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ARTICLE *in* PROTOPLASMA · FEBRUARY 2001

Impact Factor: 2.65 · DOI: 10.1007/BF01289417 · Source: PubMed

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Protoplasma (2001) 217: 77–93

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Higher-plant plasma membrane cytochrome b_{561}: a protein in search of a function

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Received June 27, 2000
Accepted July 19, 2000

Summary. During the past twenty years evidence has accumulated on the presence of a specific high-potential, ascorbate-reducible *b*-type cytochrome in the plasma membrane (PM) of higher plants. This cytochrome is named cytochrome b_{561} (cyt b_{561}) according to the wavelength maximum of its α-band in the reduced form. More recent evidence suggests that this protein is homologous to a *b*-type cytochrome present in chromaffin granules of animal cells. The plant and animal cytochromes share a number of strikingly similar features, including the high redox potential, the ascorbate reducibility, and most importantly the capacity to transport electrons across the membrane they are located in. The PM cyt b_{561} is found in all plant species and in a variety of tissues tested so far. It thus appears to be a ubiquitous electron transport component of the PM. The cytochromes b_{561} probably constitute a novel class of transmembrane electron transport proteins present in a large variety of eukaryotic cells. Of particular interest is the recent discovery of a number of plant genes that show striking homologies to the genes coding for the mammalian cytochromes b_{561}. A number of highly relevant structural features, including hydrophobic domains, heme ligation sites, and possible ascorbate and monodehydroascorbate binding sites are almost perfectly conserved in all these proteins. At the same time the plant gene products show interesting differences related to their specific location at the PM, such as potentially N-linked glycosylation sites. It is also clear that at least in several plants cyt b_{561} is represented by a multigene family. The current paper presents the first overview focusing exclusively on the plant PM cyt b_{561}, compares it to the animal cyt b_{561}, and discusses the possible physiological function of these proteins in plants.

Keywords: Ascorbate; Cytochrome b_{561}; Electron transport; Higher plant; Plasma membrane; Protein family; Redox activity.

Abbreviations: Asc ascorbate; cyt cytochrome; DHA dehydroascorbate; E_0' standard redox potential; EST expressed sequence tag; His histidine; MDA monodehydroascorbate; Met methionine; PM plasma membrane.

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Specific cytochrome *b* in plant plasma membrane

The discovery of the presence of *b*-type cytochromes in the plasma membranes (PMs) of plants is closely linked to the investigation of blue-light perception in higher plants and fungi. In the seventies it became apparent that the actinic irradiation of fungal mycelia of *Dictyostelium discoideum*, *Neurospora crassa*, and *Zea mays* coleoptiles with blue light resulted in a transient absorbance change (Muñoz and Butler 1975, Poff and Butler 1975, Brain et al. 1977). The spectra of the difference of blue light minus dark demonstrated the involvement of a *b*-type cytochrome in this reaction. Preparation of subcellular fractions from fungal mycelia and corn coleoptiles further indicated that the blue-light-mediated cytochrome reduction was possibly associated with the PM (Schmidt et al. 1977, Brain et al. 1977, Leong et al. 1981). The *b*-type cytochrome in fungi that was detected in these experiments has not been characterised in more detail so far.

At about the same time Jesaitis et al. (1977) demonstrated the presence of a "non-NADH-reducible" *b*-type cytochrome in a membrane fraction cosedimenting in sucrose density gradients with the PM marker glucan synthase II. These membrane fractions were obtained from etiolated corn coleoptiles, also supporting the presence of a specific "nonenergetic" cytochrome *b* (cyt *b*) in the PM of higher plants. A few years later, when the aqueous polymer two-phase partitioning method for the preparation of highly purified plant PMs was introduced, conclusive evidence was obtained on the localisation of a *b*-type cytochrome in

higher-plant PMs (Widell et al. 1982, Caubergs et al. 1983, Widell et al. 1983).

Analysis of sucrose density gradients also confirmed the presence of a specific PM cyt *b* in cauliflower and mung beans (Asard et al. 1987) and in beans (Scagliarini et al. 1998). The experiments on mung beans and beans also suggested the possible presence of yet another *b*-type cytochrome in membranes distinct from those derived from mitochondria and endoplasmic reticulum. This second cytochrome was also reducible by ascorbate (Asc), suggesting a high redox potential similar to that of the major PM *b*-type cytochrome (see below). However, the subcellular localisation of this additional component remains unknown.

In the past two decades it has become increasingly clear that cyt *b* is one of the specific redox constituents in the PM of plant, animal, and fungal cells. A number of redox enzymes have now been characterised, and several of them were purified and sequenced (for reviews, see Bérczi and Asard 1995, Lüthje et al. 1997, Bérczi et al. 1998, Döring et al. 1998, Bérczi and Møller 2000). It is generally accepted that these redox components play an important role in a variety of physiological processes, including iron uptake, cell defence (oxygen radical production), nitrate reduction, signal transduction, and the control of the redox status of extracellular molecules. Although detailed mechanisms of actions are still lacking for most of the PM redox proteins, considerable progress may be expected with the availability of the genes for some of these components. Most likely the PM cytochromes are involved in one or more of these PM redox processes as will be discussed in this paper.

After the discovery of the presence of *b*-type cytochromes in the higher-plant PM, characterisation studies were performed resulting in a more detailed description and identification of the cytochrome species. On the basis of redox titration analysis it was readily recognised that purified PM fractions of a number of plant species contain at least two distinct cyt *b* molecules (Caubergs et al. 1986, Asard et al. 1989, Askerlund et al. 1989). One of these has a characteristic high redox potential (E_0' around +140 mV, see also below) and an α-band maximum of the reduced protein near 561 nm wavelength. The protein is accordingly named cyt b_{561}. This component is the major component in all PM fractions tested so far and constitutes 60–80% of the cytochromes present in this membrane. The current paper concentrates almost exclusively on the properties and function of this heme protein.

In addition to cyt b_{561}, the presence of a cyt *b* with a lower redox potential was revealed by redox titration analysis (Asard et al. 1989, Askerlund et al. 1989). The latter protein was not yet studied in much detail and has been tentatively identified as a cyt b_5. A more extensive discussion on the presence and nature of other *b*-type cytochromes in the plant PM, such as the NADPH oxidase and the PM nitrate reductase, is presented in Asard et al. (1998).

A number of properties of the PM cyt b_{561} raise a particular interest in this protein. First, it is now clear that PM cyts b_{561} with very similar standard redox potentials (E_0') are present in all plant species tested so far. Second, the plant PM cyt b_{561} has been demonstrated to transfer electrons from a cytoplasmic electron donor to an extracellular electron acceptor molecule. As such it is the only PM redox component described so far for which this trans-membrane electron transport reaction has been experimentally demonstrated. Third, the PM cyt b_{561} shows homology to a mammalian protein, present in chromaffin granule membranes, with similar E_0', α-band, and electron transport characteristics. It thus seems that these proteins constitute a new protein family within the class of *b*-type cytochromes and are present at least in the kingdoms of plants and animals (Asard et al. 2000). These and other aspects of the PM cyt b_{561} will be presented in the current overview, as well as suggestions on the possible physiological function of this protein in plants.

PM cyt b_{561} is a ubiquitous plant protein

The most conclusive identification on the presence of cyt b_{561} in plant PMs relies on the full redox titration analysis of purified membrane fractions. This analysis allows the discrimination of separate cytochromes and the identification of their α-band maximum and standard redox potential (E_0'). Detailed redox titration analyses have been performed on highly purified PMs from cauliflower inflorescences, bean apical hooks, zucchini hypocotyls, spinach leaves, barley roots, and corn roots (Asard et al. 1989, Askerlund et al. 1989, S. Lüthje and H. Asard unpubl. results) (Table 1). In each of these cases, PMs were prepared by using the aqueous polymer two-phase partitioning protocol. The titration analysis for all of these fractions was very consistent with the presence of one major

Table 1. Characteristics of the PM cyt b_{561} in various plant species

Species and tissue	Cyt content (pmol/mg)	Asc reduction (%)[a]	α-Band (nm)	E_0' (mV)	Reference or source
Beta vulgaris					
leaves	290	57	560		Askerlund et al. 1989
Brassica oleracea					
inflorescences	333	70	558.8	165 ± 12	Asard et al. 1989
	280	71	560	153 ± 32	Askerlund et al. 1989
			560.7	164	Caubergs et al. 1986
			559.7[b]	135 ± 20[b]	Rich and Bendall 1975
Cucumis sativus					
fruits	220		561		N. Horemans unpubl.
Cucurbita pepo					
hypocotyls	67	32	560.7	135 ± 15	Asard et al. 1989
Glycine max					
hypocotyls			562[b]	120 ± 20[b]	Rich and Bendall 1975
			562.5[b]	105 ± 23[b]	Hendry et al. 1981
Phaseolus vulgaris					
cotyledons	94	65			N. Horemans unpubl.
primary leaves	146	57			N. Horemans unpubl.
hooks	459	64	560.7	110 ± 13	Asard et al. 1989
				220 ± 12[c]	
hypocotyls	155	76			N. Horemans unpubl.
	374				Scagliarini et al. 1998
	136	72			Asard and Bérczi 1998
roots	202	55			N. Horemans unpubl.
	144	81			Asard and Bérczi 1998
Spinacia oleracea					
leaves	420	64	561	155 ± 16	Askerlund et al. 1989
Triticum aestivum					
leaves	350		561	162	Askerlund et al. 1989
				~200[c]	
Zea mays					
roots	567	67	561	143 ± 1.5	S. Lüthje unpubl.
				267 ± 3.6[c]	

[a] Levels of Asc reduction relative to dithionite reduction
[b] Results from microsomal fractions
[c] Resolution of an additional high-potential component

high-potential *b*-type cytochrome (E_0' from +110 to +165 mV), cyt b_{561}, and one additional low-potential cyt *b* component (E_0' from −20 to −50 mV). It should be noted that in some of the PM preparations the redox titration analysis indicated the presence of an additional cytochrome component with an even higher redox potential (E_0' of +220 mV in bean, Asard et al. 1989; E_0' of +200 mV in barley roots, Askerlund et al. 1989; E_0' of +267 mV in corn roots, S. Lüthje and H. Asard unpubl. results). This component was, however, not resolved in all experiments. A very recent analysis using a partially purified bean apical-hook cyt b_{561} protein preparation suggested that the two high-potential heme centres are likely to be part of one and the same protein (Trost et al. 2000). As suggested by the authors both heme centres may be necessary to allow the trans-membrane electron transfer reaction. Experimental evidence suggests that the mammalian cyt b_{561} also contains two distinct hemes, which were resolved by redox titration analysis (Apps et al. 1984), by electron paramagnetic resonance analysis on the purified bovine cytochrome (Tsubaki et al. 1997), or by circular dichroism spectral analysis (Degli Esposti et al. 1989b). It seems probable that the presence of two hemes indeed provides a structural basis for the electron transfer across the membrane in which the cytochrome is embedded (Degli Esposti et al. 1989a, Kobayashi et al. 1998).

In addition to the results obtained by redox titration analysis using purified plant PMs, cyt b_{561} is apparently also present in a number of other species (Table 1). In these cases cyt b_{561} was discriminated from the overall

PM cytochrome pool by using Asc as an electron donor. Due to its relatively high redox potential (E_0' +58 mV; Sapper et al. 1982, Washko et al. 1992) Asc acts as an electron donor to the cytochrome with high E_0' (cyt b_{561}) but not to the low-potential cyt *b* component. At least in purified PM fractions Asc can therefore be used as a more or less selective reductant to indicate the presence of cyt b_{561}. In a number of other plant species the presence of *b*-type cytochromes in purified PMs has been demonstrated, but no attempts have been made to specifically detect the presence of cyt b_{561} (see Asard et al. 1998). The specific cytochrome concentrations in these species are in the same range as indicated in Table 1, and it seems highly likely that this cytochrome pool also includes the high-potential cyt *b*.

Redox titration analysis has also been performed on so-called microsomal membrane fractions from cauliflower and mung beans (Rich and Bendall 1975, Hendry et al. 1981). These membrane fractions are largely depleted of mitochondria and chloroplasts and contain mainly endoplasmic reticulum, Golgi apparatus, and PMs. Although it is not possible to assign specific cytochromes to particular organelles from these data, the result of these redox titrations have revealed a similar high-potential cyt *b* component. It seems very likely that this component actually represents the PM cyt b_{561}. For example, the redox titration of cauliflower microsomal fractions (Rich and Bendall 1975) and purified cauliflower PMs (Asard et al. 1989, Askerlund et al. 1989) revealed *b*-type cytochromes with redox potentials of 135 ± 20 mV, 165 ± 12 mV, and 153 ± 32 mV, respectively. No other high-potential cytochrome was found in the microsomal membranes.

From the results presented above it is clear that the high-potential cyt b_{561} is a ubiquitous PM protein, widespread in the plant kingdom. This broad occurrence supports the idea that the protein may have a general "housekeeping" function in the physiology of the plant cell. It is also interesting to note that so far no apparent organ specificity has been demonstrated. Cyt b_{561} has been detected in the PM of organs as different as cotyledons, green leaves, roots, and etiolated hypocotyls and even plant cell cultures (N. Horemans unpubl.). Only in the case of bean it is possible to compare specific cyt b_{561} concentrations in different parts of the plant (Table 1). These data seem to suggest a slightly higher cyt b_{561} concentration in the meristematic-hook region when compared to the rest of the hypocotyl and to the leaves and cotyledons. Whether this observation may be related to the physiological function of this PM protein remains an open question.

Despite the initial discovery of *b*-type cytochromes in *N. crassa* and *D. discoideum*, no conclusive information is available on the nature of the cytochrome in these species or on the presence of cyt b_{561} in the PM of these fungi. PM preparations of *D. discoideum* (Glomp and Hess 1986), *N. crassa* (Borgeson and Bowman 1985), and *Schizosaccharomyces pombe* (H. Asard unpubl. results) have been demonstrated to contain *b*-type cytochromes (see also Møller et al. 1991). In contrast, Ramirez et al. (1984) were not able to detect any *b*-type cytochrome in PM fractions from *Saccharomyces cerevisiae*. However, no data are available to positively identify cyt b_{561} in any of these fungal PM fractions. In the case of *S. pombe* no Asc-reducible component could be detected (H. Asard unpubl. results). The major cytochrome in the PM-enriched fraction obtained from *S. pombe* was readily reduced by NADH and had an α-band wavelength maximum slightly below 561 nm, suggesting that it was different from cyt b_{561}. Since flavocytochromes *b* have been demonstrated in the PM of *S. cerevisiae* (Fre1p) and *S. pombe* (Frp1p) (Dancis et al. 1992, Roman et al. 1993), the cytochrome detected in *S. pombe* PMs may be part of this protein complex. The flavocytochromes *b* show homology to the NADPH oxidase of neutrophils (Segal et al. 1998), which contains a low-potential *b*-type cytochrome (E_0' around −245 mV). The Fre1p protein has been demonstrated to be involved in the extracellular reduction of iron in yeast cells (Lesuisse et al. 1996, Askwith and Kaplan 1998), and the cyt *b* identified in *D. discoideum* was suggested to be similar to cyt *b*5 (Glomp and Hess 1986). Therefore, at present there is no biochemical evidence supporting the presence of cyt b_{561} in fungal PMs.

Even less information is available with regard to algae. The presence of *b*-type cytochromes has been reported for a PM-enriched fraction obtained from *Acetabularia mediterranea* (Caubergs et al. 1984). However, since no Asc reduction experiments have apparently been performed, it is not clear whether this protein is similar to cyt b_{561}. In contrast, no *b*-type cytochromes could be demonstrated in highly purified PMs obtained from *Dunaliella* sp. (A. Katz, Weizmann Institute of Science, Rehovot, Israel, pers. commun.). Therefore, the presence of a *b*-type cytochrome similar to cyt b_{561} in algae is still questionable.

As indicated earlier the cyt b_{561} identified in higher-plant PMs shows remarkable similarities at the bio-

chemical level to a protein identified in chromaffin granule membranes (also named cyt b_{561}). The chromaffin granules occur in the adrenal medulla tissues and are involved in the biosynthesis of adrenaline. The key enzyme in the biosynthesis of adrenaline, dopamine-β-hydroxylase, converts dopamine into noradrenaline, and this reaction requires Asc as an electron donor. This Asc is located inside the chromaffin granule, and the one-electron transfer step results in the generation of the Asc free radical, monodehydroascorbate (MDA). It was suggested by Njus and coworkers that the chromaffin granule cyt b_{561} could function in the regeneration of Asc by the reduction of intravesicular MDA (Njus et al. 1983, 1987; Kelley and Njus 1986). Cytoplasmic Asc was considered as the electron donor to this trans-membrane electron transport reaction. Thus, the α-band maximum wavelength, the standard redox potential (E_0' around +140 mV) (Flatmark and Terland 1971, Apps et al. 1984), and the trans-membrane electron-transporting capacities with Asc as an electron donor of animal chromaffin granule cyts b_{561} are properties which are strikingly similar to the plant PM cyt b_{561}. In fact, MDA was also indicated as a potential electron acceptor for the plant PM protein (Horemans et al. 1984).

The physiological function of the chromaffin cyt b_{561} in supporting dopamine biosynthesis argues for a very specific tissue localisation in the adrenal medulla. However, it turned out that this cytochrome was also expressed in other peptidergic and adrenergic vesicles and in hematopoietic tumor cells and was colocalised with dopamine-β-hydroxylase or peptidylglycine α-amidating mono-oxygenases (Srivastava 1995, 1996). Cyt b_{561} thus seems to play an essential role in the physiology of all neuroendocrine tissues. It is not yet clear whether the animal cyt b_{561} is also present in other subcellular membranes, such as the PM.

It thus seems highly interesting to further investigate the remarkable similarities between a plant PM cyt b_{561} and the mammalian chromaffin granule protein. The possible homology between these proteins strongly suggests that the trans-membrane electron transport mediated by cyt b_{561} may serve many additional and yet undiscovered physiological processes in eukaryotic cells.

Purification of plant PM cyt b_{561}

Attempts to purify the plant PM cyt b_{561} from etiolated bean apical hooks by the protocol developed by Wakefield et al. (1984) and employed by Annaert (1993) for the purification of the bovine chromaffin granule cyt b_{561} were unsuccessful (H. Asard unpubl. results). This fact suggested minor but seemingly important differences between the mammalian and plant cyt b_{561} molecules.

Recently a method using two anion exchange chromatographic steps (Mono-Q columns) was successfully employed for the partial purification of an Asc-reducible *b*-type cytochrome from etiolated bean hypocotyls (Scagliarini et al. 1998, Bérczi et al. 2001, Trost et al. 2000). After a single freeze-thaw cycle and consecutive ultracentrifugation of the phase-partition-purified PM vesicles, solubilisation of PM proteins was achieved with 1% (v/v) Triton X-100R (2 mg detergent per mg protein). Unsolubilised material was pelleted by ultracentrifugation and the solubilised material was loaded onto a Mono-Q column previously equilibrated with Tris-HCl at pH 8.0 in the presence of 0.1% (v/v) Triton X-100R. The Asc-reducible *b*-type cytochromes did not bind to the column under these conditions and were collected in the flow-through fractions. After changing the buffer of the combined flow-through fractions to a 3-(cyclohexamino)-1-propanesulfonic acid-KCl buffer at pH 9.9 plus 0.1% (v/v) Triton X-100R, the material was loaded on a second Mono-Q column previously equilibrated in the same buffer. Proteins bound to the column were eluted by a continuous KCl gradient between 0 and 0.3 M. Cyt b_{561} was eluted at about 100 mM KCl. The apparent size of the detergent-cyt b_{561} complex was 130 kDa, as determined by size exclusion chromatography on a Superdex 200 column (Scagliarini et al. 1998). After this final step the purified protein fraction contained approximately 40 nmol heme per mg of protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the cyt b_{561}-containing fractions resulted in a broad protein band between 50 and 55 kDa (Trost et al. 2000). The diffuse appearance of the protein band after silver staining may be related to the glycosylation of the plant cyt b_{561} as recently indicated by experiments from Trost et al. (2000). Although the protein fraction obtained after the Mono-Q column at pH 9.9 may only contain a single heme protein (Trost et al. 2000), in our hands this fraction was not yet fully purified and still contained several other proteins (Bérczi et al. 2001).

The observation that the bean hypocotyl cyt b_{561} bound to the Mono-Q at pH 9.9 but did not bind at pH 8 indicates that the isoelectric point (pI) of this

protein is rather high. This nicely fits to the predicted pI values (9.61, 9.48, and 9.93) on the basis of the sequences deduced from the *Arabidopsis thaliana* genes encoding for putative cyts b_{561} (*Artb561-1*, *Artb561-2*, and *Artb561-3*; see below and in Asard et al. 1998). The pI for the mammalian chromaffin granule cyt b_{561} is 6.2 (Apps et al. 1980), and the difference in the pI values itself may explain why the isolation protocol employed for purifying the mammalian protein was unsuccessful for the plant protein. The apparent molecular mass for the partially purified bean PM cytochrome, as derived by gel electrophoresis (50 to 55 kDa; Trost et al. 2000) is significantly different from that for the animal cyt b_{561}. The bovine chromaffin granule cyt *b* was estimated between 25,100 Da and 30,061 Da by SDS-PAGE (Duong and Fleming 1982, Wakefield et al. 1984) and sequence analysis (Perin et al. 1988), respectively. Whereas a molecular mass of 27.6 kDa was deduced for the human cyt b_{561} (Srivastava et al. 1994). The molecular mass derived for the bean protein also differs from the molecular mass predicted for the Arabidopsis gene products (31,024, 25,289, and 30,820 for Artb561-1, -2, and -3, respectively), which were identified as potential homologues to the chromaffin granule cytochrome (Asard et al. 1998). However, protein glycosylation may significantly affect the molecular-mass determination by SDS-PAGE, providing a possible explanation for these apparent discrepancies. For example, the well-characterized NADPH-oxidase from neutrophil PMs has a predicted molecular mass of 65 kDa (Royer-Pokora et al. 1986, Teahan et al. 1987), whereas the rough molecular mass estimated by SDS-PAGE is 91 kDa (Parkos et al. 1988).

Recent experimental results indicate that the pI values of cyt b_{561} proteins may differ significantly in different plant species. In contrast to the results from bean, the Asc-reducible cyt b_{561} from the PM of *A. thaliana* leaves and *Zea mays* roots bound to the Mono-Q column at pH 8.0 under the experimental conditions mentioned above (Bérczi et al. 2001). Interestingly, in both cases the cyt b_{561} eluted from the Mono-Q column at about the same KCl concentration (100 mM) as in the case of bean hypocotyl cyt b_{561}, independently of the fact that the pH of the buffers were different (pH 8 for the elution of the Arabidopsis and maize proteins, pH 9.9 for the elution of the bean protein). Clearly these results suggest that the bean, Arabidopsis, and maize cyts b_{561} show similarities as well as some interesting differences. It is not yet clear whether these differences are consistent with differences in tissue types or whether they are related to species differences.

One possible approach to studying the plant PM cyt b_{561}, based on the apparent homology with the chromaffin granule cyt b_{561}, is obviously through the use of antibodies which have been raised against the bovine protein (Annaert 1993). However, no cross-reaction was observed with a bovine cyt b_{561} polyclonal antibody against purified bean apical-hook PM fractions (H. Asard unpubl. results), suggesting that the bean cyt b_{561} contained no sufficiently similar epitope. With the recent availability of amino acid sequences from *A. thaliana* with significant homology to the mammalian cyt b_{561} (see below), it was possible to raise antibodies against a synthetic peptide. A peptide of 20 amino acids located near the C-terminal end of the predicted amino acid sequence for one of the predicted Arabidopsis proteins, Artb561-1 (see Fig. 1), was produced and antibodies were raised against this peptide (Eurogentec, Liège, Belgium). Again the results were negative, showing no specific cross-reactivity in either bean PMs or even in Arabidopsis membrane fractions (A. Bérczi and H. Asard unpubl. results). This result may be explained on the basis of different folding of the 20-amino-acid peptide as compared to the native protein. Further work is in progress with a different set of antibodies raised against the bovine cyt b_{561} (Hunter et al. 1982).

Homology of plant and animal cyts b_{561}

As indicated earlier, it was recognised early during the investigations on the plant PM cyt b_{561} that this protein actually showed a remarkable homology at the biochemical level to the mammalian cyt b_{561}. Probably the most striking similarity was the demonstration of the trans-PM electron transport reaction, using Asc-loaded PM vesicles (Asard et al. 1992). In much the same way as the chromaffin granule cyt b_{561}, the plant protein apparently transports reducing equivalents across the PM with Asc as an electron donor.

Using the primary structure information available for animal species, several genes have now been identified in plants that show an interesting homology to the mammalian cyt b_{561} sequences. As indicated in Table 2, homologous genes have been found in a variety of plant species, supporting the ubiquitous nature of this protein. However, since in many cases this information is only available at the expressed-

Table 2. Currently available plant sequences showing significant homology to the *A. thaliana* (Artb561-1) cyt b_{561}

Species	Gene	Type	Tissue[a]	% identity	Nr. of amino acids aligned	Accession nr.
Arabidopsis thaliana	*Artb561-1*	genomic and incomplete EST	mixed	100	280	R6513
	Artb561-2	genomic and incomplete EST	etiolated seedlings (5 days)	45.1	204	AF132115
	Artb561-3	genomic		33.8	206	AC006917
Craterostigma plantaginum	*Crpb561*	genomic		68.7	176	AJ251963
Glycine max	*Glmb561-1*	incomplete EST	immature flowers	68.0	121	AW099442
	Glmb561-2	incomplete EST	8-day-old roots	66.8	219	AI443292, AI438059
Gossypium hirsutum	*Gohb561*	incomplete EST	abscission zone	41.2	114	AI055670
Lotus japonicus	*Lojb561*	incomplete EST	roots and root nodule primordia	84.6	78	AW164178
Lycopersicon esculentum	*Lyeb671-1*	incomplete EST	leaves	71.7	223	AI780400, AI772650
	Lyeb561-2	incomplete EST	fruit (pericarp)	42.8	197	AW22220
Mesembryanthemum crystallinum	*Mecb561*	incomplete EST	roots	39.6	197	AF097661
Oryza sativa	*Orsb561*	genomic		49.3	230	AC078840
Zea mays	*Zemb561-1*	incomplete EST	roots	59.5	167	AW46627
	Zemb561-2	incomplete EST	leaf primordia	31.9	63	AW461539
	Zemb561-3	incomplete EST	mixed stage	55.2	13	AW738212

[a] Tissue sources are indicated for the ESTs

sequence-tag (EST) level, the primary sequence of putative cyt b_{561} genes is often incomplete. Nevertheless, as discussed below, the comparison of structural elements reveals striking similarities, leaving little doubt on the similarity between the derived proteins. An initial report on the comparison between the chromaffin granule cyt b_{561} and two Arabidopsis genes has recently been presented (Asard et al. 1998). In the following section this comparison is extended to several other plant species, yielding new information on the conserved properties and amino acid sequences of the proteins.

A nomenclature for the cyt b_{561} protein

Since homology searches reveal the presence of genes coding for proteins similar to the mammalian cyt b_{561} in nearly all plant species for which a considerable amount of EST or genomic information is available, there is an increased need for a systematic nomenclature. The genes in *A. thaliana* have previously been named *Atb561* (for *Arabidopsis thaliana* cyt b_{561}) (Asard et al. 1998). However, since it becomes clear that the cyt b_{561} actually represents a novel class of proteins, even with a number of homologues within the same plant species, we suggest here to adopt a more systematic nomenclature. The designation "b561" seems useful to refer to this particular class of proteins, and discrimination between different species could be achieved by a prefix of three letters. Thus, the common name for the cyt b_{561} genes and proteins would be "*Xxyb561-n*", with "Xx" representing the first two letters of the genus name, "y" the first letter of the species name, and "n" the nth gene in that species. The cyt b_{561} homologue in *A. thaliana* would thus be *Artb561-1* for the first gene (previously named *Atb561-A*) and *Hosb561* would refer to the human cyt b_{561} gene.

Homologous genes in plant species

Table 2 summarises the information that is available so far at the gene level for a number of different plant species. It is clear from these results that a large variety of plants, including monocotyledonous and dicotyledonous species, contain genes with similarity to the Arabidopsis and mammalian cyt b_{561} sequences. However, in several species only partial sequence information is available, obtained from the sequencing of ESTs. To our knowledge, information on the complete genes is available only in the case of *A. thaliana*, rice, and watermelon (K. Akashi, Nara Institute of

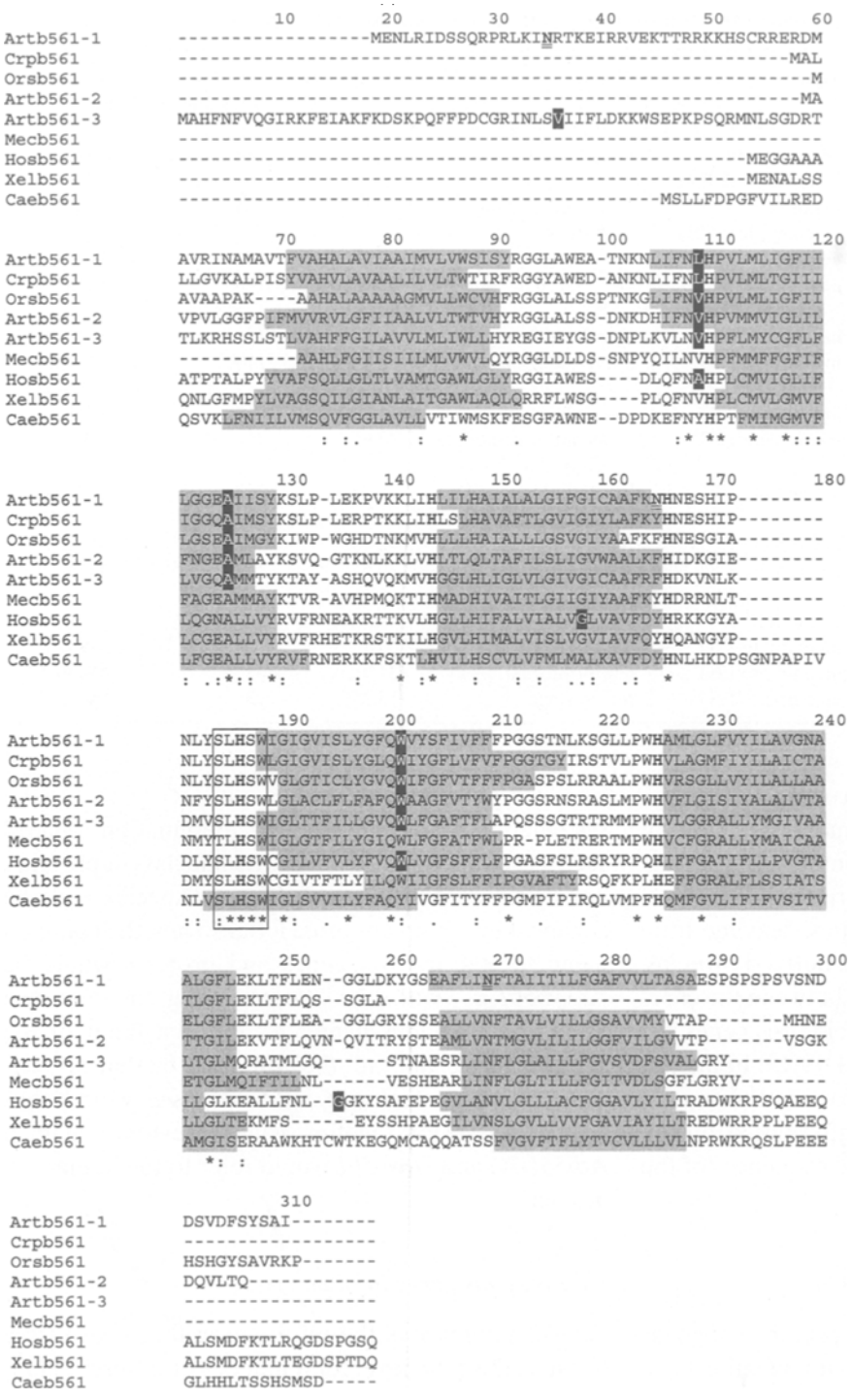

Fig. 1. Multiple amino acid alignment (CLUSTAL W; Thompson et al. 1994) of presumptive plant cyt b_{561} sequences, compared to human (Hosb561), *Xenopus laevis* (Xelb561), and *Caenorhabditis elegans* cyt b_{561} (Caeb561). The plant species are *A. thaliana* (Artb561-n), *Craterostigma plantagineum* (Crpb561), *Mesembryanthemum crystallinum* (Mecb561), and *Oryza sativa* (Orsb561). Shaded areas represent predicted transmembrane regions (TMpred; Hofmann and Stoffel 1993). The boxed areas represent the predicted Asc and MDA binding sites. Black sites are splicing sites (first amino acid of new exon, where the genomic structure is known). Putative Asn glycosylation sites are marked (double underline) at positions 35, 165, and 269 of the Artb561-1 sequence. Conserved His residues are at positions 110, 144, 148, 166, 186, and 225. See text for details

Science and Technology, Takayama-cho, Japan, unpubl. results). A multiple alignment of the available plant sequences is presented in Fig. 1. ESTs are not included in Fig. 1.

In a number of plants it is clear that there is more than one gene coding for a cyt b_{561}-like protein, suggesting the presence of multigene families. In the cases of *A. thaliana* and watermelon, respectively, three and two genes have already been identified. The third Arabidopsis gene (*Artb561-3*) was identified after a reanalysis (WebGeneMark, M. Borodovsky unpubl.) of the intron-exon organisation in a bacterial artificial chromosome clone from chromosome 1 (accession nr. AC006917) and has not been published. Each of the

plant genes shows a low but significant homology (around 30% identity) to, for example, *Hosb561*. This apparently low homology may explain the failure to show cross-reactivity of the plant proteins with antibodies against the mammalian cyt b_{561}. Interestingly, the homology between the predicted proteins within one plant species was relatively low when compared to the homology between the cyt b_{561} proteins in different mammals. For example, the level of identity between Artb561-1 and Artb561-2 is 41%, which is significantly lower than that of the human (Hosb561) with the pig (84%) or the sheep cyt b_{561} (86.5%). On the other hand, the identity between the human cyt b_{561} and a homologous protein in *Caenorhabditis elegans* is only 33.5%. It thus seems that the presumptive plant cyt b_{561} proteins show a rather low overall conservation of the primary structure, even within one species. The conservation of a number of structural elements, as will be discussed below, is therefore even more striking.

In addition to the structural homology between the Arabidopsis and the mammalian sequences at the protein level (see below), also the gene organisation shows significant similarities. The number of exons identified in the plant (*Artb561-1*) and mammalian genes (*Hosb561*) were 4 (Asard et al. 2000) and 5 (Srivastava et al. 1994, Srivastava 1995), respectively. However, two of the three splicing sites in *A. thaliana* are perfectly conserved in the human sequence (see Fig. 1). In addition, the starting amino acid residue for each of the exons seems also highly conserved in the other plant proteins.

With respect to the translation initiation site, some question remains in the *Artb561* genes. The genomic information for *Artb561-1* indicates the presence of two potential starting sites for translation, with the second one 123 bp downstream of the first. The proteins predicted from the two different open reading frames are 280 and 239 amino acids long. It thus remains an open question whether the first or second initiation site is used for the translation of the functional protein. A similar problem was encountered in the bovine protein in which two Met codons could potentially indicate the start of the protein. However, the protein analysis (Srivastava 1996) suggested that the expressed bovine protein was the short version and thus 21 amino acids shorter than the previously reported sequence (Perin et al. 1988). On the basis of the multiple alignment analysis of the plant proteins (Fig. 1) it seems likely that also in the case of *A. thaliana* actually the shorter Artb561-1 version (239 amino acids) may represent the native protein. It is interesting to note that the third Arabidopsis gene (*Artb561-3*) (see Fig. 1) also encodes a relatively long protein (275 amino acids) and has a downstream Met residue that may constitute the actual N terminus of the protein. Moreover, the first 40–50 amino acids at the N terminus of the potentially longer version of Artb561-1 and Artb561-3 show little or no homology. The true length of Artb561-1 is currently under investigation by expression analysis of the two possible open reading frames and combining this with deletion analysis of promoter fragments fused to a reporter gene.

An interesting case of gene homology is found in *Glycine max*. In this species a number of ESTs have been identified which segregate into two clusters when a multiple alignment analysis is performed (*Glmb561-1* and *Glmb561-2* in Table 2). Within each cluster the ESTs overlap at least partially and are identical in the overlapping regions. When the proteins encoded by the two sets of ESTs are compared, they show a very high similarity (98.5% identical) in the N-terminal 130 amino acids but are completely divergent towards the C terminus (no identity in 29 amino acids). The first amino acid at which the Glmb561-1 and Glmb561-2 sequences differ exactly coincides with the starting point of the exon 4 in the *Artb561* genes, suggesting the possibility of alternative splicing. Interestingly, the EST clones for *Glmb561-1* are derived from flower cDNA libraries, whereas those for the *Glmb561-2* gene are derived from root tissues. These results suggest that at least in *Glycine max* two different genes are present which may be expressed selectively in different tissues. The Arabidopsis genes were obtained from mixed-tissue cDNA libraries preventing any conclusion on their differential expression.

Finally, another remarkable observation from the BLAST and FASTA searches for homologous genes is that no significant matches are evident in *S. cerevisiae* or in prokaryotes. Since the yeast genome has been completely sequenced, this seems to suggest that no cyt b_{561} homologue exists in this species. This result is in apparent agreement with the fact that it was not possible to demonstrate the presence of an Asc-reducible cyt b_{561} in PM-enriched fractions from *S. pombe* (see above). However, the observation that no homologous protein was identified in prokaryotes so far is somewhat puzzling. It raises the question on the evolutionary origin of the cyt b_{561} protein and whether

this protein may be exclusively found in eukaryotic cells. Clearly more extensive comparisons should be made with the progressive availability of new sequence information.

Comparison of the cyt b_{561} homologues at the protein level

Although the sequence homology between the plant and animal primary structures was in general not higher than 30% identity, the homology between these proteins becomes more striking when a detailed comparison of the structural properties is made. These elements are discussed in the following section.

One remarkable homology between the plant and animal sequences is the very high conservation of the potential membrane-spanning structures (grey areas in Fig. 1). Clearly the cyt b_{561} is a highly hydrophobic protein and 6 potentially trans-membrane regions were predicted (TMPred software; Hoffman and Stoffel 1993) with both the N- and C-terminal ends facing the cytoplasm. All of these regions are well conserved among all proteins and coincide with highly hydrophobic domains revealed by a hydropathy plot analysis (Asard et al. 2000). It seems therefore likely that the plant PM cyt b_{561} proteins effectively contain 6 membrane-spanning structures. This model conforms to that predicted for the mammalian cyt b_{561} by several authors (Perin et al. 1988, Degli Esposti et al. 1989a, Okuyama et al. 1998). The 6-helix model has however been disputed by Srivastava on the basis of biochemical evidence and the intron-exon analysis (Srivastava et al. 1994, Srivastava 1996). Biochemical evidence indeed suggested that the N- and C-terminal parts of the protein may be located at opposite sides of the membrane (Kent and Fleming 1990). Srivastava also suggested that the 5 trans-membrane helices would coincide with the 5 exons identified for *Hosb561*. However, as has been argued (Degli Esposti et al. 1989a) the 6-helix model would provide a better organisation to accommodate for the possible presence of two heme molecules in the protein, both ligated by His residues. It was suggested that these His residues are located in helices II and IV as well as III and V, respectively (Degli Esposti et al. 1989a, Okuyama et al. 1998). The 6-helix model is also in agreement with circular dichroism data obtained by Burbaev et al. (1991), suggesting exclusive His-ligation for the cyt b_{561} hemes and thus disproving the involvement of a methionyl axial ligand as previously suggested (Perin et al. 1988). Clearly, the elucidation of the trans-membrane structure of cyt b_{561} in plants and mammals will need more conclusive biochemical evidence. A model depicting the current working model of 6 trans-membrane helices for the cyt b_{561} is presented in Fig. 2.

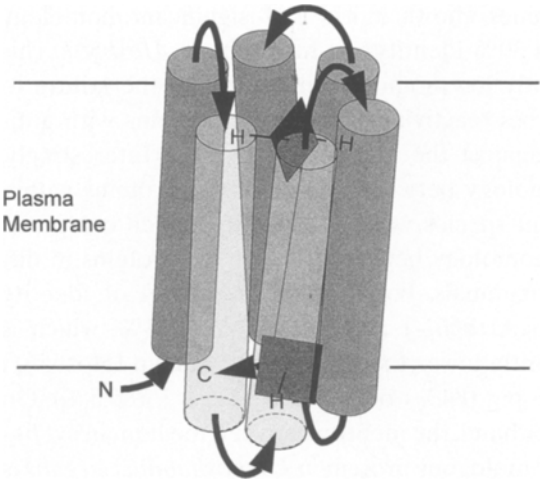

Fig. 2. Model representing the working hypothesis on the molecular structure of cyt b_{561}. Rods represent the six predicted trans-membrane helices and the parallelograms represent the hemes. The predicted cytoplasmic location of N and C termini are indicated

Considering the ligation of the two hemes in cyt b_{561} by His residues, one would expect the strong conservation of at least 4 His residues among the different proteins. As pointed out (Okuyama et al. 1998), 6 His residues were conserved in the animal cyt b_{561} proteins, of which 5, His-110, His-144, His-148, His-186, and His-225 (Fig. 1), could potentially be involved in the heme ligation, according to these authors. When comparing the mammalian sequences to those obtained from plants there is an obviously very high level of conservation of these 6 His residues (Fig. 1). These His residues were also found conserved in all the EST sequences that were identified (not shown). There is one notable exception to this conservation. In Artb561-2, the His-148 is replaced by Gln. It is therefore unlikely that this His participates in heme ligation in the other proteins. Recent evidence obtained by the modification of His residues by the reagent diethyl pyrocarbonate supports this conclusion (Tsubaki et al. 2000). It was found that modification of His-144 and His-225 resulted in the impaired transfer of electrons from Asc, suggesting the participation of these residues in the heme ligation. The high conservation of these His amino acids between the plant and animal cyt b_{561} homologues strongly supports a functional

importance for these residues in the functioning of the protein.

Two well-conserved regions in the mammalian cyt b_{561}, ^{125}ALLVYRVFR^{133} and ^{184}SLHSW^{188} (Fig. 1), have previously been considered as potential binding sites for Asc and MDA, respectively (Okuyama et al. 1998). These regions appear also to be fairly well conserved in most of the other sequences (Fig. 1). The SLHSW region is almost completely conserved in all plant sequences with exceptions in *Mesembryanthemum crystallinum* (Mecb561) and in an EST from *Zea mays* (not shown). In contrast, the ALLVYRVFR sequence is only partially conserved in the other cyt b_{561} sequences. A comparison between the available plant and animal primary structures results in the consensus sequence AhhxYcxxx, with h designating a small hydrophobic residue and c a positively charged residue. The absence of this sequence in a previous report (Asard et al. 1998) was apparently due to an error in the database and the corrected version has now been deposited. The apparent conservation of these 9- and 5-amino-acid sequences in all species not only supports the homology between the plant and animal proteins but also supports their functional importance in the action of the cyt b_{561}. It should however be kept in mind that there is no actual experimental evidence that these conserved motifs are effectively involved in Asc and/or MDA binding, and care should be taken when attributing any functional meaning to them.

Finally it should be mentioned that the plant sequences have some properties which were not found in the mammalian proteins. For example, Artb561-1 has three predicted N-glycosylation sites (Asn-35, Asn-165, and Asn-269), whereas no predicted glycosylation sites were identified in Artb561-2 or in the mammalian proteins. This prediction nicely matches the experimental evidence on the potential glycosylation of the partially purified cyt b_{561} from bean (Trost et al. 2000). However, one of the predicted glycosylation sites (Asn-35) is predicted to be located in the cytoplasm between the N-terminal part of the protein and the first trans-membrane region, which seems rather unlikely. In the shorter version of Artb561-1, this potential glycosylation site is absent.

In summary, the detailed comparison of the structural and functional elements in the primary structure of the animal cyt b_{561} to sequences obtained from plant genes strongly supports the homology between these proteins. The sequence information therefore seems to complement the biochemical evidence on the similarity of these proteins. However, direct experimental proof on the cyt *b* nature of the proteins encoded by the homologous genes still needs to be provided.

A protein in search of a function

One major question raised with respect to the plant cyt b_{561} is related to what its physiological function in the plant cell may be. Although the experimental evidence at this level is still limited, some hypotheses can be put forward.

PM cyt b_{561} and blue-light perception

As indicated at the beginning of this paper, the presence of *b*-type cytochromes in higher-plant PMs is closely related to the investigation of the action mechanism of blue-light perception. The initial discovery of the reduction of a cyt *b* by blue-light irradiation (Muñoz and Butler 1975, Poff and Butler 1975) pointed to the possible involvement of this protein in blue-light responses. Moreover, the blue-light-mediated cytochrome reduction showed an action spectrum that was very similar to that of many blue-light reactions (Widell et al. 1983), suggesting that the cytochrome may actually be tightly linked to the perception of the light signal or to the early signal transduction of this process. Several lines of experimental evidence indeed suggest that it is actually the high-potential cyt b_{561} which is reduced by the blue-light irradiation in purified PM fractions (Caubergs et al. 1988, Asard and Caubergs 1990). It was also noted that the blue-light-mediated cytochrome reduction could be mediated by an endogenous PM chromophore, i.e., without the addition of external flavins (Asard et al. 1995a).

In the meantime there has been considerable progress in the understanding of blue-light photoperception in plants. A major breakthrough was the discovery of a number of blue-light perception mutants in *A. thaliana* (Ahmad and Cashmore 1995, Liscum and Hangarter 1994, Liscum and Briggs 1995) and the subsequent identification of a 120 kDa serine-threonine protein kinase as the possible photoreceptor (Christie et al. 1998, 1999). This kinase autophosphorylates upon blue-light illumination and binds flavin mononucleotide as a chromophore. It is suggested that NPH1 is the photoreceptor for the phototropic response in plants (Christie et al. 1999).

However, it became also clear that other blue-light responses such as stomatal opening are likely controlled by a photoreceptor distinct from NPH1 and genetically separable (Lascève et al. 1999). It is clear from these results that the participation of the cyt b_{561} as the primary photoreceptor in these responses is no longer obvious. However, since it was suggested that up to four different signal transduction pathways may be operational in *A. thaliana* (Lascève et al. 1999), it is premature to fully exclude the possibility that the PM cyt b_{561} could be involved in the early signal transduction of one of these reaction mechanisms.

Cyt b_{561} transports electrons across the PM

A few years ago, a discovery was made which shed new light on the function of the PM cyt b_{561}. It was demonstrated that the cytochrome is capable of transferring reducing equivalents across the PM (Asard et al. 1992, 1995b; Horemans et al. 1994). The initial observation was that similar reductions of PM cytochromes occurred when Asc was presented either on the outside of sealed PM vesicles or in the presence of a mild detergent to disrupt the vesicle membrane. These similar reduction levels suggested that the Asc-reducible cytochrome could be a trans-membrane protein, as had been suggested for the chromaffin granule cyt b_{561} (e.g., Njus et al. 1987). Subsequent experiments using Asc-loaded PM vesicles demonstrated that electrons from the vesicle interior were effectively transferred to impermeant electron acceptors outside the vesicle (Asard et al. 1992). It was also established that this transport was electrogenic, resulting in an inside positive charge gradient which could be abolished by ionophores (Asard et al. 1995b). Since Asc is present in considerable amounts inside the plant cell (Rautenkranz et al. 1994, Foyer and Lelandais 1996, Horemans et al. 2000a), it may well serve as a natural electron donor to the trans-PM electron transport.

The demonstration of the trans-membrane electron transport capacity of both the plant and the mammalian cyt b_{561} clearly suggests that this may be the common biochemical function of this class of proteins. Asc is a common constituent in the cytoplasm and organelles of many cells and does not readily permeate through the lipid bilayer. The cytochromes b_{561} therefore provide a "cellular instrument" to transfer electrons from one compartment to another, using a ubiquitous electron donor. This biochemical reaction is thus likely to support a variety of different cellular functions, most likely determined by the nature of the electron acceptor residing on the opposite side of the membrane. In the example of chromaffin granules, the electron acceptor was suggested to be MDA (Kelley and Njus 1986, Wakefield et al. 1986). In this organelle the cyt b_{561} thus re-reduces the Asc free radical (MDA) to Asc which then functions as an electron donor in the peptide hormone biosynthesis. Although experimental evidence indicates that the plant PM cyt b_{561} may also transfer electrons to MDA (Horemans et al. 1994), it is not yet clear whether this reaction also occurs in vivo. The plant PM has recently been shown to contain specific carriers transporting Asc and dehydroascorbate (Horemans et al. 2000a), which may provide a more efficient means of supplying Asc to the apoplast.

It should be kept in mind that there is circumstantial evidence for the presence of cyt b_{561} on membranes distinct from the PM. As discussed earlier, some experiments indicated the presence of an Asc-reducible cyt *b* in nonenergetic membranes in bean hypocotyls (Asard et al. 1987, Scagliarini et al. 1998). In addition, the *A. thaliana* Artb561-2 protein was predicted to have a slightly higher probability of being located at the endoplasmic reticulum (68.5%) than at the PM (64%) (PSORT Software; Nakai and Kanehisa 1992). One could therefore cautiously speculate that the Asc-mediated trans-membrane electron transport function may also be operational in other membrane types.

Cyt b_{561} and the plant cell wall

The elucidation of the cyt b_{561} involvement in trans-PM electron transport in plants strongly suggested a role of this protein in the redox balance of the apoplast. In addition, its widespread occurrence in plants suggests a "housekeeping" function rather than a highly specialised tissue-dependent function. Since the major housekeeping in the apoplast of plant cells is related to the synthesis and modification of the cell wall, we hypothesise that the cyt b_{561} may be involved in the control of plant cells growth.

It is well know that redox reactions and in particular the redox balance of small molecules is essential in the biosynthesis and cross-linking of cell wall polymers. A considerable number of experiments demonstrated that Asc is probably a key factor in the building and cross-linking of the cell wall polymers (see discussion in Smirnoff 1996, Horemans et al. 2000b). For

example, there is convincing information on the role of Asc in cell growth and cell division (Arrigoni 1994; González-Reyes et al. 1995, 1998). The presence of Asc is apparently necessary to proceed through the cell cycle and levels of Asc and dehydroascorbate change during cell division (de Pinto et al. 1999, Kato and Esaka 1999, Potters et al. 2000). It thus seems that the redox status of molecules such as Asc and glutathione plays a central role in the control of cell growth (Reichheld et al. 1999). In addition, Asc plays an important role in the control of plant cell elongation. For example, the biosynthesis of hydroxyproline-rich cell wall proteins is catalysed by prolyl-4-hydroxylase, which is likely to depend on Asc (De Gara et al. 1991). Asc is also likely to interfere with the activity of cell wall peroxidases, thereby affecting the cross-linking of hydroxyproline-rich proteins with phenolic acids (Cooper and Varner 1984, Takahama and Oniki 1992, Sanchez et al. 1997). It is thus clear that the redox control of cell wall phenomena involves Asc and H_2O_2 as key molecules.

The elucidation of the physiological role of the PM cyt b_{561} is closely linked to the identification of the in vivo electron acceptor on the outer side of the PM. As discussed earlier, MDA may function in this role, at least in isolated PM vesicles (Horemans et al. 1994). If this reaction also occurs in vivo, the role for cyt b_{561} in the cell wall metabolism lies principally in the regeneration of Asc, thereby supporting a number of the above mentioned enzymatic reactions in which Asc is involved. However, it is also possible that the cyt b_{561} has yet another electron acceptor, more closely related to a direct enzymatic function. Of interest in this respect is the suggestion by V. Preger et al. (University of Bologna, Bologna, Italy, unpubl. result) that a specific apoplastic Asc peroxidase may be accepting the cyt b_{561} electrons. This enzyme could then reduce H_2O_2, providing a system to modulate H_2O_2 levels in the cell wall.

Another indirect and merely suggestive observation of a coupling of cyt b_{561} to cell wall metabolism is the apparent absence of this protein in the PM of the cell-wall-deficient green alga *Dunaliella* sp. (A. Katz pers. commun.). It is thus tempting to speculate that the lack of a rigid polysaccharide cell wall is correlated to the specific absence of the cyt b_{561} in the PM of this organism. However, *Dunaliella* sp. is a halotolerant alga and several other of its PM functions may show specific adaptations to these conditions.

Although a role for the cyt b_{561} in cell wall metabolism and cell growth and division can be envisaged, the exact mechanism and specific effects on the plant cell function remain to be clarified.

PM cyt b_{561} possibly linked functionally to other PM redox components

Since it has been convincingly shown that the PM contains a number of specific redox components (Bérczi and Asard 1995, Lüthje et al. 1997, Bérczi et al. 1998, Döring et al. 1998, Bérczi and Møller 2000), the possibility that the cyt b_{561} is somehow functionally coupled to the action of these enzymes should be considered. Several models have been presented in the past suggesting a functional coupling between flavin-containing oxidoreductases in the PM and a *b*-type cytochrome (Lin 1984, Marrè et al. 1988, Dahse et al. 1989, Raghavendra 1990, Lüthje et al. 1997). Initial attempts however to relate PM NADH oxidase activity to reduction levels of the *b*-type cytochrome were unsuccessful (Asard et al. 1997). Experiments directed toward the purification of PM redox components have also revealed that NAD(P)H-acceptor reductase activities were readily separated from cyt b_{561} (Van Gestelen et al. 1996, 1997), suggesting that these proteins were at least not tightly connected in protein complexes.

Nevertheless, PM redox enzyme activity and cyt b_{561} may be functionally related. An initial and most obvious suggestion is the interaction of the cytochrome with the PM MDA reductase. The latter enzyme has recently been purified and identified by Bérczi and Møller (1998). This protein is located at the cytoplasmic face of the PM and uses NADH to re-reduce the MDA radical generated in the cytoplasm. Since the one-electron transfer of Asc to reduce the cyt b_{561} generates MDA, the action of the MDA reductase may efficiently regenerate the cytoplasmic Asc at the expense of NADH.

Another coupling between the activity of PM redox enzymes and the PM cyt b_{561} may be achieved through the action of a lipid soluble "redox mediator". Since some of the PM redox enzymes actually possess quinone reductase activity (Serrano et al. 1994, Trost et al. 1997, Bérczi and Møller 2000), one could assume that the reduced quinone generated in this reaction may serve as an electron donor to the PM cyt *b*. Although some evidence exists that such a reaction may occur, at least in vitro (S. Lüthje, University of Hamburg, Hamburg, unpubl. results), the nature of this redox mediator still remains to be determined.

In any case, the affinity of cyt *b* for this mediator should be significantly higher than that for Asc since the latter molecule is present in the cytoplasm in high concentrations.

Interestingly in both cases of the coupling of the cyt *b*-mediated electron transport to the action of an NAD(P)H-dependent redox enzyme on the cytoplasmic side of the PM, the net result at the cellular level would be the apparent trans-membrane transport of electrons with the consumption of reduced pyridine nucleotides. As has been pointed out (Bérczi and Møller 2000), the PM cyt b_{561} is the only protein so far which has been experimentally shown to effectively transport electrons across the PM. However, the coupling to other redox enzymes may provide an explanation for the suggestion that NAD(P)H could also function as a an electron donor to trans-PM electron transport (Döring et al. 1998).

Prospects for future work

The increasing evidence regarding the similarity between the animal and plant cyt b_{561} and the recent availability of a number of genes potentially coding for the plant PM cyt b_{561} stimulate new approaches in the study of these proteins. Of particular interest is the possibility to employ molecular biological techniques to increase our understanding. Heterologous expression of the plant genes may result in the production of significant quantities of recombinant cyt b_{561} that could be used to experimentally verify its nature. Functional expression systems should also provide a source, for example, to raise antibodies and to study the electron transfer mechanism(s) after reconstitution in a lipid bilayer. A molecular biological approach should also help unravel the detailed functional role of the PM cyt b_{561} in plant cell physiology, either through overexpression or generation of antisense plants with altered levels of cyt b_{561}. This functional analysis should also be expanded to the detailed study of the cyt b_{561} promoter sequences and their regulation in plant development.

In addition, it will be of interest to further develop purification protocols to isolate the native cyt b_{561} from the PM. Although significant progress has been made at this level, no sequence data have yet been obtained to unambiguously identify the cytochrome in the purified protein fractions.

However, it seems likely from the available information that the PM cyt b_{561} may have a major contribution to the physiology of the plant cell, perhaps a potential constitutive role in the regulation of different components of the plant cell wall. Moreover, this protein is likely a representative of a novel protein family widespread in plant and animal cells providing trans-membrane electron transport mechanisms which may support a variety of cellular functions. Cumulative indirect evidence has led to the formulation of a working model for the protein's physiological role, and this needs to be tested with the combination of the newly emerging tools at both the molecular and biochemical levels.

Note added in proof. In a recent paper (McKie et al., Science 291: 1755–1759, 2001) a new role for the mammalian cyt b_{561} in the extracellular reduction of iron was proposed. This result opens new perspectives for the possible physiological function of the plant PM cyt b_{561} and should be investigated in the future.

Acknowledgments

This work was supported by grants from the Flemish (BIL 99/48) and Hungarian (TÉT B-4/99) governments. H.A. is a Research Associate at the Belgian Fund for Scientific Research (FWO-Vlaanderen). We acknowledge Dr. P. Trost (University of Bologna) and Dr. J. M. Stone (Massachusetts General Hospital) for helpful comments and suggestions on the manuscript.

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