MOLECULAR BIOLOGY IN SUPPORT OF AN OLD PARADIGM ABOUT THE INDUCTION OF FLOWERING

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To make a long story short

A still unsolved and important question in the field of flowering is whether floral induction in the leaf of photoperiodic species requires the expression of specific genes or not. This question is of course related to the doubts that persist about the nature of the leaf-generated floral stimulus (1). Hence, molecular analysis can be hardly targeted. Most studies concern the whole population of transcripts in the induced organs - leaves or cotyledons - and are based on the comparison of induced with non-induced or inhibited plants. The primary approach was to analyse the transcript population by 2D-PAGE of their *in vitro* translated products. The most detailed analyses concluded that photoperiodic induction results in quantitative changes only (4,13,18). Molecular cloning opened a new perspective but, despite several reports of investigations in progress (3,11,16), published results are still scarce. The lack of major advances in unravelling the molecular processes of floral induction was recently discussed (15). O'Neill and coworkers (17, 30) found by differential screening of cDNA libraries several clones whose expression was affected by the photoperiod in *Pharbitis nil*, but none in an hit or miss manner. Two clones of unknown function could be related to flowering since their expression increased in inductive conditions and was repressed when flowering was inhibited by a night break.

Lolium temulentum Ceres is regarded as a model photoperiodic monocot since it responds to a single long day (LD) even if the photoperiod extension is given at very low energy (reviewed in 6). Moreover, the leaf area can be reduced without any detrimental effect on flowering, which allows to work with unifoliated plants and, consequently, to harvest homogeneous leaf material. This strain thus seemed to offer significant advantages for the investigation of molecular processes in floral induction and the strategy described above was then undertaken. Since *L. temulentum* has been extensively studied in Canberra by Evans and co-workers, their experimental system was adopted; it consists in inducing 45-d old plants grown in 8-h short days (SD), reduced to leaf 6, with one 24-h LD whose extension is given with only incandescent light at low energy (Fig. 1). The analysis of the leaf Poly(A)$^+$RNA complement by 2D-PAGE did not detect any qualitative effect of the LD induction (paper in preparation) and was thus consistent with previous studies on *Sinapis alba* (4) and *Arabidopsis thaliana* ecotype Eil-O (13). Interestingly, most RNAs whose relative amount changed in LD followed a rhythmic pattern during a 24-h cycle in the SD regime, emphasizing the importance of the biological clock. It had been previously observed that the flowering response to light was driven by a circadian rhythm of sensitivity in *L. temulentum*, on the basis of which flowering could be induced by an 8-h SD given at an unusual time, called a 'displaced short day' (DSD) (19). However, changes in the Poly(A)$^+$RNA complement were too numerous - 50 spots were affected by the photoperiod - to target precisely further investigation. At that point, two options came to mind : either to construct a subtractive cDNA library to get rid of unspecific changes that had probably been picked up in the preceding procedure, or to

analyse the expression of known genes. Because of the tedious aspect of looking for something nobody has any idea about, the second option seemed more promising since the physiology of flowering of *L. temulentum* was deeply studied. Results shown here concern Rubisco activase and nitrate reductase : the steady-state level of their transcripts was analysed in both inductive systems - the LD and the DSD (Fig. 1) - on Northern and dot blots.

Rubisco activase (RCA-mRNA)

In the SD regime, the maximum amount of RCA-mRNA was found at the middle of the light period, and the minimum value was recorded during the night (Fig. 2). In between, the fluctuation was not superimposed to the light-on and light-off signals : the increasing phase extended from midnight to noon and *vice versa*. This timing thus suggested that an endogenous rhythmicity controlled the steady-state level of RCA-mRNA as it was confirmed in a free running 40-h period of darkness : the rhythm continued without any environmental clue with a periodicity that was shortly less than 24 h (not shown). When plants were exposed to a 24-h LD, little change was observed during the photoperiod extension but high levels were maintained throughout the following day, instead of decreasing after noon as it did in SD. When plants were exposed to an 8-h DSD, the steady-state level of RCA-mRNA increased continuously during the light period of the DSD and decreased thereafter, with a shoulder around 28-32 h that could be the manifestation of the endogenous rhythm. Thus the common feature between the LD and the DSD was that the steady-state level of RCA-mRNA increased continuously during the 8-h light period given **after** the LD and **during** the DSD, while it peaked at the middle of the light period in the SD regime.

Nitrate reductase (NR-mRNA)

Results shown in Fig. 3 are Northern blot autoradiograms because the signal obtained with the NR-cDNA probe was too weak to be reliably quantified by radioactivity counting. Several experiments were conducted and gave consistent data, of which results of Fig. 3 are representative.

The relative amount of NR-mRNA increased during the night and was very low during the light period in SD (Fig. 3A). This fluctuation was due to an endogenous rhythm since it continued in protracted dark-

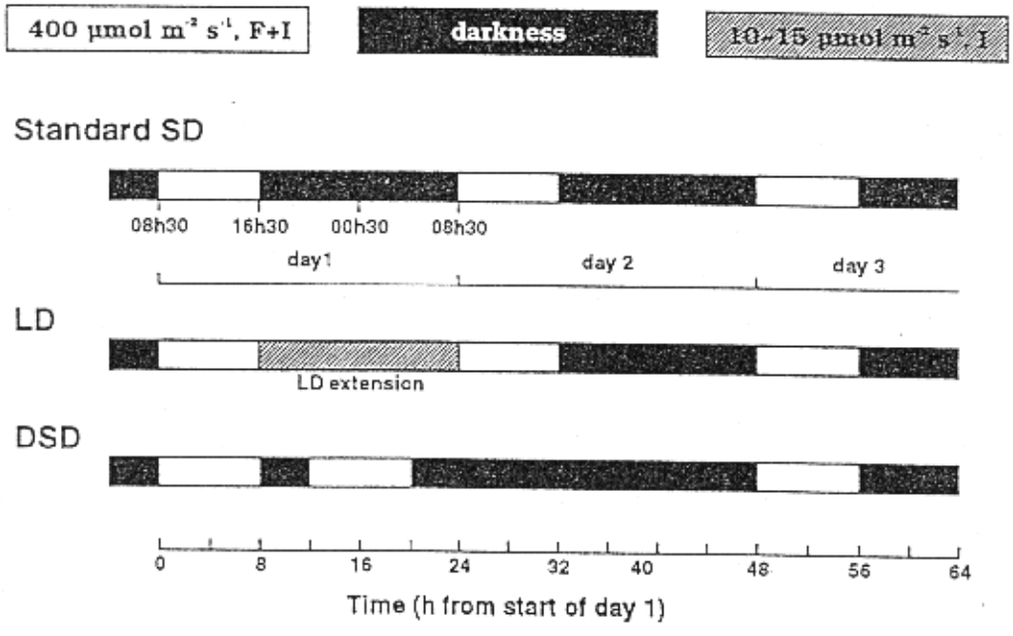

Figure 1. Experimental systems used in *Lolium temulentum* Ceres. (F = fluorescence, I = incandescence).

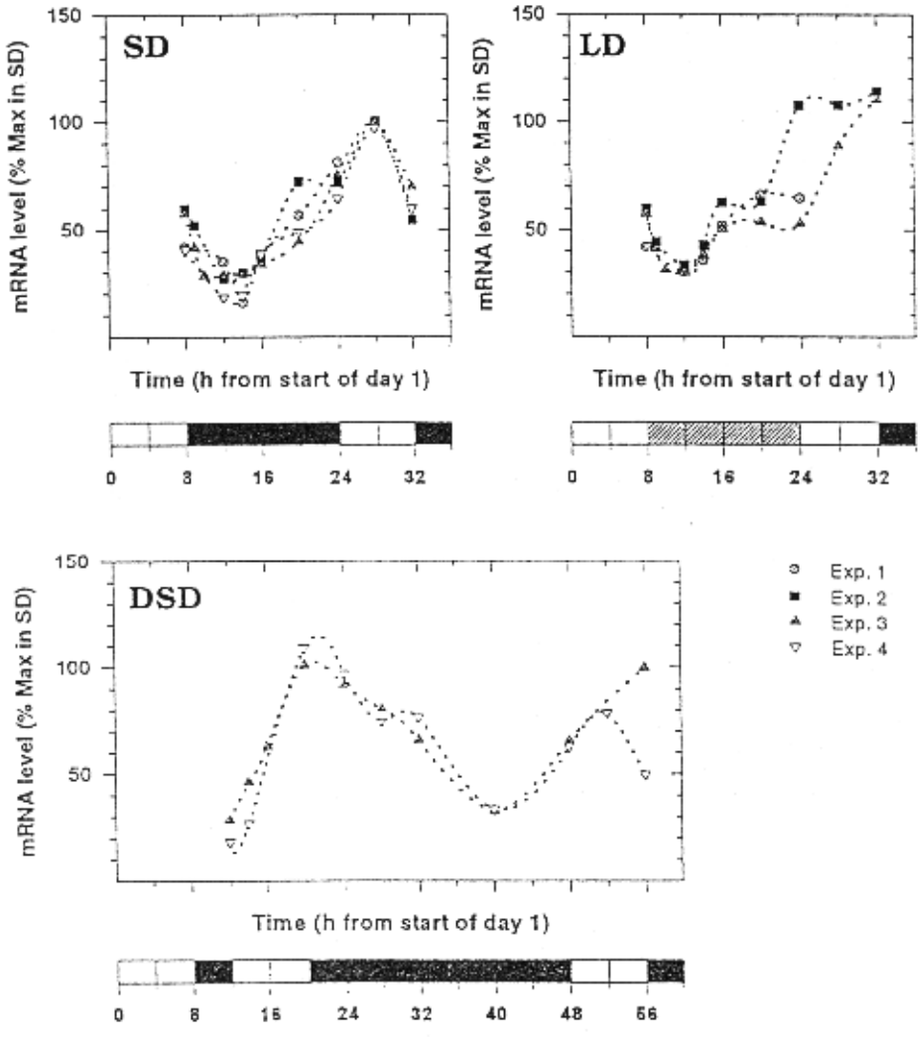

Figure 2. Steady-state level of RCA-mRNA in leaf 6 of *L. temulentum* in different light regimes. 2-µg total RNA samples were dotted onto a nylon membrane, and hybridized with ^{32}P-labelled *Eco*RI insert of pSD1 clone, encoding RCA in apple-tree and kindly supplied by Dr. B. Watillon, Faculté des Sciences agronomiques, Gembloux, Belgium. Last wash = 0.1x SSPE, 40°C. On each membrane, LD or DSD samples were dotted with control SD-samples so that the radioactivity counts could be reported to the maximum found in SD. Triplicate blots were prepared, results shown are the average values.

ness (result not shown). During the LD, NR-mRNA accumulation was decreased under the incandescent extension of the photoperiod compared to darkness (SD) but, on the opposite, the steady-state level remained as high or was even higher during the day that followed the extension, while it decreased strongly in the SD regime. Similarly in the DSD system (Fig. 3B), the relative amount of NR-mRNA increased during and after the light period of the DSD. Thus the common feature between the LD and the DSD was that the steady-state level of NR-mRNA remained high **after** the LD and **during** the DSD, while it was very low under the same light conditions in the SD-regime.

Symptoms of NR deficiency

The steady-state level of NR-mRNA was shown to be negatively controlled by some reduced N-metabolite(s), possibly glutamine (5, 28). NR-mRNA accumulation after the LD, as well as during the DSD, could thus indicate a decrease in NR activity (20). When N-metabolism is impaired by a nitrate deficiency or by the inactivation of NR, Rubisco is soon affected (14, 21); some

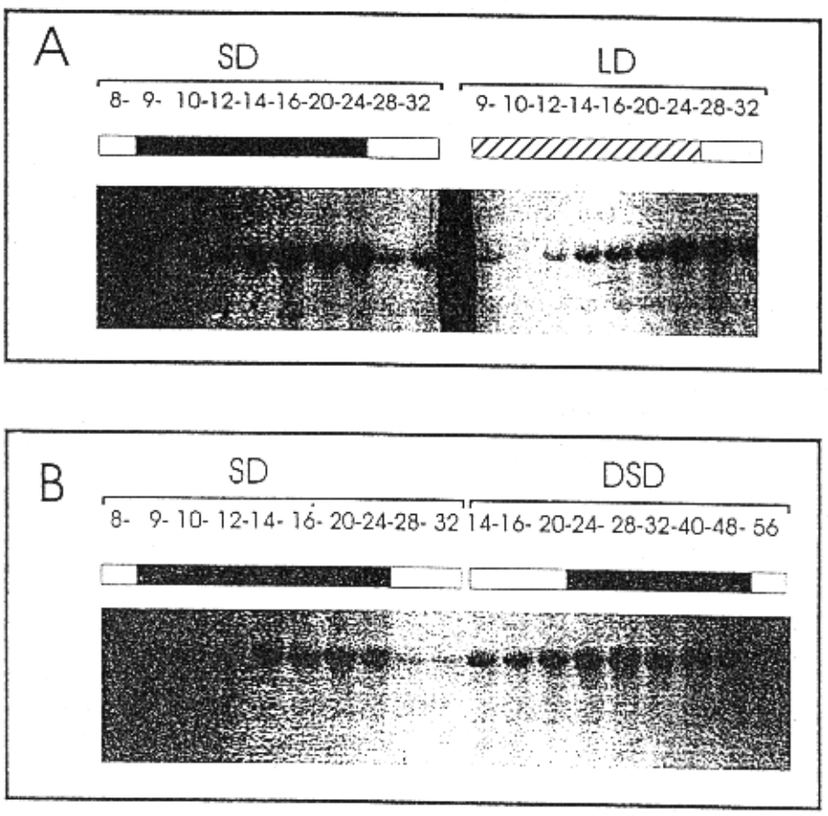

Figure 3. Steady-state level of NR-mRNA in leaf 6 of *L. temulentum* in different light regimes. A) 8-h SD and 24-h LD; B) 8-h SD and 8-h DSD. Samples of 20-µg total RNA were fractionated by electrophoresis in denaturing 1.2% agarose gel, transferred onto nylon membrane and hybridized with ^{32}P-labelled *Eco*RI insert of Zmnr1 clone encoding NR in maize and kindly supplied by Prof. H. Campbell, Michigan Technological State University, Pullman, USA. Last wash = 0.1x SSPE, 40°C; 3-d exposure.

authors postulate that a 'luxury' amount of Rubisco exists in the leaves under non limiting conditions (21).The relative amount of Rubisco SSU-mRNA was assayed but no significant effect of the inductive treatments was found (data not shown). However, many examples of post-transcriptional regulation of Rubisco are known (12). On the other hand, an increase in the relative amount of RCA-mRNA was shown above (Fig. 2). RCA is known to be implied in the increase of Rubisco efficiency that compensates for its abundance limitation (26), and RCA seems to be largely controlled at the transcriptional level (24).

Another feature commonly found in NR-deficient plants is starch accumulation (8, 25). Starch was extracted from leaf 6 and its content was found to be almost 3 times as high during the LD-extension than at the same time in SD (not shown). It is hard to believe that this difference was due to the photosynthetic activity during the LD-extension since the energy used was very low. Unfortunately, the alternate system to induce flowering did not help to clarify this point since the DSD was given at 400 µmol m^{-2} s^{-1} and caused an expected increase in starch concentration (not shown).

Conclusion

The results shown here suggest that N-metabolism may be impaired during the induction of flowering in *L. temulentum*. This is still speculative since this study was limited to the mRNA level and regulation at subsequent steps may be important, notably for NR activity (10). However, this view is especially motivating since physiological

studies mainly based on the manipulation of plant nutrition previously showed that a nitrate deficiency may induce flowering in the SD plants *Perilla crispa* (29), *Pharbitis nil* (9), *Lemna paucicostata* (27), and suggested the importance of the C/N ratio in the control of flowering (e.g. 22, 23), an old paradigm that was first known as the Klebs's theory, early in this century (reviewed in 2). This work is now joining such an hypothesis by a molecular approach and the perspective is now to look at the protein level. However, it must be kept in mind that this is probably no more than just a piece of the puzzle to be assembled with the others. In the particular case of *L. temulentum*, the implication of gibberellins in the early steps of flowering is supported by a bulk of physiological and biochemical studies (7). Anyway : is complexity not the essence of plant physiology ?

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