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ORIGINAL ARTICLE

Isolation of the *3β-HSD* promoter from *Digitalis ferruginea* subsp. *ferruginea* and its functional characterization in *Arabidopsis thaliana*

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Abstract

Background Although members of the *SDR* gene family (short chain dehydrogenase) are distributed in kingdom of life, they have diverse roles in stress tolerance mechanism or secondary metabolite biosynthesis. Nevertheless, their precise roles in gene expression or regulation under stress are yet to be understood.

Methods As a case study, we isolated, sequenced and functionally characterized the *3β-HSD* promoter from *Digitalis ferruginea* subsp. *ferruginea* in *Arabidopsis thaliana*.

Results The promoter fragment contained light and stress response elements such as Box-4, G-Box, TCT-motif, LAMP element, ABRE, ARE, WUN-motif, MYB, MYC, W box, STRE and Box S. The functional analysis of the *3β-HSD* promoter in transgenic Arabidopsis seedlings showed that the promoter was expressed in cotyledon and root elongation zone in 2 days' seedlings. However, this expression was extended to hypocotyl and complete root in 6 days' seedlings. In 20 days-old seedlings, promoter expression was distributed to the whole seedling including hydathodes aperture, vascular bundle, shoot apical meristem, trichomes, midrib, leaf primordia, hypocotyl and xylem tissues. Further, expression of the promoter was enhanced or remained stable under the different abiotic stress conditions like osmotic, heat, cold, cadmium or low pH. In addition, the promoter also showed response to methyl jasmonate (MeJA) application. The expression could not be induced in wounded cotyledon most likely due to lack of interacting elements in the promoter fragment.

Conclusions Taken together, the *3β-HSD* promoter could be a candidate for the development of transgenic plants especially under changing environmental conditions.

Keywords SDR · *3β-HSD* promoter · Functional characterization · Abiotic stress

Introduction

Digitalis plant species are the great source of cardiac glycosides which can obstruct Na^+/K^+-ATPase thereby enhancing output force of the cardiac muscles and improving its rate of contractions in order to cure heart failure [1, 2]. Most of the *Digitalis* species grow in natural habitats of Turkey [3]. The biosynthesis of cardiotonic glycosides occurs during normal growth of the plant [4]. However, the glycosides synthesis is also triggered due to various environmental [5],

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mineral [6, 7] or oxidative [8] stresses in the *Digitalis* species. 3β-hydroxysteroid dehydrogenase (3β-HSD) is a critical enzyme in the pathway of cardiac glycoside encoded by the *3β-HSD* gene [4, 5]. The gene belongs to a short-chain dehydrogenases/reductases (SDRs) which is a superfamily protein and largely dependent on NADPH reductases [9]. The *SDR* gene family have great diverse functional roles in vascular plants ranging from synthesis of secondary metabolites such as phenolics, alkaloids, terpenoids, hormone metabolism and flower development [9]. Therefore, keeping in consideration of the previous studies led our curiosity to undermine the *cis*-acting elements in the promoter of the *3β-HSD* for its transcriptional regulation under normal growth and development processes and abiotic stresses. Promoter is the structural part of the gene to which proteins bind to initiate the transcription of the gene under normal growth and development or abiotic stresses [10]. Due to unavailability of the genome sequence of the *Digitalis* species, we have used the universal genome walking approach to find the promoter sequence using known sequences of the *3β-HSD.* A recent report describes the promoter *cis*-acting elements mainly responsive to abiotic stresses distribution in the upstream regions of *SDR* genes in *Medicago truncatula* [11]. The *cis*-elements were related to wound ((WUN-motif and WRE3), drought (MBS), low temperature (LTR), phytohormones like auxin (TGA-element and AuxRR-core), gibberellin (TATC-box, GARE-motif, and P-box), abscisic acid (ABA) response element (ABRE) and salicylic acid (TCA-element). The highest number (435) of *cis*-elements related to ABA were found in the *MtSDR* gene members. While the gibberellin and MeJA responsive elements (151) were equally distributed in the gene family. Rest of the responsive elements such as auxin (128), drought responsive elements (137), LTR (89) and wound response elements (280) were differentially distributed throughout gene family [11]. It's also worth noting that a promoter's activity isn't just determined by the presence of a certain *cis*-acting elements. In some circumstances, evidence suggests that interaction between elements is required for differential promoter activity [12].

To date, isolation, sequencing and functional characterization of the promoter analysis of the *3β-HSD* has not been reported. With the evidence of the literature survey, this is the first report of the isolation, sequencing and functional characterization of the promoter fragment of the *3β-HSD* from *Digitalis* species in general and specific for *Digitalis ferruginea* subsp. *ferruginea*.

Materials and methods

Genomic DNA isolation and digestion

High quality gDNA (genomic DNA) was isolated by the method of [13] from fully expanded leaves of *Digitalis ferruginea* subsp. *ferruginea* [3]. To produce four gDNA libraries (DL), gDNA was digested with four blunt end restriction enzymes including *Dra* I (DL1), *Eco*R V (DL2), *Pvu* II (DL3) and *Stu* I (DL4). Reaction mixture was prepared according to instruction manual of GenomeWalker™ Universal Kit (Clontech, CA, USA). Four tubes were prepared for the digestion of gDNA with each enzyme in 1.5 µL micro centrifuge tubes. Digestion was carried out at 37 °C overnight.

Purification of digested gDNA and ligation to GenomeWalker™ adaptors

The digested and purified gDNA was ligated with GenomeWalker™ Adaptors (Clontech, CA, USA) and four libraries (DL1, DL2, DL3 and DL4) were constructed following manufacturer's manual. All the steps were carried out following the procedure according to instruction manual of GenomeWalker™ Universal Kit (Clontech, CA, USA). In order to improve the quality of digested DNA, phenol chloroform method was used. Briefly, 95 µL of phenol was mixed to equal volume of digested DNA. Samples were spin briefly and upper liquid phase was carefully taken and added into new microfuge tube. Then to each tube equal volume (95 µL) of chloroform was added and mixed well. Then centrifuged at room temperature and aqueous phase was transferred to new microfuge tube. Ice cold ethanol (95%) 190 µL, 9.5 µL NaOAc pH4.5, and glycogen (20 µg) was added to each tube and mixed well. After centrifugation at 14,000 RPM for 15 min, pellet was washed with ice cold 80% ethanol. After drying the pellet was dissolved in 20 µL TE buffer.

For GenomeWalker adaptors four ligation reactions were setup. For each tube, 4 µL of digested and purified DNA was added into PCR tubes. Then ligation reaction mixture was added to each tube. The ligation reaction was consisted of 1.9 µL GenomeWalker adaptor, 1.6 µL 10X ligation buffer and 0.5 µL T4 DNA ligase. The reaction tubes were incubated at 16ºC for overnight.

Amplification of the promoter fragment of the gene *3β-HSD*

To amplify promoter fragments of the *3β-HSD,* primary and secondary PCR steps were carried out according to the kit manual. Gene specific primers were designed according to

the instructions of the kit. Two gene specific primers (GSP1 and GSP2) were designed to clone promoter for primary and secondary PCR reactions, respectively. GSP1 of the *3β-HSD* gene was used in separate reactions along with AP1 (Adaptor primer 1 provided in kit) primer in primary PCR reaction.

Primers for cloning of promoter's fragment of the gene *3β-HSD*, 3β-HSD-GSP1 5′-TCATCTCTGACGTCGCAGTGGTAGTAAC-3′, AP1 5′-GTAATACGACTCACTATAGGGC-3′, 3β-HSD-GSP2 5′CTGACGTCGCAGTGGTAGTAACTTATCTTG-3′,AP2 5′- ACTATAGGGCACGCGTGGT-3′.

GSP1 primer sequence of *3β-HSD* was designed from full length ORF of the *3β-HSD* (gene bank accession KM406483) from 206 nucleotide number to 179 nucleotide number as reverse compliment. GSP2 primer sequence of the *3β-HSD* was designed from full length ORF of *3β-HSD* (gene bank accession KM406483) from 200 nucleotide number to 171 nucleotide number as reverse compliment. These primers were designed by using Primer3 (http://sim-gene.com/Primer3) with standard parameters.

T100™ Thermal Cycler (Bio-Rad, USA) was used to perform the cycle using the two-step cycle parameters following kit instructions. 5 µL of the primary PCR products were observed on a 1.5% agarose/EtBr gel using 1xTAE buffer, along with DNA size marker of 1 kb ladder. To perform secondary PCR (nested PCR) primary PCR products were 50 times diluted in 0.5 -mL tubes for each sample. Secondary PCR reaction mixture was prepared according to kit instructions.

5 µL of the secondary PCR products were observed on a 1.5% agarose gel stained with EtBr.

Table 1 The *cis*-acting elements relevant to growth and stresses in the *3β-HSD* promoter

Motif name	Number of copies	Sequence	Motif function
Box 4	1	ATTAAT	Light element
G-box	2	CACGTC	Light element
LAMP-element	1	CTTTATCA	Light element
TCT-motif	1	TCTTAC	Light element
ABRE	2	ACGTG	abscisic acid response element
ARE	1	AAACCA	essential element for the anaerobic induction
WUN-motif	1	AAATTTCCT	wound-response element
MYB	1	TAACCA	Regulator of drought stress genes, flavonoid biosynthesis
MYC	1	CATGTG	Regulator of early responsive to dehydration gene (erd1)
W box	2	TTGACC	*cis* element involved in plant defense response to pathogens
STRE	1	AGGGG	Stress response element
Box S	2	AGCCACC	elicitor-responsive element

Ligation and transformation of PCR products to *E. coli* and plasmid isolation

For sequencing of promoter fragments, PCR products were ligated into cloning vector pCR2.1 following the instructions of TA Cloning ® Kit manufacturer (Thermo Fisher Scientific, Massachusetts, USA). Ligated products were transformed into competent cells of Top10 *E. coli* by heat shock method. The cells were spread on LB media plates containing 50 µg/mL kanamycin and incubated at 37 ºC overnight. Single colonies (5–8) were selected and cultured in liquid LB medium containing 50 µg/mL kanamycin. Plasmid was isolated using a plasmid isolation kit (Thermo Fisher Scientific, Massachusetts, USA). The promoter fragments were sequenced by Sanger sequencing method. Promoter sequences were analyses by online tool PlantCare [14].

Cloning and subcloning of the *3β-HSD* promoter fragment

Promoter fragments were cloned from cloning vectors (pCR2.1) using promoter specific primers containing restriction enzymes sites of *Eco*RI and *Nco*I. To clone promoter's fragments of *3β-HSD*, primers with restrictions sites were 3β-HSD pro-F 5'-GAATTCCACGAAACGCTCGGTTTCC-3' and 3β-HSD pro-R 5'-CCATGGACGGGAGGAAACTTCGCG-3'. Sequences with underline shows *Eco*RI and *Nco*I sites, respectively. The products were confirmed on 1% agarose gel stained with EtBr. PCR products and pCambia 1381 were digested with *Eco*RI (NEB) and *Nco*I (NEB) enzymes simultaneously for 1 h. The digested products were run on 1% agarose gel stained with EtBr. Bands were cut with sharp razor and purified according to manufacturer instructions of NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany). The purified products were mixed for ligation reaction and placed at 16ºC overnight.

Transformation of ligation products into *Agrobacterium tumefaciens*

Ligation products (pCambia1381 + promoter fragment) were transformed into competent cells of *Agrobacterium*

tumefaciens strain C58C1. Transformation was performed by heat shock method. 4 µL ligation product was added into the competent cells and incubated on ice for 30 min. Then, cells were incubated at -80 ºC for 2 min. Then cells were placed at 37 ºC for 5 min. Afterward, cells were incubated on ice for few minutes and 250 µL SOC media was added. Cells were shaken at 28 ºC for 2.5 h at 175 rpm in a shaker. Cells were pelleted and spread on LB agar media plates containing 50 µg/mL kanamycin and cells were placed at 28 ºC for two days to obtain colonies.

Plant growth and transformation of the *3β-HSD* promoter into *Arabidopsis thaliana*

Arabidopsis thaliana ecotype Columbia-0 (Col-0) plants were grown in peat soil at 25 ºC under 16-h-light/8-h-dark cycle. *Agrobacterium tumefaciens* (strain C58C1) mediated transformation of Arabidopsis plants were accomplished using the floral dip protocol [15].

T_1 generation seeds were selected for resistance to hygromycin. Seeds were surface sterilized with a commercial 20% (v/v) bleach (Domestos) for 15 min, then rinsed five times with sterile distilled water. Then, the seeds were placed on one-half-strength Murashige and Skoog [16] medium solidified with 0.8% (w/v) agar and supplemented with 25 µg/mL hygromycin. At least 10 independent transgenic lines were obtained, and transgenes were transferred to sterile peat media for further growth to get T_2 seeds.

3β-HSD promoter activity in Arabidopsis

Promoter activity was determined during plant growth developmental (2, 6 and 20 days) stages [17] while for sucrose (3%) signalling effect, abiotic stresses (150 mM NaCl, 2% mannitol, 42 °C for 1 h; heat, 4 °C for 24 h; cold, room temperature for 24 h hypoxia), metal stress (20 µM $CdCl_2$ at pH5.8 for 24 h and 50 µM $AlCl_3$ at pH5.0 for 24 h) and phytohormones (50 µM IAA, 50 µM GA3, 50 µM 6-BA, 100 µM MeJA for 24 h) exposed to 2 weeks old seedlings. For hypoxia seedlings growing on MS agar media plates were submerged into water which was sonicated to remove trapped air. All the treatments were followed with minor modification as described earlier for sucrose, NaCl and mannitol [18], Al stress [19], phytohormones [20], hypoxia [21], cold and heat [22] and $CdCl_2$ [23].

GUS histochemical staining

For histochemical GUS expression analysis, the transgenic Arabidopsis seedlings were incubated in the GUS staining solution (0.2 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.0, and 2 mM 5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid) at 37 °C for 24 h, followed by washing in 70%

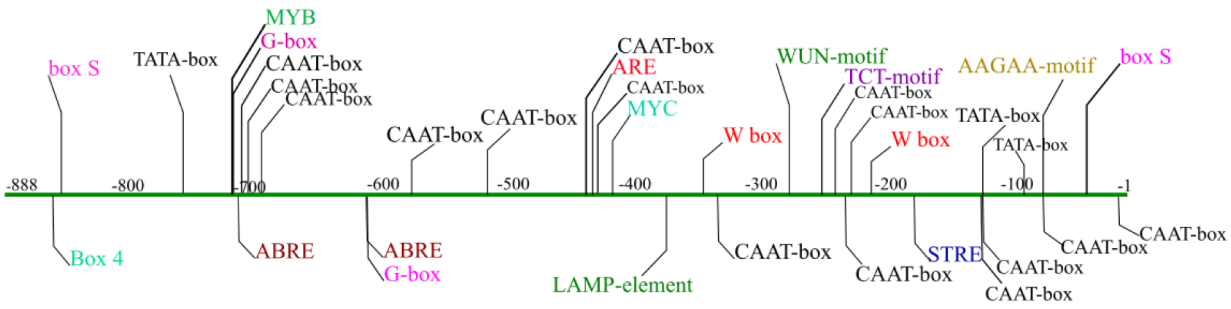

Fig. 1 Distribution of *cis*-acting elements of the *3β-HSD* promoter

ethanol according to a published method [24]. After complete removal of chlorophyll by series of ethanol 10%, 30%, 50% and 70% each 30 min and achieving transparency, the seedlings were observed and photographed were acquired with a Leica DM1000 LED microscope.

Results

3β-HSD promoter sequence analysis

The promoter fragment was amplified in a primary and secondary round of PCR (**Supplementary Fig. 1**). In brief, during amplification step of the promoter, in the primary PCR, only one bright band was appeared from DL4 library. In the second round of PCR (secondary PCR), two bands were found brightest in DL4 library having sizes of 1200 bp and 1000 bp. In other libraires medium bright or faint bands also appeared. All visible bands were cut from gel, purified, cloned into cloning vector and sequenced. The final promoter fragment (ca. 888 bp) of the *3β-HSD* (**Supplementary Fig. 2**) was obtained from gDNA using GenomeWalker™ Universal Kit (Fig. 1). The *cis* acting elements related to plant growth and development were found. Four different types of light regulating motifs were identified. Box4, G-box, LAMP-element and TCT motifs were located at -849, -605, -707, -366 and − 242 upstream to ATG sequence, respectively. Two copies of ABRE were found at -605 and − 707 position. For anaerobic induction, ARE element was determined at -428. One wound response element was present at -276 position. One MYB motif which is a regulator of drought stress and flavonoid biosynthesis was located at -703. For the early response of dehydration MYC motif was located at -409. For the plant defense against pathogens 2 motifs W-box were located at -203 and − 338. A stress response element was found at -175. Two elicitor response elements Box-S were found at -34 and − 841 **(**Table 1**)**.

Functional analysis of the *3β-HSD* promoter in Arabidopsis

In order to determine functional activity of the *3β-HSD* promoter fragment, expression construct was developed using pCambia 1381 (**Supplementary Fig. 3**). The *3β-HSD* promoter expression driven by the *GUS* reporter gene in

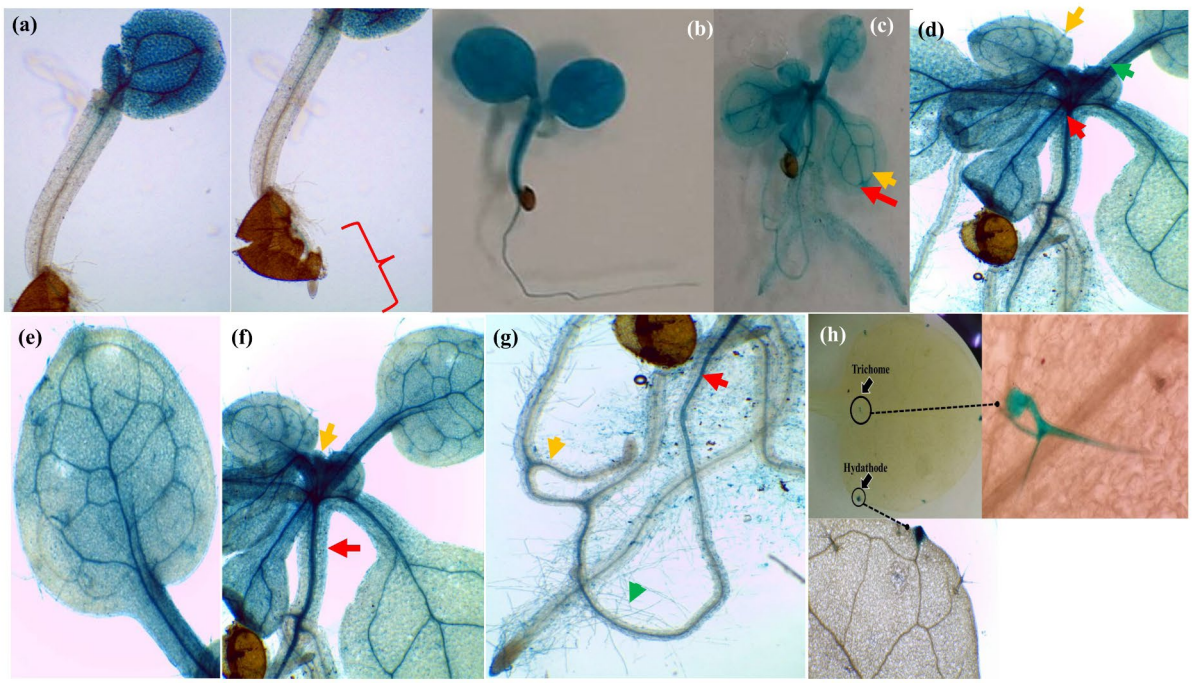

Fig. 2 *3β-HSDpro*::*GUS* activity in Arabidopsis seedlings during growth and developmental stages. (a) 2 days old seedling, red curly bracket showing expression in root elongation area, (b) A 6 days old seedling, (c) A 20 days old seedling expression in shoot, hypocotyl and root regions; red arrow shows hydathodes aperture, yellowish arrow shows vascular bundle in mature leaf, (d) Red arrow shows shoot apical meristem from where new cells emerge and yellow arrow head shows trichomes, green arrow head shows midrib of young leaf, (e) Young leaf, (f) Leaf primordia shown by yellow arrow head and red arrow head shows hypocotyl region, (g) Xylem tissues (vascular parenchyma, shown as red arrow head), lateral root vascular tissues (yellow arrow head) and root hairs shown by green arrow head, (h) Trichome and hydathode in mature leaf of one-month old seedling

Arabidopsis T_2 homozygous plants were determined under normal plant growth and development stage. GUS activity was observed in 2 days-old seedlings (Fig. 2a). The expression of the promoter was confined to cotyledon and to some extent in vascular tissue in hypocotyl region. In root zone, expression was just confined to area of root elongation. The strong expression was found in 6 days-old seedlings at cotyledonary leaf and in hypocotyl region (Fig. 2b). In 20 days-old seedlings, promoter activity was monitored by GUS histochemical staining in the leaves (both young and old), shoot apical meristem, trichome, hydathodes, hypocotyl region and root **(**Fig. 2c-h**)**. In mature leaf, GUS activity was found in vascular tissues or hydathodes (Fig. 2c). On the other hand, in young leaves, promoter activity was evident in trichomes (yellow arrowhead), hydathodes (purple arrowhead), vascular tissues, mid rib (green arrowhead) and (SAM) shoot apical meristem (red arrow head) (Fig. 2d, e). Further strong GUS activity was observed in leaf primordia (yellow arrowhead) and hypocotyl region (red arrowhead) (Fig. 2f). Shoot apical meristem (SAM) is the region in the growing shoot containing meristematic cells with multipotent stem cells and produces primordia that develop into all the above ground organs of a plant. In root tissues, promoter activity was restricted to xylem tissues (vascular parenchyma, shown as red arrowhead), lateral root vascular tissues (yellow arrow head) and root hairs shown by green arrow head (Fig. 2 g). In mature leaves, expression was merely found in trichome and hydathodes (Fig. 2 h). *AtHSD1promoter* activity was observed in shoot tissue more extensively than in root tissues [25]. Two days-old seedlings expressing *3β-HSD* promoter activity had a similar fashion of expression (Fig. 2a**)**. However, this expression activity was enhanced with the seedling growth and developmental stages of 6 days- (Fig. 2b**)** and 20 days-old seedlings **(**Fig. 2c**)**. The promoter activity was enhanced in 20 days-old seedlings in all above ground and root tissues and more intensively in vascular tissues, shoot apical meristem, hydathodes, trichomes, lateral primary root and root hairs. The promoter activity in apical meristem showed that as it is expressed in root hair and root vascular tissues, *3β-HSDpro* may be involved in seedling development and nutrient transport or signaling.

Expression activity of the *3β-HSD* promoter under stresses and hormone treatment

Under stress conditions, *3β-HSD* promoter activity was enhanced under 2% mannitol treatment, however, down-regulated in cotyledonary leaves and root under salt stress conditions compared to control with enhanced expression in

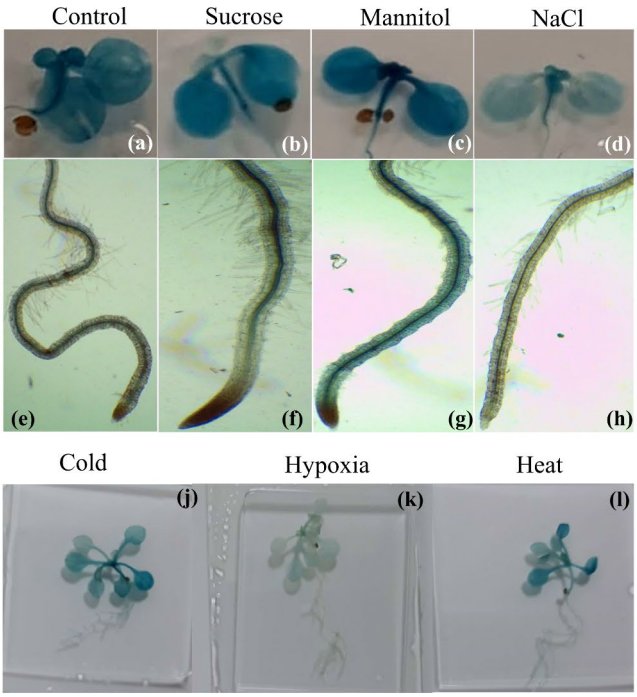

Fig. 3 *3β-HSDpro*::*GUS* activity in Arabidopsis seedlings under sugar signalling or abiotic stresses. Control seedlings (a, e) were not subjected to any treatment. For sugar signalling, 3% sucrose was supplied (b, f). For abiotic stresses, seedlings were exposed to the different stresses including 2% mannitol (c, g), 150 mM NaCl (d, h), 4 °C for cold (i), submerged in water for hypoxia (j) and 42 °C for heat (k). Sucrose, mannitol, NaCl, cold and hypoxia were imposed for 24 h and heat for 1 h

SAM cells. Under sucrose application, expression was limited to cotyledon, hypocotyl, and root zone but not in SAM (Fig. 3a-h). Our findings are consistent with the results of *AtSDR1promoter*::GUS activity where expression was enhanced with 6% mannitol in shoot and root tissues [18].

Because of cold stress, the expression activity of the promoter was weaker than control, but it was more pronounced in younger leaves than in older leaves (Fig. 3i). In hypoxia treated seedlings, expression was vanished (Fig. 3j). Under heat stress, GUS activity was enhanced in the shoot region of Arabidopsis seedlings (Fig. 3k). Upon exposure to cadmium (Cd) stress, the GUS activity in transgenic Arabidopsis seedlings was enhanced in the shoot and hypocotyl region over control plants (Fig. 4a, b). When aluminium (Al) stress was applied, promoter activity was lower under Al stress than under control where pH was adjusted to 5. Higher GUS activity under low pH shows that the *3β-HSD* promoter has transcriptional activity under acidic condition (Fig. 4c, d). IAA, BA6 and GA3 phytohormones did not alter the activity of the *3β-HSD* promoter (Fig. 5a-c). However, the expression acvitivity was confined to SAM and young developing leaf compared to control (Fig. 5d). According to these studies, the *3β-HSD* promoter is active both during normal growth and development as well as abiotic stress conditions.

Discussion

In *Digitalis* plant species, for the biosynthesis of cardiac glycoside, sterols are converted into pregnenolone. However, this step is achieved by 3β-hydroxysteroid dehydrogenase by metabolizing into isopregesterone [5]. The glycosides are the secondary metabolites which produced under the

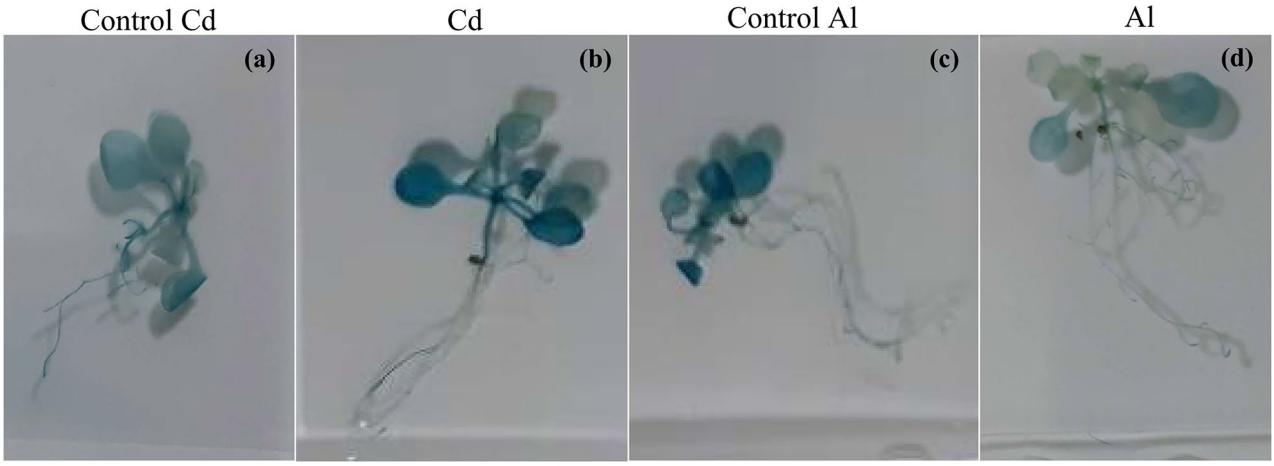

Fig. 4 *3β-HSDpro*::*GUS* activity under metal exposure stress conditions. Control seedling (a) without (0 µM Cd) and seedling treated (b) with 20 µM Cd for 24 h at pH 5.8. Control seedling (c) exposed to 0 µM $AlCl_3$ and seedling treated (d) with 50 µM $AlCl_3$ at pH5.0 for 24 h

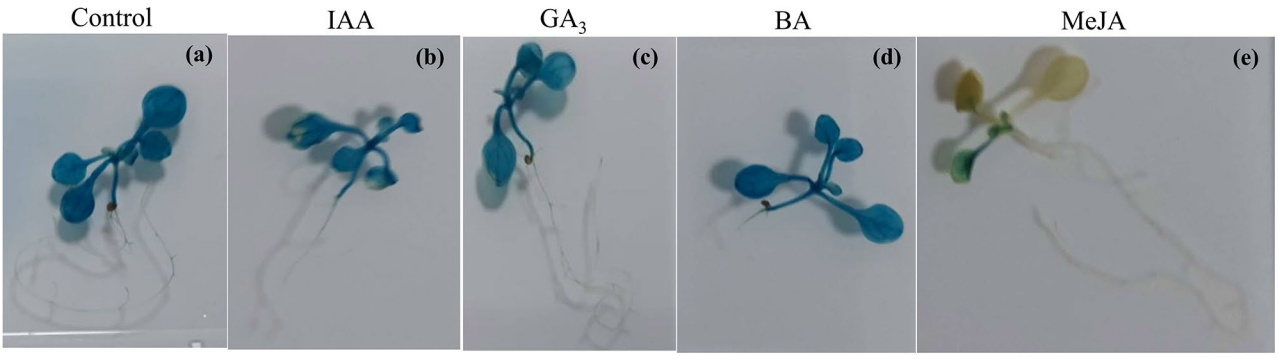

Fig. 5 *3β-HSDpro*::*GUS* activity due to phytohormones applicaiton. Control seedling treated with ½ MS (a), 50 µM IAA (b), 50 µM GA3 (C), 50 µM 6-BA (d) and 100 µM MeJA (e) for 24 h

abiotic stresses [26]. On the other hand, this plant is highly tolerant to a series of abiotic stresses such as heat, drought, cold, flooding, scorching sun light and the barren lands. Further, the plant has great potential to survive and thrive under a diverse climatic condition [27]. In this study, isolation, sequencing, and transformation of the *3β-HSD* promoter fragment were performed to study *cis*-acting elements, cues for normal growth, development, as well as stresses. Because the *cis* elements are the regulatory elements of the genes which play a role in transcriptional activities of the gene. The promoter fragment of *3β-HSD* revealed important *cis*-acting elements related to growth and development as well as abiotic stresses in the sequence. G-Box was found in light morphogenesis related genes [28, 29] and in stress related genes such as high temperature and the humidity stress responsive gene *GmSBH1* [30] or nutrient stress [31]. Plant growth and development under light conditions may be affected by G-Box present in light regulated promoters [32]. ABA responsive element play crucial role in ABA mediated signaling pathway especially under abiotic stresses [33, 34]. WUN motif was present in pathogen-responsive promoters [35] or the WRKY genes which are also induced due to biotic and abiotic stresses [36] indicate that the motif is mainly conserved in stress related promoters. Anaerobic induction elements (ARE) and the MYB jointly present in the promoter fragment which possibly would have a role in flavonoid biosynthesis under anaerobic condition. The presence of stress related *cis*-elements such as ABRE [37], ARE, WUN-motif, MYB, MYC, W box [38], STRE and Box S indicate the possible transcriptional regulation of the *3β-HSD* under the abiotic or biotic stresses. The abiotic stress response elements were also found in the recent report of *SDR* gene family of *Medicago truncatula* [11]. The *SDR* gene family contains *cis*-acting elements for drought, cold and salt stresses. The presence of abiotic stresses cues which seems evolutionary conserved in the promoter of *3β-HSD* (*Digitalis ferrruginea*) as well as in the *MtSDRs* depict their possible role in defense against harsh environmental conditions. Further, alcohol dehydrogenase gene family which is phylogenetically close to the *SDR* family shows the conservation of *cis* elements for abiotic stresses [39]. The presence of these environmental stresses' cues in the promoters of *SDR* genes could be game changer for the development of climate resilient crops in future. In addition, our recent finding of transplastomic expression of the *SDR* genes (*3β-HSD*, *P5βR1* and *P5βR2*) interpret their pivotal role in salt tolerance [40].

During the growth stages, the promoter expression in the transgenic Arabidopsis seedlings distributed in all the tissues of shoot, root, shoot apical meristem, hypocotyl, vascular bundles, root hair, trichome and hydathodes. Similar promoter expression pattern in the transgenic Arabidopsis seedlings was demonstrated for the promoter fragment of the *AtHSD1* [25]. The results showed that promoter fragment of *3β-HSD* has similar activity as *AtHSD1* during normal growth and development. Further, the enhanced expression in the vascular tissue could be possibly related to involvement of brassinosteroids (BRs) in the tissue differentiation [25, 41]. Then, transgenic Arabidopsis seedlings were exposed to various chemical or stresses and phytohormones. Under the sucrose or mannitol application, the promoter expression activity was enhanced in shoot or root tissues. However, under salt stress, the expression decreased in cotyledonary leaves but increased in apical meristem or newly emerging leaves. These findings partially corroborating the similar findings to earlier study of the *AtSDR1* promoter [18] where promoter expression was enhanced under glucose or mannitol but reduced to a negligible level under salt. In addition, our recent findings for the transplastomic expression of the *3β-HSD* strongly confirms its role in salt tolerance [40]. The *Rab16A* promoter contains *cis*-ABRE element therefore, when the rice seedlings were imposed to mannitol for the of 24-h period, the transcripts of the *Rab16A* reached to extended to higher level. The *3β-HSD* promoter also induced due to mannitol stress. Our results are closely related to previously reported findings [42]. Under cold stress, the promoter activity was slightly reduced compared to control in contrary to the *AtSDR1* promoter where noticeable GUS activity was not found. Heat stress inducing high GUS activity in transgenic seedlings might have the activity like heat shock protein promoter [43, 44]. Further, STRE does not only induce the heat shock proteins but also response to other stress factors like low pH and osmotic stress [45]. Almost complete loss of GUS activity observed under hypoxia indicates that the *cis*-element perhaps could not induce expression alone and may need other *cis*-elements to act in combination. Under cadmium (Cd) exposure, the promoter activity was enhanced in the seedlings. Among differentially expressed genes, the *SDR* gene was induced in freshwater crayfish [46] due to cadmium exposure. The Cd based inducible expression in the transgenic seedlings and the cray fish might show some unknown evolutionary conserved *cis*-acting element in both plant and fish. A control treatment of Al at pH 5.0 resulted in increased expression of the seedlings possibly due to the presence of STRE *cis* elements [45] but reduced in Al exposure. The *cis* acting elements for wound (WUN motif) could not induce expression in the transgenic Arabidopsis tissues (data not shown), although the wound element was present in the promoter region. The possible reason for this event could be due to lack of interacting *cis* element in the promoter fragment [12]. Therefore, the expected phenotype could not induce in the transgenic plant.

As a result of phytohormones application, effect of all phytohormones was stable, except for JA, which tended to limit expression to the shoot apical meristem or emerging leaf, which is consistent with the decreased expression of the *SDR* genes under JA application [5]. Furthermore, the *BjCHI1* promoter containing the W-box showed the highest GUS activity in newly developing leaves due to MeJA treatment [38], correlating with our finding of MeJA induced expression. MejA mostly response to herbivory or pathogens [38].

Conclusions

In the present study we have shown isolation and functional characterization of the *3β-HSD* promoter from *D. ferrruginea* subsp. *ferrruginea*. The sequence analysis of the promoter fragment revealed important signals for plant growth and development as well as biotic and abiotic stresses. The expression of the promoter under normal plant growth conditions and the given stresses display its possible role in the development and survival potential. In addition, the promoter fragment could be used for the development of of transgenic crop plants to combat abiotic stresses such as heat, cold, salt and metal stress like Cd. In conclusion, the *3β-HSD* promoter could have a promising usage for trangenic plant development under a variety of stress conditions, including osmotic, heat, low pH, cold, cadmium and biotic stress.

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Data Availability Whole data needed to conduct this study is provided within the manuscript.

Declarations

Conflict of interest The author confirm that this article content has no conflict of interest.

Consent to publish All the authors read the manuscript and showed their willingness to publish this study.

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