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Promoter and expression studies on an *Arabidopsis thaliana* dehydrin gene***

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Abstract A genomic clone of a group 2 *lealrabldehydrin* gene from *Arabidopsis thaliana*, *Xero2/lti30*, was cloned and sequenced. Promoter-*GUS* fusions were introduced into plants to analyse the promoter and determine expression patterns. Using root cultures, *GUS* expression was found to be moderately stimulated by abscisic acid (ABA), wounding, cold and dehydration. Results with an ABA-deficient mutant suggested endogenous ABA is required for these responses. Promoter deletion studies indicated multiple *cis*-acting elements are involved in the induction of the gene. *GUS* expression occurred in desiccated seeds, in all tissues of young seedlings and in roots (with the exception of the root tip), desiccated pollen grains, trichomes and the vascular tissues of leaves and stems in mature plants.

Key words: Arabidopsis thaliana; Dehydrins; Abscisic acid; Stress; Promoter; Tissue expression

1. Introduction

In plants exposed to stresses such as low temperature (cold), salt stress or desiccation, a large set of genes is transcriptionally activated [1]. Although no direct evidence is yet available, it is thought that the stress-induced proteins are involved in tolerance [2]. The phytohormone abscisic acid (ABA) is implicated in the control of processes resulting in tolerance [1,3–5]. Endogenous ABA concentrations increase in plants in response to stress [6] and many, but not all stress-induced genes, are responsive to exogenous ABA treatment [1,3–5]. Many genes that respond to ABA are also expressed during seed development during the late stages of embryogenesis and are thought to be involved in the development of desiccation tolerance [1].

Many proteins accumulated in response to dehydration caused by low temperature, drought, salts or during seed development belong to the group 2 LEA/Rab/dehydrin family (LEA, late embryogenesis abundant). These proteins are found in both monocots and dicots (reviewed in ref. [5]) as well as cyanobacteria [7]. A number of genes coding for these proteins have been identified in *Arabidopsis thaliana* [8–13].

A maize dehydrin gene was used to clone the *Xero1* gene from an *A. thaliana* genomic library [9] and, sequencing upstream from *Xero1*, have identified a second dehydrin-like gene [14]. We have designated this gene *Xero2/lti30* since a partial cDNA clone of the gene has been recently described and named *lti30* [12]. We present the complete genomic sequence of the *Xero2/lti30* gene including 5′ and 3′ sequences plus details of its temporal and spatial expression. Preliminary promoter analysis is also described along with the effects of various stresses on expression levels in roots.

2. Materials and methods

An *Arabidopsis thaliana* ecotype Landsberg *erecta* λEMBL 3 genomic library was screened with a radiolabelled maize *M3* cDNA insert [15]. A clone with a 15 kb insert was further analysed. Overlapping restriction fragments of the genomic insert were subcloned into pTZ18U and 19U vectors (Bio-Rad). Single-stranded DNA was generated [16] and sequenced using the dideoxy-mediated chain termination method [17].

2.1. Promoter constructs

A 1259 bp *Msp*I fragment from the 5′ region of the *Xero2* gene was ligated into *Sal*I site of the pTZ 19U vector. The *Msp*I construct contains a 1148 bp region upstream of the transcription site, 86 bp untranslated leader sequence and 25 bp coding region. Truncated promoters were generated in the pTZ 19U plasmid by digesting with *Hin*dIII, *Nsi*I, *Asu*I, *Aat*II and *Eae*I. Constructs were verified by sequencing and then cloned into the *Hin*dIII/*Xba*I sites of the pBI101 vector [18].

pBI101 plasmids containing the promoter/*GUS* fusion constructs were transferred from *Escherichia coli* MV1190 strain to *Agrobacterium tumefaciens* strain AGL1 [19] via triparental mating with an *E. coli* strain that contained the mobilisation plasmid pRK2013. Transformation of *Arabidopsis* was carried out according to Valvekens et al. [20]. Seeds (T_0) of transformed *Arabidopsis* lines were grown on germination medium (GM) phytagel (Sigma) plates containing 50 μg/ml kanamycin. After 3 weeks seedlings were transferred to soil. Seeds (T_1) were harvested after 4 weeks. Seedlings of T_1 seed lines exhibiting 100% kanamycin resistance were analysed for integration of the intact promoter/*GUS* construct by PCR [21].

2.2. Root liquid cultures

Three centimetre long root tips from kanamycin selected T_1 seed lines were placed in 75 mm deep sterile culture pots containing 50 ml of B5 medium (0.387% Gamborg's B5 medium (Sigma) and 3% sucrose, pH to 5.8). The roots were grown in the dark at 22°C with constant shaking for 5 to 8 weeks. Root liquid cultures were treated for 26 h with either 100 μM ABA, 5% (w/v) polyethylene glycol (PEG6000), 4°C (cold), or damaged with forceps (wounding). Treatments were carried out in a 10 mM MES buffer (pH 5.9). After treatment roots were removed, strained, washed with water, blotted dry, frozen under liquid nitrogen and stored at −70°C prior to quantification of GUS activity.

2.3. GUS assays

Root material was ground under liquid nitrogen, weighed and extraction buffer (0.1 M K_2HPO_4/KH_2PO_4 pH 7.0, 1.0 mM DTT and 1.0 mg/ml BSA) added. GUS activity in the extracts was assayed by fluorometric quantification of 4-methylumbelliferone metabolised from the glucuronide precursor [22]. The assay buffer contained 1.0 mM 4-methylumbelliferyl-β-D-glucuronide in extraction buffer and 20% methanol. GUS activity was expressed in nmoles of product generated per minute per gram fresh weight. Tissue localisation of

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***The GenBank accession number for the sequence reported in this paper is U19536.

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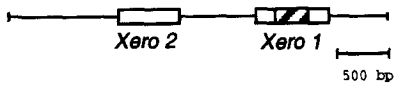
A



B

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-1750 TCTTGCTGAGCATTCCTGTCAAAGATTTGCACTCTTTAAGCAAAAATTCT
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-1650 CCTTTTTAAGAAGAACGTTCTCTAGTTTATTCGTTGTGAAATCGTCCTTG
-1600 GTTCCCAGATCCTGGAACCCAACTAGCCTATCCATCGCGACTCCCTTGCT
-1550.AAGAATGGTAAAGAAACTCGTACTTTATGAGTATACACCTAACAACAAAC
-1500.CCTGTATGTAAGAAGGAATAGAAAGAGGCGAAACCTGAAAAGCACAACGC
-1450.AAGGCAAAGTCTTTATTGCAAGCTTCGTGACAAAGAAAGGAGCATTCTGC
-1400 ATTTATTTCAGCCAAGCATTAGCTTCAGCAAAGGATTGTTGAAAAGCCAT
-1350 TTCCTTTTATAAAACTTTCATCAAATGGTTCACTAACCTCTGCGTCCACT
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-1250.GTCCATAATCCTACAGTAATGCAGTAAATATGTTTGGTTATGAGCTCATA
-1200.TACATATCAGAAGACGAACAAAAAATAAGCTGATGAGAGATCTTACTTGC
-1150.ACCGGTAAAATTCCTTATGGTAGAAATGACATATCACTTTCTCACTCCTG
-1100.GTGACTTCACCCAAGAAATCTCCTTCACTCACCTCTCGATATTCACCATG
-1050 CCCTTGTCTTTTAAACGATTCTCTCTTCTCAACCTCTCTCTGTCAAGATA
-1000 AAATCTTGAAGATTTTAGTTCATACCAAAAGCTGCATAACTATATAGGCC
-950  ACAATACAATGCCAAAGTTATAACCTTTAGTGCTGCAATCCTATCTGCGT
-900  GCAATCTTTCTAGCTCTGGATCCTACAGAACATAGACAAATAAGTCTACC
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-800  ATAAGTTCATCAAGATCAACCTCTTCATTAACAGGATTTGATCCTTGCGC
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-700  AACCATACAAGTAAGACCTCACAGGTAATCTACTCAGACAAAGAAACATC
-650  AAAAAAACTCATAAAGTCTATCACTATTTCAAAAACAAATTGAGAAACAT
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-200  TACGTCGGTCGCTAACCACTACTACACCGACGTCTTACTTGCCATGTGTG
-150  TGTGACTCTTAATCAATTACGAATTGAATATATTACTTTTACGATGTCGG
-100  CCAACACGTATTGAGTAAAATATCTATGTGATGATGAATTCCTATCCAAA
-50   ATGAAATTTGCATCTCTATAAAAGTATCATTCAAGAGATCATTAATCTTC
+1    ATCGATCAAAGTCTTGAATATTTCATCTATATATACCTAAGAAAGAAAAG
+51   AGTTAAAGAAGTATATTTTCGATTCAAAGAAGAAAAATGAATTCTCACCA
                                        M  N  S  H  Q
+101  GAATCAAACCGGAGTGCAAAAGAAGGGAATAACGGAGAAAATCATGGAGA
       N  Q  T  G  V  Q  K  K  G  I  T  E  K  I  M  E  K
+151  AGCTACCTGGTCATCATGGACCTACTAACACTGGTGTTGTTCACCACGAG
         L  P  G  H  H  G  P  T  N  T  G  V  V  H  H  E
+201  AAGAAAGGGATGACGGAGAAAGTTATGGAGCAGCTGCCAGGTCATCATGG
       K  K  G  M  T  E  K  V  M  E  Q  L  P  G  H  H  G
+251  TGCTACTGGTACCGGTGGTGTTCACCACGAGAAGAAAGGAATGACGGAAA
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       N  T  T  Y  G  T  T  N  T  G  G  V  H  H  E  K  K
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       S  V  T  E  K  V  M  E  K  L  P  G  H  H  G  S  H
+451  ATCAAACTGGGACTAACACGGCTTATGGTACTAACACTAATGTCGTTCAC
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         V  H  H  E  N  K  S  T  M  D  K  I  K  E  K  L
+651  CCCGGTGGTCATCACTAGATTGATTAACAACTTGTTTAAGCATATTTTAA
       P  G  G  H  H  *
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+751  GCTAAATAATATATGTAAGTGATGGATCTTTGAATTAATATAAAATATCT
+801  ACTTCTGGTTTCGTTTATTTATCTTTCTATCGCTTCAACTATTCCATGTT
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Fig. 1. (A) Genomic organisation of the *Xero2 (Xero2/lti30)* and *Xero1* genes. The intron in *Xero1* is indicated by cross-hatching. (B) Nucleotide and deduced amino acid sequence of the *Xero2/lti30* genomic clone. The putative TATA box and polyadenylation signal sequence are underlined and double-underlined, respectively. The lysine-rich amino acid repeat motif is double-underlined.

GUS activity was detected by incubating plant tissues in X-Gluc substrate solution (0.5 mg/ml 5-bromo-4-chloro-3-indolyl glucuronide, 50 mM $NaPO_4$ (pH 7.0) and 0.05% Triton X-100) at 37°C. Chlorophyll was removed by incubation in 100% ethanol at room temperature.

3. Results and discussion

The sequence of the *Xero1* gene has already been described [9] and the genomic organisation of the *Xero2/lti30* and *Xero1* genes is shown in Fig. 1A. The stop codon of the *Xero2/lti30* gene occurs 627 bp upstream of the translation start site of the *Xero1* gene [14]. The genomic sequence of *Xero2/lti30* is given in Fig. 1B. Southern analysis indicated that the *Xero2/lti30* gene is a single copy gene (results not shown). Primer extension studies (not shown) indicated a non-polyadenylated transcript size of 816 bp. The sequence of an incomplete cDNA clone (*lti30*) isolated from full grown plants of *A. thaliana* ecotype Landsberg *erecta* has previously been published [12]. The clone contains 712 bp homologous to most of the *Xero2* gene but lacking the 5′ untranslated region and a small region of the 5′ translated RNA. It also appears to have a frameshift mutation as a result of which the first seven amino acids of the published sequence are out of frame. The predicted amino acid sequence (Fig. 1B) indicates a molecular weight of 20912 Da. Unlike *Xero2/lti30*, most group 2 *leaI/Rab/dehydrin* genes contain a single intron, the splice site of which occurs within the DNA coding for the serine-rich motif common to the majority of group 2 LEA/Rab/dehydrin proteins. The XERO2/LTI30 protein contains neither a stretch of serines nor a DEYGNP motif typical to and found in the N-terminal region of this protein family. A lysine-rich repeat occurs six times and regions between these repeats also share similarity, threonine being the most prominent amino acid (Fig. 1B). Most group 2 LEA/Rab/dehydrin proteins possess only two lysine-rich repeats. The cold-induced spinach CAPS85 protein [23] and the wheat COR39 protein [24] contain 11 and six lysine-rich repeats, respectively. Both proteins lack the serine stretch but COR39 possesses a glycine-rich sequence that is repeated six times.

Welin et al. [12] used Northern analysis of total RNA extracted from leaves and stems of plantlets to examine the effects of low temperature, drought and ABA on *Xero2/lti30* mRNA levels. We have transformed plants with *Xero2/lti30* promoter/*GUS* gene constructs. Knowing the gene is expressed in roots (see later), we used root liquid cultures of transformed plants to examine the effects of various stresses on *GUS* expression and to identify regions of the 5′ promoter involved in these responses. Welin et al. [12] found that low temperature (4°/2°C day/night) and drought (air-drying at relative humidity of 30%) caused maximum increases of leaf and stem mRNA levels of approximately 40- and 9-fold, respectively. Some decrease occurred after 16 and 8 h. In root liquid cultures we found cold (4°C) and desiccation (5% PEG) resulted in only ca. 3-fold increases in *GUS* expression after 26 h (Fig. 2a). ABA (100 μM) and wounding both stimulated

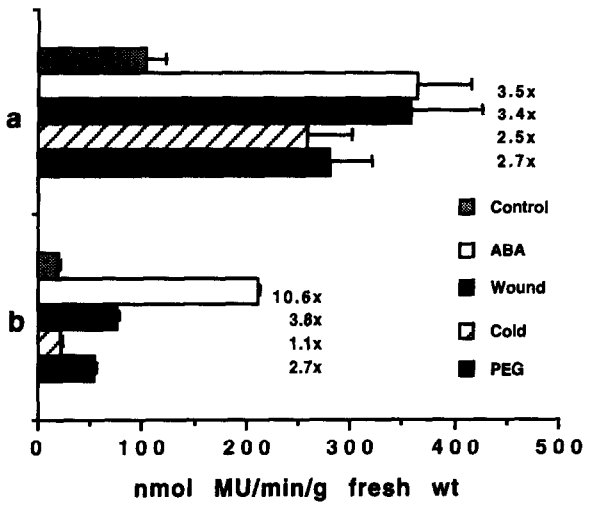

Fig. 2. Effects of ABA, wounding, cold (4°C) and PEG (dehydration) on GUS activity in transgenic roots. The *Xero2/lit30* promoter was deleted to −1148 bp. Roots were grown in liquid culture and treated for 26 h. Fold induction resulting from the various treatments is given. (a) Wild type. (b) ABA-deficient (*aba-1*) mutant.

GUS expression 4-fold (Fig. 2a). Welin et al. [12] found an ABA induction in leaves and stems of ca. 4-fold after 2 h, but this was almost halved after 16 h.

When roots from the ABA-deficient (*aba-1*) mutant were used, a ca. 11-fold stimulation of GUS activity by ABA was observed (Fig. 2b). Welin et al. [12] obtained similar results for the *rab18*, *cor47* and *Xero2/lti30* genes and suggested the ABA pathway is autoregulated, thereby reducing the sensitivity in the wild type in comparison to the *aba-1* mutant. Basal activity was reduced to 20% of wild type roots (Fig. 2b). A significant proportion of the wild type basal activity may be due to endogenous ABA. In the *aba-1* mutant wounding and PEG treatments increased *GUS* expression 4- and 3-fold, respectively (Fig. 2b). Nevertheless, absolute GUS activity was 5-fold less than in wild type roots. Cold completely failed to increase *GUS* expression in *aba-1* roots (Fig. 2b) which is contradictory to results with leaves and stems [12]. The data suggest that endogenous ABA is required to achieve maximum responses in roots to the stresses studied. We have also used Northern analysis to show that ABA increases *Xero2/lti30* mRNA levels in roots (results not shown).

A chimeric gene consisting of a deleted series of promoter regions of the *Xero2/lti30* gene fused to the *GUS* reporter gene was constructed and the fused genes transferred into *Agrobacterium* for transformation of *Arabidopsis* plants. Six gene fusions with the promoter deleted to −1148, −354, −250, −218, −167 and −104 bp were used for analysis of the effects of ABA, cold and dehydration on expression of the *GUS* reporter gene. The basal level of GUS activity in the −1148 and −354 transformants was the same but decreased ca. 50% in the −250 transformants, further dropping to 7% and 5% of the −1148 transformant activity in the −218 and −167 transformants, respectively (Fig. 3). No GUS activity was detected in −104 transformants. Hence, elements responsible for basal levels of expression are present downstream of −354 bp, in particular between −250 and −167 bp.

ABA-inducible GUS activity was not significantly influenced by shortening the promoter to −250 bp, however an 80% reduction occurred when a further 32 bp were removed (to −218) and only 5% of the original activity remained in −167 transformants. On the other hand, ABA induction increased from ca. 4-fold in −1148 and −354 transformants, to 6- and 9-fold in −250 and −218 transformants, dropping to 4-fold in the −167 transformant. The increase in fold induction suggests there may be negatively regulating elements within the promoter (Fig. 3). The promoter of the barley ABA-responsive *HVA22* gene may also possess such elements [25]. The *Xero2/lti30* promoter contains three ACGT sequences at −199, −171 and −95 bp. ACGT forms the core of sequences reported to function as ABA-responsive elements (ABREs) in many ABA-responsive genes (see ref. [25] for a summary). The sequences flanking the three ACGT cores in the *Xero2/lti30* promoter are novel and it remains to ascertain whether any of them do function as ABREs. However, the major decrease in fold induction by ABA does occur between −218 and −167 bp where two of these sequences occur.

The increase in absolute GUS activity caused by cold treatment dropped 85% between the −1148 and −354 transformants with no change when a further 104 bp were deleted (to −250), the majority of the remaining activity being lost when 32 bp (to −218) were removed. A decrease in induction from 3-fold to ca. 1.25-fold occurred when the promoter was shortened from −1148 to −354 bp (Fig. 3). Further promoter deletions had little influence on the fold induction. The sequence ACCGACG (−175 bp) differs in only one nucleotide from the low temperature element ACCGACA previously identified in *Arabidopsis* [26]. However, in *Xero2/lti30* this element is apparently not involved or insufficient on its own to confer low-temperature inducibility.

Dehydration (PEG) inducible GUS activity decreased 85% between the −354 and −250 transformants and the majority of the remaining activity was lost when a further 32 bp were removed (to −218) (Fig. 3). The major drop in fold induction occurred between −354 and −250 bp, although a complete loss of induction only occurred in the −167 transformants.

The data tend to support the concept that multiple *cis*-acting elements are involved in the induction of the gene by environmental stresses [27]. The *aba-1* mutant studies suggest the ABRE(s) must be 'activated' (e.g. transcription factors

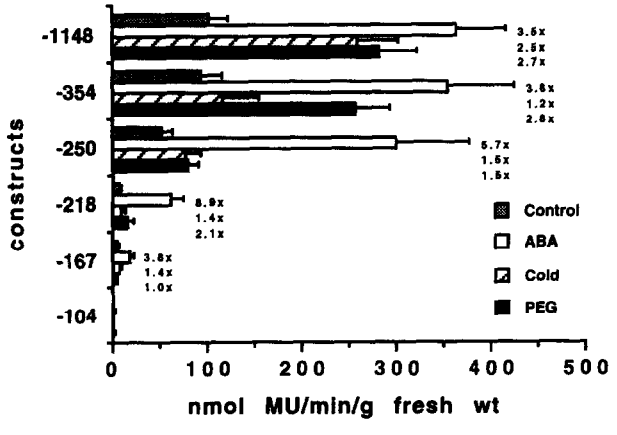

Fig. 3. 5′ deletion analysis of the *Xero2/lti30* promoter for the ABA, cold and PEG (dehydration) induction of the GUS reporter gene. Roots from transgenic plants were grown in liquid culture and treated for 26 h. The lengths of the deleted promoter are shown on the ordinate, the GUS activity on the abscissa. Fold induction resulting from the various treatments is also given.

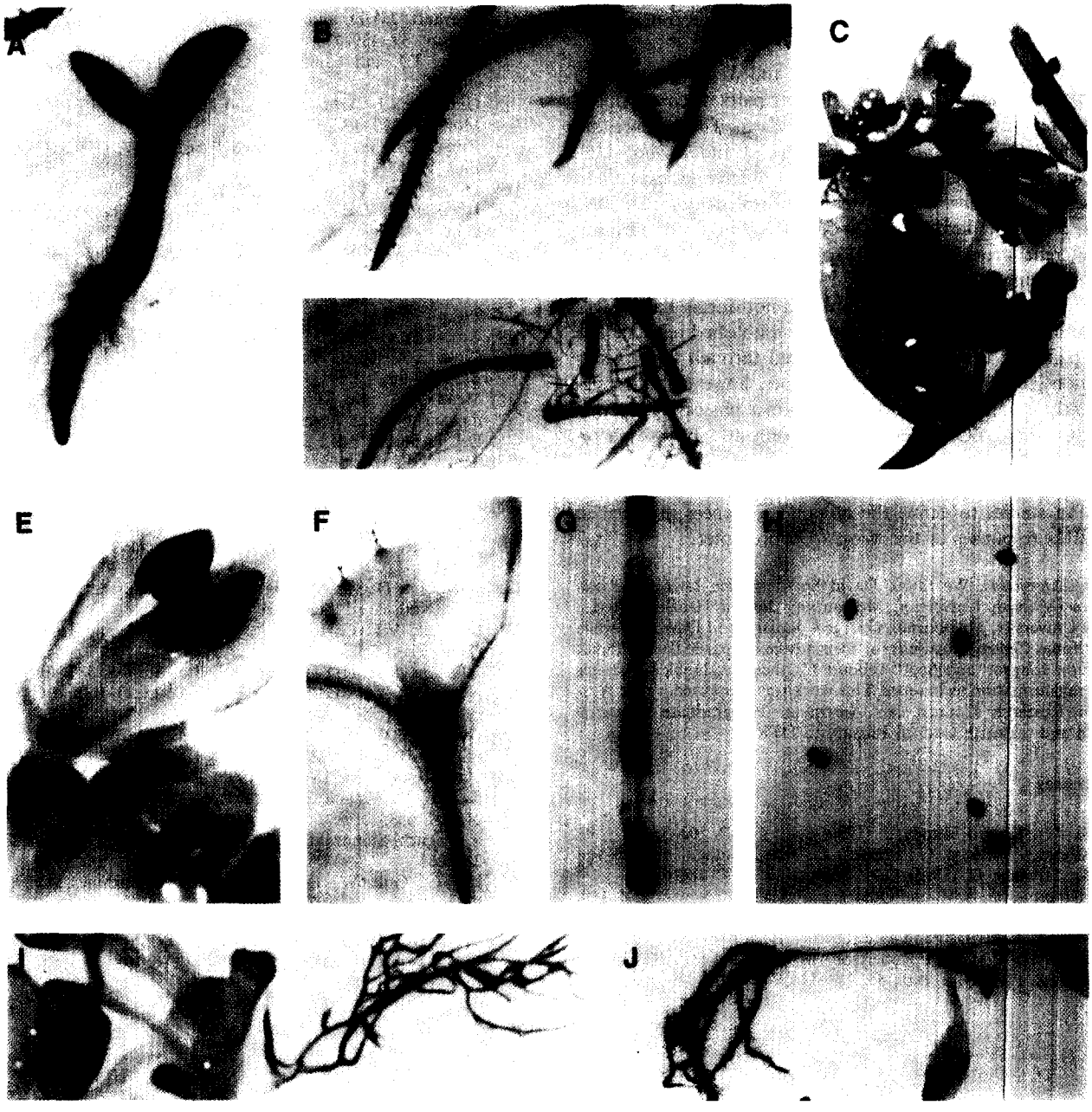

Fig. 4. Histochemical localisation of GUS activity in transgenic *A. thaliana* plants. Except for I and J, all plants are wild type. (A) Three day old −250 transformant. (B) Roots from 14 day old −354 transformants. (C) Floral tissue of 4 week old −1148 transformant. (D) Wounded roots of −218 transformant, grown in liquid culture. (E) Flower of 4 week old −1148 transformant. (F) Stem and leaf tissue of 14 day old −354 transformant. (G) Wounded root of −167 transformant, grown in liquid culture. (H) Seeds of −354 transformants; two seeds from an untransformed plant were also stained and are shown for comparison. (I) Twenty day old −1148 transformant (*aba-1* mutant). (J) Twelve day old −1148 transformant (*abi 2-1* mutant).

bound) for the elements involved in the cold and dehydration responses to be fully effective.

Histochemical analysis of the expression of the *Xero2/lti30* promoter/*GUS* gene in transgenic plants showed expression occurred in young seedlings and desiccated seeds (Fig. 4A,H). Expression occurred in all tissues of seedlings up to 3–5 days post-germination and was subsequently localised to specific tissues. In mature plants (4–5 weeks old) expression occurred in roots, desiccated pollen grains, trichomes and the vascular tissues of leaves and stems (Fig. 4B,C,E,F). Strong expression was observed in most root tissues with the exception of the root cap and root tip where no expression was detected (Fig. 4B). Tissue specificity of expression was the same as the wild type plants in the ABA-deficient (*aba-1*) and ABA-insensitive (*abi2-1*) mutants (Fig. 4I,J).

ABA, PEG and cold had no effect on spatial and temporal expression. Wounding induces expression (Fig. 4D,G), even in tissues where expression otherwise does not occur (e.g. leaf laminae and petal/sepal abscission zones). Reduction of promoter length to −167 bp, although influencing the level of *GUS* expression, had no effect on spatial and temporal expression patterns. Different length promoters are employed in the histochemical results shown in Fig. 4.

The *Xero2/lti30* gene is constitutively expressed in many tissues, notably in young seedlings and desiccated seeds, suggesting it may provide immediate protection against stress

during the time required for the plants to produce other stress response proteins.

The tandemly arranged *Xero2/lti30* and *Xero1* genes add a fifth pair of duplicate genes to the four pairs of cold-induced genes previously described in *A. thaliana*. These genes are *lti39/cor47* [13], *cor15a/b* [28], *kin1/2* [29] and *lti78/65* [26]. All of the latter four gene pairs exhibit differential expression patterns in response to low temperatures, dehydration and ABA, perhaps indicating similar functions but a specialization to different stress situations [13]. *Xero1* differs from *Xero2/lti30* in being expressed only in seeds and young seedlings (up to 3–4 days post-germination) and not during later stages of development or in mature plants (unpublished results). The gene pairs are thought to have evolved through tandem duplication [26,30]. XERO1 sequence does, however, differ significantly from XERO2/LTI30, the former possessing a serine stretch, the DEYGNP sequence and only two lysine-rich repeats.

We are currently using linker-scan analyses and gain of function studies to define the minimal sequences involved in the stress responses of the *Xero2/lti30* promoter.

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