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Sequence of a Plastocyanin cDNA From Wheat and the Use of the Gene Product to Determine Serologically Tissue Degradation after Infection with *Pseudocercosporella herpotrichoides*

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With 4 figures

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Abstract

Pseudocercosporella herpotrichoides, the causal agent of the eyespot disease of wheat, induces lesions at the stem bases. In degraded tissue a specific protein (Pc) cannot more be detected whereas it is abundantly produced in the green tissue showing no disease symptoms. In plant samples including the stem bases, the amount of protein Pc is therefore correlated with the degree of tissue degradation. A serological test (ELISA) was developed for the quantitative determination of the protein. ELISA readings can be used as estimates of disease severity.

By sequence analysis, Pc was identified as plastocyanin. Based on the nucleic acid sequence, wheat pre-plastocyanin consists of a transit peptide of 61 amino acids and a mature peptide of 97 residues. Comparison of the nucleotide sequences of plastocyanin of wheat and barley showed homology of 93.7%.

Zusammenfassung

Sequenz einer Plastocyanin-cDNA in Weizen und die Verwendung des Genprodukts zur serologischen Bestimmung des Gewebeabbaus nach Infektion mit *Pseudocercosporella herpotrichoides*

Der Pilz *Pseudocercosporella herpotrichoides* verursacht bei Weizen die Halmbruchkrankheit. In dem sich abbauenden Gewebe wird das Protein Pc nicht mehr gebildet, während es im umliegenden grünen Gewebe ohne sichtbare Läsionen noch reichlich nachzuweisen ist. Die Menge an Protein Pc ist deshalb mit dem Grad des Gewebeabbaus korreliert. Mit Hilfe eines ELISA kann über die gemessene Proteinmenge eine quantitative Beurteilung des Schädigungsgrades durch den Pilz vorgenommen werden.

Untersuchungen zur Bedeutung des Proteins Pc während der Pathogenese führten zu seiner Identifizierung als Plastocyanin. Auf Grund der Nukleotidsequenz setzt sich das Weizen-Präplastocyanin aus einem Transitpeptid mit 61 und dem reifen Peptid mit 97 Aminosäuren zusammen. Die Sequenzierung ergab, daß zwischen Weizen- und Gersten-Plastocyanin eine Homologie von 93,7% besteht.

Introduction

Eyespot of wheat is caused by the fungus *Pseudocercosporella herpotrichoides* (Fron) Deighton. It also attacks barley, rye, oats and some other grasses (Murray, 1992). When spores of *P. herpotrichoides* colonize host tissue the fungus will first germinate on the epidermis. Penetration is initiated from either appressoria, individual hyphae or the stroma. Penetration pegs pierce the cell wall of the epidermal cells or may invade the stomata. Penetration is not of a purely physical nature but is accompanied by dissolution of the outer cell wall by extracellular lytic enzymes produced by the fungus (Soulié et al., 1985). After successful penetration, the hyphae colonize successive layers of cells in the coleoptile and leaf sheaths. The fungus causes degeneration and destruction of the host cells. When all leaf sheaths are penetrated, the fungus continues to colonize the stem.

When a plant has been infected by *P. herpotrichoides*, it has some general response mechanisms which, in principle, are much the same in resistant and susceptible genotypes. Defosse (1971) found that haloes are formed at the site of penetration in the epidermal host tissue. They are due to degradation of pectin, cellulose and other polysaccharides in the cell wall and the appearance of compounds with reducing aldehyde groups. Soulié et al. (1985) found a hypersensitivity reaction upon infection which occurs more frequently in resistant than in susceptible cultivars.

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The present studies aimed at the identification of a protein synthesized in wheat. Its quantity is indicative of the damage caused in the plant by the fungus. Information is presented on the serological measurement of the protein level in differently affected tissue and the identification of the DNA sequence of the corresponding gene.

Materials and Methods

Plant cultivation and identification of protein

The experiments were carried out with winter wheat. The resistant cultivar was 'Rendezvous' carrying the gene *Pch*-1 and the susceptible cultivar was 'Granada'. Plants were sown in pots and cultivated in the greenhouse in two sets at 10°C and 12 h of light. At time of emergence, the plants of the first set were artificially inoculated by spreading wheat kernels infected with a mixture of *P. herpotrichoides* var. *herpotrichoides* and *P. herpotrichoides* var. *acuformis* on the soil surface. The plants of the second set served as controls and were grown without the fungus. After infection, lesions (eyespots) developed at the base of the stem followed by an increasing degradation of tissue. This part of the diseased plants was used for comparison with healthy plants. Differences of the pattern of native proteins were analysed by polyacrylamide gradient gel electrophoresis (PAGGE). Gels were applied with a linear acrylamide gradient of 4% C constant using 0.375 mol/l Tris-HCl buffer, pH 8.8. The pore gradient was between 3 and 24% T. As electrode buffer Tris-glycine, pH 8.3, was prepared.

At growth stage 22 (start of tillering), the total proteins from the lower 3 cm segments of the stem bases of control plants were extracted with 15 mM Tris-HCl buffer, pH 7.4. The specific protein Pc synthesized in green and healthy tissue, but not in degraded plant parts, was isolated and purified by ion exchange chromatography (DEAE-52 cellulose, Serva) and subsequent gel filtration (Sephadex G-100; Pharmacia, LKB, Uppsala, Sweden). The isolated protein was used as antigen for primary antibody production. It was injected twice into rabbits each time with 220 μg of protein. The final antiserum titer was 1:2500 as determined by Western blot analysis.

The specifity of the antiserum was tested by Western blotting after PAGGE. Electrophoretic transfer onto nitrocellulose membrane and visualization of antigen/antibody interaction was performed as described by Lind (1990) and for immunoscreening.

ELISA of plant tissue

The occurrence of protein Pc in plant tissue was verified by ELISA (Lind, 1992). In all serological tests the lower 3 cm of plant stems at growth stages 22 and 35 (middle of stem elongation) were used as samples. They included the green leaf sheaths as well as the stem within. Each sample consisted of 10 stem segments. The ELISA was repeated six times.

Poly(A) + RNA isolation

In order to identify the sequence coding for protein Pc, a cDNA-library was constructed. At stage 22 the stem bases of wheat plants were taken as samples in the same way as for ELISA tests to isolate mRNA. The isolation of total RNA followed the methods described by Sambrook et al. (1989). Poly(A) + RNA was isolated using a magnetic mRNA-isolation kit according to the instructions of the supplier (PolyATractTM mRNA Isolation Kit II, Promega, Madison, WI, USA). *In vitro* translation of poly(A) + RNA was performed in a wheat germ extract (*in vitro* translation kit; Promega).

cDNA synthesis

cDNA synthesis was performed according to standard procedures. Briefly, poly(A) + RNA was converted into double stranded cDNA (cDNA Synthesis Kit, Boehringer, Mannheim, Germany), which was ligated to *Eco*R I/*Not* I adaptors and subsequently inserted into the *Not* I site of phage vector Lambda gt11. *In vitro* packaging of phage concatamers was performed by using commercial packaging extracts (Gigapack II Gold, Stratagene, La Jolla, CA, USA). For immunoscreening, phages were plated together with *E. coli* strain Y1090r- on BBL medium (1% bacto tryptone, 1% NaCl, 0.5% yeast extract, 0.2% maltose, 10 mM MgSO4, 1.5% bacto agar).

Immunoscreening of cDNA library

The screening of the cDNA library was essentially done according to Sambrook et al. (1989). A nitrocellulose filter soaked in a solution of 10 mM isopropylthio-β-D-galactoside (IPTG) was placed over the plated *E. coli* cells and incubated at 37°C for 4 h. After blocking, it was incubated in a solution of the primary antibody directed to protein Pc, washed in Tris-HCl buffer, pH 8.0, containing 0.1% bovine serum albumin and transferred to the commercially-available secondary antibody (goat-antirabbit IgG alkaline phosphatase conjugate, Biorad). The antigen-antibody-antibody-AP complexes were visualized using the substrate 5-bromo-4-chloro-3-indolyl phosphate in combination with nitro blue tetrazolium, which detects the precipitated indoxyl group.

Sequence analysis

Immunopositive plaques were replated at least three times to ensure homogeneity of the corresponding phage populations. Phage DNA was prepared from plate lysates according to standard procedures (Promega, 1991). For sequence analysis, cDNA inserts were recovered by digestion with *Not* I and subcloned into the plasmid vector Bluescript (Stratagene). DNA sequence analysis was performed by the dideoxy chain termination procedure (Sanger et al., 1977) according to the instructions of the supplier of the Sequenase (Ver. 2.0) sequencing kit (United States Biochemicals, Cleveland, OH, USA). Sequence comparisons were performed in the EMBL database using the FASTA programme.

Results

Protein Pc

After successful penetration, the fungus *P. herpotrichoides* starts colonizing leaf sheaths and stems of wheat plants. This process induces changes of the protein

Fig. 1 Detection of protein Pc by Western blot analysis. The native proteins of samples were separated by polyacrylamide gradient gel electrophoresis and blotted onto nitrocellulose membrane. Lane 1: sample from excised lesions of infected plants; lanes 2 and 3: samples from non-inoculated plants

pattern. In Fig. 1 green stem tissue of non-infected plants was compared by Western blotting with tissue from eyespots excised from infected plants. Only in healthy tissue could protein Pc be visualized.

The content of protein Pc within different parts of the stem can be quantified by ELISA. At the site of lesions host tissue is degraded. This process appears much more rapidly in susceptible than in resistant genotypes carrying the gene *Pch*-1 which retards the development of lesions. This is indicated in Table 1. It shows the readings of a serological test with the cultivars 'Rendezvous' (resistant) and 'Granada' (susceptible). When samples were taken from plants at stage 22, most tissue in the lower part of the stem was still green in resistant but already heavily attacked in susceptible plants. Resistant genotypes therefore show high ELISA values, i.e., high quantities of protein Pc, whereas in susceptible genotypes ELISA values are very low, i.e., the content of the protein has been reduced drastically. In non-inoculated plants of 'Granada' the quantity of protein Pc was about the same as in the green and obviously unaffected tissue of 'Rendezvous'.

The quantity of protein Pc was also measured at growth stage 35. At that stage, even in resistant plants extensive tissue degradation has developed (lower part of Table 1). At the site of lesions the ELISA values have decreased and do no longer differ very much between 'Rendezvous' and 'Granada'. Above the lesions in the green and undegraded tissue the protein is still synthesized abundantly in both infected cultivars. Their values are similar to that of the non-inoculated control. ELISA, therefore, can be used to differentiate green tissue from more or less diseased tissue.

Sequence analysis

In order to identify the protein Pc, the mRNA was isolated at the same time (stage 22) from green and healthy tissue when samples for serological tests were collected. The mRNA was used to construct a cDNA library. In immunological screening of about 70 000 cDNA clones, 22 showed a positive reaction with the antiserum, i.e., 3200 clones were required to identify one insert coding for protein Pc. Because of overlapping regions, the clones N, B, Kks, Tu, To, and Uu were used for sequence analysis. An EMBL database search identified them as fractions of the gene for plastocyanin. The nucleotide sequencing strategy, the clones and the region that has been sequenced are presented in Fig. 2. The nucleotide sequence is based on clones N and B which, after being joined together, include the complete coding region of the plastocyanin gene. The remaining clones were analysed to supply additional data for sequence confirmation. Figure 3 shows the nucleotide and deduced amino acid sequence. The coding region, initiated by a methionine codon, starts at position 1 and terminates at position 462. The open reading frame comprises the pre-plastocyanin of 158 amino acid residues, the first 61 residues of which constitute the transit peptide. It is followed by a sequence encoding the N-terminal amino acid sequence of the mature protein comprising 97 residues. The cleavage site between transit peptide and mature plastocyanin was determined by comparison with the sequence known from barley (Nielsen and Gausing, 1993) which is completely homologous in that region. The calculated molec-

Table 1
ELISA values measuring the quantity of plastocyanin in the infected wheat cultivars 'Rendezvous' (resistant) and 'Granada' (susceptible) and in the non-inoculated control ('Granada')

Type of sample	Inoculated Rendezvous	Inoculated Granada	Non-inoculated Granada
Growth stage 22:			
Lower 3 cm of stem	0.586	0.096	0.638
Growth stage 35:			
3 cm above lesion	0.631	0.593	0.597[a]
Upper part of lesion	0.216	0.193	
Centre of lesion	0.011	0.014	
Lower part of lesion	0.156	0.129	

[a] Sample of the lower 3 cm of the not infected stem.

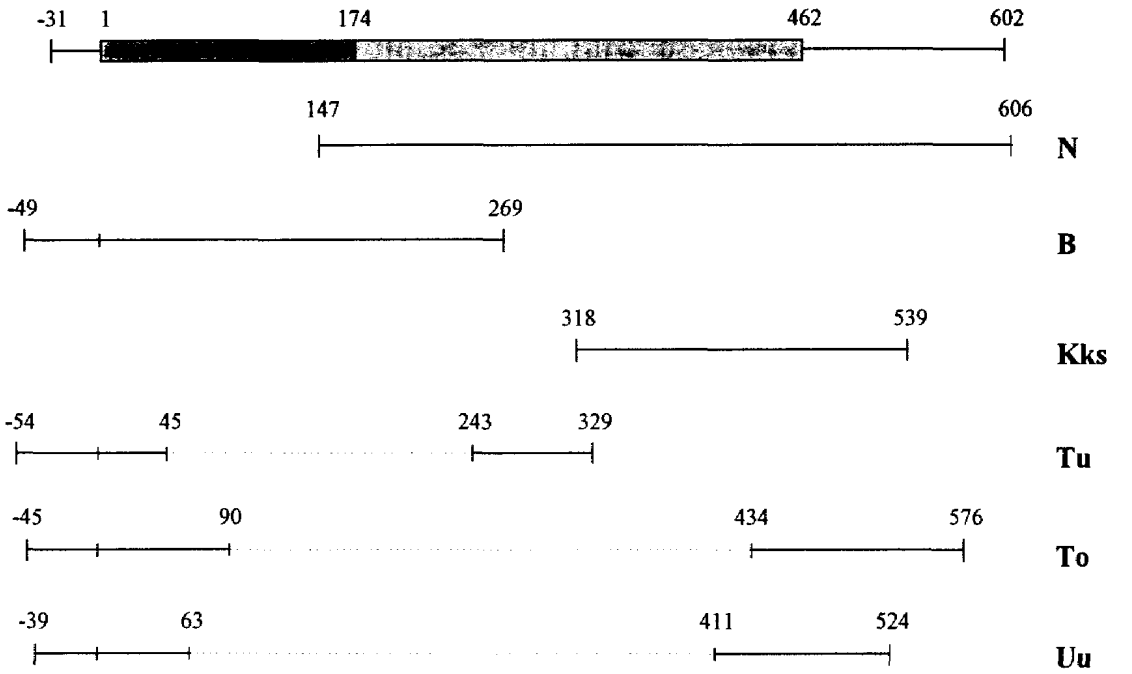

Fig. 2 Nucleotide sequencing strategy of the plastocyanin gene and the six partial cDNA clones used (N, B, Kks, Tu, To, Uu). The regions coding for the transit peptide and mature plastocyanin are represented by a dark-dotted and a light-dotted box, respectively. Non-sequenced regions of cDNA clones are indicated by a dotted line. Clone sizes are given in bp. The numbering of nucleotides is the same as in Fig. 3

ular weight for the transit peptide is 5.5 kDa and for the mature protein 10.1 kDa.

The DNA sequence in Fig. 3 also includes a 5′- and a 3′-untranslated region, comprising the nucleotides -1 to -31 and 466–602, respectively. The 5′-region contains the translation initiator consensus surrounding the methionine codon. The consensus was identified again by alignment with the corresponding sequence in barley. Characteristic polyadenylation signals (Elliston and Messing, 1988) could not be detected in the untranslated regions.

Discussion

In higher plants most chloroplast proteins are encoded by nuclear DNA. These proteins are synthesized as precursor proteins that are post-translationally taken up by chloroplasts and directed towards their functional compartment (Hageman et al., 1990). One of these proteins, plastocyanin, is a photosynthetic electron carrier encoded by the nuclear gene *petE*.

The comparison of the derived amino acid sequence of wheat with that of barley reveals a high identity of plastocyanin (Fig. 4) of 93.7%. The mature protein regions are more conserved than the transit peptide parts. In untranslated regions, however, there is no homology, except at the consensus sequence close to the methionine starter codon which is shown for wheat and is GCAGCCATGGC for barley (Nielsen and Gausing, 1993).

In Fig. 4, the few differences in transit and mature peptides are boxed and gaps introduced to maximize homology are indicated by dashes. The most notable features are the deletions of amino acids 17–19 in barley and of amino acids 24–26 and 71 in wheat. As in barley the mature plastocyanin starts with glutamine, an amino acid not hitherto found in the N-terminus of plastocyanin (Nielsen and Gausing, 1987). Three of the amino acids of the mature protein of wheat differ from barley: glutamic acid (79), serine (84) and phenylalanine (96). They are replaced in barley by the chemically closely related amino acids aspartic acid, alanine and tyrosine, respectively. The close relationship between wheat and barley plastocyanin is supported by studies of Robinson and Ellis (1984) with the enzyme that cleaves pre-plastocyanin to the mature size.

Comparisons between other plant species revealed a much lower identity of nucleotide sequences than between both cereals of wheat and barley (Smeekens et al., 1985; Rother et al., 1986; Nielsen and Gausing, 1987, 1993; Vorst et al., 1988; Detlefsen et al., 1989; Last and Gray, 1989; Merchant et al., 1990). There were, however, regions that proved to be highly conserved and were common to all plastocyanin genes.

There is no information available on the organisation of the plastocyanin gene in hexaploid wheat. With regard to the high homology between wheat and barley, a similarly high identity of sequences is expected for the plastocyanin genes of the individual wheat genomes A, B and D. Therefore, it cannot be excluded that the cDNA clones selected for the sequence analysis belonged to different genomes.

Plastocyanin could be extracted abundantly from

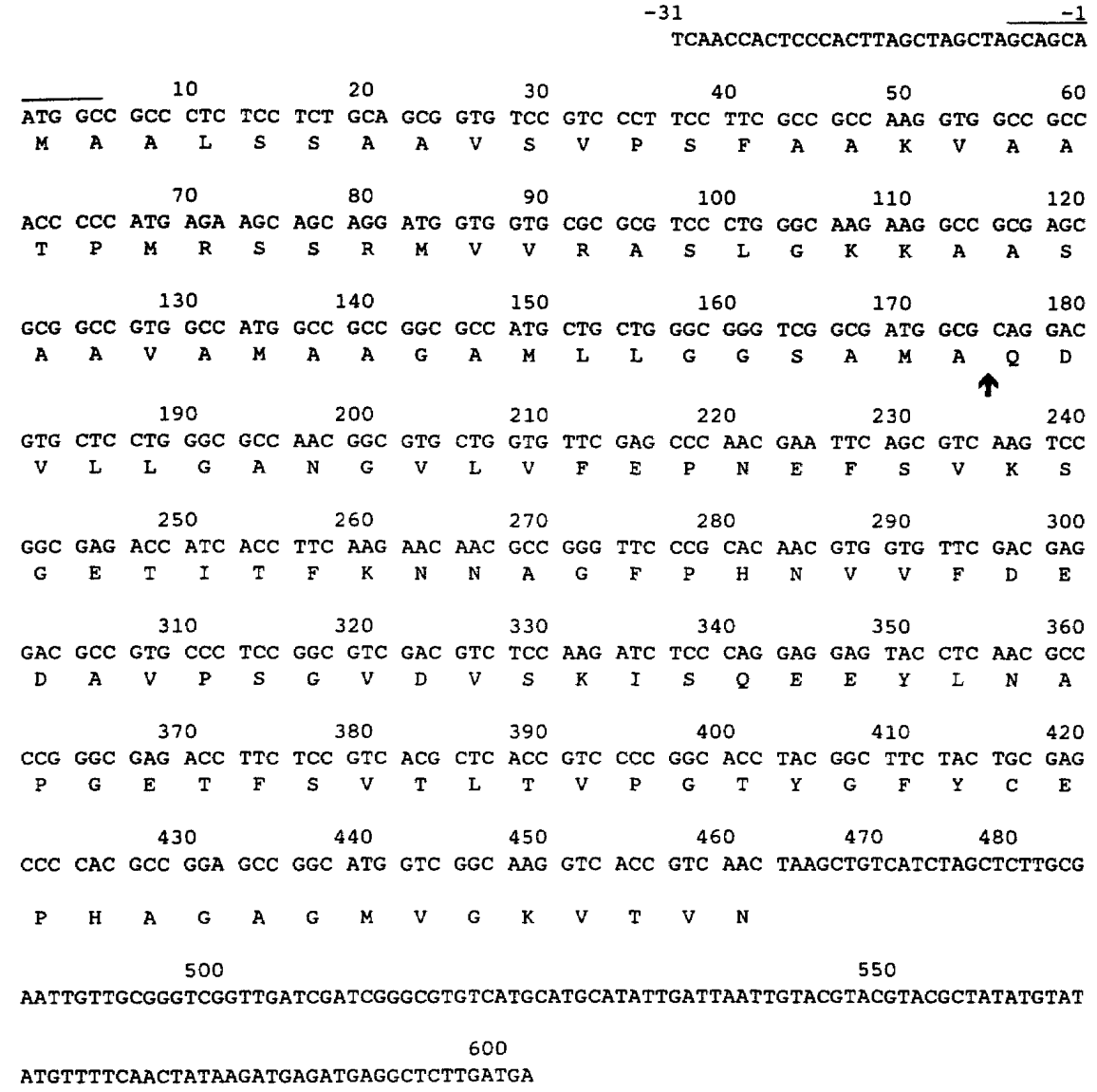

Fig. 3 Nucleotide and derived amino acid sequence of pre-plastocyanin. Numbering of the nucleotides starts at the methionine initiator codon, nucleotides upstream of the coding region have negative numbers. The putative processing site between the transit peptide and the mature plastocyanin is designated by an arrow. The translation initiator consensus is overlined

green plant parts but was less or no more existent in diseased tissue. For this reason the protein level is related with the extent of degraded tissue and with disease severity. The amount of protein in stem segments can be measured by ELISA using the antiserum directed against protein Pc. For the assessment of disease symptoms this type of assay has found widespread application for quantification of disease severity in different plant species (Foroughi-Wehr et al., 1995; Lind, 1992; Höxter et al., 1991; El-Nashaar et al., 1986).

The identification of protein Pc as plastocyanin suggests that the protein, although used as indicator for the damage caused by the fungus, is not related with a defense mechanism of the plant. Its reduction in amount occurs in all genotypes irrespective of the presence of the resistance gene *Pch*-1 and depends solely on the process of tissue degradation. The higher plastocyanin content in resistant plants is due to the reduced growth of the fungus which is affected by the gene *Pch*–1.

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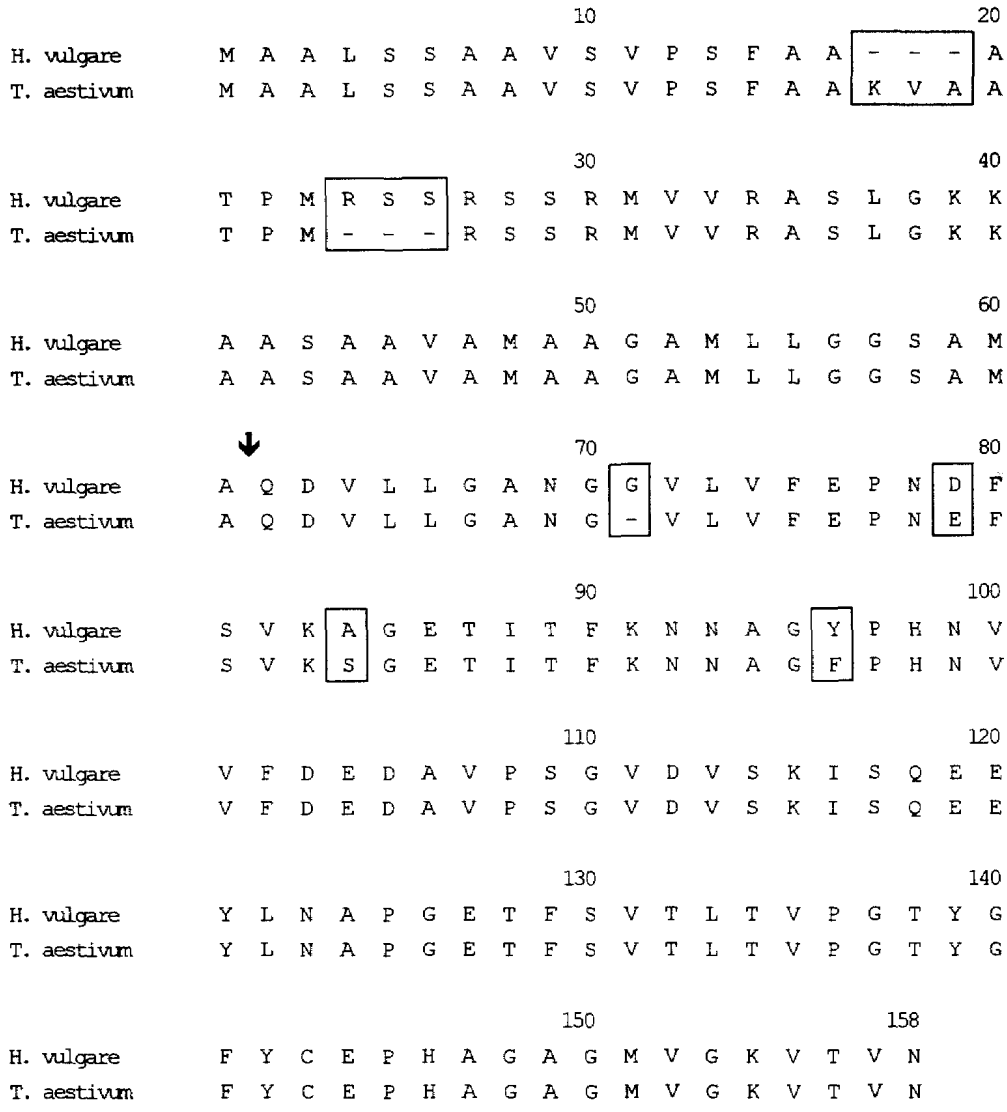

Fig. 4 Alignment of pre-plastocyanin of *H. vulgare* and *T. aestivum*. Differences in the amino acid sequences are boxed. The arrow indicates the site of cleavage to yield the mature protein

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