analysis indicates that four amino-acid residues in the ascorbate-binding site may show functional adaptation, supporting the possibility of different substrate-binding site affinities among cytochromes b561 in A. thaliana.

Plant and animal cytochromes b561 generally separate into two clusters in a phylogenetic tree (Figure 4), indicating a diversification early in evolution. The pairwise similarity between each of the A. thaliana cytochromes b561 and the animal homologs is comparable (data not shown), suggesting that they diversified within an evolutionarily short time span from a single ancestral protein, after the separation between plants and animals. Cytochrome b561 proteins from invertebrates (insects, nematode and flatworm) are not tightly linked to the plant or mammalian cluster (Figure 4).

Within the mammalian cluster of cytochromes b561, the three paralogous proteins (from chromaffin granules, duodenal
and 'ubiquitous') form separate, monophyletic groups. The presence of a cytochrome b561-like protein in C. intestinalis is interesting. Tunicates, the most primitive chordates, are considered the direct ancestors of vertebrates [36], suggesting that the protein from C. intestinalis might represent an ancestral form of the mammalian cytochromes b561. Synonymous substitution rates for the cytochrome b561 genes from C. intestinalis and different vertebrates are rather low (1-1.5 × 10^−9/position/year, Table 1), as compared to other nuclear-encoded mammalian genes (for example, 4.61 × 10^−9/position/year [37]). Nonsynonymous substitution rates are in the same range, indicating a good conservation at the protein level.

In addition to conclusions on the evolutionary relationship between cytochromes b561 in different species, the cladogram points to interesting relations between cytochrome b561 isoforms within a single species. The four paralogous proteins from A. thaliana (Arb561-1, -2, -3 and -4) form separate clusters with their respective orthologs from other plant species (Figure 4). It is thus likely that gene duplication events had already occurred in algae or primitive plants. The identification of a cytochrome b561 in the gymnosperm P. taiada (Pith561) supports this suggestion.

The clusters containing the A. thaliana isoforms Arb561-1 and Arb561-2 also contain homologous sequences from both dicotyledons and monocotyledons. In both cases, the proteins from monocots and dicots form separate sub-clusters. This observation suggests that the four different cytochromes b561 have evolved separately in dicots and monocots, and hence that the diversification between these proteins had already occurred in their common ancestor. Substitution rates in cytochrome b561 genes from monocots and dicots are comparable to those in other plant nuclear-encoded genes (Table 1). The Artb561-1 ortholog in the drought-resistant resurrection plant (Crp561), seems to have a remarkably high rate of nonsynonymous substitution (13.25/position/year). Interestingly, the Artb561-2 and -4 proteins tend to group in the same cluster (Figure 4), suggesting that they diversified more recently in evolution, which is supported by our Li93 analysis (Table 1).

Cyts b561 are possibly involved in the regeneration of ascorbate through transmembrane electron transport [8,10,11,18]. This functional conservation is supported by our likelihood ratio test (Gu99 test). As ascorbate is present in different subcellular organelles in plants and animals, it is not surprising to find members of the cytochrome b561 protein family in different organelles. In humans, the chromaffin tissue cytochrome b561 is present in the membrane of a subcellular secretory vesicle [8,10,38], whereas the duodenal cytochrome b561 is a plasma membrane protein [39]. The subcellular localization of the third human isoform (Hosb561-3) is not yet known. In A. thaliana and several other plant species, at least one of the cytochrome b561 isoforms is present in the plasma membrane [7,12]. The subcellular localization of the other isoforms remains to be determined. The association with different membranes raises the question on the subcellular localization of the ancestral cytochrome b561. The apparent absence of a cytochrome b561 in the plasma membrane of algae [7] can be taken as an indication that the primitive form of the protein was located in internal membranes.

Materials and methods
Database searches used BLAST [40] on the website of the National Center for Biotechnology Information (NCBI [41]). Multiple sequence alignments were made with Clustal W [20], and confirmed with POA [22] and T-Coffee [21], which have been reported to give more accurate alignments [23]. These data were converted into a cladogram using Treecon software [42]. Distances were calculated with Poisson correction, and for tree topology, complete linkage clustering was used. The Li93 analysis was performed with DAMBE [26], after aligning the genes pairwise with Clustal W and T-Coffee. The maximum likelihood analysis (Gu99) was performed with the DIVERGE software [31], using the phylogenetic tree from Figure 4. DIVERGE calculates a theta ML value indicative of the level of functional divergence between proteins in different clusters of the tree, and a posterior probability to trace the amino-acid positions that are likely to be responsible for the functional divergence between proteins in both clusters. Transmembrane structures were predicted with TMHMM [43].

Acknowledgements
The authors thank Etsuko Moriyama (UNL) for valuable advice and for critically reading the manuscript. This research was supported by grants from the Fund for Scientific Research (FWO) and the University of Antwerp; WV. 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. Journal Series No. 14061.

References
1. Noctor G, Foyer CH: Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 1998, 49:249-279.
2. Mittler R: Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 2002, 7:405-410.
3. Arrigoni O, De Tulio MC: The role of ascorbic acid in cell metabolism: between gene-directed functions and unpredictable chemical reactions. J Plant Physiol 2000, 157:481-488.
4. Davve MW, Van Montagu M, Inze D, Sammartin M, Kanelis A, Smirnoff N, Benzie JJ, Strain J, Fawell D, Fletcher J: Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of processing. J Sci Food Agricult 2000, 80:825-860.
5. Wheeler GL, Jones MA, Smirnoff N: The biosynthetic pathway of vitamin C in higher plants. Nature 1998, 393:365-369.
6. Agius F, González-Lamothe R, Caballerio JL, Muñoz-Blanco J, Botella MA, Valpuesta V: Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. Nat Biotechnol 2003, 21:177-181.
7. Asard H, Kapila J, Verelst W, Bérczi A: Higher-plant plasma membrane cytochrome b561: a protein in search of a function. Protoplasma 2001, 217:77-93.
8. Wakefield LM, Cass AEG, Radda GK: Functional coupling between enzymes of the chromaffin granule membrane. J Biol
9. Asard H, Horemans N, Preger V, Trost P: Plasma membrane b-type cytochromes. In Plasma Membrane Redox Systems and Their Role in Biological Stress and Disease. Edited by: Asard H, Bérczi A, Caubergs RJ. Dordrecht: Kluwer Academic Publishers; 1998:1-31.

10. Njus D, Kelley PM, Harnadek GJ, Pacquing YV: The role of ascorbate free radical as an electron acceptor to cytochrome b₅₅₁ in plasma membranes of higher plants. Plant Physiol 1994, 104:1455-1458.

11. Horemans N, Asard H, Caubergs RJ: The role of ascorbate free radical as an electron acceptor to cytochrome b₅₅₁ in plasma membranes of higher plants. Plant Physiol 1989, 90:1077-1083.

12. Asard H, Venkovskyy M, Caubergs R, Reijnders W, Oltmann FL, De Greef JÁ: b-Type cytochromes in higher plant plasma membranes. Plant Physiol 1989, 86:123-134.

13. Asard H, Venkovskyy M, Caubergs R, Reijnders W, Oltmann FL, De Greef JÁ: b-Type cytochromes in higher plant plasma membranes. Plant Physiol 1989, 86:123-134.

14. Perin MS, Fried VA, Slaughter CA, Südhof TC: The role of ascorbate free radical as an electron acceptor to cytochrome b₅₅₁ in plasma membranes of higher plants. Plant Physiol 1989, 90:1077-1083.

15. Wang Y, Gu X: Functional diversity of the ascarcase gene family and altered functional constraints: statistical analysis and prediction. Genetics 2001, 158:1311-1320.

16. Wang Y, Gu X: Functional diversity of the ascarcase gene family and altered functional constraints: statistical analysis and prediction. Genetics 2001, 158:1311-1320.

17. Wang Y, Gu X: Functional diversity of the ascarcase gene family and altered functional constraints: statistical analysis and prediction. Genetics 2001, 158:1311-1320.