# The heart of oxygenic photosynthesis illuminated

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# *Chapter* 3

## $^{15}$N Photo-CIDNP MAS NMR reveals functional heterogeneity in the electron donor of PSI across plant species

In plants and cyanobacteria two light-driven electron pumps, PSI and PSII, facilitate electron transfer from water to ferredoxin with quantum efficiency close to unity. While similar in structure and function, the reaction centers of PSI and PSII operate at widely different potentials with PSI being the strongest reducing agent known in living nature. Photochemically induced dynamic nuclear polarization (photo-CIDNP) in magic-angle spinning (MAS) NMR measurements provides direct access to the heart of large photosynthetic complexes (Alia *et al.* 2004; Diller *et al.* 2005). By combining the dramatic signal increase obtained from the solid-state photo-CIDNP effect with $^{15}$N isotope labeling of PSI, we were able to map the electron spin density ($\rho_i$) in the active cofactors of PSI and study primary charge separation at the atomic level. We compare data obtained from two different PSI proteins, one from spinach (*Spinacia oleracea*) and other from the aquatic plant duckweed (*Spirodela oligorrhiza*). Results demonstrate a large flexibility of the PSI in terms of its electronic architecture while their electronic ground states are strictly conserved.

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## 3.1 INTRODUCTION

Photosynthesis in cyanobacteria, algae and plants involves the participation of two reaction centers (RCs), PSI and PSII, located in the thylakoid membrane of chloroplasts. The coupling of these two photosystems allows for pumping of electrons across the photosynthetic membrane from water molecules finally into $CO_2$ in order to achieve the buildup of organic material. The oxidized primary electron donor of PSII, $P680^{\bullet+}$ is the strongest oxidizing agent known in living nature, with a redox potential of at least 1.2 V (Gorkom and Schelvis 1993). On the other hand, the electronically excited electron donor of PSI, P700, is a strongly reducing agent, at a potential of approximately -1.2 V. Thus, P700* probably is the most reducing compound found in natural systems (Webber and Lubitz 2001). The redox properties of PSI are highly optimized in order to provide a strongly reducing species.

The X-ray structure of cyanobacterial PSI has been solved with 2.5 Å resolution and shows high resemblance to plant PSI (Fromme *et al.* 2001; Jordan *et al.* 2001). From plant PSI currently only X-ray structures at a resolution down to 3.4 Å are available (Amunts *et al.* 2010), leaving the exact electronic structure of plant P700 a matter of debate (Webber and Lubitz 2001, Amunts *et al.* 2010). All PSI systems, as in other RCs, have two symmetric branches of chlorophyll-type cofactor molecules, and two quinones (Figure 3.1). In addition, PSI contains three iron-sulphur [4Fe-4S] clusters as terminal intrinsic electron acceptors (Romberger and Golbeck 2010). The primary electron donor P700 is a heterodimer consisting of one chlorophyll *a* (Chl *a*, $P_B$) and one Chl *a′* ($P_A$) which is the $13^2$-epimer of Chl *a*. While $P_A$ forms hydrogen bonds to its protein environment, no hydrogen bonds are found on the $P_B$ side (Jordan *et al.* 2001). EPR studies showed most of the positive charge to be localized on the Chl *a* (the $P_B$ donor) molecule (Käss *et al.* 1995; Holzwarth *et al.* 2006), however a consensus about the extent of asymmetry has not yet been reached (Alia *et al.* 2004; Santabarbara *et al.* 2010).

In contrast to PSII, where electron transfer (ET) is known to be primarily on a single branch, data from transient EPR indicate that both branches are active in PSI (Setif and Brettel 1993; Poluektov *et al.* 2005; Müller *et al.* 2010). However, the conservation of the occurrence of bidirectional ET among different species and varying experimental conditions remains under discussion (Santabarbara *et al.* 2010). The biological significance of bidirectional ET in PSI is also not yet understood. Hence, despite the

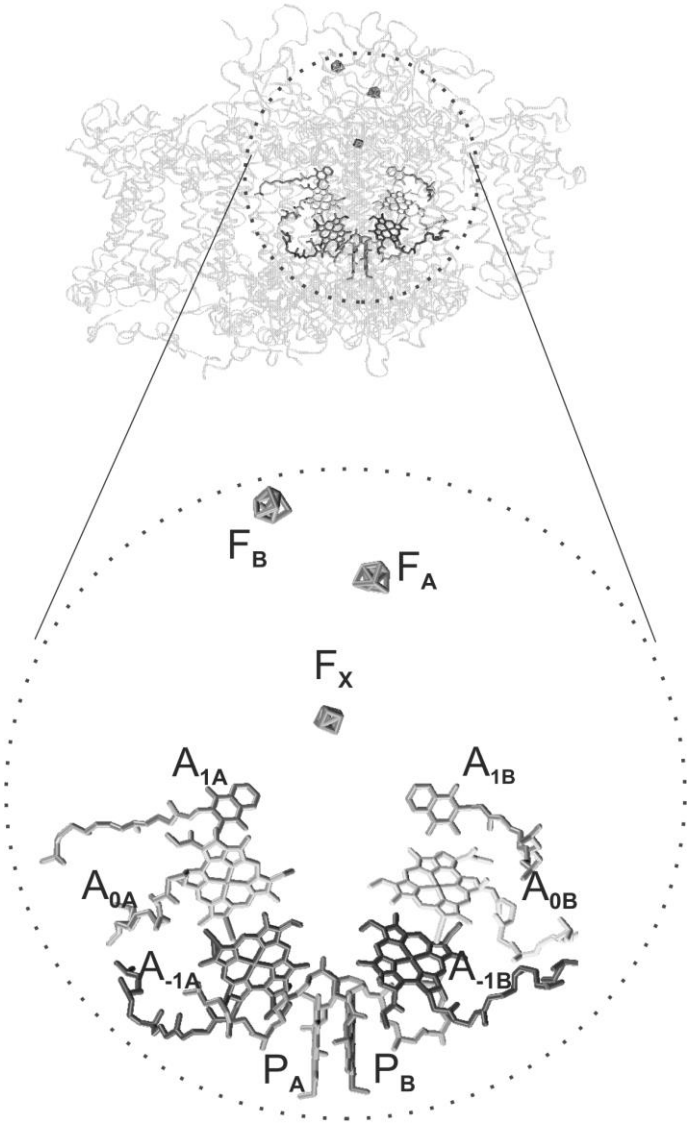


**Figure 3.1:** Arrangement of the cofactors in the RC of PSI (bottom) located in the heart of the PSI protein (top). Based on the X-ray crystal structure of cyanobacterial PSI (Jordan *et al.* 2001), visualized using VMD (Beckman Institute, University of Illinois at Urbana-Champaign).

highly optimized redox properties of P700, PSI appears to be functionally heterogeneous, *e.g.* in terms of the relative participation of the two branches in ET. The question addressed here is whether these variations affect P700 in different plant systems from different habitats, containing the highest evolved photosystems.

Photochemically induced dynamic nuclear polarization (Photo-CIDNP) is well known in liquid NMR as a method that increases NMR intensities (Hore and Broadhurst 1993; Goez 1997). In solid-state NMR upon illumination with continuous white light, photo-CIDNP has been observed by applying $^{15}$N magic-angle spinning (MAS) NMR to four photosynthetic RCs: (i) *Rhodobacter (Rb.) sphaeroides* R26 (Zysmilich and

McDermott 1994; Zysmilich and McDermott 1996; Prakash *et al.* 2006) and wild type (WT) (Matysik 2000; Schulten *et al.* 2002; Prakash *et al.* 2005), PSI (Alia *et al.* 2004) and PSII (Diller *et al.* 2007). Photo-CIDNP is caused by the strong electron polarization in the initial radical pair and subsequently transferred to nuclei, where it is detected by NMR as signal enhancement up to a factor of about 10,000 (Prakash *et al.* 2005; Prakash 2006).

The origin of photo-CIDNP in bacterial RCs is now understood (Jeschke and Matysik 2003; Daviso *et al.* 2009). Two coherent mechanisms running in parallel, called 'three spin mixing' and 'differential decay', transfer electron spin order to nuclear spin order. A third mechanism, called 'cyclic reaction' or 'differential decay', requires a long lifetime of the donor triplet state and is not expected to be relevant in PSI (Jeschke 1998). Since the appearance of the photo-CIDNP signals improves sensitivity and selectivity of NMR dramatically, this method allows the study of small changes in the photochemically active region of the RCs in great detail and is therefore particularly suitable for the study of packing effects on P700. In this study we compare PSI proteins of spinach (*Spinacia oleracea*) and of the aquatic plant duckweed (*Spirodela oligorhizza*), using $^{15}N$ photo-CIDNP MAS NMR. Our results demonstrate that the electronic ground state of the donor cofactor is highly conserved and reveal differences in the overall electronic architecture in the two different plant systems, which may represent a common denominator across species (Santabarbara *et al.* 2010).

## 3.2 MATERIALS AND METHODS

### *3.2.1 Photosystem I Particle Preparation*

$^{15}N$-labeled spinach plants were grown as described by Diller et al 2007 (Diller *et al.* 2007). $^{15}N$-labeled duckweed plants were grown under aseptic conditions on half-strength Hunter's medium (Posner 1967) under continuous light (20 $\mu Em^{-2}s^{-1}$) at 25 °C. The medium was continuously bubbled with sterile air containing 5% $CO_2$. As a source of isotope labeled nitrogen $KNO_3$ was substituted by 5.5 mM of $^{15}NH_4{}^{15}NO_3$, and in addition $Ca(NO_3)\bullet 4H_2O$ was replaced by 4 mM $CaCl_2$. After 7 days plants were harvested, frozen in liquid nitrogen and stored at -80 °C until use. The PSI complex containing ~110 Chl/P700 (PSI-110 particles) was prepared according the method described by Alia *et al.* 2004.

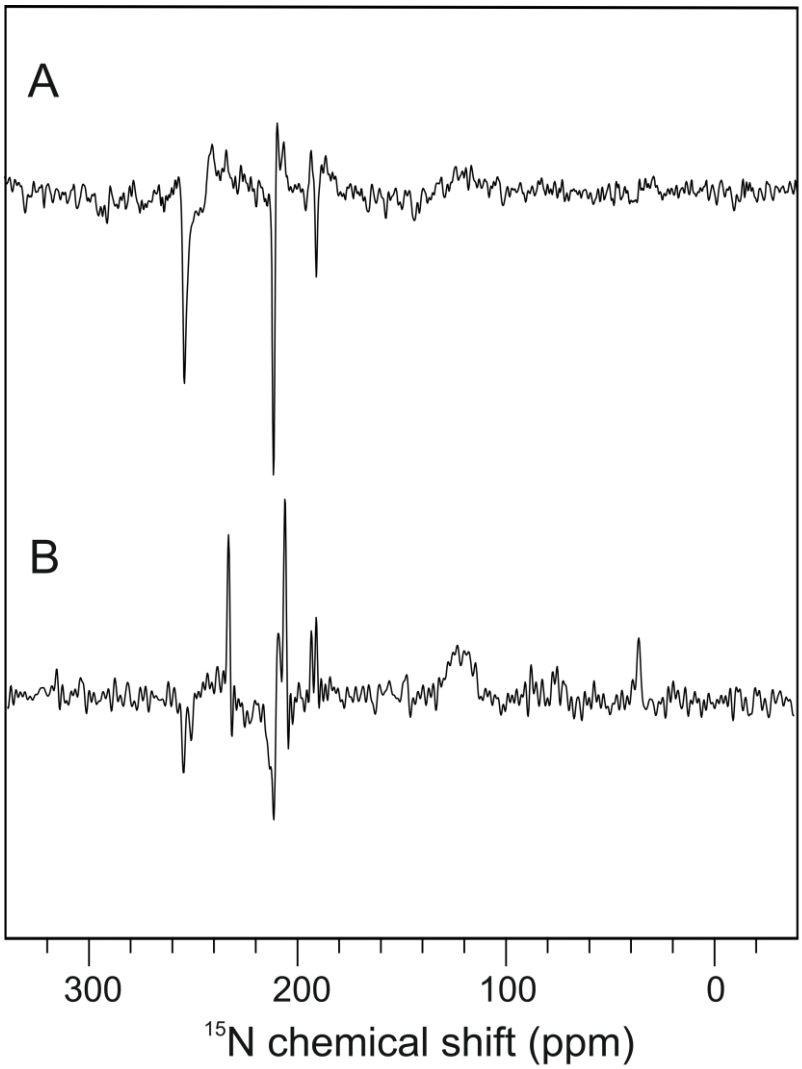


**Figure 3.2:** $^{15}$N photo-CIDNP MAS NMR spectra of PSI of duckweed (A) and spinach (B), obtained by continuous illumination at 4.7 T, with a cycle delay of 4 s, a spinning frequency of 8 kHz and at a temperature of 240 K.

### *3.2.2 Photo-CIDNP MAS NMR experiments*

The NMR experiments were performed using a DMX-200 NMR spectrometer (Bruker GmbH, Karlsruhe, Germany). The samples were loaded into optically transparent 4-mm sapphire rotors. The PSI samples were reduced by the addition of an aqueous solution of 10 mM sodium dithionite solution and 40 mM glycine buffer (pH 9.5) in an oxygen free atmosphere. Immediately following the reduction, slow freezing of the sample was performed directly in the NMR probe inside the magnet with liquid nitrogen-cooled gas under continuous illumination with white light. The temperature was kept at 235 K. The used illumination set-up was specially designed for the Bruker MAS probe (Matysik 2000). The light and dark spectra were collected with a CPMG-echo pulse sequence and two pulse phase modulation (TPPM) proton decoupling. The number of scans was 20 k,

unless stated differently. Fitting of the spectra was performed with Igor Pro 6.01, based on the relative intensity of the signals the electron spin density was calculated for the nitrogen assigned to the donor.

## 3.3 RESULTS AND DISCUSSION

### *3.3.1 The occurrence of photo-CIDNP in different PSI preparations from duckweed and spinach*

Figure 3.2A shows the $^{15}$N MAS NMR spectrum of uniformly $^{15}$N labeled PSI of duckweed obtained under continuous illumination. The very weak and broad positive signals arising at about 120 ppm originate from the amide nitrogen of the protein backbone and are also present in spectra obtained in the (data not shown). Upon illumination six absorptive, positive, and five emissive, negative, photo-CIDNP signals can be resolved in the spectrum. No spinning side bands are observed at the applied magnetic field of 4.7 T and with a spinning frequency of 8 kHz. The narrow linewidth of the photo-CIDNP signals reveal a highly defined packing of the cofactors in the RC that are involved in the light-induced NMR response. The photo-CIDNP effect in PSI has been observed previously for another plant species, spinach (Alia *et al.* 2004; Diller *et al.* 2005; Roy *et al.* 2007) as well as for *Synechocystis* sp. PCC 6803 (chapter 2, (Janssen *et al.* 2010)). Here, we demonstrate that the solid-state photo-CIDNP effect on PSI can also be observed in duckweed. Hence most likely the effect occurs in all natural photosynthetic systems (Matysik *et al.* 2009). Being an aquatic plant, duckweed has the advantage over spinach that it is easier to introduce isotope labels, which allows for more detailed and selective $^{15}$N and $^{13}$C solid state photo-CIDNP studies (chapters 4 and 5). Duckweed previously has shown to successfully incorporate isotope labeled amino acids into its RC (Alia 2003).

Spectra A and B in Figure 3.2 compare the $^{15}$N MAS NMR spectra of uniformly $^{15}$N-labeled PSI of duckweed and spinach obtained by continuous illumination, with light induced signals occurring between 180 and 280 ppm. Figure 3.3 shows detailed spectra of duckweed (spectrum 3.3A) and spinach (spectrum 3.3B) PSI. Both the linewidth and the chemical shift of the photo-CIDNP signals observed for the two plant systems are very similar. However the intensity and sign of the signals are different for the two species.

**Table 3.1** $^{15}$N chemical shifts of the photo-CIDNP signals observed in duckweed in comparison with published chemical shift data

| Assignment | Solution data | PSI spinach | PSI duckweed |
|---|---|---|---|
| atom | $\sigma_{LS}$ [a] | $\sigma_{SS}$ [b] | $\sigma_{SS}$ [c] |
| N-I | 186.0 | **186.2** e | **186.3** a |
| | | 190.9 a | 188.6 a |
| N-II | 206.5 | 206.1 a | 206.3 a |
| | | | 210.0 a |
| | | **211.5** e | **211.4** e |
| N-III | 189.4 | | **191.0** e |
| | | 193.2 a | 193.3 a |
| N-IV | 247.0 | 233.3 a | 233.6 a |
| | | 250.3 e | 250.1 e |
| | | | 253.0 e |
| | | **254.9 e** | **254.3 e** |

All shifts are referenced to liquid ammonia with use of an external standard of solid $^{15}NH_4NO_3$ ($\delta$ = 23.5).
Bold printed shifts are assigned to the primary Chl *a* donor of the B-branch ($P_B$)
*a*, Absorptive (positive); *e*, emissive (negative).
[a] Chemical shift in ppm. Free Chl *a* measured in $CDCl_3$. Source: Boxer 1974.
[b] Chemical shift in ppm. PSI-110 complex from spinach. Source: Diller 2007
[c] Chemical shift in ppm. PSI-110 complex from duckweed Source: this work.

### *3.3.2 Signal assignment and electron spin density distribution in the donor $P_B$*

The $^{15}$N chemical shift assignments of the photo-CIDNP signals observed in duckweed PSI are summarized in Table 3.1, and are compared with $^{15}$N chemical shifts assignments for spinach PSI (Diller *et al.* 2007) and liquid NMR data of monomeric Chl *a* in solution (Boxer *et al.* 1974) . If both branches participate in the ET, photo-CIDNP signals from up to three active Chl *a*, and one active Chl *a´* cofactors can be expected from PSI with a total maximum of sixteen nitrogens. In photo-CIDNP spectra of PSI in duckweed, a total of eleven signals has been observed, as opposed to eight signals assigned previously based on data obtained from spinach PSI (Diller *et al.* 2005). In duckweed PSI the four signals with chemical shifts between 242 ppm and 255 ppm have been assigned to the N-IV nitrogens of four different cofactors. In addition, the observation of three signals within the chemical shift range expected for the N-II nitrogens indicate that both branches may be active in PSI of duckweed. Based on the expectation

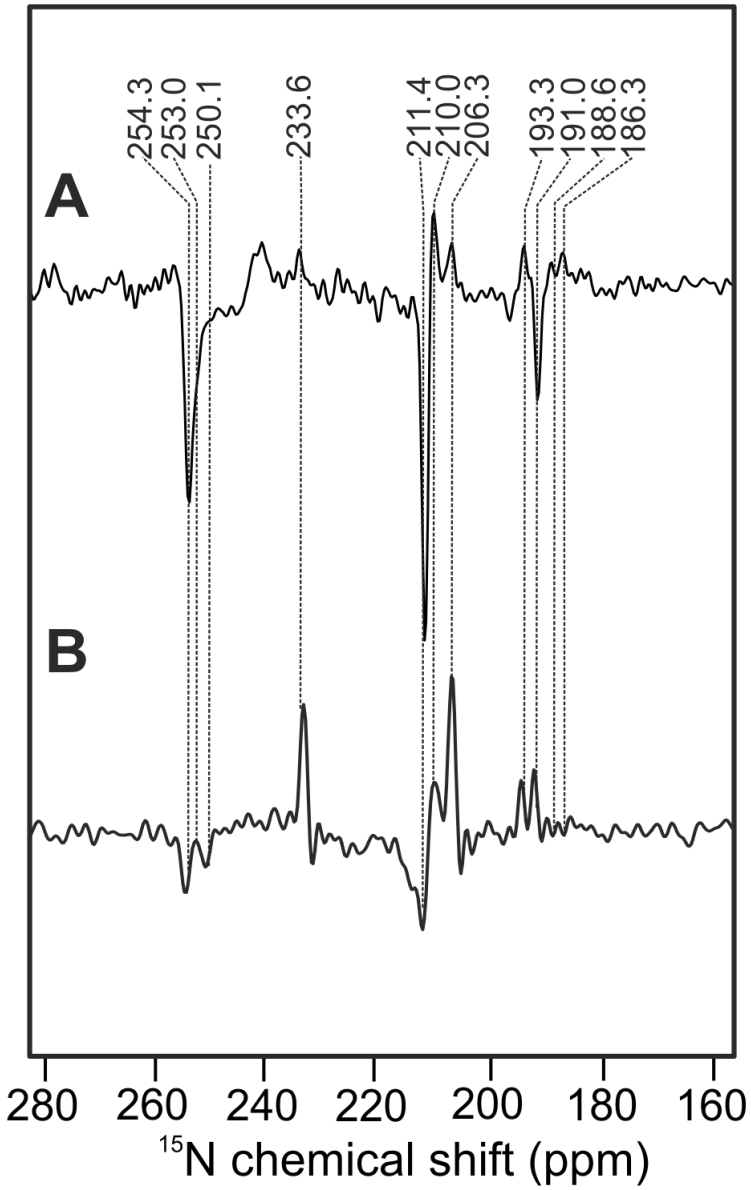


**Figure 3.3:** Detailed $^{15}$N photo-CIDNP MAS NMR spectra of the light induced signals obtained from PSI of duckweed (A) and spinach (B). Spectra were obtained by continuous illumination at 4.7 T, with a cycle delay of 4 s, at a temperature of 240 K.

that most electron spin density ($\rho_i$) is located on the primary Chl *a* donor of the B-branch ($P_B$) (Plato *et al.* 2003), four signals in the spectrum obtained from duckweed PSI have been assigned to the nitrogen of $P_B$; N-I at 188.6 ppm, N-II at 211.4 ppm, N-III at 191.0 ppm and N-IV at 254.3 ppm (bold printed in Table 3.1). The signal at 186.3 ppm, which is assigned to the N-I of $P_B$ (see Figure. 3.3 and Table 3.1) is absorptive in duckweed and emissive in spinach while in general the absorptive signals observed in the spinach PSI spectrum are much more pronounced. The relative intensities of the emissive signals assigned to the $P_B$ cofactor are stronger in duckweed compared to the corresponding signals observed in spinach.

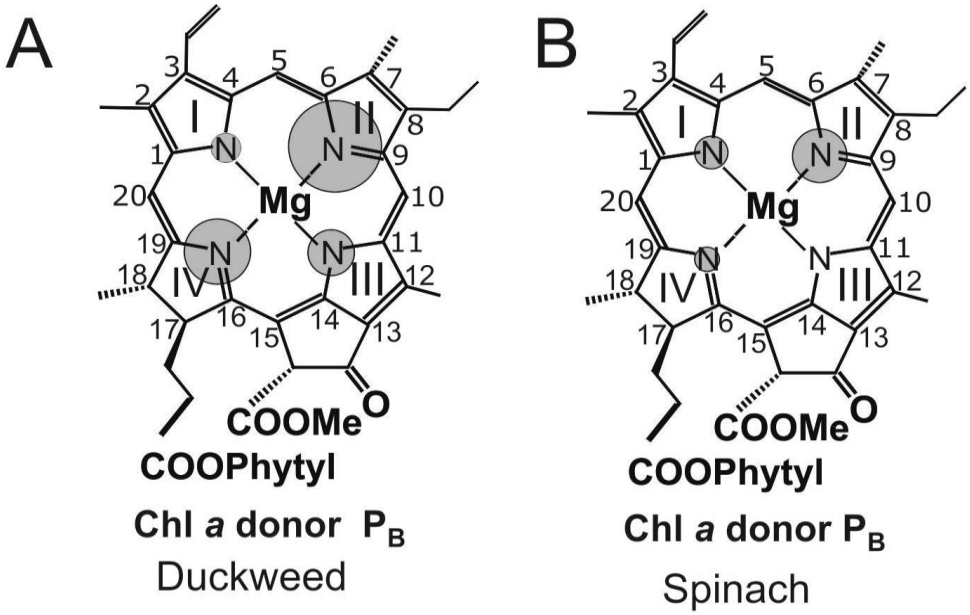


**Figure 3.4:** Estimated electron spin density ($\rho_i$ ) patterns in the steady state based on the integrated $^{15}N$ photo-CIDNP signal intensities for the primary donor of the B branch ($P_B$) of PSI of duckweed (A) and spinach (B).

The chemical shifts observed for duckweed appear very similar to those observed for spinach (see Table 3.1 on page 57). For both PSI systems the donor and primary acceptor cofactors, are plant Chl *a* or Chl *a′*. Hence, the $^{15}N$ shift data indicate that for the RCs of duckweed and spinach, the packing effects on the cofactors and their electronic ground states are quite comparable for both species, while the signal intensities reveal differences at a higher level in the protein machinery.

Based on the intensities of the observed photo-CIDNP signals, the distribution of $\rho_i$ in the $2p_z$ orbitals can be estimated (for details, see Diller *et al.* 2005). In Figure 3.4 the steady-state electron spin density map of the $P_B$ cofactor of duckweed (Figure 3.4A) is compared to the relative $\rho_i$ distribution observed for the primary donor of spinach PSI (Figure 3.4B). Both for the duckweed and for the spinach PSI most of the $\rho_i$ is located on the ring II nitrogens, which is in line with the electron spin distribution observed for free Chl *a* in solution (Käss *et al.* 1995; Käss *et al.* 1996; Käss *et al.* 1998). Likewise, similar to undisturbed Chl *a* in solution, a small $\rho_i$ is observed on the N-I for both spinach and duckweed PSI. A prominent difference is that the signal assigned to the ring I nitrogen of $P_B$ of spinach, with a chemical shift of 186.2 ppm, is emissive in nature while in duckweed it is observed as an absorptive signal with almost the same chemical shift (186.3 ppm, see

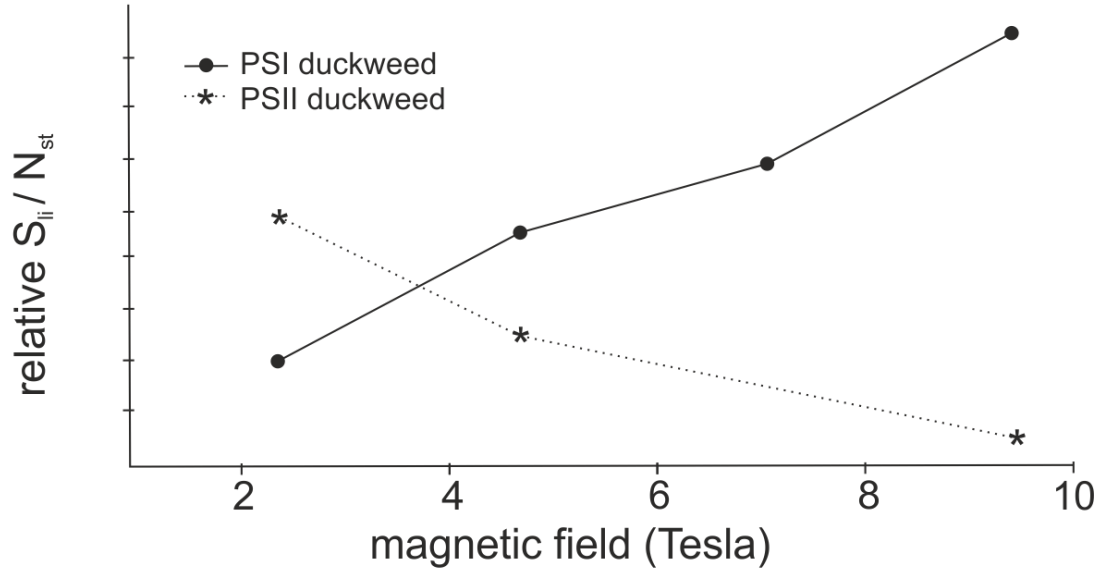


**Figure 3.5:** The relative magnitude of the ratio of the light-induced signal ($S_{li}$) to the standardized noise ($N_{st}$) for PSI and PSII of duckweed.

Table 3.1 and Figure 3.3). Compared to spinach PSI, a much higher $\rho_i$ on the ring IV nitrogen is observed in the duckweed PSI preparation (Figure 3.4A), while a moderate $\rho_i$ is found to be located on N-III. None of the signals in the spectrum obtained from spinach PSI (Table 3.1, Figure 3.3B) was assigned to the N-III of the primary donor $P_B$ which is expected to procduce an emissive signal around 190 ppm (Table 3.1) (Diller *et al.* 2007).

In contrast with the similar chemical shifts, the intensity patterns between duckweed and spinach appear substantially different. Such changes in the intensity pattern suggest fundamental differences between the radical pairs and electron delocalization in both plant systems. It is possible that this difference is related to a different balance of the photo-CIDNP enhancement mechanisms. This would imply that parameters ruling dynamics, or the architecture of the radical pair, are also different. Such an interpretation implies that there is a large flexibility in PSI systems as proposed earlier (Santabarbara *et al.* 2010). The differences in the electronic architecture may be due to different patterns of activation of two branches in two systems leading to more asymmetric radical pair architectures having other coupling parameters. The question then remains if (i) the functionality of PSI differs between species or (ii) if functional flexibility is an inherent property of the PSI system itself, independent of the species or (iii) a combination of both options. This will be discussed further in chapter 6 of this thesis.

### 3.3.3 The magnetic field effect of photo-CIDNP in different plant PSI

The field dependence of the solid state photo CIDNP effect in PSI from spinach has been measured previously and it was shown that the signal strength increases from 2.4T to 9.4 T (Roy *et al.* 2007). Figure 3.5 shows the relative signal to noise intensity of the photo-CIDNP signals obtained from uniformly $^{15}$N-labeled PSI and PSII of duckweed at different magnetic field strengths. In line with previous observations in spinach PSI (Roy *et al.* 2007), the light induced signals of duckweed PSI increase in intensity with an increasing magnetic field strength from 2.4 T up to 9.4 T. PSII on the other hand seems to show the opposite trend with signal intensity decresing upon increasing the field from 2.4 T to 9.4 T. Within the lower range of observed fields, the field dependence of the solid state photo-CIDNP effect in duckweed thus appears to be similar to the signal enhancement for spinach. This observation contrasts with the significant differences in the intensity patterns of the light induced signals, which suggest different electronic architecture of the radical pairs, while the field dependence appears to be similar in the two systems.

### *3.3.4 Outlook*

To explain the differences between the radical pairs for the two PSI systems and determine if the functionality of PSI differs between species or functional flexibility is an inherent property of the PSI system itself, further experimental and theoretical studies have been performed. In chapter 5 time-resolved experiments on $^{13}$C and $^{15}$N labeled PSI and theoretical simulations will be discussed. In chapter 6 preliminary data obtained from selectively $^{13}$C- labeled PSI from duckweed at different fields will be presented and the discussion about the functional flexibility of PSI will be continued.