SCFOsFBK1 mediates JA induced turn-over of OsATL53 and OsCCR14 to regulate rice anther lignification

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Article

SCF^{OsFBK1} mediates JA induced turn-over of OsATL53 and OsCCR14 to regulate rice anther lignification

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ABSTRACT

We have shown earlier the F-box protein, OsFBK1, mediating turn-over of a cinnamoyl CoA-reductase, OsCCR14, to regulate rice anther lignification. Currently, we have identified OsATL53, a member of ATL family of RING-H2 proteins interacting with OsCCR14 in cytoplasm. OsFBK1 mediates turn-over of OsATL53 in cytoplasm and nucleus, while of OsCCR14 in the nucleus. In presence of jasmonic acid (JA), which plays a role in anther dehiscence, FLIM analyses demonstrate OsATL53-OsCCR14 undergoing conformational changes that trigger the complex to accumulate around the nuclear periphery and signals OsFBK1 to initiate degradation of the proteins in respective cellular compartments. Biochemically, OsATL53 decreases enzymatic activity of OsCCR14 and sequesters it in the cytoplasm. Knock-down transgenics of *OsATL53* display increased lignin deposition in the anthers vis-a-vis wild-type, while those of *OsCCR14* have decreased lignin content. These data show OsATL53 affects activity of OsCCR14, and their JA induced degradation by SCF^{OsFBK1} regulates lignification of rice anthers.

INTRODUCTION

Homeostasis in protein levels in all biological processes is a critical and essential regulatory mechanism. The onus of this regulation falls vastly on the ubiquitin 26S proteasome-mediated system (UPS) that is conserved across species. The ligases in the UPS participate in a range of concerted processes including substrate identification to mediating controlled ubiquitination of target proteins. The specificity in target protein recognition is particularly expanded in plants where there is a presence of an enormous number of E3 ligases as compared to animals (1), (2). Plant E3 ligase genes vastly outnumber those of the other non-plant eukaryotes, where approximately 1400 E3 ligase encoding genes have been annotated in *Arabidopsis thaliana* genome and about 600 in the human genome (3), (4). Of the different classes of E3 ligases available, the RING-type family forms the largest group. The RING family of E3 ligases is characterized by the presence of

the canonical RING domain that functions as an intermediary between E2-ubiquitin (Ub) and the E3 ligases. RING-based E3 ligases could be either single-subunit or multi-subunit. The cullin-RING E3 ligases (CRLs) are complex RING-type E3 ligases and amongst them the most abundant type found in plants is the SCF type (3), (5).

The RING-finger domain found in RING-type E3 ligases is a specific type of zinc finger that binds two Zn ions in a 'cross-brace' structure using a defined pattern of cysteine (C) and histidine (H) residues found in its conserved sequence (6). The C₃HC₄ (RING-HC) and C₃H₂C₃ (RING-H2) are the most common RING domains where RING-HC domain has been found in single polypeptide E3 ligases and RING-H2 domain is associated with complex multi-subunit E3 ligases (7). Apart from these domains, the presence of less frequent domains, like RING-v, RING-C2, RING-D, RING-S/T and RING-G type, has also been described (8). A staggering 551 potential E3 ligases have been reported in *Arabidopsis thaliana* where 451 correspond to RING-H2 variant and the proportion of this variant in species like *Brassica rapa* and *Oryza sativa* are 55% and 57%, respectively (9, 10). It has been previously reported that some RING zinc finger protein genes are rapidly induced in response to elicitors and these genes are members of a family known as ATL (*Arabidopsis* Tóxicos en Levadura) (11). Mutants of ATLs have been previously isolated and analysed to reveal a link between these putative ubiquitin (Ub) ligases and plant defence signalling pathways (12). It was also reported that the *Arabidopsis* family consisted of 80 members and rice had 121 ATL genes (13). In the past years, two new classes of RING E3 ligases have been identified in addition to ATLs, based on RING-H2 domain variations and RING-outside conserved motifs: BCA 2 Zinc-Finger (BZF) ATLs (BTLs) (14) and CTLs containing the YEELL conserved motif (15).

Several E3 RING-finger protein genes have been characterized over the years for their role in diverse processes; these include *CONSTITUTIVE PHOTOMORPHOGENIC 1* (*COP1*) in light signalling (16, 17), *SINA of Arabidopsis thaliana 5* (*SINAT5*) in accentuating auxin signalling (18,

19), *HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1)* in cold response (20), *ACCUMULATION AND REPLICATION OF CHLOROPLASTS 1* in self-incompatibility (21), *SHOOT APICAL MERISTEM ARREST 1* involved in shoot apical meristem maintenance (22), *ABI1-INTERACTING PROTEIN 2* in ABA signalling (23), *RING-H2 GROUP F1A/RING-H2 GROUP F2A* in gametogenesis (24) and *DEFECTIVE IN ANTHR DEHISCENCE 1 (DAD1)- ACTIVATING FACTOR (DAF)* that affects jasmonic acid (JA) biosynthesis (25), to name a few. Several *ATL* genes that have been studied include *ARABIDOPSIS TÓXICOS EN LEVADURA 2 (ATL2)*, which participates in plant defence (12), *ELICITOR 5 (EL5)* that regulates cell death during root development, and *DAY NEUTRAL FLOWERING (DFN)/AthATL62*, in repressing photoperiod response (26). Some of the important CTLs characterized include *Arabidopsis* E3 ligase *BIG BROTHER (BB)* regulating organ size (27); the human *RNF11/ARKADIA* that is a TGF-signalling and DNA damage responsive SUMO E3 ligase gene (28); *RNF 165/ARK2C* (29) and the p53 regulator *RNF38* (30).

In the present study, we have investigated the rice *ATL* gene *OsATL53* that is a component of E3 ligase and interacts with *OsCCR14* (*OsCCR20* (31)), the substrate of the F-box protein, *OsFBK1*. These two proteins were identified as putative substrates of *OsFBK1* in a Y2H anther library screening, and degradation of *OsCCR14* by *SCF^{OsFBK1}* has been described to regulate lignification in rice anthers and roots (32). We have tried to explore the degradation mechanism of protein factors (involved in lignification) in response to hormones like jasmonate, as well as provide a new insight into the function of RING-H2 *ATL* proteins.

RESULTS

OsATL53 is a component of E3 ligase and interacts with both OsFBK1 and OsCCR14

OsATL53 (RING-H2 protein) is one of the 121 odd RING-H2 *ATLs* found in rice with a distinct signature sequence (13). Sequence alignment between *OsATL53* and known RBX1 proteins studied in humans, yeast and *Arabidopsis* has also shown that there is homology amongst these

proteins (Fig. S1A), even though OsATL53 is not the direct homologue of the canonical RBX1 in reverse blast hits. ATL proteins are also known to comprise of an N-terminal region that is rich in hydrophobic amino acids, and the module generally comprises of a single hydrophobic region of at least 18 residues (13). OsATL53 also has been found to contain one N-terminal hydrophobic transmembrane domain of 22 amino acid residues (Fig. S1B).

To confirm whether OsATL53 could be a component of SCF E3 ligase, in vitro immunoprecipitation assay was carried out between GST-CUL5g (32) and 6xHis-OsATL53, and a weak interaction was detected (Fig. 1A). We had previously shown that the SCF^{OsFBK1} E3 ligase complex comprises of OSK1/20, CUL5g (non-standard nomenclature) and OsFBK1 employing Y2H assay (32). With the current data it appears that the complete SCF^{OsFBK1} also harbours OsATL53, probably as the RBX1 component (Fig. S1D), although it cannot be ruled out that OsATL53 can be a part of another RING-based E3 ligase.

A detailed study on the architecture of ATLs has suggested that there are two distinct regions in ATLs that are responsible for mediating protein-protein interactions; region I located at the amino-terminal end that interacts with components of E3 ligase, and region VII positioned towards the carboxy-terminal end that might be involved in target recognition (33). To test this hypothesis with OsATL53, we generated GST-tagged deletion constructs having a truncated N-terminus, Δ OsATL53 (region I, including the transmembrane domain, amino acids from 1 to 32) and a truncated C-terminal end, OsATL53 Δ (region VII, amino acids from 138 to 202), respectively (Fig. S1B).

ATLs like DAF (DEFECTIVE IN ANTER DEHISCENCE1-Activating Factor) have been previously described to have auto-ubiquitination activity (25). First, we investigated the auto-ubiquitination activity of OsATL53 using a modified protocol described earlier (25). As is evident from Fig. 1B, OsATL53 has auto-ubiquitination activity and can probably function as an E3 ligase, while the truncated constructs functioning as negative controls showed little or no ubiquitination activity.

ΔOsATL53 failed to display any auto-ubiquitination property, while OsATL53Δ showed diminished activity. These results also show that region I of OsATL53 is involved in ubiquitination activity. We also observed a slight shift in size without an apparent smear in the ubiquitinated form of OsATL53 (lane 4 of Fig. 1B) indicating that the addition of ubiquitin is probably in the mono form rather than poly-ubiquitination.

In general, RING-H2 proteins have been found to localise to different cellular compartments (34, 35) and OsATL53 has been found to specifically localize to the cytoplasm of onion epidermal cells and absent in the nucleus (Fig. 1C and Fig. S3). For all localisation experiments using confocal microscopy, we chose to work with onion epidermal cells as it is a monocot system, has a visibly distinguishable nucleus and is a standardized and widely used transient expression system. As stated earlier, OsATL53 was identified as one of the putative substrates of OsFBK1 in the Y2H anther library screening along with OsCCR14 (32). This interaction was further confirmed by in vitro immunoprecipitation assay between GST-OsFBK1 and 6xHis-OsATL53 (Fig. 1D) and by split EYFP BiFC (Fig. 1E). However, the BiFC interaction was found to be labile and required short incubation time after bombardment (10-12 h). While OsATL53 is localised solely to the cytoplasm, BiFC between OsFBK1 and OsATL53 showed a dominant cytoplasmic interaction along with a slight presence of the complex in the nucleus (nuclear plane image, Fig. 1E); implying that the interaction between these proteins takes place in both cellular compartments, albeit with a cytoplasmic preference.

In a serendipitous experiment, OsATL53 was found to interact with OsCCR14 with BiFC EYFP fluorescence found scattered throughout the cytoplasm of the onion peel cells (Fig. 1E). BiFC was found to be stable even after 16 h post incubation after particle bombardment. This scattered appearance of fluorescence, however, did not match with any organellar localisation (data not shown) and resembles protein aggregates. This interaction was further confirmed by in vitro immunoprecipitation (Fig. 1F). OsATL53, however, does not interact with OsCCR18 (OsCCR19

(31), Fig. S1E), the closest homologue of OsCCR14 (32), indicating that the OsATL53-OsCCR14 interaction is specific.

Next, in vitro immunoprecipitation assays were carried out with OsFBK1 and OsCCR14 separately using both OsATL53 deletion constructs. Western blot analyses show Δ OsATL53 interacting weakly with OsFBK1, but its interaction with OsCCR14 is unaffected, indicating that region I is involved in associating with the components of the functional ubiquitin ligase. Whereas for OsATL53 Δ , the interaction with OsCCR14 (the target of SCF^{OsFBK1}) is vastly hampered, indicating that the C-terminal domain of OsATL53 is involved in target recognition as reported earlier (33). These experiments also strengthen the idea that RING-H2 proteins might have multiple roles apart from their property to function in the ubiquitination process *per se*. Putative models of OsATL53-OsCCR14 and OsFBK1-OsATL53-OsCCR14 interactions were generated by ab initio modelling (Fig. S2), and the models were checked by Ramachandran plot analyses.

OsATL53 is a substrate of OsFBK1 and is preferentially degraded in the cytoplasm

Cell-free degradation assays using the total plant protein extracts of 7-day-old seedlings of wild-type (WT), vector control (VC) and *OsFBK1* transgenics as described earlier (32) demonstrate that OsATL53 degradation is mediated by OsFBK1 *via* the 26S proteasome pathway (Fig. 2A and 2B). BiFC fluorescence showed OsFBK1-OsCCR14 interaction preferentially visible in the nuclei, with weak signals detectable in the cytoplasm (Fig. 2C; see also (32)), while that of OsFBK1-OsATL53 was detected predominantly in the cytoplasm with weak signals visible in the nucleus (Fig. 1E and 2E). Such interactions in specific cellular compartments could provide hint at the target proteins' sites of degradation mediated by SCF^{OsFBK1} E3 ligase. As proof, we carried out separate cell-free degradation assays of 6xHis-OsCCR14 and 6xHis-OsATL53 in both nuclear and cytoplasmic protein extracts of *OsFBK1*^{OE} and WT lines. Fig. 2C and 2D show degradation of OsCCR14 by SCF^{OsFBK1} taking place specifically in presence of nuclear protein-enriched extracts under the given experimental duration; while Fig. 2E and 2F show that OsATL53 is degraded in both protein

fractions but preferentially in the cytoplasmic protein fraction. Hence, the BiFC signals observed for both OsCCR14 and OsATL53 with OsFBK1 indeed indicate the sites of interaction where their subsequent degradation takes place. The labile split-EYFP signals observed for OsFBK1-OsATL53 interaction (Fig. 1E) also correspond to the faster degradation kinetics of OsATL53 mediated by SCF^{OsFBK1}. That cytosolic enzymes like OsCCR14 are degraded specifically in the nucleus and mediated by F-box proteins find support from some earlier studies in animal systems (36).

Jasmonic acid changes the dynamics of the OsCCR14-OsATL53 complex

The process of anther dehiscence preceding pollination is characterised by three distinct events (37). During anthesis, auxin regulates commencement of anther dehiscence by controlling the timing of the lignification at the start of late stamen development (38), while JA is thought to initiate dehydration and opening of anther at the final stages of anther development (39-43). Thus, it has been suggested that an auxin maximum at early floral stages in *Arabidopsis* blocks premature endothecium lignification, and its decline in the later stages triggers endothecium lignification. This reduction in the auxin concentration also leads to the increase in JA concentration, thereby causing stomium breakage and anther dehiscence (44).

Though the degradation of OsCCR14 (the enzyme catalysing the second rate-limiting step in lignin formation) by SCF^{OsFBK1} is not affected by auxin, its transcription is induced by auxin (32). However, as JA affects the process of anthesis, it might regulate the degradation of OsCCR14 to affect anther lignification. Thus, we first carried out particle bombardment on onion peel cells using various constructs (Fig. S3) and treated the peels with 6.4 mM JA for 4 h to check whether JA affects the localization of these proteins (Fig. S3). The concentration of JA used in our assay was as deployed earlier (44) that triggered precocious anthesis in *Arabidopsis* faster than that observed before (42). Since 6.4 mM JA is considerably higher than normal physiological conditions, we first tested the effect of this concentration of JA on various constructs. We found that the localization of

OsFBK1, OsCCR14 and the vector controls were unaffected by JA, even after 4 h of incubation. F-boxes are known to function as hormone receptors (3), but since localization of OsFBK1 was unaffected after JA treatment (Fig. S3), we can presume that OsFBK1 does not function as a JA sensor. However, the EYFP fluorescence of OsATL53 appeared to accumulate around the nuclear periphery (Fig. S3 and Fig. S4; for cells with nuclear stain). This accumulation of OsATL53 around the nucleus was seen to initiate as early as 2 h post incubation (data not shown). In order to rule out the effect of stress caused by a high JA concentration in the bombarded cells during the experimental time frame, we checked the changes in lifetime of EYFP-OsATL53 on exposure to a 6.4 mM JA by FLIM (Fluorescence Lifetime Imaging) confocal microscopy (Fig. S5B), as the lifetimes of fluorophores are also affected by the cellular pH and ionic changes (45). Even after 4 h of incubation with 6.4 mM JA, the lifetime of EYFP-OsATL53 did not change (Fig. S5C). A representative stressed or plasmolysed cell expressing EYFP-OsATL53 displays a decrease in lifetime due to changes in cell integrity (Fig. S5B and S5C). Further, we also checked the changes in localization of EYFP-OsATL53 with 100 μ M JA, as done earlier (42) and found changes in its localization initiating only after 8 h of incubation (Fig. S4A). Due to the shorter incubation time and that the concentration does not affect the cellular integrity, we chose to use 6.4 mM JA for all further experiments.

Interaction between OsATL53 and OsCCR14 was also analysed by co-localization using respective constructs and subjecting the bombarded cells to JA treatment for 4 h. Fig. 3A shows a distinct accumulation of both EYFP (OsATL53) and ECFP (OsCCR14) fluorescence around the nucleus after 4 h of JA treatment. The localization of OsCCR14 is otherwise unaffected by JA (Fig. S3). This implies that JA affects localization of the OsCCR14-OsATL53 complex, presumably mediated by the movement of OsATL53 towards the nucleus on exposure to JA (Fig. 3A). On the other hand, co-localization of OsFBK1-OsATL53 and OsFBK1-OsCCR14 were unaffected even in the presence of JA (Fig. S6). However, OsATL53 was found to be present slightly in the nucleus when co-localized with OsFBK1 with or without JA (Fig. S6). This observation for OsCCR14-

225 OsATL53 complex localization in response to/without JA was also repeated by BiFC (Fig. 3B).
226 However, no BiFC fluorescence beyond 4 h of JA exposure was observed.

227 We also investigated whether JA also induces structural changes in the OsATL53-OsCCR14
228 complex. For this we adopted the FLIM-FRET technique to examine any changes in the lifetime of
229 the ECFP-donor construct with/without JA. We used FRET probes that have both the fluorescent
230 probes (ECFP and EYFP) in the N-terminal regions as opposed to the combination used in BiFC,
231 and technically will be separated beyond the required F $\ddot{O}$ rster distance (<10 nm). As expected, the
232 lifetime of the donor ECFP (~3 ns in solution, <https://www.fpbases.org/>, (46)) did not change under
233 FLIM-FRET condition (Fig. 3C and 3D). However, when we added JA and incubated the peel cells
234 for 2 hours, we noticed that the lifetime of the donor ECFP had decreased indicating a positive
235 FRET (Fig. 3C and 3D). Positive FRET was not only observed around the nucleus where the
236 ECFP-OsCCR14 and EYFP-OsATL53 fluorescence were found to accumulate, but also throughout
237 the cell (Fig. 3D). This meant that the addition of JA induces a certain conformational change to
238 the OsCCR14-OsATL53 complex that enables the two FRET probes to come within the minimum
239 F $\ddot{O}$ rster distance and cause energy transfer. Such a change in donor lifetime was not observed in
240 the control (OsFBK1-EGFP donor, EYFP-OsCCR14 acceptor) (Fig. S7). Such conformational
241 changes in the OsCCR14-OsATL53 complex might also explain why BiFC fluorescence
242 disappeared after 4 h of JA exposure (Fig. 3).

243 We had also mentioned that the BiFC interaction between OsCCR14-OsATL53 was stable for long
244 duration post incubation after particle bombardment, but its conformation and localization was
245 affected by JA. Therefore, we wanted to investigate whether JA affects the stability of the
246 OsCCR14-OsATL53 complex in rice. We carried out cell-free degradation of 6xHis-OsCCR14 and
247 6xHis-OsATL53 in whole protein extracts of WT rice seedlings as described previously (32). Figure
248 3C clearly shows JA influencing the stability of OsCCR14-OsATL53 complex after 4 h of treatment
249 by hastening the degradation of OsATL53, without affecting OsCCR14 during the experimental

timeframe. However, in absence of JA, OsATL53 is seen to be stable in the cell-free experiments when present as a complex with OsCCR14 (Fig. 3E). This is a stark contrast to the observation where the half-life of OsATL53 has been found to be considerably less when present alone (Fig. 2A). The results of the cell-free experiments have been quantified and graphically represented in Fig. 3F.

Jasmonic acid induces changes in the OsFBK1/OsCCR14-OsATL53 interaction and localization dynamics

As demonstrated above, OsFBK1 interacts with OsCCR14 and OsATL53 individually to mediate their degradation via the 26S proteasome pathway in different cellular components. We had also observed that OsATL53 probably enters the nucleus while forming a complex with OsFBK1 (Fig. 1E and Fig. S6). It was thus, imperative to check the nature of interaction between OsFBK1 and OsCCR14-OsATL53 complex and determine whether JA affects interaction amongst all these proteins. For this, we carried out particle bombardment for a three-way interaction amongst ECFP-OsFBK1 and OsCCR14-OsATL53 BiFC. The upper panel in Fig. 4 shows that under normal untreated conditions, OsFBK1 does not seem to overly interact with OsCCR14-OsATL53 complex and follows its normal localization pattern, although some minor interactions could also be seen. However, when the bombarded peels were treated with JA (+MG132) and incubated for 4 h, a drastic change in the dynamics of the interactions amongst the three proteins was observed (lower panel, Fig. 4A). The green colour shows the overlapping regions between the ECFP and EYFP fluorescence and it also shows that OsFBK1 interacted with OsCCR14-OsATL53 complex while accumulating around the nucleus, as observed earlier (Fig. 3B). At the same time, in most cells we could also see that OsFBK1 has migrated out of the nucleus into the cytoplasm to interact with the OsCCR14-OsATL53 complex (Fig. 4A and B) which we did not observe for OsFBK1 alone when treated with JA (Fig. S3). Further, we also saw that on exposure to JA (-MG132), there was a loss of EYFP signal in the target cells indicating the disruption of the OsCCR14-OsATL53 complex and the possible degradation of the components in the respective compartments, as also proved by the

cell-free studies (Fig. 4B, Fig. S8). Also, the localization of OsFBK1 also seemed to change back to its original state after this process of degradation is over and its fluorescence appeared throughout the cell and in the nucleus (Fig. 4B). We also noticed some target cells exhibiting faster movement and degradation kinetics, some initiating as soon as 40 min (+JA, - MG132, Fig.4B) as compared to the cell-free studies (Fig. 3E and F); as expected since the cell-free studies had considerably higher substrate concentration (5 μ g) than available in native cellular conditions.

OsATL53 attenuates enzymatic activity of OsCCR14

Since OsATL53 forms a stable cytosolic complex with OsCCR14, we investigated whether it affects the enzymatic activity of OsCCR14. OsCCR14 is a cytosolic enzyme and preferentially reduces feruloyl-CoA esters into coniferaldehyde (47). Similarly, SbCCR1 (the sorghum ortholog of OsCCR14) also has a higher affinity for feruloyl-CoA ester as a substrate as opposed to p-coumaroyl CoA and sinapoyl-CoA (48). Thus, we chose feruloyl-CoA as a substrate for investigating the enzymatic activity of OsCCR14 alone, and in complex with OsATL53 (with/without JA). Feruloyl-CoA ester was prepared following the protocol described earlier (47, 48) and used as substrate for the reaction mix comprising of: a) OsCCR14, b) OsCCR14 + OsATL53, c) OsATL53, d) OsCCR14 + OsATL53 + JA, and e) OsCCR14 + JA. As evident from Fig. 5A, OsCCR14 shows a K_m of 6 μ M, whereas with OsATL53 it demonstrates a significant reduction in the substrate affinity (K_m 20 μ M). The addition of JA to either OsCCR14 or to the complex, however, does not affect the K_m values but slightly affects both their K_{cat} values (Fig. 5B), thereby signifying a very marginal decrease in enzyme activity.

Our data clearly show that OsATL53 attenuates the enzymatic activity of OsCCR14 by significantly reducing its affinity for feruloyl-CoA, without totally inhibiting its enzymatic activity. We might speculate at this juncture that OsATL53 acts as an allosteric effector molecule rather than an inhibitor and might sequester OsCCR14 in the cytosol as aggregates (as demonstrated by BiFC) to regulate lignification. However, it would require detailed biochemical experimentation to confirm

this assumption. Additionally, the presence of JA does not further reduce the substrate affinity of OsCCR14 as a complex with OsATL53 but changes the conformation of the complex in its presence (Fig. 3) triggering the translocation of the complex towards the nucleus, and for OsFBK1 to recognise the complex as a substrate to initiate its degradation via the 26S proteasome pathway (Fig. 4).

Rice transgenics of *OsCCR14* and *OsATL53* demonstrate differential lignin accumulation in roots and anthers

Knock-down rice transgenics of *OsATL53* were generated by RNAi, keeping in concert to the *OsCCR14^{KD}* transgenics reported earlier (32). The downregulation of *OsATL53* in these transgenics were analysed by real-time PCR (Fig. S9A). *OsCCR14* transcript levels in *OsCCR14^{OE}* rice transgenics were checked by real-time PCR (Fig. S9C). The roots of 30-day-old WT, *OsCCR14^{OE}*, *OsCCR14^{KD}* and *OsATL53^{KD}* transgenics were measured and observed for morphological changes. The roots of *OsCCR14^{KD}* were of similar length to WT, although they displayed lesser root density w.r.t. WT (Fig. 6A, upper panel). The roots of *OsCCR14^{OE}* and *OsATL53^{KD}*, however, were shorter and less thick as compared to WT (Fig. 6A, middle and bottom panels respectively). The roots lengths are graphically represented in Fig. 6B. Lignin estimation of the same 30-day-old roots of the transgenics and WT showed that *OsCCR14^{OE}* and *OsATL53^{KD}* accumulated more lignin as compared to WT, while those of *OsCCR14^{KD}* had a lesser amount of lignin (Fig. 6C). These results show correlation with the enzyme kinetics data where *OsATL53* seem to attenuate the enzymatic activity of *OsCCR14* in producing lignin.

The anthers of *OsCCR14^{OE}*, *OsCCR14^{KD}*, *OsATL53^{KD}* transgenics and WT were cleared and their lignin visualised as described earlier (32). While the anthers of *OsCCR14^{KD}* displayed reduced thickenings as compared to WT, those of *OsCCR14^{OE}* and *OsATL53^{KD}* showed significantly more lignin. The accumulation of U-shaped thickenings in the anther bases were more evident than in apices (Fig. 6D). Our data thus proves that *OsATL53* regulates the lignification activity of

OsCCR14 in anthers. Additionally, the anthers of *OsATL53^{KD}* transgenics are comparatively shorter in length as compared to WT (Fig. S9E).

However, when we also checked the expression levels of *OsCCR14* in the same *OsATL53^{KD}* transgenics, we found that *OsCCR14* transcript levels were more abundant than WT (Fig. S9B). However, the transcript levels of *OsATL53* in *OsCCR14^{OE}* plants were comparable to WT (Fig. S9D). Therefore, we also believe that *OsCCR14* is regulated by *OsATL53* even at the transcript level via an unknown mechanism. Whether *OsATL53* physically regulates the transcription of *OsCCR14* or does so by interacting with other protein partners is being investigated.

DISCUSSION

The ATL family of RING-H2 proteins are a dynamic group that has been studied in the recent years. Several ATL proteins have been explored in *Arabidopsis* and other plant species and diverse functions have been attributed to them (26), many thought to be functioning as putative RING-H2 type E3 ligases (13). The diversity in the architecture of the ATLs has been proposed to recognise and target substrates in different cellular environments (33). We found *OsATL53* to be a component of a putative SCF^{OsFBK1} and to have auto-ubiquitination properties (Fig. 1), and we also confirmed *OsATL53* interacting with the F-box protein *OsFBK1* and with its substrate, cinnamoyl CoA-reductase enzyme, *OsCCR14* (Fig. 1). However, the cellular localization of these two interactions differed, indicating that *OsATL53* interacts with these two proteins differently. These variations in the interactions could be due to the different domains present in *OsATL53* as described earlier (33) and have been proved true by deletion studies (Fig. 1).

Seeing that both *OsCCR14* and *OsATL53* are substrates of SCF^{OsFBK1} and, *OsATL53* itself is likely to be a component of the same E3 ligase, it appears that *OsATL53* performs other functions apart from participating in the ubiquitination process. *OsCCR14* has been found to be the closest homologue to *AtCCR1* and has been suggested to be primarily involved in actively lignifying tissues like stems and roots (47). It was also found that *OsCCR14* expresses constitutively

351 throughout the various developmental stages of rice (47). Since OsCCR14 and OsATL53 were
352 both identified as single target clones in the same anther library, we speculate that these two
353 proteins might be together involved in the lignification processes in anthers and in other target
354 tissues. Jasmonic acid has been previously studied extensively for its role in anther dehiscence
355 (39, 40, 44). Additionally, the ubiquitin system and jasmonate signalling pathway are closely linked
356 where the ubiquitin system contributes to the regulation of the synthesis, perception and signal
357 transduction of JA (49). JA was found to induce conformational changes in an erstwhile stable
358 OsCCR14-OsATL53 complex triggering the accumulation of the complex along the nuclear
359 periphery that initiated their degradation (Fig. 2, 3, 4B, S8). We believe that this translocation could
360 be attributed to OsATL53 (Fig. S3) via an unknown mechanism. RBX1 RING-H2 proteins have
361 been quoted to “function as allosteric activators of E2 enzymes and play an essential role in
362 ubiquitination, although their precise mechanism of action is unknown” (7, 50, 51), which made us
363 wonder whether plant ATLs might also participate in other enzymatic processes apart from
364 ubiquitination of target substrates. Thus, we provide evidence that the RING-H2/ATL protein,
365 OsATL53, interacts with OsCCR14 and might function to decrease its enzymatic activity.

366 To account for the role of OsFBK1 in this entire process, we found that in an uninduced state,
367 OsFBK1 does not interact with the OsCCR14-OsATL53 complex but does so with the proteins
368 individually (Fig. 4A). But, as soon as JA is applied, OsFBK1 not only interacts with the OsCCR14-
369 OsATL53 complex in the cytoplasm but also travels with the complex towards the nucleus (albeit
370 not appearing to enter the nucleus with the OsCCR14-OsATL53 complex) (Fig. 4A). SCF^{OsFBK1}
371 then mediates the degradation of OsATL53 in the cytoplasm (Fig. 2E and 3E) thereby destabilising
372 the OsCCR14-OsATL53 complex and enabling OsCCR14 and the remaining OsATL53 to enter the
373 nucleus to be degraded via the 26S proteasome. We also believe that the JA-induced
374 conformational changes brought about in the OsCCR14-OsATL53 complex (Fig. 3C and D) might
375 be the trigger for the movement and subsequent degradation of the complex by OsFBK1. Such

conformational changes brought about by JA in proteins and their subsequent degradation by the 26S proteasome have been previously reported for the JAZ degron (52).

Since OsCCR14 catalyses the first limiting step in lignin formation in the cell cytoplasm, our data reveals that OsATL53 slows down the enzymatic processes of OsCCR14, probably sequesters OsCCR14 in the cytosol as aggregates as demonstrated by BiFC, and, thereby, functions to regulate lignification. Microarray data obtained from previously conducted experiments (1) have shown that the expressions of *OsCCR14* and *OsATL53* overlap in tissues like roots of 7-day-old seedlings, inflorescence stages like P4 and P6 and seed stage S5, whereas their expression differs in the other stages of development examined (Fig. S10). Based on these data, it can be suggested that OsATL53 affects the lignification processes catalysed by OsCCR14 in different target tissues depending on the requirements of that development stage of rice plant. The presence of JA in tissues like late anther stages, however, changes the dynamics of the enzymatic processes and signals the recognition of the OsATL53-OsCCR14 as substrates for destruction via the SCF^{OsFBK1} E3 ligase. As proof of concept that OsATL53 regulates the activity of OsCCR14, the roots and anthers of *OsATL53^{KD}* transgenics display increased lignin thickenings as compared to WT and was comparable to the increased lignification displayed by the anthers and roots of *OsCCR14^{OE}* transgenics. However, as expected, the roots and anthers of *OsCCR14^{KD}* transgenics have less lignin (Fig. 6). Surprisingly, OsATL53 also seems to regulate the transcript abundance of *OsCCR14* via an unknown pathway that we are currently exploring (Fig. S9C).

Finally, we propose the following model (Fig. 6): During the initial anther development stages where lignification is controlled by auxin, OsCCR14 executes its enzymatic processes in the cytoplasm whereas the increased presence of OsATL53 in the later stages reduces this enzymatic activity. However, towards the later stages of anther maturation preceding dehiscence, when the level of JA starts to increase, the process of lignification needs to cease, and the proteins involved need to be turned over. At this stage, the presence of increasing levels of JA in the cell might

change the conformation of the OsCCR14-OsATL53 complex thereby triggering OsATL53 to travel towards the nucleus, while carrying OsCCR14 along with it as a payload. The change in conformation in the complex is recognised by SCF^{OsFBK1} as substrates and initiates the degradation of OsATL53, thereby disassociating the complex and enabling OsCCR14 to enter the nucleus where it is finally degraded by the 26S proteasome. Also, some of the remaining OsATL53 might enter the nucleus as an OsFBK1-OsATL53 complex where OsATL53 would also be finally degraded. OsATL53 might also regulate the transcription of *OsCCR14* by an unknown pathway.

To summarise, we have reported a divergent role of ATL RING-H2 proteins in interacting with other proteins, regulating their activities, and in turn become substrates of E3 ligases, thereby highlighting the diversity in their functions apart from participating as a bridging partner between the E3 and E2 ligases. Additionally, the modulation of OsCCR14 enzymatic function by OsATL53 could have application in terms of manipulating the lignin content in cash crops. At the same time, we provide an insight into the functioning of the F-box protein, OsFBK1, in JA-induced targeted substrate degradation that would in turn regulate anther development in rice with precision.

METHODS

Gene constructs

The 609 bp of the *OsATL53* gene (LOC_Os04g48310) was cloned in the following vectors: pET28a (*EcoRI/SacI*) (Novagen); pGEX4T1 (*EcoRI/SalI*) (GE); pENTR-D-TOPO (5' CACC overhang) entry vector and BiFC GatewayTM vectors, pSITE-3CA-EYFPC₁ and pSITE-3CA-EYFPN₁ using GatewayTM technology (pENTRTM Directional TOPO[®] cloning kit, and LR clonase Enzyme mix II kit; Invitrogen Inc. USA). *CUL5g* (LOC_Os05g05700) was cloned in pGEX4T1 vector (*BamHI/SalI*). The deleted constructs of *OsATL53* (510 bp of Δ *OsATL53*, 411 bp of *OsATL53* Δ) were cloned in the pGEX4T1 vector (*EcoRI/XhoI*). The cloning of *OsFBK1* (LOC_Os01g47050) and *OsCCR14* (LOC_Os08g34280), in their respective vectors has been described earlier (32). For localization and FLIM-FRET experiments, pSITE-1CA/pCAMBIA1302

was used for the donor constructs and pSITE-3CA for the acceptor constructs. Additionally, OsFBK1 was cloned in the pSITE-1CA vector for CFP fluorescence by Gateway™ cloning. The amplification of the genes was carried out using Phusion™ high-fidelity Taq polymerase (Finnzymes, Finland) as per manufacturer's instructions (see Table S1 for primers).

Particle bombardment

Biolistics of the various constructs on onion epidermal peel cells for BiFC, intercellular localization and hormone assays were carried out using Biolistic PDS-1000/He particle delivery system (Bio-Rad, USA) as described earlier (32). For hormone-related visualisation, the bombarded and incubated onion peels (12-16 h) were treated with 6.4 mM JA for the requisite time-points before observing for fluorescence under confocal microscope (Leica TCS, SP5). For FLIM-FRET experiments, all observations and analyses were carried out using the Leica SP8 FALCON confocal microscope at the Central Instrumentation Facility (CIF), University of Delhi South Campus. FLIM was carried out at 40 MHz and curve-fitting was carried out using the n-Exponential Reconvolution fit model. For all experiments, the tissue was mounted in water and observed at 20X magnification. All experiments were carried out at least five times, with an average of 3 cells being visualised in each repetition.

Protein induction, western blotting and immunoprecipitation

Induction and western blotting of 6xHis-OsATL53, 6xHis-OsCCR14, GST-OsATL53, GST-OsFBK1 and GST-Cul5g was carried out as described earlier (32). Immunoprecipitation assays were performed using 100 µg of unpurified bait and prey protein lysates as per the protocol described earlier (32). Pre-stained markers used for all blots were Puregene NEX-GEN-PinkADD pre-stained protein ladder (Cat. # PG500-0500PI, Genetix Asia Pvt. Ltd., New Delhi, India). Western blotting was carried out with anti-His (Cat. # H1029-5ML, Sigma, USA) and anti-GST (Cat. # G7781-2ML, Sigma, USA) antibodies.

Auto-ubiquitination assay

The auto-ubiquitination activity of OsATL53 was carried out following a modified protocol described by (25). An E1 and E2 ligase mix (Cat. # U8382, Sigma, USA) was used for the activation and conjugation of ubiquitin. Negative controls used were generated in the laboratory. Probing of ubiquitin was done using monoclonal anti-ubiquitin antibodies (Cat. # U0508-50UL, Sigma, USA) at a dilution of 1:8,000+1% BSA.

Cell-free degradation

For cell-free degradation assays, total plant protein was extracted from 7-day-old seedlings of WT and OsFBK1 transgenics and cell-free experiments were carried out using 5 µg 6xHis-OsATL53/5 µg 6xHis-OsCCR14 in 30 µg total plant protein extracts/nuclear-enriched protein extracts/cytoplasmic protein extracts by following the protocol described before (32). Nuclear protein extracts from 10-day-old seedlings (5 g tissue) were obtained as per protocol described by (53). The cytoplasmic extracts were aliquoted after filtration and the first centrifugation step. For loading controls, the same blots were stained with Ponceau for the detection of the Rubisco protein large subunit band for cell-free with total plant protein and cytoplasmic extracts whereas for cell-free with the nuclear-enriched protein extracts, Ponceau stained histone H3 band (~15kDa) was used as loading control. For cell-free experiments with JA, the cell-free degradation cocktail was mixed with 6.4 mM JA and incubated at 30 °C for the requisite time points. The degradation data was graphically represented by measuring the intensities of the bands of all blots in Image Studio™ Lite ver. 5.2 (https://www.licor.com/bio/products/software/image_studio_lite/). The 2 h/6 h western band for each blot of OsATL53 and OsCCR14, respectively, was used as the reference and measured intensities were normalised against it and multiplied by 100. Representation of the data is in percentage intensity ratio. All experiments were carried out at least thrice and error bars denote standard deviation across replicates.

Synthesis of feruloyl-CoA thioester and purification

The protocol for synthesis of the substrate feruloyl-CoA thioester was modified from the processes described earlier (47, 48). Briefly, the *Arabidopsis 4-coumarate: CoA ligase 1* gene, *At4CL1*, was amplified from leaf cDNA and cloned in pET28a vector (see Table S1 for primers). The clone was transformed in BL21-RIL competent cells and the protein induced and purified as described earlier (32). For the synthesis of the thioester, 410 µg of purified At4CL1 protein was added to a 40 mL solution containing 50 mM sodium phosphate buffer, pH 7.0, 800 µM CoA (Cat. # C4282, Sigma, USA), 2.5 mM ATP (Cat. # A-7699, Sigma, USA), 2 mM ferulic acid (Cat. # 128708, Sigma, USA) and 5 mM MgCl₂. The reaction mix was incubated at room temperature for 20 h at low rpm in dark conditions. Thermal denaturation of the enzyme was carried out at 80°C for 10 min, and pelleted at 15,000g for 30 min at room temperature. The supernatant was dried under vacuum centrifugation to collect solutes. The solutes/substrates were purified by ethanol for 6 h at -20 °C and the supernatant was dried under vacuum centrifugation. The obtained pellet/substrate was stored at -20 °C for later use. A standard curve of the obtained ester was generated using A₃₆₆ for measurement using a spectrophotometer.

Enzyme kinetic assay of OsCCR14

The activity of OsCCR14 was measured according to the protocols described (47, 48). To a final volume of 210 µL of each reaction mixture consisting of 100 mM sodium phosphate buffer, pH 6.2, 0.25 mM NADPH (Cat. # 144935, SRL Pvt. Ltd., India), thioester substrate (5 µM, 10 µM, 20 µM, 50 µM and 100 µM), 3 µg purified OsCCR14 was added, and the enzyme reactions carried out at 30°C. For reactions with OsCCR14 and OsATL53 complex, equal amounts of OsCCR14 and OsATL53 were first incubated at 4 °C for at least 2 h, quantified and 3 µg used per reaction. For reactions with JA, 100 µM JA was added to each reaction and incubated at 4 °C for 3 h. Reactions were initiated by the addition of the enzyme for 20 min with an interval of 1 min between reactions (20 reactions for one substrate concentration per reaction condition). Reactions were stopped by the addition of 40 µL of 15% (w/v) trichloroacetic acid and mixed well. These were then centrifuged briefly and 200 µL of each reaction mix was loaded onto a Corning clear 96-well plate. Decrease in

A₃₆₆ (substrate concentration) was recorded using a Tecan M200 microplate reader (Tecan Austria GmbH) and the i-control 1.7 software (Tecan Austria GmbH). K_m, V_{max} and K_{cat} values were determined by extrapolation from Lineweaver-Burk plots. Each enzyme assay was carried out for five times and the average values represented the mean ± standard deviation.

Transgenic analyses

OsATL53^{KD} and *OsCCR14^{OE}* transgenic lines were generated in Pusa Basmati 1 by RNAi and overexpression respectively, and their expression levels quantified by real-time PCR as described earlier (32). Lignin estimation of roots of 30-day old seedlings were carried out as described earlier (32). Pre-dehiscent anthers were harvested from at least 3 lines, cleared in lactic acid and visualised using confocal microscopy (Leica SP8 FALCON) as earlier (32). See Table S1 for primers.

Author contributions: P.B. designed the experiments and wrote the paper. P.B. and A.S. carried out the experiments. J.P.K. supervised the work and critically edited the article.

SUPPORTING INFORMATION

Fig. S1. Structural analyses of OsATL53.

Fig. S2. Predicted putative protein complexes.

Fig. S3. Sub-cellular localization of proteins under control and JA treatments.

Fig. S4. Sub-cellular localization of OsCCR14+OsATL53 BiFC complexes under control and JA treatments with nuclear stained cells.

Fig. S5. Sub-cellular localization of OsATL53 under different JA concentrations.

Fig. S6. Sub-cellular co-localization of OsFBK1-OsATL53 under control and JA treatments.

Fig. S7. Additional FLIM-FRET data.

Fig. S8. Time-lapse confocal images depicting the targeted degradation of EYFP-OSATL53 in the presence of JA.

Fig. S9. Real-time expression analyses.

526 Fig. S10. Microarray expression data of *OsCCR14* and *OsATL53* at different stages of rice
527 development.

528 Table S1. Primers used in the study.

529 Materials and Methods

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532 spatial gene expression during panicle and seed development, and regulation by light and
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FIGURE LEGENDS

Fig. 1. Interaction studies of OsATL53. **A.** In vitro immunoprecipitation assay between GST-CUL5g and 6xHis-OsATL53. Lanes 2, 3 and 4 are the unpurified input bacterial extracts of GST, GST-CUL5g and 6xHis-OsATL53, respectively. GST wash was used as a negative control for IP with 6xHis-OsFBK1 (lane 5). GST-tagged proteins were probed by rabbit anti-GST mAb, while 6xHis-OsATL53 was detected by using mouse anti-His mAb. These blots were parallelly processed with the same samples. M - Marker lane with the designated protein bands. **B.** In vitro auto-ubiquitination assay to determine E3 property of OsATL53. Controls used are GST-OsATL53 Δ and GST- Δ OsATL53 respectively. Arrows determine the positions of ubiquitinated OsATL53/GST-OsATL53 Δ in top panel, red asterisks indicate non-specific bands. Bottom panel shows the input proteins as probed by anti-GST mAb. **C.** Representative image of cellular localization of OsATL53 in onion epidermal peel cells. Control: pSITE-3CA vector. Bright field images show the location of the nuclei in all panels. Bar- 50 μ m. Red boxes denote nucleus. **D.** In vitro immunoprecipitation assay between GST-OsFBK1 and 6xHis-OsATL53. GST wash (lane 2) was used as the negative control. Lanes 3 and 4 show the input bacterial extracts of GST-OsFBK1 and 6xHis-OsATL53 respectively. Lanes 5 and 6 are the IP lanes. **E.** YFP fluorescence showing the BiFC between OsATL53/OsFBK1 (upper panel) and OsATL53/OsCCR14 (middle panel) respectively in a representative cell. Bright field images show the location of the nuclei. Negative control: BiFC between OsATL53 and EFTu (lower panel). Bar- 50 μ m. Red boxes denote nucleus. **F.** IP between GST-OsATL53 and 6xHis-OsCCR14. **G.** Western blot showing the IPs between GST- Δ OsATL53/ GST-OsATL53 Δ and OsCCR14 and OsFBK1 respectively. Red asterisks denote non-specific binding. For all IPs, GST

and GST-tagged proteins were probed by rabbit anti-GST mAb, His-tagged proteins were detected by mouse anti-His mAb. M- marker lane for both blots. See also Fig. S1.

Fig. 2. Cell-free degradation of OsATL53 in plant protein extracts. **A.** Time-scale degradation assay of 5 µg 6xHis-OsATL53 in 30 µg cell-free whole seedling protein extracts of control and *OsFBK1* transgenics both with and without MG132. Loading control depicted by the Ponceau stained Rubisco large subunit band (55 kDa) for all. The bottom-most panel shows the level of OsFBK1 in the plant materials used, as probed by anti-OsFBK1 antibodies described earlier (Borah and Khurana, 2018). **B.** Graphical representation of degradation kinetics data of OsATL53. Error bars denote SD. **C.** Degradation assay of 6xHis-OsCCR14 in the nuclear protein-enriched and cytoplasmic protein extracts of WT and *OsFBK1^{OE}* seedlings without MG132. The time points used were as described before (Borah and Khurana, 2018). The confocal image on the left shows the BiFC interaction between OsFBK1 and OsCCR14 in onion peel cells. Loading controls are Ponceau stained Rubisco (55 kDa) for cytoplasmic extracts and histone H3 band (15 kDa) for nuclear protein-enriched extracts respectively. **D.** Graphical representation of the same where error bars denote SD. **E.** Cell-free degradation time kinetics of 6xHis-OsATL53 in the nuclear protein-enriched and cytoplasmic protein extracts of WT and *OsFBK1^{OE}* seedlings without MG132. Image of BiFC between OsFBK1 and OsATL53 in onion epidermal peel cells is shown on the left. Loading controls, histone H3 and Rubisco large subunit as mentioned above. **F.** Percentage intensity ratio graph of OsATL53 degradation in the cell-free systems. Error bars denote SD.

Fig. 3. Jasmonic acid induced changes in sub-cellular location and conformation of OsATL53-OsCCR14 complex. **A.** Co-localization of OsCCR14-ECFP and OsATL53-EYFP in onion peel cells. Upper panel shows ECFP and EYFP fluorescence without JA treatment, while lower panel shows changes in EYFP fluorescence after 4 hours of JA treatment. Red boxes show the accumulation of ECFP and EYFP fluorescence around the target cell's nucleus before and after JA treatment. **B.** Movement of OsATL53-OsCCR14 BiFC complex in response to 6.4mM JA observed after 4 hours.

Bright field images show the location of the nucleus in the representative target cell. Bar- 50 μm . **C.** Change in lifetime of ECFP-OsCCR14 donor construct upon exposure to JA under FLIM-FRET conditions. Error bars denote SD of at least 5 cells observed. **D.** Representative Fast FLIM images of target cells in normal and JA-induced conditions. Positive FRET is present through-out the cell. Bar represents the range of lifetimes from 0.5-3.5 ns. **E.** Cell-free degradation of 6xHis-OsCCR14 and 6xHis-OsATL53 in the cytoplasmic protein extracts of WT seedlings, in the absence and presence of JA respectively. Time kinetics performed in the absence of MG132. Loading control: Ponceau stained Rubisco large subunit band (55 kDa). Red arrows denote non-specific binding. M - marker lane. **F.** Graphical representation of cell-free degradation kinetics where error bars denote SD. See Fig. S3-S6.

Fig. 4. Changes in protein interaction and localization dynamics in the presence of JA. **A.** OsFBK1 ECFP fluorescence was observed to merge with the BiFC (OsATL53-OsCCR14) EYFP fluorescence (Z-stacked) after 2 hours of JA exposure and accumulate around the nuclear boundary after 4 hours, in the presence of MG132. **B.** Changes in localization pattern of OsFBK1 after JA without MG132 treatment in presence of OsCCR14-OsATL53 complex. After degradation of OsATL53 and the disruption of the OsCCR14-OsATL53 complex and loss of EYFP signal, the normal localization pattern of OsFBK1 is seen. The same target cell was observed after the requisite time points after JA treatment. A loss in signal strength was observed due to repeated laser exposure, and the ECFP and EYFP fluorescence were adjusted accordingly. The orange boxes and inset pictures below each panel focusses the nucleus. Bright field images show the location of the nucleus in each target cell. Merged pictures are shown in both dark and bright field background. Green colour denotes overlapped regions of ECFP and EYFP fluorescence. Bar - 50 μm . See Fig. S7.

Fig. 5. A: Kinetic parameters of the enzymatic activities of OsCCR14 under different conditions. Graphical representation of the K_m values of OsCCR14 either alone or in a complex with OsATL53,

with or without JA. **B:** Tabular representation of the observed kinetic parameters of the various enzyme combinations.

Fig. 6. Transgenic morphological data. **A:** Root morphology of 30-day-old seedlings. **B:** Graphical representation of root length of 30-day-old seedlings. Error bars - SD. **C:** Lignin estimation in roots of 30-day-old seedlings. Error bars - SD. **D:** Observation of wall morphology in representative rice pre-dehiscent anthers of wild type and transgenics. Confocal microscopy of cleared anther apices (top panels) and bases (bottom panels) of *OsCCR14^{KD}* and *OsATL53^{KD}* transgenics and wild type showing the differences in the dispersal of the U-shaped wall thickenings in the endothecium. The U-shaped thickenings are indicated by arrows. Bar = 50 μ m. Asterisk denotes separate locules of a single anther in the same frame. The globular structures are pollen grains.

Fig. 7. Proposed model of the signalling pathway involved in the jasmonic acid-mediated degradation of *OsATL53* and *OsCCR14* by SCF^{OsFBK1} and the 26S proteasome. OsFBK1 interacting with OSK1/OSK20 has been reported earlier (Borah and Khurana, 2018). See text for more details.

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A

B

Sample	K _m (μM)	K _{cat} (s ⁻¹)	K _{cat} /K _m (s ⁻¹ μM ⁻¹)
OsCCR14	6.006 ± 1.1075	3.8978e -4 ± 0.0029	6.41e -5 ± 4.45e-5
OsCCR14 + OsATL53	20.0461 ± 1.2642	3.5456e -4 ± 6.27e-5	2.1234e -5 ± 1.39e-5
OsCCR14 + JA	6.9571 ± 0.7292	2.28e-4 ± 1. 68e-5	3.3124e -5 ± 4.28e-6
OsCCR14 + OsATL53 + JA	21.0866 ± 1.2854	2.8972e -4 ± 1.79e-4	1.4256e -5 ± 9.12e-6
OsATL53	40.7378 ± 5.5711	3.3370e -4 ± 4.96e-5	1.0132e -5 ± 2.49e - 6

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