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Uclacyanins, stellacyanins, and plantacyanins are distinct subfamilies of phytocyanins: Plant-specific mononuclear blue copper proteins

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Abstract

The cDNAs encoding plantacyanin from spinach were isolated and characterized. In addition, four new cDNA sequences from *Arabidopsis* ESTs were identified that encode polypeptides resembling phytocyanins, plant-specific proteins constituting a distinct family of mononuclear blue copper proteins. One of them encodes plantacyanin from *Arabidopsis*, while three others, designated as uclacyanin 1, 2, and 3, encode protein precursors that are closely related to precursors of stellacyanins and a blue copper protein from pea pods. Comparative analyses with known phytocyanins allow further classification of these proteins into three distinct subfamilies designated as uclacyanins, stellacyanins, and plantacyanins. This specification is based on (1) their spectroscopic properties, (2) their glycosylation state, (3) the domain organization of their precursors, and (4) their copper-binding amino acids. The recombinant copper binding domain of *Arabidopsis* uclacyanin 1 was expressed, purified, and shown to bind a copper atom in a fashion known as "blue" or type 1. The mutant of cucumber stellacyanin in which the glutamine axial ligand was substituted by a methionine (Q99M) was purified and shown to possess spectroscopic properties similar to uclacyanin 1 rather than to plantacyanins. Its redox potential was determined by cyclic voltammetry to be +420 mV, a value that is significantly higher than that determined for the wild-type protein (+260 mV). The available structural data suggest that stellacyanins (and possibly other phytocyanins) might not be diffusible electron-transfer proteins participating in long-range electron-transfer processes. Conceivably, they are involved in redox reactions occurring during primary defense responses in plants and/or in lignin formation.

Keywords: blue copper protein; cDNA; lignin formation; oxidative burst; plant cell wall; plantacyanin; stellacyanin; uclacyanin

Plantacyanin, stellacyanin, umecyanin, and mavicyanin are plant specific proteins that contain a single copper ion bound in a type 1, or "blue" configuration. They display common spectroscopic properties that differentiate them from another plant monocopper blue protein, plastocyanin. Based on this spectroscopic difference, it was originally proposed that they be combined into a separate family named phytocyanins. This classification was further supported by a high degree of identity in their amino acid sequences (Ryden & Lundgren, 1979; Ryden & Hunt, 1993). In addition, two members of the phytocyanin family, cucumber basic protein (CBP, plantacyanin[6]) and cucumber stellacyanin have been recently char-

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Abbreviations: EPR, electron paramagnetic resonance; IPTG, isopropyl-β-D-thiogalactopyranoside; EST, expressed sequence tag; PCR, polymerase chain reaction; LMCT, ligand-to-metal charge transfer; CV, cyclic voltammetry; CST, cucumber stellacyanin; UMC, umecyanin; BCB, blue copper binding protein; STC, *Rhus vernicifera* stellacyanin; MAV, mavicyanin; PEA, pea pods blue copper protein; UCC1, UCC2, and UCC3, uclacyanins 1, 2, and 3, respectively; PINE, protein encoded by a water stress-related gene from Loblolly Pine; CBP, cucumber plantacyanin; PNC, spinach plantacyanin; ARPN, *Arabidopsis* plantacyanin.

[6]The name cucumber basic protein (CBP) indicates that this protein is specific only for cucumber. However, similar proteins possessing identical spectroscopic features and physico-chemical properties have been characterized from other plant species, in particular, from spinach (Aikazyan & Nalbandyan, 1981; Nersissian & Nalbandyan, 1988). We present in this report the amino acid sequences for plantacyanins from spinach and *Arabidopsis* as derived from their cDNA sequences. The precursors of these proteins have identical features, and in addition, the sequences of the mature proteins share more than 60% identity to the known sequence of the protein from cucumber. Hence, we use plantacyanin as a common name for these proteins as proposed in 1974 (Markossian et al., 1974).

acterized by high-resolution X-ray crystallography (Guss et al., 1996; Hart et al., 1996). Although both of these crystal structures reveal a β-barrel fold that resembles other crystallographically characterized blue copper proteins including plastocyanins, they also exhibit an interesting "unclosed" feature of the barrel that arises from an unusual twist of the two β-sheets that form the barrel. Two additional structural features of cucumber plantacyanin and cucumber stellacyanin support their classification as members of a distinct family of cupredoxins. First, both His ligands are entirely exposed to the solvent with their copper-distal imidazole nitrogens oriented toward the surface of the protein molecule. This structural arrangement results in a significantly more accessible copper site in phytocyanins relative to other cupredoxins, in which the copper center is more buried within the protein, with only one His ligand exposed to the surface. Second, phytocyanins are characterized by the presence of three invariant cysteine residues, two more than are found in most cupredoxins. One cysteine serves as a copper ligand, while the two others form a disulfide bridge. This disulfide bridge may play a crucial role in maintaining the tertiary structure of the protein and/or the formation of the copper binding center because one of the His ligands of copper is followed directly by a bridging Cys residue.

Stellacyanins are distinguished from plantacyanins and other cupredoxins by the nature of the amino acid residue coordinated to the axial site of copper. The refined structure of cucumber stellacyanin shows that the fourth ligand (in addition to two His and one Cys) is provided by the ϵ-carbonyl oxygen of a Gln (Hart et al., 1996). At a distance of 2.2 Å, this residue is bound more tightly than the weak thioether sulfur from Met (2.6–3.1 Å) that characterizes the binding site in other blue copper proteins.

It is generally accepted that cupredoxins serve as mobile electron carriers in a variety of charge-transport systems. For example, it is well established that plastocyanins act as diffusable electron-transfer proteins in the lumenal site of the thylakoid membranes between cytochrome b6/f complex and photo-oxidized reaction center of photosystem 1 (Hippler et al., 1996). Interestingly, the same cupredoxin may have more than one reaction partner, i.e., a given cupredoxin can mediate electron transfer between different donors and acceptors in vitro as long as the partners display compatibility with respect to their redox potentials and protein–protein interactions.

In contrast, little is known about the function of phytocyanins. Preliminary data on their biochemical/biophysical properties and unique structural features, for example, highly exposed copper site, suggest that phytocyanins are likely involved in redox reactions with small molecular weight compounds, rather than with protein electron mediators (Hart et al., 1996; Nersissian et al., 1996).

The pronounced differences between phytocyanins and plastocyanins are also evidenced by their distinct subcellular locations, suggestive of diverse evolutionary origin. Plastocyanins are chloroplast proteins. Based on the endosymbiont hypothesis, it is believed that they originated from an archaic photosynthetic bacterium, which was domesticated by a protoeucaryotic cell as a separate organelle, the chloroplast. Throughout evolution the plastocyanin gene, like many other protochloroplastic genes, was transferred into the nucleus and acquired a N-terminal transit peptide that targets the precursor protein into the chloroplasts. In contrast, phytocyanins harbor classical signal peptides in their precursors that direct them into the endoplasmic reticulum.

We previously isolated a full-length cDNA encoding cucumber stellacyanin (Nersissian et al., 1996). The protein in its mature form, isolated from the plant, is a chimeric protein comprised of a 109-amino acid copper-binding domain (domain II) and a hydroxyproline- and serine-rich domain (domain III), which resembles the motifs found in plant cell-wall structural glycoproteins (Mann et al., 1992). The precursor stellacyanin sequence differs from the mature protein in that two additional hydrophobic domains flank the mature protein. Its N-terminus is a 23-amino acid peptide with features characteristic of the endoplasmic reticulum targeting signal peptides (domain I). Its C-terminus is a 22-amino acid hydrophobic polypeptide (domain IV) (Nersissian et al., 1996). This indicates that the stellacyanin precursor undergoes a unique post-translational maturation including processing of both the C- and N-termini.

To date, three genes have been isolated from various plants that encode polypeptides resembling the four-domain organization of the stellacyanin precursor: (1) a blue copper binding protein (BCB) from *Arabidopsis* (Van Gysel et al., 1993); (2) a blue copper protein from pea pods (Drew & Gatehouse, 1994); and (3) a water deficit-inducible gene product from loblolly pine (Chang et al., 1996). Domain II in all of these polypeptides shares a high degree of sequence identity with the copper-binding domain of stellacyanin and plantacyanin, including residues that have been identified as copper-binding ligands via X-ray crystallography. Although the putative blue copper binding sites in these new proteins are apparent, no attempts have yet been made to isolate them from the plant, nor to heterologously express them in order to study their metal binding abilities, spectroscopic properties, and their relationships to other cupredoxins.

In this report we present the sequences of full-length cDNAs that encode the entire precursors of plantacyanins from both spinach and *Arabidopsis*, as well as the cDNAs of three novel copper-binding proteins from *Arabidopsis*, designated uclacyanin[7] 1, 2, and 3. In addition, we present expression and purification protocols for the copper binding domain of uclacyanin 1 and a comparison of its spectroscopic properties with those of other phytocyanins including a Q99M axial ligand mutant of stellacyanin. We propose a further classification of phytocyanins as well.

Results

cDNAs encoding plantacyanin from spinach

Poly(A)$^+$ RNA isolated from green spinach leaves was used to synthesize an oligo-(dT) primed cDNA pool, which was subsequently used for the construction of a λgt11 based library. The same cDNA pool was used as a template, and two degenerate oligonucleotides were designed as primers for amplification of a partial clone by PCR. The first primer was derived from the N-terminal amino acid sequence of plantacyanin isolated from spinach green leaves. The second primer was based on the sequence of a proteolytic peptide, which has been identified to be proximal to the C-terminus of the protein from its comparison with that of known amino acid sequence of plantacyanin from cucumber (Murata et al., 1982). A single 229 bp PCR product with the expected size and correct nucleotide sequence was obtained. We used this partial cDNA clone as a radioactively labeled hybridization probe to screen 600,000 plaques. Two positive plaques were

[7]The name uclacyanin is derived from the abbreviation of University of California Los Angeles.

identified and purified by two rounds of rescreening. The nucleotide sequences of the cDNAs revealed that the inserts are 670 bp (*pnc1*) and 688 bp (*pnc2*) long and possess open reading frames identical in sequence that encode a 121-amino acid precursor plantacyanin polypeptide. They differ only slightly from each other in their 5′ and 3′ untranslated regions (Fig. 1). The precursor protein is composed of a 30-residue hydrophobic N-terminal domain followed by a 91-residue copper binding domain. The N-terminal hydrophobic domain is unusually long for signal peptides (30 residues compared to the 18–25 for others), although it possesses all of the features characteristic of signal peptides. The predicted cleavage site matches the N-terminal amino acid residue of the mature protein isolated from spinach, as determined by protein sequencing. Unlike cucumber stellacyanin, which is a chimeric protein consisting of a copper-binding domain (domain II) and a hydroxyproline and serine-rich sequence domain (domain III) resembling plant cell-wall structural glycoproteins, the mature form of spinach plantacyanin is a single domain protein of 91 amino acid residues. Thus, plantacyanin from spinach is the smallest cupredoxin characterized to date, with a calculated molecular weight of 9.7 kDa.

Plantacyanins are extremely basic proteins, with pIs of approximately 11. Accordingly, spinach plantacyanin possesses a large number of positively charged residues (6 Lys and 7 Arg) but only two negatively charged (Asp) side chains. It displays 53 and 24% amino acid sequence identity to cucumber plantacyanin and to the copper-binding domain of cucumber stellacyanin, respectively. The four putative copper ligands in spinach plantacyanin, an upstream His34, and the triad of Cys74, His79, and Met84 align with similar residues in cucumber plantacyanin that have been previously identified from X-ray crystallography to be involved in copper coordination (Guss et al., 1988). Northern blot analysis on poly(A)$^+$ RNA from both spinach green leaves and etiolated seedlings revealed an approximately 0.75-kb-long single transcript that was equally presented in both preparations and corresponded to the size of the isolated cDNAs (Fig. 2A). This indicated that the isolated cDNAs encode a full-length precursor and that the spinach plantacyanin gene is not a light-regulated gene. Southern blot analysis on nuclear DNA that was digested with different restriction enzymes reveals a simple pattern with one strongly hybridizing band and other weakly hybridizing bands (Fig. 2B). This result implies that the plantacyanin gene exists as a single-copy gene in the spinach haploid genome. The presence of the weak bands suggests that at least three sequence-related genes are present as well.

Arabidopsis cDNAs encoding proteins homologous to phytocyanins

To identify genes that resemble phytocyanins, we employed spinach plantacyanin and cucumber stellacyanin sequences to carry out a homology search with currently available sequences in the GenBank database (TBLASTN program; Altschul et al., 1990). The screening enabled us to identify at least four different partial cDNA sequences from the *Arabidopsis* EST (expressed sequence tag) collection. We have completed the nucleotide sequences of these anonymous cDNA clones that have been generously provided to us by the *Arabidopsis* Biological Resource Center at Ohio State University. The analyses of these sequences show that they encode putative polypeptides with a remarkable similarity to the phytocyanins.

One of the four new sequences (EST stock I.D. 129L9T7) displays a high degree of sequence identity with plantacyanins from both cucumber (67%) and spinach (51%), and includes the four putative copper binding residues (two His, one Cys, and one Met) as well as the disulfide-bridged cysteines. Similar to spinach plantacyanin, the predicted precursor protein consists of only two domains, a 33-amino acid hydrophobic leader peptide and a 96-amino acid copper-binding domain. We have, therefore, assigned it as the gene that encodes plantacyanin from *Arabidopsis*. In Figure 1 its nucleotide and deduced amino acid sequences are compared with those of two cDNAs, *pnc1* and *pnc2*, encoding spinach plantacyanin.

The three other *Arabidopsis* sequences resemble the previously characterized phytocyanins with the four-domain arrangement of their precursors. Domain I, a 21–28-amino acid hydrophobic N-terminal peptide with features characteristic of the classical tripartite signal sequences of secretory proteins (von Heijne, 1986) (e.g., a positively charged N-terminus, correlation of the putative cleavage sites with the -3, -1 rule, and the presence of secondary structure breakers (Pro or Gly) at positions $-4/-5$ after the hydrophobic core of the signal peptide). Domain II, a 96–101-amino acid sequence, shares 23–44% amino acid identity with the copper-binding domains of phytocyanins. Domain III in two cases are Pro and Ser rich 82 and 119 amino acid long peptides, while in one case it is rich in His and Pro and composed of 37 amino acid residues. In these sequences, designated as uclacyanins 1, 2, and 3 (UCC1, UCC2, and UCC3), EST stock I.D. ATTS2364, 116K21T7, and 137J6T7, respectively, domain III closely resembles the peptide motifs found in cell-wall structural proteins. Domain IV, in all three sequences, is a 16–20 amino acid long hydrophobic peptide with hydrophobicity values equal to those determined for signal peptides. This domain is presumably post-translationally processed as observed in the case of cucumber stellacyanin (Nersissian et al., 1996). Hence, we believe that the maturation of these proteins could involve processing of Domains I and IV. All four sequences possess the disulfide-bridged Cys residues matching the analogous residues in plantacyanin and stellacyanin. The four postulated copper ligands (two His, a Cys, and a Met) match in sequence alignment with the residues that have been identified from crystal structures as copper ligands in plantacyanin (cucumber basic protein) (Guss et al., 1988) and stellacyanin (where a Gln residue substitutes Met) (Hart et al., 1996).

In Figure 3, schematic representations of the precursors of two stellacyanins, two plantacyanins, and four uclacyanins are shown. Figure 4 illustrates the multiple sequence alignment of the copper-binding domains of 13 phytocyanins. In addition to the conserved C-terminal copper binding loop, there is another conserved sequence motif near the N-terminus. It is comprised of 11 amino acids, and extends between two aromatic residues, Phe31 and Phe41 (numbers refer to the sequence of cucumber stellacyanin). Five of these 11 residues are invariant in all 12 sequences (the sequence information in this region for loblolly pine protein is not complete). This conserved sequence motif is located in the so-called "southern" end of the protein molecule, opposite the copper binding site ("northern" end of the protein) and consists of Phe ^{31}-X_1-X_2-Gly-Asp^{35}-X_3-Leu-X_4-Phe-X_5-Ar^{41}, where the X_1 in 10 sequences is a positively charged residue Arg or His; X_2 is a hydrophobic residue Val, Ile or Ala; X_3, X_4, X_5 are nonspecific residues; and Ar is an aromatic residue (Phe or Tyr). This conserved motif appears to be homologous to one described in other cupredoxins (SCR4) (Murphy et al., 1997). Similar to what is observed in the SCR4 motif, the side-chain OD1 of Asp35 in stellacyanin (Asp28 in plantacyanin, CBP) hydrogen bonds to the backbone amide nitrogen from the upstream third residue of the motif (X_1 above), which

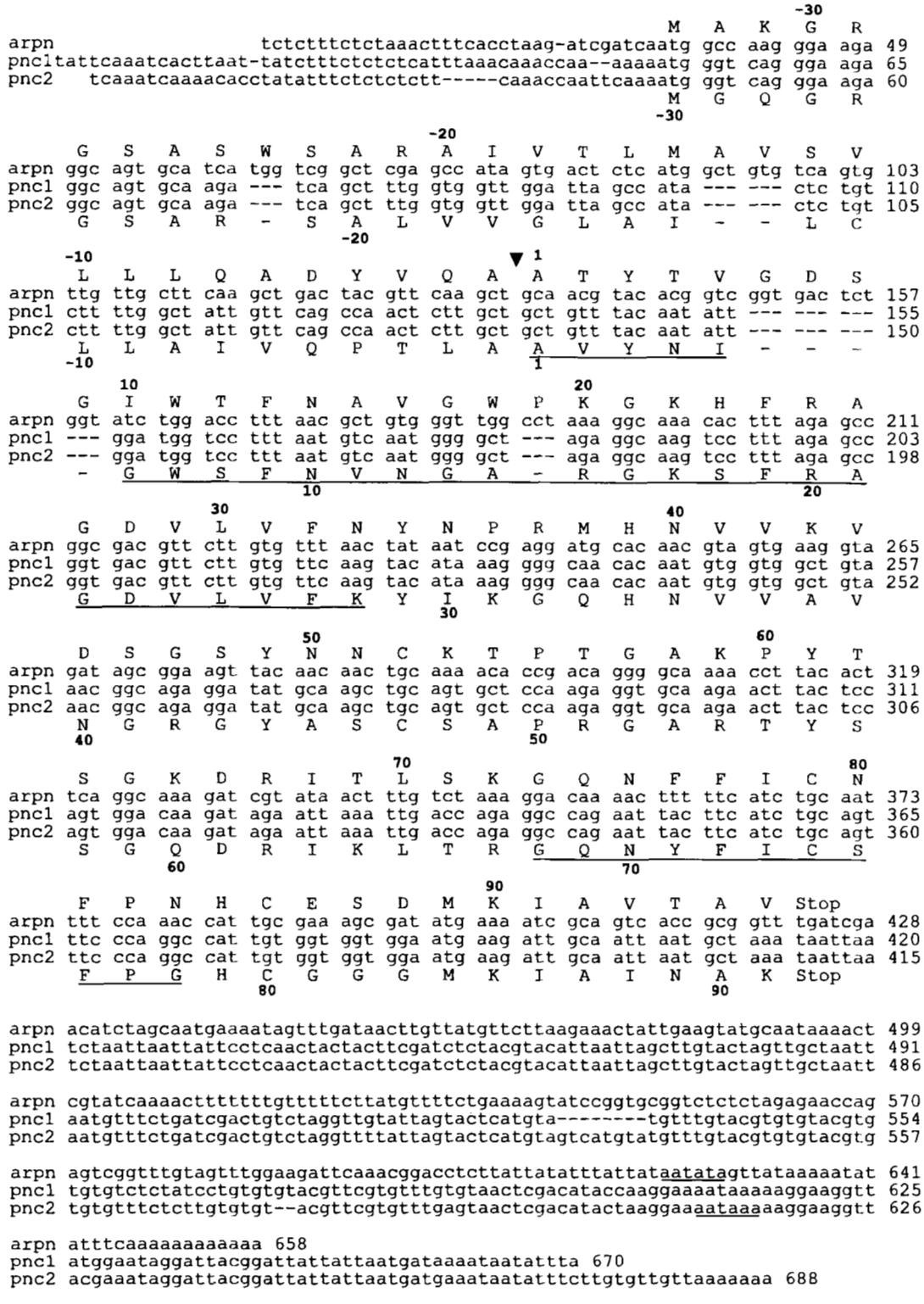

Fig. 1. Nucleotide sequences of two cDNAs encoding plantacyanins from spinach[8] (*pnc1* and *pnc2*) and *Arabidopsis* (*arpn*). The number of nucleotides is given at the right of each line. The deduced amino acid sequence (single-letter code) of spinach plantacyanin is shown below the nucleotide sequence, while that of *Arabidopsis* plantacyanin is shown above the nucleotide sequence and are numbered beginning with the first residue of the mature protein. The amino acid sequences determined by protein sequencing are underlined, as are the potential polyadenylation signals. The inverted solid triangle marks the leader peptides cleavage sites.

[8]The nucleotide sequences of the cDNAs encoding spinach plantacyanin have been previously presented at the EUCHEM Conference on Bioinorganic Chemistry of Copper, July 1994, Göteborg/Hindäs, Sweden.

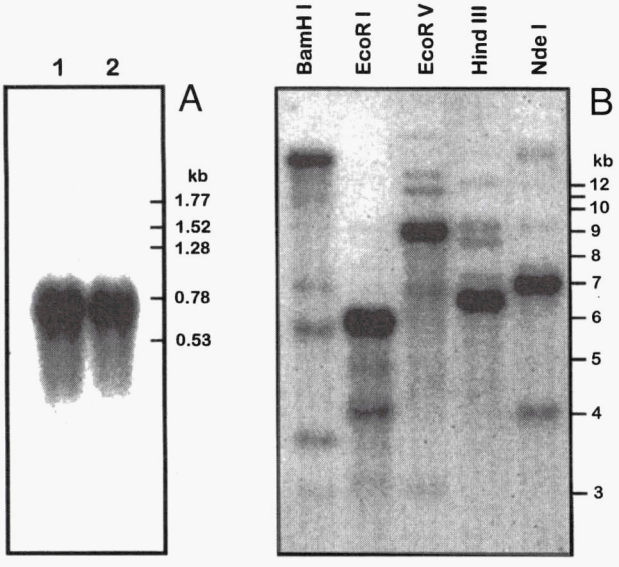

Fig. 2. RNA blot (**A**) and DNA blot (**B**) analysis of spinach plantacyanin gene. Two micrograms of poly(A)$^+$ RNA isolated from green spinach leaves (lane 1) and etiolated spinach seedlings (lane 2) were electrophoresed and blotted onto nitrocellulose. Twenty micrograms of spinach genomic DNA was digested with *BamH I*, *EcoR I*, *EcoR V*, *Hind III*, or *Nde I* and separated on a 0.7% agarose gel and transferred to nitrocellulose. Both blots were hybridized with the 229 bp radiolabeled partial cDNA clone.

is Arg32 in stellacyanin (Arg25 in plantacyanin, CBP). This hydrogen bond apparently stabilizes an important turn in the cupredoxin fold. On the other hand, the conserved basic residue, X_1 (Arg or His), seems to be specific for phytocyanins. The side chain NH1 of Arg32 in stellacyanin hydrogen bonds to the backbone carbonyl oxygen of Ala107 connecting the copper-binding domain to the C-terminal extension, which we believe should provide additional structural stability. Another invariant residue in all 12 sequences is the Trp13, which is thought to help position the axial ligand (Met in plantacyanins and Gln in stellacyanins) close to the copper because of its close contact to that ligand (Guss et al., 1988; Hart et al., 1996).

The BCB gene has been shown to contain two exons separated by a single 192 bp intron, which is located between the last nucleotide of the codon CTC for Leu35 and the first nucleotide of the codon GAA for Glu36 (Van Gysel et al., 1993). We have identified an EST sequence, stock I.D. 123N22T7, which could be derived from an alternately spliced transcript of BCB. This sequence appears to be truncated at its 5′ end, although the available sequence information shows that it encodes BCB starting from Glu36. Thus, the clone 123N22T7 features the heptanucleotide sequence ATTACAG at its 5′ end, which corresponds to the 3′ end of the intron found in BCB gene. The sequence following this heptanucleotide is identical to that of the second exon of the BCB gene, which encodes the part of the BCB protein starting from Glu36. In addition, another EST sequence, stock I.D. 125A3T7, contains a 5′

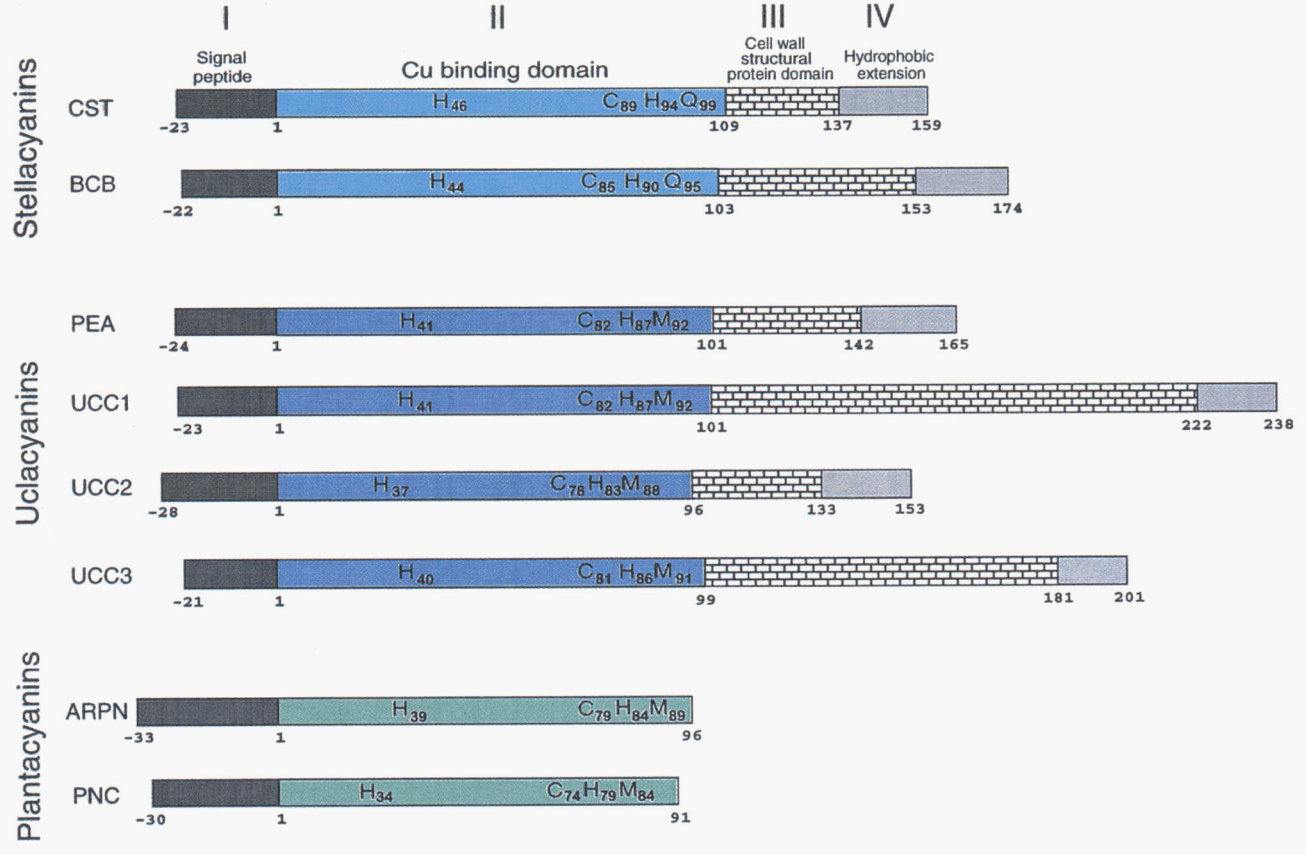

Fig. 3. Schematic diagrams of the entire precursors of eight members of phytocyanin family proteins. The abbreviations are the same as in Figure 4.

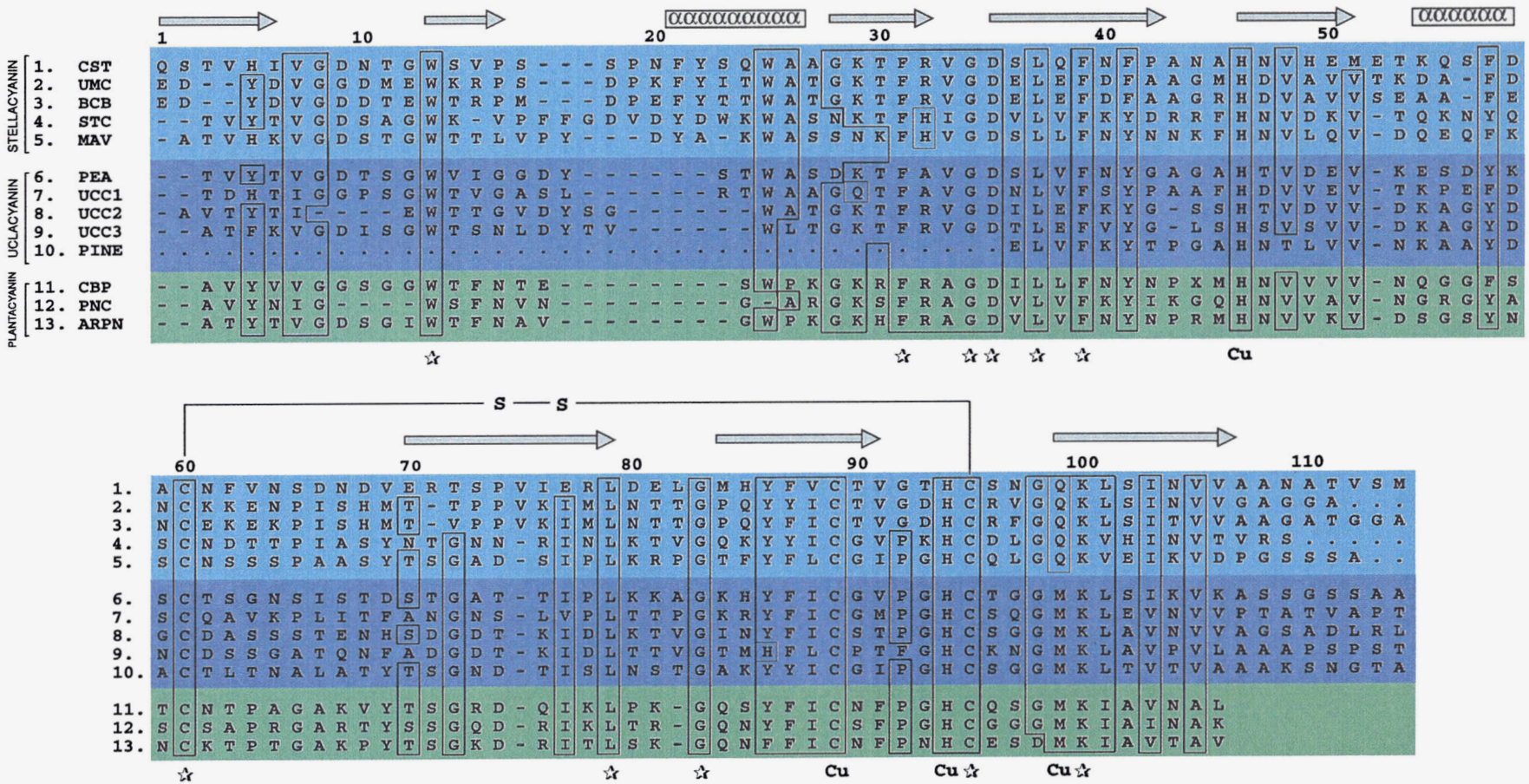

Fig. 4. Multiple alignment of the amino acid sequences of copper binding domains of 13 members of phytocyanin family cupredoxins. CST—cucumber stellacyanin (Nersissian et al., 1996). UMC—umecyanin from horseradish roots (Van Driessche et al., 1995). BCB—blue copper binding protein encoded by a negatively light-regulated gene from *Arabidopsis* (Van Gysel et al., 1993). STC—stellacyanin from *R. vernicifera* (Bergman et al., 1977). MAV—mavicyanin from green zucchini (Schinina et al., 1996). PEA—pea pods blue copper protein (Drew & Gatehouse, 1994). UCC1, UCC2, and UCC3—uclacyanins 1, 2, and 3 from *Arabidopsis*[9] (this study). PINE—protein encoded by a water stress-related gene from Loblolly Pine (Chang et al., 1996). CBP—plantacyanin from cucumber, cucumber basic protein (Murata et al., 1982). PNC—plantacyanin from spinach (this study). ARPN-Plantacyanin from *Arabidopsis* (this study). Three distinct subfamilies are grouped and separated. Residue numbering is indicated for cucumber stellacyanin. The residues that display a conserved side chain character in nine and more sequences are boxed, and those that are invariant in all 13 sequences are indicated in addition by asterisks. Cu- indicates the known or postulated copper ligands. -S-S- indicates disulfide bridge. Dashes indicate gaps that were introduced to optimize the alignment. Dots indicate that the sequence information is not available. The secondary structure, as adopted from the crystal structures of cucumber plantacyanin and stellacyanin, is displayed above the corresponding regions (α, indicates helices while arrow, indicates β-strands).

[9]The sequence of UCC1, UCC2, UCC3, and APNC have been previously presented at the Tenth Symposium of the Protein Society, August 1996, San Jose, California.

sequence GGGATATGAAACAG, which is absent in the UCC2 cDNA sequence and is followed by a nucleotide sequence that is identical to the UCC2 cDNA, and in its second reading frame, encodes a polypeptide starting from Glu30. Thus, the putative intron in UCC2 spans the first and second nucleotides of the codon GAG of Glu30. It is important to note that the putative alternate splicing in both cases occurs in the regions that encode the above mentioned N-terminal conserved sequence motif.

Spectroscopic characterization of the copper-binding domain of Arabidopsis uclacyanin 1 and the Q99M mutant of cucumber stellacyanin

A number of examples from the plantacyanin and stellacyanin subfamilies have been extensively characterized. In contrast, while the putative blue copper binding sites in uclacyanins are apparent, there have been no attempts to isolate these proteins from the plant or to express them heterologously. Using the same expression system and purification procedure that we have previously employed for cucumber stellacyanin (Nersissian et al., 1996), we expressed and purified the 101 amino acid copper-binding domain (domain II) of *Arabidopsis* UCC1 (the sequence of this domain is shown in Fig. 4). The expressed protein forms insoluble aggregates (inclusion bodies), as has been also observed in the case of cucumber stellacyanin and its Q99M mutant. Refolding of the Q99M mutant of cucumber stellacyanin requires a simple procedure consisting of a gradual dilution of the aggregates, which were solubilized in 8 M urea. This allows almost full recovery of the protein in its correctly folded form. This procedure, however, fails to give any detectable correctly folded protein when it was applied to the refolding of *Arabidopsis* UCC1. Hence, we have introduced a minor modification to the refolding procedure. Instead of the gradual, stepwise dilution, a single 10-fold dilution of the solubilized aggregates was applied. This modification yields correctly folded UCC1, as determined from the intense blue color of the protein solution upon reconstruction with copper, and its spectroscopic properties (see below). It accounts, however, for only 2–3% (compared to almost 100% in the case of the Q99M mutant of cucumber stellacyanin) of the total amount of the protein present in the original solution of the solubilized aggregates. The identity of the purified protein has been confirmed by partial N-terminal amino acid sequencing.

Purified UCC1 displays spectroscopic properties (UV-vis and EPR) characteristic of Type 1 or blue copper proteins. For example, its electronic absorption spectrum features an intense band at 600 nm (ϵ = 3,150 M^{-1} cm^{-1}) that is responsible for its intense blue color and corresponds to the Cys thiolate sulfur to copper charge transfer (LMCT). Additionally, the small hyperfine splitting in the $g_{\|}$ region of its EPR spectrum, $A_{\|} = 45 \times 10^{-4}$ cm^{-1}, is another typical feature of blue copper sites. By analogy to the crystallographically characterized phytocyanins, the following four amino acid residues, His41, Cys82, His87, and Met92, are likely to be involved in copper coordination in UCC1, with Cys54 and Cys88 forming the disulfide bridge. The spectroscopic properties of UCC1 more closely resemble those of the Q99M mutant of cucumber stellacyanin than plantacyanin (see Table 1). Figure 5 summarizes the electronic absorption and EPR spectra of cucumber plantacyanin, cucumber stellacyanin, its Q99M mutant form, and *Arabidopsis* UCC1.

A unique characteristic of stellacyanins is their low redox potentials (~+180–280 mV vs. NHE) relative to other blue copper proteins (~+310–680 mV). As shown in Figure 6A, substitution of the axial Gln ligand to Met in cucumber stellacyanin, a ligand that is found in all other blue copper proteins, results in a significant increase in the redox potential (+420 mV for Q99M compared to +260 mV for the wild-type protein). This substitution does not drastically alter the features in the electronic absorption spectrum of the protein as no shifts in the main LMCT bands at 600 and 450 nm occur, although the intensity of the band at 600 nm decreases by nearly 20%, while at 450 nm it increases by the same degree. The only significant change occurs in the region of the band at 820 nm in wt protein, which shifts to the higher energy (750 nm), in the Q99M mutant. Simultaneously, its extinction coefficient increases by a factor of 2, from 800 to 1,500 M^{-1} cm^{-1}. The result is that the Q99M mutant spectrum closely resembles that of UCC1. Interestingly, in spite of the similarities in the spectroscopic properties of UCC1 and the Q99M mutant, the redox potential of UCC1 was measured to be +320 mV, a value that is

Table 1. *Spectroscopic properties of three different subfamilies of phytocyanins and Q99M mutant of cucumber stellacyanin*

	$\epsilon(\lambda_{max})$ [M^{-1} cm^{-1} (nm)]			$g_{\|}$	$g_{\perp}$[a]	$A_{\|}$ (10^{-4} cm^{-1})	$A_{\perp}$[a] (10^{-4} cm^{-1})
Plantacyanin	1,600 (440 nm)	2,800 (595 nm)	1,450 (750 nm)	2.21	2.05	57	40
Stellacyanin (wt)	700 (450 nm)	4,100 (600 nm)	800 (820 nm)	2.29	2.06	36	35
Q99M mutant	890 (450 nm)	3,300 (600 nm)	1,500 (750 nm)	2.23	2.04	45	35
Uclacyanin 1	1,000 (450 nm)	3,150 (600 nm)	1,250 (750 nm)	2.24	2.06	45	40

[a]Approximate values are reported as the average of g_x and g_y (or A_x and A_y) values that were used in simulations of the experimental spectra. More definitive assignments of g_x and g_y (or A_x and A_y) will require further experiments at variable frequencies (see Antholine et al., 1993).

comparable to those determined for cucumber plantacyanin, plastocyanin, and other cupredoxins that possess Met as an axial ligand (except rusticyanin).

We have previously noted that during the routine preparative isolation of blue copper proteins from various plant sources, including spinach and cucumber, plastocyanins were always eluted from an ion exchange column in their reduced form, while plantacyanins and stellacyanins were always oxidized. Moreover, unlike plantacyanins and stellacyanins, highly purified plastocyanins (oxidized with ferricyanide) were found to spontaneously reduce after a few days of storage. In addition, reduced plantacyanins were found to be susceptible to autooxidation in the absence of excess reductant (Nersissian et al., 1991). Such behavior was unexpected, as both plantacyanins and plastocyanins have virtually the same redox potentials (+340 mV) (Nersissian et al., 1985; Sakurai et al., 1996). These observations prompted us to carry out similar experiments with wild-type cucumber stellacyanin and its Q99M mutant. These experiments revealed that both proteins are oxidized by molecular oxygen after their reduction by ditionite and removal of excess of reductant by gel filtration on a Sephadex G-25 sf column. Nevertheless, oxidation of the wild-type protein was ~10 times faster than the Q99M mutant (2.2 vs. 0.23 $mM \cdot h^{-1}$, see Fig. 6B). The kinetics of oxidation therefore correlate with the measured redox potentials indicating that reduced stellacyanin is a stronger reducing agent than its Q99M mutant or other cupredoxins.

Discussion

Uclacyanins, stellacyanins, and plantacyanins are distinct subfamilies of phytocyanins

The availability of the new sequences described in this report and their comparison with those of other members of the phytocyanin family have enabled us to further categorize these proteins into three, presumably functionally distinct, subfamilies designated as plantacyanins, stellacyanins, and uclacyanins. Our classification is based on (1) the domain organization of the precursor and mature proteins, (2) known or inferred glycosylation state, (3) the identity of copper ligand residues, and (4) spectroscopic properties. It is also supported by the fact that a representative from each of the subfamilies has now been characterized in the same plant species (*Arabidopsis*), which excludes interpretation of such a sequence diversity in phytocyanins as simply a result of their various plant origins. Moreover, the pairwise comparison of these sequences shows that the proteins are indeed clustered into three different groups, exhibiting in their copper-binding domains a higher degree of sequence identity between the members of the same group (see Table 2). On the other hand, uclacyanins are much more closely related to stellacyanins than to plantacyanins with regard to their four-domain organization and sequence identity. The characteristic features of all three subfamilies of phytocyanins are summarized below and displayed in Table 3.

Plantacyanins (PNC) are extremely basic, nonglycosylated, single-domain proteins in their mature form. The protein from cucumber was first described in 1974 (Markossian et al., 1974) and over the past years they have been characterized from various plant species (Aikazyan & Nalbandyan, 1981; Sakurai, 1986; Nersissian & Nalbandyan, 1988). Plantacyanin from cucumber seed-

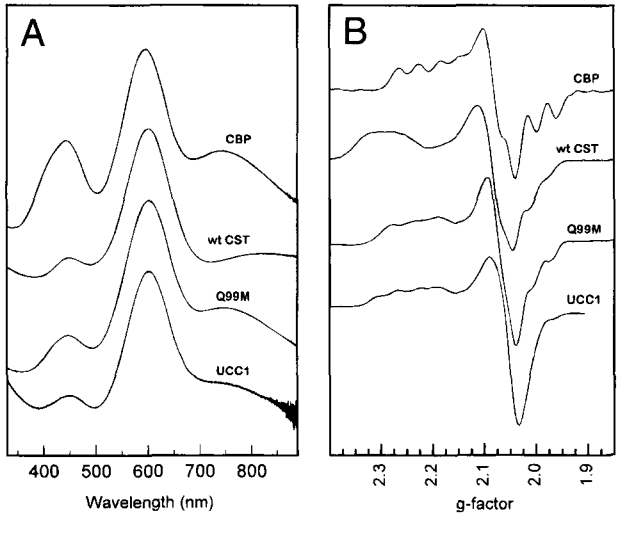

Fig. 5. Comparison of the optical absorption (**A**) and X-band EPR (**B**) spectra of the copper binding domains of cucumber stellacyanin (wt CST) and its Q99M mutant, *Arabidopsis* uclacyanin 1 (UCC1), and plantacyanin isolated from cucumber peelings (CBP).

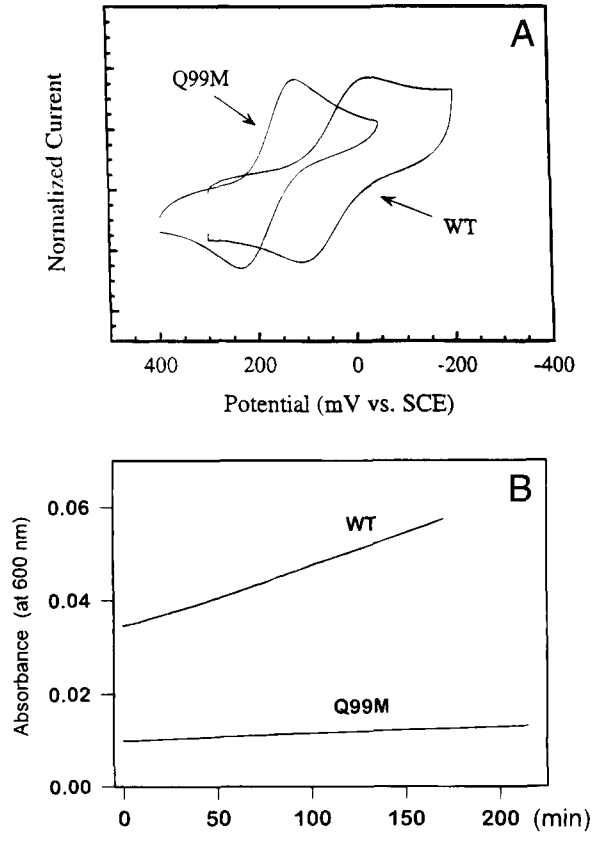

Fig. 6. Cyclic voltammograms (**A**) and kinetics of the autooxidation (**B**) of wild-type cucumber stellacyanin and its Q99M mutant. Voltammetric measurements were recorded using 1 mM protein samples in 0.1 M phosphate buffer, pH 7.0. All experiments were carried out under an atmosphere of purified argon. The autooxidation reactions were followed by the increase in absorption at 600 nm. The proteins were in 10 mM acetate buffer, pH 5.5, containing 100 mM NaCl.

Table 2. *The amino acid sequence identities between 12 phytocyanins expressed as percentages of pairwise comparison*[a]

	UMC	BCB	STC	MAV	PEA	UCC1	UCC2	UCC3	CBP	PNC	ARPN
CST	44	43	29	31	38	34	30	26	24	24	23
UMC		82	36	30	38	36	37	33	28	27	25
BCB			33	32	40	34	38	37	28	27	27
STC				46	43	37	34	26	32	37	35
MAV					44	39	34	32	40	32	31
PEA						47	47	38	30	36	29
UCC1							40	36	30	33	27
UCC2								62	30	38	29
UCC3									31	25	29
CBP										53	67
PNC											51

[a]These values are derived from the version of a multiple sequence alignment shown in Figure 4.

lings, under the name cucumber basic protein (CBP), has been sequenced (Murata et al., 1982). Its crystal structure was determined, revealing a copper binding site similar to other cupredoxins, and consisting of four ligands two His, one Cys, and one Met (Guss et al., 1988, 1996). In addition, the amino acid sequence of spinach plantacyanin, determined by protein sequencing, has been recently reported (Mann et al., 1996). The N-terminal leader sequences in both *Arabidopsis* and spinach plantacyanin precursors are unusually long, 33 and 30 amino acids, respectively (compared to the typical 18–25 residues for others) for the endoplasmic reticulum targeting signal peptides, although they possess the features ascribed to such peptides.

Uclacyanins (UCC) presumably are chimeric proteins in their mature form consisting of a copper-binding domain and a domain resembling a cell-wall structural protein. In spite of the fact that the copper binding site in these proteins is predicted to be arranged from the same residues as found in plantacyanins (two His, one Cys, and one Met), their spectroscopic properties appear to be closely related to the Q99M mutant form of cucumber stellacyanin rather than plantacyanin (see Fig. 5 and Table 1). *Arabidopsis* UCC1 is the only uclacyanin for which the copper binding abilities and spectroscopic properties have been characterized.

Stellacyanins (STC) are distinguished from plantacyanins and uclacyanins by having a Gln residue as a copper ligand in the position of Met found in plantacyanins and predicted for uclacyanins. However, they resemble uclacyanins in their assembly as chimeric proteins consisting of a copper-binding domain and a cell-wall structural glycoprotein-like domain. Additionally, stellacyanins and uclacyanins are glycoproteins, while plantacyanins are not. They carry several N-linked glycosylation sites in their copper binding domains through an Asn residue from a consensus Asn-X-Thr/Ser sequence, and numerous O-linked glycosylation sites in their cell-wall structural protein domains through Ser and hydroxyproline residues (Mann et al., 1992; Van Driessche et al., 1995).

Three members of stellacyanin subfamily, classical stellacyanin from *Rhus vernicifera* (Bergman et al., 1977), mavicyanin from green zucchini (Schinina et al., 1996), and umecyanin from horseradish roots (Van Driessche et al., 1995), lack domain III, as determined by protein sequencing. The gene sequences of these proteins are not known, and therefore no information is currently available concerning domain organization of their precursors. Nevertheless, it has been found that a umecyanin preparation applied for protein sequencing has a heterogeneous C-terminus, which may be indicative of a nonspecific proteolysis that could occur during the preparative isolation of the protein (Van Driessche et al., 1995). This might therefore account for the absence of domain III in its protein sequence. We speculate it will ultimately be found that the precursors of these proteins contain both domain III and IV, when their gene sequences become available. Stellacyanins are also distinguished from other cupredoxins, including plantacyanins and uclacyanins, by a low redox potential, +180–280 mV (versus +340–680 mV for other blue copper proteins) (Sakurai et al., 1996). Therefore, Cu(I) stellacyanins are much stronger reducing agents than other Cu(I) cupredoxins.

EPR spectrum of UCC1 appears to be axial, in contrast to the rhombic spectra of wild-type stellacyanins, Q99M mutant, and

Table 3. *The distinctive properties of plantacyanins, stellacyanins, and uclacyanins*

	Number of domains in precursors	Number of domains in mature proteins	Glycosylation	Amino acid composition of the metal-binding site	Redox potential (mV)
Plantacyanins (PNC)	2	1	No	2His, 1Cys, 1Met	+340
Stellacyanins (STC)	4	2	Yes	2His, 1Cys, 1Gln	+180–280
Uclacyanins (UCC)	4	2	Yes	2His, 1Cys, 1Met	+320

plantacyanins. One of the characteristic spectroscopic properties of stellacyanins is an unresolved feature of the four-line pattern of the hyperfine splitting in $g_{\|}$ region (Peisach et al., 1967). This pattern, however, is resolved to a certain degree in both the Q99M mutant and UCC1, simultaneously shifting the $g_{\|}$ value from 2.29 in wild-type stellacyanin to 2.23 in its Q99M mutant and to 2.24 in UCC1.

It has been suggested that an equatorial trigonal plane formed by two His and a Cys is a prerequisite for the generation of naturally occurring blue copper sites (Holm et al., 1996). The role and contribution of the fourth axial ligand in blue copper binding have been the subject of extensive studies (Lowery & Solomon, 1992). To date, two different naturally occurring axial ligands have been identified in blue copper proteins: a hydrophobic Met residue found in the vast majority of blue copper centers, typically only weakly bound at a distance ranging between 2.6 Å in cucumber plantacyanin (Guss et al., 1996) to 3.15 Å in *Alcaligenes denitrificans* azurin (Baker, 1988), and a hydrophilic Gln, found only in stellacyanins, bound much more strongly at a distance of 2.2 Å (Hart et al., 1996). In some other cases, the protein does not provide any ligand residue at that position. For instance, a recently determined crystal structure of human ceruloplasmin reveals that one of the three blue copper sites in this multicopper oxidase is formed by only three equatorial ligands, two His and a Cys, while no residue has been found to contribute at its fourth, axial coordination position (Zaitseva et al., 1996). Similar to the ceruloplasmin, several fungal laccases (Xu et al., 1996) , and tobacco laccase as well (Kiefer-Meyer et al., 1996), possess nonliganding residues, such as Leu or Phe.

A number of azurin mutants have been engineered in which the axial Met ligand has been substituted by other residues with various side-chain characteristics (Pascher et al., 1993). It has been shown that a hydrophobic residue at the axial position tends to increase the redox potential, while a hydrophilic residue has the reverse effect. Both plastocyanin and azurin mutants where the Met was substituted by Gln display stellacyanin-like spectroscopic properties (Romero et al., 1993; Hibino et al., 1995), for example, rhombic EPR spectra, and an increase in the intensity of the LMCT band at 450 nm, which is thought to correlate with the degree of copper displacement from the trigonal plane (Lu et al., 1993). Simultaneously, the redox potentials of these mutants of azurin and plastocyanin decrease by 25 and 35 mV, respectively.

Additionally, several lines of evidence suggest that other residues located in the copper binding loop or its second coordination sphere may contribute to the fine tuning of the redox potential. Substitution of Pro by Ala at position 80 adjacent to the His81 ligand in *Alcaligenes faecalis* S-6 pseudoazurin alters the hydrogen-bonding network in the vicinity of copper and brings about a significant increase (139 mV) in the redox potential (Nishiyama et al., 1992). The redox potentials (400–500 mV) of two fungal laccases from *Myceliophthora thermophila* and *Scytalidium thermophilium* that lack the axial Met in their sequences are comparable with the potential of lacquer tree *R. vernicifera* laccase (440 mV), which presumably carries a Met as an axial ligand (Xu et al., 1996).

All phytocyanins have a common folding topology: The phytocyanin fold

All cupredoxins (including phytocyanins and related sequence domains found in multicopper oxidases and nitrite reductases) possess a similar folding topology, designated as a Greek key β-barrel, even though some examples show only very slight sequence identity (less than 10%) (Adman, 1991). A single copper binding site is located at the so-called "northern" end of the barrel (Guss & Freeman, 1983). Three liganding amino acids are positioned in a loop that is organized by a conserved copper-binding sequence motif. The amino acid composition of this loop and the spacing between the copper binding ligands may determine the phylogenetic relationships between blue copper proteins. Thus, the substitution of the entire loop in a copper protein by a loop derived from an evolutionary distinct protein can create, on the scaffold of the host's β-barrel, a new copper-binding site that displays spectroscopic properties similar to that of the donor protein. An example of such a conversion is the generation of a binuclear Cu_A site of the subunit II of cytochrome *c* oxidase on the protein scaffoldings of azurin and amicyanin (Dennison et al., 1995; Hay et al., 1996). In this context, it is important to note that the copper-binding loop in phytocyanins, in both length and the four-residue spacing between the liganding amino acids, resembles the loops of multicopper oxidases, ceruloplasmin, ascorbic acid oxidase, laccase, and very likely blood coagulation factor VIII, although in the latter, the presence of a blue copper remains unproven (Tagliavacca et al., 1997).

The structures of cucumber plantacyanin and stellacyanin are very similar. The root-mean-square deviation between the positions of the α-carbon atoms of these proteins is only 1.1 Å (Guss et al., 1996; Hart et al., 1996). They differ from other cupredoxins by an unusual twisted shape of the two β-sheets that form the barrel. Three other common structural features of plantacyanin and stellacyanin distinguish them from the other cupredoxins (Fig. 7). First, the imidazole rings of both His ligands are completely exposed on the surface of the protein, resulting in a metal binding site that is much more accessible to the solvent. Second, phytocyanins are characterized by the presence of three invariant cysteine residues, two more than are found in most cupredoxins. One cysteine residue serves as a ligand of copper, while two others form a disulfide bridge. This disulfide bridge may play a crucial role in maintaining the tertiary structure of the protein and/or formation of the copper binding center because one of the His ligands of copper is followed directly by a bridging Cys residue. Third, in addition to the seven β-strands, there are two α-helices that are tightly assembled with the β-sheets and encompass the barrel, presumably providing more structural stability.

These observations, together with the fact that the UCC1 and Q99M mutant of cucumber stellacyanin display very similar spectroscopic properties, lead us to conclude that uclacyanins possess a folding topology similar to plantacyanins and stellacyanins, known as the phytocyanin fold.

We have performed structure prediction studies of UCC1 using the UCLA-DOE Structure Prediction Server at the URL—http://www.doe-mbi.ucla.edu/people/frsvr/frsvr.html (Fischer & Eisenberg, 1996). In support of our conclusion, the highest scoring similarity was observed for the stellacyanin fold giving a Z-SCORE value of 22.77, a value far greater than the proposed reliability threshold value of 4.4 ± 1. Surprisingly, among other cupredoxin folds included in the structure prediction library, only rusticyanin displays some similarity, with a Z-SCORE value of 6.18.

Possible involvement of phytocyanins in lignin formation and the early events of plant defense responses

Phytocyanins are apparently plant-specific proteins. To date, no proteins have been found in organisms other than plants that dis-

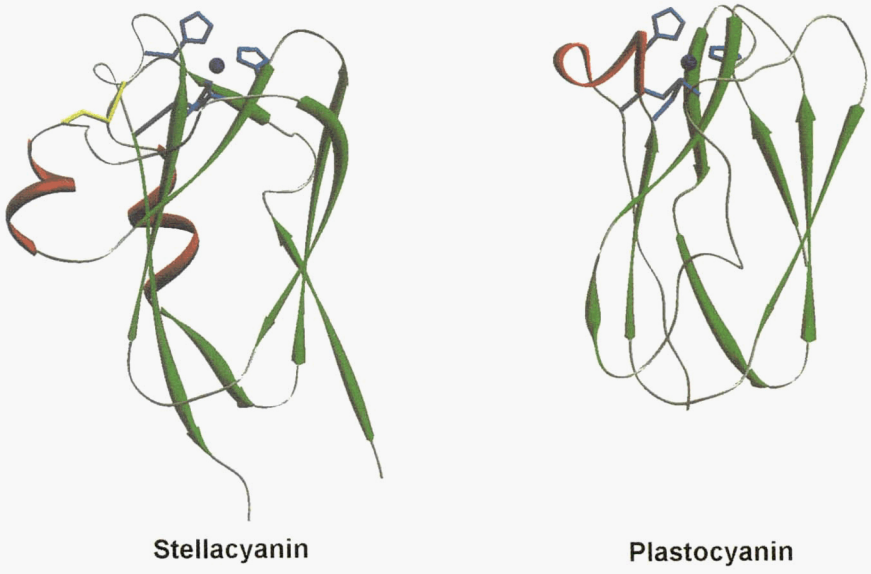

Fig. 7. Ribbon diagrams of cucumber stellacyanin and poplar plastocyanin. The copper atoms and the side chains of the ligand-amino acids (2His, Cys, and Met in plastocyanin, and 2His, Cys, and Gln in stellacyanin) are shown in blue. The disulfide bond in stellacyanin is shown in yellow. β-Strands are in green, α-helices are in red. The plastocyanin molecule is shown in an orientation equivalent to that of stellacyanin based on the structure alignment procedure described in Materials and methods. Note that the stellacyanin molecule has a twisted or "open" β-barrel, and a more exposed copper-binding site than does plastocyanin.

play homology to phytocyanins. This includes yeast *Saccharomyces cerevisiae*, and cyanobacterium *Synechocystis* sp. PCC6803, organisms for which the complete genome sequences are available.

Several lines of evidence suggest that uclacyanins and stellacyanins are cell-wall proteins. First, the presence in their precursors of N-terminal hydrophobic sequence domains possessing all features characteristic for secretory signal peptides indicates that these proteins are targeted to the endoplasmic reticulum. They also lack the endoplasmic reticulum retention signals for soluble proteins, the tetrapeptide sequences KDEL or HDEL (Pelham, 1990; Napier et al., 1992), which suggests that they are not bound to the compartments of the endoplasmic reticulum and most likely translocate through the Golgi apparatus to the exterior. Second, a C-terminal hydrophobic domain similar to the Domain IV in stellacyanins and uclacyanins has been found in the precursors of several cell-wall arabinogalactan proteins (Chen et al., 1994; Du et al., 1994; Pogson & Davies, 1995; Li & Showalter, 1996) and two xylem specific gene products of loblolly pine (Loopstra & Sederoff, 1995). Furthermore, the signal peptide cleavage sites in these proteins and stellacyanins feature similar amino acids, for example, Ala or Gly at the position -1 and Gln or Glu at the position $+1$. The signal sequences of five arabinogalactan proteins and two stellacyanins are compared below:

	-20 . . . . . -10 . . . . . -1 $+1$		
AGP*Pc*1	MKMGFAGFQVLMVLGLLATSCIA	QAPGAAP	(Chen et al., 1994)
AGP*Na*1	MAYSRMMFAFIFALVAGSAFA	QAPGASP	(Du et al., 1994)
PtX3H6	MARSTAMTMVLFLLAGFLVSSMA	QSPSASP	(Loopstra & Sederoff, 1995)
PtX14A9	MEKIMVLVCIMSFTILGLSSA	QEINHAA	(Loopstra & Sederoff, 1995)
LeAGP	MDRKFVFLVSILCIVVASVTG	QTPAAAP	(Pogson & Davies, 1995)
CST	MAAGSVAFVLGLIAVVFLHPATA	QSTVHIV	(Nersissian et al., 1996)
BCB	MAGVFKTVTFLVLVFAAVVVFA	EDYDVGD	(Van Gysel et al., 1993)

One could speculate that the phytocyanins (probably with the exception of the plantacyanin subfamily) and the cell-wall arabinogalactan proteins are utilizing the same post-translational secretory pathway, which processes a C-terminal hydrophobic polypeptide in addition to the N-terminal signal peptide. Third, uclacyanins and stellacyanins contain hydroxyproline- and serine-rich sequence domains that are often found in cell-wall structural glycoproteins. In addition, there are several palindromic sequences in these domains, such as PPSSSPPSSSPP in CST, SGKGS in PEA, TTGTT in UCC1, HPHPPHPH in UCC2, SPPSPPSPPS in UCC3, which are considered to be a prerequisite for self-assembly of these proteins into the cell-wall macromolecular structures, according to the criterion of Kieliszewski and Lamport (1994).

Two phytocyanin genes have been identified as stress-inducible genes. The first is the light-negatively regulated gene from *Arabidopsis*, BCB, which according to our criteria belongs to the stellacyanin subfamily (Van Gysel et al., 1993). It has been shown that this *Arabidopsis* gene is developmentally regulated, and a 20–25-fold increase in the steady-state mRNA level has been observed upon dark treatment of the light-growing plant. The second is a cDNA isolated as a water deficit stress-inducible gene in loblolly pine (*Pinus taeda*) (Chang et al., 1996). Although this clone appears to be truncated and lacks a large portion at its 5′ end, the available sequence shows that it encodes a blue copper protein with Met as an axial ligand to copper. It has been therefore ascribed to the uclacyanin subfamily. Another member of uclacyanin subfamily, the pea pod blue copper protein, has been isolated as a gene specifically related to lignification in pea pods (Drew & Gatehouse, 1994). It is possible that these genes are involved in plant defense processes because one of the defense responses in plants is the synthesis and deposition of lignin in peripheral tissues. In addition, it is noteworthy that the stress related genes are activated in response to wounding or a pathogen infection of the plant.

One example includes the water deficit related proteins, so-called osmotins (Neale et al., 1990).

Two different processes are currently considered to be orchestrated by redox active enzymes localized in the plant cell wall. First is the oxidative burst, a mechanism that plants have developed as a rapid primary defense response to resist pathogen infection or to repair mechanical wounding (Low & Merida, 1996; Mehdy et al., 1996). During the oxidative burst hydrogen peroxide is generated with concomitant oxygen consumption, which mediates rapid crosslinking and insolubilization of the cell-wall structural proteins presumably making the damaged site invulnerable for further penetration of pathogens to interior (Bradley et al., 1992; Levine et al., 1994). Very little is known about the mechanism and origin of oxidative burst in plants in spite of the intensive research since its discovery by Doke in 1983 (Doke, 1983). The plant defense response related genes characterized to date fail to identify features that could be related to the redox active enzymes or proteins capable of reducing molecular oxygen. Recently, however, an acidic isozyme of peroxidase, which is capable of catalyzing inter-molecular crosslinking reaction of extensins through isodityrosine formation, has been partially purified from tomato cell suspension cultures (Schnabelrauch et al., 1996).

Second is the formation of lignins, the heterogeneous and hydrophobic cell-wall polymers composed of phenylalanine derived hydroxycinnamyl alcohol units, monolignols (p-coumaryl, coniferyl, and sinapyl alcohols) (Whetten & Sederoff, 1995). A phenoxy radical coupling mechanism has been proposed for the polymerization of monolignols to form lignins or for their dimerization to form lignans (Lewis & Yamamoto, 1990). Laccases and peroxidases have been considered to be responsible for generation of monolignol radicals which are prerequisites for the proposed coupling mechanism (Bao et al., 1993; O'Malley et al., 1993). A homotrimeric protein composed of 27 kDa subunits was recently characterized, which controls the stereo-selectivity of bimolecular phenoxy radical coupling of coniferyl alcohol to give preferentially one enantiomeric form, (+) pinoresinol (Davin et al., 1997). As we have mentioned previously, one of the uclacyanins, pea pod blue copper protein, has been shown to be specifically related to the lignification in pea pods (Drew & Gatehouse, 1994). In addition, the sequences related to phytocyanins are present in ESTs obtained from the cDNA library from loblolly pine differentiating xylem, the highly specified cells involved in the formation of lignins and their accumulation (data generously provided by Dr. Ron Sederoff from a survey of nearly 1000 EST sequences).

Stellacyanins and uclacyanins are unique among the cell-wall proteins characterized to date, in possessing a redox active copper-binding domain. We speculate that the highly solvent accessible nature of the copper site in phytocyanins relative to other cupredoxins such as azurin and plastocyanin may help these proteins to be involved in redox reactions with small molecular weight compounds rather than with protein electron mediators. Furthermore, the structure of the reduced form of cucumber stellacyanin, which was recently determined, reveals alteration in the copper coordination geometry compared to that of its oxidized form (P.J. Hart, unpubl. obs.). This finding supports our hypothesis because according to the rack-induced or entatic concepts (Malmström, 1994; Williams, 1995) the uniform geometry of the redox center in both its oxidized and reduced states is considered to be one of the prerequisites for a metalloprotein to function as a long-range electron transfer protein. It has been also recently shown that the *bcb* gene encoding *Arabidopsis* stellacyanin (BCB) is induced by an oxidative stress in concert with other oxidative stress-inducible genes such as those that encode superoxide dismutase, peroxidase, and glutathione S-transferase (Richards et al., 1998), suggesting that stellacyanins are possibly involved in oxygen activation. These data also support our hypothesis that stellacyanins and possibly also other phytocyanins are unlikely to be long-range electron transfer proteins interacting with protein electron mediators. Most likely they participate in redox processes occurring during the primary defense response and/or lignin formation in plants.

Materials and methods

Library construction and screening

Total RNA from either three-week-old spinach green leaves or six-day-old etiolated seedlings were isolated and applied for purification of poly(A)$^+$ RNA on a oligo-(dT) cellulose column (Pharmacia, Uppsala, Sweden). After examination of the quality of spinach green leaves poly(A)$^+$ RNA preparation by in vitro translation using wheat germ extract (Stratagene, La Jolla, California), 2 μg was used to construct a cDNA library using a commercial kit (Pharmacia). The cDNA was ligated with an *EcoR I* linker containing an internal *Not I* site and introduced into the *EcoR I* digested λgt11 phage DNA (Pharmacia), which was subsequently packed *in vitro* using Gigapack Gold packaging extract (Stratagene). An aliquot of the cDNA pool for library construction was applied as a template for PCR to amplify a partial clone. Primer 1, a sense 28-mer with a 5′ overhang *EcoR I* site (underlined) TGAATTCGCNGTNTAYAAYATHGGNTGG, corresponds to the N-terminal amino acid sequence of plantacyanin isolated from spinach Ala^1-Val-Tyr-Asn-Ileu-Gly-Trp^7. Primer 2, an antisense 18-mer RCADATRAARTARTTYTG, was designed based on a sequence of a proximal to the C-terminus proteolytic peptide, and corresponds to the following sequence, Gln^{69}-Asn-Tyr-Phe-Ileu-Cys^{74}. In the above sequences, N represents any nucleotide A, T, C, or G; H - A, C or T; D - T, G or A; Y - C or T; R - A or G. PCR conditions were: denaturing for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 50 °C, and extension at 72 °C for 2 min. Final extension was 10 min. The reaction was carried out in 100 μL with *Taq* DNA polymerase, 1 μM of each primer, and 2 mM $MgCl_2$ in a buffer recommended by manufacturer (Promega, Madison, Wisconsin). A single 229-bp amplification product was purified on a 2% low melting agarose gel and extracted from the gel by phenol. The purified DNA was treated with T4 DNA-polymerase at room temperature, digested with E*coR I*, and ligated into the *EcoR I/Sma I* sites of Bluescript KS$^+$ (Stratagene). The nucleotide sequence shows that the amplified DNA fragment corresponds to a partial cDNA clone encoding spinach plantacyanin. This partial cDNA was labeled with [α^{32}P]dCTP by a random priming technique and applied as a riboprobe for screening of the library.

Standard cDNA library screening was carried out with approximately 600,000 plaques at high stringency conditions. Two positive plaques were identified and purified by two additional rescreening cycles. The cDNA inserts were excised from the phage DNA by *EcoR I*, ligated to *EcoR I* digested and dephosphorylated Bluescript KS$^+$, and sequenced.

DNA sequencing was carried out using [α^{35}S]dATP and a U.S. Biochemicals Sequenase kit employing the dideoxynucleotide chain termination method. The ultimate sequence of all DNA fragments was confirmed for both strands. Primer walking, subcloning, and

restriction fragment analysis (where it was applicable) strategies were applied to obtain complete sequences.

All standard molecular biology procedures and Northern and Southern blot analysis with the 229 bp partial cDNA clone as a hybridization probe were performed as described in (Sambrook et al., 1989).

Construction of plasmids and expression of Arabidopsis uclacyanin 1 and Q99M mutant of cucumber stellacyanin

A DNA fragment that corresponds to the copper-binding domain of *Arabidopsis* uclacyanin 1 (UCC1) was amplified by PCR using UCC1 cDNA as a template. The primers were designed so that the sense primer 1, containing a 5′ overhang *Nde I* restriction site (underlined), TAACACATATGACTGACCATACCATTGGTGG TCC, would generate an AUG transcriptional initiation codon next to the putative signal peptide cleavage site. The antisense primer 2 with a 5′ overhang *BamH I* restriction site (underlined), GGT TGGATCCACGGTTCAGGTTGGAACAACGTTTACTTCAAGT TTC, would replace the GCA codon for Ala102 with a UGA termination codon upstream of the *BamH I* restriction site. The amplified 332-bp PCR product, which encodes the 101 amino acid copper binding domain (Thr1–Thr101) of UCC1, was digested by *Nde I* and *BamH I* and ligated into the corresponding sites of the pET3a bacterial expression vector (Stratagene), which directs the expression of the target gene under the control of the T7 gene 10 promoter (Studier et al., 1990).

The mutational substitution of the copper ligating residue Gln99 with a Met in cucumber stellacyanin was achieved by an overlap extension PCR technique on a template of the pET3a plasmid harboring the gene encoding copper binding domain of cucumber stellacyanin (Ho et al., 1989). For details concerning the construction of this plasmid see (Nersissian et al., 1996). Two primers were designed to flank the coding region of the gene and corresponded to the nucleotide sequences of the pET3a vector upstream of the *Nde I* site, CGACTCACTATAGGGAGACC (sense-flanking primer), and downstream of the *BamH I* site, CCCGTTTAGAGGCCCC AAGGGG (antisense-flanking primer). Two other primers, CATTG TTCAAATGGTATGAAGTTATCCATCAATG (sense mutagenic), and CATTGATGGATAACTTCATACCATTTGAACAATG (antisense mutagenic), were complementary overlapping to each other and carrying the requested substitution of a CAA codon for residue Gln99 with ATG (underlined). Two different overlapping fragments were amplified by PCR applying the sense flanking primer and antisense mutagenic primer for one PCR product, and sense mutagenic primer and antisense flanking primer for the other PCR product. The amplified fragments were purified on a 2% low melting agarose gel, extracted by phenol from the gel, mixed, and used as a template for the final round of the amplification of the entire gene using sense and antisense flanking primers. The final PCR product was digested by *Nde I* and *BamH I* and inserted into the corresponding sites of pET3a. Both DNA fragments that encode the copper-binding domain of UCC1 and the Q99M mutant of cucumber stellacyanin were sequenced for verification of the integrity of the entire gene and the occurrence of the desired mutation prior to protein expression.

The proteins were expressed in *Escherichia coli* BL21(DE3), which harbors a genomic copy of T7 RNA polymerase under the control of the IPTG-inducible lac UV5 promoter. The expression and purification procedures for the copper binding domains of UCC1 and Q99M mutant of cucumber stellacyanin were virtually identical to that of wild-type cucumber stellacyanin described in (Nersissian et al., 1996), with one minor modification. The refolding of the copper-binding domain of UCC1 was achieved by fast, simultaneous, 10-fold dilution of solubilized inclusion bodies instead of a gradual slow dilution. The identities of the expressed proteins were confirmed by N-terminal protein sequencing and electrospray ionization mass spectrometry.

The molar extinction coefficients (ϵ) at 281 nm for the apoproteins were estimated from the Trp and Tyr contents, using 5,500 M^{-1} cm^{-1} for a Trp residue and 1,490 M^{-1} cm^{-1} for a Tyr residue according to (Pace et al., 1995). The ϵ values for the copper chromophores were calculated based on the ratio A_{281}/A_{600}, after the complete reconstitution of apoproteins with copper (see Nersissian et al., 1996).

Plantacyanins from green spinach leaves and cucumber peelings were isolated essentially according to (Nersissian & Nalbandyan, 1988). The ratio of A_{281}/A_{595} for the fully oxidized and electrophoretically pure preparations were 6.0 and 4.8 for the proteins from cucumber and spinach, respectively.

Spectroscopic and electrochemical measurements

Electronic absorption spectra were recorded at room temperature on a Cary 3 spectrophotometer. The EPR spectra were recorded either at 77 or 90 K using a Bruker ER200D-SRC spectrometer, operating at X-band frequency (9.287–9.645 GHz). The modulation amplitude typically was 10 G, while the microwave power 10.4 mW. The magnetic field was calibrated using the powder of a stable free radical 2,2-diphenyl-1-picrylhydrazyl ($g = 2.0036$) as a field standard. The protein samples for both EPR and electronic absorption measurements were in 10 mM acetate buffer, pH 5.5.

Electrochemical experiments were performed using either a BAS CV-50W electrochemical analyzer, or a Princeton Applied Reseach Model 173 potentiostate/galvanostat driven by a Model 175 Universal Programer. Cyclic voltammetry was carried out at ambient temperature with a normal three-electrode configuration consisting of either an edge-plane graphite or modified gold working electrode, a saturated calomel reference electrode, and a platinum-wire auxiliary electrode. The working compartment of the electrochemical cell was separated from the reference compartment by a modified Luggin capillary. All three compartments contained a 0.1 M solution of supporting electrolyte.

The edge-plane graphite electrode was lightly sanded (1000 grit sand paper, 3M), then polished successively with 0.3 and 0.05 μm alumina (Buhler) prior to use. After sonication, the electrode was rinsed and the tip was dried using a heat gun. Modified gold electrodes were prepared by incubating pre-polished gold-disk electrodes (0.5 μm alumina) in a saturated pyridine-4-aldehyde thiosemicarbazone solution for 10–60 min.

3D Structural alignment

The structural alignment of poplar plastocyanin (PDB1PLC) (Guss & Freeman, 1983) and cucumber stellacyanin (PDB1JER) (Hart et al., 1996) was accomplished using a modified version of the program ALIGN (Satow et al., 1986). The 2His and Cys ligand side chains of plastocyanin were superimposed on the corresponding ligand side chains of stellacyanin. The resulting rotation and translation vectors were applied to all the atoms of plastocyanin to position them on the same coordinate basis as stellacyanin. Figures were created with the program SETOR (Evans, 1993).

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