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Different Role of the Jα Helix in the Light-Induced Activation of the LOV2 Domains in Various Phototropins

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ABSTRACT: Phototropins (phot) are blue light receptors in plants which are involved in phototropism, stomatal opening, and chloroplast movements. Phototropin has two LOV domains (LOV1 and LOV2), and the LOV2 domain is responsible for activation of Ser/Thr kinase. There is an α-helix at the C-terminal side of the LOV2 domain, which is called the Jα helix. The functional importance of the Jα helix has been established for *Arabidopsis* phot1, where light-induced structural perturbation takes place in the Jα helix during the photocycle of LOV2 domains. However, the present FTIR study reports a different role of the Jα helix in light-induced signal transduction of LOV2 domains. Here we construct LOV2 domains with (LOV-Jα) and without (LOV-core) the Jα helix for *Arabidopsis* phot1 and phot2 and *Adiantum* neochrome 1 and compare their light-induced difference FTIR spectra. Light-induced protein structural changes differ significantly between LOV-Jα and LOV-core for *Arabidopsis* phot1 [Yamamoto, A., Iwata, T., Sato, Y., Matsuoka, D., Tokutomi, S., and Kandori, H. (2009) *Biophys. J. 96*, 2771−2778]. In contrast, the difference spectra are identical between LOV-Jα and LOV-core for *Adiantum* neochrome 1. In *Arabidopsis* phot2, the protein structural changes are intermediate between *Arabidopsis* phot1 and *Adiantum* neochrome 1. These results suggest that the conformational changes of the Jα helix and the interaction between the LOV-core and the Jα helix are different among phototropins. The role of the Jα helix for signal transduction in phototropins is discussed.

Phototropins (phot),[1] blue light receptors in plants, are involved in phototropism (*1*), chloroplast movements (*2*−*4*), stomata opening (*5*), cotyledon expansion (*6*) and leaf expansion (*7*), and rapid inhibition of hypocotyl elongation (*8*), all of which are related to optimize the efficiency of photosynthesis. Most higher plants have two phot isoforms, phot1 and phot2 (*9*). Stomatal opening is mediated redundantly by both phot1 and phot2 (*5*). In contrast, phot1 and phot2 share tropic responses and chloroplast accumulation, depending on the fluence rate of light in *Arabidopsis* (*10*), whereas chloroplast avoidance is regulated by only phot2 (*11*). Thus, they share these responses via their different sensitivities to light. In addition to phot1 and phot2, ferns and green algae have a fusion protein of phot in its C-terminus with a chromophoric domain of phytochrome in its N-terminus, which is named neochromes (neo) (*12*, *13*) and acts as a red light sensor. Neo1 from *Adiantum* can also act as a blue light sensor (*14*), although neo1 and 2 from *Mougeotia scalaris* cannot (*13*).

Phototropin consists of about 1000 amino acid residues and two prosthetic FMN molecules. The FMN-binding domains are located at the N-terminal half, and the C-terminal half has a serine/threonine (Ser/Thr) kinase domain. The photochemical reaction of FMN yields kinase activation through a change in the domain−domain interaction, although the mechanism is not yet clear. The two FMN-binding domains (ca. 100 residues) are named LOV1 and LOV2, since they have primary (*15*) and tertiary (*16*) structures highly homologous to bacterial light-sensor PYP (photoactive yellow protein), oxygen-sensor FixL, and voltage-sensor HERG of a channel protein so that the domain is called the LOV (light, oxygen, and voltage sensing) domain. The protein fold belongs to the PAS (Per−Arnt−Sim) superfamily. X-ray crystallography showed that the structures of various LOV domains, LOV1 domains from *Chlamydomonas* phot (*17*) and *Arabidopsis* phot1 and phot2 (*18*) and LOV2 domains from *Adiantum* neo1 (*16*, *19*) and oat phot1 (*20*), all reported similar protein architectures (Figure 1).

The photoreaction in the LOV domain is an adduct formation between FMN and a nearby cysteine (*19*, *21*−*24*) through a triplet excited state of FMN (Figure 1 (*23*, *25*)). Although various models have been proposed for the reactive cysteine, previous FTIR studies revealed that cysteine is protonated in both the ground (*26*−*30*) and triplet excited (*31*) states of FMN.[2] S390 is the only ground-state intermediate during the photocycle of LOV

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[1]Abbreviations: phot, phototropin; neo, neochrome; LOV, light−oxygen−voltage; FMN, flavin mononucleotide; PAS, Per−Arnt−Sim; FTIR, Fourier-transform infrared; NMR, nuclear magnetic resonance; CBP, calmodulin binding peptide.

[2]In Sato et al. (*31*), the difference FTIR spectra at 77 K monitored the S−H stretch of the reactive cysteine in the triplet excited state. Recent time-resolved IR studies by Kottke et al. (*47*) and Alexandre et al. (*48*) reported difference spectra similar to those by Sato et al. at $< 1800\ cm^{-1}$ on the microsecond and nanosecond time scales, respectively. These observations imply protonation of the reactive cysteine in the triplet excited state at room temperature.

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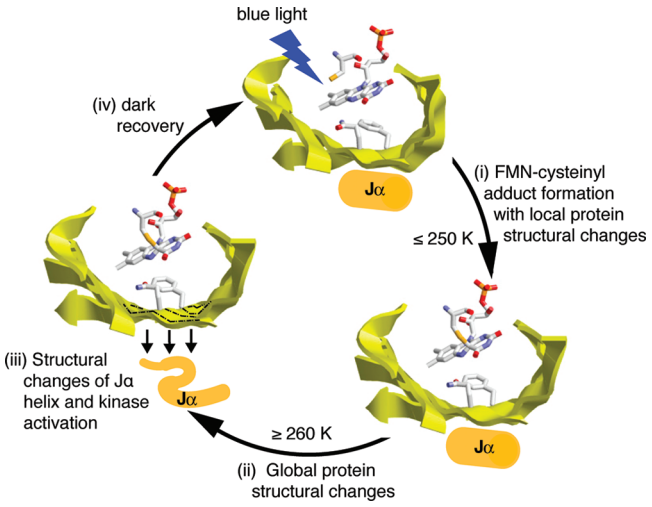

FIGURE 1: Protein structure of the LOV2 domain and light-induced structural changes during the photocycle. The structures of the unphotolyzed (*16*) and S390 (*19*) states of *Adiantum* neo1-LOV2 are shown. The ribbon drawing in yellow represents the β-sheet of LOV2-core, while stick drawings highlight FMN and three key residues in signal transduction, Cys966, Phe1008, and Gln1029. This illustration is modified from Figure 6 in ref *42*.

domains which possesses an adduct between FMN and Cys. Therefore, it is believed that S390 is the active state for the light-sensing function of phototropin. Molecular biological experiments using expression in insect cells and transgenic *Arabidopsis* reported that only the photochemical reaction of LOV2 is necessary for kinase activation and that phototropin works even if LOV1 did not respond to light for both phot1 and phot2 (*3*). An *in vitro* study found that LOV2 regulates kinase activity by binding and dissociating to the kinase domain directly in *Arabidopsis* phot2 (*32*). Thus, the LOV2 domain is only essential for activation of the kinase domain.

By means of light-induced difference FTIR spectroscopy at various temperatures, we revealed the presence of progressive protein structural changes in the S390 intermediate of neo1-LOV2 (Figure 1) (*33*−*35*). In particular, structural perturbation of the β-sheet was observed at physiological temperatures, which was absent for neo1-LOV1 (*29*). Thus, it was implicated that a structural change of the β-sheet is important for the signal transduction activity of neo1-LOV2. In parallel, NMR and X-ray crystallographic studies of extended LOV2 fragments from oat phot1 revealed that an extra α-helix (named Jα helix) associates with the surface of LOV2 in the dark state (*20*, *36*, *37*). Since the interaction between Jα and LOV2 is disrupted upon formation of S390, an important role of the Jα helix in the signal transduction of LOV2 has been recognized. In addition, light-induced conformational changes of the Jα helix were also observed by a transient grating method (*38*−*41*) in *Arabidopsis* phototropins. Our recent FTIR study also detected structural changes of the Jα helix in *Arabidopsis* phot1-LOV2 by comparing the spectra of LOV2 with and without the Jα helix (*42*).[3] Thus, the light-signal transduction pathway inside LOV2 can be described as follows: (i) adduct formation causes local structural changes of the loop region, (ii) structural changes of the β-sheet then take place, (iii) the Jα helix is detached from the β-sheet, and kinase activity is switched on (Figure 1).

[3]After submission of the present paper, Alexandre et al. reported similar FTIR results on *Avena sativa* phot1 LOV2 (*49*), and reached the same conclusion by Yamamoto et al. (*42*).

The scheme explains well the mechanism of activation of LOV2, because the β-sheet is structurally sandwiched between FMN and the Jα helix (Figure 1). Nevertheless, we have to be careful to accept the scheme as a common mechanism in phototropin because it was made from limited data based on species (neo1, phot1, phot2, etc.) and methods (FTIR, NMR, transient grating, etc.). For instance, our FTIR study of *Arabidopsis* phot1-LOV2 observed prominent bands at 1650 (−)/1625 (+) cm^{-1} only in the presence of the Jα helix (*42*). Since the frequency at 1650 cm^{-1} is characteristic of the α-helix in amide I vibrations, structural perturbation of the Jα helix is consistent with other observations. It should however be noted that the previous FTIR studies of *Adiantum* neo1-LOV2 (*26*, *29*−*31*, *33*−*35*) were also applied to the construct containing the Jα helix, but we observed a negative band at 1643 cm^{-1} and not at ~1650 cm^{-1}. We interpreted these results as being a structural perturbation of the β-sheet, although we never measured neo1-LOV2 in the absence of the Jα helix. Thus, a comprehensive FTIR analysis is required for better understanding of the role of the Jα helix, particularly comparing the spectra of neo1-LOV2 with and without the Jα helix.

The above motivation is related to an important question as to whether protein structural changes in activation of LOV2 domains are common or unique. From similar protein structures of LOV domains, one may tend to believe that the activation mechanism is common among LOV domains. Is this true? Matsuoka and Tokutomi measured the activity of *Arabidopsis* phot2-LOV2 in a pull-down assay using the construct even in the absence of the Jα helix (*32*), suggesting that the Jα helix is not a prerequisite for activation of phot2-LOV2. A unique signal transduction may be important, since LOV domains are able to interact with so many proteins (*43*), in which each mechanism should be unique. Although the interaction partner of LOV2 domains is common (Ser/Thr kinase), a different activation mechanism may exist. In this paper, we report the comprehensive FTIR analysis of LOV2 domains among phot1, phot2, and neo1. To reveal the role of the Jα helix domain, we constructed LOV-core and LOV-Jα, which are equivalent to those of *Arabidopsis* phot1-LOV2 in our recent study (*42*) (Figure 2). Very interestingly, the Jα-helix-dependent FTIR spectra differ among species. Light-induced protein structural changes are significantly different for phot1 between LOV-core and LOV-Jα as reported recently (*42*). That was also the case for phot2, but the effect was somehow different. On the other hand, the differences in spectra were identical for neo1 between LOV-core and LOV-Jα. These results suggest that the conformational changes of the Jα helix and the interaction between the LOV-core and the Jα helix are different among phototropins. Roles of the Jα helix for signal transduction in phototropins will be discussed.

MATERIALS AND METHODS

Expression and Purification of Recombinant Proteins. LOV2 domains with and without the Jα helix from *Arabidopsis* phot2 and *Adiantum* neo1 were prepared as for phot1-LOV2 (*42*). Using a plasmid which includes DNA of the *Arabidopsis* phot2 LOV2 linker (Asp363-His575) (*44*) or *Adiantum* neo1-LOV2 (Pro905-Pro1087) (*33*) as a template, required DNA fragments with appropriate restriction sites were synthesized with PCR and oligonucleotide primers as described previously (*32*, *44*). Amplified DNA was isolated, digested, and cloned into a pGEX4T1 bacterial expression vector (GE Healthcare) as a translational

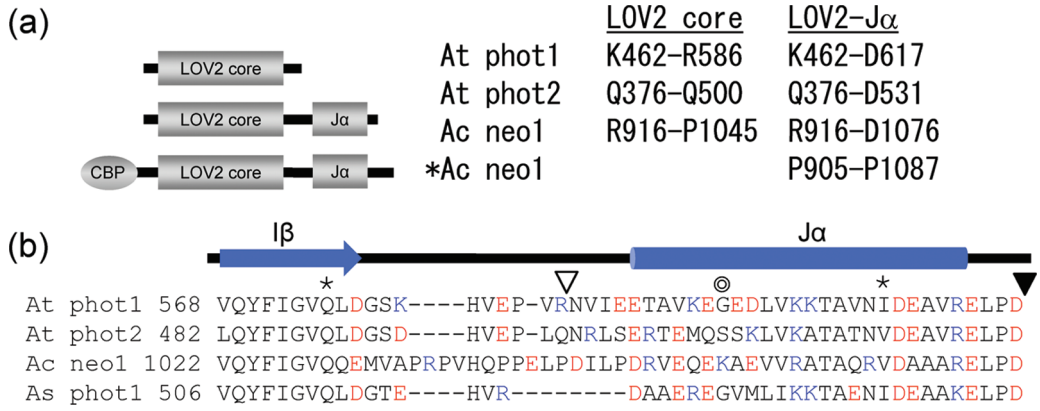

FIGURE 2: (a) Schematic illustration of the LOV2 constructs of phototropin used in this study. In the previous studies of *Adiantum* neo1-LOV2 (*Ac neo1) (*26*, *29*−*31*, *33*−*35*, *45*), we used the P905−P1087 construct containing the CBP tag at the N-terminus. In this study, we used the constructs encoding the same region of the LOV2-core and LOV2-Jα for *Arabidopsis* (At) phot1 and phot2 and *Adiantum* (Ac) neo1, where the GST tag at the N-terminus is digested with thrombin. (b) Amino acid sequences of the C-terminal side of LOV2-core and LOV2-Jα. Open and closed triangles show the positions of the C-termini of the LOV2-core and LOV2-Jα constructs, respectively. Two asterisks (*) show the functionally important amino acids, Gln575 and Ile608, in *Arabidopsis* phot1 (*42*). In this study, we mutated Gly597 of *Arabidopsis* phot1 to Lys and Lys1056 of *Adiantum* neo1 to Gly, which are located in the Jα helix (◎).

fusion to glutathione *S*-transferase (GST). The following two polypeptides, fused with GST in the N-terminus, were prepared: *Arabidopsis* phot2-LOV2-core (Q376-Q500) and phot2-LOV2-Jα (Q376-D531); *Adiantum* neo1-LOV2-core (R916-P1045) and neo1-LOV2-Jα (R916-D1076) (Figure 2). The G597K mutant of phot1-LOV2-Jα and the K1056G mutant of neo1-LOV2-Jα were constructed by PCR using the QuickChange site-directed mutagenesis method (Stratagene). These amino acid substitutions were confirmed by DNA sequencing.

GST fusion proteins were prepared by overexpression systems with *Escherichia coli* as described previously (*32*, *44*). Briefly, the GST-LOV fusion proteins expressed in *E. coli* were purified by glutathione−Sepharose 4B (GE Healthcare), and the GST tag was digested with thrombin. The purified LOV domain leaves a linker of five amino acid residues, Gly-Ser-Pro-Glu-Phe, on the N-terminus of each LOV construct. The protein solution was concentrated to 2.5 mg/mL by using an Amicon Ultra PL-10 (Millipore) and dialyzed against 1 mM potassium phosphate buffer (pH 7). 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride, which works as an inhibitor for thrombin protease, was added to give a final concentration of 0.1 mM. The solution (70−80 μL) was placed on a BaF_2 window, and the dry films were hydrated by dropping H_2O next to the film on the window.

FTIR Spectroscopy. Infrared spectra of the hydrated films were measured using an FTS-40 (Bio-Rad) spectrophotometer as described previously (*29*, *30*, *33*, *42*). Low-temperature spectra were measured by using a cryostat (Optistat DN, Oxford, U.K.) and a temperature controller (ITC 4, Oxford, U.K.) with liquid nitrogen as the coolant. Hydrated films were illuminated by a >400 nm light for 1 min, which was supplied with a combination of a halogen−tungsten lamp (1 kW) and a long-pass filter (L42; Asahi Techno Glass). For the measurement of phot2-LOV2 at 295 K, light-induced difference spectra were measured under continuous illumination with >400 nm light because of the short lifetime of S390.

RESULTS

Light-Induced Difference FTIR Spectra in Various LOV2 Domains. Figure 3 shows light-minus-dark difference FTIR spectra of LOV2-core (dotted lines) and LOV2-Jα (solid lines) for *Arabidopsis* phot1 (a, reproduced from ref *42*), *Arabidopsis* phot2 (b), and *Adiantum* neo1 (c) in the 1750−1600 cm^{-1} region. The light-induced difference spectra were measured in a wide temperature range (150−295 K). In addition to the amide I vibrations, the C2=O and C4=O stretching vibrations of FMN also appear in this frequency region. Our previous study of neo1-LOV2 identified the frequencies at 1677 and 1711 cm^{-1} (Figure 3c) as the C2=O and C4=O stretching vibrations by use of ^{13}C2=O- and ^{13}C4=O-labeled FMN, respectively (*45*). Similarly, negative bands at 1676 and 1712 cm^{-1} in Figure 3a and at 1678 and 1713 cm^{-1} in Figure 3b presumably originate from these vibrations in the unphotolyzed states of phot1 and phot2. It is likely that a higher frequency shift of both C2=O and C4=O stretches occurs in Figure 3, implying common structural changes of FMN among phot1, phot2, and neo1. Similar changes between LOV2-core and LOV2-Jα also indicate that the presence of the Jα helix does not affect structural perturbation of FMN.

All other vibrations in this frequency region originate from those from protein, particularly amide I modes reflecting C=O stretches of the peptide backbone. It is well-known that the amide I frequencies are characteristic of secondary structures, such as a loop (1670−1690 cm^{-1}), an α-helix (~1650 cm^{-1}), and a β-sheet (1620−1640 cm^{-1}). Figure 3a shows prominent spectral alterations at 1650−1600 cm^{-1} between LOV2-core and LOV2-Jα. At 150 and 250 K, there are no significant differences at this frequency (Figure 3a), implying structural changes of neither the α-helix nor the β-sheet. On the other hand, prominent peaks newly appeared at 1650 (−) and 1625 (+) cm^{-1} at 260, 273, and 295 K for LOV2-Jα (solid lines). The negative 1650 cm^{-1} band, characteristic for the α-helix, was not observed for LOV2-core, and we interpreted that disruption of the α-helix structure presumably originates from the Jα helix (*42*). This interpretation was further supported by the fact that such spectral changes were diminished by point mutation of functionally important amino acids such as Phe556 between FMN and the β-sheet, Gln575 being hydrogen-bonded with FMN, and Ile608 on the Jα helix (Figures 1 and 2) (*42*).

Light-Induced Difference FTIR Spectra of LOV2-core and LOV2-Jα in Arabidopsis Phot2. Essentially similar spectral features were observed for *Arabidopsis* phot2-LOV2 (Figure 3b). At 150 and 250 K, solid and dotted spectra are similar, particularly in the amide I region, indicating that the

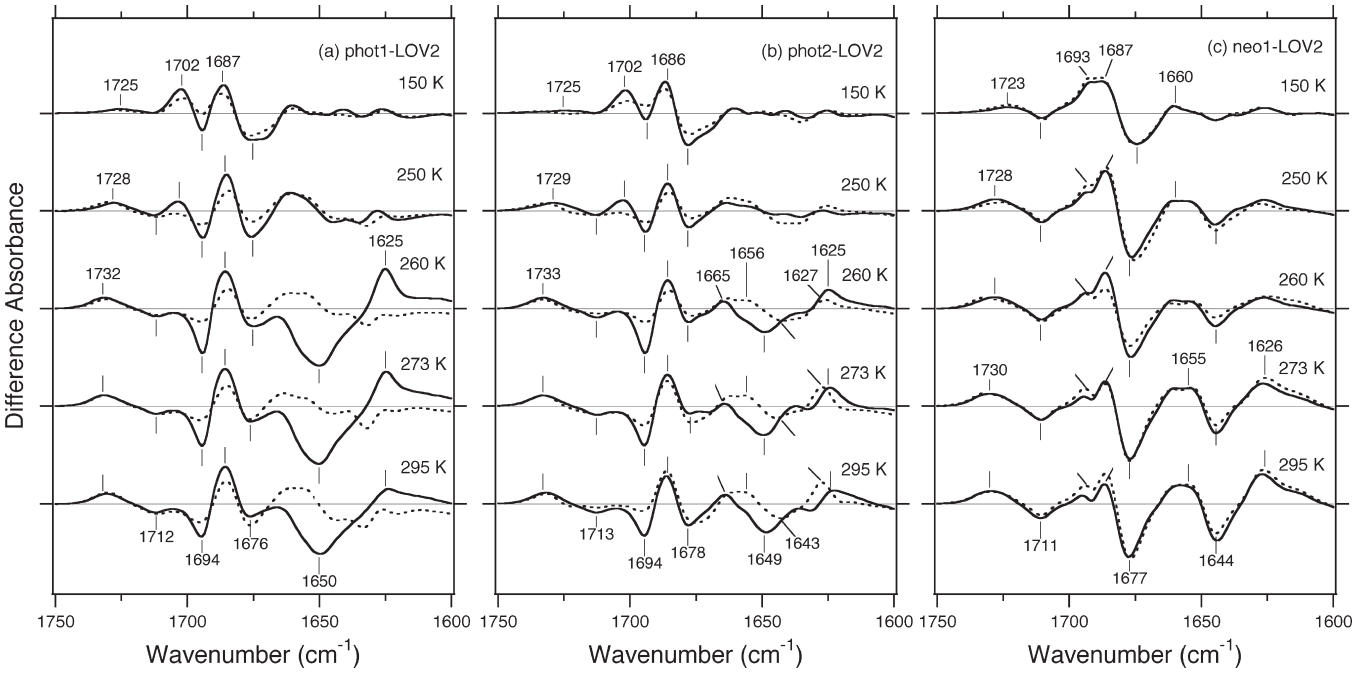

FIGURE 3: Light-induced difference FTIR spectra for the LOV2 domain of *Arabidopsis* phot1 (a), *Arabidopsis* phot2 (b), and *Adiantum* neo1 (c) in the 1750−1600 cm^{-1} region. LOV2-core (dotted lines) and LOV2-Jα (solid lines) are shown. The spectra were recorded at 150, 250, 260, 273, and 295 K. One division of the *y*-axis corresponds to 0.02 absorbance unit. Figure 3a was reproduced from ref *42*.

structural changes in phot2-LOV2 are not affected by the existence of the Jα helix. In particular, little spectral difference at 1650−1600 cm^{-1} is the same as in phot1-LOV2 (Figure 3a), indicating little effect of Jα at 150 and 250 K. In contrast, a clear spectral difference was observed between LOV2-core and LOV2-Jα at ≥260 K. A negative peak at 1649 cm^{-1} only appears in LOV2-Jα, but not in LOV2-core. The corresponding positive peak is presumably at 1625 cm^{-1}, and these spectral features are similar between phot1 (Figure 3a) and phot2 (Figure 3b).

Although the appearance of a peak pair at 1649 (−)/1625 (+) cm^{-1} in LOV2-Jα is common, an obvious difference was also seen between *Arabidopsis* phot1 and phot2. The most noticeable observation in phot2 was a positive peak at 1627 cm^{-1} for LOV2-core. This peak is close in frequency to that at 1625 cm^{-1} in LOV2-Jα, and the intensity in LOV2-core is smaller at 260 K, equal at 273 K, and larger at 295 K than that in LOV2-Jα (Figure 3b). Such a spectral feature was never observed for phot1 (Figure 3a). The corresponding negative band of the band at 1627 (+) cm^{-1} must be located at 1643 cm^{-1}, and there is an additional positive peak at 1656 cm^{-1} (Figure 3b). It should be noted that the bands at 1656 (+)/1643 (−)/ 1627 (+) cm^{-1} for phot2 LOV2-core were observed in neo1-LOV2 (Figure 3c; see below) (*33*, *45*). Thus, it is likely that in phot2-LOV2 structural changes in the α-helix and *β*-sheet regions take place without the Jα helix.

Influence of the Jα helix can be seen for the negative band at 1694 cm^{-1} for both phot1 (Figure 3a) and phot2 (Figure 3b). In both cases, the negative 1694 cm^{-1} band intensified in the presence of the Jα helix. This is also the case at 150 and 250 K, which can be seen more clearly in the double difference spectra (see below; Figure 5). Since the C2=O and C4=O stretching vibrations of FMN are located at 1676−1678 and 1712−1713 cm^{-1}, respectively, the negative band probably originates from an amide I vibration that corresponds to the loop structure.[4] The corresponding positive band is presumably located at 1686−1687 cm^{-1}, where a lower frequency indicates the strengthened hydrogen bond of the loop structure.

Light-Induced Difference FTIR Spectra of LOV2-core and LOV2-Jα in Adiantum neo1. Entirely different spectral features on the influence of the Jα helix were obtained for *Adiantum* neo1-LOV2. Figure 3c shows almost identical differences in FTIR spectra between LOV2-core (dotted lines) and LOV2-Jα (solid lines) at any temperature. This indicates neither structural changes of the Jα helix itself nor Jα-dependent structural changes of other parts upon photoactivation of neo1-LOV2. By using ^{13}C labeling, we identified the bands at 1730−1723 (+)/1711 (−)/1687 (+)/1677 (−) cm^{-1} as C=O stretching vibrations of FMN and the bands at 1693 (+)/1677 (−)/1660 (+)/1655 (+)/1644 (−)/1626 cm^{-1} as amide I vibrations (*45*). As already reported, a temperature-dependent nature is obvious; the amide I band of the loop (1677 cm^{-1}) is shifted up (1693 cm^{-1}) at a low temperature but shifted down (1655 cm^{-1}) at room temperature (Figure 3c) (*45*). In addition, the bands at 1644 (−)/1626 (+) cm^{-1} are intensified as temperature increases, and we previously interpreted the negative 1644 cm^{-1} band as structural perturbation of the *β*-sheet (*33*). It should be noted that the negative peak at 1644 cm^{-1} could also be assignable as an α-helix only from the frequency, but the band was also observed for LOV2-core, which excludes the possibility of the negative 1644 cm^{-1} band from the Jα helix.

It should also be noted that the construct of neo1-LOV2 in the present study is different from those reported previously. We previously used neo1-LOV2 containing P905-P1087 with a calmodulin binding peptide (CBP) tag (Figure 2a). In contrast, the present construct is slightly shorter (R916-D1076 for LOV2-Jα; Figure 2a), and no tags are involved. Since such a difference

[4]Alexandre et al. proposed the negative 1694 cm^{-1} band as the C4=O stretch of FMN, suggesting multiple C4=O conformations (1694 and 1712 cm^{-1}) in the dark state of *A. sativa* phot1 LOV2 (*49*). Since Figure 3a,b shows different Jα-dependent negative intensities between the bands at 1694 and 1712 cm^{-1}, influence of the Jα helix may differ between the two C4=O conformations. It should however be noted that the negative 1694 cm^{-1} band has not been assigned by use of isotope labeling.

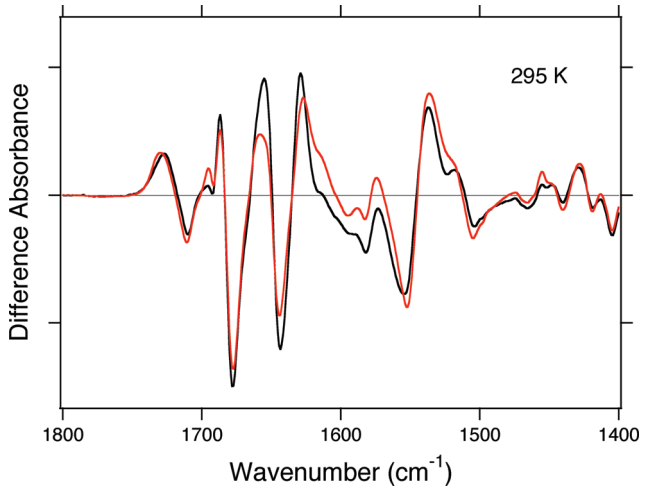

FIGURE 4: Light-induced difference FTIR spectra for the LOV2 domain of *Adiantum* neo1 in the 1800−1000 cm^{-1} region at 295 K. LOV2-Jα of this study (R916-D1076) (red line) and of the previous study (CBP-P905-P1087) (black line) are compared. One division of the *y*-axis corresponds to 0.02 absorbance unit.

might influence the spectra, we thus compared the spectra. Figure 4 shows similar spectral features at 1800−1400 cm^{-1}. Thus, we concluded that the Jα helix does not affect light-induced protein structural changes in *Adiantum* neo1-LOV2, which is in high contrast to *Arabidopsis* phot1 and phot2.

Double Difference Spectra of LOV2-core and LOV2-Jα. The influence of the Jα helix on protein structural changes of LOV2 domains was very different among phot1, phot2, and neo1. Such a difference is obviously seen by subtracting the dotted spectrum from the solid one in Figure 3. Figure 5 shows such double difference spectra of phot1 (black lines), phot2 (blue lines), and neo1 (red lines). It should be noted that each difference spectrum can be quantitatively compared among phot1, phot2, and neo1, because the difference spectra in Figure 3 are normalized by use of vibrational bands at 1500−1200 cm^{-1} (Supporting Information Figure S1). In phot1, the bands at 1653 (−)/1625 (+) cm^{-1} at 260−295 K originate from the Jα-dependent α-helical perturbation (Figure 5), which is presumably owing to the Jα helix itself. There are additional bands in the 1700−1680 cm^{-1} region: bands at 1702 (+)/1694 (−)/1688 (+) cm^{-1} at 150 and 250 K and at 1694 (−)/1688 (+) cm^{-1} at 260−295 K (Figure 5). It is likely that such a spectral feature at 1700−1680 cm^{-1} is also seen in phot2 (blue lines in Figure 5). In phot2, the influence of the Jα helix is clear (Figure 3b), but the spectral feature at 1650−1600 cm^{-1} is more complex than in phot1. Figure 5 shows the presence of a negative band at about 1650 cm^{-1} in phot2 at 260−295 K, but the amplitude is about half of phot1. In addition, a peak pair was seen at about 1625 cm^{-1} in Figure 5 (blue lines), which originates from two positive peaks at 1627 and 1625 cm^{-1} in Figure 3b.

Red lines in Figure 5 were featureless when compared with black and blue lines, indicating no effect of the Jα helix on protein structural changes of neo1-LOV2. In summary, three phototropins exhibited different effects of the Jα helix. In *Arabidopsis* phot1-LOV2, the Jα helix is structurally perturbed upon activation as shown by the Jα-dependent 1650 (−)/1625 (+) cm^{-1} bands. In contrast, no changes were gained between LOV2-Jα and LOV2-core for *Adiantum* neo1. Interestingly, *Arabidopsis* phot2-LOV2 exhibits an intermediate property between *Arabidopsis* phot1-LOV2 and *Adiantum* neo1-LOV2. Namely, LOV2-core shows

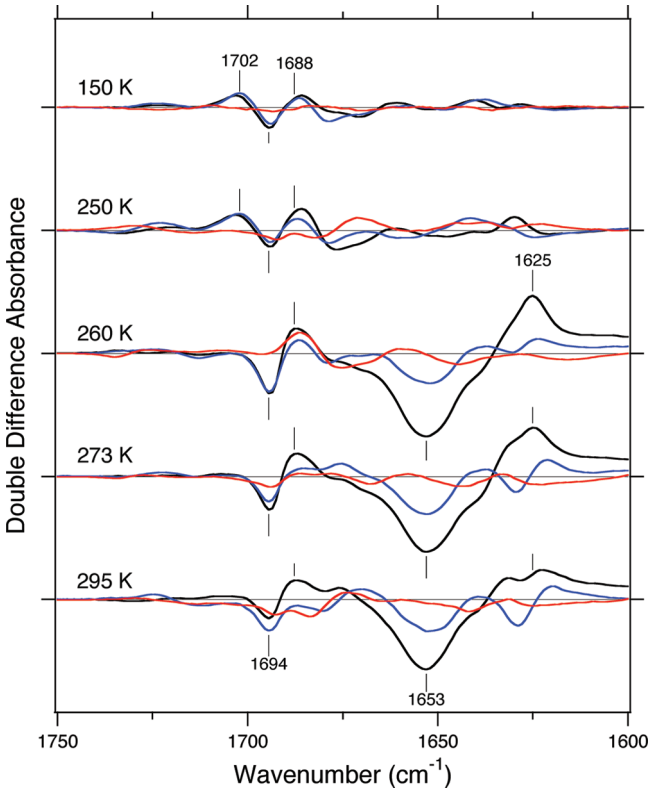

FIGURE 5: Double difference spectra between the LOV2-Jα and LOV2 core in the 1750−1600 cm^{-1} region. Spectra of phot1 (black lines), phot2 (blue lines), and neo1 (red lines) are shown at 150, 250, 260, 273, and 295 K. One division of the *y*-axis corresponds to 0.02 absorbance unit.

1643 (−)/1627 (+) cm^{-1} bands (dotted lines in Figure 3b) that resemble those in neo1-LOV2 (Figure 3c). In contrast, LOV2-Jα shows Jα-dependent 1649 (−)/1625 (+) cm^{-1} bands (solid lines in Figure 3b) that resemble those in phot1-LOV2 (Figure 3a).

Light-Induced Difference FTIR Spectra of LOV2-core and LOV2-Jα in the G597K phot1-LOV2 and K1056G neo1-LOV2 Mutants. Lacking influence of the Jα helix on protein structural changes in neo1-LOV2 can be explained alternatively: (i) the Jα helix does not interact with LOV2-core, or (ii) the Jα helix interacts with LOV2-core like *Arabidopsis* phot1 and phot2, but light-induced protein structural changes do not occur. The construct for the crystal structure of neo1-LOV2 does not include the Jα domain (*16*). For the 31 amino acids representing the Jα helix (Figure 2b), there are 17 identical amino acids between phot1 and phot2, 11 between phot1 and neo1, and 15 between phot2 and neo1. Certainly a high identity suggests the intrinsic ability of α-helix formation for the Jα domain in *Adiantum* neo1. Nevertheless, introduction of an amino acid might cause an unfavorable binding surface only in *Adiantum* neo1. It was suggested that LOV2-core and Jα-helix bind through hydrophobic interactions, in which Ile608 is involved in phot1-LOV2 (*46*). Figure 2b shows that Gly597 is aligned to the same surface of Ile608 in the α-helix (facing the β-sheet of LOV-core), whose corresponding residue is Ser511 in phot2-LOV2 and Lys1056 in neo1-LOV2. A positive charge at the interaction surface might be unfavorable for the interaction in neo1-LOV2. We thereby designed (i) G597K phot1-LOV2 and (ii) K1056G neo1-LOV2, both of which may change the properties of the Jα dependence.

Figure 6a compares light-induced difference FTIR spectra of the wild-type (black solid line) and G597K (red line) mutant

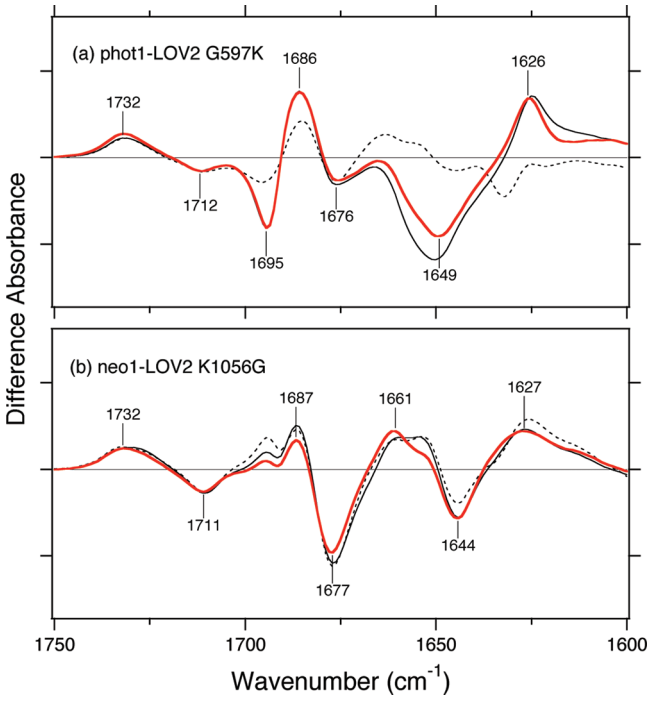

FIGURE 6: (a) Light-induced difference FTIR spectra of LOV2-Jα of the wild-type (black solid line) and G597K mutant (red line) proteins of *Arabidopsis* phot1. The black dotted line represents the spectrum of LOV2-core of the wild type. (b) Light-induced difference FTIR spectra of LOV2-Jα of the wild-type (black solid line) and K1056G mutant (red line) proteins of *Adiantum* neo1. The black dotted line represents the spectrum of LOV2-core of the wild type. These spectra were obtained at 295 K. One division of the *y*-axis corresponds to 0.02 absorbance units.

proteins of phot1 LOV2-Jα. In spite of a slight decrease (~20%), a similar negative band was observed at 1650 cm^{-1} for G597K, indicating that introduction of Lys at the interaction surface does not perturb proper light-induced protein structural changes. It is likely that the Jα helix is bound to LOV2-core even when Gly597 is replaced to Lys, and structural perturbation occurs upon light as in the wild type. Figure 6b compares light-induced difference FTIR spectra of the wild-type (black solid line) and K1056G (red line) mutant proteins of neo1 LOV2-Jα. No negative band newly appeared at 1650 cm^{-1} for K1056G, indicating that removal of Lys at the interaction surface does not lead to the phot1-like protein structural changes of LOV2-Jα. Thus, a single amino acid mutation did not convert specific protein structural changes.

DISCUSSION

This paper reports different protein structural changes among LOV2 domains of *Arabidopsis* phot1 and phot2 and *Adiantum* neo1. In *Arabidopsis* phot1-LOV2, the Jα helix is structurally perturbed upon activation as shown by the Jα-dependent 1650 (−)/1625 (+) cm^{-1} bands (Figure 3a),[5] which is consistent with other spectroscopic observations (*36*−*41*). In contrast, light-induced structural changes were never affected by the presence of the Jα helix in *Adiantum* neo1-LOV2. While Jα-dependent structural changes were observed in *Arabidopsis* phot2-LOV2 as shown by the 1649 (−)/1625 (+) cm^{-1} bands (solid lines in Figure 3b), phot2-LOV2 also exhibits an intermediate property between *Arabidopsis* phot1-LOV2 and *Adiantum* neo1-LOV2. In fact, phot2 LOV2-core exhibits the 1643 (−)/1627 (+) cm^{-1} bands (dotted lines in Figure 3b) that resemble those in neo1-LOV2. Thus, a different role of the Jα helix suggests different signal transduction mechanisms among various phototropins. Below we describe the activation mechanism of kinase on the basis of the present FTIR observations.

Kinase Activation Mechanism in phot1-LOV2. The signal transduction mechanism in phot1-LOV2 was discussed in a recent paper based on the results shown in Figure 3a (*42*). The LOV2 domain is composed of a U-shaped β-strand that holds the FMN chromophore in it, where Gln575 and Phe556 on the β-sheet are in contact with FMN in a different manner (Figure 1). Light-induced adduct formation between FMN and Cys causes environmental changes for these residues, presumably leading to structural perturbations on the β-sheet region. A negative band at 1640−1620 cm^{-1} for LOV2-core (Figure 3a) presumably corresponds to the β-sheet. Such structural perturbations on the β-sheet region consequently yield interaction changes between the β-sheet and the Jα helix, eventually releasing the Jα helix from LOV-core. This scenario for phot1-LOV2 was further supported by the mutation study, because the negative 1650 cm^{-1} bands were diminished by a point mutation of functionally important amino acids such as Phe556 between FMN and the β-sheet, Gln575 being hydrogen-bonded with FMN, and Ile608 on the Jα helix (*42*). We can safely state that this scenario is established, because this is consistent with other spectroscopic and biochemical observations (*36*−*41*).

Kinase Activation Mechanism in phot2-LOV2. The structural changes of phot2-LOV2 were essentially similar to those of phot1-LOV2 (Figure 3a,b), particularly for LOV2-Jα, but structural changes of LOV2-core were different. The spectral feature of LOV2-core was rather closer to that of neo1-LOV2 (dotted curves in Figure 3b,c). Structural perturbation of the β-sheet was observed at high temperatures in phot2 and neo1, where hydrogen bonds are strengthened. Matsuoka and Tokutomi reported that the *Arabidopsis* phot2-LOV2 domain without the Jα helix showed light-dependent dissociation to the kinase domain (*34*), suggesting that the Jα helix may not be necessary for signal transduction. The present FTIR results may be able to explain the mechanism, because phot2 LOV2-core shows protein structural changes similar to those in neo1-LOV2. Thus, a neo1-LOV2-like signal transduction mechanism is used for protein structural changes in phot2-LOV2.

Kinase Activation Mechanism in neo1-LOV2. It is likely that *Adiantum* neo1-LOV2 does not use the Jα helix region for the activation of kinase domain, because of no influence of the Jα helix. This may be related to the specific protein construct of neo1, which contains a chromophoric domain of phytochrome in addition to LOV1 and LOV2 domains. It is known that both LOV2 domains and the chromophoric domain of phytochrome can regulate kinase activity in neo1 (*14*). Consequently, the LOV2 domain may somehow reorganize the structure so that LOV2 can activate the kinase domain without the Jα helix.

In a mechanistic point of view, lacking influence of the Jα helix in neo1-LOV2 is intriguing. The main questions are if the "Jα"

[5]While the frequency of the negative band at 1650 cm^{-1} is characteristic of an α-helix, the 1625 cm^{-1} frequency is too low for an α-helix. The possible origin of the positive band at 1625 cm^{-1} was discussed in Yamamoto et al. (*42*). An additional possibility is noted here. In the low-temperature FTIR study of bacteriorhodopsin, we previously identified the bands at 1625 (+)/1618 (−) cm^{-1} as the amide I vibration of Val49 by use of [1-^{13}C]Val labeling and mutations (*50*). Val49 is involved in the transmembrane α-helix, and unusual low frequency was interpreted by hydration of internal water molecules. These results for bacteriorhodopsin suggest that the helical structure is possible for Jα even after activation of LOV domains, though unfolding of the Jα helix is more consistent with other spectroscopic observations (*36*−*41*).

domain forms an α-helix or not and if the "Jα" domain interacts with LOV2-core. If the Jα helix interacts strongly with the β-sheet region of LOV2-core like other LOV2 domains, then structural changes of the β-sheet region would accompany those of the Jα helix. On this basis, strong binding between the Jα helix and LOV2-core may be excluded, because structural changes of the β-sheet take place (1644 (−)/1626 (+) cm^{-1}), regardless of the presence or absence of the Jα helix. It is reasonable that the "Jα" domain forms an α-helix from amino acid sequences and that dissociation may be possible because of a specific amino acid. Lys1056 in neo1-LOV2 was one of the candidates because a positive charge at the interaction surface might be unfavorable for the interaction in neo1-LOV2. However, differences in FTIR spectra of G597K phot1-LOV2 and K1056G neo1-LOV2 were similar to those of the wild type (Figure 6). Thus, the origin of no influence of the Jα helix in neo1-LOV2 remains elusive. Amino acid residues between the LOV core and the Jα helix are more numerous in *Adiantum* neo1-LOV2 than the LOV2 domains in oat phot1 and *Arabidopsis* phot1 and phot2 (Figure 2b), so that the conformation between the LOV2 core and the Jα helix of *Adiantum* neo1 may be different from that of oat phot1-LOV2, whose X-ray crystal structure has been reported (*20*).

SUPPORTING INFORMATION AVAILABLE

Light-induced difference FTIR spectra of LOV2-Jα of *Arabidopsis* phot1 and phot2 and *Adiantum* neo1 in the 1500−1200 cm^{-1} region at 150 K (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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