Drought-induced protein (Di19-3) plays a role in auxin signaling by interacting with IAA14 in Arabidopsis

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Abstract
The members of early auxin response gene family, Aux/IAA, encode negative regulators of auxin signaling but play a central role in auxin-mediated plant development. Here we report the interaction of an Aux/IAA protein, AtIAA14, with Drought-induced-19 (Di19-3) protein and its possible role in auxin signaling. The AtDi19-3 mutant seedlings develop short hypocotyl, both in light and dark, and are compromised in temperature-induced hypocotyl elongation. The mutant plants accumulate more IAA and also show altered expression of NIT2, ILL5, and YUCCA genes involved in auxin biosynthesis and homeostasis, along with many auxin responsive genes like AUX1 and MYB77. AtDi19-3 seedlings show enhanced root growth inhibition when grown in the medium supplemented with auxin. Nevertheless, number of lateral roots is low in AtDi19-3 seedlings grown on the basal medium. We have shown that AtIAA14 physically interacts with AtDi19-3 in yeast two-hybrid (Y2H), bimolecular fluorescence complementation, and in vitro pull-down assays. However, the auxin-induced degradation of AtIAA14 in the AtDi19-3 seedlings was delayed. By expressing pIAA14::mAIAA14-GFP in AtDi19-3 mutant background, it became apparent that both Di19-3 and AtIAA14 work in the same pathway and influence lateral root development in Arabidopsis. Gain-of-function slr-1/iaa14 (slr) mutant, like AtDi19-3, showed tolerance to abiotic stress in seed germination and cotyledon greening assays. The AtDi19-3 seedlings showed enhanced sensitivity to ethylene in triple response assay and AgNO₃, an ethylene inhibitor, caused profuse lateral root formation in the mutant seedlings. These observations suggest that AtDi19-3 interacting with AtIAA14, in all probability, serves as a positive regulator of auxin signaling and also plays a role in some ethylene-mediated responses in Arabidopsis.

Significance Statement: This study has demonstrated interaction of auxin responsive Aux/IAA with Drought-induced 19 (Di19) protein and its possible implication in abiotic stress response.

Keywords
abiotic stress, Arabidopsis, Aux/IAA, auxin signaling, Di19, ethylene signaling
Hormones play a major role in coordinating various developmental processes in plants as well as in adaptation of plants to both abiotic and biotic stresses. There is a complex network of signaling components that act as a link between the signaling pathways of various hormones, thus relaying the signal to the nucleus, eliciting changes in gene expression, and leading eventually to more overt developmental responses. Auxin is one of the major phytohormones that has been studied extensively and is involved in many developmental responses, including embryonic and post-embryonic development and tropic movements in plants. Relative content of auxin in a particular tissue and at a specific stage of development is decisive in defining the fate of cells in that tissue. However, high levels of indole-3-acetic acid (IAA) at the cellular level induce various growth abnormalities like reduction in shoot growth, epinasty, reduction in leaf area, and root proliferation (Grossmann & Scheltrup, 1998); this also forms the very basis of herbicidal action of auxins. Moreover, in coordination with other hormones like ethylene, gibberellin, abscisc acid, and cytokinin, auxin plays an essential role in many fundamental processes in plants (Chandler, 2009; Munne-Bosch & Müller, 2013), conferring phenotypic plasticity. Synergy between auxin and ethylene can be explained by positive regulation of auxin synthesis by ethylene (Stepanova, Hoyt, Hamilton, & Alonso, 2005; Stepanova, Yun, Likhacheva, & Alonso, 2007) along with involvement of ethylene in polar auxin transport (Lewis, Negi, Sukumar, & Muday, 2011; Prayitno, Rolfe, & Mathiesius, 2010). Moreover, auxin regulated AUX1 has been implicated as a central player in ethylene-induced inhibition of lateral root (LR) formation (Lewis et al., 2011; Negi, Ivanchenko, & Muday, 2008). Recently, the role of SOR1, an E3 ubiquitin ligase, in ethylene-auxin-mediated inhibition of root growth in rice seedlings has been demonstrated (Chen et al., 2018). Auxin has been shown to induce ethylene production by upregulating ACC synthase expression or by redirecting the available pool of ACC towards ethylene synthesis (Stepanova et al., 2007; Wei, Cheng, & Hall, 2000). Auxin also stimulates the expression of NCED encoding gene leading to ABA accumulation (Grossmann & Scheltrup, 1998; Hansen & Grossmann, 2000; Kraft, Kuglitsch, Kwiatkowski, Frank, & Grossmann, 2007). In fact, in earlier studies from our laboratory (Borah et al., 2017; Jain & Khurana, 2009), many of the auxin responsive genes in rice were found to be expressed differentially under abiotic stress conditions like drought, salinity, cold, and heat stress. Many other recent studies have also provided evidences for the involvement of auxin in abiotic stress response pathways (Jung, Lee, Choi, & Kim, 2015; Shani et al., 2017; Sharma, Sharma, Borah, Jain, & Khurana, 2015; Uga et al., 2013).

The changes in the endogenous level of auxin can cause both repression and enhanced expression of auxin responsive genes. Among the genes that are upregulated, members of Aux/IAA, GH3, and SAUR family genes have been the subject of innumerable studies (Hagen & Guilfoyle, 2002; Jain, Kaur, Garg, et al., 2006; Jain, Kaur, Tyagi, & Khurana, 2006; Jain & Khurana, 2009; Jain, Tyagi, & Khurana, 2006; Paponov et al., 2008). Auxin-induced changes in gene expression are mediated by two well-studied gene families encoding Aux/IAAs and auxin response factors (ARFs). The Aux/IAA family represents auxin-induced short lived proteins that act as negative regulators of auxin signaling (Gray, Kepinski, Rouse, Leyser, & Estelle, 2001; Jain, Kaur, Garg, et al., 2006; Ramos, Zenser, Leyser, & Callis, 2001; Santos Maraschin, Memelink, & Offringa, 2009). The expression of most of the Aux/IAA genes is enhanced upon auxin treatment and at the same time it signals the activation of proteasome pathway. Auxin finds a binding pocket in its receptor TIR1/AFB, acting as a “molecular glue,” thus providing an extended interface for the protein–protein interaction between Aux/IAA and TIR1/AFB (Dharmasiri, Dharmasiri, & Estelle, 2005; Kepinski & Leyser, 2005; Tan et al., 2007). Auxin enhances TIR1/AFB protein binding to Aux/IAA for its degradation that hitherto has sequestered the ARFs, thus leaving the ARFs free to bind AuxREs in the upstream promoter region of auxin responsive genes and regulate their expression. Considering the complexity of auxin signaling and its participation in a wide diversity of physiological processes and developmental events, it is not surprising that many more components may be involved in auxin signaling pathways. Recent studies have led to the identification of components that serve as chaperones and chromatin modifiers, affecting auxin signaling (Lavy & Estelle, 2016). A mechanism involving the Mediator complex, which becomes functional only on degradation of IAA14 at higher levels of auxin, due to dissociation from TPL and CKM, thus allowing ARF7- and ARF19-mediated gene expression to commence, has been deciphered recently (Ito et al., 2016).

In an effort to identify Aux/IAA interacting proteins and define their cellular functions, in the present study, we initially identified an interacting partner, Di19, for one of the Aux/IAA proteins (OsIAA13) in Oryza sativa through Y2H library screening and subsequently confirmed it by in vitro pull-down and bimolecular fluorescence complementation (BiFC) assays. Based on the lead available from rice work, we could identify the rice homologs of Di19 and Aux/IAA in Arabidopsis thaliana and also demonstrated their interaction. Di19 family proteins represent a class of Cys2/His2 zinc finger transcription factors that are induced upon drought stress (Milla, Townsend, Chang, & Cushman, 2006). In Arabidopsis as well as rice, there are seven hydrophilic members comprising the Di19 family. The transcript levels of Di19 family genes are enhanced upon dehydration and high salinity stress (Milla et al., 2006; Wang et al., 2014). There are also reports showing association of AtDi19-1 with pathogenesis response (Liu et al., 2013). To pursue the work on Arabidopsis, we searched for an Arabidopsis mutant of the closest homolog of OsDi19-5 in Arabidopsis; AtDi19-3 emerged as the closest match to OsDi19-5. Subsequent analysis of overexpression, complementation and knock down mutant seedlings of AtDi19-3 clearly revealed its involvement in various
developmental events, like hypocotyl growth, LR growth, and development. Furthermore, the roles of AtDi19-3 in auxin- and ethylene-mediated responses have also been analyzed. Overall, our work provides evidence that Di19-3 is involved in hormonal interplay for regulating plant development besides the role it might play in stress response pathways.

2 | RESULTS

2.1 | OsIAA13 interacts with OsDi19-5 and AtIAA14 interacts with AtDi19-3

In pursuit of identifying different components in Aux/IAA-mediated auxin signaling, we performed library screening in yeast using one of the rice Aux/IAAs as bait. With prior knowledge that OsIAA13 (earlier designated as OsIAA1; Thakur, Tyagi, & Khurana, 2001) has high expression in coleoptiles of 3-day-old etiolated seedlings of rice and it is induced by auxin (Thakur, Jain, Tyagi, & Khurana, 2005; Thakur et al., 2001), we generated cDNA library from 3-day-old etiolated rice seedlings for yeast two-hybrid (Y2H) screening with OsIAA13 as bait. In addition to OsDi19-5, we found few ARFs, a dnaJ, and a CaMK to be interacting with OsIAA13; in this study, we kept our focus on OsDi19-5. The interaction between OsIAA13 and OsDi19-5 was further analyzed by Y2H, in vitro pull-down, and BIFC assays (Figure 1a–e). For in vitro pull-down assay, Sepharose 4B pre-cleared 6X-His tagged OsDi19-5 was passed through GSH-Sepharose 4B beads bound GST-OsIAA13 or GST. Immuno-detection with anti-His antibody revealed that OsDi19-5 could be affinity purified by GST-OsIAA13 protein; GST alone did not show any affinity to OsDi19-5 (Figure 1b). To confirm the interaction under in vivo conditions, BIFC assay was performed using split YFP. Full-length sequence of OsIAA13 fused to N-terminal of YFP and OsDi19-5 fused with C-terminal of YFP were co-bombarded into onion epidermal cells and viewed under confocal microscope after incubation for the appropriate duration. The detection of YFP fluorescence in the nucleus (Figure 1d,e; Figure S8) confirmed the physical interaction between these two proteins.

In an earlier study, the Arabidopsis Di19 family genes coding for Cys2/His2 zinc finger class of transcription factors, have been shown to be upregulated upon exposure to dehydration and high salinity stress (Milla et al., 2006). Since genetic resources are readily available for Arabidopsis, we extended this work on Arabidopsis and first identified the homologs of OsIAA13 and OsDi19-5 in Arabidopsis. Using Matrix Global Alignment tool (MatGAT) (Campanella, Bitincka, & Smalley, 2003), AtDi19-3 showed the closest match to OsDi19-5 (Table S1). Likewise, phylogenetic analysis revealed that OsIAA13 shares the clade with AtIAA14 (Figure S3).

The physical interaction between AtDi19-3 with AtIAA14 was confirmed in yeast (Figure 1a); AtIAA16, another Aux/IAA protein belonging to the same clade, however, did not show any interaction with AtDi19-3, and thus served as a negative control. Among the seven members of AtDi19 family, AtIAA14 was found to interact predominantly with AtDi19-3 (Figure 1c). Affinity purification of 6X-His-AtIAA14 using GST-AtDi19-3 bound to GSH-Sepharose and subsequent immunodetection confirmed their interaction by in vitro pull-down assay (Figure 1c). The presence of YFP fluorescence in the nucleus of onion epidermal cells during BIFC assay further substantiated the interaction between AtIAA14 and AtDi19-3 (Figure 1d,e; Figure S8). These observations provide sufficient evidence for the interaction between AtIAA14 and AtDi19-3 in Arabidopsis. Thus, further work was only confined to Arabidopsis; work on rice we intend to pursue later.

2.2 | Altered hypocotyl elongation in Atdi19-3 mutant seedlings grown in light and dark and in response to changes in temperature

To elucidate whether the interaction of AtDi19-3 with AtIAA14 is physiologically relevant for auxin-regulated responses, mutant of AtDi19-3 (SALK_072390) was obtained from ABRC. Following the standard procedure for SALK T-DNA insertion lines, homozygous line for AtDi19-3 was identified and the insertion site was mapped near the 3’ end of the fifth exon of the coding sequence (Figure S2a). The transcript level of AtDi19-3 was considerably reduced in Atdi19-3 homozygous line as revealed by real time-quantitative PCR (RT-qPCR) (Figure S2b). In addition, we constitutively expressed AtDi19-3 in wild-type Col-0, as well as complemented Atdi19-3 mutant with AtDi19-3 and OsDi19-5 genes from Arabidopsis and rice, respectively. The AtDi19-3 transcript accumulation in Atdi19-3/35S:AtDi19-3 complimented lines was almost same as in the wild-type, whereas the overexpression lines, 35S:AtDi19-3 (L2 and L5), showed significant increase in transcript level compared to the wild-type plants (Figure S2b).

Seedlings/plants were grown in white light (50–60 µmol m−2 s−1) for comparative phenotypic analysis. The rosette size of 14-day-old plants of Atdi19-3 mutant was smaller in comparison to plants of the wild-type and the complemented lines; however, in overexpression lines the rosette was somewhat larger in size (Figure 2a). The fully mature Atdi19-3 plants were also short in height (Figure S2c). The phenotypic differences were more apparent in young Atdi19-3 seedlings grown in white light. The hypocotyl length was shorter than the wild-type, whereas the complemented lines had hypocotyl length similar to the wild-type (Figure 2b); however, 35S:Di19 (lines L2 & L5) seedlings showed slight increase in hypocotyl length (Figure 2b). The cotyledon size was also relatively smaller in Atdi19-3 seedlings (Figure 2c). The epidermal cell size of Atdi19-3 mutant seedling hypocotyl was also smaller than the wild-type. The cell size of complemented lines as well as overexpressing lines was more or less similar to the wild-type (Figure 2d,e; Figure S4a).

When grown at higher temperature in light, the wild-type seedlings were more responsive to temperature-induced hypocotyl elongation in comparison to the Atdi19-3 mutant seedlings (Figure 2b); the hypocotyls of the wild-type seedlings were longer when grown at 28°C instead of 22°C. However, the Atdi19-3 mutant seedlings also
showed increase in hypocotyl length at 28°C but to a lesser extent than the wild-type. The 3-day-old dark grown seedlings of Atdi19-3 mutant were taller than the wild-type (Figure 3a,b), whereas the hypocotyl of overexpressing lines was somewhat shorter than the wild-type. Interestingly, when the growth of wild-type and mutant seedlings was monitored over a period of 7 days in dark, the 3-day-old mutant seedlings initially showed elongated hypocotyl as compared to the wild-type seedlings but eventually the wild-type seedlings surpassed the growth of Atdi19-3 mutant seedlings and developed longer hypocotyls (Figure 3a,b).
observed that the apical hook in mutant seedlings is relatively open, whereas the apical hook is still maintained tightly in the wild-type seedlings (Figure 3c). These observations indicate that AtDi19-3 plays an important role in at least some of the auxin-mediated responses in Arabidopsis.

2.3 Higher IAA content in AtDi19-3 mutant seedlings

It has been reported earlier that auxin plays a role in seed germination (Liu et al., 2007, 2013; Wang et al., 2016). In the Atdi19-3 mutant, seed germination was also faster as compared to the wild-type (Figure 4a). The role of auxin in germination of AtDi19-3 mutant seeds was further analyzed by imbibing the seeds in the presence of auxin transport inhibitors. There was a decline in the germination rate for both the wild-type and AtDi19-3 mutant seeds but the effect of auxin transport inhibitors, NOA and TIBA, on the suppression of seed germination was significantly greater in the mutant than the wild-type (Figure 4b). Furthermore, we observed higher transcript levels of gene encoding auxin influx transporter, AUX1 in Atdi19-3 dry seeds (Figure 4c).

To find out whether the auxin-associated phenotypes displayed by Atdi19-3 mutant seedlings are due to any change in auxin levels, we adopted a two-pronged approach. First, auxin-responsive reporter DR5::GUS was introduced into the Atdi19-3 mutant background (see Materials and Methods). Enhanced DR5::GUS activity was observed in the cotyledons and roots of the 10-day-old Atdi19-3 seedlings in comparison to the wild-type (Figure 4e; Figure S5a-c). Hence, we quantified IAA in 2-week-old light grown Arabidopsis seedlings using UPLC. The UPLC data revealed that the IAA level in AtDi19-3 seedlings is more than two-fold higher than in the wild-type seedlings (Figure 4f,g).
To examine the sensitivity of explants from Atdi19-3 plants to auxin, wild-type and mutant seeds were grown in light for 2 weeks, and root explants excised and grown on 1/2 MS medium supplemented with 2,4-D and kinetin. Atdi19-3 mutant root explants formed profuse callus in comparison to the wild-type root explants (Figure 4h). This result indicated that either the mutant has high auxin content or increased sensitivity to auxin in the root explants. Thus, IAA level in the callusing root tissue was also measured and it was indeed higher in the Atdi19-3 callusing root tissue than that in the wild-type (Figure 4i).

Keeping in view that Atdi19-3 mutant seedlings have higher auxin content, the transcript levels of some known auxin responsive genes were quantified by RT-qPCR. The transcript levels of AUX, WES1, and some YUCCA genes were significantly higher in Atdi19-3 mutant seedlings as compared to the wild-type (Figure 4d), although we did not find any significant change in transcript levels of PIN2, AXR2, AXR3, ARF7, and ARF10 genes (Figure S5e).

### 2.4 Control of LR number by AtDi19-3

The antagonistic effect of auxin on growth of primary root and LRs has been documented earlier (Overvoorde, Fukaki, & Beeckman, 2010; Stepanova et al., 2007; Swarup et al., 2007). In the present study, Atdi19-3 mutant seedlings showed increased sensitivity to auxin in primary root growth inhibition assay (Figure 5a–c, Figure S5d). The Atdi19-3 mutant seedlings when grown in the presence of 2,4-D for 3 days showed enhanced inhibition of primary root growth as compared to the wild-type (Figure 5a,b). Even when grown in 1/2 MS medium for 5 days and then transferred to IAA containing medium, the extent of root growth inhibition by IAA was greater in the Atdi19-3 mutant seedlings (Figure 5c; Figure S5d).

When both wild-type and Atdi19-3 seedlings were grown on 1/2 MS medium for 14 days in light, the LR density and number of LR primordia (LRPs) in the mutant seedlings were relatively less (Figure 5d,e). But when the wild-type and Atdi19-3 mutants were subjected to IAA treatment, the number of LRs was found to be higher in both the wild-type and mutant seedlings (Figure 5f,g), indicating responsiveness of mutant seedlings to exogenous IAA for the emergence of LRs. The LR staging of Atdi19-3 showed significant decrease in the number of Stage I and Stage II LRP compared to wild-type indicating that the initial stages of LR development are affected significantly in Atdi19-3 mutant seedlings (Figure 5d,e; Figure S9). We did not find any significant difference in LR density in the overexpression lines 35S:AtDi19 (L2 and L5) when compared with the wild-type (Figure S4b).

Our initial study gave an indication of interaction of OsIAA13 with OsDi19-5 in rice. According to an earlier report on rice gain-of-function mutant, Osiaa13, the mutant plants displayed auxin-related phenotype, like reduced LR formation (Kitomi, Inahashi, Takehisa, Sato, & Inukai, 2012). Osiaa13 has single amino acid mutation in domain II resulting in stabilized OsIAA13 protein, causing reduced LR formation. Phylogenetic analysis has revealed that rice OsIAA13 and Arabidopsis AtIAA7, AtIAA14, and AtIAA16 belong to the same clade (Figure S3). In Arabidopsis, the LR formation is regulated by different Aux/IAA-ARF modules; one such module represented by SLR/IAA4-ARF7-ARF19 has a role in LR initiation and hence LR formation (Fukaki, Tameda, Masuda, & Tasaka, 2002; Goh, Kasahara, Mimura, Kamiya, & Fukaki, 2012; Vanneste et al., 2005). The present study has revealed that AtIAA14 interacts with AtDi19-3 and Atdi19-3 mutants are also compromised in LR density.
According to a study performed on the dynamics of LR development (Guseman et al., 2015), the rate of auxin-induced IAA14 degradation quantitatively determines the LR development; LR density is directly correlated to the rate at which IAA14 degradation occurs.

To assess the turnover rate of AtIAA14 in the Atdi19-3 mutant seedlings, we expressed AtIAA14 in E. coli BL21 (RIL) cells and purified the protein. The degradation rate of recombinant AtIAA14 was ascertained in the presence of protein extract from 2-week old wild-type
and Atdi19-3 mutant seedlings over a period of 600 min (Figure 5h,i). The rate of AtIAA14 degradation when incubated with protein extract from the mutant seedlings was somewhat slower as compared to the protein extract from the wild-type seedlings, although it eventually degrades completely when incubated for longer duration. In the above assay, the degradation of AtIAA14 was abolished in the presence of MG132, a proteasome inhibitor (Figure 5h,i), indicating that this degradation is mediated by the 26S proteasome.

To ascertain the behavior of Atdi19-3 mutant in the presence of stabilized mIAA14-GFP in comparison to Atdi19-3 plants (Figure 6a). When subjected to NAA treatment, the roots of Atdi19-3 and Atdi19-3 expressing plAA14::mIAA14-GFP showed LR formation, but Atdi19-3 plants showed relatively more LR formation and profuse root hair formation (Figure 6a–c; Figure S6c). The LR density in both wild-type and Atdi19-3 genetic background (Figure 6d). The GFP signal was less prevalent in cells toward the tip of the root in Atdi19-3 seedlings in comparison to the wild-type, and Atdi19-3 transgenic lines was further repressed in the presence of stabilized mIAA14-GFP in comparison to Atdi19-3 plants (Figure 6a).
although GFP signal was comparable in the cells of the upper part of the root, away from the root tip (Figure 6d; Figure S6d). Notably, the root tips in Atdi19-3 seedlings also showed higher auxin level (Figure 4e). Thus, reduced LR density in the presence of stabilized mIAA14 in Atdi19-3 or a delayed degradation of IAA14 in Atdi19-3, both indicate that in all probability, AtDi19-3 acts as a positive regulator of auxin signaling.

2.5 | Differential expression of auxin, ethylene, and stress-associated genes in Atdi19-3 and congruous stress response of slr mutant

Transcriptome analysis of 10-day-old Atdi19-3 mutant and wild-type seedlings under control conditions using microarray showed that the differentially expressed genes (DEGs) were enriched in
KEGG pathways such as proteasome, photosynthesis, glyoxylate metabolism, and superoxide radical degradation (File S2 and File S3). In consonance with an earlier report claiming that Di19 acts as a transcriptional regulator of pathogenesis-related (PR) genes in Arabidopsis (Liu et al., 2013), we found PR1 (PATHOGENESIS-RELATED GENE 1) expression was downregulated in Atdi19-3 seedlings (Figure 7a). Gene ontology (GO) analysis showed that the DEGs in Atdi19-3 were enriched for biological process terms, such as response to salt stress, glycolytic process and water transport, and molecular function terms like mRNA binding, copper ion binding, structural constituent of ribosome and protein binding (Figure 7b, Figure S11). Genes associated with either oxidative stress response (CSD2, CAT3, FSD2), dehydration stress (ERD3, FBS1, SZF1) or auxin signaling (IAA9 and IAA8) were upregulated in Atdi19-3 mutant seedlings (Figure 7a). We also observed perturbation in the expression of genes associated with IAA biosynthesis and homeostasis involving pathway components like indole-3-acetamide, Indole-3-acetonitrile, indole-3-acetaldoxime, and indole-3-acetylatedamine (Figure 7c, File S2 and File S3). Recently, glucosinolate breakdown products have also been implicated in the root development, their levels being regulated by Aux/IAA proteins under drought stress in Arabidopsis (Katz et al., 2015; Salehin et al., 2019). In Atdi19-3 mutant seedlings, genes associated with the pathway involving indolylmethyl glucosinolate aglycone biosynthesis from tryptophan were upregulated (Figure 7c). Some of the genes associated with auxin biosynthesis and signaling, such as NIT2, ILL5, IAA9, MYB77, ARF2, whose expression were found to be altered in Atdi19-3 mutant seedlings in the microarray profile, were validated by RT-qPCR wherein the transcript levels of most of these genes were found to be more than two-fold higher in Atdi19-3 mutant as compared to the wild-type (Figures 7d and 8a).

2.6 | Impaired apical hook development in Atdi19-3 mutant is indicative of altered hormone response

In the transcriptome analysis described above, the expression of genes (SAM1, MAT3, ACS6, and ACO2) involved in ethylene biosynthesis from its precursor methionine was upregulated in Atdi19-3 mutant (Figure 7a,c). Their transcript levels along with those involved in ethylene signaling response (ETR2, ERF1, and ERF4) were validated by RT-qPCR wherein the transcript levels of most of these genes were found to be more than two-fold higher in Atdi19-3 mutant as compared to the wild-type (Figures 7d and 8a).

The interactive effect of ethylene and auxin in manifestation of various developmental processes, like maintaining apical hook and root development in the young dark grown seedlings is well known. These observations led us to assess the effect of ethylene on Atdi19-3 mutant seedlings. Ethylene has a major role to play in the maintenance of apical hook in dark. However, 3-day-old dark grown seedlings of Atdi19-3 displayed not so prominent apical hook. In order to examine the effect of ethylene on apical hook formation, both wild-type and Atdi19-3 mutant seeds were germinated and allowed to grow in the presence of ethylene precursor ACC in dark. Although at lower ACC concentration the response of Atdi19-3 was not striking, the Atdi19-3 mutant seedlings showed hypersensitivity to ACC at higher ACC concentrations (Figure 8bc). Normally, the hypocotyls of 3-day-old dark grown Atdi19-3 mutant seedlings were longer and slender as compared to wild-type seedlings but in the presence of 5 and 10 µM ACC, the inhibition in hypocotyl growth and apical hook thickening was quite remarkable in the Atdi19-3 mutant in comparison to wild-type seedlings.

The phenotype of ethylene signaling and biosynthesis mutants is well documented (Negi et al., 2008; Stepanova et al., 2007; Swarup et al., 2007; Wang, Li, & Ecker, 2002). We examined the behavior of seedlings in the presence of ethylene antagonist, AgNO3 (Negi et al., 2008). In response to AgNO3, the Atdi19-3 mutant seedlings showed considerable increase in the number of LRs, whereas wild-type seedlings showed slight, although statistically insignificant increase in the number of LRs (Figure 8d,e); as stated earlier too, the number of emerged LRs in mutant seedlings was less when grown on basal 1/2 MS medium. Since AgNO3 also inhibits aquaporins, increased LR number in Atdi19-3 upon aquaporin inhibition can partly explain the resistant behavior of Atdi19-3 mutant under water deficit conditions (Figure 7f; present study). These observations presented above are indicative of alteration in hormonal interaction that is manifested eventually in the altered phenotypic behavior of the Atdi19-3 mutant.

3 | DISCUSSION

Involvement of Cys2/His2 (C2H2) zinc finger transcription factor family in regulating the plant’s response to abiotic and biotic stress has been reported previously (Huang et al., 2009; Kim et al., 2001; Mukhopadhyay, Vij, & Tyagi, 2004; Sakamoto et al., 2004; Xu,
Wang, & Chen, 2007). Di19 has atypical ZZ-type zinc finger motif that has the potential for protein–protein interaction (Kang, Chong, & Ni, 2005) and contains putative nuclear localization signals (NLS). The expression of Di19 genes is induced upon dehydration and high salinity stress (Milla et al., 2006; Wang et al., 2014). AtDi19-1 and AtDi19-3 transcript levels increased in Arabidopsis plants subjected to dehydration stress, whereas those of AtDi19-2 and AtDi19-4 were upregulated by high salinity stress (Liu et al., 2013; Qin et al., 2014). In rice, OsDi19-3 and OsDi19-4 were induced both by salt stress and dehydration (Wang et al., 2014). In contrast, AtIAA14 was also found to be downregulated by DREB and AP2 class of TFs under desiccation stress (Shani et al., 2017). In addition, PR1, PR2, and PR5 expression is modulated by AtDi19-1 on dehydration stress (Liu et al., 2013); drought stress induces the expression of AtDi19-1 that in turn leads to enhanced expression of PR genes. In the present study too, PR1 was downregulated in AtDi19-3 mutant (Figure 7a). Moreover, Di19
The Di19 family members are also involved in pathways other than those associated with stress response. For example, the expression of AtDi19-7, also called HRB1, is regulated by light (Kang et al., 2005). In the present study, Arabidopsis AtDi19-3 mutant seedlings developed short hypocotyls when grown in light. However, when grown in dark, the hypocotyl elongation of AtDi19-3 mutant seedlings was more than that of the wild-type till 3-day stage of seedling development but the growth was virtually arrested thereafter and that of the wild-type seedlings was sustained at least for the duration of the experiment. As a result, the 7-day-old wild-type seedlings were much taller than the AtDi19-3 mutant seedlings. It has been shown that when IAA is supplied exogenously to the wild-type seedlings grown in light, hypocotyl growth is reduced. It has been postulated that auxin level in the hypocotyl of wild-type seedlings is optimal for its elongation and any additional auxin is inhibitory (Collett, Harberd, & Lesyer, 2000). Faster seed germination in AtDi19-3 could partly explain the faster hypocotyl growth observed initially for 3 days, however, virtual decline in AtDi19-3 hypocotyl elongation growth thereafter, as compared to wild-type, also points toward some developmental changes occurring in the due course.

When the growth of wild-type and AtDi19-3 seedlings grown in light was compared, the hypocotyl length of the mutant was found to be shorter. The AtDi19-3 mutant plants are less responsive to temperature-induced changes in hypocotyl elongation growth in comparison to the wild-type plants. It has been previously reported that hypocotyl elongation at higher temperature is dependent on auxin. The seedlings of mutants of auxin receptor tir1 are less responsive to temperature-induced hypocotyl elongation (Gray, Ostín, Sandberg, Romano, & Estelle, 1998). The apical hook in 3-day-old etiolated AtDi19-3 seedlings was found to be loose as compared to the wild-type. The hookless phenotype has been observed in auxin over-accumulating mutants and also in mutants with altered auxin response (Abbas, Alabadí, & Blázquez, 2013). Auxin levels were indeed high in the callusing root explants derived from the AtDi19-3. Moreover, the DR5::GUS harboring lines of AtDi19-3 seedlings also exhibited more GUS activity in the root tip.

In the present study, we also found that Arabidopsis Di19-3 physically interacts with AtIAA14 and the degradation kinetics of AtIAA14 in AtDi19-3 mutant seedling extract is slowed down to some extent (Figure 1; Figure 5h,i). The degradation rate of Aux/IAAs is also influenced by the transcriptional complex between Aux/IAAs and ARFs (Guseman et al., 2015; Korasick et al., 2014). In Arabidopsis, SLR/IAA14-ARF7-ARF19 module is one such module that is involved in LR emergence and density (Lavenus et al., 2013; Romano, & Estelle, 1998). The apical hook in 3-day-old etiolated AtDi19-3 seedlings was found to be loose as compared to the wild-type. The hookless phenotype has been observed in auxin over-accumulating mutants and also in mutants with altered auxin response (Abbas, Alabadí, & Blázquez, 2013). Auxin levels were indeed high in the callusing root explants derived from the AtDi19-3. Moreover, the DR5::GUS harboring lines of AtDi19-3 seedlings also exhibited more GUS activity in the root tip.
Although, the root elongation in \textit{AtDI19-3} mutant seedlings was more strongly inhibited by 2,4-D and IAA as compared to wild-type (Figure 5a–c), however, the number of LRs in \textit{AtDI19-3} mutant seedlings was relatively less under control conditions (on basal medium, without any auxin supplementation). But when subjected to exogenous IAA treatment, LR formation was enhanced in both wild-type and mutant seedlings. It is worth mentioning here that IAA8, which is involved in LR formation (Arase et al., 2012), was upregulated in the \textit{AtDI19-3} mutant. Moreover, the root phenotype displayed by the seedlings could also be explained by the combined effect of ethylene and auxin (Figures 5 and 8). The \textit{AUX1} gene expression is altered in the \textit{AtDI19-3} mutant seedlings.
seedlings (present study) and previous reports also suggest that AUX1 plays a critical role in ethylene regulated root development, thus linking auxin and ethylene signaling pathways for their possible role during root development. Ethylene promotes polar IAA transport through AUX1 resulting in negative effect of ethylene on LR formation (Negi et al., 2008). However, IAA can reverse the negative effect of ethylene on LR formation. Although both ethylene and auxin affect primary root elongation in a similar manner, but auxin and ethylene act antagonistically for LR development (Lewis et al., 2011; Negi et al., 2008). In Arabidopsis, SLR/IAA14-ARF7-ARF19 module plays a major role in LR emergence and density (Lavenus et al., 2013; Okushima et al., 2007). The gain-of-function mutation in IAA14 (sir-1) leads to complete loss of LR formation (Fukaki et al., 2002). The present study also, we observed that the auxin responsiveness of Atdi19-3 transgensics expressing pIAA14::mIAA14-GFP (stabilized mIAA14-GFP protein) is compromised when compared with Atdi19-3 seedlings (Figure 6a–c; Figure S6c); expressing pIAA14::mIAA14-GFP in Atdi19-3 mutant background resulted in reduced repression of LR formation in comparison to Atdi19-3 (Figure 6). The expression of mIAA14-GFP was detected more at the region away from the root tip and not at the root tip in Atdi19-3 (Figure 6d; Figure S6d). Although these observations do suggest involvement of Di19-3 in Aux/IAA-mediated auxin signaling pathway, whether there are other factors that facilitate this interaction remains to be elucidated.

Difference in auxin level between the wild-type and Atdi19-3 mutant seedlings prompted us to find out which genes are probably responsible for elevated auxin content. Microarray analysis showed that NIT2 is upregulated in Atdi19-3 mutant by almost seven-fold; it was also validated by RT-qPCR (Figure 7c,d). Incidentally, NIT2 is involved in IAOX-dependent auxin biosynthesis pathway, which is Brassicaceae species-specific pathway among plants (Mano & Nemoto, 2012). YUCCA gene family encodes flavin monoxygenases that are key enzymes in auxin biosynthesis (Cheng, Dai, & Zhao, 2007; Zhao et al., 2001). The expression of some of the YUCCA genes in the Atdi19-3 mutant seedlings was indeed upregulated when checked using RT-qPCR (Figure 4d). In many instances, expression of YUCCA genes has also been associated with drought tolerance in Arabidopsis (see Sharma et al., 2015). Among those responsible for auxin transport, as mentioned before, we found only AUX1 coding for an auxin influx transporter to be upregulated by two- to three-fold in Atdi19-3 mutant seeds as well as seedlings (Figure 4c,d). Recently, Wang et al. (2016) have shown that transgenic plants over-expressing AUX1 have an enhanced rate of germination. However, this influence of auxin on seed germination is dose dependent. It may positively or negatively affect the rate of seed germination (Liu et al., 2007, 2013; Wang et al., 2016). Even low level of IAA has been reported to facilitate germination (He et al., 2012). Atdi19-3 mutant seeds showed faster germination as compared to wild-type seeds (Figure 4a), which was significantly reduced in the presence of auxin transport inhibitors, TIBA and 1-NOA (Figure 4b), substantiating the role of auxin in germination of Atdi19-3 seeds.

It has also been documented previously that auxin promotes ACC synthase activity, thus stimulating ethylene production (Hansen & Grossmann, 2000). In Atdi19-3, among other genes for ethylene biosynthesis and signaling response, genes coding for ethylene biosynthetic enzymes ACC synthase and ACC oxidase were upregulated (Figures 7 and 8). The mutant showed sensitivity to ethylene in triple response assay and AgNO₃, an ethylene inhibitor, caused profuse LR formation in the mutant seedlings (Figure 8d,e). These findings thus support the view that high IAA content influences the ethylene biosynthesis pathway and vice-versa. Thus, Di19-3 can be postulated as an important component in balancing the two pathways.

The present study indicates that drought stress-induced Di19-3 influences auxin as well as ethylene response pathways in Arabidopsis (Figure 8f). Di19-3 promotes stress-related responses, such as production of LRs, by interacting with IAA14, and possibly aids in further processing of auxin response signal through destabilizing the repressor proteins. Expressing stabilized mIAA14 in Atdi19-3 mutant background resulted in further reduction of LR formation in comparison to Atdi19-3 mutant which also corroborates that IAA14 and Di19-3 most likely work in the same pathway. Since ethylene also inhibits the number of LRs and promotes seed germination, these phenotypes in Atdi19-3 mutant can also be partly attributed to the effect of ethylene. Although we did not focus on ethylene, the overlap between ethylene and auxin signaling is well known. Physiologically, Di19-3 appears to regulate ethylene levels that are usually induced under stress and repression of the same allows auxin action to be de-repressed as indicated by promotion of LRs by ethylene antagonist. More work of course needs to be done to decipher the molecular link between Di19-3, auxin, and ethylene. However, from these observations it can be inferred that interaction of Drought-induced 19 (Di19-3) with IAA14 is important for auxin signaling leading to promotion of LRs and maintaining a balance in auxin level in the cellular milieu, which is necessary for plant growth and more so under stress conditions.

4 | MATERIAL AND METHODS

4.1 | Plant material, growth conditions, and genetic transformation

The T-DNA insertion mutant of At03g05700 (SALK_072390) was obtained from ABRC. Plants were grown in 1/2 MS medium with 1% (w/v) sucrose and 0.8% (w/v) agar in light (50–60 µmol/m² s⁻¹) under 16 hr light and 8 hr dark cycle, at 22 ± 1°C, after 72 hr of stratification at 4°C. For raising overexpression lines and complementation lines, AtDi19-3 and OsDi19-5 were cloned in PMDC32 vector and transformed in wild-type Col-0 and Atdi19-3 mutant, respectively, using floral dip (Burman, Bhatnagar, & Khurana, 2018). DR5::GUS/Col-0 lines were crossed with Atdi19-3 plants. F2 lines were screened for mutant phenotype and then analyzed for GUS induction using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Sigma Aldrich) as substrate (Jefferson, 1987). F3 seeds were used for all
physiological and molecular analyses. To introduce pIAA14::mIAA14-GFP in AtDi19-3 mutant background, we crossed AtDi19-3 mutant with pIAA14::mIAA14-GFP lines. F2 lines were screened for mutant phenotype showing GFP expression and T-DNA insertion (Figure S6). For ACC treatment, seeds were allowed to grow in different concentrations of ACC (Sigma) for 3 days in dark. All the photographs were taken using a stereo microscope (Leica DFC295) and measurements were done using ImageJ software.

For root inhibition assay, seeds were allowed to germinate in 1/2 MS medium, supplemented with 0.1 µM 2,4-D (Sigma) for 3 days in light and the primary root length was measured. In another set, 5-day-old light grown seedlings were transferred to specified concentrations of IAA containing medium and grown vertically for 3 days. For LRs, 3-day-old light grown seedlings were transferred to 1 µM IAA/NAAl containing medium and grown for 10 days; following this, number of LRs was counted. For germination assay, seeds were plated on filter paper (spread in 90 mm plates) pre-soaked in water, TIBA (Sigma) or NOA (Sigma) solution, stratified at 4°C for 72 hr, and then transferred to a growth room maintained at 22 ± 1°C before scoring for germination after defined duration. Radicle emergence was considered as the sign for seed germination.

Cotyledon greening assay was performed where 3-day-old wild-type, AtDi19-3 and sir seedlings were transferred to 150 mM NaCl, 300 mM mannitol, and 2 µM ABA (Qin et al., 2014) and grown for 10 days. Germination percentage was also assessed in the presence of 300 mM mannitol, and 2 µM ABA (Qin et al., 2014) and grown for 10 days. For root inhibition assay, seeds were allowed to germinate in 1/2 MS medium, supplemented with 0.1 µM 2,4-D (Sigma) for 3 days in light and the primary root length was measured. In another set, 5-day-old light grown seedlings were transferred to specified concentrations of IAA containing medium and grown vertically for 3 days. For LRs, 3-day-old light grown seedlings were transferred to 1 µM IAA/NAAl containing medium and grown for 10 days; following this, number of LRs was counted. For germination assay, seeds were plated on filter paper (spread in 90 mm plates) pre-soaked in water, TIBA (Sigma) or NOA (Sigma) solution, stratified at 4°C for 72 hr, and then transferred to a growth room maintained at 22 ± 1°C before scoring for germination after defined duration. Radicle emergence was considered as the sign for seed germination.

4.2 | Yeast Two-Hybrid assay

For cDNA library screening, RNA was isolated from 3-day-old dark grown coleoptile tissue and cDNA library was made in pGADT7-rec and transformed in Y187 strain following manufacturer’s protocol (Clontech). OsIAA13 was cloned in pGBK7 and checked for autoactivation (Figure S1a,b). The cells with bait plasmid were allowed to mate with the cDNA library transformed cells and plated on SD-AHLW (+X-α-Gal) plates for screening, following manufacturer’s protocol (Matchmaker™ Gold yeast two-hybrid System user manual, Clontech).

AtDi19-3 and OsDi19-5 clones were subcloned in pGADT7 (Clontech), whereas AtIAA14, AtIAA16, and OsIAA13 were cloned in pGBK7 (Clontech). Individual constructs were checked for autoactivation prior to performing Y2H experiments (Figure S1a,b). The plasmids were co-transformed in AH109 cells and selected on SD/-LW media. Interaction between pAD-GAL-Di19 and pBD-GAL-IAA was analyzed by the expression of HIS3 reporter gene and MEL1 reporter gene.

4.3 | Callus induction

Root explants were excised from 2-week-old light grown seedlings and kept in callus induction medium containing 0.25 µM 2,4-D (Sigma) and 2 µM kinetin (Sigma) for one week (Laxmi, Paul, Raychaudhuri, Peters, & Khurana, 2006) under a daily cycle of 16 hr light and 8 hr dark, in a culture room maintained at 22 ± 1°C. Observations for callus induction were made periodically.

4.4 | RNA isolation, RT-qPCR, and microarray analysis

Total RNA was isolated from 10-day-old light grown seedlings using Trizol as described earlier (Jain, Nijhawan, Tyagi, & Khurana, 2006). Total RNA from dry seeds was isolated following protocol by Meng and Feldman (2010). One microgram of total RNA was used for cDNA synthesis using ABI High capacity cDNA synthesis kit following manufacturer’s protocol. RT-qPCR was performed using LightCycler® 480 SYBR Green I Master (Roche) in the LightCycler® 480II Real Time Machine (Roche). Three different control primer sets (UBQ, ACT2, UBC) were taken for relative expression analyses. The experiment was repeated three times taking three technical replicate each time. Mean and standard error were calculated from three different experiments. All the primers used for RT-qPCR are listed in Table S2. The 10-day-old light grown seedlings were processed for microarray analysis. Microarray analysis was performed following Borah et al. (2017) and Sharma, Jain, and Khurana (2019).

4.5 | Quantification of free IAA

One gram tissue, each of 10-day-old light grown wild-type Col-0 and AtDi19-3 mutant seedlings, was homogenized and IAA was extracted in the solvent containing methanol: isopropanol: glacial acetic acid (20:79:1) overnight at 4°C in dark (Müller & Munné-Bosch, 2011). All the components were of HPLC grade. Extract was passed through C-18 Sep-Pak Cartridge (Waters). Eluates were evaporated at 4°C and reconstituted in 50 µl ammonium formate. UPLC was performed using Waters Acquity UPLC, with the following parameters: Solvent A: 5 mM ammonium formate, pH 4.0, Solvent B: Methanol, Column: Waters BEH C18 2.1 x 50 mm, particle size 1.7 µm, Column Temperature 40°C, Detector: PDA Detector at 260 nm; total run for 12 min. Sample chromatogram was compared with standard IAA (Sigma).

4.6 | In vitro pull-down assay and protein degradation assay

Recombinant 6-X His tagged proteins were expressed in E. coli (BL21-RIL) cells by cloning full length coding sequence of OsDI19-5 and AtIAA14 in pET28a(+) vector (Novagen). Similarly, GST-tagged recombinant proteins were obtained by cloning the CDS of OsIAA13 and AtDi19-3 in pGEX4T1 vector (Amersham Biosciences) followed by induction in E. coli (BL21-RIL) cells. In vitro pull-down assay was performed following Kepinski (2009). Bacterial lysate expressing
GST, GST-OsIAA13, and GST-AtDi19-3 fusion proteins were immobilized onto GSH-Sepharose (GE Healthcare) by gently mixing at 4°C for 30 min. Following sedimentation, column was washed thrice in 10 bed volumes of ice cold PBS containing 0.5% Triton X-100, 1 µM PMSF, and 10 µM DTT (Kepinski, 2009). Bacterial lysate of OsDi19-5 and AtIAA14 were pre-cleared by incubation with Sepharose-4B beads (GE Healthcare). About 4 µg of pre-cleared protein extracts of 6-X-His tagged OsDi19-5 and AtIAA14 were incubated with the fusion protein immobilized Sepharose 4B beads, respectively, for 1h at 4°C in EB (150 mM NaCl, 100 mM Tris–Cl-pH 7.5, 0.5% NP40, protease inhibitor, MG132) in the presence of 5 µM IAA and 50 µM MG132. Following this, the immobilized beads were washed thrice with EB. Proteins were eluted from the beads by adding 1X loading buffer for SDS-PAGE. Proteins were transferred to PVDF membrane (GE Healthcare) and probed with anti-His Ab (Sigma) and developed with Chemiluminiscence kit (MERCK) following manufacturer’s protocol.

In vitro protein degradation assay was carried out following Thakur et al. (2005). Full length sequence of AtIAA14 was cloned in pET28a (+) vector (Novagen) and expressed in E. coli BL21 (RIL) cells and then purified using Ni-NTA agarose beads following manufacturer’s protocol (Qiagen). For in vitro protein degradation assay, plant proteins were extracted from 2-week-old light grown seedlings in extraction buffer (200 mM Tris–Cl pH 8.0, 100 mM NaCl, 400 mM sucrose, 10 mM Na₂EDTA, 14 mM β-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride, 1% plant protease inhibitor cocktail, 0.05% Tween-20, and 5% glycerol) followed by incubation on ice for 20 min; debris was then pelleted down. About 40 µg plant protein extract was incubated with 7.5 µg of purified recombinant AtIAA14 in the presence of 5 µM IAA and 50 µM MG132 separately for specified duration. Samples were resolved on SDS-PAGE and immunoblotting carried out as mentioned previously.

4.7 | BiFC assay

OsDi19-5, OsIAA13, AtDi19-3, and AtIAA14 were amplified using Phusion high fidelity Taq polymerase (NEB Inc.) and cloned in pENTR/D-TOPO vector (Invitrogen Inc.) as per the manufacturer’s protocol. OsIAA13 and AtIAA14 were then transferred to pSITE-ceYFPC1 and OsDi19-5 and AtDi19-3 into pSITE-nEYFPC1 BiFC Gateway vectors (Chakrabarty et al., 2007) using Gateway LR clonase enzyme mix. Thereafter, for particle bombardment, 3–5 µg DNA was used to coat 0.5 mg of gold particle and onion epidermal cells were bombarded with the constructs following the protocol as described earlier (Burman et al., 2018). The onion peels were viewed for YFP expression under confocal microscope (Leica TCS SP5).

4.8 | Tissue staining

Hypocotyl of 7-day-old seedlings were excised and kept in tissue clearing solution (70% lactic acid) for 3–4 days at 50°C with regular changes (Herr, 1993). The tissue was then stained with 0.01% toluidine blue and viewed under fluorescence microscope (Leica DFC450C).

4.9 | Accession numbers

OsDi19-5: Os01g73960; OsIAA13: Os03g53150; AtDi19-3: At3g05700; AtIAA14: At4g14550. The normalized microarray data were submitted to NCBI GEO (GSE104975).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

SMM designed and contributed to most of the experiments. SMM, ES, and JPK raised the AtDi19-3 mutant lines in pIAA14::mIAA14-GFP background. SMM and ES performed the stress assays. SMM and BS did onion peel bombardment experiments and UPLC analysis. ES and BS did the microarray analysis and ES helped in designing RT-qPCR experiments. SMM and ES drafted the manuscript. JPK supervised the research work, gave inputs in designing the experiments and finalized the manuscript. All authors read and approved the contents of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.