Modulation of *GmFAD3* Expression Alters Abiotic Stress Responses in Soybean

Ajay K Singh (ajaysingh73ansh@gmail.com)
National Institute of Abiotic Stress Management https://orcid.org/0000-0002-4533-8705
Susheel Kumar Raina
: Government of Jammu and Kashmir
Mahesh Kumar
National Institute of Abiotic Stress Management
Lalitkumar Aher
National Institute of Abiotic Stress Management
Milind B Ratnaparkhe
ICAR Indian Institute of Soybean Research
Jagadish Rane
National Institute of Abiotic Stress Management
Aardra Kachroo
University of Kentucky

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Abstract

Fatty acid desaturases (FADs) are a class of enzymes that mediate desaturation of fatty acids by introducing double bonds. They play an important role in modulating membrane fluidity in response to various abiotic stresses. However, a comprehensive analysis of FAD3 in drought and salinity stress tolerance in soybean is lacking. We used Bean Pod Mottle Virus (BPMV)-based vector for achieving rapid and efficient overexpression as well as silencing of *Omega-3 Fatty Acid Desaturase* gene from *Glycine max* (*GmFAD3*) to assess the functional role of FAD3 in abiotic stress responses in soybean. Higher levels of recombinant BPMV-GmFAD3A transcripts were detected in overexpressing soybean plants. Overexpression of *GmFAD3A* in soybean resulted in increased levels of jasmonic acid and higher expression of *GmWRKY54* as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants under drought and salinity stress conditions. FAD3A overexpressing plants showed higher levels of chlorophyll content, efficient photosystem-II, relative water content, transpiration rate, stomatal conductance, proline content and also cooler canopy under drought and salinity stress conditions as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants. Results from the current study revealed that GmFAD3A overexpressing soybean plants exhibited tolerance to drought and salinity stresses. However, soybean plants silenced for GmFAD3 were vulnerable to drought and salinity stresses.

Key Message

This study focused on enhancing resilience of soybean crops to drought and salinity stresses by overexpression of *GmFAD3A* gene, which play an important role in modulating membrane fluidity and ultimately influence plants response to various abiotic stresses.

Introduction

Soybean [*Glycine max* (L.) Merr] is an important crop contributing to the protein and oil requirement of humans as well as animals. It is also used as a raw product for human health and industrial applications. Therefore, the demand of soybean is increasing continuously worldwide. Growth, development, reproduction and survival of plants are greatly compromised by abiotic stresses such as drought and salinity which may occur individually or in combination (Choudhury et al. 2017; Gupta et al. 2020; Lamers et al. 2020; Rane et al. 2021). Drought and salinity also cause severe losses to soybean productivity worldwide by adversely affecting plant growth, development and yield potential. Thus, enhancing resilience of soybean crops to abiotic stress in order to maintain genetic yield potential are extremely demanding areas in agricultural research.

Fatty acids are vital cellular constituents of plants since they contribute to cellular membrane architecture, suberin and cuticular waxes as well as to meeting the energy requirements of cells. Fatty acids also play an important role during signal transduction (Wang 2004). Linoleic acid and α-linolenic acid are crucial polyunsaturated fatty acids for most plant seed oils (Napier and Graham 2010). The role

of linolenic acid as a stress signaling compound and precursor of oxylipin and jasmonic acid biosynthesis in plants has also been emphasized (Upchurch 2008; Martin *et al.* 2018). Jasmonic acid is synthesized from 12-OPDA with different metabolic conversions and physiological processes and triggers defense response as well as responses to abiotic stresses (Wasternack 2007; Wang et al. 2021).

Commodity soybean oil contains about 10% palmitic acid, 4% stearic acid, 18% oleic acid, 55% linoleic acid and 13% linolenic acid. Genes controlling contents of oleic acid and polyunsaturated fatty acids have been reported in soybean (Pham et al. 2012). Fatty acid desaturase 3 (FAD3) enzymes convert linoleic acid to α-linolenic acid and this enzymatic catalytic process involves three active members viz. FAD3A, FAD3B and FAD3C (Singh et al. 2011). However, FAD3A has been reported to have a higher expression in seeds compared to FAD3B and FAD3C (Singh et al. 2011).

In tobacco plants, expression of *FAD3*/ *FAD8* enhanced tolerance to osmotic stress (Zhang et al., 2005). In another study, silencing *FAD7* in tobacco plants reduced the levels of linolenic acid and tolerance to drought and salinity stress (Im et al. 2002). In recent years, some novel genes/transcription factors modulating various abiotic stress responsive genes have been characterized by genome-wide characterization (Li et al. 2019; Do et al. 2019). Efforts have also been made to enhance abiotic stress tolerance in soybean by modulating expression of several genes/transcription factors (Jumrani and Bhatia 2019; Zhang et al. 2019; Chen et al. 2019). Previously, Singh et al. (2011) silenced FAD3 employing BPMV-based VIGS vector and FAD3-silenced plants were analyzed for biotic stress responses, but plants response to abiotic stress was not studied. Flores et al. (2008) silenced *FAD3* gene employing siRNA-mediated approach in soybean, however, comprehensive analysis of their role in drought and salinity stress responses was not carried out. In the present investigation, we assessed the functional role of FAD3A to abiotic stress responses in soybean and it was observed that the *FAD3A* overexpressing plants exhibited tolerance to drought and salinity stresses. On the contrary, *GmFAD3* silenced *plants* were vulnerable to drought and salinity stresses.

Materials And Methods

Construction of BPMV-based viral vectors for overexpression of FAD3A and silencing FAD3 gene in soybean

For construction of BPMV-based viral vector for overexpression of *GmFAD3A* gene in soybean, a full-length coding sequence (1128 bp) without stop codon of *GmFAD3A* gene was PCR amplified using soybean cDNA as template with primers (forward primer- 5'-AAAACGCCTATGGTTAAAGACACAAAG-3' and reverse primer- 5'-AAAAGGCCTGTGTCGTTGCGAGTGGAG-3'). Primers were designed to amplify full-length coding sequence of *GmFAD3A* as retrieved from the database (AY204710). In the present investigation, BPMV-based viral vector (Zhang and Ghabrial 2006) comprising of pGHopRNA1-BPMV harbouring native BPMV-RNA1 (Figure 1a) and pGG7RNA2-BPMV (Figure 1b) was used for cloning

GmFAD3A gene. For construction of GmFAD3A overexpression vector, the PCR product representing full-length coding sequence without stop codon of *GmFAD3A* digested with *Stu*I was cloned into *Msc*I-digested pGG7RNA2-BPMV vector. The BPMV-based viral vector for overexpressing GmFAD3A was designated as pGG7RNA2- BPMV-OE-GmFAD3A (Figure 1c). Construction of FAD3 silencing vector was described previously (Singh et al. 2011). *In vitro* transcription followed by rub-inoculation of soybean plants was performed as described previously (Zhang and Ghabrial 2006; Diaz-Camino et al. 2011; Singh et al. 2011; Kachroo and Ghabrial 2012; Rao et al. 2014; Shine et al. 2016).

Plant materials, growing conditions, in vitro **transcription and plant inoculation with** in vitro **transcripts**

Soybean cultivars Essex, Harosoy, Williams, NRC-37 and JS-335 were grown in the greenhouse with day and night temperatures of 27 and 24°C, respectively. *In vitro* transcripts were prepared as described previously (Kachroo and Ghabrial 2012). Soybean plants at unrolled unifoliolate leaves (VC stage) were dusted with carborundum followed by rub-inoculation with *in vitro* transcripts derived from plasmid pGHopRNA1-BPMV containing a full-length infectious BPMV-RNA1 cDNA and empty vector plasmid pGG7RNA2-BPMV, recombinant pGG7RNA2-S-FAD3-BPMV viral vector harbouring FAD3 silencing fragment, and pGG7RNA2-OE-FAD3A-BPMV having a full length coding sequence of *FAD3A* gene without stop codon, respectively for generating vector-infected control plants, FAD3-silenced and FAD3A overexpressing plants. *In vitro* transcripts were prepared separately from plasmid pGHoRNA1-BPMV, pGG7RNA2-BPMV, pGG7RNA2-BPMV-S-FAD and pGG7RNA2-FAD3A. *In vitro* transcripts prepared from pGHoRNA1-BPMV was mixed separately with transcripts derived from pGG7RNA2-BPMV, pGG7RNA2-S-FAD3-BPMV and pGG7RNA2-OE-FAD3-BPMV before rub-inoculation on VC stage soybean plants for generating vector-infected control, FAD3-silenced and FAD3A overexpressing plants, respectively. Soybean plants raised through *in vitro* transcripts inoculation were used to verify silencing *FAD3A,B,C* gene or overexpression of *FAD3A* gene. Soybean plants with four unfolded trifoliolate leaves (V3 growth stage), which were previously inoculated on the unifoliate leaves with fine ground freeze-dried leaves of vector-infected, FAD3-silenced and FAD3A overexpressing plants raised from *in vitro* transcripts inoculation, were used to study response of soybean plants to drought and salinity stresses at physiological and molecular levels. The experiments were replicated three times and plants used for testing abiotic stress responses were verified for *GmFAD3A* mRNA level by semi-quantitative RT-PCR and also RT-qPCR.

RNA extraction, Reverse Transcriptase PCR and quantitative Real-Time PCR

Total RNA extraction from leaf tissues of mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants 21 days post-infection was performed using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) as per manufacturer's instructions. First-strand cDNA synthesis was performed using SuperScript® II Reverse Transcriptase (Invitrogen). Relative differences in FAD3A transcript accumulation in mock-inoculated, vector-infected and FAD3 overexpressing soybean plants were analyzed by 25- cycle semi-quantitative Reverse Transcriptase-mediated PCR (RT-PCR) in three independent RNA preparations

using forward primer-5'-AAAACGCCTATGGTTAAAGACACAAAG-3' and reverse primer- 5'-AAAAGGCCTGTGTCGTTGCGAGTGGAG-3' to amplify a 1128 bp PCR product. Expression profiling of *GmWRKY54* transcription factor in mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants under well-watered (no stress), drought and salinity stress conditions was conducted employing semi-quantitative RT-PCR. Transcript accumulation of *β-Tubulin* from mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants was also performed using forward primer 5'-CAATTGGAGCGCATCAATG-3' and reverse primer 5'-ATACACTCATCAGCATTCTC-3' to check equal quantity loading of cDNA on a 1% agarose gel. Relative differences in transcript levels in mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing soybean plants were also evaluated by quantitative RT-PCR (RT-qPCR) using forward primer 5'-CACTGTGCCATTTCCATTGTT-3' and reverse primer 5'- GGTGGGAACAGATTGCTGTA-3'. The cDNA was synthesized and DNA amplification was performed in the presence of SYBR green Real-Time Bio-Rad PCR master mix on the Bio-Rad CFX 96 Touch Real-Time PCR detection system. The relative mRNA levels of FAD3A were determined by normalizing the PCR threshold cycle number with that of *β-Tubulin*. All experiments were repeated three times independently and the average was calculated.

Assessment of tolerance to drought and salinity stress in FAD3-silenced and FAD3A overexpressing soybean plants

Nine individual soybean plants (cvs. Essex, Harosoy, Williams, NRC-37 and JS-335) were tested for each experiment. Tolerance to drought stress at whole plant level in mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants was evaluated by uniformly withholding watering for 4 days under greenhouse conditions. For salinity stress tolerance assessment, mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants were watered with 100 and 150 mM NaCl for four days.

Moisture measurements in pot experiment

Mock-inoculated, vector-infected, FAD3-silenced and FAD3 overexpressing soybean plants were exposed to drought stress by withholding watering for four days in green house. Soilrite samples from pots for drought stress and well-watered (no stress) treatments were collected in aluminum boxes with lids fitted securely. The samples were weighed immediately after collection and oven dried at 105°C for 72 h to determine moisture content of soilrite by gravimetric method. Moisture content of soilrite mix was calculated using formula: Moisture content (%) = Weight of moist soilrite - weight of dry soilrite / weight of dry soilrite x 100.

Chlorophyll assay, leaf SPAD value, photosystem II efficiency and relative water content

For chlorophyll content estimation, 250 mg of finely-ground fresh leaf tissue of mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants was extracted with 10 ml of DMSO in a test tube and incubated at 60°C for 6 hours. Absorbance was measured at 665 nm and 649 nm using UV–VIS spectrophotometer (UV-1800, Shimadzu, Japan) and total chlorophyll contents were estimated as

described by Barnes et al. (1992). The SPAD meter (SPAD-502, Konica Minolta Optics, Inc. Japan) was used to measure the greenness or relative chlorophyll content in leaves of mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants under no stress, drought stress and salinity stress conditions (100 and 150 mM NaCl). A total 9 plants were analyzed for SPAD value with 3 readings recorded from each plant. Chlorophyll fluorescence was measured in the excised leaves at V3 stage of mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing soybean plants after 21 days for evaluating photosystem II efficiency. The leaf images were captured by chlorophyll fluorescence measuring system (PSI, Czechoslovakia) and image analysis was performed using fluorochrom7 software. The relative water content (RWC) was determined in mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing soybean plants at V3 stage under no stress and drought stress condition imposed by withholding watering for four days. The RWC was calculated using following equation, RWC = (FW-DW) X 100 / (SW-DW), where FW is the fresh weight, SW is the water-saturated weight and DW is the dry weight (Turner, 1981). For RWC estimation, trifoliate leaves were excised and were weighed immediately to record fresh weight. Then turgid weight was determined by soaking leaves in water for 6 h in distilled water at room temperature and then surface water was removed and leaves were weighed. Dry weight was measured after drying the leaves in oven at 65°C for 72 hours.

Canopy temperature, leaf stomatal conductance and transpiration rate

The canopy temperature was measured with a hand-held IR-Gun thermometer under no stress and drought stress condition imposed by withholding watering for four days at V3 stage of mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing soybean plants after 21 days. The data were recorded approximately 50 cm above the canopy. A porometer (Delta-T AP4, Delta-T Devices, Cambridge, UK) was used to estimate stomatal conductance of leaves with 3rd trifoliate leaf of mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants and 9 replicates were used for measurement. Transpiration rate was measured on trifoliate leaves of mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants with a portable photosynthesis system (GFS 3000, WALZ) at a photosynthetic photon flux density 900 µmol m^{-2} s^{-1}, and air temperature of 25 ± 1°C to 28 ± 2°C.

Proline content

Free proline content of fresh leaf samples (500 mg) of mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants was estimated using the ninhydrin method as described by Bates et al. (1973). Optical density was measured at 520 nm using toluene as blank using UV–VIS spectrophotometer (UV-1800, Shimadzu, Japan). The amount of proline was determined from a standard curve.

Fatty acid and jasmonic acid analysis

Fatty Acid (FA) analysis was performed as described previously (Kachroo et al. 2008). For FA analysis, leaves from 3-4 weeks-old mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing

soybean plants were transferred in 2 ml of 3% H_2SO_4 in methanol containing 0.001% butylated hydroxytoluene. One ml of hexane with 0.001% butylated hydroxytoluene was added after 30 min of incubation at 80°C. The hexane phase was then collected in vials for gas chromatography (GC), and samples were analyzed by GC on a Varian FAME 0.25-mm 650-m column and were quantified with flame ionization detection. The identities of the peaks were determined by comparing the retention times with known FA standards. Mole values were obtained by dividing peak area by molecular weight of the FA.

For extraction of jasmonic acid, one gram leaf tissue of mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants was ground in cold 100% methanol and dihydrojasmonic acid was used as an internal standard. The methanol extract was passed through Seppak-18 column (Waters: Sep-Pak Classic C18 cartridge). The column purified extract was processed as described earlier by Xia et al. (2009) and used to inject into gas chromatograph attached to an electron ionization detector (Hewlett Packard GCD Systems).

Statistical Analysis

Data obtained for the soil moisture, biochemical, physiological and molecular analyses was subjected to Analysis of Variance (ANOVA). Each experiment was repeated three times and the data shown are the average of three replicates ± Standard Errors. Significant differences among the mean values were compared using the Student's t-Test. For fatty acid profiling of leaf, immature and mature seeds from mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants, experiments were repeated three times. The data depicted are the average of three replicates ± Standard Error. One way ANOVA was used for statistical analysis of data. Least Significant Difference (LSD) at $p<0.05$ was calculated for making comparison of mean values.

Results

Higher accumulation of BPMV-RNA2-GmFAD3A recombinant transcripts in GmFAD3A-overexpressing plants

For overexpression of *GmFAD3A*, full length cDNA (1128 bp) coding for *GmFAD3A* lacking the termination codon, was inserted in *Msc*I site of the pGG7RNA2-BPMV vector. Semi-quantitative RT-PCR analysis using primers designed to amplify the full-length coding sequence of GmFAD3A was performed to study accumulation of endogenous *GmFAD3A* or recombinant BPMV-RNA2:GmFAD3A transcripts, was performed. Densitometry analysis revealed about 5 times higher accumulation of *GmFAD3A* transcripts in overexpressing plants compared to mock-inoculated and vector-infected soybean plants (Figure 2a). The RT-qPCR was also conducted to analyze endogenous *GmFAD3A* or recombinant *BPMV-RNA2:GmFAD3A* transcripts. The RT-qPCR analysis revealed about three-fold decrease in FAD3mRNA levels in FAD3-silenced soybean plants, while about five-fold higher FAD3A-mRNA levels in FAD3A overexpressing soybean plants **(**Figure 2b**).** Plants infected with recombinant vector carrying a full-length cDNA of *GmFAD3A* showed distinct phenotype compared to the vector-infected and FAD3-silenced soybean plants

(Figure 2c). To further validate the transcript abundance for *GmFAD3A*, RNA blots were probed using *GmFAD3A* specific probe (Figure 2d). *GmFAD3A* overexpressing plants revealed two bands corresponding to endogenous *GmFAD3A* transcripts and recombinant *BPMV-RNA2-GmFAD3A* transcripts. In mock-inoculated and vector-infected, as expected, a single RNA band corresponding to endogenous *GmFAD3A* was detected. Due to the strong hybridization signals from recombinant *BPMV-RNA2:GmFAD3A*, the exposure time for overexpression treatments was kept very short (< 30 minutes). Since, exposure time was different, autoradiogram of mock-inoculated and vector-infected control was separated by doted lines from FAD3A overexpression treatment (Figure 1d). Because of lower exposure time, lower abundance of endogenous *GmFAD3A* was detected in FAD3A overexpressing plants when compared to mock-inoculated and vector-infected plants (Figure 2d). Otherwise, levels of endogenous *GmFAD3A* were similar in mock-inoculated, vector-infected and *FAD3A* overexpression treatments.

Silencing FAD3 and overexpression of GmFAD3A **gene alter fatty acid profile in soybean Plants.**

Since FAD3 protein desaturates 18:2 to 18:3 in soybean, we analyzed mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing soybean plants for their fatty acid (FA) profiles in leaves, immature and mature seed. In line with our earlier reports (Kachroo et al. 2008; Singh et al. 2011), infection with BPMV did not significantly alter FA levels in soybean plants. FAD3A overexpression significantly increased 18:3 levels in leaves and immature seeds compared to mock-inoculated and vector-infected soybean plants. On the other hand, FAD3-silenced plants exhibited significant reduction in 18:3 levels in their leaf tissues as well as immature seeds as compared to mock-inoculated and vector-infected soybean plants. Leaf tissue from FAD3A overexpressing plants showed 69.5 percent mole level of 18:3 as compared to 31.2 percent mole level in S-FAD3, 41.5 percent mole level in vector-infected and 42.5 percent mole level in mock-inoculated soybean plants **(Table 1).** Furthermore, immature seeds from FAD3A overexpressing plants revealed 39.5 percent mole level of 18:3 compared to 19.1 percent mole level in S-FAD3, 28.1 percent mole level in vector-infected and 26.5 percent mole level in mock-inoculated soybean plants **(Table 1).** The level of 18:2 and 18:3 was almost similar in mature seed from mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing soybean plants (Table 1).

Table 1: Fatty acid profile in mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing soybean plants. Fatty acid content ± SE. Experiments were repeated three times. n=3.

	Fatty acid content (mol%)

Plants used	Tissue used	16:0	18:0	18:1	18:2	18:3
Mock-inoculated	Leaf	17.4±0.5	4.1±0.5	2.5±0.2	16.8±1.8	42.5±1.5
	Immature seed	11.5±1.1	4.8±0.4	9.2±0.7	31.1±0.7	26.5±1.5
	Mature seed	15.1±0.5	3.5±0.2	16.5±0.4	58.5±3.6	10.9±1.5
Vector- infected	Leaf	16.5±1.2	4.5±0.5	2.8±0.3	16.2±1.3	41.5±1.5
	Immature seed	11.0±0.6	4.0±0.3	8.8±0.8	32.1±0.7	28.1±1.5
	Mature seed	14.3±1.1	2.8 ±0.6	15.6±0.5	56.5±1.8	11.2±2.5
FAD3-silenced	Leaf	18.5±0.3	5.5±0.2	5.4±0.1	38.0±1.4	31.2±1.7
	Immature seed	12.4±1.2	4.9±0.5	8.5±0.6	45.5±1.9	19.1±2.1
	Mature seed	16.3±1.4	2.5 ±0.7	15.1±0.8	55.5±1.5	10.2±2.3
FAD3A overexpressing	Leaf	17.8±0.5	4.7±0.4	4.1±0.2	12.0±1.6	69.5±2.7
	Immature seed	10.4±0.5	4.8±0.3	9.5±0.3	20.5±1.6	39.5±2.8
	Mature seed	15.9±0.8	3.1±0.3	16.1±0.3	55.5±2.5	11.5±2.5
LSD<0.05	Leaf	ns	ns	0.58	1.65	3.39
	Immature seed	2.21	ns	ns	1.75	2.48
	Mature seed	ns	1.70	ns	ns	ns

ns=Non significant

Overexpression of GmFAD3A does not alter soybean seed size

Our earlier work (Singh et al. 2011) had demonstrated increase in soybean seed size and weight in *FAD3*-silenced plants in comparison to mock-inoculated and vector-infected plants. In the present investigation also, seed weight and seed size were higher of FAD3-silenced plants compared to mock-inoculated, vector-infected and FAD3A overexpressing plants (Table 2). To study the impact of *GmFAD3A* overexpression, these seed traits were analyzed in mock-inoculated, vector-infected and FAD3A overexpressing plants of soybean cvs. Essex, Harosoy, Williams, NRC-37 and JS-335. Although the *GmFAD3A* overexpressing plants exhibited BPMV associated symptoms, they produced pods with seeds that were similar to those of mock-inoculated and vector-infected soybean plants in size and weight (Table 2).

Table 2
Yield related attributes in mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants. Mean ± SE. Experiments were repeated three times. n=3

Soybean genotypes	Plant	Seed yield related traits			
		Number of seeds/plant	Seed width (mm)	Seed length (mm)	100 seed weight (g)
Essex	Mock-inoculated	203±1.72	4.0±0.24	5.0±0.37	12.0±0.62
	Vector-infected	200±1.51	4.2±0.11	5.2±0.48	12.5±0.54
	FAD3-silenced	198±1.43	5.9±0.30	6.8±0.54	17.0±0.43
	FAD3A overexpressing	203±0.61	4.1±0.21	5.1±0.21	12.7±0.48
LSD<0.05		ns	0.55	0.95	1.06
Harosoy	Mock-inoculated	212±1.84	4.1±0.42	5.5±0.51	12.2±0.57
	Vector-infected	210±1.57	4.3±0.31	5.7±0.47	12.6±0.35
	FAD3- silenced	205±0.94	6.2±0.54	7.0±0.64	17.5±0.94
	FAD3A overexpressing	212±1.04	4.2±0.31	5.4±0.34	12.8±0.45
LSD<0.05		ns	0.62	0.92	1.26
Williams	Mock-inoculated	255±1.92	4.2±0.37	4.9±0.22	11.5±0.54
	Vector-infected	250±2.01	4.3±0.24	5.1±0.11	12.1±0.70
	FAD3- silenced	247±1.52	6.5±0.41	6.7±0.34	16.5±1.15
	FAD3A overexpressing	255±1.34	4.1±0.30	5.2±0.27	12.5±0.54
LSD<0.05		ns	0.65	0.88	1.38
NRC-37	Mock-inoculated	155±1.22	4.0±0.45	5.0±0.52	11.0±0.54
	Vector-infected	150±1.51	4.2±0.51	5.3±0.32	11.5±0.43
	FAD3- silenced	145±0.83	6.0±0.32	6.9±0.25	17.0±0.81
	FAD3A overexpressing	155±0.75	4.3±0.25	5.1±0.42	11.8±0.47
LSD<0.05		ns	0.75	0.82	1.42

ns=Non significant

Soybean genotypes	Plant	Seed yield related traits			
		Number of seeds/plant	Seed width (mm)	Seed length (mm)	100 seed weight (g)
JS-335	Mock-inoculated	156±1.54	4.1±0.21	4.8±0.12	12.0±0.78
	Vector-infected	152±1.25	4.3±0.35	5.2±0.23	12.5±0.57
	FAD3- silenced	158±0.96	5.9±0.48	6.8±0.36	16.5±0.94
	FAD3A overexpressing	163±1.31	4.5±0.56	5.1±0.46	12.8±0.45
LSD<0.05		ns	0.74	0.95	1.34
ns=Non significant					

Moisture level under well-watered (no stress) and drought stress conditions

Moisture level was measured by gravimetric method for soilrite used to grow mock-inoculated, vector-infected, FAD3-silencd and FAD3A overexpressing, soybean plants. For imposing drought stress watering withheld for four days in green house. Under no stress condition, the moisture content of soilrite was about 30%, while under drought stress condition for four days the moisture content depleted up to 16% (Table 3). It was observed that FAD3A overexpressing pants survived and grew normally, while mock-inoculated, vector-infected and FAD3-silenced plants showed severe wilting phenotype at depleted moisture conditions (16%).

Table 3
Soiltrite moisture after withholding watering for different time periods (0-4 days) of mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants. Mean ± SE. Experiments were repeated three times. n=3.

Plants	Moisture content in soilrite after withholding watering (%)				
	0-day	1-day	2-day	3-day	4-day
Mock-inoculated	30.2±0.69	26.9±0.11	23.2±0.22	20.0±0.19	16.1±0.11
Vector-infected	30.8±0.65	27.4±0.56	23.9±0.30	20.2±0.40	16.4±0.22
FAD3-silenced	29.3±0.58	26.0±0.51	22.3±0.77	19.4±0.56	15.6±0.11
FAD3A- overexpressed plants	29.8±0.60	25.9±0.59	22.1±0.29	19.0±0.19	16.0±0.20
LSD<0.05	ns	ns	ns	ns	ns
ns: Non significant					

Enhanced tolerance to drought and salinity stresses in plants with higher FAD3A Expression

Overexpression of *FAD3* or *FAD8* in tobacco plants is known to increase osmotic stress tolerance (Zhang et al. 2005). This investigation impelled us to investigate impact of *GmFAD3A* overexpression on drought and salinity stress tolerance in soybean plants. Mock-inoculated, vector-infected, FAD3-silenced and *GmFAD3A* overexpressing soybean plants were subjected to drought and salinity stress. With increasing water deficit, mock-inoculated, vector–infected and FAD3-silenced plants started wilting followed by drooping of leaves, although very less drooping was observed in OE-FAD3A plants when subjected to drought stress conditions (Figure 3a). For salinity stress tolerance evaluation, mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing soybean plants were watered with 100 and 150 mM NaCl solution in pots filled with soilrite (mixture of peat moss, perlite and vermiculite). Within four days of salt stress, mock-inoculated, vector-infected and FAD3-silenced plants exhibited leaf scorching and this scorching progressively led to leaf necrosis with increasing exposure to salt stress. Interestingly, FAD3A overexpressing plants did not develop any leaf scorching (Figure 3b). Under no stress mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing soybean plants grew well (Figure 3c).

Higher chlorophyll content, SPAD Value, maximum Quantum Yield (QY_max) and relative water content under drought and salinity stress conditions

Chlorophyll content was significantly higher in FAD3A overexpressing soybean plants compared to mock-inoculated, vector-infected and FAD3-silenced under no stress as well as drought and salinity stress conditions (150 mM NaCl) (Figure 4a). In drought and salinity stress condition, reduction in chlorophyll content was less in FAD3A overexpressing plants as compared to mock-inoculated, vector-infected and FAD3-silenced. When FAD3A overexpressing plants were subjected to 150 mM NaCl, chlorophyll content decreased from 3.07 ±0.18 mg/g FW to 2.19 ±0.07 mg/g FW. On the other hand, when mock-inoculated, vector-infected, and FAD3-silenced plants were treated with 150 mM of NaCl for 4 days, chlorophyll content decreased from 2.14±0.14 mg/g FW to 0.99 ±0.07 mg/g FW, 1.89 ±0.12 mg/g FW to 0.76 ±0.07 mg/g FW, 2.16 ±0.13 to 1.30 ±0.09 mg/g FW, respectively. Upon exposure to drought stress, the chlorophyll content decreased from 2.14 ±0.14 mg/g FW to 1.37 ±0.25 mg/g FW, 1.89 ± 0.12 mg/g FW to 1.04 ±0.24 mg/g FW, 2.16 ±0.13 mg/g FW to 1.64 ±0.17 mg/g FW, respectively in mock-inoculated, vector-infected and FAD3-silenced. However, in case of FAD3A overexpressing plants, chlorophyll content reduced from 3.07 ±0.18 mg/gFW to 2.41 ±0.22 mg/g FW under drought stress conditions.

FAD3A overexpressing plants exhibited increase in SPAD value as compared to mock-inoculated, vector-infected plants and FAD3-silenced plants under no stress, drought and salinity stress conditions (Figure 4b). The SPAD value decreased markedly in mock-inoculated, vector-infected, and FAD3-silenced plants as compared to FAD3A overexpressing plants under drought and salinity stress conditions. Under drought stress condition, the SPAD value decreased from 48.66 ±3.30 to 38.33 ±2.44, 45.66 ±4.52 to 35.22 ± 3.06, 53.55 ±1.59 to 41.33 ±2.11, respectively in mock-inoculated, vector-infected and FAD3-silenced plants, but from 64.34 ±2.43 to 52.88 ±2.18 in FAD3A overexpressing plants. When mock-inoculated, vector-infected

and FAD3 silenced plants were exposed to 150 mM salt stress, the SPAD value decreased from 48.66 ±3.30 to 33.22 ±3.03, 45.66 ±4.52 to 31.00 ± 2.30, 53.55 ±1.59 to 37.55 ±1.40, respectively. By contrast, SPAD value in FAD3A overexpressing plants decreased from 64.33 ±2.43 to 49.55 ±1.19, when subjected to 150 mM NaCl salt stress.

Maximum quantum yield (QYmax) in terms of Fv/Fm value indicates efficiency of photosystem II which is adversely affected under drought and salinity stress condition. In the present study, Fv/Fm values was higher in FAD3A overexpressing plants as compared to mock-inoculated, vector-infected and FAD3-silenced plants under no stress, drought and salinity stress condition (Figure 4c). Maximum quantum yield (Fv/Fm) value markedly decreased in mock-inoculated, vector-infected and FAD3-silenced plants as compared to FAD3A overexpressing plants under drought and salinity stress conditions (Figure 4c). Mock-inoculated, vector-infected, and FAD3-silenced plants exposed to 150 mM NaCl salt stress recorded a reduction in the Fv/Fm values from 0.76 ±0.03 to 0.45 ±0.01, 0.72 ±0.026 to 0.38 ±0.02, 0.75 ±0.02 to 0.48 ±0.02, respectively. In contrast, Fv/Fm values in FAD3A overexpressing plants under salt stress decreased from 0.89 ±0.02 to 0.64 ± 0.03. Upon exposure to drought stress, the Fv/Fm value decreased from 0.76 ±0.03 to 0.45 ±0.01, 0.72 ±0.03 to 0.42 ±0.01, 0.75 ±0.02 to 0.51 ±0.03, respectively in mock-inoculated, vector-infected and FAD3-silenced plants, but from 0.89 ±0.02 to 0.69±0.03 mg/g-FW in FAD3A overexpressing plants.

Under well-water, drought and salinity stress condition, FAD3A overexpressing plants exhibited higher RWC as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants (Figure 4d). It was observed that decrease in RWC was less in FAD3A overexpressing plants as compared to mock-inoculated, vector-infected and FAD3-silenced under drought and salinity stress conditions. Under drought stress conditions, the RWC decreased from 72 ±2.04 to 56.89 ±4.31, 67.11 ±2.12 to 51.44 ± 2.55, 74.00 ±2.56 to 57.89 ±2.18, respectively in mock-inoculated, vector-infected and FAD3-silenced plants, but from 88.78 ±2.38 to 74.22 ±1.92 in FAD3A overexpressing plants. When mock-inoculated, vector-infected and FAD3-silenced plants were exposed to salt stress, the RWC decreased from 72 ±2.04 to 51.88 ±2.54, 67.11 ±2.552 to 45.44 ±1.84, 74.00 ±2.56 to 51.67 ±1.73, respectively. By contrast, when FAD3A overexpressing plants were exposed to 150 mM NaCl for 4 days, the RWC decreased from 88.78 ±2.38 to 65.22 ±2.26. These results suggested that the overexpression of *FAD3A* gene had a strong effect in maintaining the water status as compared to mock-inoculated, vector-infected and FAD3-silenced plants.

FAD3-overexpressing plants have lower canopy temperature

FAD3A overexpressing plants showed lower canopy temperature as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants under no stress and drought stress conditions (Figure 5a). Under drought stress condition, canopy temperature markedly increased in mock-inoculated, vector-infected and FAD3-silenced plants compared to FAD3A overexpressing plants (Figure 5a). The canopy temperatures under drought stress condition increased from 27.78 ± 0.92°C to 31.68 ± 0.72°C, 28.43 ± 0.77°C to 32.33 ± 0.84°C, 26.9 ± 0.84°C to 29.03 ± 0.57°C, respectively in mock-inoculated, vector-infected, FAD3-silenced plants. However, in FAD3A overexpressing soybean plants canopy temperature raised from 23.91 ± 0.89°C to 26.15 ± 0.79°C. The results showed that under drought stress condition,

FAD3A overexpressing plants maintained comparatively cooler canopy as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants.

FAD3A-overexpressing soybean plants have higher transpiration rate and stomatal conductance

Transpiration controls water absorption from roots and regulates water status of plants. FAD3A overexpressing plants exhibited significantly higher transpiration rate compared to mock-inoculated, vector-infected and FAD3-silenced plants under drought and salt stress conditions (Figure 5b). When mock-inoculated, vector-infected, and FAD3-silenced plants were subjected to salinity stress, transpiration rate decreased from 2.02 ± 0.21 mmol $m^{-2}s^{-1}$ to 1.00 ± 0.08 mmol m^2s^{-1}, 1.73 ± 0.12 mmol $m^{-2}s^{-1}$ to 0.87 ± 0.06 mmol $m^{-2}s^{-1}$, 2.44 ± 0.83 to 1.37 ± 0.03 mmol $m^{-2}s^{-1}$, respectively. In contrast, when FAD3A overexpressing plants were subjected to salt stress, the transpiration rate decreased from 3.5 ± 0.26 mmol $m^{-2}s^{-1}$ to 2.10 ± 0.12 mmol $m^{-2}s^{-1}$. Under drought stress condition, the transpiration rate decreased from 2.02 ± 0.21 mmol $m^{-2}s^{-1}$ to 1.43 ± 0.11 mmol $m^{-2}s^{-1}$, 1.73 ± 0.12 mmol $m^{-2}s^{-1}$ to 1.24 ± 0.11 mmol $m^{-2}s^{-1}$, 2.44 ± 0.0.08 mmol $m^{-2}s^{-1}$ to 1.68 ± 0.22 mmol $m^{-2}s^{-1}$, respectively in mock-inoculated, vector-infected and FAD3-silenced plants. However, in FAD3A overexpressing plants, transpiration rate decreased from 3.5 ±0.21 mmol $m^{-2}s^{-1}$ to 2.41 ± 0.25 mmol $m^{-2}s^{-1}$.

Under no stress and drought stress conditions, FAD3A overexpressing plants exhibited higher stomatal conductance as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants (Figure 5c). Stomatal conductance in mock-inoculated, vector-infected and FAD3-silenced plants under salinity stress decreased from 0.21 ±0.01 mmol $m^{-2}s^{-1}$ to 0.12 ±0.005 mmol $m^{-2}s^{-1}$, 0.18 ±0.01 mmol $m^{-2}s^{-1}$ to 0.11 ±0.01 mmol $m^{-2}s^{-1}$, 0.23 ±0.005 to 0.14 ±0.02 mmol $m^{-2}s^{-1}$, respectively. In contrast, stomatal conductance in FAD3A overexpressing plants decreased from 0.32 ±0.05 mmol $m^{-2}s^{-1}$ to 0.26 ±0.01 mmol $m^{-2}s^{-1}$. Stomatal conductance under drought stress condition reduced from 0.21 ±0.01 mmol $m^{-2}s^{-1}$ to 0.15 ±0.01 mmol $m^{-2}s^{-1}$, 0.18 ±0.01 mmol $m^{-2}s^{-1}$ to 0.13 ±0.01 mmol $m^{-2}s^{-1}$, 0.23 ±0.005 mmol $m^{-2}s^{-1}$ to 0.17 ±0.01 mmol $m^{-2}s^{-1}$, respectively in mock-inoculated, vector-infected and FAD3-silenced plants, but from 0.32 ±0.005 mmol $m^{-2}s^{-1}$ to 0.26 ±0.01 mmol $m^{-2}s^{-1}$ in FAD3A overexpressing plants.

GmFAD3-overexpressing plants accumulate higher levels of jasmonic acid under drought and salinity stress conditions

Jasmonic acid and its metabolites collectively known as jasmonates and play an important role in plant development and stress responses (Wasternack et al. 2013). FAD3 mediates unsaturation of linoleic acid to produce α-linolenic acid. Since α-linolenic acid is a precursor for JA biosynthesis, JA levels were quantified in mock-inoculated, vector-infected, FAD3-silenced as well as FAD3A overexpressing plants under no stress and also under drought and salinity stress conditions. JA levels were not significantly altered under no stress, drought and salinity stress conditions in mock, vector-infected and FAD3-silenced soybean plant. Compared with vector-infected soybean plants, GmFAD3A overexpressing plants showed

approximately 3-fold and 6-fold higher levels of JA under no stress, and drought and salinity stress conditions, respectively (Figure 6a). These results indicate that JA plays an important role in drought and salinity stress tolerance in soybean.

Increase in proline level in FAD3A overexpressing plants under drought and salinity stress conditions

Earlier reports demonstrated that proline contents increase in response to drought and salinity stress (Trovato et al. 2008; Goel et al. 2010, Goel et al. 2011). These finding prompted us to measure proline level in mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants under no stress, drought and salinity stress conditions. The level of proline accumulated in FAD3A overexpressing plants under drought and a salinity stress condition were much higher than that in mock-inoculated, vector-infected and FAD3-silenced soybean plants (Figure 6b). Under no stress condition, the proline level was 2.17 ±0.35 mg/g FW, 1.94 ±0.43 mg/g FW, 2.61 ±0.54 mg/g FW, 2.77 ±0.53 mg/g FW, respectively in mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants. Under drought stress conditions, proline content increased from 2.17 ±0.35 mg/g FW to 3.04 ±0.45 mg/g FW, 1.94 ±0.43 mg/g FW to 3.43 ±0.48 mg/g FW, 2.61 ±0.54 to 4.01 ±0.54 mg/g FW, 2.77 ±0.53 to 6.79 ±0.48 mg/g FW, respectively in mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants. When mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants were subjected to salt stress, proline content increased from 2.17 ±0.35 mg/g FW to 4.1 ±0.48 mg/g FW, 1.94 ±0.43 mg/g FW to 4.3 ±0.55 mg/g FW, 2.61 ±0.54 to 5.5 ±0.65 mg/g FW, 2.77 ±0.53 to 7.5 ±0.55 mg/g FW, respectively.

Higher expression of GmWRKY transcription factor in FAD3A overexpressing plants under drought and salinity stress

Differential tolerance to abiotic stress was achieved in Arabidopsis when transformed with *GmWRKY* transcription factor (Zhou et al. 2008). This prompted us to study the expression of *WRKY* transcription factor in FAD3A overexpressing plants. Mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants were analyzed for expression of *WRKY54* transcription factor under no stress, drought and salinity stress conditions. Higher expression of *GmWRKY54* transcription factor was detected in FAD3A overexpressing plants as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants under drought and salinity stress condition. Interestingly, in non-stressed plants, expression of *GmWRKY-54* transcription factor did not change in mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing plants (Figure 6c).

Discussion

Abiotic stresses have a profound impact on almost all aspects including growth, development and reproduction in plants (Devireddy et al. 2021, Rane et al. 2021). Therefore, it is crucial to understand abiotic stress responses in plants in order to enhance abiotic stress resilience to maintain genetic yield potential. Fatty acids are vital constituents of cellular membrane architecture. The cell membrane acts as a prime sensor for abiotic stresses and its stabilization is vital for the survival of the plant (Zhang et al. 2005; Shi et al. 2008). Membrane stability and its integrity maintenance is largely affected by lipid

content and fatty acid desaturation (Mikami and Murata. 2003; Shi et al. 2008). Hence, fatty acid desaturation by fatty acid desaturases and increase in linolenic acid are considered critical factors for tolerance of plants to multiple abiotic stresses (Upchurch 2008). Fatty acid desaturase 3 (FAD3) is known to mediate conversion of linoleic to α-linolenic acid, a polyunsaturated fatty acid whose levels are altered under abiotic stress conditions (Napier et al. 1999). In the present study, the transcript levels of the *GmFAD3A* gene were manipulated in soybean by overexpression as well as silencing of FAD3 using a BPMV-based viral vector. Overexpression of the *GmFAD3A* gene was confirmed by semi-quantitative RT-PCR and RT-qPCR. Overexpression of FAD3 employing BPMV-based viral vector resulted very high level of α-linolenic acid which in turn resulted drought and salt stress tolerance. Im et al. (2002) reported that tobacco plants with antisense expression of omega-3 fatty acid desaturase from Arabidopsis had reduced salt tolerance. Shi et al. (2018) reported that overexpression of *Chorispora bungeana* microsomal *ω-3 FAD3* gene (*CbFAD3*) increased linolenic acid (C18:3) in both leaves and roots, which in turn enhanced plant tolerance to drought and salt stresses in tobacco and correlated it with activation of reactive oxygen species scavenging system, plasma membrane Ca^{2+}-ATPase and stress-induced Ca^{2+} signaling. Similarly, yeast transformed with ω-6 desaturases from sunflower had increased salt tolerance (Upchurch 2008). Recently, Rane et al. (2021) studied implications of reactive oxygen species and antioxidative system with respect to effective use of water in crop plants.

We show here that higher expression of *GmFAD3A* led to enhanced drought and salt stress tolerance in soybean, while FAD3-silenced plants were vulnerable to drought as well as salinity stresses. These results may be explained based on changes occurred at physiological, biochemical and molecular levels under non-stress, drought and salinity stress conditions. We observed that FAD3A overexpressing soybean plants showed significantly higher chlorophyll content, maximum quantum yield (Fv/Fm), RWC as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants under no stress as well as under drought and salinity stress conditions. Less decrease in chlorophyll content and leaf SPAD value were observed in FAD3A overexpressing plants as compared to mock-inoculated, vector-infected, FAD3-silenced plants under drought and salinity stress conditions. The ability of FAD3A overexpressing soybean plants to tolerate drought and salinity stress could be associated with chlorophyll content and protection of chlorophyll from degradation under drought and salt stress conditions. Leaf chlorophyll content is considered to be a good indicator of photosynthetic capability in terms of Photosystem-II (PS-II, Fv/Fm) efficiency. Maximum quantum yield (QYmax) in terms of Fv/Fm value, which indicates photochemical efficiency varies with severity of drought and decreased drastically during prolonged drought stress (Zivcak et al. 2008; Rane et al. 2019; Rane et al. 2021). Kumar et al. (2017) reported that photosynthetic efficiency in soybean was closely associated with canopy greenness reflected by higher chlorophyll content. Markedly decrease in photosynthetic efficiency (PS-II) due to lower chlorophyll content may be the reason for reduced drought and salt stress tolerance in mock-inoculated, vector-infected, FAD3-silenced plants than that of FAD3A overexpressing soybean plants under drought and salinity stress conditions. Jamil et al. (2007) also reported reduction in chlorophyll content in radish due to salt stress. Water status of plants is also crucial for plants response to various abiotic stresses especially drought and salinity. FAD3A overexpressing plants showed relatively higher RWC compared to

mock-inoculated, vector-infected and FAD3-silenced plants under no stress, drought and salinity stress condition. It was observed that RWC decreased markedly in mock-inoculated, vector-infected and FAD3-silenced plants as compared to FAD3A overexpressing plants under drought and salinity stress condition. Drought and salinity stress induced reduction in the relative water content indicated a decrease in turgor that resulted in limited water availability for cellular process in mock-inoculated, vector-infected and FAD3-silenced plants as compared to FAD3A overexpressing soybean plants.

Canopy temperature was found lower in FAD3A overexpressing plants as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants under no stress, drought and salt stress conditions. Several researchers demonstrated canopy temperature as an indicator to assess variation in transpiration rate and stomatal conductance in crop plants (Jones et al., 2002; Rebetzke et al. 2013; Kumar et al. 2017; Taria et al 2019). Lower canopy temperature could be due to higher stomatal conductance and transpiration rate in FAD3A overexpressing plants compared to mock-inoculated, vector-infected and FAD3-silenced plants under drought and salinity stress conditions.

The FAD3A overexpressing soybean plants had higher JA levels under drought and salinity stress conditions as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants. This result suggested important roles of jasmonate in coping with drought and salt stress in FAD3 overexpressing soybean plants. Regulation of JA synthesis is altered in stressed as well as non-stressed plants, which is associated with a variety of metabolic pathways including signal transduction and abiotic stress responses (Ahmad et al. 2016). A large-scale expression profiling in barley revealed considerable overlapping for genes regulated by salinity stress and JA application (Walia et al. 2007). Several researchers reported enhanced drought stress tolerance upon exogenous application of MeJA; for instance, in tobacco by improving Fv/Fm, alleviating degradation of chlorophyll and protecting PS-II under drought stress (Wei-Wei et al. 2011), in wheat by increasing photosynthesis rate, delayed senescence and improving water status (Ma et al. 2014). Salinity stress tolerance was also improved by exogenous application of MeJa in several plants, for example, in tomato (Enteshari and Jafari, 2013) and in soybean by improving photosynthesis, transpiration rate, chlorophyll and proline content (Yoon et al. 2009). *Arabidopsis thaliana* plants with duplication of a genomic region having *FAD3* locus had elevated levels of linolenic acid, a precursor of JA, content in seed oil (O'Neill*et al*. 2011). It may be worthy of mentioning here that JA biosynthesis involves two pathways; via an octadecanoid pathway involving addition of molecular oxygen to linolenic acid and/or a hexadecanoid pathway that uses oleic acid as a precursor.

Proline is an important organic compatible solute, which protects plants against free radical-induced damage under stress condition. Many plants accumulate proline in response to stresses such as drought and salinity (Trovato et al. 2008). In the present study, it was observed that drought and salinity stresses resulted in significant increase in proline content in FAD3A overexpressing plants as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants. Rice roots exposed to NaCl stress resulted in accumulation of proline with increasing NaCl concentrations (Morant *et al* 2004). Increase in proline content under drought and salinity stress might be due to activation of proline syntheses from glutamate

or decrease in its utilization during protein syntheses. Gad (2005) reported that proline may be the major source for energy and nitrogen during metabolism after stress and accumulated proline supplies that energy for growth and survival, thereby enhancing drought and salinity stress tolerance.

In the present study, FAD3A overexpressing plants showed higher expression of *WRKY54* transcription factor as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants under drought and salinity stress conditions. The JA-responsive TFs like *WRKY* regulate the expression of many genes associated with growth and development of plants, and especially the responses and adaptation of plants to the environmental stress. The WRKY transcription factors play pivotal role in the regulation of abiotic stress responses in plants. The *WRKY* gene involved in multiple pathways induced by stresses and JA signal network (Finatto et al. 2018). Zhou et al. (2008) reported differential abiotic stress tolerance by transforming Arabidopsis with *GmWRKY* transcription factors. In *Arabidopsis thaliana*, overexpression of *GsJAZ*, a novel JAZ family gene from *Glycine soja*, enhanced the salt and alkali stress tolerance (Zhu et al. 2012). It may be worth noting that JAZ proteins act as repressors of JA signaling. The endogenous bioactive form of JA, a JA-isoleucine conjugate (JA-Ile) mediates the binding of Jasmonate ZIM (JAZ) proteins to the F-box protein CORONATINEINSENSITIVE1 (COI1) and forms the Skp1/Cullin/F-box (SCFCOI1) complexes. Upon degradation of JAZ proteins via the 26S proteasome pathway, transcription factors, including WRKY, MYC, bHLH/MYB, are relieved from JAZ-proteins and activate their respective downstream responses (Cheng et al. 2011; Song et al. 2011; Qi et al. 2011). In rice, a signaling module consisting of *OsbHLH148–OsJAZ–OsCOI1* mediates jasmonate-regulated gene expression under drought stress. Jasmonate mediated degradation of *OsJAZs* and activation of *OsbHLH148* leads to downstream drought stress responses (Seo et al. 2011). Protection of FAD3A overexpressing soybean plants against drought and salinity stresses was observed in the investigation of physiological, biochemical and molecular changes. The enhanced tolerance of the FAD3A overexpressing plants to drought and salinity stress may be due to higher chlorophyll content, protection of PS-II, higher water retention capacity, lower canopy temperature, higher transpiration and increased level of JA under drought and salinity stress condition as compared to mock-inoculated, vector-infected and FAD3-silenced soybean plants. Taken together, enhanced drought and salinity stress tolerance in GmFAD3A overexpressing plants may be correlated with linolenic acid (C18:3) induced membrane stabilization and the increased expression of stress-responsive genes such as *WRKY54*.

Declarations

Author contributions

AKS and AK conceptualized and planned experiments; AKS, MK, SKR performed experiments; JR, MBR provided resources; AKS, JR, MK and AK contributed to organizing and analyzing data and drafting the manuscript.

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Conflict of interest

Authors declare no competing interests

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Figures

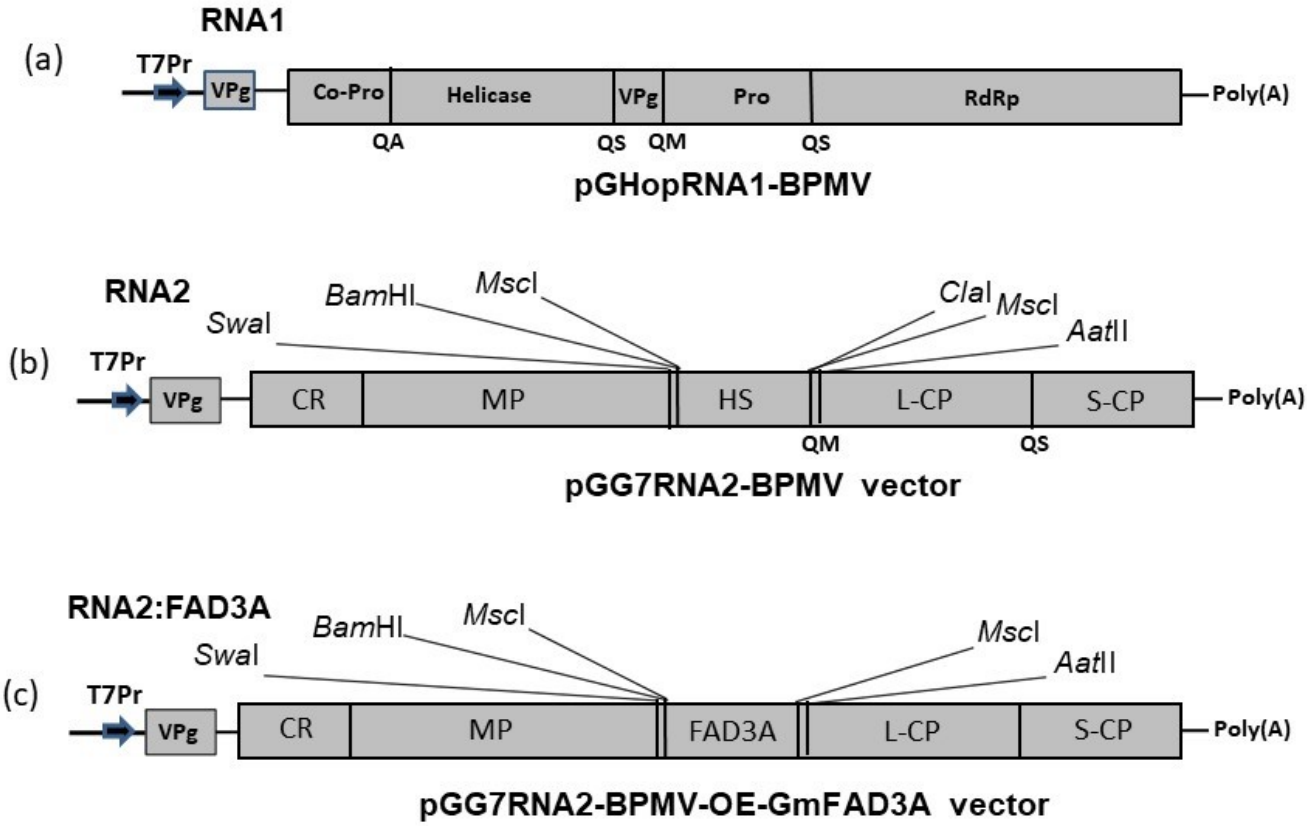

Figure 1

Schematic presentation of BPMV-based viral vector used for overexpression of *GmFAD3A* gene in soybean. *In vitro* transcripts from native BPMV-RNA1 and recombinant BPMV-RNA2 are required together for overexpressing *GmFAD3A* gene employing BPMV-based viral vector in soybean. (a) The pGHopRNA1-BPMV vector used for *in vitro* transcript preparation. The BPMV RNA1 encodes Helicase and RNA dependent RNA Polymerase (RdRp). The BPMV RNA1 was cloned under control of the T7 promoter (T7Pr). (b) The pGG7RNA2-BPMV vector used for *in vitro* transcript preparation. The BPMV RNA2 encodes cell to cell movement protein (MP), Large coat protein (L-CP), the Small Coat Protein (S-CP) and the RNA2 replication factor (CR). The cleavage site QM. RNA2 was cloned under control of the T7 promoter (T7Pr). (c) The pGG7RNA2-GmFAD3A-BPMV vector used for *in vitro* transcript preparation. The full-length cDNA of GmFAD3A without stop codon (1128 bp) was inserted into pGG7RNA2-BPMC vector using *Msc*I restriction site.

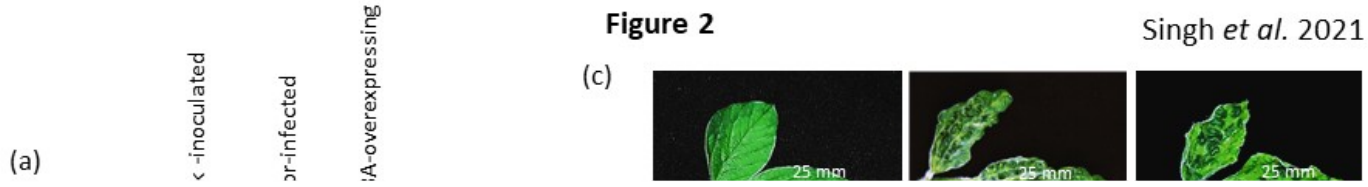

Figure 2

Expression analysis of the GmFAD3 in the leaf tissue and morphological phenotype in the leaf and at whole plant level. (a) Reverse-transcription polymerase chain reaction analysis showing expression of the *GmFAD3* in the leaf tissue from mock-inoculated (M), vector-infected (V) and *GmFAD3A* overexpressing soybean plants. *β-Tubulin* levels were used as internal control for cDNA amounts. (b) Real Time Polymerase Chain Reaction analysis showing relative mRNA level of the *GmFAD3* in the leaf tissue from mock (M), vector-infected (V), GmFAD3-silenced and *GmFAD3A* overexpressing soybean plants. Values were represented as mean ± standard errors. Each experiment was repeated three times. Significant differences among the mean values were compared using Student's t Test ($P < 0.05$). Asterisk denotes significant difference. (c) Morphological phenotype of leaves from of vector infected, *GmFAD3* silenced and FAD3A overexpressing soybean plants. (d) RNA blot analysis of mock-inoculated, vector-infected and GmFAD3A overexpressing plants. Ethidium bromide staining of rRNA was used as a loading control. RNA blot was probed with GmFAD3A probe.

Figure 3

Response of FAD3-silenced and overexpressing plants to drought and salinity stress. (a) Difference in drought tolerance at the whole plant level between mock-inoculated, vector-infected, GmFAD3-silenced and *GmFAD3A* overexpressing soybean plants. Watering was withheld for 4 days. (b) Difference in salinity stress tolerance (150 mM NaCl) at the whole plant level between mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing soybean plants. (c) Non-stressed mock-inoculated, vector-infected, GmFAD3-silenced and GmFAD3A overexpressing soybean plants.

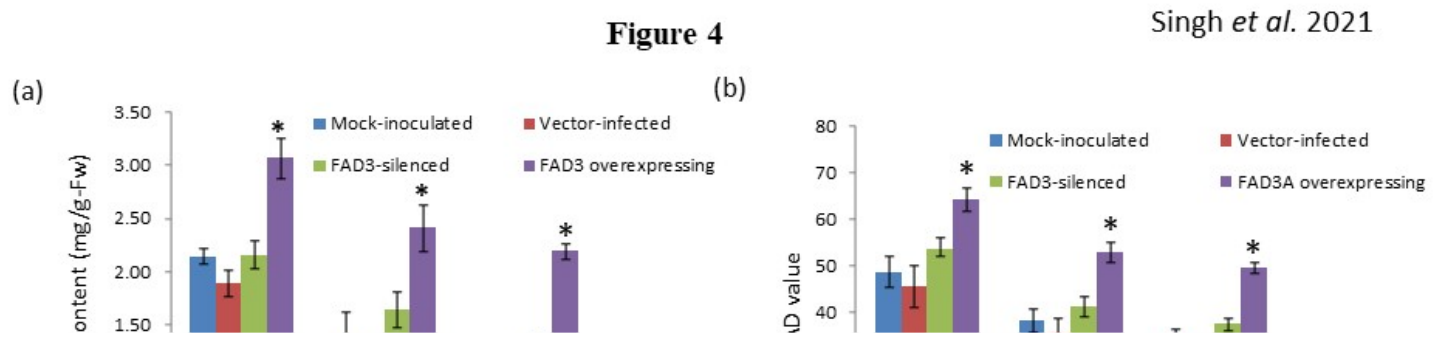

Figure 4

Effect of drought and salinity stress on various physiological processes in mock-inoculated, vector-infected, GmFAD3-silenced and GmFAD3A-overexpressing soybean plants. Effect of drought and salinity stress on chlorophyll content (a), SPAD value (b), maximum quantum yield (chlorophyll fluorescence) (Fv/Fm) value (c), relative water content (d) in mock-inoculated, vector-infected, GmFAD3-silenced and GmFAD3A overexpressing soybean plants. Values were represented as mean ± standard errors. Each experiment was repeated three times. Significant differences among the mean values were compared using Student's t Test ($P < 0.05$). Asterisk denotes significant difference.

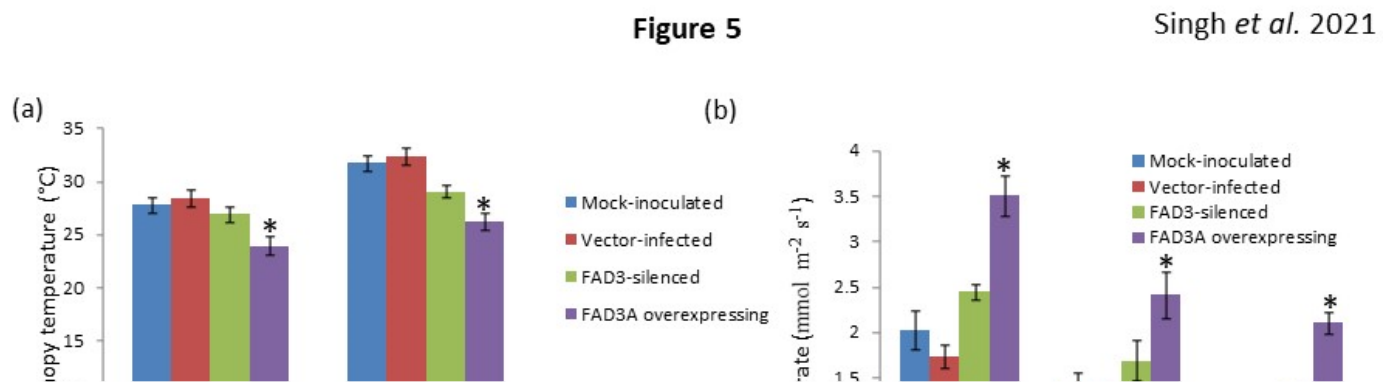

Figure 5

Effect of drought stress and salinity stress on canopy temperature (a), transpiration rate (b), stomatal conductance (c) in mock-inoculated, vector-infected, GmFAD3-silenced and GmFAD3A overexpressing soybean plants. Values were represented as mean ± standard errors. All the experiments were repeated at least three times. Significant differences among the mean values were compared using by Student's t Test ($P < 0.05$). Asterisk denotes significant difference.

Figure 6

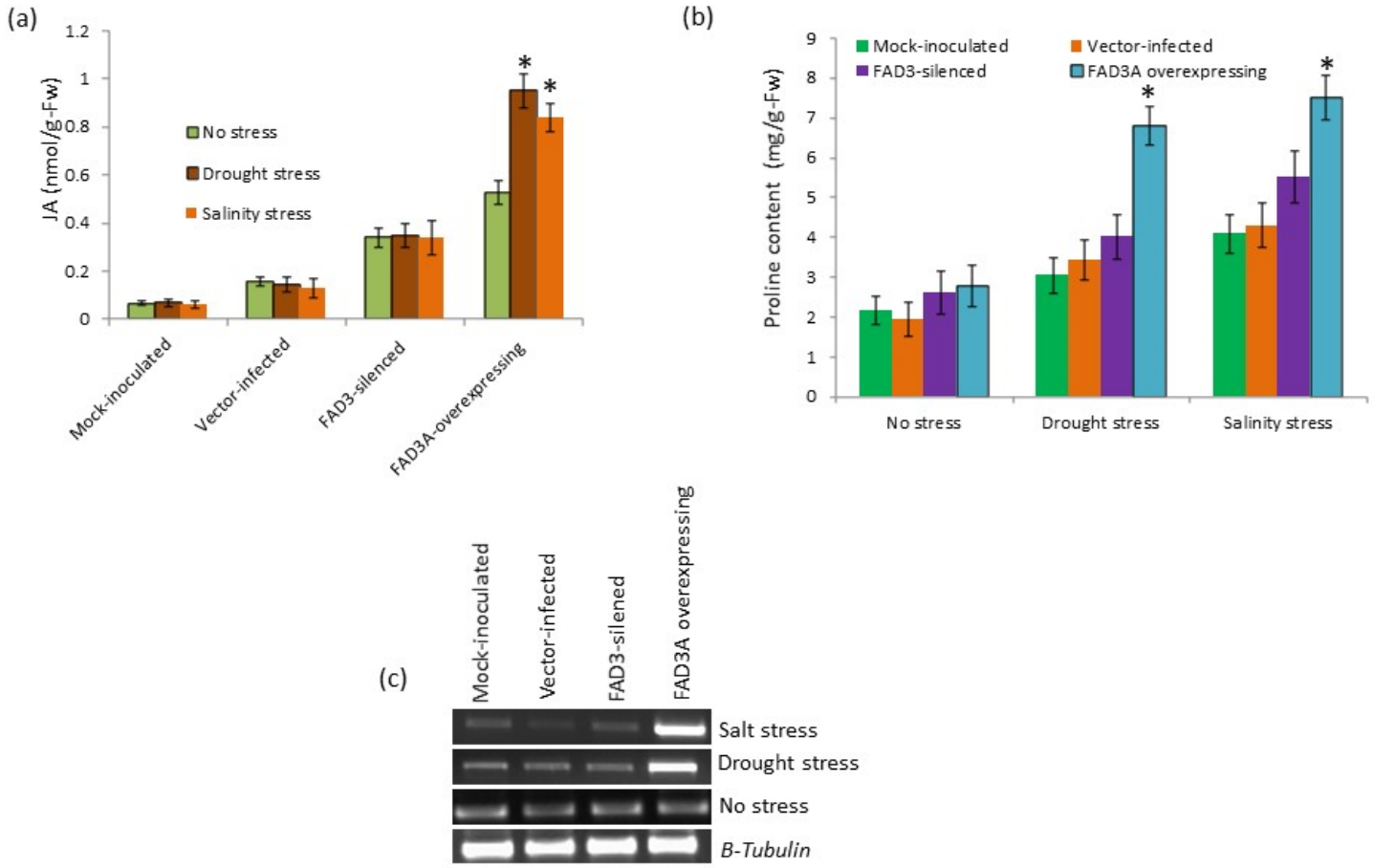

Figure 6

Effect of drought stress and salinity stress on JA level in mock-inoculated, vector-infected, GmFAD3-silenced and GmFAD3A overexpressing soybean plants under no stress, drought and salinity stress conditions (150 mM) NaCl (a), Proline content in mock-inoculated, vector-infected, GmFAD3-silenced and GmFAD3A overexpressing soybean plants under no stress, drought and salinity stress conditions (b). Values were represented as mean ± standard errors. All the experiments were repeated at least three times. Significant differences among the mean values were compared using Student's t Test ($P < 0.05$). Asterisk denotes significant difference. (c) Reverse-transcription polymerase chain reaction analysis showing expression level of *WRKY54* transcription factor in mock-inoculated, vector-infected, FAD3-silenced and FAD3A overexpressing soybean plants under no stress, drought and salinity stress conditions. Expression of *β-tubulin* was used to check equal quantity loading of cDNA.