Short Communication

High Affinity of the 5'-Upstream Region of a Winged Bean Chymotrypsin Inhibitor Gene for Nuclear Matrix

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The 5'-upstream region of a winged bean chymotrypsin inhibitor gene (WCI-3b) was found to have a high affinity for nuclear matrix. The region, named WCI-3b MAR (matrix attachment region), is highly A+T-rich and contains multiple sites interacting with nuclear matrix. A MAR was also found in the corresponding region of the WCI-x gene, another active gene of the WCI family. Several MAR-binding proteins were detected in the wheat nuclear matrix.

Key words: MAR — MAR-binding proteins — WCI-3b promoter — Wheat nuclear matrix — Winged bean.

Nuclear matrix, a proteinaceous structure involved in the organization of the nucleus, has been implicated in various nuclear functions such as transcription, mRNA maturation, recombination, and replication (Berezney and Jeon 1995). The chromosomal DNA interacts with the nuclear matrix through special regions called matrix attachment regions (MARs). In animals, some MARs are known to be located at the base of chromatin loops and are involved in establishment of a sort of functional domain (Gasser et al. 1989, Laemmli et al. 1992), and other MARs are in or near promoters, enhancers, and origins of replication, to facilitate their respective functions (Bode et al. 1996, Getzenberg 1994).

The study of MARs in plants has focused mainly on chromosomal organization and position-independent gene expression; localization of MARs between genes has suggested their involvement in defining boundaries (e.g., Avramova et al. 1998, van Drunen et al. 1997) and some, but not all, have been shown to be able to normalize the expression levels of the nearby genes (as a review, Spiker and Thompson 1996). However, little is known about nuclear matrix-MAR interactions at the molecular level or their role in gene regulation.

Kunitz-type chymotrypsin inhibitors accumulate abundantly in mature seeds and tuberous roots in winged bean (Psophocarpus tetragonolobus (L.) DC.) (Shibata et al. 1986). The winged bean chymotrypsin inhibitors (WCIs) are encoded by a multigene family and several members have been identified (Habu et al. 1992, 1997). The WCI-3b gene, a gene encoding the major species, has been studied most extensively with respect to gene expression and regulation, and a region of a few hundred base pairs upstream of the transcription initiation site has been shown to have cis-acting regulatory elements responsible for its transcriptional regulation during seed maturation (Habu et al. 1992, Sakata et al. 1994, 1997, Umemoto et al. 1992). In a search for MARs of plant origin, we found that a 1.9-kb region just upstream of WCI-3b exhibited a strong affinity for nuclear matrix. Here, we report the initial characterization of this region in relation to nuclear matrix-MAR interactions and gene regulation in plants.

Preparation of wheat nuclear matrix capable of binding to MARs—Protoplasts were isolated from suspension-cultured cells of wheat (Triticum aestivum L. cv. HY1) as described by Nakayama et al. (1992) and lysed on ice in a modified Honda buffer [2.5% Ficoll, 5% dextran T-40, 25 mM Tris-HCl (pH 8.5), 5 mM MgCl₂, 0.1% Triton X-100, 0.44 M sucrose] supplemented with 2.5 mM dithiothreitol (DTT), 0.5 mM spermine, 0.125 mM spermidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64). The nuclei were collected by centrifugation at 3,000 × g for 5 min, washed twice, and homogenized in the same buffer without spermine and spermidine with a Dounce homogenizer (5–20 gentle strokes). Two cycles of 3-step Percoll gradient centrifugation were performed to purify the nuclei. The top and middle layers
consisted of 0.44 M sucrose, 25 mM Tris-HCl (pH 8.5), 10 mM MgCl₂ plus 60% (v/v) and 80% (v/v) Percoll. The bottom layer consisted of 2 M sucrose, 25 mM Tris-HCl (pH 8.5), and 10 mM MgCl₂. After centrifugation at 3,000 × g for 30–60 min, the nuclei were recovered from the interface between the middle and bottom layers. The purified nuclei were finally suspended in a stock solution [50 mM Tris-HCl (pH 8.5), 5 mM MgCl₂, 50% glycerol] and stored at −70°C. The amount of nuclei present was determined as described by Breyne et al. (1992).

Nuclear matrix was prepared by the method originally developed by Mirkovitch et al. (1984) with modifications. The purified nuclei were stabilized by incubation at 42°C for 15 min in a buffer consisting of 37.5 mM Tris-HCl (pH 7.5), 0.2 M KCl, 0.1% digitonin, 0.5 mM spermine, 0.125 mM spermidine, 1 mM PMSF, and 10 mM EDTA. Without heat stabilization, nuclear matrix proteins could not be recovered by low-speed centrifugation. Core histones were extracted with 25 mM Li diiodosalicylate (LIS) at room temperature for 10 min in a solution containing 20 mM HEPES-KOH (pH 7.5), 0.1 M Li acetate, 1 mM EDTA, and 0.1% digitonin. The nuclear matrix preparation contained a variety of proteins (Fig. 1A), like other plant nuclear matrices capable of interacting with MARs (Moreno Díaz de la Espina 1995). Histone-depleted nuclear halos thus obtained were washed thoroughly and exposed DNAs were digested with restriction enzymes (BamHI, HindIII, and XhoI). After the removal of solubilized DNA and restriction enzymes by centrifugation, the pellet was suspended in the binding buffer [20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 mM KCl, 10 mM DTT, 0.1 mg ml⁻¹ bovine serum albumin, 0.1% digitonin, 25 mM EDTA, 1 mM PMSF] and used as wheat nuclear matrix.

To verify the capability of the wheat nuclear matrix to bind to MARs, two MAR-containing fragments were used, considering that nuclear matrix-MAR interactions are evolutionarily conserved (Phi-Van and Strätling 1990); one was a DNA fragment derived from downstream of the pea plastocyanin gene (Slatter et al. 1991), and the other a fragment containing the soybean Ibcj gene, which has been described to have a high affinity for nuclear matrix (Izaurrealde et al. 1988). A 3 fmol portion of the MAR-containing DNA was labeled at the 3' ends and incubated with freshly prepared wheat nuclear matrix (equivalent to 0.3–1.0 A₂₆₀ unit of the original nuclear DNA) in 0.3 ml of the binding buffer containing 10 μg of sheared E. coli DNA (at least a 1,000-fold excess over the labeled fragments by weight) at 37°C for 15 h. After the reaction, nuclear matrix-bound and free DNAs were separated by centrifugation and subjected to agarose gel electrophoresis. The DNA fragment containing the Ibcj gene was found almost exclusively in the pellet fraction, whereas a vector-derived fragment, included in the reaction as a non-MAR control,
was found in the supernatant (Fig. 1B), indicating that the wheat nuclear matrix retained MAR-binding activity. The affinity of the MAR from the pea plastocyanin gene for the nuclear matrix was rather weak under the conditions employed, which had been optimized for WCI-3b MAR (see below).

High affinity of the region upstream of the WCI-3b gene for nuclear matrix—Since the WCI-3b promoter is A+T-rich, we examined whether it bound to wheat nuclear matrix. As shown in Fig. 1B, the 1.9-kb fragment derived from the 5'-upstream region of WCI-3b showed a high affinity for the nuclear matrix. This fragment was found to bind also to tobacco nuclear matrix prepared by the LIS extraction method, and to both wheat and tobacco nuclear matrices prepared by the high salt method (not shown). Thus, the affinity of the 1.9-kb region for nuclear matrix was considered to be an intrinsic feature, and we named it WCI-3b MAR.

In order to define the subregion(s) within the 1.9-kb fragment responsible for binding to nuclear matrix, various parts of this fragment were subcloned and subjected to the in vitro binding assay (Fig. 2, 3). Most subfragments were recovered from the nuclear matrix fraction, showing that there are multiple nuclear matrix-binding sites in WCI-3b MAR. High affinity was observed in the region between -200 and -1,600 relative to the transcription initiation site.

The features of WCI-3b MAR are summarized in Fig. 2. On the whole, WCI-3b MAR is highly A+T-rich (77% on average) and it contains many motifs often found in known MARs, such as A-box, T-box, the consensus sequence for the topoisomerase II recognition site, and the yeast autonomously replicating sequence (ARS), although their importance in binding to the nuclear matrix has not been clarified. Regions of intrinsically curved DNA and those with base-unpairing propensity were also found in WCI-3b MAR. These motifs are widely dispersed in the region corresponding to the fragments with high affinity for nuclear matrix. A MAR signature recently deduced through analysis of the Arabidopsis genome containing the plastocyanin gene (van Drunen et al. 1997) is absent in the case of WCI-3b MAR.

Presence of a MAR in another expressed gene of the WCI family—The WCI proteins are encoded by a multigene family and several other members of the family, in addition to WCI-3b, have been cloned (Habu et al. 1992, 1997). To gain further insight into the relationship between the presence of a MAR in the region upstream of the MAR in the WCI-3b gene

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**Fig. 2** Features of the 5'-upstream region of the WCI-3b gene and the fragments used for MAR-binding assays. Fragments shown below the scale were obtained from pUC-3b1.9 (CE, from −1,891 to −1,580 relative to the transcription initiation site; CS, from −1,891 to −1,396; EE, from −1,579 to −1,078; SS-1, from −1,395 to −1,168; SS-2, from −1,167 to −677; M, from −1,513 to −630), pWCI-1.0G (3b-1.0, from −1,043 to +34), and its deletion derivatives (Sakata et al. 1994). Shown in the middle of the figure are the positions of motifs often found in known MARs (Bode et al. 1992, Boulikas 1995, Gasser and Laemmli 1987): A-box (AATAAAYAAA), T-box (TTWTWTTWTT), ARS (WTTTATRTTNR), the recognition sequence of topoisomerase II (topoII, GTNWAYATTNNR), intrinsically curved portions (Bend, AAAANAAAAAN AAAA and TTTA AAAA), and regions with base-unpairing propensity (ATC, >20 bp of a G-less stretch with the core sequence ATATAT). Shown at the bottom is the A+T content of the 1.9-kb promoter region.

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**Fig. 3** Interaction of the region upstream of the WCI-3b gene with the wheat nuclear matrix. DNA fragments shown in Fig. 2 were applied to the MAR-binding assay as described in Fig. 1 and in the text. V, the vector fragment pBluescript SK(+) used as an internal non-MAR control; I, insert DNA derived from the WCI-3b promoter.
promoter and gene regulation, the 5'-flanking region of an active gene, \textit{WCI-x}, and that of a pseudogene, \textit{WCI-P1}, were applied to the in vitro binding assay, taking into account that the promoters of \textit{WCI-3b}, -x, and -P1 have diverged from each other considerably at the sequence level and appear to be of different evolutionary origins (Habu et al. 1997). As shown in Fig. 4, the region upstream of the \textit{WCI-x} promoter displayed a high affinity for the nuclear matrix like that of \textit{WCI-3b}, whereas the corresponding region of \textit{WCI-P1} showed a weak affinity, comparable with the non-MAR controls, i.e. an \textit{E. coli}-derived fragment and a vector fragment. Although such difference may be primarily due to the difference in the A+T content (77% for \textit{WCI-3b} and -x, and 64% for \textit{WCI-P1}), it is possible that interactions of the promoters with the nuclear matrix may be involved in correct regulation of the \textit{WCI} genes in vivo, because a \textit{\beta}-glucuronidase chimeric gene with the \textit{WCI-P1} promoter used in this work has been shown to be expressed in leaves of transgenic tobaccos but not correctly regulated during seed development (Habu et al. 1997), and because the functioning of the upstream region of \textit{WCI-3b} was remarkable only in stable transformants and in the presence of the proximal promoter region (Sakata et al. 1994, 1997). In relation to this, chromosomal integration has been shown to be required for correct tissue-specific regulation of tomato \textit{rbcS} and bean \textit{\beta}-phasesolin genes, and, in each case, a MAR present in the promoter has been implicated in the regulation (Frisch et al. 1995, Meier et al. 1997).

\textit{MAR-binding proteins in the wheat nuclear matrix—}

While a plant nuclear matrix preparation contains a variety of proteins (Moreno Díaz de la Espina 1995, and see Fig. 1), only a few are known to be MAR-binding components (Meier et al. 1996, Nomura et al. 1997). In an effort to identify the MAR-binding proteins involved in nuclear matrix-MAR interactions in plants, southwestern analysis was carried out. In this experiment, fragment M, derived from the middle portion of \textit{WCI-3b} MAR, was used as a probe. This fragment contains many motifs characteristic to MARs (Fig. 2) and lacks proximal regulatory elements responsible for promoter activity (Sakata et al. 1994, 1997). As shown in Fig. 5, about 10 protein bands in the range of \(~30\text{--}100\text{ kDa}\) were detected, some of which became faint or disappeared when A+T-rich competitors were included in the reaction. The corresponding proteins each having a different affinity for A+T-rich sequences might be involved in the interactions with MARs in vitro, and further, in the formation of higher-order chromatin structure and nuclear reactions in vivo. Their identification and characterization could help towards unraveling the organization inside the plant nucleus.

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\textbf{References}


MAR in the WCI-3b gene


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