FEBS Letters 423 (1998) 324–328 FEBS 19883

# The wheat *wcs120* promoter is cold-inducible in both monocotyledonous and dicotyledonous species

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Received 5 December 1997; revised version received 26 January 1998

**Abstract The *wcs120* gene is specifically induced by low temperature (LT) and encodes a protein that is thought to play an important role in the cold acclimation process in wheat. To identify the regulatory elements involved in its LT responsiveness, the transient expression activity of different promoter regions was determined using the luciferase reporter gene. The data indicate the involvement of putative enhancer elements, negative and positive regulatory regions in the transcriptional regulation of this gene. The promoter was found to be cold-inducible in different freezing-tolerant and -sensitive monocot and dicot species, suggesting that universal transcription factors responsive to LT may be present in all plants. This promoter could be used to drive the genes needed for LT tolerance in sensitive species.**



*Key words:* Cold inducibility; Dicotyledon; Freezing tolerance; Monocotyledon; Promoter

## 1. Introduction

The cold acclimation process in plants is genetically programmed and is associated with an increased freezing tolerance (FT) resulting from the expression of many genes [1]. Among these, *wcs120* is a specifically LT-regulated gene encoding a highly abundant protein which appears to play an important role during cold acclimation of wheat [2]. The accumulation of both the mRNA and protein was shown to correlate closely with the capacity of wheat cultivars to develop FT. The *wcs120* gene copy number and organization are identical in freezing-tolerant and less tolerant wheat cultivars, and the expression is regulated mainly at the transcriptional level. On the other hand, the homologs of *wcs120* (and other cold-regulated genes) in chilling-sensitive Gramineae species such as rice and corn are not expressed. This lack of expression may result from inefficient *cis*-acting elements or from the absence of cold-specific transcription factors and could explain the inability of the sensitive plants to cold acclimatize. Few *cis*-acting elements responsive to LT (LTRE) have been identified so far, the best known being the $^{A}/_{G}$CCGAC core motif in the promoters of *cor15A*/*rd29A* from *Arabidopsis*, and *bn115* from *Brassica napus* [3–5]. In our efforts to identify the nuclear events regulating the cold-specific expression of the *wcs120* gene, we have characterized its promoter region. DNA-protein interaction studies revealed the presence of multiple DNA binding proteins in nuclear extracts from non-acclimatized plants which interact with several elements in the promoter [6]. The binding of these factors, which may act as transcriptional repressors, is decreased when they are phosphorylated upon LT exposure. In this report, we present the deletion analysis of the *wcs120* promoter region using transient expression in wheat leaves. In addition, the promoter strength was determined in different monocot and dicot species.

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*Abbreviations:* FT, freezing tolerance; FI, fold induction; LT, low temperature; $LT_{50}$, temperature required to kill 50% of the plants; WCS, wheat cold-specific

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Seeds of winter (*Triticum aestivum* L. cv Fredrick) and spring wheat (*T. aestivum* L. cv Glenlea), barley (*Hordeum vulgare* L. cv Sophie) and winter rye (*Secale cereale* L. cv Puma) were germinated in moist vermiculite for 7 days. Control plants were maintained under controlled environment at a 24°C/20°C (day/night) regime with a 15 h photoperiod and a 75% relative humidity. For rice (*Oryza sativa* L. cv Nipponbare), seeds were germinated in a water-saturated mixture of soil, peat and vermiculite at 28°C with a 12 h photoperiod. Rapeseed (*Brassica napus* L. cv Jet Neuf), alfalfa (*Medicago sativa* ssp. *falcata* L. cv Anik), sweet pepper (*Capsicum annuum* L. cv Supersett), cucumber (*Cucumis sativus* cv Vertige) and tomato (*Lycopersicon esculentum* Miller cv Floramerica) were grown at 24°C in the same soil, peat and vermiculite mixture. For the cold treatment, plants were grown for 1 week with a 12 h photoperiod at 4°C for wheat, barley, rye, alfalfa and rapeseed, or at 10°C for rice, cucumber, pepper and tomato. Freezing tolerance of these species, expressed as the $LT_{50}$, varies as follows: rye (−25°C), Fredrick wheat (−16°C), *Brassica* (−16°C), alfalfa (−15°C), Glenlea wheat (−6°C), barley (−4°C), rice (4°C), pepper, tomato and cucumber (5–10°C).

### 2.2. Transient expression experiments

The 942 bp upstream of the ATG translation initiation codon of *wcs120* was cloned and sequenced, and the transcription initiation start site was determined [6]. A deletion series was generated by exonuclease III and exonuclease VII digestion and the subclones were sequenced (T7 sequencing kit, Pharmacia). Constructions bearing transcriptional fusions of the promoter fragments with the luciferase reporter gene and the *nos* terminator were prepared in pBluescript. For the 3′ deletions, the TATA box and transcription start site were provided by the 90 bp proximal fragment of the CaMV 35S promoter. Our results showed that, even though this promoter is much more efficient in dicots, it is active in wheat and its capacity to promote transcription is temperature-independent (Fig. 2B, MIN). All the plasmids used were purified from *Escherichia coli* cultures by alkaline lysis and CsCl centrifugation.

For transformation, plasmid mixtures were prepared by mixing equal amounts of each *wcs120* promoter-luciferase construct with a ubiquitin promoter-glucuronidase construct (pAHC27 [7]). Plasmid DNA was coated on 0.9 μm tungsten beads (M-10, Bio-Rad) by ethanol precipitation [8] and delivered to the leaf tissues using a microprojectile bombardment apparatus [9]. The tissues were placed on an agar plate and bombarded with a 50 ms 85 PSI helium discharge under a vacuum of 25 inches of Hg. They were then floated on a nutrient solution (0.5 g/l 20:20:20, N:P:K; CIL) in a Petri dish and incubated for 2 days at 24°C or 3 days at 4° or 10°C, as specified for each experiment. Soluble proteins were extracted by grinding each


*PII* S0014-5793(98)00116-1

sample (~50–60 mg) with a mortar and pestle in 400 μl of ice-cold extraction buffer (50 mM Na-phosphate pH 7.0, 10 mM DTT, 0.1% Triton X-100, 10 mM EDTA) and centrifugation at 10 000×*g* for 10 min. The supernatant was used directly for enzymatic determination of luciferase (LUC; assay kit from Promega) and β-glucuronidase (GUS [10]) activities.

In all cases the results are expressed as LUC to GUS ratios, and are means ± S.D. of at least four independent extracts. Prior to LUC/GUS calculation for the 4°C samples, we found that a correction of the GUS values was absolutely necessary since the GUS activity obtained at 4°C was constantly 2–4-fold lower than the activity at 24°C. A correction factor was thus calculated for each construct by dividing the average GUS activity at 24°C by the average activity at 4°C. The individual GUS values at 4°C were then multiplied by this factor to obtain the corrected values used in the LUC/GUS calculation. If this correction had not been made, the activity of the promoter at LT would have been overestimated.

## 3. Results and discussion

### 3.1. Deletion analysis

Western analyses were performed to evaluate the capacity of the transformed tissues to express the endogenous *wcs120* gene family upon LT exposure. The results show that the sections of untransformed leaves (Fig. 1A) and those of leaves transformed with the beads only (Fig. 1B) accumulate the WCS120 proteins in a similar manner. The conditions tested were those used for the post-bombardment incubation period in the transient expression assays. These results show that the wounding stress caused by the bombardment had no effect on the level of expression of the endogenous *wcs120* genes, and should not affect the activity of the promoter in the transient assays.

Wheat leaf sections were transformed with constructs bearing the different deletions of the promoter transcriptionally fused to the luciferase (*luc*) reporter gene. The chimeric constructs are represented schematically in Fig. 2A. The results show that LT treatment increases LUC activity 8-fold when the full length *wcs120* promoter is used (Fig. 2B, FL860). A control experiment was performed using the pAHC18 (*Ubi-luc*) vector under the same conditions. The results showed no increase in LUC activity at 4°C when the gene is driven by the *Ubi* promoter. This confirms that when the *wcs120* promoter is used, the increase in activity observed at 4°C is due to the LT inducibility and not to an increased *luc* transcript stability at this temperature.

The 5′ deletion up to −590 leads to an increase of the fold induction (FI) from 8- to 55-fold. This is mainly due to the almost complete loss of basal activity at 24°C. The region between −860 and −590 contains a 52 bp direct repeat composed of two elements separated from each other by 4 bp, and four of the five CGTCGG elements which do not show any homology with known motifs. The loss of activity at both 4° and 24°C upon deletion of this region suggests that either or both elements could act as transcriptional enhancers. It was reported that repeated sequences of 127–337 bp from *Arabidopsis* could act as enhancers in tobacco transgenic plants [11]. A further deletion to −415 increases the activity at both temperatures, suggesting that the region from −590 to −415 contains elements that repress transcription. This region contains the two GGGTATA elements of unknown function. The fact that the effect of the putative negative elements was not apparent in the FL860 construct suggests that the putative enhancers located between −860 and −590 may overcome or inactivate the negative factors of the −590 to −415 region. If

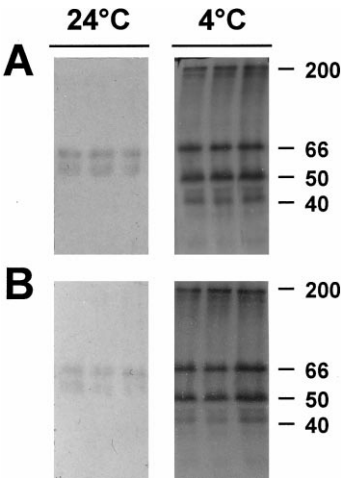


Fig. 1. Effect of bombardment on the expression of the endogenous WCS120 proteins. The proteins were extracted and analyzed by Western blotting using the polyclonal antibody directed against the members of the WCS120 family. Numbers on the right indicate the MW (in kDa) of the major members of the family. Three independent samples were analyzed for each condition. A: Protein accumulation in non-transformed wheat leaves sections after 48 h at 24°C and 72 h at 4°C. B: Protein accumulation after bombardment of wheat leaves with tungsten beads without DNA and incubation at 24°C for 48 h or at 4°C for 72 h.

the region between −415 and −215 is deleted, a decrease in activity is observed at both temperatures, suggesting the presence of enhancer elements. However, the significant decrease in activity at 4°C suggests that this region could contain cold-inducible positive regulatory elements. In fact, this region contains a CCGAC LTRE previously identified in the promoters of *cor15A*/*rd29A* from *Arabidopsis* and *bn115* from *Brassica napus* [3–5]. The presence of a motif identical to this LTRE in the *wcs120* promoter may suggest a similar role in wheat. A cDNA clone corresponding to a protein from *Arabidopsis* which can bind the CCGAC motif has been isolated recently [12]. A more detailed characterization of this transcription factor will help to determine the implication of this motif in the LT response.

The deletion of the region between −215 and −72 abolishes almost all promoter activity. The decrease of activity at 4°C suggests that the deleted region contains an LTRE. This region contains the other GCCGAC LTRE and two of the three CACCTGC elements. The latter elements contain the CANNTG motif, which forms the core of several *cis*-acting elements such as the ABA response elements (ABRE) [13]. This motif was identified as the preferred binding site for the bHLH proteins, the common plant regulatory factors (CPRFs) and for the G-box binding factors (GBFs) belonging to the bZIP class of proteins [13]. Another element, CACTCAC, is repeated two times and was identified as a binding site for the transcriptional activators GCN4 from yeast and *zeste* from Drosophila [14,15]. Taken together, these observations suggest that the *wcs120* promoter possesses putative *cis*-acting elements that could bind known transcription factors, present in both the plant and animal kingdoms.

The promoter region of the *wcs120* gene was also analyzed by 3′ deletions. The deletion of the proximal 67 bp of the *wcs120* promoter leads to a decrease of the FI from 8.0- to 4.0-fold, suggesting the presence of an LTRE in the region between −67 and +1. A further deletion to −178 decreases

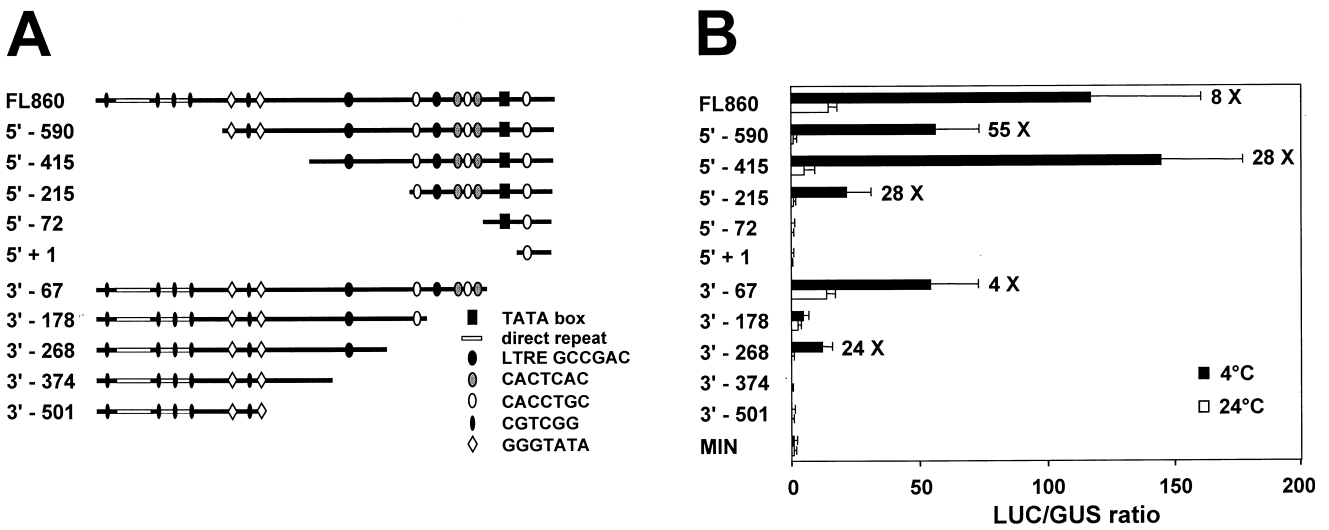


Fig. 2. Deletion analysis of the *wcs120* promoter by transient expression. A: Schematic representation of the promoter fragments and relative positions of the repeated DNA motifs deduced from the sequence analysis (GenBank accession number AF031235). The constructions were named according to the first (5′ deletions) or last (3′ deletion) nucleotide of the promoter fragment. B: Effect of cold treatment on the activity of the *wcs120* promoter fragments. The leaf sections were transformed with the different promoter-*luc* fragments and *Ubi-Gus* (pAHC27), and incubated at 4°C for 3 days or at 24°C for 2 days. Soluble proteins were extracted and enzymatic activities of LUC and GUS were determined. Numbers at the right of the error bars indicate the induction factors (4°C/24°C relative activity ratio).

the FI from 4.0- to 1.8-fold, suggesting the presence of an LTRE. This supports the observation made from the analysis of the −215 to −72 deletion. Upon removal of the initial 268 bp of the promoter, the activity at 4°C increased whereas that at 24°C decreased compared to −178 construct. The relative increase of the FI suggests the existence of negative regulatory elements between −268 and −178 that would be mainly active at 4°C. Deletion to −374 completely abolishes the *wcs120* promoter activity. Since the most important loss of absolute activity is that at 4°C, an LTRE may be present between −374 and −268. On the other hand, we cannot rule out the possibility of the existence of an enhancer element in this region. Further deletion to −501 does not reactivate the promoter, indicating that most of the elements responsible for the promoter activity were removed.

Together, the results from the transient expression assays suggest that the transcription of the *wcs120* gene is dependent on the presence of multiple regulatory elements. Footprinting experiments are required to identify more precisely the DNA motifs that bind the different proteins. It will then be possible to perform the mutation analyses needed to confirm the importance of these motifs in the LT responsiveness.

### 3.2. Activity of the wcs120 promoter in different species

The transient activity of the full length promoter (FL860) was determined in different freezing-sensitive and -tolerant species. The results show that the promoter activity is similar in the more tolerant Fredrick and less tolerant Glenlea cultivars (Fig. 3). This suggests that even though Glenlea is less tolerant, the plant possesses the *trans* regulatory elements needed to express this important gene for FT. This result was expected since Northern analysis had shown no significant differences between the levels of expression of the *wcs120* gene in Glenlea and Fredrick, during the first week of acclimation [1]. The kinetic analyses of WCS120 protein accumulation and the close inverse relationship between WCS120 protein levels and $LT_{50}$ indicate that the FT of cereals is determined by the degree and duration of LT gene expression. These results and other evidence, such as increased transcript stability and alternative splicing, support a role for post-transcriptional regulation events in the differential accumulation of proteins at LT [1,16].

The promoter shows a similar LT inducibility in wheat and barley (Fig. 3). Barley, a species closely related to wheat, possesses a gene (*dhn5* [17]) that is almost identical to

Table 1
Activity of the *wcs120* full length promoter in different dicotyledonous species after cold exposure

| Species | Family | Tolerance to freezing | LUC/GUS ratio | | FI[a] |
|---|---|---|---|---|---|
| | | | 24°C | cold | |
| Alfalfa | Leguminosae | Tolerant | 0[b] | 1031 ± 109 | (ND)[b] |
| *Brassica* | Cruciferae | Tolerant | 0[b] | 39 ± 2 | (ND)[b] |
| Cucumber | Cucurbitaceae | Sensitive | 197 ± 44 | 5117 ± 743 | 26 |
| Tomato | Solanaceae | Sensitive | 73 ± 21 | 59 ± 29 | 1[c] |
| Pepper | Solanaceae | Sensitive | 18 702 ± 11 557 | 1957 ± 405 | 0.1 |

The leaf sections were transformed with the FL860LUC construct and *Ubi-Gus* (pAHC27), and incubated at LT for 3 days or at 24°C for 2 days. The LT treatment was performed at 4°C or at 10°C for the cold-tolerant and -sensitive species, respectively. Soluble proteins were extracted and enzymatic activities of LUC and GUS were determined.
[a]The fold induction (FI) is the cold/24°C relative activity ratio.
[b]The FI was difficult to determine due to the undetectable activity of LUC at 24°C.
[c]The difference between the two conditions is not significant.

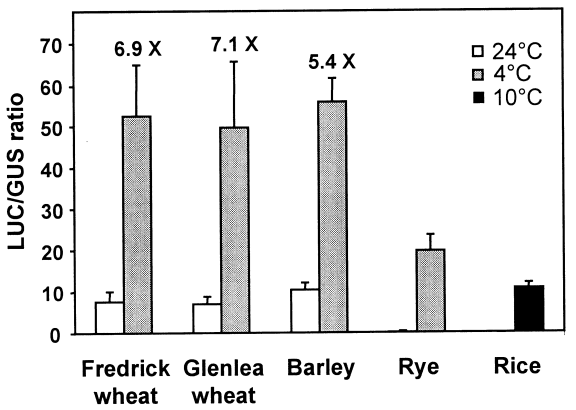


Fig. 3. Activity of the *wcs120* full length promoter in different monocotyledonous species after cold exposure. The leaf sections were transformed with the FL860LUC construct and *Ubi-Gus* (pAHC27), and incubated at LT for 3 days or at 24°C for 2 days. The LT treatment was performed at 4°C except for rice, a sensitive species, which was exposed at 10°C. Soluble proteins were extracted and enzymatic activities of LUC and GUS were determined. Numbers above the error bars indicate the fold induction (FI; 4°C/24°C relative activity ratio). In the case of rye and rice, the FI was difficult to estimate due to the undetectable activity of LUC at 24°C. The LUC/GUS ratio was almost zero.

*wcs120*. Winter rye, a highly tolerant Graminea that also possesses homologs of the *wcs120* family members, showed a 20-fold increase in activity following exposure to 4°C. Rice, a cold-sensitive monocot species that possesses an inactive gene homologous to *wcs120*, showed an 11-fold increase at 10°C compared to 24°C. A much higher LT inducibility was observed in the freezing-tolerant dicot species alfalfa and *Brassica* at 4°C (Table 1). The fold increase was very difficult to estimate due to the undetectable activity of *wcs120*-LUC at 24°C. The *wcs120* promoter activity was also evaluated in cold-sensitive dicots (Table 1). In cucumber, a 26-fold induction of the activity at 10°C was observed. On the other hand, in tomato, no difference in activity was observed between the samples treated at the two temperatures. Pepper is the only species for which a decrease (10-fold) of the promoter activity was observed upon LT exposure. These results indicate that the level of promoter activity is not correlated with the capacity of these species to develop FT. For example, the promoter is less active in rye (the most tolerant species tested) than in wheat, a result that is supported by previous genetic analyses. Amphiploids from rye/wheat crosses show a FT equivalent, but not superior, to that of wheat [1]. Furthermore, the level of expression of the *wcs120* family genes in these individuals does not exceed that observed in wheat. This suggests that the elements important for FT in rye are silenced in a predominant wheat background, and would suggest the existence of differences in the *cis*- and/or *trans*-acting elements involved in the transcription of *wcs120* and perhaps of other LT-induced genes.

Our data indicate that the *wcs120* promoter is LT-inducible in the Gramineae (wheat, barley, rice and rye), Cruciferae (*Brassica*), Leguminosae (alfalfa) and Cucurbitaceae (cucumber), but not in the Solanaceae family (tomato, pepper). The reason for the absence of LT induction in the latter family is not known. The results provide evidence of the existence of common transcription factors in both monocots and dicots, and suggest that these factors have the capacity to recognize the *cis*-acting elements of a cold-inducible heterologous promoter. The data presented here indicate that the cold sensitivity of some species (such as rice and cucumber) is apparently not due to inefficient or absent transcription factors but may possibly result from the inefficiency of the promoters of the homologous genes. This hypothesis could be verified with the characterization of the promoter of the *wcs120* homologs in sensitive species.

The observations from the transient assays do not, of course, necessarily represent what would happen in transgenic plants. Nevertheless, the identification of such a promoter is interesting from an application point of view since it could be a useful tool in the elaboration of strategies aimed at the improvement of FT in sensitive monocot and dicot species. Until now, plants modified to overexpress genes potentially implicated in FT have been transformed with constructions bearing the genes under the control of constitutive promoters. The most widely used promoters are those of the maize ubiquitin gene (*Ubi*) and of the CaMV 35S gene, for monocots and dicots respectively. Efforts are now being focused towards the identification of promoters that are more efficient in different plants. For example, several constructions bearing, in different combinations, fragments of the CaMV 35S promoter, an intron of the bean phaseolin gene, the Ω sequence of TMV and terminating sequences from the CaMV 35S or *nos* genes have been tested in rice (monocot) and tobacco (dicot) [18]. It was shown that the most efficient constructions for rice were not the same as for tobacco, suggesting differences in the specificity of gene expression between monocot and dicot plants. On the other hand, the overexpression of a gene resulting from the use of a constitutive promoter is not necessarily an objective to achieve in all cases. Indeed, few studies have focused on the physiological consequences related to the constitutive expression of genes that are normally inducible and thus expressed only when needed. The use of a cold-inducible promoter such as that of the *wcs120* gene would allow the expression of the genes only when the plant is under cold stress conditions.

*Acknowledgements:* We are grateful to Sylvain Dallaire for the construction of the microprojectile apparatus. This work was supported by a Natural Sciences and Engineering Research Council of Canada research grant (A7199) to F. Sarhan. F. Ouellet was supported by a Ph.D. scholarship from the Fonds pour la Formation de Chercheurs et l'Aide à la recherche, Gouvernement du Québec.

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