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Full Length Research Paper

Co-suppression of sterol-regulatory element binding protein mediates etiolation in *Arabidopsis thaliana*

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Arabidopsis plants were transformed with a chimeric construct containing expression cassettes for GFP election marker and CaMV 35S promoter-driven *At5g35220* cDNA, via Agro bacterium-mediated method. Two transformants produced pigmentation deficient phenotype. Analysis revealed the decrease of chlorophyll in all etiolated plants. RT-PCR showed that, total *At5g35220* mRNA levels were greatly inhibited in co-suppression lines. *PORA* and *PORB* mRNA expression were influenced also in the mutants. It is found that, the *At5g35220* gene is responsive to both inhibitor and some hormone **with regard to MVP/MEP pathway in our study.**

Key words: Etiolation, co-suppression, protochlorophyllide oxidoreductase gene (*POR*), light regulation.

INTRODUCTION

Isoprenoids, the largest family of natural products, play essential roles in both plant and human life. So the regulation of isoprenoids pathway is generally of great concern. In animal cholesterol and fatty acids biosynthetic pathways, the key transcriptional modulators of sterol-responsive genes that is subject to feedback regulation by cholesterol and fatty acids are a common family of transcription factors designated as sterol regulatory element binding proteins (SREBPs) (Briggs et al., 1993, Wang et al., 1993). SREBPs belong to a large class of transcription factors containing bHLH-Zip domains, which are synthesized as membrane-bound precursors that require a two-step proteolytic process of cleavage in order to release their amino-terminal bHLH-Zip containing domains into the nucleus to bind to a specific DNA sequence, sterol regulatory element (SRE) and activate their target genes in a sterol-regulated manner (Brown and Goldstein; 1997; Yokoyama et al., 1993; Wang et al., 1994; Hua et al., 1993). In plant, two independent isoprenoids pathways located in separate intracellular compartments are involved in the biosynthesis of the common precursors of isoprenoids isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) presently. The study of SREBPs in plant isoprenoids pathway is scarcely reported. In Arabidopsis, *At5g35220* gene being sterol regulatory element-binding protein site 2, protease and metalloendopeptidase activity were required for chloroplast development and play a role in regulation of endodermal plastid size and number that are involved in ethylene-dependent gravitropism of light-grown Arabidopsis hypocotyls (Chen G et al., 2005; Guo et al., 2008). Two transgenic lines(*35SI-a,-b*) were disco-

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Abbreviations: POR, Protochlorophyllide oxidoreductase gene; **Pchlide,** protochlorophyllide; **MVA,** mevalonate; **MEP,** 2-C-methyl-d-erythritol 4-phosphate; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **ABA,** abscisic acid; **IAA,** indole-3-acetic acid; **GA_3,** gibberellic acid; **PCR,** polymerase chain reaction; **RT-PCR,** reverse transcriptase-mediated-PCR; **GFP,** green fluorescent protein; **EGFP,** enhanced green fluorescent protein; **HMGR,** 3-hydroxy-3-methylglutaryl coenzyme A reductase; **DXP,** deoxyxylulose 5-phosphate; **PTGS,** post-transcriptional gene silencing; **SRE,** sterol regulatory element; **SREBPs,** sterol regulatory element binding proteins; **IPP,** isoprenoids isopentenyl diphosphate; **DMAPP,** isomer dimethylallyl diphosphate.

Table 1. Primers used in RT-PCR.

Gene name (acc. #)	Primer sequence	Fragment size (bp)
Actin (NM_112764.3)	F:5'-CACTGTGCCAATCTACGAGGGT-3' R:5'-CACAAACGAGGGCTGGAACAAG-3'	318
At5g35220 NM_122913.3	F:5'-ATCTCTGACTTATGTTCTGTTTTGT -3' R:5'- TATTAACTTGACATCTGCAGGATCT-3'	232
PORA (NM_124799.3)	F:5'-AATGCCGCAGTCTATCAG -3' R:5'- AAAGTTATGCCGGTGTCT-3'	389
PORB (NM_001036654.1)	F:5'-TACCACCGAAGGCGAATC -3' R:5'- GAAAGGGAGGGAAGAGGG-3'	272
PORC (NM_100243.3)	F:5'-AGGCTTAGCGTCAGGATT -3' R:5'- GACTAGGGTCACTCACAAC-3'	318

F, Forward primer; R, reverse primer.

when a *pEGAD-35220* transgenic Arabidopsis was expected to express a transgene SREBP2 (*At5g35220*) with regard to regulation of isoprenoid synthesis pathway at high level. *35SI* co-suppression lines, instead expressed neither the transgene nor the related endogenous gene, exhibits pigmentation-deficient phenotype and had big cotyledon compare to wild type. The average level of *POR* [light-dependent NADPH-protochlorophyllide (Pchlide) oxido-reductases] gene induction by dark was reduced in the mutants. In addition, a feedback mechanism of *At5g35220* by end products of the 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway was investigated.

MATERIALS AND METHODS

Plant materials and different treatments

Arabidopsis thaliana wild type seeds were treated with 70% ethanol for 1 min, then with 30% household bleach for 10 min and washed five times with sterile water and were plated on MS medium solidified with 0.8% agar incubated at 4°C for 4 days before being placed at 23°C under long-day conditions. Different concentrations of Mevacor (Singer et al., 1984; Rodríguez-Concepción et al.,2004) or Lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, blocks the formation of mevalonate and its metabolites in the mevalonate (MVA) pathway (MEV: 0, 0.5, 5 mM) and Fosmidomycin (Steinbacher et al., 2003; Rodríguez-Concepción et al., 2004) (a specific competitive inhibitor of DXR, enzyme catalyzing the NADPH-dependent production of MEP from deoxyxylulose 5-phosphate (DXP) in the first committed step of the MEP pathway) (FSM : 0, 20, 50 µM) were added to the medium and 6 days old seedlings were analyzed.

Three weeks old *Arabidopsis thaliana* wild type seedlings grown on MS medium were treated with 100 µM abscisic acid (ABA), gibberellic acid (GA_3), indole acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) for 6 h. The seedlings were sprayed thoroughly to ensure the total coverage of the foliage with the chemicals.

Southern blot analysis

Genomic DNA was extracted from wild type and 35SI mutant (Khanuja et al., 1999) and digested with HindIII and BamHI, respectively. The digested DNA (10 µg per lane) was separated on a 0.6% (w/v) agarose gel and transferred onto a nylon membrane (Hybond N+, Amersham Biosciences, Piscataway, NJ) under vacuum. The membranes were hybridized with digoxigenin-labeled enhanced green fluorescent protein (EGFP) or full-length *At5g35220* cDNA probes at 65°C and immersed in solution containing alkaline phosphatase-conjugated anti-digoxigenin antibodies (Wang and To, 2004). The hybridization signals were visualized using CDP-Star (Amersham Biosciences).

Reverse transcriptase-mediated PCR for expression analysis of *At5g35220* gene

To analyze the expression of *At5g35220* by reverse transcriptase–mediated (RT) PCR, total RNA was extracted from Arabidopsis tissues with EasyWay RNA Plant Mini Kit (Ambiogen Life Science Technology Ltd., China). Total RNA (2 µg) was heated to 65°C for 7 min and then subjected to reverse transcription reaction using Moloney murine leukemia virus reverse transcriptase (200 units per reaction; MBI) for 60 min at 42°C. PCR amplification was performed with initial denaturation at 95°C for 5 min followed by different (20 to 27) cycles of incubations at 95°C for 30 s, 55 to 60°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min using the *At5g35220*, *PORA, PORB and PORC* specific primers (Table 1). Actin2 gene expression level was used as a quantitative control. 16 µl of individual PCR products were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide under UV light.

RESULTS

Isolation and classification of the transgenic line that knock out the *At5g35220* gene transcriptional level

Two etiolated transformants (*35SI-a,-b*) were discovered as shown in Figure 1a. DNA extracted from the transgenic-plant *35SI-a, 35SI-b* and WT, were analyzed by Southern hybridization, using the digoxigenin-labeled *At5g35220* cDNA and enhanced green fluorescent protein (EGFP) cDNA, respectively (Figure 1b). The co-suppression line exhibited pigmentation-deficient phenotype

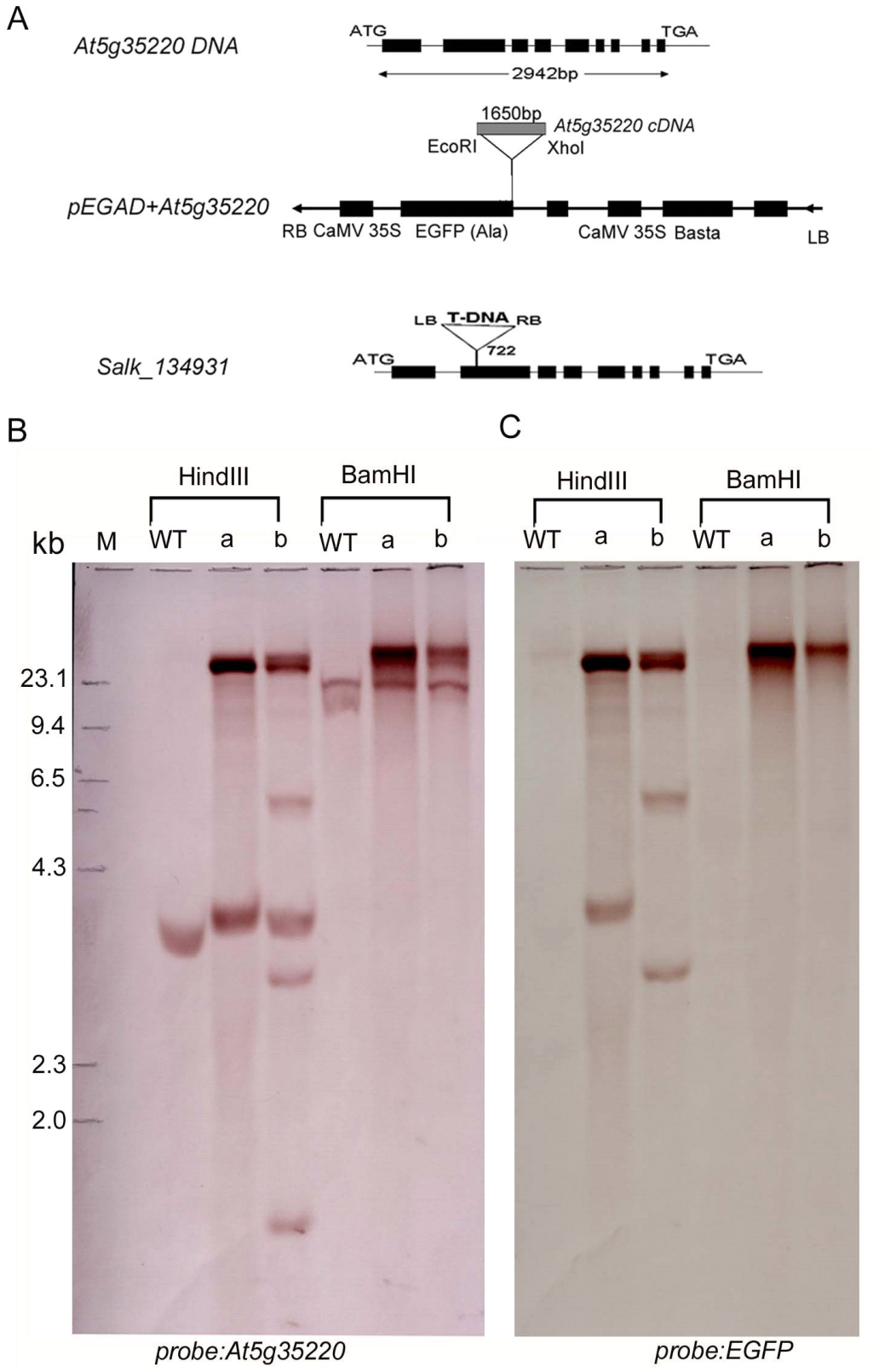

Figure 1. Transgenic plant verification. (A) The scheme of *At5g35220* gene and *pEGAD-At5g35220*; (B) Southern blot analysis of transformants. Total DNA from wild type, transformants 35SI-a and 35SI-b were digested with HindIII and BamHI, transferred to nylon membrane and hybridized with digoxigenin-labeled *At5g35220* and EGFP probes. Sizes of marker DNA are shown in kb (lane M). M, λHindIII mark; WT, col-4; a, 35SI-a; b, 35SI-b.

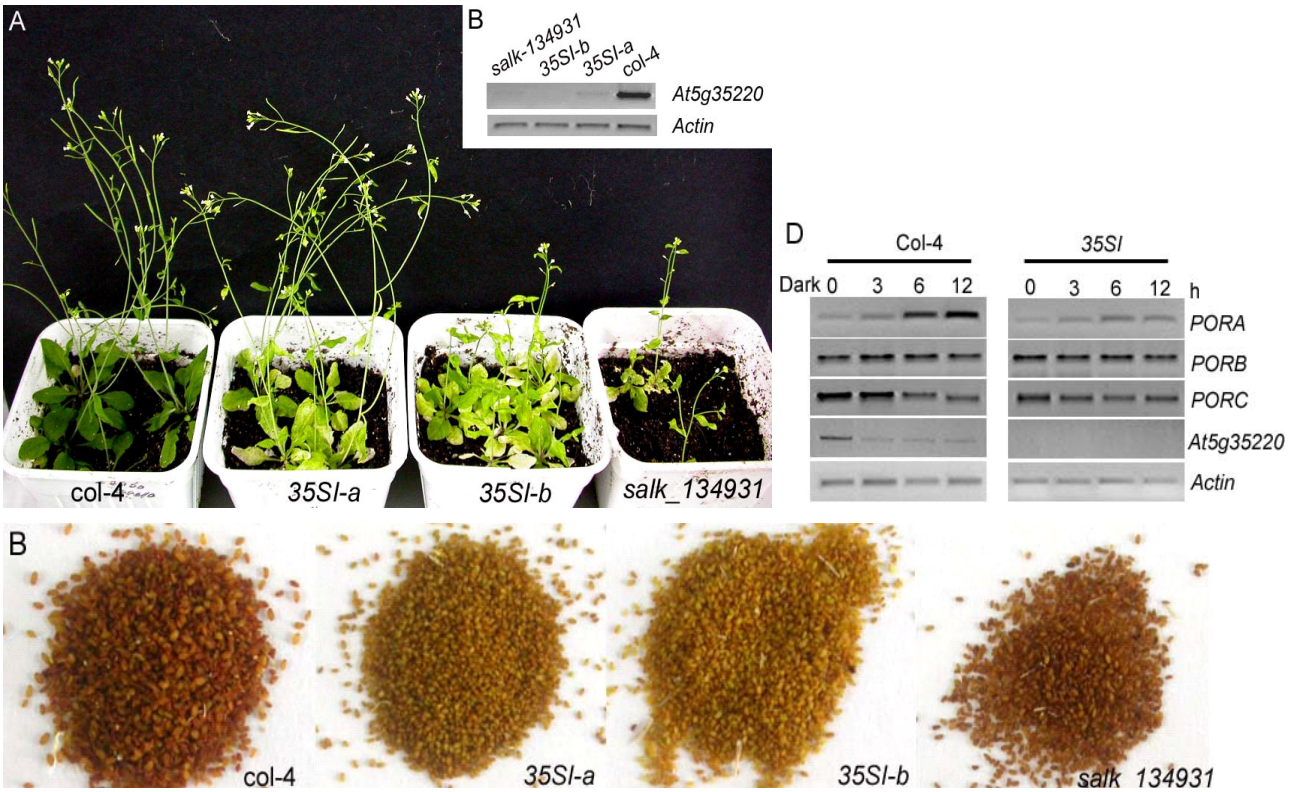

Figure 2. Morphology and classify the Arabidopsis co-suppression mutant the *At5g35220* and *POR* gene transcriptional level. (A) Show the morphology of wild type (col-4, left 1), co-suppression mutants and T-DNA insertion mutant. 26 day old plant growing in greenhouse was used; (B) Seeds of wild type (col-4, left 1), co-suppression mutants and T-DNA insertion mutant. plant growing in greenhouse was used; (C) Co-suppress the *At5g35220* gene transcription in transgenic line and classify the T-DNA insertion mutant by RT-PCR assay. Results were presented as average values ± SE from three experiments; (D) The transcripts of *POR* genes in col-4 and *35SI* which grown in natural conditions for 15 day then transferred to dark for figured times by RT-PCR analysis Results were presented as average values ± SE from three experiments.

(Figure 2a, b). The physiological and morphological characterization of *35SI* mutant is described in Table 2. The transcripts of *At5g35220* in the mutants were significantly inhibited by RT-PCR analysis (Figure 2c). To further determine the synthesis of chlorophyll, the transcripts of *POR* genes in WT and *35SI* which grown in natural conditions for 16 day then transferred to dark for figured times by RT-PCR analysis (Figure 2d). As reported previously (Qingxiang et al., 2001), the concentration of *PORC* mRNA rapidly declined, after these seedlings were transferred to the dark. While the average level of *PORC* induction was reduced to about 40 to 60% in the co-suppression lines. Contrarily, *PORA* mRNA was barely detected in continuous light and become elevation in seedlings transfer to dark. From the Figure 2d we could find that *PORA* induction in the wild type was thrice as much as in the mutant when their peak of *PORA* mRNA accumulation appeared at the same time. The similar expression pattern of *PORB* in wild type and co-suppression lines was found in our study. The dark regulation of *At5g35220* gene was confirmed by Figure 2d also. One T-DNA insertion lines, *SALK_134931*, contained inserts at nucleotide positions 722 bp in the second exon of the *At5g35220* gene (Figure 1), were obtained as control for further study.

Distribution and expression of *At5g35220* gene

We determined *At5g35220* mRNA levels using RT-PCR in different tissues. Analysis in Figure 3A showed that, leaf organs expressed the highest levels of *At5g35220* mRNA. To analyze the expression of *At5g35220* gene in different development status (Figure 3b), we found that significant transcription exhibited in the young seedling (cotyledon, two true leaves seedling and before florescence). These results were in accord with The Arabidopsis Information Resource (http://www.arabidopsis.org) and previously reports (Chen et al., 2005; Guo et al., 2008) that *At5g35220* is required for chloroplast development.

Interestingly**,** when the two isoprenoids pathway was blocked by Mevacor and Fosmidomycin, the inhibitor to 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) in mevinolin (MEV) pathway (Singer et al., 1984; Rodríguez-Concepción et al., 2004) and DXR in MEP

Table 2. Characterization of col-4, 35SI and salk_134931.

Plant	Hypocotyl (mm)	Stem (cm)	Root (mm)	Fresh weight (mg/seedling)	Flowering time (day)	Seeds amount (mg/plant)	Chlorophyll a content (μg g^{-1} FW)	Chlorophyll b content (μg g^{-1} FW)	Chlorophyll ratio a/b
Col-4	2.56	71.12	21.05	75.66	25	278.79	407.75	155.44	2.62
35SI-a	2.49	70.98	20.15	73.98	25	240.56	178.17	56.92	3.13
35SI-b	1.80	59.64	17.12	40.56	25	121.06	160.17	46.92	3.43
Salk_134931	1.78	58.09	16.98	40.43	26	120.86	180.56	60.35	2.99

Hypocotyl length of 6-day-old light-grown seedlings on paper towels; stem length of 42-day-old plants; root length of 5-day-old seedlings grown on MS medium; fresh weight of 21-day-old soil-grown plants; flower time was measured as the day when the first flower opened after seeds were sown in soil; chlorophyll content of 21-day-old soil-grown plants.

pathway (Steinbacher et al., 2003; Rodríguez-Concepción et al., 2004), respectively, the higher inhibitor used the more transcripts of *At5g35220* observed which is revealed in Figure 3c. At the same time, the transcripts of *At5g35220* gene were markedly down regulated by exterior ABA and GA_3 treat-ment. The expression level becomes stabilization in base line only after 1 h/ABA and 0.5 h/GA_3, respectively. IAA and 2, 4-D treated for only 6 h seemed having no influence on *At5g35220* expression in our study as showing in Figure 3d.

DISCUSSION

To investigate the effect of the regulation of isoprenoids pathway in transgenic Arabidopsis plants, *At5g35220* gene was introduced into Arabidopsis in this study. However, two etiolated transgenic plants (*35SI-a, 35SI-b*) were found in which the transcripts of *At5g35220* were significant inhibited by RT-PCR analysis (Figure 2c). As the previously reported (Depicker and Van Montagu 1997; Metzlaff et al., 2000), the introduction of additional copies of *At5g35220* gene into Arabidopsis plants results in events of *At5g35220* gene post-transcriptional gene silencing (PTGS) or “co-suppression” in our study. Multiple models of PTGS have been proposed, including roles for RNA thresholds and DNA repeats (Baulcombe, 1996; Vaucheret et al., 1998; Chicas and Macino, 2001; Vaucheret et al., 2001).

In the present study, abnormal transcript of *POR* gene was found in *35SI* mutants (Figure 2d). This was consistent with the fact that chlorophyll (Chl) synthesis in Arabidopsis is controlled by two light-dependent NADPH-protochlorophyllide (PChlide) and oxidoreductases (PORs) (Nikolai et al., 1995). Two co-suppression transgenic plants (*35SI-a,-b*) showed phenotypic of loss of pigmentation in leaf (Figure 2a). The etiolated and retarded growth phenotypes (Figure 2a and Table 2) of the mutants may result from the compromised chloroplast function as they severely reduced levels of chlorophyll (Table 2). Significant *At5g35220* transcription exhibited in leaf organs and the seedling stage finding in our study gave further revelation that this SREBP2 is required for chloroplast development (Figure 3a, b).

In higher plants, isoprenoids play numerous vital roles in basic plant processes, including respiration, photosynthesis, growth, development, reproduction, defense and adaptation to environmental conditions (Gershenzon and Kreis, 1999, Rodriguez and Boronat 2002). Two independent pathways for the biosynthesis of isoprenoid precursors co-exist within the plant cell: the cytosolic mevalonic acid (MVA) pathway and the plastidial methylerythritol phosphate (MEP) pathway. The main MVA-derived isoprenoid end products in plants are sterols, brassinosteroids, dolichol and the prenyl groups used for protein prenylation and cytokinin biosynthesis. Primary metabolites, such as phytol group of chlorophylls, carotenoids and the plant hor-mones abscisic acid (ABA) and gibberellins (GAs) are derived directly from the MEP pathway. Interestingly, two co-suppression transgenic plants (*35SI-a,-b*) showed phenotypic of variation of pigmentation in seed (Figure 2b) in our study, suggesting that *At5g35220* gene is closely linked to carotenoid development. We arrested seedling development by specifically blocking the MVA pathway with mevinolin (MEV) or the MEP pathway with fosmidomycin (FSM) and searched for *At5g35220* gene feedback control for inhibitor adjustment. It turns out that, the induction of *At5g35220* gene in the wild type plants was signi-ficantly raised in the presence of inhibitor of MVP pathway (Figure 3c). Simultaneously, the transcription level of *At5g35220* gene was influenced by exogenous ABA, GA_3 (Figure 3d) by further Study, Showing that *At5g35220* gene is regulated in a sterol-regulated manner

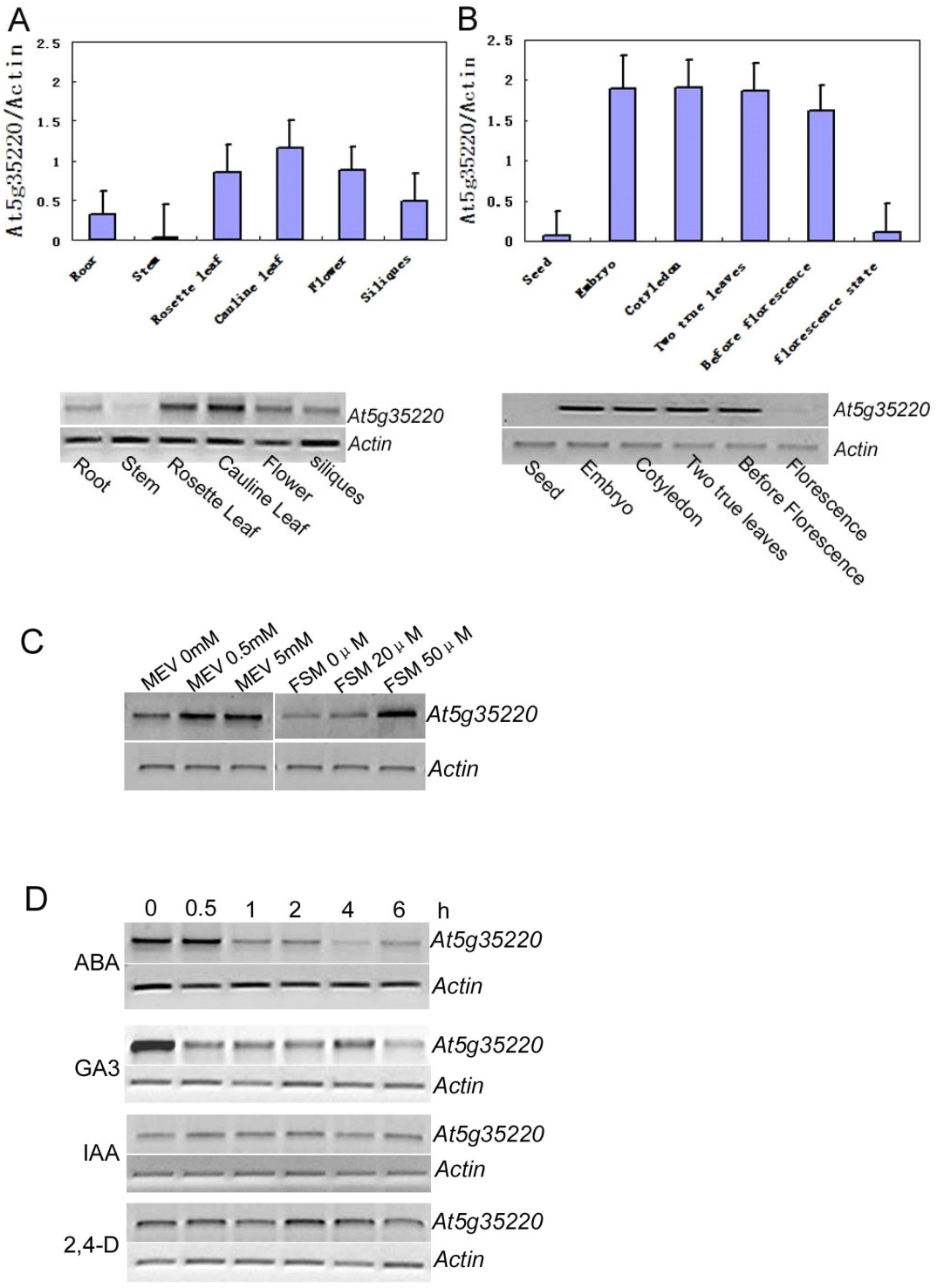

Figure 3. Expression Patterns of the *At5g35220* gene. (A) RT-PCR analysis of *At5g35220* transcripts during different development status; (B) in different organs of Arabidopsis plants Total RNA was isolated from various tissues (root, leaf, stem, flower and silique) of wild-type plants grown under long-day conditions or from seeds. Embryo seedling, cotyledon seedling and two true leaves seedling before florescence state and florescence state plants. RT-PCR was performed with either *At5g35220* -specific primers (top gel) or Actin-specific primers (bottom gel). The corresponding histogram was offered; (C) The expression of *At5g35220* under exterior MEV and FSM treatment by RT-PCR assay. MEV, Mevacor; FSM, Fosmidomycin; (D) The expression of *At5g35220* under exterior 100 μM ABA, GA3, IAA and 2, 4-D treatment by RT-PCR assay. 15 day old seedling was used. The numbers indicate hours after each treatment. Results were presented as average values ± SE from three experiments.

in *Arabidopsis*. Although, *At5g35220* gene works in the MEP pathway as a regulatory factor which remained to be further investigated.

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