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Alternative polyadenylation generates three low-pI α-amylase mRNAs with differential expression in barley

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Abstract Specific low-pI α-amylase genes from barley (*Hordeum vulgare* L.) produced alternative mRNAs with a 17-base 3′ extension (extension 1) or a 17-base extension beyond this (extension 2). The extended mRNAs do not arise from splicing of downstream sequences, and not all low-pI genes contain the extended sequences. All three mRNAs occur in aleurones and shoots, while extension 2 is missing from scutella. Also, the unextended mRNAs predominate in total mRNA, but the extended mRNAs predominate in membrane-bound polysomes. The extended sequences do not occur in previously characterized α-amylases, but 16 of 18 bases, mainly in extension 1, are identical with a sequence in the 3′-UTR of PAPI, a putative inhibitor of α-amylase. These observations suggest that the extended sequences could play a functional role in α-amylase expression.

Key words: α-Amylase; Membrane-bound polysome; Alternative polyadenylation; Barley

1. Introduction

α-Amylase enzyme activity in kernels of germinated barley results from the activities of two isozymes, high-pI (AMY2) and low-pI (AMY1) α-amylases. The high-pI genes (*Amy1*) are represented by 6 to 7 genes and/or pseudogenes [1], while the low-pI genes (*Amy2*) are represented by 2 to 3 genes and/or pseudogenes [2]. To understand their tissue- and temporal-specific expression, it is necessary to determine which, if any, are differentially regulated under different conditions. However, this is impossible unless sequence heterogeneity occurs between transcripts and can be assigned to particular nuclear genes within the family. Using primer extension with total RNA from GA-stimulated aleurone cells of the Himalaya cv., it was determined that low-pI α-amylase genes produce two major transcripts varying in length at the 5′-end [3,4]. The major extended products represented the cloned [5,6] low-pI mRNAs. Although sequence differences in low-pI transcripts occur, all respond coordinately to the phytohormones gibberellin (GA) and abscisic acid, and only slight differences occur in their temporal expression in the grain of developing seedlings [3,7].

Plant mRNAs are often heterogenous at the 3′ end due to multiple polyadenylation sites [8]. The combined effect of polyadenylation signals and downstream GT-rich motifs can strongly influence chimeric gene expression in plants [9]. Sequences at the 3′ end can also influence mRNA processing efficiency and/or mRNA stability in plants [10]. In animal systems, the 3′-UTR (untranslated region) influences the intracellular routing of mRNAs and their association with polysomes [11–13].

Little study has been devoted to possible 3′ terminal heterogeneity in barley α-amylase mRNAs. In GA-stimulated isolated aleurones of the Himalaya cv., polyadenylation at downstream or cryptic signals [15] has not been detected for the *Amy2* gene. Expression of α-amylase transcripts has not been studied in other barley tissues, although translatable α-amylase mRNA has been detected in scutella [16]. Thus, it is not known whether heterogeneity exists between the 3′-ends of α-amylase transcripts in other tissues or cultivars and whether the 3′-end may contribute to differential expression.

The following study describes the cloning of barley seedling low-pI α-amylase mRNAs which contain 3′ extensions beyond the known polyadenylation site. These sequences were used in a PCR approach to study alternative polyadenylation and relative expression levels between seedling tissues and between total and polysomal mRNAs in the Morex and Himalaya cultivars. The extended sequences may have functional significance and may represent low-pI genes whose expression has not been studied.

2. Materials and methods

2.1. RNA and DNA sources

Messenger RNA was prepared from aleurones, scutella, shoots and developing seeds of the malting barley (*Hordeum vulgare* L.) cultivar Morex. Seedlings were grown as previously described [17]. Isolated aleurones were prepared from deembryonated half-seeds and treated with GA_3 or buffer alone, as described [18] and modified [17]. Messenger RNA was extracted with guanidium thiocyanate [19] and isolated by oligo d(T)-cellulose chromatography [20]. Membrane-bound polysomes were purified from the kernels of 2- through 6-day-old Morex and Himalaya cv. seedlings [21,22]. Prior to oligo d(T)-cellulose chromatography, polysomal RNA was purified by phenol/chloroform extraction. Genomic DNA was purified from etiolated 8-day-old shoots by centrifugation through CsCl [17].

2.2. Polymerase chain reactions

Single-stranded cDNA was synthesized from poly(A)$^+$ mRNA by priming with oligo $d(T)_{12-18}$ and extending with AMV reverse transcriptase [23] and was used for polymerase chain reactions (PCRs; [24]). Taq polymerase and buffer were used according to the supplier's specifications (Perkin Elmer). One ηg of non-radiolabeled single-stranded cDNA was used in reaction volumes of 100 μl for all tissue PCRs, except scutella (2 ng). cDNA amounts were based upon yields from separate radiolabeled reverse transcriptase reactions. The upstream primer (LOWMIDR, 5′HO-AAGGGATACTGAACGCTG) corresponded to bases 823 to 841 (middle coding region) of barley low-pI α-amylase cDNA clone E [5]. The downstream primer LOWEND2

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(5'HO-ACCTCTTCGTATCCTCAGTACT) corresponded to the reverse complement of bases 1466 to 1487 near the 3' end of clone E. Additional downstream primers were synthesized to correspond to the reverse complement of our cloned 17-base extension, which immediately follows the 3' end of the clone E sequence (LOWEXT1, 5'HO-GGTACAAACTCGAATTT) and our cloned 17-base extension immediately following LOWEXT1 (LOWEXT2, 5'HO-TTCACCCCCA-CACAGAT) (Fig. 1). A downstream primer was also synthesized corresponding to the reverse complement of GenBank bases 2618 to 2638 of barley low-pI genomic clone Amy32b [6]. This primer (AMY32bEXT, 5'HO-CAAATGCCGTAATTGCCTCTT) extends 18 bases downstream from the terminus of clone E (Fig. 1). All oligonucleotides were synthesized by the University of Wisconsin Biotechnology Center. For PCRs with genomic DNA (200 ng), 20 pmol of primer was annealed for 2 min at 48°C for LOWEND2, 40°C for LOWEXT1, 45°C for LOWEXT2 and 46°C for AMY32bEXT. Extension was conducted at 72°C for 1 min, and melting of hybrids was conducted at 95°C for 1 min. After 30 cycles, products were extended for 7 min. Thirty-five μl of reaction products was electrophoresed 5% polyacrylamide gels in 1 × TBE buffer and detected with EtBr. The linear range of cDNA amplification was determined by conducting PCRs with increasing amounts of GA-treated aleurone cDNA and with clone Amy 14 plasmid. Amplification levels peaked with 5 ng of cDNA and declined with higher amounts. Amplification plateaued when 1 ng of Amy 14 was reached. PCRs were therefore conducted with 2 ng or less of cDNA and 0.25 ng of Amy 14 plasmid.

2.3. cDNA cloning and sequencing

A cDNA library was produced from mRNA purified from polysomes of 3- and 4-day-old seedling tissues, including kernels, scutella and shoots. cDNAs was synthesized [25], ligated to *Eco*RI linkers and cloned into the *Eco*RI site of λZapII (Stratagene). Low-pI α-amylase clones were detected by plaque hybridization [26] with insert DNA from clone E, uniformly labeled with [α-^{32}P]dCTP [27]. Clones E and Amy32b were generously provided by John Rogers (Univ. of Missouri, Columbia). Plaques were converted to plasmids by in vivo excision with R408 helper phage. Plasmid DNA was purified by lysis in alkali, phenol/chloroform extraction and PEG precipitation [23]. DNA was sequenced at both ends by dideoxy chain termination [28]. Sequence analyses, RNA folding and GenBank searches were conducted with the GCG software package [29].

2.4. Hybridization analyses

For analysis of alternative 3' end frequencies, mRNAs (1 μg) were slot-blotted to Nytran membranes (Schleicher and Schuell) in SSC/formaldehyde [30]. Membranes were hybridized with oligonucleotides in a SSPE-buffered solution [31]. Oligonucleotides (16 pmol) were end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol) with T4 polynucleotide kinase 32] and precipitated with ethanol. All hybridizations of slot blot membranes were conducted with 1 × 10^6 dpm/ml of oligonucleotides in 5 × SSPE, 1% SDS at 37°C. Filters were washed four times at 37°C in 2 × SSPE, 1% SDS for 15 min each.

Genomic DNA (10 μg) for Southern blot analysis was restricted to completion with either *Eco*RV, *Bam*HI or *Hin*dIII and electrophoresed through 0.8% agarose gels in TAE buffer. DNA was stained with EtBr and blotted [33] onto Nytran membranes. Hybridization and filter washing were conducted as above, but 2 to 4 × 10^6 dpm/ml of probe was used. For hybridization with the 1550 bp insert of clone E, 1 × 10^6 dpm/ml of DNA, uniformly labeled with [α-^{32}P]dCTP, was used, and filters were hybridized and washed at 62.5°C, as previously described [17]. Filters were autoradiographed by exposure to XAR-5 film, at exposure times within the film's linear response range, and scanned densitometrically with a soft laser.

3. Results

3.1. Cloning of extended low-pI transcripts

Low-pI clones from the seedling cDNA library were partially sequenced to about 240 bases from the 5'- and 3'-termini. Two clones (Amy 3 and 4) had 3'-tail sequences which ended and were polyadenylated at the same position as clone E (Fig. 1). All sequences were identical to that of clone E. Two clones

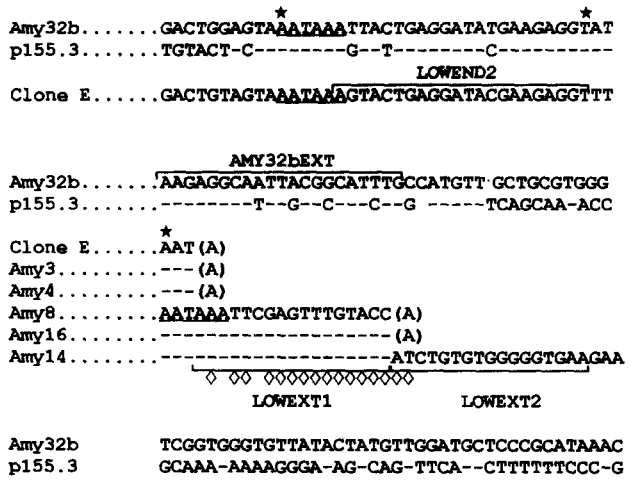

Fig. 1. Comparison of cloned low-pI α-amylase 3' end sequences with existing low-pI cDNA and genomic clones. Genomic clones Amy32b and p155.3 are compared with cDNA clone E and newly cloned cDNAs, Amy 3, 4, 8, 14, and 16. Nucleotides identical to those above are marked with a dash (–). Canononical polyadenylation signals (AATAAA) are underlined, and all potential polyadenylation signals are denoted at the beginning with a star (★). Sequences corresponding to constructed oligonucleotides LOWEND2, LOWEXT1 and 2 and AMY32bEXT are marked with brackets. Sequences identical to PAPI are noted with a ◇. Gaps are marked with a dot.

(Amy 8 and 16) represented mRNAs which had 17-base extensions beyond the 3' terminus of clone E, and one clone (Amy 14) had the same 17-base extension plus an additional 17- (possibly 19-) base GT-rich extension. Because the library was prepared from polysomal poly(A)$^+$ mRNA, the extended mRNAs which were probably actively translated. The known low-pI mRNA 3'-UTR ends with an AA sequence which marks the beginning of the 3' tail extension corresponding to primer LOWEXT1. These sequences begin with a canononical polyadenylation signal, AATAAA. However, no similar polyadenylation signal occurs downstream from this, as shown by the Amy 14 terminal sequence.

The 3'-terminal extensions have no homology with corresponding regions of the low-pI genomic clones Amy32b [6] or p155.3 [2] (Fig. 1). They also have little homology to any of the sequences stored in GenBank. Thus, the extended transcripts originated from low-pI genes which have not been previously cloned. Intriguingly, one of the best uninterupted matches is to PAPI, a putative amylase and protease inhibitor of barley [34]. In LOWEXT1 and the beginning of LOWEXT2, 16 of 18 bases are identical to an 18-base sequence in the 3'-UTR of PAPI (Fig. 1). The extended sequences provide possible opportunities for increasing the secondary structure of the 3'-UTR, although these structures would not be highly stable. The optimal secondary structure for the 3' 244 bases of Amy14 predicts a structure with a minimum free energy of −49.5 kJ/mol. Without the LOWEXT2 sequence ΔG would decline to −43.4, and without the LOWEXT1 and 2 sequences ΔG would decline to −38.1.

3.2. Relative levels of extended mRNAs in various tissues

To determine the incidence of 3'-tail extensions in various tissues, total mRNAs were applied to replicate slot blots and probed with 3' end oligonucleotides LOWEND2, LOWEXT1 and LOWEXT2. Plasmid Amy 14, containing all three se-

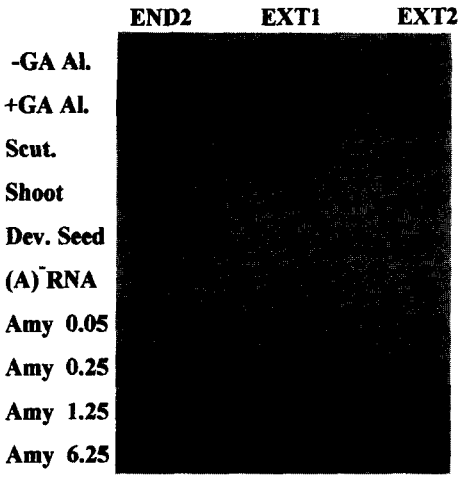

Fig. 2. Slot-blot analysis of alternative polyadenylation frequencies in various tissues. One μg of poly(A)$^+$ mRNA from each tissue was applied to each of three slot blots and probed with end-labeled LOWEND2, LOWEXT1 or LOWEXT2 oligonucleotides. Tissues included isolated aleurones treated with buffer (−GA) or with gibberellin (+GA), scutella of 4-day-old intact seedlings, shoots of same, and developing seeds (12–18 days after anthesis). One μg of poly(A)$^-$ total RNA from 4-day-old seedlings was applied as a background control. Amy 14 plasmid was applied at 0.05, 0.25, 1.25 and 6.25 ng to serve as hybridization controls. The autoradiogram was exposed for 16 h.

quences, was used as a hybridization control. Signal levels were normalized for each probe, relative to the signal strength of Amy 14 probed with LOWEND2. Values specific for LOWEND2 (representing unextended mRNAs) were computed by subtracting LOWEXT1 and 2 values and for LOWEXT1 by subtracting LOWEXT2 values. Unextended low-pI mRNAs prevailed in all tissues - 73% in GA-treated and untreated aleurones and 82–89% in scutella, shoots and developing seeds (Fig. 2). In GA-treated aleurones, 18% utilized the second polyadenylation signal. Slightly more mRNA was polyadenylated at the second site in untreated isolated aleurones, but far less was polyadenylated at the second site in scutella, shoots and developing seeds (less than 9%). Little mRNA was extended beyond the second polyadenylation site (8–9% in GA-treated aleurones and scutella and none in shoots and developing seeds).

To provide an alternative quantitation of extended mRNAs and confirm the specificity of the oligonucleotide probes, PCRs were performed with cDNA from mRNA of scutella, shoots and GA-treated aleurones. Products from upstream primer LOWMIDR to LOWEND2, LOWEXT1 and LOWEXT2 were of the expected lengths (665, 687 bp and 704 bp, respectively; Fig. 3A). Autoradiogram scanning of radiolabeled PCRs (Fig. 3B) revealed that 61% of the low-pI mRNA in aleurones is extended beyond the first polyadenylation site, while in scutella and shoots only 31 and 40%, respectively, were similarly extended. In aleurones, slightly more low-pI mRNA was polyadenylated at the third polyadenylation site, compared with the second site. This differed considerably from the slot blot analysis, perhaps from differences in the efficiencies of the oligonucleotides in priming cDNA for PCRs relative to their abilities to simply hybridize to mRNA. The scutellar cDNA yielded no PCR products with LOWEXT2, even with longer film exposures.

Approximately 15% of shoot transcripts utilized the third polyadenylation site, less than half of aleurone levels. These immature shoots were isolated from 4-day-old etiolated seedlings and included coleoptiles. To determine the pattern of expression in more developed leaves, we analyzed poly(A)$^+$mRNA from leaf blades of 8-day-old seedlings grown in complete darkness or under continuous fluorescent light for the final two days. mRNAs from green and etiolated leaves were polyadenylated at all three sites (Fig. 4A). As with 4-day-old shoots and aleurones, most mRNAs from green leaves were polyadenylated at the first site, with a much smaller but significant fraction polyadenylated at the second and third sites. Etiolated leaves favored polyadenylation at the second site.

Extended sequences beyond the first polyadenylation site of low-pI α-amylase were previously unknown. As previous analyses of Himalaya were conducted with Amy32b sequences [14], we synthesized a 21-base oligonucleotide (AMY32bEXT) which extends 18 bases beyond the Amy32b polyadenylation site (Fig. 1). Using GA-treated aleurone mRNA, we detected a very low level of PCR-amplified DNA of the expected size (Fig. 3A), confirming that appreciable polyadenylation from a secondary site does not occur with this particular low-pI gene [14].

To determine if Himalaya utilizes the same extended sequences as Morex and to examine expression in polysomal mRNA, we conducted PCRs with cDNA from membrane-bound polysomal poly(A)+mRNA from seedling kernels. In both cultivars, the same products resulted with all primers (Fig. 4B). In contrast to total aleurone mRNA, most of the polysomal mRNA in both cultivars was extended at the 3′ end (Figs. 2 and 4B). In both cultivars, a similar amount of polysomal mRNA was polyadenylated at the first and second sites.

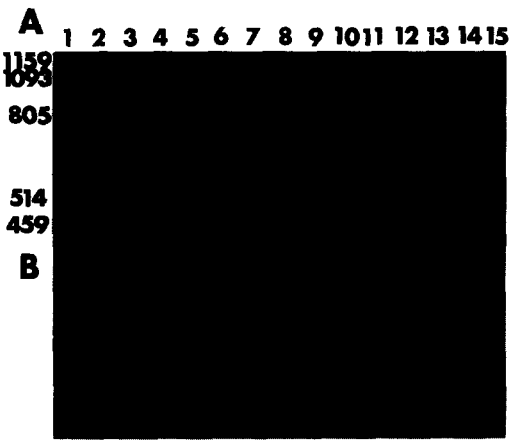

Fig. 3. PCR analysis of alternative polyadenylation frequencies in seedling tissues. Single-stranded cDNAs from aleurone (+GA; 1 ng; lanes 2, 6 and 10), scutellum (2 ng; lanes 3, 7 and 11) and shoot (1 ng; lanes 4, 8 and 12) poly(A)$^+$ mRNAs and Amy14 plasmid (0.25 ng; lanes 5, 9 and 13) were amplified with the LOWMIDR upstream primer in combination with either the LOWEND2 (lanes 2–5), LOWEXT1 (lanes 6–9) or LOWEXT2 (lanes 10–13) primers. [α^{32}-P]dCTP was added to the PCR. Panel A: Amplified products were displayed on polyacrylamide gels and stained with ethidium bromide. Aleurone cDNA (lane 14) and Amy32b (lane 15) were also amplified using AMY32bEXT as the downstream primer but were not radiolabeled. Lambda phage DNA cut with *Pst*I was used as molecular size markers, given in base pairs (bp, lane 1). Panel B: Autoradiogram of panel A gel, exposed for 1 h.

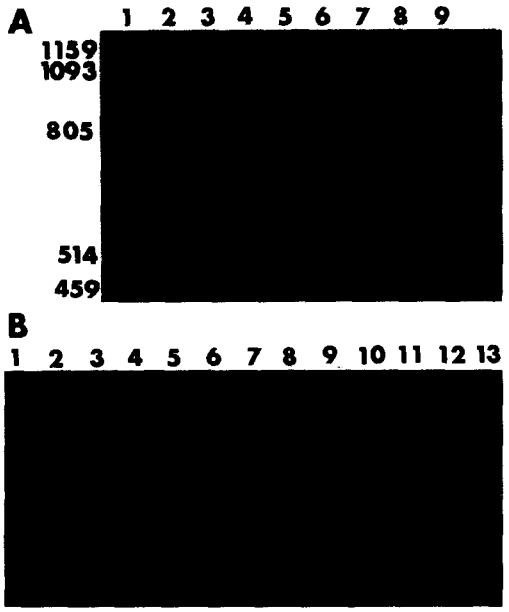

Fig. 4 PCR analysis of alternative polyadenylation in leaf mRNA and nRNA from membrane-bound polysomes of seedling kernels. Panel A: Single-stranded cDNAs from poly(A)$^+$mRNAs of 8-day-old green eaves (lanes 2–4) and 8-day-old etiolated leaves (lanes 5–7) were amplified with LOWEND2 (lanes 2 and 5), LOWEXT1 (lanes 3 and 6) or LOWEXT2 (lanes 4 and 7). Controls consisted of +GA cDNA PCRd without primers (lane 8) and LOWMIDR+LOWEXT1 primers PCRd without template DNA (lane 9). *Pst*I/Lambda molecular size markers are in lane 1, given in base pairs. Panel B: Single-stranded cDNAs from membrane-bound polysomal RNA of Himalaya (lanes 2–4) and Morex (lanes 5–7) were PCRd and compared to PCR products from Morex total poly(A)$^+$ mRNA (lanes 8–10). Amy14 plasmid (lanes 11–13) was PCRd as a primer efficiency control. Templates were amplified with downstream primers LOWEND2 (lanes 2, 5, 8 and 11), LOWEXT1 (lanes 3, 6, 9 and 12) or LOWEXT2 (lanes 4, 7, 10 and 13). *Pst*I-cut lambda molecular size markers are in lane 1. In all reactions in both panels, 1 ng of cDNA was used, and 35 μl of reaction products was electrophoresed.

3.3. Nuclear genes containing extended sequences

If the extended sequences arose from alternative transcript splicing from distant 3′ sequences or from unspliced transcripts, the lengths of PCR products from genomic DNA would be larger than those from cDNA, including the length of possible introns. In genomic clones Amy32b and p155.3, similar introns of 95 and 92 bp, respectively, occur in the same position near the 3′ end of the coding sequence [2,6]. PCR analysis with upstream primer LOWMIDR and downstream primer LOWEND2, using both Morex and Himalaya genomic DNA, produced 760 bp products, as expected (Fig. 5). Using LOWEXT1 and LOWEXT2 downstream primers, product lengths were increased by only 22 and 39 bp, as they were when cDNA was used as the template. Thus, the extended mRNA sequences did not arise from distant splicing events. Also, because similar products occur with Himalaya, they represent genes which have not previously been cloned from this cultivar.

We have attempted to determine which of the low-pI genes of Morex may produce the extended transcripts. Southern blots of genomic DNA were probed with insert DNA from low-pI cDNA clone E and with oligonucleotides LOWEXT1 (Fig. 6) and LOWEXT2 (data not shown). DNA restricted with *Hin*dIII and probed with clone E produced bands of 12, 7.4 and 3.4 kbp, as was previously found for the Himalaya cv. [3]. LOWEXT1 hybridized to the 12 kbp *Hin*dIII band (Fig. 6, lane 4 arrow) but not to the 7.4 and 3.4 kbp bands. These bands, and the uppermost bands produced with *Eco*RV (11 kbp) and *Bam*HI (10 kbp), were superimposable upon the band pattern resulting from probing with the clone E. Apparently artifactual bands were found in the *Eco*RV lane (4.0 kbp) and in the *Hin*dIII lane (2.8 kbp), perhaps through hybridization with a PAPI-like sequence. Autoradiograms from LOWEXT2 probing were too weak to discern banding patterns. Thus, at least one low-pI gene, represented by the *Bam*HI 12 kbp band, contains extended (LOWEXT1) sequences.

4. Discussion

These studies demonstrate that polyadenylation at alternative sites occurs in low-pI α-amylase genes and is common to most tissues, to varying degrees. Alternative polyadenylation occurs in many plant genes [8] but has not previously been found in the low-pI α-amylase genes, which encode the most abundant protein synthesized by isolated aleurones responding to GA [1]. The 3′-terminal sequences revealed by the extended mRNAs are specific to at least one low-pI gene, which has not previously been cloned. Other low-pI genomic clones, such as Amy32b [6] and p155.3 [1], do not contain sequences homologous to LOWEXT1 or 2. The size distribution of cDNA and genomic DNA PCR products eliminates the possibility that the LOWEXT1 and 2 extensions arose from splicing of distant downstream sequences. These sequences could thus be used to locate genomic clones representing additional low-pI genes whose transcripts are actively translated in aleurones, scutella and shoots. The extended sequences may have escaped previous detection because of the low frequency of extended low-pI mRNA in total mRNA, relative to polysomal mRNA.

The absence of LOWEXT2 PCR products from scutella indicates that, unlike other tissues, scutella do not permit polyadenylation from a third (cryptic) site or that these mRNAs are rapidly degraded. Alternatively, scutella may only transcribe low-pI genes without LOWEXT2 sequences. Due to the sensitivity of the PCRs, even leaky expression or the accumulation of unprocessed transcripts containing the LOWEXT2 sequence would have been detected.

The sequence of the 3′ end of mRNAs can greatly affect gene expression in plants [9, 10] and animals, influencing cytoplas-

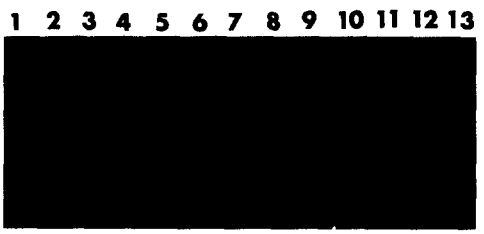

Fig. 5. Size-comparison between genomic DNA and cDNA PCR products with primers from normal and extended low-pI α-amylase mRNA 3′ sequences. Morex genomic DNA (lanes 2, 5, and 8), Himalaya genomic DNA (lanes 3, 6 and 9) and +GA aleurone cDNA (lanes 4, 7 and 10) were amplified with downstream primers LOWEND2 (lanes 2–4), LOWEXT1 (lanes 5–7) and LOWEXT2 (lanes 8–10). Control PCRs included LOWMIDR+LOWEND2 primers with no template DNA (lane 11) and Morex genomic DNA with no primers (lane 12). *Pst*I-cut Lambda size markers are in lanes 1 and 13 (sizes are given in Fig. 3).

Fig. 6. Southern blot analysis of nuclear genes containing the LOWEXT1 sequence. Morex genomic DNA was cut to completion with *Eco*RV (E, lane 2), *Bam*HI (B, lane 3) or *Hin*dIII (H, lane 4), electrophoresed through agarose gels and Southern blotted. Panel A: Filter probed with the full low-pI clone E insert DNA. Lambda DNAs cut with *Pst*I (lane 1) or *Hin*dIII (lane 5) were used for molecular size markers. Three-day exposure. Panel B: Filter probed with end-labeled LOWEXT1. Eleven-day exposure. Arrows: *Hin*dIII fragment with LOWEXT1 sequence.

mic compartmentalization, nuclear export, association of the mRNA with polysomes and distribution to either free or cytoskeletal-bound polysomes [11–13]. Likewise, we found an increased frequency of extended low-pI mRNAs in membrane-bound polysomes, relative to their distribution in total mRNA from GA-treated aleurones.

The high proportion of extended low-pI mRNAs in membrane-bound polysomes and in etiolated leaves suggests that the extended 3′ sequences may have a functional role. In plants and animals, GT-rich sequences (as in LOWEXT2) downstream from the polyadenylation signal are required for efficient 3′ end formation [9]. However, this would lead to removal of the LOWEXT2 sequence during processing. It also appears that the extended sequences would not promote formation of 3′ end secondary structures with regulatory functions, such as stem-loops. Finally, it is intriguing that the extended low-pI mRNA sequence is homologous to a similar region in the 3′-UTR of PAPI mRNA, which encodes a probable amylase/protease inhibitor [34]. As suggested by these authors, cereals may guard against precocious germination and early α-amylase activity by placing α-amylase and its inhibitor under control by the same factor, at least during late seed development. If so, this must give way to separate controls as germination proceeds.

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