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Genetic mapping of genes for twelve nuclear-encoded polypeptides associated with the thylakoid membranes in *Beta vulgaris* L.

Klaus Pillen[1], Jörg Schondelmaier[2], Christian Jung[3], Reinhold G. Herrmann*

Botanisches Institut der Ludwig-Maximilians-Universität München, Menzinger Str. 67, D-80638 München, Germany

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Abstract Thylakoid membranes of chloroplasts are composed of approx. 75 polypeptide species. Nearly 60% originate in nuclear genes, the remainder in plastid genes. In order to localize representatives of the nuclear-encoded gene complement in a eukaryotic plant genome (sugar beet, *Beta vulgaris* L.), we have investigated the RFLP patterns of 21 cDNAs from spinach that code for thylakoid proteins or proteins peripherally associated with thylakoid membranes. Differences in gene dosage were noted between both related species. Polymorphism was found for 12 cDNA loci in a segregating sugar beet F_2 population. These loci were mapped along with genomic RFLP, isozyme, and morphological markers, and shown to be distributed in six of the nine sugar beet linkage groups. The lack of positional clustering even of genes that encode components of the same supramolecular membrane assembly is commensurate with phylogenetically independent gene translocations from the plastid (endosymbiont), and raises the question of the functional integration of various translocated genes into common signal transduction chains.

Key words: RFLP mapping; Photosynthesis; Nuclear gene; Evolution; Gene dosage; *Beta vulgaris*

1. Introduction

Thylakoid membranes in chloroplasts, which catalyze the fundamental process of the solar energy conversion in photosynthesis, are of dual genetic origin (summarized in [1]). Approx. 40% of the protein complement that constitutes this membrane, about 75 major polypeptide species, originates in organelle chromosomes and the remainder in nuclear genes. Most of these proteins are assembled into five membrane-embedded multisubunit assemblies, photosystems I and II with their chlorophyll *a/b*-binding antenna, the cytochrome *b/f* complex, ATP synthase, and a putative NAD(P)H dehydrogenase, which along with mobile, peripheral extrinsic (plastocyanin, ferredoxins, thioredoxins) and intrinsic (plastoquinone) electron carriers, ensure efficient photosynthetic electron transport.

During the past decade, research in this field has focused primarily on the isolation and physical mapping of the plastid-encoded genes for thylakoid proteins, and subsequently on the isolation of cDNAs for nuclear partner genes (e.g. summarized in [1]). Although more than three dozen nuclear genes for thylakoid proteins have been isolated and characterized, their genetic mapping has thus far been limited to members of two pauci-gene families of the photosynthetic machinery, the genes of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*Rbc*S) and those of the chlorophyll *a/b*-binding proteins (*Lhc*). In pea, both gene families are each located at a single locus [2]. Two or more loci have been found for each family in tomato [3,4], maize [5], *Petunia* [6], and *Arabidopsis* [7,8]. Comparably, Chao et al. [9] have localized seven genes encoding enzymes of the Calvin/Benson cycle on wheat chromosomes. The genes for five of nine enzymes were classified as single copy, yet in spite of the fact that they belong to a common pathway there was no detectable positional linkage between them.

The work presented here was initiated to extend our knowledge on nuclear-encoded genes for thylakoid proteins. The recent construction of an RFLP map from sugar beet [10], a close relative of spinach, the availability of a substantial number of cDNAs encoding thylakoid and membrane-peripheral proteins from the latter organism [1] as well as the development and the perspectives of megabase and of novel cytological technologies ([11] and references therein) provided the incentive to localize genes by genetic linkage analysis.

2. Materials and methods

2.1. Screening plants for DNA polymorphism and estimation of gene dosage of photosynthesis genes

The spinach cDNAs [1] were mapped among 96 F_2 plants, originating from an intraspecific sugar beet cross [10]. Leaf DNA extracted as outlined in Pillen et al. [10] was digested with one of eight restriction enzymes (*Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Ssp*I, and *Xba*I) according to the manufacturer. Electrophoresis of digested DNA and Southern analysis with radiolabelled cDNA (Table 1; [α-^{32}P]dCTP plus [α-^{32}P]dATP, Amersham/Braunschweig) were performed according to [10]. The gene nomenclature used follows the recommendations outlined in [12].

2.2. Mapping of RFLPs

cDNAs exhibiting restriction fragment length polymorphism (RFLP) between the parents of the mapping population were hybridized to Southern blots of digested DNA of the complete set of F_2 individuals using appropriate restriction enzymes. After checking for correct 1:2:1 segregation of the allelic RFLP fragments in the population by χ^2 analysis ($\alpha = 0.01$), the cDNA markers were mapped along with random genomic RFLP markers, isozymes, and two morphological genes [10] using the MAPMARKER computing package [13]. Linkage criteria for associating a marker with a discrete linkage

*Corresponding author: Fax: (49) (89) 171683.

[1]*Present address:* Institut für Pflanzenbau, Rheinische Friedrich-Wilhelms-Universität Bonn, Katzenburgweg 5, D-53115 Bonn, Germany.

[2]*Present address:* Institut für Pflanzenbau und Pflanzenzüchtung, Christian-Albrechts-Universität Kiel, Olshausenstr. 40, D-24118 Kiel, Germany.

[3]*Present address:* Institut für Pflanzenbau und Pflanzenzüchtung, Christian-Albrechts-Universität Kiel, Olshausenstr. 40, D-24118 Kiel, Germany.

Abbreviations: cM, centimorgan; RFLP, restriction fragment length polymorphism; Fd, ferredoxin I; FNR, ferredoxin NADP$^+$ oxidoreductase; PC, plastocyanin; TRF, thioredoxin *f*; TRM, thioredoxin *m*

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group included a minimum LOD score of 4.0 and a maximal genetic distance of 0.21 recombination units. The order of markers in each linkage group was re-found using the MAPMARKER function *order* with LOD = 3.0. Recombination distances were transformed to centimorgans (cM) according to Kosambi [28].

3. Results

3.1. Gene dosage

21 cDNAs encoding thylakoid membrane proteins from spinach (Table 1) were screened for gene dosage and restriction polymorphism in sugar beet. Depending on the number of DNA fragments detected in Southern analysis, the genes related to these cDNAs were grouped into three classes: single-copy genes that were characterized by one or two signals, low-copy genes by three to six, and multi-copy genes in the case of more than six signals. According to these criteria and considering signal intensity, three cDNAs (loci) were classified as single copy, 12 as low copy and six as multi copy (Table 1). Low-copy sequences hybridized in all but one case to two different loci as indicated by three to four signals. Clone cTRF generated five signals suggesting the existence of at least three genes in sugar beet. Three low-copy clones (*Psa*F, *Psa*L, *Pet*E) generated both strong and weak hybridization signals (cf. Section 4).

3.2. Mapping of photosynthesis genes

12 of the 21 cDNAs generated polymorphism between the chosen sugar beet parents after screening with the eight restriction enzymes listed above. These cDNAs were then hybridized to restricted, filter-bound DNA of all 96 F_2 plants of the mapping population. The individual F_2 genotypes were scored from autoradiograms for (i) homozygosity of the male or female RFLP fragment, or for (ii) heterozygosity (both RFLP fragments present). Only one of the clones (*Pet*H) displayed distorted segregation. For this cDNA, the genotype classes segregated 9:59:25 within the population, presumably due to weak linkage to a gametophytic lethal gene [10]. The skewed segregation did not interfere with linkage analysis, however [14]. All other cDNAs segregated in a 1:2:1 ratio as expected.

The linkage analysis for the 12 cDNAs encoding thylakoid and membrane-peripheral proteins was computed with a set of 164 single-locus markers [10], including 155 random genomic DNA markers, seven isozymes, and two morphological markers. The genes are spread on six of the nine sugar beet linkage groups corresponding to the individual chromosomes (for designation see [10]; Fig. 1). Four are located on chromosome II (*Psb*O, cTRF, *Psa*L, and *Psb*R), two each on groups IV (*Psa*H and *Pet*C), V (*Psb*S and *Pet*E) and VII (*Pet*H and *Psb*P), and single loci mapped on chromosomes VI and IX (*Psa*E and *Psb*Q, respectively). The genetic distance between the loci on the same linkage group varies up to 45.8 cM (*Psa*H and *Pet*C) implying that these genes are loosely linked. Tight linkages have been noted between anonymous genomic DNA probes and various cDNA loci that could serve as initiating targets for the isolation of genes utilizing the available beet megabase (YAC) library [15]. For instance, the gene *Psa*H is flanked by markers pKP1162 and pKP563 with distances of 0.0 and 0.6 cM, respectively (see Fig. 1).

Table 1
Assessment of gene dosage and genomic location of nuclear cDNAs encoding proteins of the thylakoid membrane in *B. vulgaris*

Protein	Position in thylakoid membrane	Gene	Gene dosage	Mapped on linkage group
Photosystem II				
33 kDa	luminal	*Psb*O	low copy	II
23 kDa	luminal	*Psb*P	low copy	VII
22 kDa	intrinsic	*Psb*S	low copy	V
20 kDa	intrinsic	*Lhcb*6	single copy	
16 kDa	luminal	*Psb*Q	low copy	IX
10 kDa	intr. (stroma)	*Psb*R	single copy	II
Cytochrome *b/f* complex				
Rieske	intrinsic	*Pet*C	single copy	IV
Photosystem I				
PSI-2	intr. (stroma)	*Psa*D	low copy	
PSI-3	intr. (lumen)	*Psa*F	low copy	
PSI-4	intr. (stroma)	*Psa*E	low copy	VI
PSI-5	intr. (stroma)	*Psa*G	multi copy	
PSI-6	intr. (stroma)	*Psa*H	low copy	IV
PSI-11	intrinsic	*Psa*L	low copy	II
ATP-synthase				
CF1-γ	intr. (stroma)	*Atp*C	multi copy	
CF-δ	intr. (stroma)	*Atp*D	multi copy	
CFo-II	intrinsic	*Atp*G	multi copy	
Peripheral proteins				
PC	luminal	*Pet*E	low copy	V
Fd	stromal	*Pet*F	multi copy	
FNR	intr. (stroma)	*Pet*H	low copy	VII
TRM	stromal		multi copy	
TRF	stromal		low copy	II

The nomenclature of proteins and their membrane localization are depicted from [12]. No approved designation of thioredoxin genes has yet been released. The stroma or lumen orientation of extrinsic or peripheral proteins is given in parentheses.

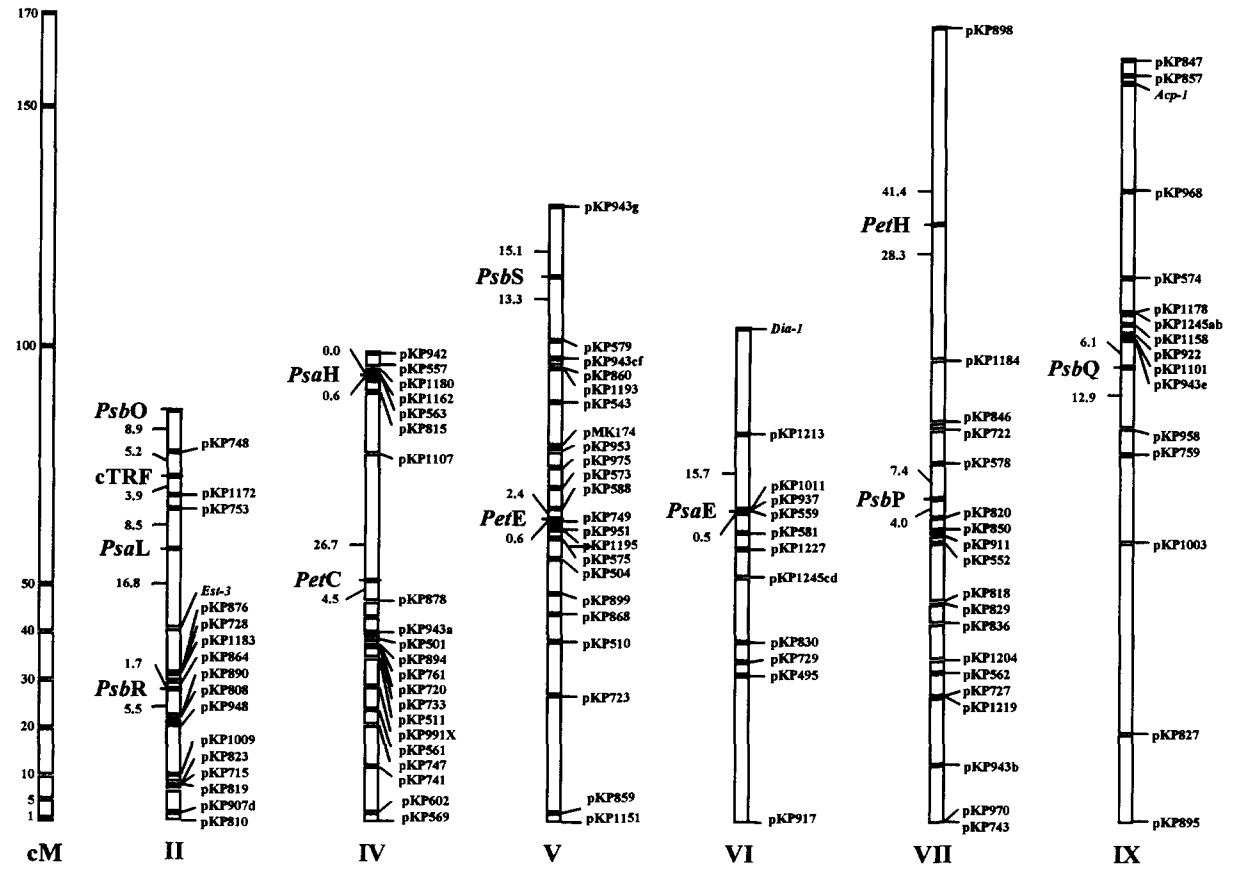

Fig. 1. Genetic location of 12 genes for proteins involved in photosynthesis on the sugar beet linkage map (Table 1 and [10]). Genes and genetic distances to adjacent loci in centimorgans (cM) are placed to the left of each linkage group. RFLP markers are placed to the right.

4. Discussion

One of two remarkable findings of this study is the difference in gene dosage noted between sugar beet and its relative spinach which do not correspond in various cases. For instance, genes of the ATP synthase and of the peripheral proteins ferredoxin (*Pet*F) and thioredoxin *m* are multi copy in sugar beet deviating significantly from gene dosages (1–2 copies) in spinach (cf. [1], Table 1). The analysis of the nuclear gene dosage for the 21 proteins studied revealed only three single-copy genes in sugar beet compared to 10 in spinach [1]. Single genes have been found for the *Psb*R-derived polypeptide, the CP24 apoprotein (*Lhcb6*) and the Rieske Fe/S protein (*Pet*C). *Psb*Q, *Psb*P, *Psb*O, *Atp*D, *Atp*G, *Pet*E, and *Pet*H, single copy in spinach, were identified as low-copy or even multi-copy genes in the haploid sugar beet genome. Alternative explanations to account for this difference between both species, which both belong to the genus of the Chenopodiaceae, include different evolution, the existence of pseudogenes, and in some instances even artifacts due to cross-hybridization of the spinach cDNAs with partially homologous DNA sequences in sugar beet under the chosen hybridization stringency (see below).

Digestion of the DNA from the mapping population with four restriction endonucleases detected polymorphism for nearly 50% of randomly selected genomic DNA markers (unpublished results). An increase of the number of restriction endonucleases to 8 was necessary to uncover polymorphism for cDNAs and to increase the ratio of polymorphic signals to approx. 60% of the cDNAs. Collectively, this is a relatively low figure for an outbreeder like sugar beet. Further improvement could probably be achieved by extending the number of restriction enzymes and/or by screening additional mapping populations.

Another remarkable finding of the outlined study is that clustering and co-transcription of genes, a strategy widely used in prokaryotic organisms (as well as in plastid chromosomes) to coordinate the expression of genes for different subunits of a distinct protein complex, is not adopted in the genome. This is consistent with both the phylogeny and the functional organization of eukaryotic genomes and reminiscent of data obtained from wheat for components of the Calvin/Benson cycle [9], but differs from those of *Rbc*S and *Lhc* gene families (cf. Section 1 and below). These findings bear three aspects of general interest that are crucial for the biology of eukaryotes.

(i) The scattered arrangement of nuclear genes for thylakoid proteins most likely finds a phylogenetic explanation [16]. The endosymbiont theory claims three distinct categories of nuclear genes involved in the biology of organelles, including both loss and gain of function. One of these classes is represented by plastid genes or ptDNA segments that were retained in the cell but translocated to the nucleus and/or to mitochondria during evolution (summarized in [16,17]), a process that is probably still ongoing. The coincidence between prokaryotic and plastid operon design, and a substantial sequence conservation leave no doubt about the common ancestry and the adoption of genes missing in plastid transcription units in the

nucleus. Information is relatively scarce of how and how often such translocations have occurred as well as what happened to a gene/DNA segment or a gene cluster after its transfer. Whatever the mechanism and frequency, the loss of a sequence from an individual plastid chromosome is without risk to the cell [18], due to the high degree of plastome reiteration (e.g. [16]). The existence of apparently organelle-derived, generally non-functional, promiscuous DNA [19] suggests that intracellular gene translocation processes are more frequent than generally anticipated, specifically if the possibility is considered that at least part of the translocated DNA is lost ([16], see below), and that only a fraction of the translocated sequences manages in evolution. To be of selective advantage, indispensable prerequisites are that a translocated gene acquires nuclear DNA segments which could operate as promoters and also possess the potential to develop into transit peptides. Not only the scattered chromosomal positions of genes for thylakoid polypeptides, but also the observations that the promoters and transit peptides of different genes even of those that code for the same membrane assembly, but not of the same gene in different organisms, lack homology [16], are consistent with the idea that translocation occurred individually for each gene. The *atp* genes encoding thylakoid-located F-ATP synthases provide an instructive example. They are generally organized in one or two operons in prokaryotes and plastids with a highly conserved order of arrangement throughout the eubacterial and plant kingdoms (e.g. [20–22]). In eukaryotes, some of these genes are now nuclear, subunit γ in all plants studied to date, subunits δ and CFo-II only in the chlorophyll *a/b* but not *a/c* lineages of plant organisms [21]. In prokaryotes, the three genes are separated by other *atp* genes from each other. It is possibly not pure coincidence that the intercistronic regions flanking *atp*C are remarkably prone to phylogenetic instability [22], that the transit peptides and promoters [23] of all three subunits display no obvious homology, and that in fungi proteolipid genes (*atp*H) are either of nuclear or of mitochondrial origin [24].

Genetic mapping of photosynthesis genes, in particular of the *Rbc*S and *Lhc* genes [2–8], has uncovered two further aspects of general interest related to the origin and the evolution of gene families. (ii) In principle, genes could be integrated into the genome and maintained individually [3]. They could be duplicated and distributed within the genome to form gene families [4–8], but repeated translocation is conceivable as well [18]. *Lhc* and related genes encode multiple biologically active peptides that perform coordinated function in light collection, energy transfer or stress protection. They represent at least five subfamilies, encoding the LHCI and II apoproteins, ELIPs, the apoproteins of the minor antenna CP24/CP26/CP29, and the recently described *Psb*S product, respectively. The former three originate in gene clusters, the latter in single-copy genes in spinach and tomato [18,25]. Only *Psb*S appears to possess an equivalent in prokaryotes, and for this and other reasons has been proposed to be original relative to the others. The observations that the structural genes display a remarkable degree of general homology and predicted secondary structure [25] but that the transit peptides (and flanking sequences, where known) between (not within) subfamilies are entirely different [18,26] have suggested that the *Lhc* family, unlike the *Rbc*S family, must represent a superfamily [16,18] that resulted from the repeated transfer of an ancestral gene or of part of it from the organelle to different chromosomes. If so, the subfamilies evolved subsequently by a series of duplications, rather than one member being initially transferred once which then spread within the genome. That *Lhc* genes and subfamilies, respectively, occupy different positions within the genome is in line with separate translocation events independent for each subfamily. (iii) Current ideas about gene duplications suggest the existence of stringent mechanisms for keeping to a minimum the number of genes, especially if the genes have a vital role [26,27]. Duplications should be tolerated only where they are of functional use, for example, to adapt or facilitate the synthesis of a component in response to different environmental or developmental stimuli. This 'elimination strategy' of genetic material, for instance amply documented in polyploid material (e.g. [9]), appears to operate not only in the nucleus but also between compartments (summarized in [16,18]) since a single translocated gene once functional in the nucleus manages both the streamlining of the acquired sequence interval and the elimination of the corresponding highly reiterated organelle segment, probably relatively fast. However, this mechanism cannot be strict, and in fact exhibits enigmatic and virtually contradictionary facets. Apart from the as yet unexplored fact that reiteration of plastid chromosomes persists, sequence comparison of independently selected cDNAs (and genomic DNA) suggests that nuclear genes for an organelle protein may be present in two (or a few) copies that can be much less diverged than members of *Rbc*S and *Lhc* gene families, since they may differ by only a few often even silent mutations [16]. It will therefore be of considerable interest to decipher the basis for the discordant gene dosage between sugar beet and spinach by synteny analysis, to explore whether '*pauci*-copy' genes possess similar regulatory sequences, whether they are clustered in tandem or occupy dispersed positions within the genome or a chromosome, and whether the chromosomal positions of the duplicated genes are related to spatial aspects of chromosome arrangement, to function, reflect solely their evolutionary history, or both.

It is obvious that nuclear genes encoding or involved in the management of chloroplast structures represent an increasingly important source of information towards a better understanding of the eukaryotic cell in general and of photosynthesis in particular, from both a phylogenetic and functional point of view. For instance, a persistently intriguing and as yet barely discussed central aspect in eukaryotic cell biology is of how the transcription for the translocated nuclear genes with so different regulatory sequences between genes is coordinated per se, and also with those originating in the organelle, to ensure the correct biogenesis of the thylakoid membrane complexes in time, space, quantity and induceability, and how during evolution these different promoters have been integrated into the network of signal pathways that control their (often concerted) expression. Furthermore, physiological adaptation or specialization (e.g. C3 vs. C4 plants) may not only reside in phylogenetic promoter changes, but also in changes of chromosomal position (cf. [16]). Thus, genetic mapping of loci for proteins involved in photosynthesis can substantially extend our knowledge on nuclear genes in general. Combined with novel approaches, it can provide access to crucial questions that were not accessible before, and thus open a wide field of experimentation. In particular, megabase techniques that allow the isolation of genes with

an extended chromosomal context, along with the recently developed techniques of high-resolution field emission electron microscopy of plant chromosomes, and refined protocols for in situ hybridization (cf. [11]) may aid in exploring the significance of the chromosomal context of a gene or of the dimensions between the DNA thread and the cytological entity 'chromosome' for gene expression that, for instance, becomes obvious in the so-called position effects of transgenes. They may also aid in unraveling the significance of phylogenetic positional relationships (and their changes) or relationships between genetic, physical and topographic distances as well as genetic processes that cause or contribute to the distribution of sequences within genomes.

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