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RESEARCH PAPER

An early auxin-responsive *Aux/IAA* gene from wheat (*Triticum aestivum*) is induced by epibrassinolide and differentially regulated by light and calcium

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Abstract

The plant hormone auxin plays a central role in regulating many aspects of plant growth and development. This largely occurs as a consequence of changes in gene expression. The *Aux/IAA* genes are best characterized among the early auxin-responsive genes, which encode short-lived transcriptional repressors. In most plants examined, including *Arabidopsis*, soybean, and rice, the *Aux/IAA* genes constitute a large gene family. By screening the available databases, at least 15 expressed sequence tags (ESTs) have been identified from wheat (*Triticum aestivum*), which exhibit high sequence identity with *Aux/IAA* homologues in other species. One of these *Aux/IAA* genes, *TaIAA1*, harbouring all the four conserved domains characteristic of the Aux/IAA proteins, has been characterized in detail. The expression of *TaIAA1* is light-sensitive, tissue-specific, and is induced within 15–30 min of exogenous auxin application. Also, the *TaIAA1* transcript levels increase in the presence of a divalent cation, Ca^{2+}, and this effect is reversed by the calcium-chelating agent, EGTA. The *TaIAA1* gene qualifies as the primary response gene because an increase in its transcript levels by auxin is unaffected by cycloheximide. In addition to auxin, the *TaIAA1* gene is also induced by brassinosteroid, providing evidence that interplay between hormones is crucial for the regulation of plant growth and development.

Key words: Auxin, *Aux/IAA*, brassinosteroid, calcium, light regulation, wheat (*Triticum aestivum*).

Introduction

Plant growth and development is a carefully orchestrated event regulated by both environmental and endogenous signals. Phytohormones are a vital part of this developmental process and provide cues to regulate this process in a spatio-temporal manner. The importance of auxin for plant sustenance is both vital and readily apparent: auxin elicits a plethora of plant responses including embryogenesis, lateral root development, vascular differentiation, apical dominance, tropic responses, and flower development, along with cell division, elongation, and differentiation (Cleland, 1999; Quint and Gray, 2006). The cellular responses to auxin involve changes in gene regulation and stimulation of the transcription of numerous genes. The most well characterized auxin-responsive genes are represented by the members of the *Aux/IAA* (auxin/indoleacetic acid), *GH3*, and *SAUR* (small auxin up RNA) gene families (Guilfoyle, 1999; Jain *et al*., 2006*a*, *b*, *c*).

The *Aux/IAA* gene family is comprised of at least 29 members in *Arabidopsis* (Dharmasiri and Estelle, 2004) and of 31 members in the rice genome (Jain *et al*., 2006*a*). The Aux/IAA proteins harbour four conserved domains. Domain I has been assigned a repressor function (Tiwari *et al*., 2004), domain II is responsible for rapid degradation of the Aux/IAA proteins, while domains III and IV are responsible for homo- and heterodimerizataion among the

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Abbreviations: ARF, auxin response factor; BR, brassinosteroid; CHX, cycloheximide; 2,4-D, dichlorophenoxyacetic acid; EBL, epibrassinolide; EST, expressed sequence tag; GSP, gene-specific primer; GUS, β-glucuronidase; IAA, indole acetic acid; NLS, nuclear localization signal; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

various members of the Aux/IAA and auxin response factor (ARF) proteins (Kim *et al*., 1997; Ouellet *et al*., 2001). The *Aux*/*IAA* genes were originally identified from soybean as mRNAs that are rapidly up-regulated in response to auxin (Ainley *et al*., 1988; Abel and Theologis, 1996). Within the promoters of these genes, *cis*-elements that confer responsiveness (referred to as auxin response elements or AuxREs) have been identified, and a family of *trans*-acting transcription factors (ARFs) that bind with specificity to AuxREs has been characterized (Hagen and Guilfoyle, 2002). Although ARFs, a plant-specific family of DNA-binding proteins, positively regulate the expression of *Aux*/*IAA* genes, the Aux/IAA proteins dimerize with ARFs to repress their activity. In fact, auxin initiates cascading events that lead to proteolysis of Aux/IAA proteins via the ubiquitin-ligase SCF^{TIR1} complex, thus allowing ARFs to dimerize and promote transcription of auxin-responsive genes (Gray *et al*., 2001; Kepinski and Leyser, 2004). The mechanism for auxin perception remained elusive for a long time, although recent studies demonstrated auxin action in a cell-free system (Dharmasiri *et al*., 2003; Thakur *et al*., 2005), pointing towards a soluble protein being the auxin receptor. In a pioneering work, very recently, TIR1 (an F-box protein) has been shown to be one of the auxin receptors mediating transcriptional responses to auxin (Dharmasiri *et al*., 2005*a*; Kepinski and Leyser, 2005). Evidence has also been provided whereby auxin may also interact with other F-box proteins (Dharmasiri *et al*., 2005*b*).

The transcript levels of many auxin-responsive *Aux*/*IAA* genes increase in response to the protein synthesis inhibitor cycloheximide (CHX; Abel *et al*., 1995; Thakur *et al*., 2005), suggesting that some short-lived proteins repress *Aux*/*IAA* transcription. Because Aux/IAA proteins are extremely short lived *in vivo* (Gray *et al*., 2001), these proteins themselves may act as repressors of the auxin-mediated transcriptional responses. The *Aux*/*IAA* genes have been identified in dicots (soybean, pea, mungbean, *Arabidopsis*, tobacco, cucumber, tomato, and *Populus*), cereals (maize and rice), and pine tree (Abel *et al*., 1995; Fujii *et al*., 2000; Thakur *et al*., 2001; Hagen and Guilfoyle, 2002; Moyle *et al*., 2002; Goldfarb *et al*., 2003; Jain *et al*., 2006*a*). Despite intensive studies on the role of Aux/IAA proteins in the regulation of auxin-mediated gene expression, their function is not yet fully understood. Some Aux/IAA proteins are involved in light signalling (Liscum and Reed, 2002), and mutants defective in Aux/IAA proteins are insensitive to multiple phytohormones (Wilson *et al*., 1990; Leyser *et al*., 1996; Rogg *et al*., 2001). An *Aux*/*IAA* gene from tomato has been shown to respond to ethylene (Jones *et al*., 2002) and its mRNA accumulation during ripening coincides with their ethylene regulation in immature green fruits. Brassinosteroids (BRs) also interact synergistically with auxin in hypocotyl elongation in several plant species (Sasse, 1999) and regulate changes in expression of *Aux*/*IAA* genes (Nakamura *et al*., 2006). The *Aux*/*IAA* genes have not been found in bacterial, animal, or fungal genomes, and are therefore probably unique to plants.

Earlier studies on the isolation and characterization of an auxin-induced cDNA from rice (*Oryza sativa*), *OsiIAA1* (Thakur *et al*., 2001, 2005), and identification of the *Aux*/*IAA* family in rice (Jain *et al*., 2006*a*), prompted the identification of its homologues in wheat as well. A database search revealed the existence of at least 15 cDNAs in wheat corresponding to homologues of *Aux*/*IAA* genes present in other plants. Simultaneously, an *Aux*/*IAA* cDNA (*TaIAA1*) was isolated by screening the wheat cDNA library using rice *OsiIAA1* (Thakur *et al*., 2001) as a probe. This study provides evidence that this primary auxin-responsive gene encodes a nuclear-localized protein, whose levels are up-regulated by auxin and BR, and down-regulated by light. Calcium ions also stimulate *TaIAA1* expression and probably mediate the action of auxin and/or BR in regulation of gene expression.

Materials and methods

Plant material and growth conditions

Wheat (*T. aestivum*) seeds were obtained from the Directorate of Wheat Research of the Indian Council Agricultural Research Institute, Karnal. Seeds were washed thoroughly with reverse osmosis (RO) water after disinfecting with 4% sodium hypochlorite for 30 min. Seedlings were grown on cotton saturated with RO water at 28 °C, either in the dark or in constant light provided by a bank of fluorescent tube lights (Philips TL 40 W/54, 6500 K) with a fluence rate of 70 μmol m^{-2} s^{-1}, as per experimental requirements.

Coleoptile elongation assay

Wheat seedlings were grown in complete darkness for 72 h. The elongation zone (the middle segment) of 5 mm length of the etiolated coleoptile was excised. The segments were incubated in KPSC buffer (10 mM potassium phosphate, pH 6.0, 2% sucrose, 50 μm chloramphenicol) for 12 h, to deplete endogenous auxins; the buffer was replaced every 1 h (Thakur *et al*., 2001). The segments were then transferred to fresh KPSC buffer containing different concentrations of IAA, 2,4-dichlorophenoxyacetic acid (2,4-D) or epibrassinolide (EBL). Control segments were incubated in the KPSC buffer for the same duration. Similarly, the segments were first incubated in KPSC buffer for 14 h and then transferred to fresh KPSC buffer supplemented with 320 μM equivalent salt of the divalent cation, Ca^{2+}, with or without 30 μM IAA/2,4-D. The calcium-chelating agent, EGTA, was used to demonstrate the specificity of the effect of Ca^{2+}. The length of the coleoptile segments was recorded after the desired duration using a measuring scale. For every treatment, the length of at least 20 coleoptile segments was measured and the values plotted indicate the mean ±SE. The whole experiment was performed in a dark room under a green safe-light. Each experiment was performed at least twice and the data of only a representative experiment are presented.

Isolation of TaIAA1 *cDNA and sequencing*

The dark-grown 4-d-old wheat seedling cDNA library was made using the cDNA synthesis kit, ZAP express™ and Gigapack III gold (Stratagene Cloning Systems, USA), according to the manufacturer's

instructions (Kulshreshtha *et al*., 2005), and screened by using radiolabelled full-length *OsiIAA1* cDNA from rice as probe (Thakur *et al*., 2001). The hybridization was carried out at 58 °C in the buffer containing 6× SSC, 5× Denhardt's solution, 0.5% SDS, and 100 μg ml^{-1} denatured herring sperm DNA. After screening ~5×10^6 recombinant plaques, 24 putative clones were selected and further purified through three rounds of successive screening. Single clone excision was done to obtain recombinant pBK-CMV phagemids according to the manufacturer's instructions. T3 and T7 primers were used for sequencing, and the sequence from the 5′ end of one of the clones showed significant homology to *Aux*/*IAA* cDNAs. The clone was thus designated as *TaIAA1*. Complete sequencing was done using an automated ABI Prism 3700 DNA Analyzer (Applied Biosystems, USA), with the ABI Prism Big Dye Terminator V 2.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA), as per the manufacturer's instructions.

Completion of the 5′ untranslated region (UTR) by rapid amplification of 5′ cDNA ends (5′ RACE)

First-strand cDNA was synthesized from total RNA (1 mg) isolated from leaf bases of 13-d-old light-grown wheat seedlings treated with 2,4-D (30 μM) for 1 d, using a gene-specific primer (GSP), 5′-GGAAGATCTCTTCCTCTTACTGCCCCATC-3′ and Stratascript II reverse transcriptase (Gibco-BRL, USA). This was purified using a gel extraction kit (Qiagen) and a T3 adaptor, 5′-CCCTTTAGT-GAGGGTTAATTTC-3′ (3AC7), and was ligated to the 5′ end of the single-stranded cDNA by using T4 RNA ligase (Ambion Biochemicals, USA). The adaptor was modified at its 5′ end by phosphorylation and at its 3′ end by amino modification. The ligated product was again purified using a Qiagen column and then used as a template for PCR amplification with the T3 adaptor and GSP. The PCR product was cloned in pGEMT-easy vector and sequenced using T7 and SP6 primers.

Northern analysis

Total RNA was isolated from different tissues (Nagy and Schafer, 2002) and resolved on a 1.2% agarose gel containing 1.1% formaldehyde, at 120 V. After alkaline blotting to a Hybond-N^+ membrane (Amersham), hybridization was carried out in 50% formamide, 5× SSC, 5× Denhardt's solution, 0.1 M sodium phosphate buffer, pH 6.5, 10% dextran sulphate, and 250 μg ml^{-1} of denatured herring sperm DNA at 42 ° C, using [α-^{32}P]dATP-labelled full-length *TaIAA1* cDNA as a probe. The membrane was then subjected to three successive washes for 5, 15, and 15 min, with 2× SSC/0.5% SDS, 2× SSC/0.1% SDS, and 0.1× SSC/0.5% SDS, respectively, at room temperature. Autoradiography and X-ray film development were performed as described above, and ethidium bromide-stained rRNA served as a control to estimate the relative amounts of rRNA in each lane.

Identification of Aux/IAA *homologues in wheat (*T. aestivum*)*

To identify *Aux*/*IAA* homologues in wheat (*T. aestivum*), the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nim.nih.gov/BLAST/nr/EST), and the Institute for Genomic Research (TIGR) database (http://www.tigrblast.tigr.org/euk-blast) resources were used. The amino acid sequences of all the rice Aux/IAA proteins were downloaded and used to search for their homologues in wheat using the TBLASTN program in the NCBI (nr and est) and TIGR databases, the redundant sequences were removed by the ClustalX program (version 1.83), and the full-length cDNA sequences and partial expressed sequence tags (ESTs) recovered. The search was limited to the identification of at least three domains in these ESTs to avoid retrieving the ARF sequences (Jain *et al*., 2006*a*).

RNA isolation and real-time PCR analysis

Total RNA was extracted using the RNeasy Plant mini kit (Qiagen, Germany) according to the manufacturer's instructions, followed by DNase I treatment to remove any genomic DNA contamination. The quantitative real-time PCR analysis was performed as described earlier (Jain *et al*., 2006*b*). In brief, the cDNA samples synthesized from 3 μg of the total RNA using the High Capacity cDNA Archive kit (Applied Biosystems, USA) were used as template and mixed with 200 nM of each primer and SYBR Green PCR Master Mix (Applied Biosystems) for real-time PCR analysis, using the ABI Prism 7000 Sequence Detection System and Software (PE Applied Biosystems) according to the manufacturer's instructions. Each pair of primers designed by using Primer Express 2.0 software (PE Applied Biosystems) was checked by the BLAST program in wheat sequences available in the TIGR database to ensure that the primers amplify a unique and desired cDNA segment. The primer sequences are listed in Supplementary Table 1 available at JXB online. The specificity of the reactions was verified by melting curve analysis. The relative mRNA levels for the *TaIAA1* and other *Aux*/*IAA* genes in RNA isolated from various tissue samples were quantified with respect to the internal standard, actin. At least two independent RNA isolations were used for cDNA synthesis, and each cDNA sample was subjected to real-time PCR analysis in triplicate.

Results

Cell elongation is one of the most rapid and well studied auxin-responsive phenomena that is generally accompanied by activation of a set of primary or early induced genes (e.g. *Aux*/*IAA*, *GH3*, and *SAUR*s). These genes further influence the expression of secondary and/or late responsive genes governing the end product/phenotype controlled by auxins (Cleland, 1999). Although a number of early auxin-inducible genes have been sequenced and characterized in various dicots, *OsiIAA1* from rice was the first early auxin-inducible gene isolated and characterized from monocots, which may also have a probable role in cell elongation (Thakur *et al*., 2001, 2005). The work on the rice *Aux*/*IAA* family was extended further (Jain *et al*., 2006*a*), and the scope of the work was also enlarged to another important and rather more complex cereal crop, i.e. wheat.

Spectrum of Aux/IAA *genes in wheat*

In an attempt to identify Aux/IAA protein-coding genes in wheat, a TBLASTN search of cDNA clones or ESTs of wheat available at TIGR and NCBI (nr and est) was performed using 31 OsIAA (Jain *et al*., 2006*a*) proteins as query. In this search, 15 non-redundant clones (among a total of 64; including *TaIAA1*; DR740490) having high sequence similarity to OsIAA proteins could be identified. Their GenBank accession numbers, nucleotide length, and the conserved domains are given in Table 1 (see also Supplementary Fig. 1 available at JXB online). The absence of domain I in most of the sequences (10 out of 15) may be due to the incomplete cDNA/EST sequences available. Some of these genes were also examined for their auxin inducibility (described later). Simultaneously, one of the auxin-inducible cDNAs from wheat was isolated

Table 1. Aux/IAA *gene family in wheat*

Accession no.[a]	Length (bp)[b]	Domains present[c]
DR741441	1107	I, II, III, IV
AL808316	524	II, III, IV
CK170519	1170	I, II, III, IV
CK207694	1113	I, II, III, IV
BJ225591	587	I, II, III, IV
AL810891	1015	II, III, IV
B1751049	559	II, III, IV
CK163783	1030	II, III, IV
CD894763	700	II, III, IV
CF132953	775	II, III, IV
DN829348	910	II, III, IV
DR740490	1084	I, II, III, IV
DR741584	1093	II, III, IV
DR732955	1159	II, III, IV
CK210695	1124	II, III, IV

[a] GenBank accession number.
[b] Length of cDNA/EST in bp.
[c] Domains present in the predicted Aux/IAA proteins (some are partial clones).

by screening a cDNA library made from 4-d-old etiolated wheat seedlings (Kulshreshtha *et al*., 2005), using rice *OsiIAA1* (Thakur *et al*., 2001) as a probe. The cDNA (accession no. AJ575098) thus isolated has been designated as *TaIAA1* (*T. aestivum* IAA 1) and its characteristic features are described below.

Characterization of TaIAA1 *cDNA*

The *TaIAA1* cDNA isolated originally by library screening was 1104 bp long with an open reading frame of 702 bp. The cDNA was further extended by 5′ RACE increasing its size to 1209 bp. A 181 bp 5′ UTR precedes the initiation codon, ATG. The 3′ UTR of 527 bp contains a poly(A) site and two potential polyadenylation signals at 59 bp and 201 bp upstream of the poly(A) site (see supplementary Fig. 2 available at JXB online). The cDNA encodes a protein of 234 amino acids with a predicted molecular mass of ~24.88 kDa. The predicted amino acid sequence of TaIAA1 showed significant identity (32–45%) with known Aux/IAA proteins. The TaIAA1 protein contains all the four domains (I–IV) that are highly conserved in Aux/IAA proteins, with the amino acid homology reaching up to 77% in these regions (see supplementary Fig. 2 available at JXB online). Invariant amino acids of the variant region (Thakur *et al*., 2001) are also present in TaIAA1, and the basic amino acids located in between domains I and II (…KR…RSYR….) may constitute a bipartite nuclear localization signal (NLS) (Robbins *et al*., 1991; Gorlich and Mattaj, 1996) (see supplementary Fig. 3 available at JXB online). A basic cluster KRLRIMK, resembling SV40 (Abel and Theologis, 1995) and a MAT α2-like NLS (Raikhel, 1992) is also present at the end of domain IV (see supplementary Fig. 3 available at JXB online) (Abel *et al*., 1994). TaIAA1 protein contains a βαα amphipathic region in a stretch of 130–170 amino acids, which covers domain III. This βαα amphipathic region constitutes a DNA-binding domain in Arc and MetJ prokaryotic repressors. The particle bombardment of onion epidermal cells with a β-glucuronidase (GUS) reporter gene fused in-frame with the complete *TaIAA1* cDNA or harbouring one of the two NLS sequences suggests that both the bipartite NLS present in the first half and the SV40-type NLS present in the second half can independently drive the protein to the nucleus, and may have an additive effect in driving the protein to the nucleus (see supplementary Fig. 4 available at JXB online). Overall, these observations suggest that TaIAA1 is a nuclear-localized protein and both types of NLS sequences present are functional.

Southern analysis

To determine whether the gene corresponding to *TaIAA1* cDNA belongs to a multigene family or is represented as a single copy, the wheat genomic DNA was digested with different restriction enzymes and processed for Southern analysis at high stringency (50% formamide, 42 °C) using complete *TaIAA1* cDNA as a radiolabelled probe. The autoradiogram shows that either two or three fragments prominently hybridized after restriction digestion with *Bam*HI, *Pst*I, *Eco*RI, and *Hin*dIII (see supplementary Fig. 5 available at JXB online). However, under low stringency conditions for hybridization, some more bands could be detected in the autoradiogram (data not presented). This indicates that *TaIAA1* belongs to a multigene family, which is similar to the situation in most of the other plant species examined (Abel *et al*., 1995; Thakur *et al*., 2001; Moyle *et al*., 2002; Jain *et al*., 2006*a*).

Auxin-induced changes in expression of wheat Aux/IAA *genes, including* TaIAA1

The effect of the natural auxin, IAA, and/or the synthetic auxin, 2,4-D, was investigated on the expression of *Aux/IAA* genes in the etiolated wheat coleoptiles excised from 3-d-old seedlings displaying maximum elongation. The endogenous auxin was depleted by floating the coleoptiles in the depletion buffer (KPSC buffer) for 14 h in the dark. The explants were then transferred to fresh KPSC buffer supplemented with various concentrations of 2,4-D or IAA for 20 h in the dark. The coleoptiles exhibited a 1.5- to 2-fold increase in length at 30 μM concentration compared with the controls (Fig. 1A), while at higher concentrations there was relatively less elongation. Also, IAA in general caused a greater increase in coleoptile elongation than did 2,4-D (Fig. 1A).

The elongation response of three different segments of coleoptile towards auxins was also examined at the optimal concentration, i.e. 30 μM (Fig. 1B). The coleoptile was excised into 5 mm segments each at the base, middle, and the tip region, and the middle segment was observed to show the maximum response (~2.5-fold increase over the

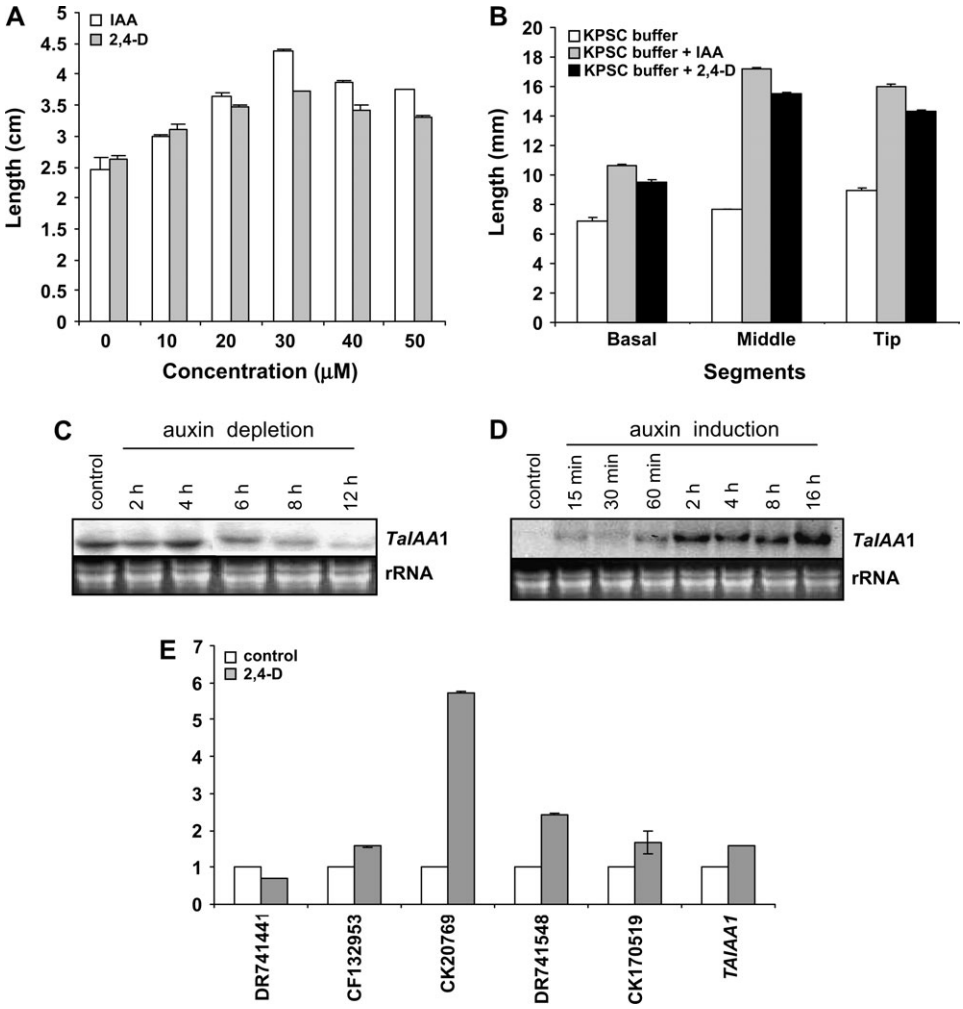

Fig. 1. (A) Effect of various concentrations of IAA and 2,4-D on elongation growth of 3-d-old etiolated wheat coleoptiles after 14 h of endogenous auxin depletion in KPSC buffer. (B) Effect of IAA and 2,4-D on elongation of segments (basal, middle, and tip) of coleoptiles from 3-d-old dark-grown wheat seedlings. The measurements were taken after 20 h of auxin treatment and are expressed as the mean ±SE (C) The kinetics of decrease in *TaIAA1* transcript abundance during depletion of endogenous auxin in the excised coleoptile segments in KPSC buffer. (D) Northern blot hybridization showing the kinetics of increase in *TaIAA1* transcript abundance in excised coleoptile segments of 3-d-old etiolated wheat seedlings by 30 μM IAA, after 14 h of auxin depletion. As a control, RNA was isolated from 14 h auxin-depleted tissue. Each lane contains 20 μg of RNA, and ethidium bromide-stained rRNA represents the control in both (C) and (D). (E) The relative mRNA levels of five Aux/IAA cDNAs in control (14 h auxin depleted) and 2,4-D- (30 μM) (2 h) treated coleoptile segments of 3-d-old etiolated wheat seedlings. The error bars represent the mean ±SD of two biological replicates, each analysed with three technical replicates.

control). The basal and tip segments also showed an increase in length, but not as great as that displayed by the middle segment (Fig. 1B).

To determine the changes in *TaIAA1* transcript abundance after depletion of endogenous auxin, the total RNA was isolated from etiolated segments floated in KPSC buffer at varying time intervals and subjected to northern analysis. As compared with the undepleted control, there was a slight decrease within 2 h in the steady-state transcript levels of *TaIAA1*, which gradually declined and became negligible after 12 h (Fig. 1C). For induction experiments, the endogenous auxin was depleted by floating the excised coleoptile segments in KPSC buffer for 14 h and then treated with 30 μM IAA for various times. The increase in transcript abundance was apparent within 15 min of incubation, increasing further with extended duration of IAA treatment (Fig. 1D). The expression of six other *Aux*/*IAA* genes of wheat was also examined by real-time PCR and all but one of these genes could be induced by auxin in etiolated coleoptile segments (Fig. 1E); maximum induction was recorded in clone CK20769.

Auxin-induced TaIAA1 *expression is insensitive to CHX*

CHX is known to induce the early auxin-responsive genes (Abel *et al*., 1995; Thakur *et al*., 2005). The steady-state transcript accumulation was monitored in excised etiolated wheat seedlings treated with 50 μM CHX for various times. The *TaIAA1* transcript levels increased within 30 min in the presence of exogenously supplied CHX, and a sustained

increase was registered for up to 5 h (Fig. 2A). The CHX treatment could not abolish the inductive effect of IAA and, in fact, their effects on the increase in *TaIAA1* transcript accumulation were additive (Fig. 2B).

Light-sensitive and tissue-specific expression of TaIAA1

The changes in *TaIAA1* transcript abundance were examined by northern analysis using the total RNA extracted from roots and shoots of both light-grown and etiolated wheat seedlings (Fig. 3). In etiolated wheat seedlings, the transcript level of *TaIAA1* was most in the upper portion of shoots, followed by the lower portion, and least in the root. This expression was negligible in light-grown roots although moderately present in the shoot (Fig. 3A). In addition, the transcript was detectable in 1-month-old leaf base calli cultured on 2,4-D medium (Fig. 3B).

Light and phytohormones profoundly influence plant growth and development. There are some compelling results that suggest that light, besides its effect on plant growth and development, can regulate *Aux/IAA* expression at the molecular level (Colon-Carmona *et al*., 2000; Thakur *et al*., 2001, 2005). To determine whether *TaIAA1* is up- or down-regulated by light, the kinetics of accumulation of the *TaIAA1* transcript were examined in seedlings exposed to white light for various times. There was a distinct decrease in *TaIAA1* transcript levels within 3–4 h in both 3- and 5-d-old etiolated wheat seedlings exposed to light (Fig. 3C, D).

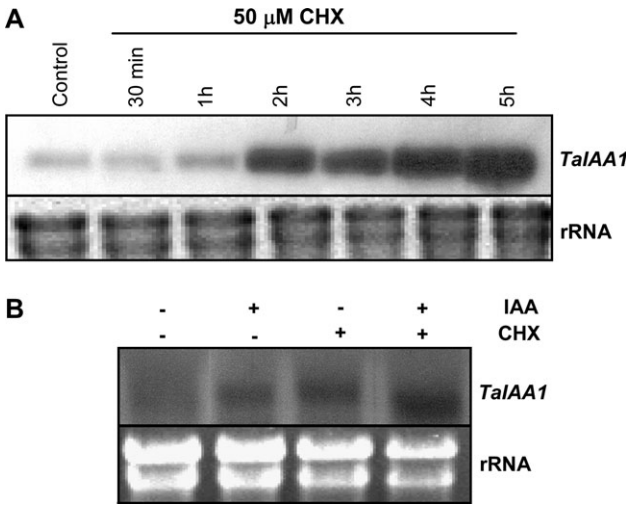

Fig. 2. Northern blot analysis to study the effect of cycloheximide (CHX) on *TaIAA1* transcript abundance. After auxin depletion in KPSC buffer for 14 h, the excised coleoptile segments of 3-d-old etiolated wheat seedlings were treated with 50 μM CHX for different times. Control indicates RNA isolated from etiolated seedlings after auxin depletion. (B) Northern blot hybridization to show the effect of CHX in the presence or absence of IAA (indicated on the top of each lane). The coleoptile segments were incubated in KPSC buffer after auxin depletion in either the presence or absence of IAA or CHX or both, as indicated. Each lane was loaded with 20 μg of total RNA. Ethidium bromide-stained rRNA represents the control.

Calcium-induced changes in TaIAA1 *expression and elongation growth*

Calcium, a second messenger in many hormone-regulated responses, plays a key role in various cellular and physiological processes of higher plants (Harper, 2001). In the present study, the effect of calcium was examined on *TaIAA1* gene expression and elongation growth of coleoptile segments. The middle segments of 3-d-old etiolated coleoptiles of wheat were first incubated in KPSC buffer for 14 h and then transferred to fresh KPSC buffer supplemented with 30 μM 2,4-D and/or 320 μM $CaCl_2$ for 20 h in the dark. There was increased elongation in the presence of both auxin and calcium. The chelating agent, EGTA, virtually arrested the elongation growth of cut segments in the presence of auxin alone or even when incubated together with calcium (Fig. 4A). The etiolated wheat coleoptile segments showed a more conspicuous curving response when both 2,4-D and $CaCl_2$ were supplied simultaneously (Funke and Edelmann, 2000). Northern studies also revealed essentially a similar picture with respect to changes in *TaIAA1* transcript levels on treatment with Ca^{2+} and/or auxin. The *TaIAA1* transcript abundance in the excised etiolated coleoptiles treated with 320 μM $CaCl_2$ increased with or without auxin, which could be checked by EGTA application (Fig. 4B).

Induction of TaIAA1 *transcripts by BR*

Plants exhibit different BR sensitivities, depending on endogenous or exogenous factors, such as organ type, environment, and growth stage (Nakamura *et al*., 2006). To determine the optimal effective concentration of EBL,

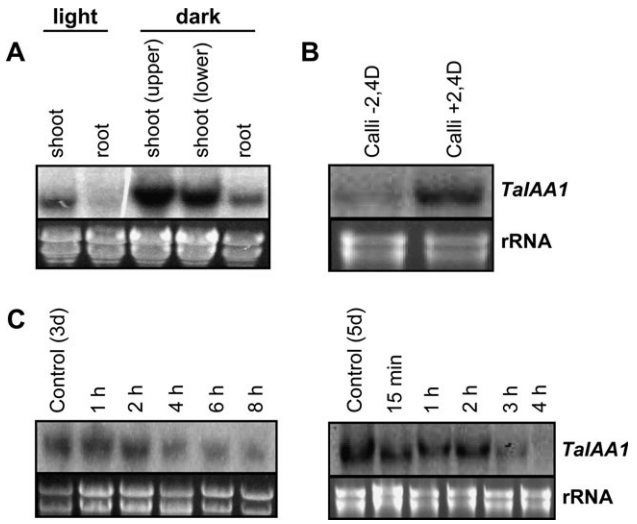

Fig. 3. The steady-state transcript levels of *TaIAA1* in various tissues of wheat seedlings grown (A) in the dark or light for 5 d and (B) in calli placed on MS basal and MS basal+30 μM 2,4-D medium. (C) Down-regulation of *TaIAA1* transcripts in 3-d-old dark-grown (left panel) and 5-d-old dark-grown (right panel) wheat seedlings by light. Each lane was loaded with 20 μg of total RNA. rRNA represents the control.

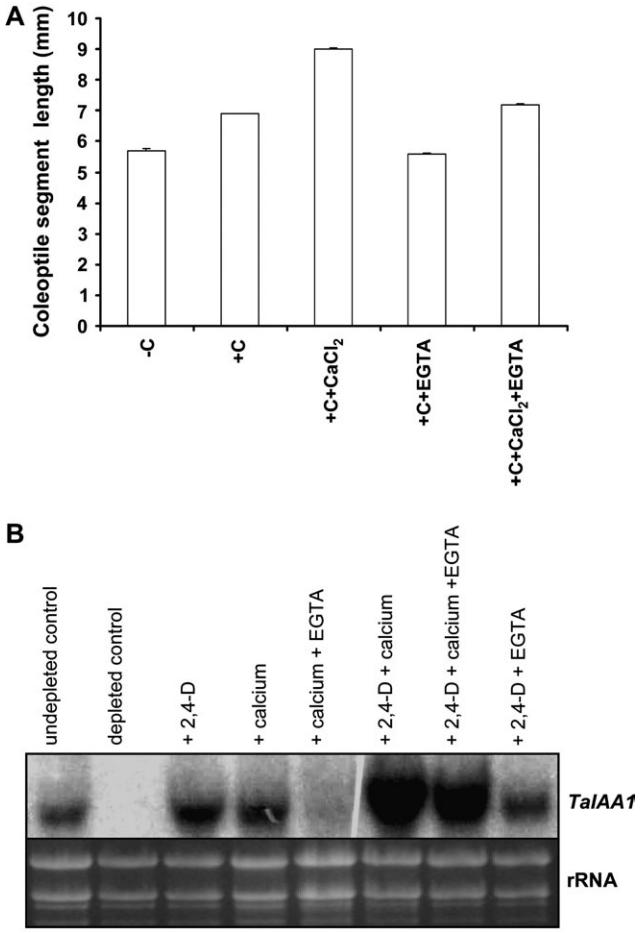

Fig. 4. (A) Effect of Ca^{2+} ions on the elongation growth of the middle segment of the coleoptile from 3-d-old dark-grown wheat seedlings. The segments were first incubated in KPSC buffer for 14 h and then transferred to fresh KPSC buffer supplemented with 30 μM 2,4-D and 320 μM equivalent salts of Ca^{2+} or the chelating agent, EGTA. The segment length was measured after 20 h of treatment in the dark. –C, control segments incubated in KPSC buffer only, +C, control segments incubated in KPSC buffer containing 30 μM 2,4-D. (B) Northern blot hybridization showing the effect of $CaCl_2$ (320 μM) and 2,4-D (30 μM) on *TaIAA1* transcripts and its reversal by EGTA (320 μM). Each lane was loaded with 20 μg of total RNA, and ethidium bromide-stained rRNA represents the control.

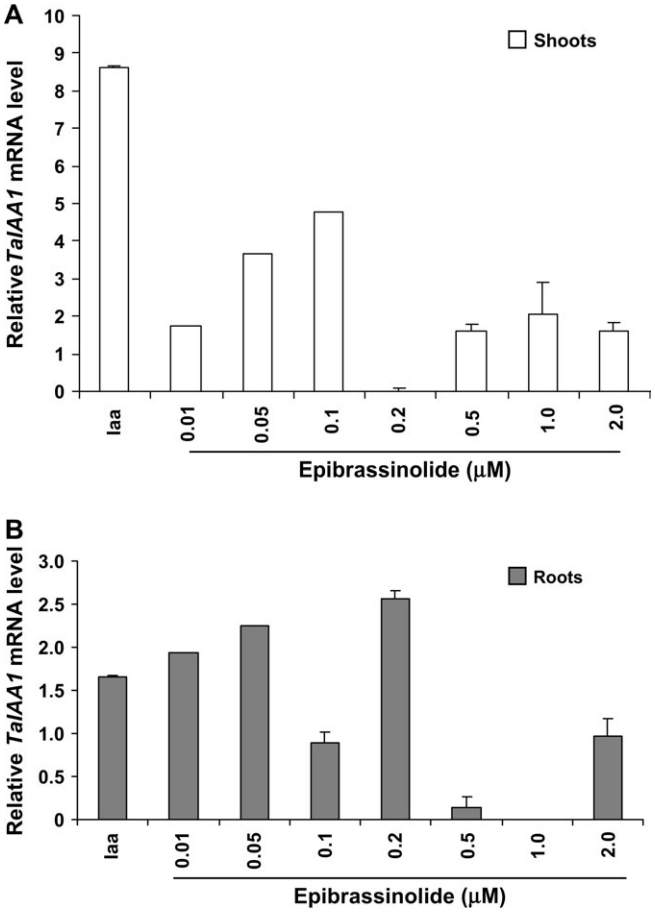

Fig. 5. Changes in *TaIAA1* mRNA levels after exposure to different concentrations of epibrassinolide (in μM) in 4-d-old etiolated wheat (A) shoots and (B) roots. The relative mRNA levels were normalized with respect to the house-keeping gene, actin. The error bars represent the mean ± SD of two biological replicates, each analysed with three technical replicates.

real-time PCR was performed with total RNA isolated from etiolated shoots and roots of the seedlings treated with varying concentrations of EBL. This analysis revealed that concentrations which were inhibitory to shoots (200 nM) were promoting expression of *Aux/IAA* transcript in roots, and concentrations which were inhibitory to roots were promoting expression of *Aux/IAA* transcript in shoots (100 nM and 1 μM) (Fig. 5A, B). Northern analysis revealed increased *TaIAA1* transcript accumulation in etiolated shoots in the presence of EBL, although it was less effective as compared with auxin (at least at 100 nM) (Fig. 6A). The expression was undetectable in roots of dark-grown seedlings (Fig. 6B). The kinetic studies revealed that the pattern of *TaIAA1* transcript accumulation was essentially similar to auxin; the increase in transcript abundance was visible within 1 h and, thereafter, a gradual increase was observed up to 16 h (Fig. 6C, D). Subsequently, the etiolated shoots were depleted of endogenous auxin in KPSC buffer for 14 h and then incubated with BR (10 nM and 100 nM) alone and/or with auxin (30 μM) for 2 h. The level of *TaIAA1* transcript increased much more in the combined presence of both auxin and BR (Fig. 6E).

Discussion

Aux/IAA *gene family in wheat*

The *Aux/IAA* genes are present as a multigene family in nearly all plants examined, including soybean (Ainley *et al.*, 1988), pea (Oeller *et al.*, 1993), mungbean (Yamamoto *et al.*, 1992), tobacco (Dargeviciute *et al.*, 1998), tomato (Nebenfuhr *et al.*, 2000), *Arabidopsis*

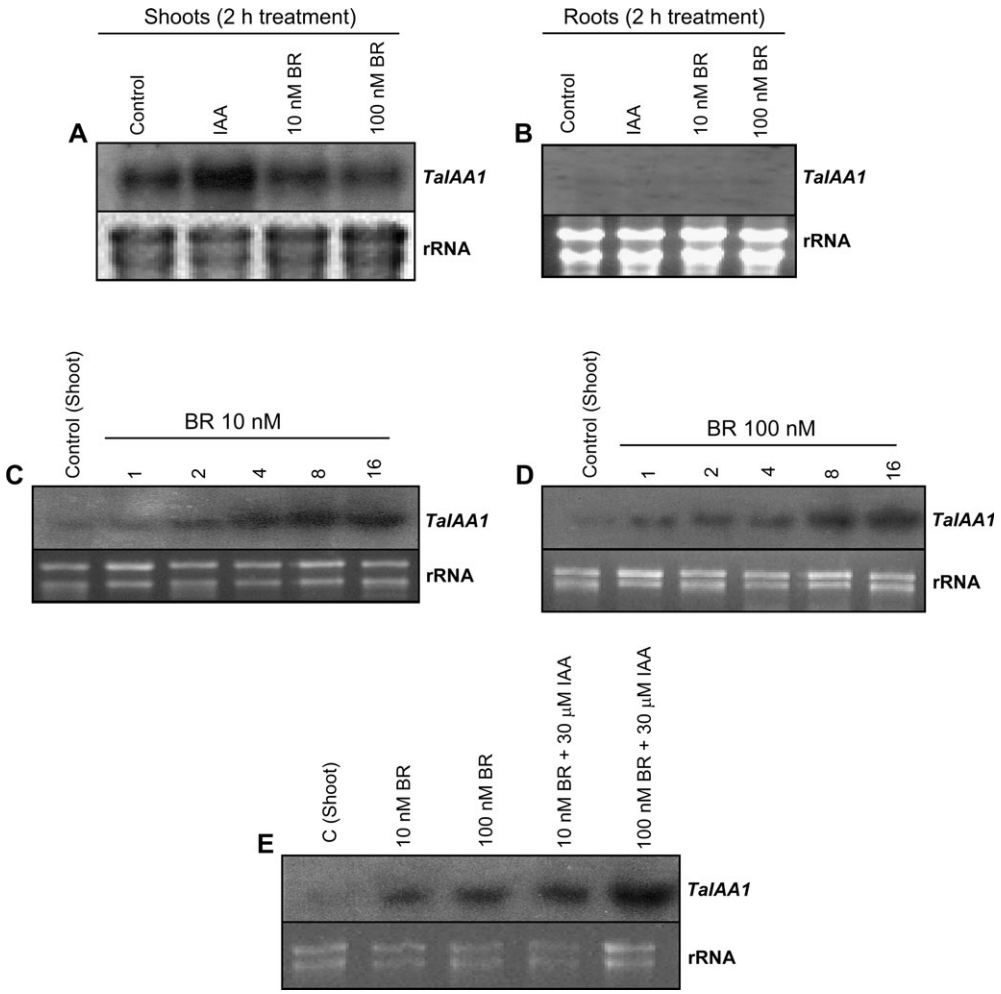

Fig. 6. Brassinosteroid and auxin induction of *TaIAA1* in 4-d-old etiolated wheat seedlings. EBL (10 nM and 100 nM) and auxin (30 μM) were used to induce *TaIAA1* transcript accumulation for 2 h in (A) shoots and (B) roots in the dark. Control indicates RNA from etiolated seedlings without auxin depletion. The lower panel of the figures shows northern blot analysis to study the effect of EBL on *TaIAA1* transcript abundance. After auxin depletion in KPSC buffer for 14 h, the excised coleoptile segments of 4-d-old etiolated wheat seedlings were treated with 10 nM (C) and 100 nM (D) of EBL for the different times indicated on the top of the lanes (in hours). (E) Effect of BR in the presence or absence of IAA (as indicated on the top of each lane). The coleoptile segments were incubated in KPSC buffer after depletion in either the presence or absence of IAA or BR, or both, as indicated. Control indicates RNA isolated from auxin-depleted etiolated seedling coleoptiles. Each lane was loaded with 20 μg of total RNA. Ethidium bromide-stained rRNA represents the control.

(Liscum and Reed, 2002), *Populus* (Moyle *et al*., 2002), and loblolly pine (Goldfarb *et al*., 2003). Very recently, the rice genome has been shown to contain 31 *Aux*/*IAA* genes (Jain *et al*., 2006*a*). Southern analysis indicated that *Aux*/*IAA* genes may be represented as a multigene family in the wheat genome, as has been shown in the case of rice (Thakur *et al*., 2001; Jain *et al*., 2006*a*). The overall *in silico* analysis (present study) also revealed that there are at least 15 ESTs (or cDNAs) of auxin-inducible *Aux*/*IAA* genes present, although the expected number in the hexaploid genome of wheat may be much more, bearing in mind the fact that rice has >30 members of this family (Jain *et al*., 2006*a*). For the present, the *TaIAA1* cDNA has been isolated and characterized in detail from the hexaploid wheat (*T. aestivum*).

The molecular mass of Aux/IAA proteins in general ranges from 19 kDa to 36 kDa (Abel and Theologis, 1995; Guilfoyle *et al*., 1998). The *TaIAA1*-encoded protein also falls in this range, with a calculated molecular mass of 24.88 kDa. The *TaIAA1*-encoded polypeptide shares four conserved domains, I, II, III, and IV, and seven invariant residues in the intervening region. Overall sequence identity between the conserved domains is highly variable (36–87%). The amino acid alignment of TaIAA1 with known Aux/IAA proteins shows conserved regions of basic amino acids. Domain III, along with five invariant hydrophobic residues, forms the βαα structure which shows similarity to the βαα DNA-binding domain of prokaryotic repressor Arc and MetJ (Pabo and Saver, 1992; Gray *et al*., 2001). This putative prokaryotic βαα

DNA-binding motif is required for protein dimerization (Kim and Harter, 1997) and protein–protein interaction. The Aux/IAA proteins are short-lived nuclear proteins, and domain II is critical for rapid degradation via the SCF^{TIR1} complex. In a very recent study in *Arabidopsis*, it has been shown that regions outside of Aux/IAA domain II could independently regulate the proteolysis of specific Aux/IAA family members (Dreher *et al*., 2006). Domain III and IV are dimerization domains that are conserved not only among the Aux/IAA proteins but also among most ARFs (Kim and Harter, 1997; Ulmasov *et al*., 1999).

The TaIAA1 protein, like other Aux/IAA proteins (Abel *et al*., 1995; Jain *et al*., 2006*b*), has a Matα2-like putative NLS sequence and an SV40-type NLS sequence. Both these sequences were found to be functional in targeting the GUS–protein fusion to the nucleus in onion epidermal cells. The Aux/IAA proteins are known to interact with ARFs (known transcription factors) to function as repressors of auxin-induced gene(s) (Ulmasov *et al*., 1999).

The TaIAA1 *is a primary auxin-responsive gene*

Besides a fast induction phenomenon, early auxin-responsive genes are characterized by increased mRNA accumulation even when *de novo* protein synthesis is blocked. The mechanism for such induction is supposed to involve both stabilization of mRNAs and derepression of transcription (Koshiba *et al*., 1995). Earlier studies employing nuclear run-on transcription assays and metabolic inhibitors also indicate that many *Aux*/*IAA*s are transcriptionally regulated (Guilfoyle, 1999). In the present study, although CHX itself up-regulated *TaIAA1* transcript accumulation, the addition of IAA further augmented the response. This CHX insensitivity towards auxin action indicates that *TaIAA1* is a primary auxin-responsive gene and that its expression is up-regulated by auxin independently of *de novo* protein synthesis.

Tissue-specific and auxin-induced changes in TaIAA1 *expression and coleoptile elongation growth*

Auxin-mediated cell elongation is one of the fastest known hormonal responses (with a lag period of 15–25 min) (Abel *et al*., 1995). Exogenously supplied auxin affects the elongation of excised coleoptiles, and this is supported by the present study. The *TaIAA1* transcripts declined on auxin depletion and this is consistent with most of the auxin-inducible genes that have a short half-life. An increase in elongation of the middle segment of the excised coleoptiles occurred with the application of 30 μM 2,4-D or IAA. The ability of exogenous auxin to promote cell elongation in excised stem and hypocotyls has been studied extensively previously (Hagen *et al*., 1984). Most *Aux*/*IAA* genes respond to exogenous auxin within the first 30 min of treatment, similar to the *SAUR* genes, the fastest responding genes known to be induced by auxin (Gee *et al*., 1991). In the case of *TaIAA1* too, transcripts started accumulating as early as 15–30 min after auxin application. The *TaIAA1* transcripts were present in shoots of 5-d-old light-grown wheat seedlings but were almost undetectable in roots, whereas its expression in the dark could be detected in roots, although it was still lower than in shoots. Essentially a similar tissue-specific profile was reported for rice *OsiIAA1* (Thakur *et al*., 2001).

Light-mediated down-regulation of TaIAA1

Light and auxins are known to interact with each other in various stimulus–response processes in plants. A direct interaction between light and auxin has been demonstrated by phosphorylation of Aux/IAA proteins (SHY2/IAA3, AXr3/IAA17, and AXR2/IAA7) by phytochrome A (Colon-Carmona *et al*., 2000). In the present study, the *TaIAA1* expression in wheat seems to be downregulated by light, as its transcripts do not accumulate in light-grown tissues, leaf blades, and coleoptiles, while the transcript is abundant in the dark, in both root and shoot. This was further corroborated by northern analysis of the RNA isolated from 3- or 5-d-old coleoptile samples harvested at various time points upon transfer from the dark to white light. The down-regulation of *TaIAA1* transcript accumulation in 3/5-d-old etiolated seedlings became visible as early as 3–4 h following irradiation with white light. The *OsiIAA1* transcript levels are also affected by white light in a similar manner in rice (Thakur *et al*., 2001). In a subsequent study, the role of red, far-red, and blue light has also been demonstrated in triggering the down-regulation of *OsiIAA1* expression (Thakur *et al*., 2005).

Brassinosteroid-mediated responses

While both auxin and BR promote elongation, their induction kinetics are quite different, with auxin generally showing a short lag time of 10–15 min (Sasse, 1999; Bao *et al*., 2004) and BR showing a gradual and continuous increase (Nakamura *et al*., 2006). This difference in kinetics is also seen at the level of gene expression in the case of *TaIAA1* (present study) and also in *Arabidopsis*, where auxin induces members of the *IAA*, *SAUR*, and *GH3* gene families generally much more rapidly than BR (Goda *et al*., 2002; Müssig *et al*., 2002; Nakamura *et al*., 2006). Earlier studies also reported that in a BR-deficient *det2* mutant of *Arabidopsis*, *Aux*/*IAA* levels were lower than in the wild type, even though the endogenous auxin levels per gram of fresh weight were higher in the *det2* mutant than in the wild type (Nakamura *et al*., 2003). The interactions of the BR and auxin signalling pathways in the best characterized auxin-insensitive mutants *iaa7*/*axr2* and *iaa17*/*axr3* have revealed that these genes are regulated by the Aux/IAA proteins in response to both auxin and BR (Nakamura *et al*., 2006). Whether auxin and BRs interact to regulate expression

of *TaIAA1* remains to be worked out, but exogenous IAA induced accumulation of *TaIAA1* transcripts quickly and transiently, whereas exogenous BR induced it gradually and in a sustained manner.

Calcium-regulated changes in TaIAA1 *expression and coleoptile elongation*

Plant cells are reported to contain all the elements essential for a calcium-based messenger system that couple the external stimuli to various physiological responses (Pleith, 2005). There is increasing evidence that auxin action is also mediated by an intracellular change of calcium levels and that calcium acts as a second messenger during auxin-mediated cellular responses (Yang and Poovaiah, 2000).

The present study too substantiates the role of calcium in auxin-mediated cell elongation. The presence of calcium, along with exogenously supplemented 2,4-D, induced curving of the excised coleoptiles (data not presented), suggesting a differential accumulation/distribution of auxin in the presence of calcium. Northern analysis revealed that the transcript levels of *TaIAA1* increased when the endogenous auxin-depleted explants were incubated with calcium alone or in combination with 2,4-D, suggesting the positive influence of calcium on the relative abundance of the *TaIAA1* transcripts. EGTA, when supplied in the presence of auxin and/or calcium, caused a decrease in the *TaIAA1* transcript levels, which was concomitant with its inhibitory influence on coleoptile elongation in the dark. These data provide evidence for a role for calcium in auxin-inducible gene expression in wheat, but whether this effect is exerted at the transcriptional or post-transcriptional level, remains to be established. In an earlier study with a calmodulin (CaM) antagonist, it was suggested that CaM mediates regulation of *ZmSAUR1* not at the transcriptional level but rather at the post-transcriptional level (Yang and Poovaiah, 2000).

The curving of coleoptile segments in the presence of calcium is an interesting observation because such a response is usually quite apparent when hypocotyl segments or internodes are treated with a BR (Sasse, 1999). Moreover, recent studies have also shown that some of the *Aux*/*IAA* genes are indeed induced by BR (Nakamura *et al*., 2003, 2006) and BR action is mediated by calcium (Du and Pooviah, 2005). It is thus imperative to find out if Ca^{2+} is involved in auxin or BR signalling, or in both, for coleoptile elongation and curving, and induction of *Aux*/*IAA* genes.

Although the precise role of *TaIAA1* remains to be elucidated, there is correlative evidence for its probable role in cell elongation in seedling coleoptiles. It is intended to try, in the near future, to validate *TaIAA1* functionally by modulating its expression in transgenics. There are probably chances that the *Aux*/*IAA* family will be much larger in this hexaploid wheat but its complexity will be unravelled only as the wheat sequencing project advances and high throughput genome data become available for computational analysis.

Supplementary data

Supplementary data can be found at JXB online.

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