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Protein Differences between Two Isogenic Cultivars of Barley (*Hordeum vulgare* L.) that Differ in Sensitivity to Photoperiod and Far-Red Light[1]

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ABSTRACT

A photoperiodically sensitive cultivar of barley (*Hordeum vulgare* L. Shabet) (BMDR-8) and an isogenic, single-gene recessive mutant of this genotype that is insensitive to photoperiod (BMDR-1) were grown under continuous cool white light with or without supplemental far-red fluorescent light. BMDR-1 initiates flowers 6 days after germination, irrespective of light treatment, whereas BMDR-8 remains vegetative for at least a week longer, even in continuous light. When far-red light is added, the delay of flowering in BMDR-8 is overcome and both genotypes initiate floral primordia at the same time. Total phenol extracted proteins of seedlings of both genotypes were resolved by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. No protein differences were found between the genotypes when isoelectric focusing gels were run in the first dimension. Two qualitative genotypic differences were found when nonequilibrium pH gradient gel electrophoresis was run in the first dimension. An 85-kilodalton polypeptide (A) and a 26-kilodalton polypeptide (B) were always present in BMDR-8 but never found in BMDR-1. The levels of A appeared to decrease from the BMDR-8 during the first 3 days of far-red treatment but did not disappear completely even after 6 days of growth in the presence of far-red. Polypeptide B decreases rapidly in continuous cool white light but is stabilized by far-red. The phytochrome content of BMDR-1 was found to be greater than that for BMDR-8. This increase appears to be caused by the type I (etiolated-tissue abundant) phytochrome pool, even in plants grown in continuous light.

Despite the fact that the control of flowering by daylength has been intensively investigated for more than 70 years (13), the fundamental cellular and biochemical basis for this regulation remains unresolved. Nevertheless, photoperiodically-sensitive plants provide an important experimental tool that can be used to manipulate the onset of floral induction. The events that lead to floral induction take place in the leaves (28); however, the consequences of inductive treatments can only be assayed through measurements of the degree of floral transformation at apical and axillary meristems. Even in those instances where very early events in floral evocation, such as the enlargement of the apex or changes in carbohydrate and mitotic activity (2), are used to establish when this transition between vegetative and reproductive development occurs, they are still temporally and spatially separated from the inductive events in the leaves.

Photoperiod sensitivity in barley appears to be controlled by at least four genes at the *Ea* maturity locus (9, 10, 14). The homozygous recessive ea_{sp}, ea_c, ea_k, and ea_7 genotypes located on chromosomes 3, 4, 5, and 6, respectively, individually confer extreme earliness of flowering under short daylengths and relative insensitivity to photoperiod (11). A dominant enhancing allele (*En*) at another locus interacts with the ea_k recessive homozygote to confer even earlier flowering than ea_k alone. Preliminary crosses with BMDR-1 indicate that it may represent a fifth *ea* allele that behaves like ea_{sp} but is nonallelic with it (L.W. Gallagher, personal communication).

The photoperiodic induction of flowering in barley is promoted by the addition of FR[3] light (8), which appears to be mediated by phytochrome (6, 19). The question of whether this promotion is the result of the production of a floral promoter, the suppression of the synthesis of a floral inhibitor, or both, is examined using two isogenic barley genotypes that differ at a single genetic locus. Genotype BMDR-1 is a single-gene recessive mutant that lacks photoperiod sensitivity. It was derived from an M2 diethyl sulfate-treated dwarf genotype, and made isogenic by backcrossing seven times into the cultivar Betzes and twice into the commercial cultivar replacement for Betzes, known as Shabet (BMDR-8), which is a facultative long-day plant (7). This paper presents evidence for qualitative differences in total (soluble and solubilized membrane) proteins between the two genotypes that can be resolved on nonequilibrium pH gradient/SDS-polyacrylamide two-dimensional gel electrophoresis. It also reports an unanticipated difference in the total phytochrome pools of these genotypes.

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[3] Abbreviations: FR, far-red light; CW, cool white; IEF, isoelectric focusing; NEPHGE, nonequilibrium pH gradient gel electrophoresis; TMB, 3,3′,5,5′- tetramethylbenzidine.

MATERIALS AND METHODS

Seed Material

The barley (*Hordeum vulgare* L.) seeds were supplied by Dr. Virgil Smail (Agricultural Utilization Research Institute, Crookston, MN) in 1985 and were derived from a breeding program at the University of Montana. The pedigrees of BMDR-1 (isotype-1) and BMDR-8 (isotype-8), which are two selections of a series of 10 isogenic lines of Shabet barley, are reported in Table 17.1 in Deitzer (7).

Growth Chamber Conditions

Twenty-five seeds were sown on coarse vermiculite in 10-cm plastic containers. Deionized water was added to start imbibition in a light-tight box in a dark room at 20°C for 4 d. On the fourth day, at which point the coleoptiles are about 1 cm above the vermiculite, the plants were transferred to full-strength Hoagland No. 1 solution containing a chelated iron source in growth chambers kept at 20°C under continuous CW light with or without supplemental FR light. The CW light was supplied by GTE-Sylvania F48T12/CW/VHO fluorescent lamps and the FR by GTE-Sylvania F48T12/232/VHO single phosphor fluorescent lamps (GTE Products Corp., Danver, MA). The photosynthetic photon flux (400–700 nm) was maintained at 100 μmol m^{-2} s^{-1} under all conditions and monitored using a Li-Cor Li-190SB Quantum sensor (Li-Cor, Inc., Lincoln, NE). Phytochrome photoequilibria (P_{fr}/P_{tot}) were calculated by multiplying the spectral distribution of the light from 350 to 800 nm, measured using an EG&G Gamma model C-3 spectroradiometer (EG&G Gamma Scientific Instruments, Inc., San Diego, CA) by the *in vivo* quantum yield spectra for P_r and P_{fr} according to the procedure of Gardner and Graceffo (12) after correction for 86% maximal photoconversion. This yields photoequilibria of 74% P_{fr} under CW and 46% P_{fr} under CW + FR.

Floral Development

Beginning on day 0 (at the time of transfer to continuous light), one container of plants was removed from the growth chamber at 9:00 AM each day. The roots and remaining scutellum were removed and discarded and the average weight of the aerial portion of the seedlings was determined. Ten seedlings were chosen that were within 10% of the mean fresh weight. These were then dissected under a dissecting microscope to determine the stage of floral development of the inflorescence (3). Each experiment was repeated at least once ($n = 20$–30). The time of floral initiation (floral stage 2 defined as the presence of double ridges on the shoot apex) was used to define the time prior to which leaf samples should be taken for the protein analysis.

Protein Extraction and Two-Dimensional Gel Electrophoresis

For each sample, 2 g of seedling leaf tissue was ground in a mortar and pestle with liquid nitrogen. The powdered, frozen tissue was transferred to a ground glass Tenbroeck tissue grinder and total protein was extracted according to Slovin and Tobin (27), except that 2% Triton X-100 was added to the extraction buffer (10 mM Tris-HCl at pH 7.4, 1% 2-mercaptoethanol and 1 mM PMSF) to solubilize membrane-associated proteins. After centrifugation, 2 M sucrose was added to the supernatant to increase the density, and buffer-saturated phenol containing 2 M sucrose was used to separate proteins from nucleic acids (17). The protein was precipitated from the phenol phase with ammonium acetate in methanol as described by Hurkman and Tanaka (18). After centrifugation at 20,000 *g* for 15 min, the pellet was resuspended in lysis buffer (9.5 M urea, 2% Triton X-100, 5% 2-mercaptoethanol) containing 2% ampholytes at a 1:4 ratio of ampholytes, pH 3.5 to 10 and 4 to 8, respectively (24). The samples were stored at −70°C.

IEF gel electrophoresis was performed according to O'Farrell (24). The gels were prerun at 200 V for 15 min, 300 V for 30 min, and 400 V for 30 min to set up a pH gradient. Approximately 100 μg protein was loaded onto each IEF gel, and the gels were electrophoresed at 400 V for 16 h and 800 V for 1 h. NEPHGE was performed according to O'Farrell *et al.* (25). The conditions used for electrophoresis were 300 V for 1 h and then 400 V for 4 h. Both IEF and NEPHGE gels were equilibrated in SDS buffer for 30 min and then stored at −70°C. The second dimension was SDS-PAGE using 12% acrylamide (24). Both the first and second dimension were electrophoresed at 12°C. When the second dimension was complete, the two-dimensional gels were fixed in 50% methanol and silver stained according to Wray *et al.* (30).

Computer Analysis

The computer analysis, utilizing a Grinnell 270 image processor hosted by a Hewlett-Packard 1000F computer, has been described by Hruschka *et al.* (16). Color slides of the gels were viewed by a videcon camera, digitized, and summed. The images were smoothed by Gaussian filter and the background was removed with a Laplacian filter. Two images to be compared were displayed on a color monitor, one in red and the other in green. For each spot, the green image was scrolled over the red so that the matching spots appeared in yellow and the pair was marked. A two-dimensional quadratic least-squares polynomial was calculated between the corresponding spot locations and applied to all green pixels to bring them into registration with the red image. In the resulting image, common spots appear bright yellow, whereas unique spots were red or green, allowing quick identification of gel similarities and differences.

Phytochrome Measurements

For the spectrophotometric analysis of phytochrome concentration *in vivo,* seeds of BMDR-1 and BMDR-8 were germinated and allowed to grow in the dark for 7 d. The tissue was harvested under dim green light. Ten shoots (coleoptile + leaves), 100 coleoptile tips, or 20 whole coleoptiles were chopped into 5-mm pieces and packed into a vertical aluminum cuvette on ice. Mean phytochrome (P_{tot}) levels were measured using a custom-built Ratiospect as the difference between the absorbance at 660 ± 2.5 and 730 ± 2.5 nm.

Because phytochrome cannot be measured spectrophoto-

metrically in green tissue, a set of samples harvested from seedlings grown for 3 d under continuous CW, CW + FR, or exposed to supplemental FR for only a 6-h period, were lyophilized, ground to a fine powder, and stored in air-tight containers at −20°C for extraction. The samples were homogenized under dim green light in a cold room at 4°C in 15 mL g^{-1} of extraction buffer (50 mM Tris-HCl, 14 mM 2-mercaptoethanol at pH 7.8). The homogenate was filtered through two layers of Miracloth and centrifuged at 13,000*g* for 30 min at 4°C. Immediately following homogenization, the samples were assayed for phytochrome by means of ELISA according to Hilton and Thomas (15). The plates were coated for 2 h at 25°C with 50 μL/well of a polyclonal anti-phytochrome rabbit antibody (39/6) at a concentration of 4 mg L^{-1} in 50 mM sodium carbonate buffer at pH 9.6. After the coating was removed, the plates were blocked with 200 μL/well of 2% (w/v) BSA in PBS buffer (20 mM phosphate buffer, 150 mM NaCl at pH 7.4) overnight at 4°C. Samples were applied at a range of dilutions in a 1% BSA (w/v) solution in PBS with 0.5% (v/v) Tween-20, and 50 μL/well of antigen solution was incubated on the plates for 4 h at 4°C. After this, 50 μg/well of a rat monoclonal antibody (MAC 56), which is specific for type I phytochrome (15), was added at a concentration of 5 mg L^{-1} in BSA-PBS-Tween-20. The plates were incubated overnight at 4°C and then 50 μL/well of goat anti-rat antibody, labeled with horseradish peroxidase (ICN Biomedicals, High Wycombe, UK), was applied to the plates at a dilution of 1:300 (v/v) in BSA-PBS-Tween-20 and incubated for 2 h at 25°C. TMB was diluted to a concentration of 10 g L^{-1} in DMSO and 100 μL of the TMB solution, plus 10 μL of H_2O_2, were diluted in 10 mL of acetate buffer (100 mM sodium acetate adjusted to pH 6.0 with citric acid); 100 μL/well of this diluted solution was added to the plates. The reaction was stopped after 30 min with 25 μL/well of 2 M H_2SO_4 and the plate was then read at 450 nm using a Bio-Rad EIA Reader.

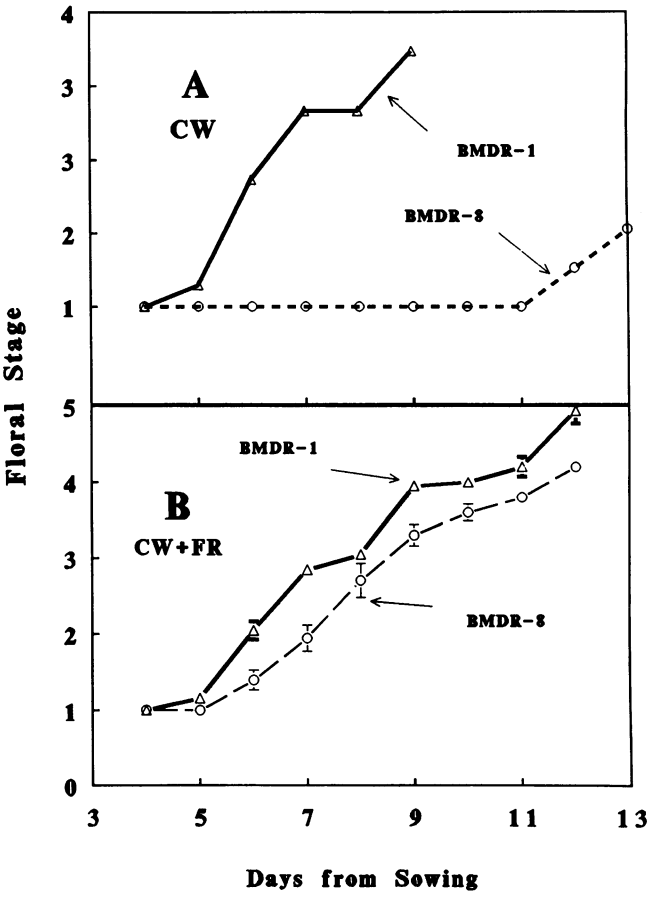

Figure 1. Time course of floral initiation and development in BMDR-1 (△) and BMDR-8 (○) grown in continuous CW light (A) alone or in continuous CW + FR light (B). Day 0 is the time of transfer from the dark to the light treatment.

RESULTS

Rate of Floral Development

In continuous CW light, BMDR-1 flowers very rapidly, reaching floral stage 2 (double ridges) by day 6. However, BMDR-8 remains vegetative until day 13 (Fig. 1A). When FR is added to the CW light, BMDR-1 flowers as rapidly as it did in continuous CW alone but BMDR-8 now flowers almost as rapidly as BMDR-1 (Fig. 1B), reaching floral stage 2 by day 7. Each point represents the mean of two experiments with 10 dissections per harvest (n = 20). There was no difference in accumulation of dry weight between BMDR-1 and BMDR-8 as a result of adding FR to the CW light (Fig. 2), so there was no significant effect of FR on photosynthesis during these experiments.

Gel Electrophoresis

On the IEF gels, there was minimal streaking with very little background, and the pH gradient ranged from 4.5 to 7.1. There were over 300 polypeptides resolved on the gels, with molecular masses ranging from 14 to over 97 kD. No differences were found on gels of BMDR-1 under either light treatment (data not shown). This was also the case for BMDR-8, where no differences in the protein patterns were seen between gels of vegetative and induced plants. Furthermore, the gels of BMDR-8 were identical to gels of BMDR-1. Thus, at this level of resolution, no differences were found between the genotypes or between light treatments on IEF gels.

The NEPHGE gels (Fig. 3) contained a pH gradient ranging from 4.6 to 8.3 with highly repeatable patterns. Gels of extracts from days 4 to 6 had a large amount of streaking due to the large subunit of ribulose 1,5-bisphosphate carboxylase, which made computer analysis difficult on these gels. No qualitative differences were found between gels of BMDR-1 in the two light treatments. There were also no qualitative differences found between gels of BMDR-8 in the light treatments. However, when gels of BMDR-8 (Fig. 3A) were compared with gels of BMDR-1 (Fig. 3B), two qualitative differences were found. Polypeptide A, a high molecular mass polypeptide (85 kD), was always present in BMDR-8 (Fig. 3A), even prior to the transfer to light. Although difficult to quantify, polypeptide A does appear to decrease in amount from darkness through the first 3 d in continuous CW + FR light (Fig. 4, A–D). Nevertheless, polypeptide A was still present on day 4, which is 2 d before floral initiation takes

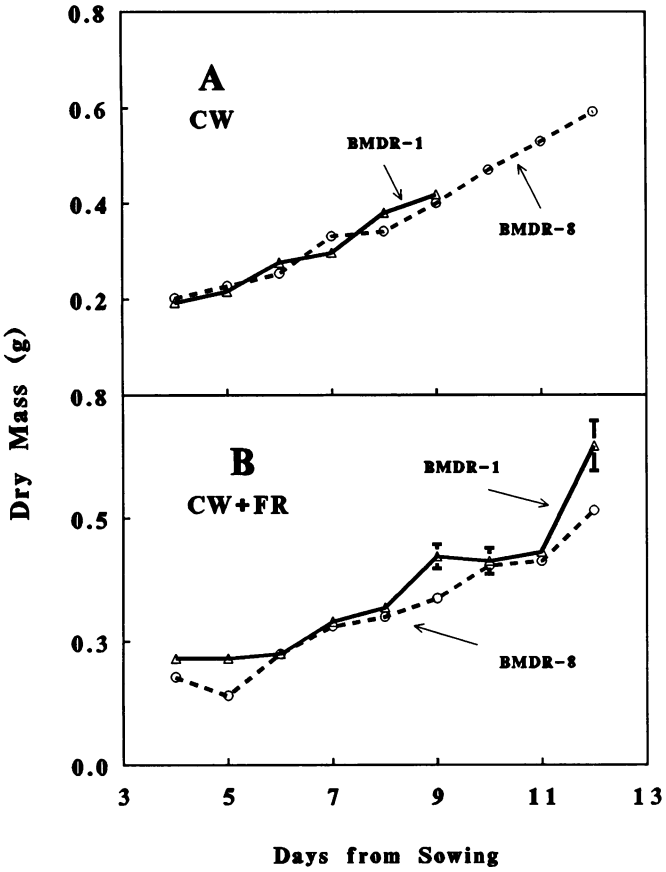

Figure 2. Time course of dry-mass increase in BMDR-1 (△) and BMDR-8 (○) grown in continuous CW light (A) alone and continuous CW + FR light (B). Day 0 is the time of transfer from the dark to the light treatment.

place at the apex (Fig. 1B). On days 5 and 6, that area on the gels becomes obscured by vertical streaking (data not shown) but the spot appears to be present. Polypeptide A was never seen in BMDR-1 (Fig. 3B).

A smaller polypeptide (polypeptide B in Fig. 3) with a molecular mass of 26 kD was also found in BMDR-8 (Fig. 3A) at all times, including darkness (Fig. 5A). This polypeptide was also never found in BMDR-1 (Fig. 3B and Fig. 5, B and F). It was still present on gels of extracts of BMDR-8 from days 4 to 6 in CW + FR (data not shown), but was too faint under CW alone to establish a quantitative change in intensity. However, this band decreases in intensity during the first 2 d in BMDR-8 plants grown under CW conditions alone (Fig. 5, C and D) but remains at a stable high level when BMDR-8 is grown under CW + FR (Fig. 5E).

Computer analysis identified only polypeptide B as a qualitative difference between BMDR-1 and BMDR-8. Because polypeptide A, a very small spot, was closely surrounded by larger spots, it was not initially detected by computer analysis. However, when the area around polypeptide A was magnified on the monitor, as shown in Figure 4, the polypeptide difference between the genotypes was then identified and confirmed.

Phytochrome Measurements

Table I shows the relative amounts of spectrophotometrically detectable phytochrome in BMDR-1 and BMDR-8 measured *in vivo* in etiolated barley seedlings. Due to different amounts of tissue in the different samples, the phytochrome levels between tissue types cannot be compared. However, in all of the samples measured, BMDR-1 contained 55 to 62% more spectrophotometrically detectable phytochrome than BMDR-8. This difference has been confirmed by ELISA analysis (Table II) using monoclonal and polyclonal antibodies raised against oat phytochrome. Here the increase appears to be on the order of twofold, but the absolute difference cannot be determined due to the nonlinearity of the ELISA color reaction. Interestingly, the addition of FR appears to cause an additional increase of 15 to 25% in both genotypes, even when only added for 6 h during the 72-h period, but the increase does not depend on when, or for how long, during this period the FR is added. Because the ELISA test utilized a type I-specific monoclonal antibody, these increases appear

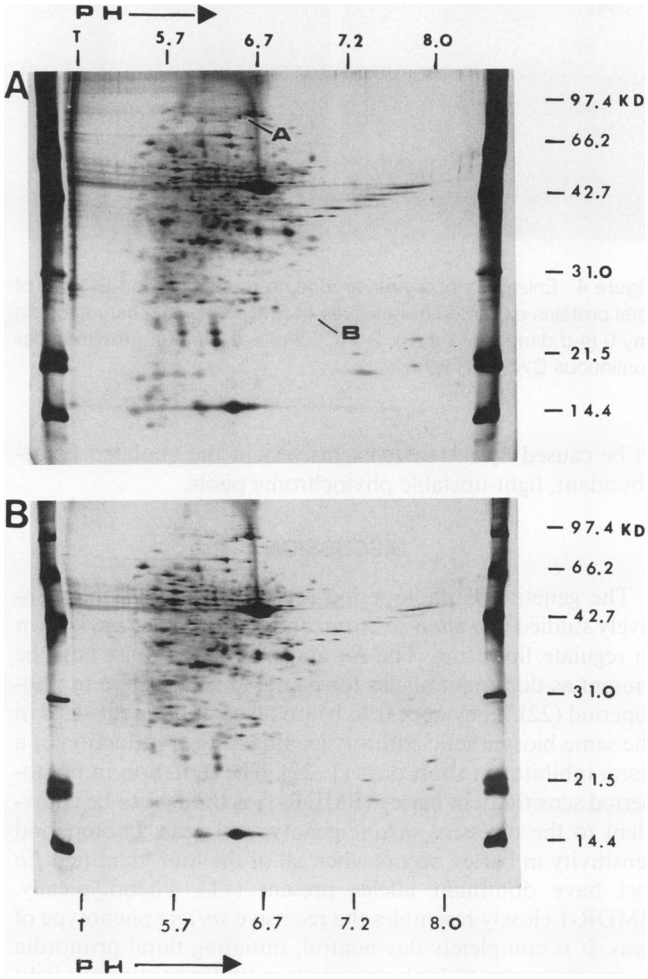

Figure 3. Representative two-dimensional NEPHGE separations of total proteins extracted from leaves of BMDR-8 (A) and BMDR-1 (B). Samples were taken on day 2 in continuous CW + FR light. Top of first dimension NEPHGE gel (T), pH gradient run in acidic to basic direction.

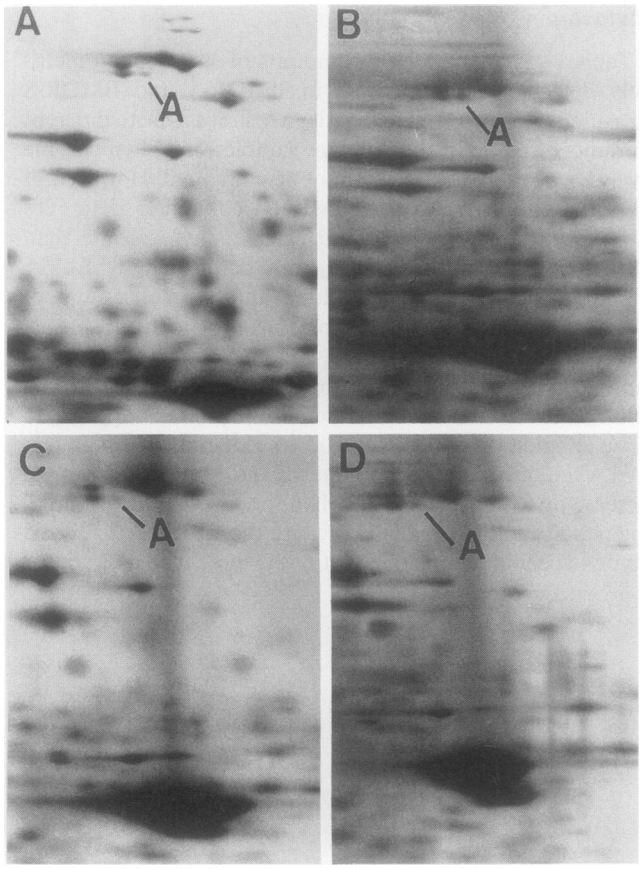

Figure 4. Enlargement of area around polypeptide A on NEPHGE of total proteins extracted from leaves of BMDR-8 plants harvested on day 0 (4-d dark) (A), 1-d (B), 2-d (C), and 3-d (D) after growth under continuous CW + FR light.

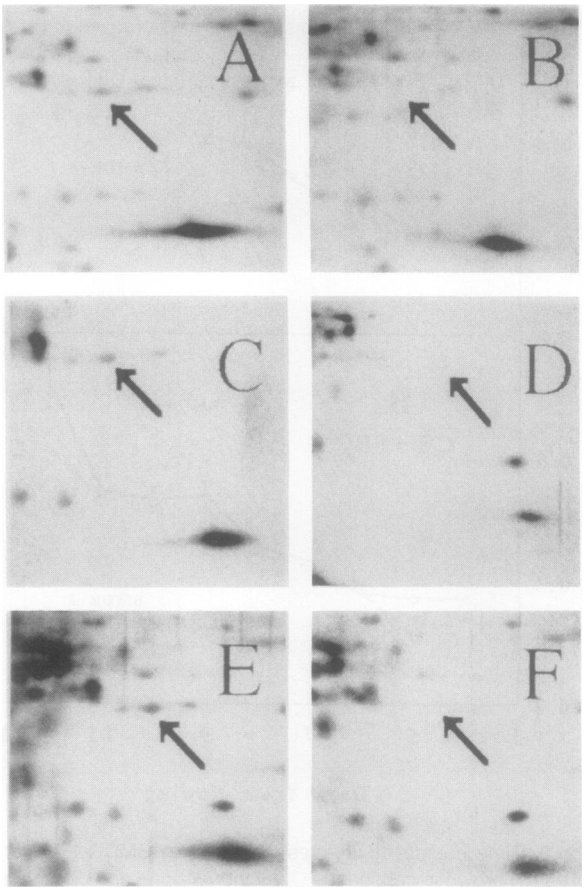

Figure 5. Enlargement of area around polypeptide B on NEPHGE of total proteins extracted from leaves of both BMDR-8 (A, C, D, E) and BMDR-1 (B, F) grown for 4 d in darkness (A, B), 4 d dark + 1 d CW (C), 4 d dark + 2 d CW (D), 4 d dark + 2 d CW + FR (E). Arrows indicate the positions of the 26-kD polypeptide B.

to be caused by increases exclusively in the etiolated tissue-abundant, light-unstable phytochrome pools.

DISCUSSION

The genetics of photoperiod sensitivity have been extensively studied in *Pisum sativum,* and several genes are known to regulate flowering. The *Sn* and *Dne* genes must both be present as dominant alleles for plants to be sensitive to photoperiod (22). They appear to be involved at different steps in the same biosynthetic pathway leading to the production of a floral inhibitor in short days (1, 22). The mutation in photoperiod sensitivity in barley (BMDR-1) is thought to be equivalent to the recessive *sn/dne* genotype of peas. Photoperiod sensitivity in barley occurs when all of the four identified *Ea* loci have dominant alleles present (11). Phenotypically, BMDR-1 closely resembles the recessive *sn/dne* phenotype of peas. It is completely day-neutral, initiating floral primordia as rapidly under 12-h photoperiods as under continuous light (7). The parental genotype BMDR-8 behaves exactly like the dominant *Sn/Dne* phenotype of peas, which is strongly inhibited by short daylengths. This inhibition is overcome in both by extended daylengths and the addition of FR light. We suggest that the mutation in BMDR-1 occurs at one of the

Table I. *In Vivo Measurements of Total Phytochrome in Etiolated BMDR-8 and BMDR-1 Barley Tissues*

Six-day-old barley seedlings were germinated and grown in complete darkness and harvested under a dim green safe light. Samples consisted of whole shoots (coleoptile + leaves), 5-mm tips of coleoptiles, or whole coleoptiles from which the leaves had been removed. All tissues were cut into 5-mm pieces and placed in cylindrical aluminum cuvettes. Measurements were made by vertical irradiation in a custom-built Ratiospect using 660 and 730 nm (± 2.5 nm) actinic and measuring wavelengths, respectively.

Tissue	Genotype	$P_{tot}(\Delta\Delta A)$	±SE
Shoots	BMDR-8	15.28	0.43
	BMDR-1	27.65	0.46
Coleoptile (tips)	BMDR-8	24.65	0.32
	BMDR-1	45.22	0.17
Coleoptile (whole)	BMDR-8	10.16	0.01
	BMDR-1	16.22	0.20

Table II. *Phytochrome Measurements by ELISA in Light-Grown Barley Leaves*

Tissue was harvested in the light after various treatments, lyophilized, and ground to a powder before extraction. ELISA tests were performed using a monoclonal anti-phytochrome antibody from oat that is specific for type I phytochrome (MAC 56) (15) and visualized as the color reaction produced by a horseradish peroxidase, linked to a monoclonal antibody, developed with TMB, and read at 450 nm with a Bio-Rad EIA Reader. Values are mean ± SE.

Genotype	72 h − FR	72 h + FR	6 h + FR hour 3–9	6 h + FR hour 15–21
BMDR-1	0.262 ± 0.02	0.324 ± 0.04	0.333 ± 0.03	0.333 ± 0.04
BMDR-8	0.133 ± 0.003	0.153 ± 0.004	0.156 ± 0.004	0.148 ± 0.003

Ea loci in barley and that the consequence of this mutation is the loss of the ability to produce a floral inhibitor under noninductive conditions, as appears to be the case in peas (1, 22). We further suggest that the synthesis of this putative floral inhibitor is regulated by phytochrome and decreased by FR light. Addition of FR to continuous CW causes BMDR-8 to reach floral stage 2 much earlier than with continuous CW alone. However, BMDR-8 lags behind BMDR-1 by approximately 24 h, even under continuous CW with FR light. It is possible that BMDR-8 is not yet able to respond to FR when it is first transferred to the light. Alternatively, it may take some period of time to remove any floral inhibitor that may have been present in the dark.

Numerous attempts have been made to isolate a floral stimulus (either promotory or inhibitory). Extracts of phloem sap from *Xanthium* produced an active substance, which was identified as salicylic acid, that caused flowering in *Lemna gibba* (5). Recently, Chailakhyan *et al.* (4) prepared extracts from leaves of flowering Maryland Mammoth tobacco plants which, when added to terminal apices of *Chenopodium rubrum,* caused them to flower under noninductive daylengths. Both the phloem sap and tobacco leaf extracts cause flowering in bioassays but have not been shown to cause flowering in the host plants from which the extracts were prepared. Differences at the protein level have been found between induced and noninduced leaves of a number of species. Kohli *et al.* (20) found two new proteins in *Amaranthus* that appeared at the same time that inflorescence primordia were initiated. Under noninductive treatments, these proteins were not found. Warm (29), using *in vitro* translation of isolated mRNA and two-dimensional gel electrophoresis, found both qualitative and quantitative differences in translation products between induced and noninduced *Hyoscyamus* leaves. However, because different photoperiods were used in both of these comparisons, it is difficult to conclude that the changes were specifically related to floral induction. Lay Yee *et al.* (21) examined translation products of *Pharbitis nil* cotyledons and, using a 10-min night-break with red light to inhibit flowering, found only one detectable quantitative difference between induced and noninduced plants. A 28-kD polypeptide was present at a higher concentration in induced plants than in the night-break controls. O'Neill (26) cloned cDNAs from *P. nil* cotyledons whose mRNA expression changes during inductive short days. One cDNA is quantitatively down-regulated, and two other cDNAs appear to be quantitatively up-regulated under inductive conditions. However, no qualitative differences were found at this level of screening.

In the experiments reported here, two isogenic lines that differ from one another at only one locus were compared under the same treatments to minimize the number of nonspecific differences. Two qualitative differences were found between the two genotypes. If flowering is promoted by the suppression of a gene coding for a floral inhibitor, BMDR-1 would be expected to be lacking such a functional gene. The product of this gene in BMDR-8 would be expected to decrease or disappear under inductive conditions (*i.e.* continuous CW + FR light). Polypeptide A appeared to follow this general hypothesis, but it did not disappear completely. Thus, although this polypeptide occurs only in the photoperiodically-sensitive cultivar, it may not be directly related to floral induction. However, it is possible that the messenger RNA for this polypeptide is no longer produced when FR is added to the CW conditions but the protein is relatively stable. Such a polypeptide would still be visualized on the two-dimensional gels of total protein extracts. *In vitro* translation experiments are currently being investigated to determine if the mRNA is regulated by the FR light.

Polypeptide B, although too weak to establish quantitative relationships with certainty, does appear to be stabilized by FR light. This could be a candidate for a floral promoter except that it is never found in BMDR-1. It may be that, in the absence of an inhibitor, a very low level of promoter is required for induction that is below the level of resolution on these gels. It should be stressed that these differences represent only correlations of proteins that can be resolved on SDS-PAGE with the two genotypes examined, and may not represent differences that are directly related to the sequence of events leading to flowering. Neither polypeptide behaves as would be expected during the FR promotion of flowering, but both appear to be affected by the FR. Neither polypeptide A nor B could be resolved on IEF gels, which emphasizes the necessity for carrying out both types of electrophoresis when analyzing for differences in total protein extracts.

A newly described mutant in pea, which has a phenotype reminiscent of the Maryland Mammoth strain of tobacco, flowers very late under 8-h photoperiods, producing extremely large (>4 m) plants (23). This mutant, which has been called *gigas,* behaves very strangely when transferred to long photoperiods. It flowers earlier under 18-h photoperiods, although still much later than the wild type. However, when returned to 24-h photoperiods, it flowers transiently and then reverts indefinitely to the vegetative condition. It appears that some

substance essential for flowering is deficient in these plants. Thus, there is genetic evidence for both floral promoting and floral inhibiting substances that are required for flowering in the same plant. There are no polypeptides that have been found in BMDR-1 that are absent in BMDR-8, but the 26-kD polypeptide (B) does appear to be more strongly expressed in BMDR-8 when FR is added (Fig. 5E). Unfortunately, as mentioned above, this spot is too weak to resolve quantitatively, so no speculation can be made about its role in the synthesis of a floral promoter. It is certainly possible that both floral promoters and inhibitors are involved in floral induction, and their levels may be coordinately regulated in opposite directions. A single mutation in the synthesis of one could thereby affect the levels of both.

In vivo spectrophotometric phytochrome measurements and *in vitro* ELISA analysis of the phytochrome protein were made in BMDR-1 and BMDR-8 to determine if BMDR-1 contains significantly less phytochrome than BMDR-8. This could explain the lack of FR control of floral induction in BMDR-1. However, BMDR-1 appears to contain more, rather than less, phytochrome than BMDR-8. Although these results were somewhat surprising, they do show that photoperiod insensitivity in BMDR-1 does not appear to be caused by a decreased level of phytochrome. Although the ELISA results show that the increase in spectrophotometrically detectable phytochrome can be accounted for entirely on the basis of increases in the etiolated type I phytochrome pool, we cannot rule out the possibility that there may be differences between the etiolated and green tissue-abundant phytochrome pools. The spectrophotometric test cannot distinguish these pools and the ELISA used only monoclonal antibodies raised against the etiolated type I phytochrome. Further studies require use of monoclonal antibodies specific for both types of phytochrome. Differences between these two pools of phytochrome might account for the increase in P_{tot} in response to FR that was detected by ELISA. However, it is unlikely that this response can account for the difference in sensitivity, because both genotypes show the same amount of increase.

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