A Genome-Wide Analysis of the Chloroplast NADH Dehydrogenase-like Complex in *Zostera Marina*: Identification, Splicing, Editing and Profiles of Expression

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Abstract

Background: The chloroplast NADH dehydrogenase-like (NDH) complex, homologous to respiratory complex I, participates in photosystem I cyclic electron flow (PSI-CEF) and chlororespiration in photosynthesis. However, little information was available about the *ndh* genes in *Zostera marina* which is one of the most productive and wide-distributed seagrasses.

Results: The phylogenetic analysis indicated that *Z. marina* has a complete NDH complex which is rarely observed in marine macrophytes. We identified all 31 *ndh* genes necessary for the functional NDH complex, with *ndhB* and *pnsb3* occurring as duplication events. Secondary structural analyses of antiporter-like subunits showed that the long amphipathic helix of NdhF was lost in *Z. marina*, suggesting an alternative mode in the generation of trans-thylakoid proton gradient. The splicing pattern in *ndh* genes exhibited tissue-specific patterns but no response to photo-regulation. RNA editing in *Z. marina* presented the ancestral pattern with many of the primitive editing sites and types. The partial editing in *ndhF* reflected the link between light stress and RNA editing. Moreover, predominant expression in the leaves of most *ndh* genes implied their major function in photosynthesis. The dynamic profiles of expression in response to light stress suggested that there were two diverse responsive and regulatory mechanisms of the NDH complex in PSI-CEF and chlororespiration.

Conclusions: In this study, we performed a systematic analysis of the identification, splicing, editing and pattern of expression of the *Z. marina* NDH complex. Our study help elucidate the further evolutionary and functional exploration of the NDH complex.

Background

The chloroplast NADH dehydrogenase-like (NDH) complex is one of the thylakoid membrane protein complexes that participates in photosynthetic electron transport chains. The NDH complex catalyzes the transfer of electrons from ferredoxin (Fd) to plastoquinone resulting in translocation of protons from the stroma to lumen across the thylakoid membrane. This process leads to the generation of trans-thylakoid proton gradient, which drives the production of ATP [1, 2]. Cyanobacterial NDH-1 can be divided into two major parts that contain the membrane segments (NdhA-NdhG) that participate in proton translocation and the peripheral segments (NdhH-NdhK) that carry redox centers, Fe-S clusters and flavin mononucleotide. The two parts form the L-shape structure which is conserved in both photosynthetic and respiratory NDH complexes [3–6]. Additionally, some new subunits that are required to stabilize the NDH complex in higher plants were found. Thus, the complete structure of the chloroplast NDH complex is composed of five subcomplexes, including subcomplex M (NdhA-NdhG), subcomplex A (NdhH-NdhO), subcomplex B (PnsB1-PnsB5), subcomplex L (PnsL1-PnsL5) and subcomplex EDB (NdhS-NdhV) [7].

Since the defective *ndhB* gene was found to influence photosystem I cyclic electron transport (PSI-CEF) in *Synechocystis* sp. PCC 6803 [8], substantial evidence indicates that chloroplast NDH is involved in PSI-CEF and chlororespiration [1, 2, 9]. Although NDH-dependent PSI-CEF subtly contributes to the total PSI-CEF under optimal growth conditions, the chloroplast NDH complex plays essential roles in the regulation of plant tolerance to abiotic stress. For example, the chloroplast NDH complex functions to alleviate the oxidative stress caused by heat, low irradiance, humidity, high light stress, and so on [10–17]. It also functions in the regulation of proton motive force at low light intensity and temperature [18]. Moreover, NDH-dependent PSI-CEF is necessary for the normal growth of plants [19]. Additionally, based on the fact that the 11 plastid-encoded Ndh subunits (NdhA-NdhK) are homologous to their counterparts in the mitochondrial NADH dehydrogenase [20, 21], the chloroplast NDH complex has been experimentally demonstrated to couple with the plastid terminal plastoquinone oxidase to mediate respiratory electron transport in chloroplasts which is referred to chlororespiration [22].

A general consensus is that the chloroplast NDH complex originated from the cyanobacterial NDH-1 complex [23]. To enhance the turnover of the electron flux via Fd and effectively resist stress, the NDH complex has attained dozens of novel subunits during the evolution of land plants [19]. Subcomplex M and several subunits in Subcomplex A (NdhH-NdhK), which forms the skeleton of NDH complex, are highly conserved in *Escherichia coli*., cyanobacteria and land plants [3]. Subcomplex B, which participates in the maintenance of the complex stability, emerged during the early stage of evolution of land plants [24]. *Marchantia polymorpha*, which is considered to be the earliest case of divergence from the land plant lineage, merely contains a portion of Subcomplex L [25], indicating this subcomplex is unique to terrestrial higher plants. NdhS in combination with NdhV in Subcomplex EDB form the Fd-binding site and is conserved in cyanobacteria and terrestrial higher plants, while other subunits in Subcomplex EDB are specific to terrestrial higher plants [26–28]. The integral chloroplast NDH complex primarily found in terrestrial higher plants, although some species in the Orchidaceae, Pinaceae, Gnetaceae and Geraniaceae have lost their chloroplast NDH complex during evolution [29–32]. The reason for the drastic variation in evolution of chloroplast NDH complex still remains unclear.

Zostera marina (Alismatales: Zosteraceae), one of the most productive species of seagrass, is widely distributed in the northern Pacific and northern Atlantic Oceans [33]. As the main component of seagrass meadows, it has crucial ecological values in nutrient cycling and sediment stabilization, as well as the provision of habitats and food provision for numerous organisms [34]. Seagrasses migrated to terrestrial conditions approximately 200 million years ago and returned to the sea approximately 140 million years ago [35]. During the migration to ocean, *Z. marina* occurred numerous gene losses and gains. Additionally, it evolved many unique structural and physiological characters to completely adapt to the marine environment. In this study, all 31 *ndh* genes of the entire NDH complex were identified in *Z. marina*, although they have rarely been detected in marine macrophytes even among the seagrasses in Alismatales [36]. Correspondingly, our previous research suggested that *Z. marina* possesses a highly efficient NDH-dependent PSI-CEF and chlororespiration [15, 37]. Although the structure, physiological function and assembly of chloroplast NDH has been reported in other species [19, 38], the specifically-evolved and highly efficient *Z. marina* NDH has yet remained to be investigated to our knowledge. The whole genome sequences that have been reported enabled us to conduct a systematic research of *ndh* genes in *Z. marina* [33, 39]. We comprehensively analyzed the phylogeny, gene structures, motif compositions, secondary structures, alternative splicing, RNA editing events, patterns of expression and upstream regulation of *ndh* genes in *Z. marina*. The pattern of expression also provided clues to understand how *ndh* genes coordinate with each other to drive the highly efficient NDH-dependent PSI-CEF in response to light exposure and chlororespiration during the subsequent recovery from being subjected to darkness.

Results

Identification of the Ndh subunits

A total of 31 Ndh subunits homologous to their *Arabidopsis thaliana* counterparts were obtained from *Z. marina* (Fig. 1, Table 1). NdhB and PnsB3 occurred duplication events during evolution. The annotations of these subunits were validated using the available transcriptome data of *Z. marina*. Gene *Zosma266g00030.1* encoding the NdhV subunit and *Zosma266g00040.1* encoding a hypothetical protein were contained in one transcript (designated *Zosma266g0003040.1*). Following the confirmation by reverse transcription-PCR (RT-PCR) and sequencing, in silico translation revealed that *Zosma266g0003040.1* translated a 191 amino acid while *Zosma266g00030.1* translated a 189 aa-long protein, suggesting that *Zosma266g00040.1* might be located in the 3′ UTR region of *Zosma266g0003040.1*. Furthermore, the misannotated coding sequence of *ndhB* was observed during the analysis of sequenced cDNA fragments in RNA editing. The duplicated *ndhB* had a uniform coding sequence with a 509 amino acid.

Table 1
Features of *ndh* genes identified in *Zostera marina*

Group	Gene Name	Gene ID	Size (aa[1])	MW (Da)	pI	Encoded Location	Chromosome No.	Gene span	Strand	Domain Type
Subcomplex M	*ndhA*	CRD45_pgp012	366	41058	6.42	Chloroplast	NC_036014.1	113255–115486	-	NADHdh
	ndhB-1	CRD45_pgp021	541	60531.28	5.69	Chloroplast	NC_036014.1	132775–135002	+	Proton_antipo_M
	ndhB-2	CRD45_pgp006	476	53088.81	5.79	Chloroplast	NC_036014.1	92137–94313	-	Proton_antipo_M
	ndhC	CRD45_pgp058	120	13869.39	4.19	Chloroplast	NC_036014.1	49652–50014	-	Oxidored_q4
	ndhD	CRD45_pgp017	495	55997.58	8.02	Chloroplast	NC_036014.1	108739–110226	-	Proton_antipo_M
	ndhE	CRD45_pgp015	101	11267.32	9.43	Chloroplast	NC_036014.1	111014–111319	-	Oxidored_q2
	ndhF	CRD45_pgp009	627	71389.28	8.53	Chloroplast	NC_036014.1	118195–120078	+	Proton_antipo_M,
	ndhG	CRD45_pgp014	184	20539.59	5.01	Chloroplast	NC_036014.1	111617–112171	-	Oxidored_q3
Subcomplex A	*ndhH*	CRD45_pgp011	404	47130.71	5.48	Chloroplast	NC_036014.1	115458–116672	-	Complex1_49 kDa
	ndhI	CRD45_pgp01	208	24405.6	8.77	Chloroplast	NC_036014.1	112589–113215	-	4Fe-4S ferredoxin-type
	ndhJ	CRD45_pgp060	164	19399.37	6.96	Chloroplast	NC_036014.1	48341–48835	-	Complex1_30 kDa
	ndhK	CRD45_pgp059	254	28713.9	9.07	Chloroplast	NC_036014.1	48918–49682	-	Oxidored_q6
	ndhL	Zosma137g00200.1	178	20545.08	10.28	Nuclear	scaffold_137	166629–167827	+	NdhL
	ndhM	Zosma57g00680.1	195	22336.32	6.43	Nuclear	scaffold_57	643639–644318	+	NdhM
	ndhN	Zosma31g01120.1	213	23958.89	9.59	Nuclear	scaffold_31	691078–692835	-	NdhN
	ndhO	Zosma196g00030.1	151	17295.97	9.32	Nuclear	scaffold_196	14822–15619	-	NdhO
Subcomplex EDB	*ndhS*	Zosma89g00850.1	250	27012.9	5.86	Nuclear	scaffold_89	385211–385963	+	NdhS
	ndhT	Zosma161g00490.1	246	28246.91	6.24	Nuclear	scaffold_161	203503–204402	-	DnaJ_domain
	ndhU	Zosma87g00270.1	218	24501.81	5.31	Nuclear	scaffold_87	167738–172875	-	DnaJ_domain
	ndhV	Zosma266g00030.1	189	20813.9	9.01	Nuclear	scaffold_266	58607–59816	+	Unkown
Subcomplex B	*pnsb1*	Zosma16g01560.1	468	51745.35	6.43	Nuclear	scaffold_16	985167–987045	+	Unkown
	pnsb2	Zosma39g00680.1	345	37684.75	6.97	Nuclear	scaffold_39	735514–738322	+	Unkown
	pnsb3-1	Zosma5g02060.1	200	22198.17	8.82	Nuclear	scaffold_5	1067520–1068382	-	2Fe-2S ferredoxin-type
	pnsb3-2	Zosma74g01180.1	229	25810.87	9.72	Nuclear	scaffold_74	611531–612956	-	2Fe-2S ferredoxin-type
	pnsb4	Zosma248g00070.1	197	22725.65	4.89	Nuclear	scaffold_248	19172–20349	+	Unkown
	pnsb5	Zosma13g00670.1	218	24800.02	5.16	Nuclear	scaffold_13	574318–576258	+	Unkown

[1]aa, amino acid

Group	Gene Name	Gene ID	Size (aa[1])	MW (Da)	pI	Encoded Location	Chromosome No.	Gene span	Strand	Domain Type
Subcomplex L	*pnsl1*	Zosma22g00960.1	233	26187.86	9.27	Nuclear	scaffold_22	705701–707101	+	PsbP
	pnsl2	Zosma30G00320.1	186	20883.08	9.05	Nuclear	scaffold_30	171042–171602	+	PsbQ
	pnsl3	Zosma19G01210.1	253	27865.71	5.47	Nuclear	scaffold_19	809801–810173	+	PsbQ
	pnsl4	Zosma241G00220.1	213	22654.18	8.99	Nuclear	scaffold_241	129967–131195	+	FKBP_C
	pnsl5	Zosma225G00240.1	249	26927.66	9.08	Nuclear	scaffold_225	229925–234960	-	Pro_isomerase

[1]aa, amino acid

Phylogenetic analysis of ndh **genes**

To investigate the evolutionary relationship among Viridiplantae NDH complexes, the amino acid sequences of 31 Ndh subunits were concatenated to construct a phylogenetic tree. As shown in Fig. 2, the phylogeny of NDH complex was almost consistent with the phylogeny of species. The complexity of NDH complex appeared to be increasing from algae to bryophytes to land higher plants with the exception of gymnosperms. Therefore, the most integrated NDH complex was mostly found in the terrestrial angiosperms with an obvious diversification between monocots and dicots. Unlike other marine species, *Z. marina* had a complete NDH complex, which indicated its particular evolutionary status.

The phylogenetic tree of the entire NDH complex was also compared with those constructed by individual Ndh subunits. The phylogenies of most Ndh subunits, including NdhA, NdhE, NdhF, NdhG, NdhI, NdhJ, NdhN, NdhT, NdhU, PnsL1, PnsL2, PnsL3 and PnsL4, were consistent with the concatenated phylogeny (Additional file 1A), indicating that these individual subunits co-evolved with the NDH complex. NdhB, PnsB3, NdhM, NdhO, and NdhS of *Z. marina* were at the basal branches of angiosperms (Additional file 1B), indicating their earlier evolution status.

Motif composition of the ndh **gene**

The motifs composition of each Ndh subunit and details of the motifs in conserved domain are shown in Additional file 1 and 2, respectively. The results revealed that closely related species usually shared a similar motif composition. The motifs in conserved domain were widely distributed, while the motifs in the N- and C-terminal regions varied, particularly for nuclear-encoded Ndh subunits. Certain motifs are clearly unique to the *Z. marina* Ndh subunits, i.e., motif 20 in NdhG, motif 13 in NdhH and motif 14 in NdhI, whereas certain conserved motifs were absent, i.e., motif 11 in NdhD and motifs 7, 14 and 17 in NdhF (Fig. 3). It was worth noting that the lost motifs in NdhD and NdhF were either located in the functional domain of the subunits or predicted to be a transmembrane helix.

Given the distinguishing motif composition of *Z. marina* in the functional domain of NdhD and NdhF, known as antiporter-like subunits, the multiple sequences of these subunits were aligned in six representative species of angiosperms. As shown in Fig. 4, NdhD harbors fourteen conserved TM helices, and NdhF harbors sixteen conserved TM helices. Unlike other species, *Z. marina* lost TM1 and TM13 in NdhD, which was ascribed to the substitution from motif 11 to 5. Moreover, it lacked the long amphipathic helix in NdhF that was attributed to the absence of motifs 7, 14 and 17.

The splicing pattern of ndh **genes in different tissues and light stress**

The alternative splicing (AS) types of the *ndh* genes that contained introns, including the chloroplast-encoded genes (*ndhA*, *ndhB*) and most of the nuclear-encoded genes (with the exception of *ndhS* and *pnsl2*), were analyzed using RT-PCR (Fig. 5 and Additional file 3). Ten *ndh* genes (*ndhB*, *ndhL*, *ndhM*, *ndhU*, *ndhV*, *pnsl1*, *pnsl4*, *pnsb2*, *pnsb3-1* and *pnsb3-2*) showed tissue-specific AS patterns (Fig. 5A), indicating that the AS of *ndh* genes possibly participated in the regulation of functional specification in different tissues. Remarkably, the AS transcripts of *ndhB* (*ndhB-AS-2*) that consisted of exons 1 and 2 and intron 1 exhibited opposite pattern of expression with the fundamental transcript (*ndhB-1*). Transcript of *ndhB-AS-2* were the primary isoform in stems and rhizomes, while *ndhB-1* was preferentially expressed in leaves. However, the AS transcripts in the ten genes described above did not response to the exposure of light according to the analysis of AS events in different light treatments (Fig. 5B). Subsequently, the gene structures of different transcript isoforms and their influence on protein translation were analyzed. As shown in Fig. 6, the AS transcripts either led to functionally impaired (*ndhB-AS-2*, *ndhL-2*, *ndhM-2*, *ndhU-2*, *3* and *4*, *ndhV-2*, *pnsb2-2*, *pnsb3-2-2* and *3*, *pnsb5-2*, *pnsl1-2*, *3* and *4*), or untranslated mRNAs (*ndhL-3* and *psnb3-1-2*).

RNA editing analysis of the chloroplast-encoded ndh **genes**

As shown in Table 2, a total of 35 editing sites were detected in eleven plastid-encoded *ndh* genes, with partial editing of *ndhD* and *ndhF* and a lack of editing for *ndhC*. Compared with other species, *Z. marina* possessed more editing sites than those in *Spirodela polyrhiza* (the close relative of the seagrass), *A. thaliana* (the model dicot) and *Zea mays* (the model monocot) but fewer than those in *A. trichopoda* (the basal angiosperm). *Z. marina* shared more editing sites with *Amborella trichopoda* (12) than with *S. polyrhiza* (10), *A. thaliana* (8) and *Z. mays* (6). Furthermore, more types of editing, including C to U (48.6%), U to C (20%), A to G (14.3%), G to A (8.6%), C to A (5.7%) and G to T (2.8%), existed in *Z. marina*, while only the C to U editing existed in the other plants. Additionally, most of the editing events tended to occur in the third bases of codons which led to silent editing. The second bases of codons were also edited, which resulted in the alteration of identity of amino acids. The majority of non-synonymous editing events in *Z. marina* either restored evolutionarily conserved

amino acid sequences, such as the methionine to isoleucine conversion of the *ndhB* 72th codon, the isoleucine to methionine conversion of the *ndhD* 435th codon and the asparagine to asparticacid conversion of the *ndhH* 50th codon, or generated lineage-specific residues in *Z. marina* compared with *S. polyrhiza*, *A. thaliana* and *Z. mays*, i.e., the *ndhD* 220th, *ndhI* 45th and *ndhK* 51th codons (Fig. 7A and B and Additional file 4 and 5).

Table 2
Comparison of identified RNA editing sites in *ndh* genes of *Z. marina* with other species

Gene	Codon position	Bases	Codon[1]	Amino acid change	Sp[2]	At[2]	Zm[2]	Am[2]
ndhA	44	A-G	Auu-Guu	I-V	-	-	-	-
	199	C-U	uCa-uUa	S-L	+	-	-	+
	323	U-C	uaU-uaC	Y-Y	-	-	-	-
ndhB	72	G-A	auG-auA	M-I	-	-	-	-
	155	C-U	cCa-cUa	P-L	+	+	+	+
	195	C-U	Cau-Uau	H-Y	+	+	+	+
	245	C-U	cCa-cUa	P-L	+	-	+	+
	276	C-U	uCa-uUA	S-L	+	+	+	+
	493	C-U	cCa-cUa	P-L	+	+	+	+
ndhD	43	U-C	acU-acC	T-T	-	-	-	-
	50	C-U	uuC-uuU	F-F	-	-	-	-
	123	C-U	uCa-uUa	S-L	+	+	-	+
	220	C-U	uCa-uUa	S-L	-	-	-	+
	225	C-U	Cua-Uua	L-L	-	-	-	+
	259	U-C	auU-auC	I-I	-	-	-	-
	288	C-U	uCa-uUa	S-L	+	+	+	+
	346	G-U	acG-acU	T-T	-	-	-	-
	393	C-U	uCu-uUu	S-F	+	-	-	-
	435	A-G	auA-auG	I-M	-	-	-	-
ndhE	43	U-C	auU-auC	I-I	-	-	-	-
ndhF	254	U-C	gcU-gcC	A-A	-	-	-	-
	361	C-A	uCu-uAu	S-Y	-	-	-	-
	468	C-A	gCa-gAa	A-E	-	-	-	-
ndhG	107	C-U	guC-guU	V-V	-	-	-	-
ndhH	50	A-G	Aau-Gau	N-D	-	-	-	-
	53	G-A	ccG-ccA	P-P	-	-	-	-
	224	U-C	gaU-gaC	D-D	-	-	-	-
	238	C-U	ucC-ucU	S-S	-	-	-	-
ndhI	45	G-A	auG-auA	M-I	-	-	-	-
	47	C-U	acC-acU	T-T	-	-	-	-
	146	U-C	ucU-ucC	S-S	-	-	-	-
ndhJ	49	C-U	uCa-uUa	S-L	+	-	-	+
ndhK	51	C-U	uCg-uUg	S-L	-	-	-	+
	99	A-G	agA-agG	R-R	-	-	-	-
	234	A-G	gaA-gaG	E-E	-	-	-	-

[1] The bases affected by RNA editing are shown in upper case

[2]At, *Arabidopsis thaliana*; Am, *Amborella trichopoda*; Sp, *Spirodela polyrhiza*; Zm, *Zea mays*.

The RNA editing events in different illumination conditions were also detected in the *ndh* genes of *Z. marina*. Interestingly, partial editing of the 361th codon in *ndhF* resulting in the incomplete serine to tyrosine conversion was observed in the dark adaptation period but subsequently disappeared in the light exposure and recovery periods (Fig. 7C and Additional file 5).

Patterns of expression of the ndh **genes in different tissues**

The expression of all 31 *ndh* genes derived from the five major tissues of *Z. marina* were also investigated, with the level of expression of the *ndhB* represented the sum of duplicated *ndhB* owing to their consensus coding sequences. As revealed in Fig. 8, plastid-encoded genes (*ndhA-ndhK*) were preferentially expressed in leaves but barely expressed in roots and rhizomes. Moreover, nuclear-encoded genes exhibited various patterns of expression across the five tissues. Five genes (*ndhL, ndhU, ndhS*, *pnsl2* and *pnsl4*) in the roots, six genes (*pnsb1, pnsb2, pnsb3-1, pnsb4, pnsb5* and *pnsl1*) in the flowers and seven genes (*ndhM, ndhN, ndhO, ndhT, ndhV, pnsb3-2* and *pnsl3*) in the leaves exhibited relatively high transcript abundances, while *pnsl5* was constitutively expressed. It was notable that *pnsb3-1* was highly expressed in flowers. In contrast, its duplicated sister (*pnsb3-2*) was particularly abundant in leaves. Unlike the other *ndh* genes, *pnsl4* and *ndhL* showed different patterns of expression with their counterparts in *A. thaliana* (Additional file 6), suggesting their functional divergence in different species.

Patterns of expression of the ndh **genes across high light stress and dark recovery**

To analyze the dynamic expression of *ndh* genes, the eleven undetectable (*ndhA-K*) and eight differentially expressed *ndh* genes (*ndhL*, *ndhM*, *ndhS*, *ndhT*, *ndhV*, *pnsb3-1*, *pnsb3-2* and *pnsl5*) in the available transcriptome were selected (Additional file 7). As shown in Fig. 9, four *ndh* genes in conserved Subcomplex M (*ndhA*), Subcomplex A *(ndhJ* and *ndhK*) and Subcomplex EDB (*ndhV*) were rapidly induced and maintained a relatively high level of expression throughout their exposure to light. Moreover, three other genes in Subcomplex M (*ndhC*, *ndhD* and *ndhE*), as well as the two genes in Subcomplex B (*pnsb3-2*) and Subcomplex L(*pnsl5*) that existed exclusively in terrestrial plants, were up-regulated during the middle and late stage of light exposure. Furthermore, these genes were also up-regulated during subsequent dark recovery period. In contrast, *ndhB*, *ndhH*, *ndhI*, *ndhL*, *ndhM* and *pnsb3-1* were repressed during light exposure and then significantly induced during the recovery period. The dynamic patterns of expression of the *ndh* genes indicated that there were two distinctive responsive and regulatory mechanisms of the NDH complex during the periods of light exposure and the subsequent dark recovery.

Stress-related cis-elements in ndh **genes promoters**

To explore the possible regulatory mechanism of the *Z. marina ndh* genes, related *cis*-elements were scanned. There were three types of *cis*-elements, as shown in Fig. 10. Different amounts of light-responsive elements, including Box4, G-box, the GATA, GT1 and TCT motifs, were present in each *ndh* gene, with the G-box comprising the most abundant element. Among the 31 *ndh* genes, *pnsl5* and *ndhM* had more light responsive elements. The second type of *cis*-elements were related to hormones, including those responsive to salicylic acid-, abscisic acid- and auxin, which were detected among 11 genes (*ndhA*, *ndhG*, *ndhI*, *ndhM*, *ndhO*, *ndhS*, *pnsb1*, *pnsb2*, *pnsb3-2*, *pnsb5* and *pnsl4*). In addition, defense- and stress-responsive and WUN-motif and TC-rich repeats associated with wound responses were identified in nine genes (*ndhF*, *ndhH*, *ndhI*, *ndhM*, *ndhU*, *pnsb3-2*, *pnsl1*, *pnsl2* and *pnsl5*). These results indicated that the *ndh* genes can be regulated by numerous factors with light serving as the primary factor.

Discussion

The loss of *ndh* genes was universal in marine macrophytes [40, 41], which was consistent with that the complexity of NDH gradually increased during the transition of marine plants to land plants. Considering that most of the *ndh* mutants resulted in impaired NDH activity [42–46], it could be hypothesized that each Ndh subunit was indispensable, functioning either in the stability or the assembly of the NDH complex. *Z. marina*, as a marine seagrass, possessed a complete NDH that could possibly have been retained during the migration from land to sea. This phenomenon, taken in combination with the observations that NDH complex was prominently activated in light exposure and dark recovery examined in our previously studies [15, 37], suggested that the *Z. marina* NDH complex could have played a crucial role both in PSI-CEF and chlororespiration.

The comparison of the phylogeny between individual Ndh subunits and the entire NDH complex revealed that some genes had been vertically or horizontally transferred among Viridiplantae together with the entire NDH complex, although some *ndh* genes evolved independently. The earlier diversification of NdhM, NdhO and NdhS suggests a possible more primitive evolutionary status of these subunits in *Z. marina*.

Among the 31 *ndh* genes, two gene pairs (*ndhB-1* and *ndhB-2*, chloroplast genome encoded; *pnsb3-1* and *pnsb3-2*, nuclear genome encoded) probably originated from gene duplication events. It is commonly accepted that the majority of gene duplications in chloroplast genomes are caused by the expansion of large inverted repeat regions (IRs) [47]. In this study, only a single *ndhB* could be observed in the outgroup species, while the duplicated *ndhB* paralogs in *Cycas* (the most ancient extant plants) and the angiosperms were all located on the chloroplast IRs [39, 48], which implied that the duplication of *ndhB* might emerge in the common ancestors of seed plants through the expansion of IRs. However, the phenomenon that some gymnosperms (Ginkgoaceae and Pinaceae) also possessed a single copy of *ndhB* [49] could be attributed to the loss or contraction of IRs. In the case of nuclear encoded *pnsb3* paralogs, the duplication mode was identified at the Plant Duplicate Gene Database (PlantDGD, http://pdgd.njau.edu.cn:8080) as “single gene transposition-duplication” [50]. This mode of duplication, prevalent in plant genomes, generated two gene copies that are neither neighboring nor colinear. The non-duplicated *pnsb3* in algae, bryophytes, lycophytes and gymnosperms, together with the independent orthologous groups of the duplicated *pnsb3* (*pnsb3-1* and *pnsb3-2*) in the monocots and dicots, indicated that the duplication events happened after the divergence of gymnosperms and angiosperms but before the divergence of monocots and dicots. In *Z. marina*, both paralogs possessed the core angiosperm motifs 1, 2, 3 and 4, while the additional motifs 5, 10 and 15 were specific to *pnsb3-1*. The divergent motif composition combined with the different tissue-specific pattern of expression observed in our study suggested that the neo-functionalization and/or sub-functionalization of *pnsb3* paralogs could help plants to adapt better to the dynamic and fluctuating environment [51].

Motifs in the functional domains were stringently conserved in most of the *Z. marina* Ndh subunits with the exception of NdhD and NdhF. Previous studies showed that a near-continuous long amphipathic α-helix between the 15th and 16th TM helices of NdhF were connected to other antiporter-like subunits [3]. This connection could transmit conformational changes in the hydrophilic domain of NDH, resulting in proton translocation. Thus, the absence of a TM helix in the N-terminus of NdhD and the loss of the long helix NdhF implied a possible modified mechanism for proton transmission in *Z. marina*. Moreover, the non-photochemical quenching value, which depends on the pH gradient generated in the thylakoid, was at a low level in most of the seagrass species [52]. Thus, it was possible that the special proton transmission mechanism was related to the low non-photochemical quenching capacity.

AS, as a post-transcriptional regulatory mechanism, could generate diverse transcripts and played crucial roles in plant development and the resistance to different biotic and abiotic stresses [53–56]. In this study, many AS transcripts were predominantly observed in leaves that primarily contained chloroplasts. Clearly, the AS events in *ndh* genes primarily participated in photosynthetic-related regulation. Many splice variants generate small interfering peptides that participated in the formation of multi-protein complex but lacked functional domain to compete with functional complex [53, 57]. Intriguingly, the AS transcripts of both *ndhB* and *pnsb2*, which produced a truncated protein without functional domain, exhibited opposite patterns of expression with their fundamental transcripts, indicating a dominant-negative regulation of the AS events in these *ndh* genes. As for light treatments, the AS events of *ndh* genes displayed analogous splicing patterns across light exposure and the subsequent recovery, implying that the AS events of *ndh* genes were not sensitive to light exposure.

RNA editing, another post-transcriptional process, resulted in numerous base transitions [58]. In addition, the canonical C to U editing and the abundant uncanonical U to C, A to G and G to A conversions that had been reported to exist exclusively in ancestral land plants [58, 59] were unexpectedly present in *Z. marina*. Moreover, the minor G to T and C to A editing events which had never been reported in other plant species were detected. It is possible that *Z. marina* retained numerous ancestral editing patterns that had a monophyletic origin but were followed by lineage-specific losses and gains. As suggested by Jobson et al., (2008) the RNA editing events could change the protein structure or interaction through the non-synonymous replacement of conserved amino acids [60, 61]. Therefore, the editing events adjacent to the charged lysine residue in the TM7 helix of NdhD which was crucial for energy transduction [3], as well as in the second helix of NdhK and the N-terminal amphiphilic helix of NdhI which had an impact on quinone-binding [5], could influence the functional NDH in *Z. marina*.

The incomplete conversion from serine to tyrosine in NdhF caused by the partial C to A editing was detected exclusively during the period of dark adaptation. The 361th serine found in the NdhF TM11 helix was conserved among most of the green plants. Accordingly, the partial non-synonymous editing of this codon was likely to alter the NdhF functions in NDH complex. It could be explained that, during the dark adaption, the partial 361th serine to tyrosine conversion could inhibit the NDH activity because there was no need to establish powerful NDH functions. During the subsequent light exposure and dark recovery, the partial editing disappeared and integrated NDH molecules were then rebuilt to address the light stress through CEF-PSI and photorespiration. The whole process could be viewed as a post-transcriptional regulation of the functions of NDH complex in response to light exposure in *Z. marina*.

Similar to *A. thaliana*, most of the *ndh* genes in *Z. marina* were primarily expressed in leaves, which implied the conserved function in photosynthesis of NDH complex during evolution. However, the prolyl cis/trans isomerase *pnsl4* and *pnsl5* exhibited a high level of expression in root and constitutive expression, respectively. The prolyl *cis/trans* isomerase could be involved in various physiological processes in addition to the assembly of NDH complex [62], such as hormone-mediated plant development and brassinosteroid-mediated flowering [63, 64], thereby leading to the unbiased expression in leaves. Additionally, the *ndhL* in *Z. marina* was predominantly expressed in roots and flowers, which differed from that in *A. thaliana*. Enriched bicarbonate in seawater can be converted to CO_2 in the surface of leaves to support the concentration of limited inorganic carbon (Ci) in fully marine conditions [65]. Therefore, the high level of expression of *ndhL* which functioned in the concentration and transport of Ci [66, 67], in roots and flowers might contribute to the transport of Ci to the leaves in *Z. marina*.

Most of the *ndh* genes that encoded components in Subcomplex M, Subcomplex A and Subcomplex EDB were significantly induced or repressed during different light periods. Thus, the three subcomplexes could be the most sensitive parts involved in the activated of the NDH complex. More light responsive elements in *pnsl5* and *ndhM* might related to the highly and continuously level of expression of these genes responded to light stress. In addition, the up regulated *ndh* genes differed during the light exposure and dark recovery period. This suggested that there were two diverse responsive and regulatory mechanisms in the NDH-dependent PSI-CEF and chlororespiration.

Conclusions

In this study, we performed a systematic analysis of the identification, splicing, editing and pattern of expression of the *Z. marina* NDH complex. These results establish the foundation for further studies in the evolution, function and regulatory mechanism of the NDH complex.

Methods

Plant materials and treatments

Z. marina with intact rhizome systems were collected from subtidal seagrass beds in Yandunjiao, Rongcheng (37° 91´N, 120° 73´E), Shandong Province, China, during their growth season. No specific permits were required to collect such samples. Samples were identified on the basis of their morphology by experienced taxonomists. Identifications were also confirmed by sequence similarity with the finished whole genome sequencing of *Z. marina* [33]. The specimen (HY202005) were deposited in the Herbarium of Ocean School of Yantai University, Shandong Province, China. Collection of plant materials complied with the institutional, national and international guidelines. Samples were cultured in an aquarium with seawater that was continuously aerated and renewed daily. Before experimentation, the plants were pre-cultivated for 3 days under 15 °C with a photoperiod of 10/14 h (light/dark) in minimum saturation light intensity (100 μmol photons $m^{-2}s^{-1}$).

The leaves obtained from dark-adapted overnight plants were exposed to 300 μmol photons $m^{-2}s^{-1}$, followed by recovery under darkness. For RT-PCR assays, the samples were collected after light exposure and recovery for 3 h, respectively. For quantitative real time-PCR (qRT-PCR) assays, the samples were collected after light exposure and recovery for 10 min, 30 min, 1 h and 3 h, respectively. The leaves under dark-adapted overnight were used as the control. Moreover, the roots, leaves, flowers, stems and rhizomes were collected at the flowering stage for tissue specific expression analyzes using the RT-PCR and qRT-PCR. There were three biological repeats for each sample.

Identification of the Ndh subunits and sequence analysis

BLASTP analyses were performed against the *Z. marina* database from the NCBI (https://www.ncbi.nlm.nih.gov/) and Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) databases using the *A. thaliana* Ndh protein sequences. The reannotated and misannotated genes were submitted to GenBank and can be retrieved with accession numbers MW051562 and MW051563. The number of amino acids, molecular weights and theoretical isoelectric points of Ndh subunits were attained by submitting protein sequences to the ExPasy website (http://web.expasy.org/protparam/). The conserved domains of each *ndh* gene were confirmed using Pfam and SMART (http://smart.embl-heidelberg.de/). The exon-intron compositions of the *ndh* genes were constructed using DNAMAN software by comparing the cDNA with genomic sequences.

Phylogenetic analysis

Phylogenetic trees were generated from the amino acid alignments of both entire concatenated complex (31 subunits) and the individual Ndh subunits using MEGA5.0 with the following settings: Poisson model, pairwise deletion and 1000 bootstrap replications. Trees containing evolutionary model species in Monocots, Dicots, Amborellales, Lycophytes, Bryophytes, Charophyceae, Chlorophyta and Cyanobacteria were analyzed, using *E. coli* as an outgroup. The protein sequences of each species were acquired from the Phytozome and NCBI databases. All of the sequences were aligned using ClustalW2 [68]. The Interactive Tree of Life (https://itol.embl.de/) was used to visualize the trees.

Structural characterization

The motifs of Ndh subunits were identified using the MEME online program (http://meme.nbcr.net/meme/intro.html) with the following parameter sets: number of repetitions-any, maximum number of motifs - 20 and the optimum motif widths - 6 to 200 amino acid residues [69]. Trans-membrane helices in the Ndh subunits were predicted using TMHMM v2.0 [70]. The multiple sequence alignments and structure analysis were conducted with ESPript 3 in six species, including *Thermosynechococcus elongatus* BP-1, *Z. mays*, *Sorghum bicolor*, *A. thaliana*, *Populus trichocarpa* and *Z. marina*. [71].

Nucleic acid isolation and cDNA synthesis

Total DNA was extracted from 100 mg leaf tissue using the cetyltrimethylammonium bromide (CTAB) protocol [61]. Total RNA was isolated using a FastPure Plant Total RNA Isolation Kit (Vazyme, Nanjing, China). The quality of RNA was examined by electrophoresis on 1% agarose gel and quantified using NanoQuant (TECAN Group Ltd., Männedorf, Switzerland). After wiping off the residual DNA, the cDNA was synthesized with HiScript® II 1st Strand cDNA Synthesis Kit (Vazyme) using 1μg of total RNA.

PCR amplification and sequencing

Primers were designed for polymerase chain reaction (PCR) and RT-PCR amplification using the DNA sequences obtained from GenBank (Additional file 8). DNA and cDNA templates were amplified using PrimeSTAR® Max DNA Polymerase (TaKaRa, Japan) with the following procedure: 98 °C for 10 s, 35 cycles of 55 °C for 10 s and 72 °C for 30 s. The amplification products were separated on 1% agarose gel and purified using a FastPure Gel DNA Extraction Mini Kit (Vazyme) before Sanger sequencing.

Analysis of splicing patterns

The RT-PCR products of *ndh* genes from five tissues and different light periods were visualized using a Gel Doc XR+ system (Bio-Rad, Hercules, CA, USA). The specific electrophoresis bands corresponded to differently spliced products. The possible protein isoforms were predicted by DNAMAN.

RNA editing analysis of chloroplast-encoded *ndh* genes

To confirm the RNA editing sites in the *Z. marinandh* genes, the sequenced cDNA and DNA from different light periods were aligned using Vector NTI. The editing sites for *S. polyrhiza* [72], *A.thaliana* [73], *Z. mays* [74] and *A. trichopoda* [75] were obtained from respective publications.

Analysis of gene expression

QRT-PCR assays of the *ndh* genes obtained from five tissues and different light periods were conducted on a Bio-Rad CFX96 Real Time PCR System with AceQ Universal SYBR qPCR Master Mix (Vazyme). The housekeeping gene *gapdh* from *Z. marina* was used as an internal control. The qRT-PCR was programmed as follows: 95 °C for 10 s, followed by 40 cycles of 56 °C for 10 s and 72 °C for 30 s. The qRT-PCR data were calculated using the $2^{-\Delta\Delta CT}$ method. Sequences of the primers used in qRT-PCR are described in Additional file 8. The tissue specific expression of *A. thaliana* was download as the publicly available RNA-Seq data in the CoNekT-Plants database (www.conekt.plant.tools). The RNA Seq of *Z. marina* under light stress was analyzed in our previously study [37]. The differentially expressed genes were selected according to $p < 0.05$ and fold change > 1.3 (for up-regulation) or fold change < 0.75 (for down-regulation). Heatmaps were constructed using the transformed log2 (TPM+ 1) or log2 (FPKM + 1) values by TBtools software [76].

Analysis of *cis*-acting elements in the *ndh* gene promoters

To obtain the possible *cis*-acting elements in the promoter regions of the *ndh* genes, 1500 bp genomic sequences upstream of the start code ATG were analyzed online using Plantcare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Statistical analysis

Statistical analysis was analyzed using SPSS 22.0. All data were analyzed by one-way ANOVA and Tukey's tests with $p < 0.05$ set to be statistically significant.

List Of Abbreviations

NDH
NADH dehydrogenase-like; PSI-CEF:photosystem I cyclic electron flow; RT-PCR:reverse transcription-PCR; AA:amino acid; TM:transmembrane; AS:alternative splicing; IRs:inverted repeat regions; Ci:inorganic carbon; qRT-PCR:quantitative real time-PCR; CTAB:cetyltrimethylammonium bromide; PCR:polymerase chain reaction

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Availability of data and materials

Individual sequences were submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and can be retrieved with accession numbers MW051562 and MW051563. Voucher specimen (specimen number: HY202005) is available in Herbarium of Ocean School of Yantai University, Shandong Province, China.

Competing interests

The authors declare that they have no competing interests.

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Contributions

MYM performed the experiments. MYM, MYZ analyzed the data. MMY wrote the manuscript. MMY and QSZ designed the study. All authors have read and approved the final manuscript.

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Figures

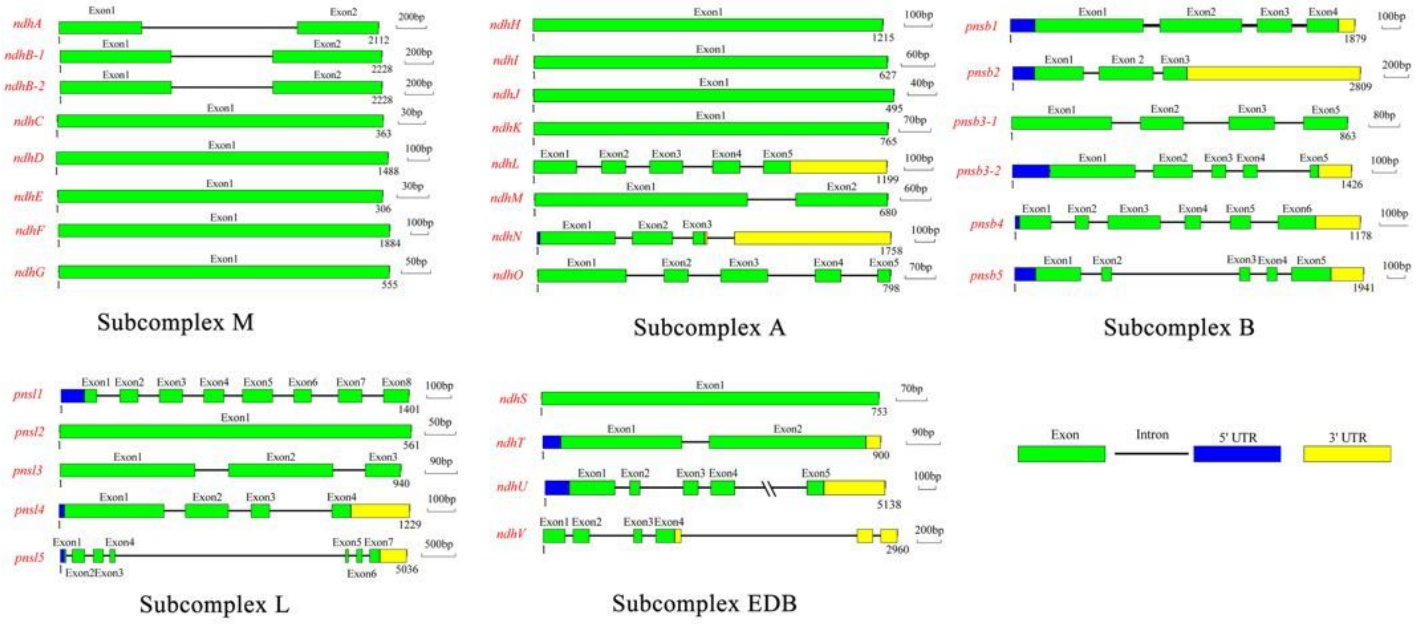

Figure 1

Gene model of ndh genes in Zostera marina. Blue boxes indicate untranslated 5′ UTR, yellow boxes indicate 3′ UTR, black lines indicate introns and green boxes indicate exons. The reannotated genes were highlighted by asterisk. The number at the bottom of each model represents gene length.

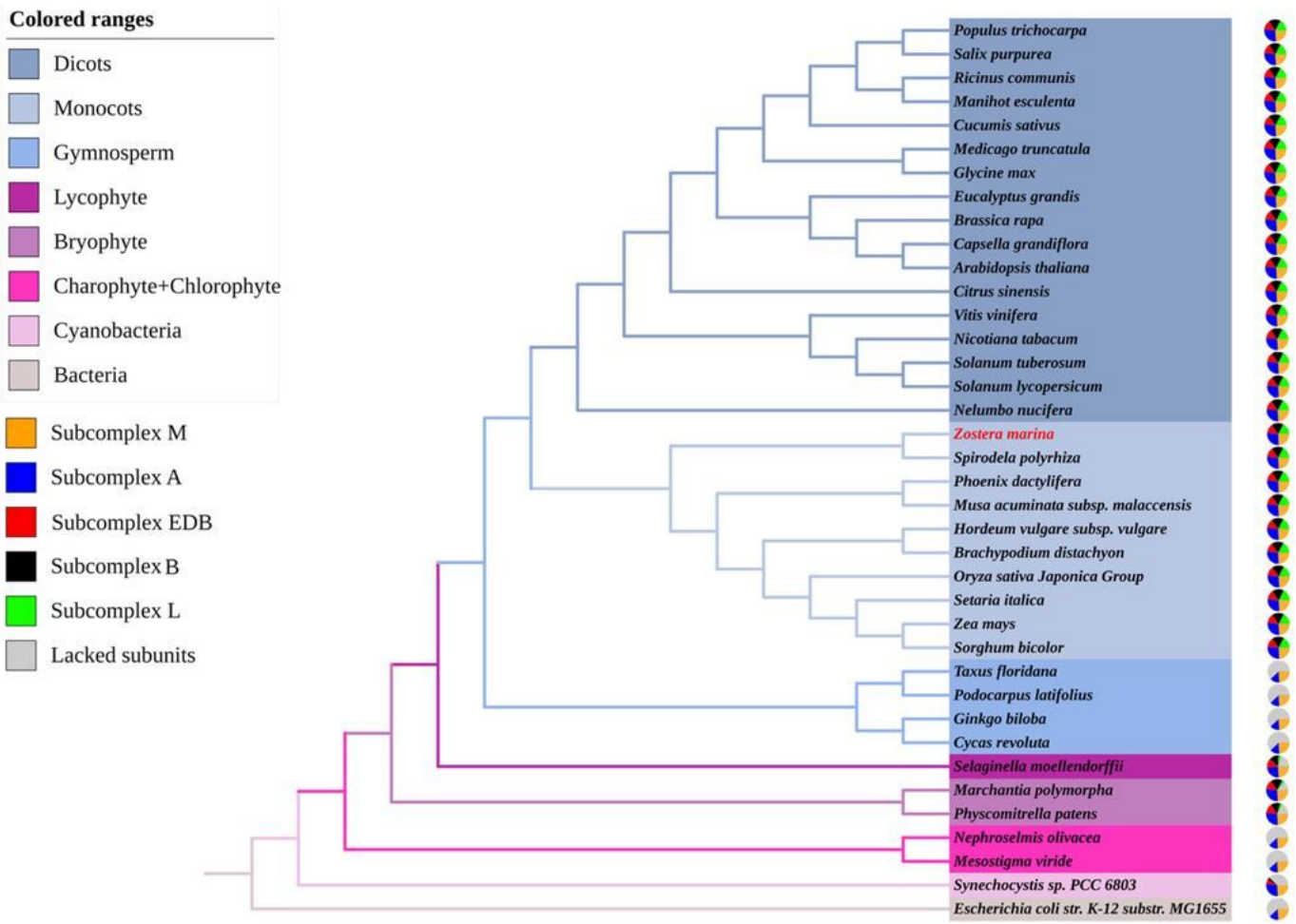

Figure 2

Phylogenetic relationship of NADH dehydrogenase-like (NDH) complex among Viridiplantae. The phylogenetic tree was constructed using concatenated amino acid sequences of 31 Ndh subunits by MEGA 5.0 software. Pie charts was divided into five part in which orange, blue, red, black and green represented Subcomplex M, Subcomplex A, Subcomplex EDB, Subcomplex B, Subcomplex L, respectively. Gray in pie charts indicating the lacking of Subcomplexes.

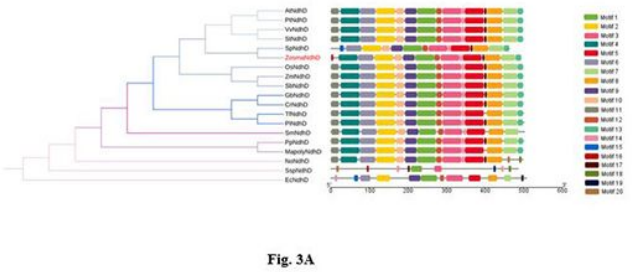

Fig. 3A

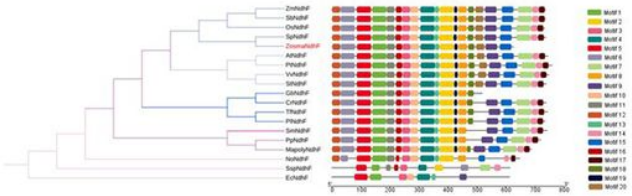

Fig. 3B

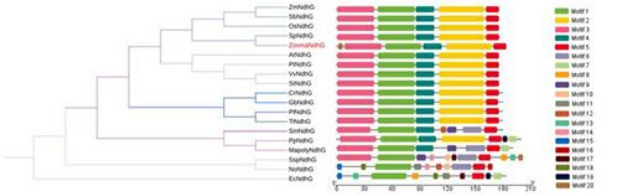

Fig. 3C

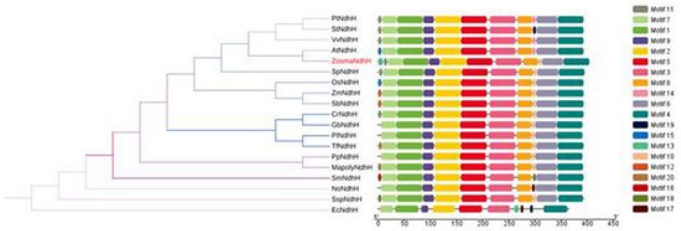

Fig. 3D

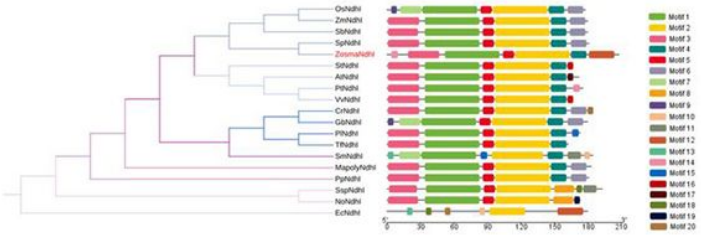

Fig. 3E

Figure 3

Phylogenetic trees and motif compositions of NdhD (A), F (B), G (C), H (D) and I (E) from nineteen different species. At, Arabidopsis thaliana; Cr, Cycas revoluta; Ec, Escherichia coli.; Gb, Ginkgo biloba; Mapoly, Marchantia polymorpha L.; No, Nephroselmis olivacea; Os, Oryza sativa Japonica; Pl, Podocarpus latifolius; Pp, Physcomitrella patens; Pt, Populus trichocarpa; Sb, Sorghum bicolor; Sm, Selaginella moellendorffii; Sp, Spirodela polyrhiza; Ssp, Synechocystis sp. PCC 6803; St, Solanum tuberosum; Tf, Taxus floridana; Vv, Vitis vinifera; Zm, Zea mays; Zosma, Zostera marina. The motifs numbered 1-20 indicate the motif composition of Ndh proteins, the scale at the bottom can be used as a reference of protein length.

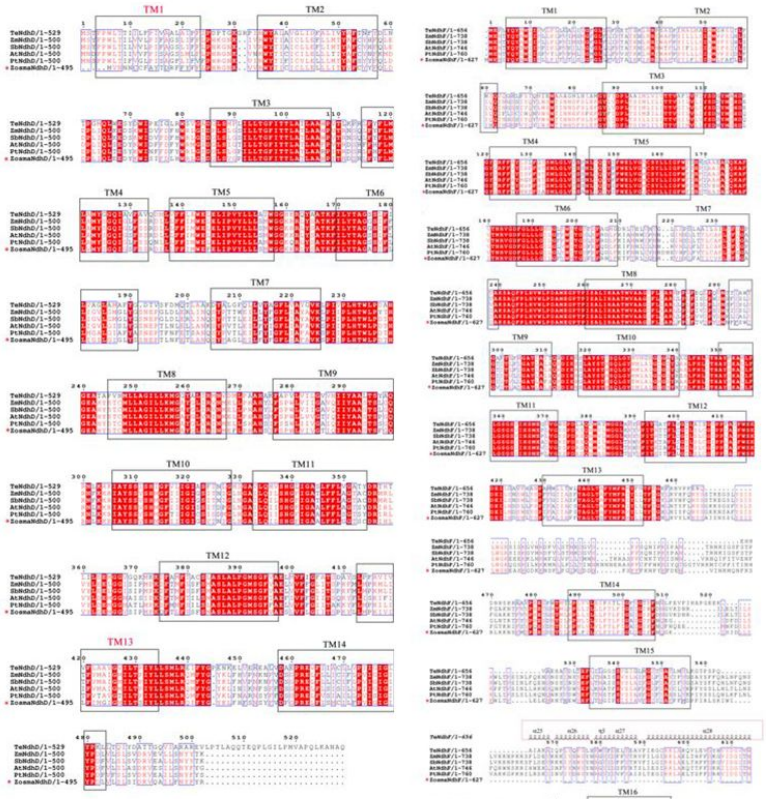

Fig. 4A

Fig. 4B

Figure 4

ClustalW amino acid alignment of the amino acid sequence of NdhD (A) and NdhF (B) from Z. marina with other five species. At, A. thaliana; Pt, P. trichocarpa; Sb, S. bicolor; Te, Thermosynechococcus elongatus BP-1; Zm, Zea mays. The conserved transmembrane (TM) helices are indicated. The particular TM helix in Z. marina are labeled in red font. The long amphipathic helix in TM15 and TM16 were indicated above the sequences with red box.

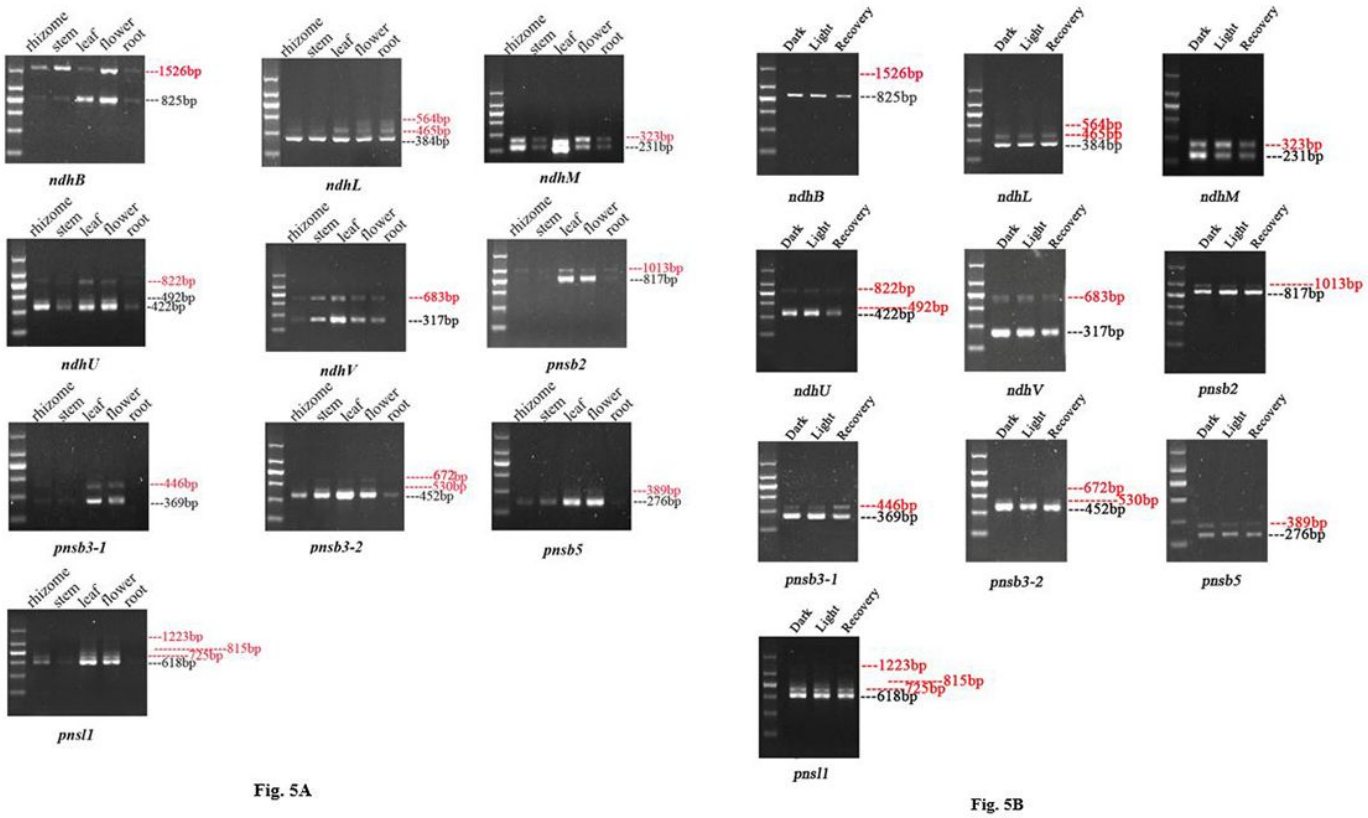

Figure 5

Splicing variants of the Z. marina ndh gene transcripts. Alternative splicing (AS) events in five tissues including rhizome, stem, leaf, flower and root (A). AS events in the period of light exposure and the subsequent dark recovery (B). The first lane left is 200bp ladder (200bp, 400bp, 600bp, 800bp, 1000bp, 1500bp). AS transcripts are highlighted in red. The fundamental transcripts are labeled in black. Full-length gels are presented in Additional file 9 and 10.

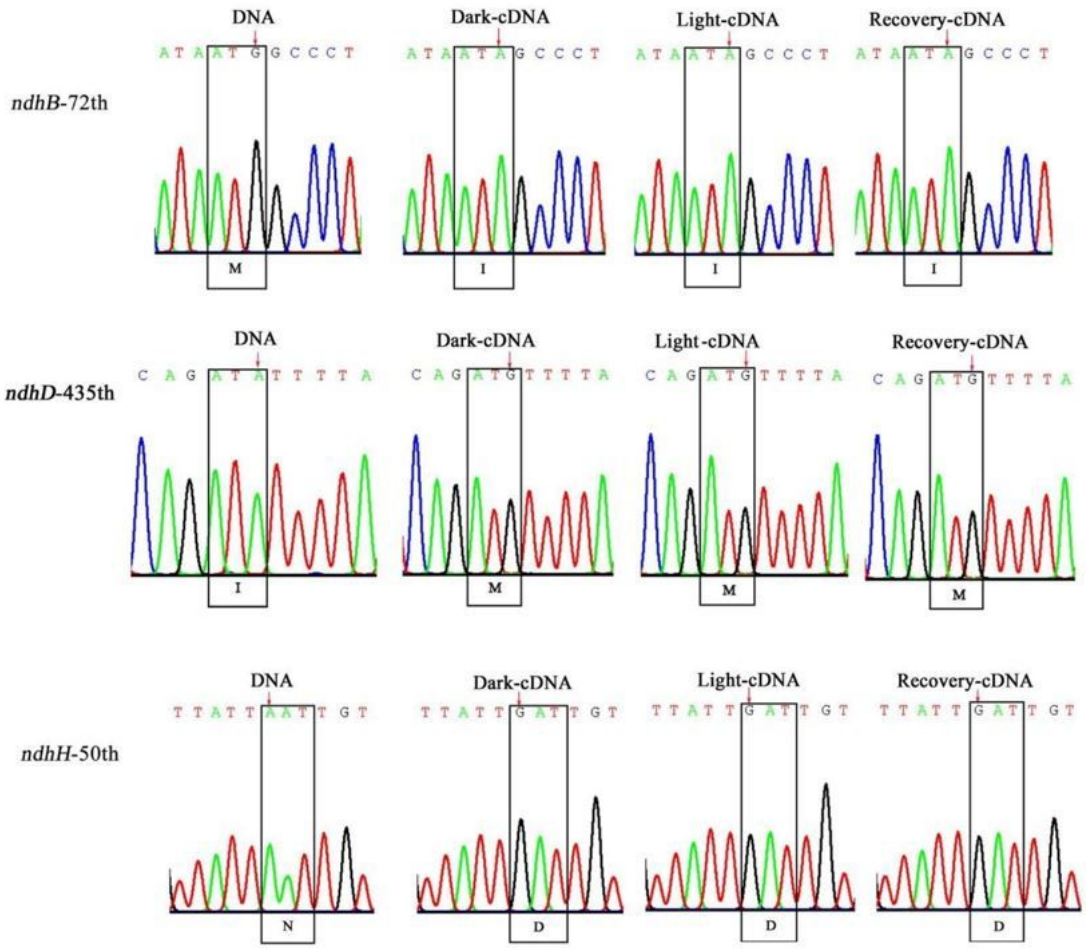

Figure 6

Gene model of the different isoforms of ndh genes occurring AS events. Blue boxes indicate untranslated 5' UTR, yellow boxes indicate 3' UTR, black lines indicate introns, green boxes indicate exons and intron retention is labeled red line. The number at the bottom of each model represents gene length. The length of amino acids is indicated after the gene models.

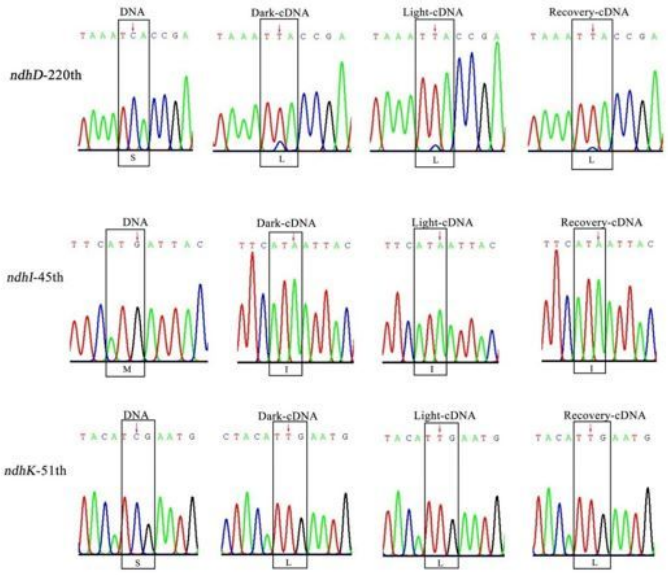

Figure 7

Gene model of the different isoforms of ndh genes occurring AS events. Blue boxes indicate untranslated 5′ UTR, yellow boxes indicate 3′ UTR, black lines indicate introns, green boxes indicate exons and intron retention is labeled red line. The number at the bottom of each model represents gene length. The length of amino acids is indicated after the gene models. Sequencing chromatograms of RNA editing sites that restored evolutionarily conserved amino acid sequences (A), generated lineage-specific residues in Z. marina (B) and influenced by light stress (C). The editing bases are indicated by arrows. The amino acids corresponding to the codons are indicated in the boxes.

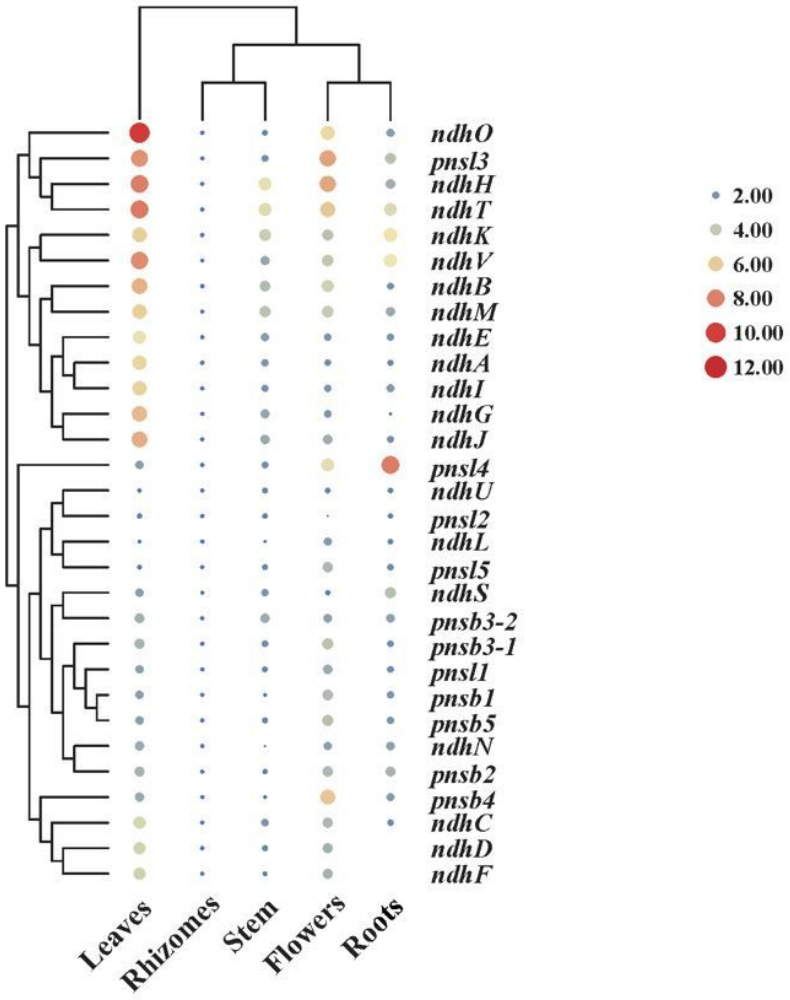

Figure 8

Patterns of expression of ndh genes in five tissues were analyzed by quantitative real time-PCR (qRT-PCR). To calculate the relative expression level, the expression of ndh gene in rhizomes was set as control. Gapdh was used as reference gene. Relative level of expression was transformed by log2 and can assessed by the size and color of the circle.

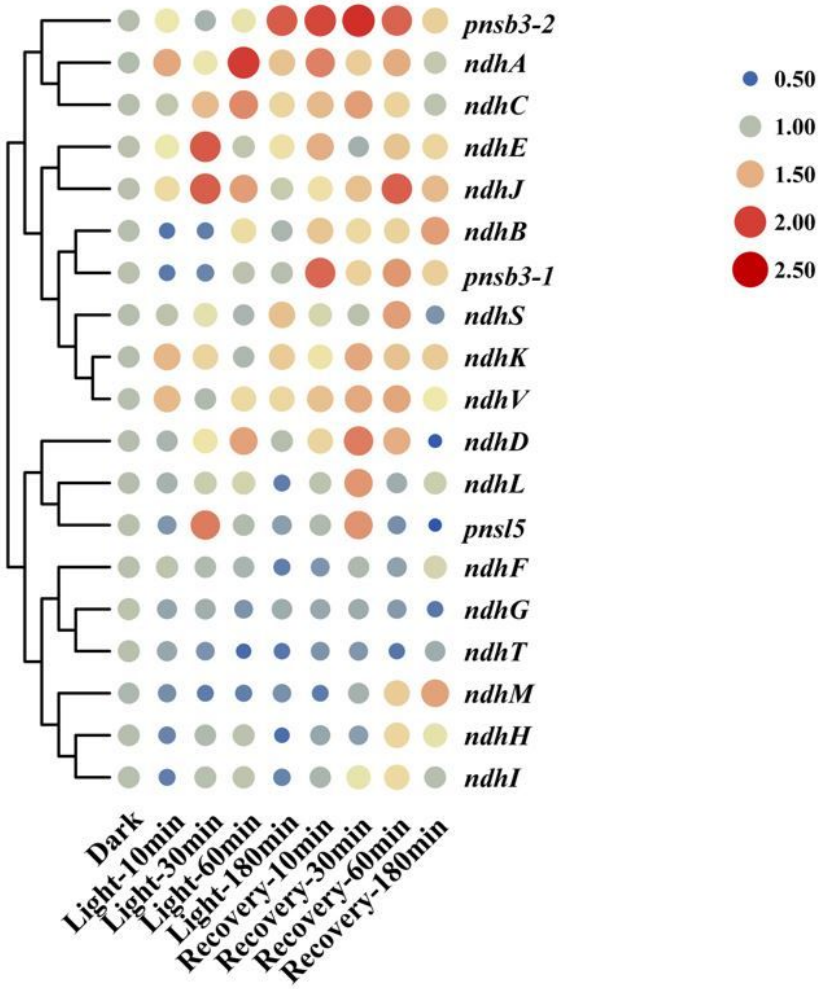

Figure 9

Patterns of expression of ndh genes in the period of light exposure and the subsequent recovery were analyzed by qRT-PCR. To calculate the relative expression level, the expression of ndh gene under dark condition was set as control. Gapdh was used as reference gene. Relative level of expression was transformed by log2 and can assessed by the size and color of the circle.

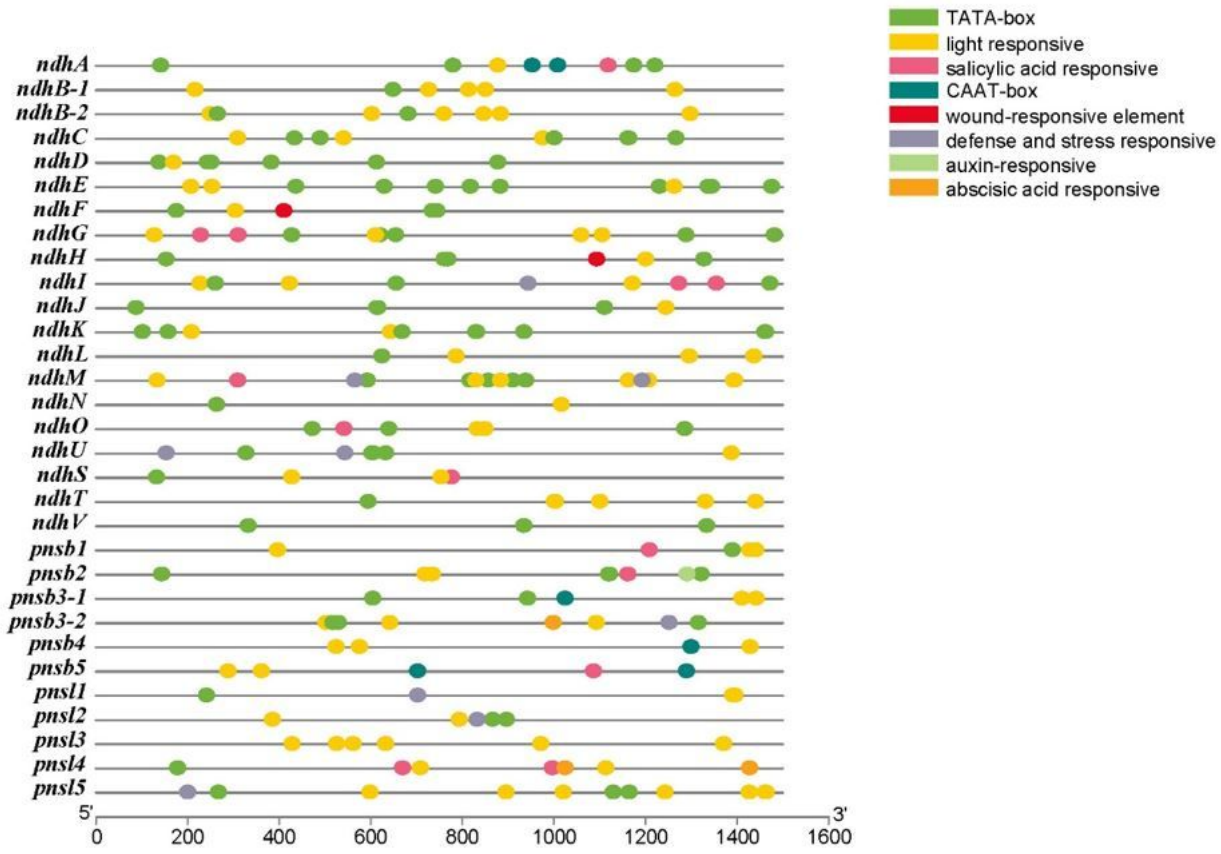

Figure 10

Predicted cis-elements in ndh genes promoters. Promoter sequences (−1500 bp) of 31 ndh genes are detected by PlantCARE. The upstream length to the translational start site can refer to the scale at the bottom.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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- Additionalfile10.docx
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- Additionalfile4.docx
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- Additionalfile6.docx
- Additionalfile7.docx
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- Additionalfile9.docx