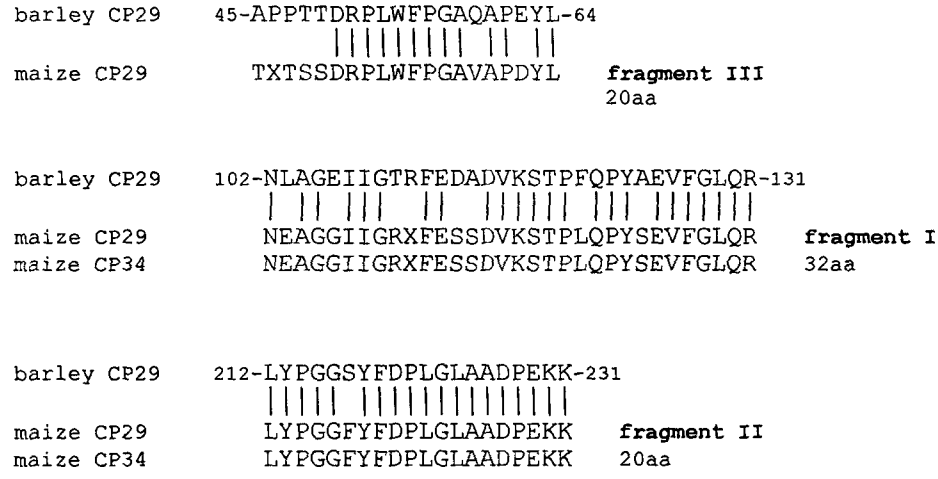

FIG. 2. **N-terminal sequences of tryptic fragments from digestions of CP29 and CP34 apoproteins.** The alignment was obtained with the FASTA program. *Vertical bars* stress amino acid identities. Amino acid positions on the primary sequence of the barley protein (20) are indicated. *X*, undetermined amino acid.

In Vivo Labeling of Thylakoid Proteins with [^{35}S]Methionine—In the light of the above results, we could then address questions on the biogenesis of CP34, *i.e.* whether it was (*a*) the product of an alternative splicing of the *lhcb4* gene transcript, (*b*) the unprocessed precursor of CP29, or (*c*) a post-translationally modified CP29. The first two hypotheses imply that the cold-induced protein is synthesized *de novo* following cold stress, while a post-translational modification would act on a protein already existing in the thylakoids.

To test these possibilities, we examined the patterns of thylakoid proteins synthesized at 25 and 4 °C in the presence of light. We labeled plastid proteins *in vivo* by feeding [^{35}S]methionine to seedlings. Maize seedlings, loaded with [^{35}S]methionine for 3 h at 25 °C, were then incubated for 6 h at either 25 or 4 °C under 200 μE m^{-2} s^{-1} illumination. Following this treatment, thylakoids were isolated from mesophyll chloroplasts, and their polypeptide content was analyzed by two-dimensional gel electrophoresis and autoradiography. Membranes were first fractionated on non-denaturing Deriphat-PAGE (13, 21) and then by SDS-urea-PAGE in the second dimension. This combined electrophoretic system allowed a clear-cut separation of CP29 from the D1 apoprotein, a PSII core component comigrating with CP29, which is known to have a very fast turnover, and was therefore expected to be heavily labeled (22, 23). As for their amino acid content, CP29 and D1 proteins, respectively, contain 3 and 12 methionines in their primary sequences in barley (20, 24, 25).

Fig. 3, *a* and *b*, show the results of this analysis. In particular, it was clear that the chilling treatment did not cause *de novo* synthesis of the CP34 protein, since the labeling of this polypeptide was as low as that of CP29 on a protein quantity basis, as determined by densitometry of gels and relative autoradiographs. The low [^{35}S]methionine incorporation in CP34 following induction by cold shows that this polypeptide is neither the precursor of CP29 nor the product of an alternative splicing of the *lhcb4* maize messenger, since both cases imply *de novo* synthesis and consequent incorporation of radioactivity.

The autoradiographic pattern was very similar in chilled and control samples. However, incorporation in all of the polypeptides was lower in the cold-exposed plants as a result of the effect of low temperature on protein synthesis (26, 27). The D1 protein is an exception to this rule; its labeling was higher in the cold-treated membranes, consistent with the photoinhibitory conditions applied (28, 29).

In Vivo Labeling of Thylakoid Proteins with $H_3^{33}PO_4$—We then checked the remaining hypothesis, aiming at identifying the specific post-translational modification of CP29. It has been already reported that the cold-induced polypeptide is not glycosylated (7). Moreover, the putative modification appeared to be rapidly inducible and reversible. This was shown by the rate of appearance of the 34-kDa band on exposing leaves to chilling and high light,[2] as well as the rate of degradation of the polypeptide on returning leaves to 25 °C (7) (Fig. 1). These kinetics are consistent with a phosphorylation-dephosphorylation mechanism that could also explain the electrophoretic upshift, a behavior common to other phosphoproteins (30). *In vitro* phosphorylation of the purified, non-denatured form of the CP29 protein by various kinases was not successful (not shown). We then proceeded to *in vivo* labeling of maize leaves with $H_3^{33}PO_4$. The radioactive phosphate was fed to excised seedlings as described for [^{35}S]methionine. Seedlings were then exposed to chilling treatment in the light, and thylakoid membranes were analyzed by two-dimensional PAGE and autoradiography. The results are shown in Fig. 3*c*; CP34, but not CP29, was heavily labeled. The two-dimensional analysis allowed a sharp distinction between CP34 and the phosphorylated form of the D2 apoprotein, which comigrate in one-dimensional PAGE.

In the autoradiograph, all of the known major thylakoid phosphoproteins (31, 32) could be identified: the light-harvesting chlorophyll *a*/*b* proteins (three between 28 and 30 kDa) (8) and the four proteins of the PSII core CP43 (43 kDa), D2 (34 kDa), D1 (32 kDa), and the 9-kDa product of the *psbH* gene (not visible in the figure but evident at the lower edge of the gel in the original film). Apart from CP34, the autoradiographic pattern of control and cold-treated samples also differed with respect to the relative intensity of the labeling of the other subunits; LHCII apoproteins were more heavily phosphorylated in the cold-treated samples by a factor of 2.5, as determined by densitometry of the film. Moreover, labeling of the PSII core subunits CP43, D1, D2, and 9 kDa was barely detectable in the control sample, while it was very strong in the cold-treated one. It is worth noting that only the PSII core subunits deriving from the monomeric form of the complex are phosphorylated, while those associated to the dimeric form are not.

When we performed the above experiment with maize lines, which produce very little or any CP34 upon cold stress in the light, essentially no differences were observed in the phosphorylation pattern apart from the lower labeling of the CP34 band (not shown).

Limited Proteolysis of CP29 and CP34 in Destacked Thylakoids—Which is the effect of the phosphorylation on CP29? Protein phosphorylation has been shown to be one of the most general mechanisms in the regulation of protein function