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1 **Title: Exogenously applied ABA induced changes in physio-biochemical**
2 **attributes in selected rice varieties under osmotic stress**

3 **Running title: Effect of ABA on rice cultivars under moisture stress**

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31
32

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35

36 **Abstract**

37 Drought is the most catastrophic abiotic stress that affect plant growth and
38 development. Plant initiates alterations at physiological, biochemical and molecular levels to
39 combat deleterious effects of drought in which abscisic acid, play a pivotal role. The present
40 investigation was conducted to investigate how abscisic acid modulates different biochemical
41 traits under osmotic stress. Twenty-one days old seedlings were exposed to osmotic stress,
42 exogenously applied abscisic acid and the combination of both osmotic stress and abscisic
43 acid to study the influence of abscisic acid on biochemical traits under moisture stress. The
44 results reveals that abscisic acid has no influence on leaf water potential, however, it has
45 active role in increasing osmolytes like proline. Δ^1-pyrroline-5-carboxylate reductase and
46 proline dehydrogenase remained unresponsiveness to ABA that indicates ABA-independent
47 regulation of these two enzymes but the activity of Δ^1-pyrroline-5-carboxylate synthesase and
48 Ornithine-δ-aminotransferase was enhanced (3 fold) in response to abscisic acid.
49 Antioxidative enzymes increased (catalase-2.5 fold; peroxidase- 3 fold and SOD- 6 fold) in
50 response to abscisic acid and osmotic stress, gave an insight of the regulation of these traits
51 under the influence of abscisic acid. Aldehyde oxidase remained unresponsive to abscisic
52 acid but showed enhanced expression when both abscisic acid and osmotic stress was applied
53 suggest its ABA-independent regulation. The information provided here has significance in
54 understanding the regulation of different catabolite that play important role in drought
55 tolerance and can be implemented for the development of drought-tolerant varieties.

56 ***Keywords:*** ABA, aldehyde oxidase, biochemical traits, Osmotic stress, P5CS and P5CR.

57 **Abbreviations:**

58 P5CS- Δ^1-pyrroline-5-carboxylate synthase; P5CR- Δ^1-pyrroline-5-carboxylate reductase;
59 OAT - Ornithine-δ-aminotransferase; PDH- proline dehydrogenase; M- Mannitol; ABA-
60 Abscisic acid; LWP- Leaf water potential; AO- Aldehyde oxidase; MTT- 3[4,5-
61 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide; GSH- reduced glutathione; DTT-
62 dithiothreitol; PAGE- polyacrylamide gel electrophoresis

63

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64 ***Introduction***

65 Drought is the most significant environmental stress in agriculture that affect plant
66 growth and productivity (Xoconostle-Cazares et al. 2010). It impairs physiological and
67 biochemical processes and causes severe damage to the plant. To nullify the effect of
68 drought, plants develop certain morphological (Farooq et al. 2010; Dash et al. 2017),
69 physiological (Sibounheuang et al. 2006), biochemical and molecular mechanisms by altering
70 its cellular metabolism and defense mechanism that includes closing of stomata to reduce
71 water loss, accumulation of osmo-regulators to retain water inside the cell and production of
72 reactive oxygen species (ROS) scavenging enzymes to neutralize superoxide radicals etc. The
73 phytohormone abscisic acid plays an important role in drought tolerance mechanism which is
74 accumulated mainly in response to drought stress. Increase in endogenous abscisic acid
75 (ABA) level with the commencement of water stress and gradually degradation upon release
76 of stress has been well documented (Zhang et al. 2006). ABA is thought to be an important
77 signalling factor involved in drought tolerance. Plants having high endogenous ABA level are
78 considered to reduce water loss through transpiration and exhibit efficient drought tolerance
79 mechanism (Kholová et al. 2010). Evidence of growth promotion and an increase in leaf
80 water content by foliar spraying of ABA under drought condition has been documented
81 (Sansberro et al. 2004). ABA enhances leaf relative water content through a simultaneous
82 increase in water uptake by aquaporins at root level and transpiration check at leaf level (Lian
83 et al. 2006). Besides activating aquaporins, ABA increases accumulation of osmo-regulators
84 that help in lowering water potential of the cell thus generating water potential gradient that
85 drives uptake of water from the soil even when stomata are closed and production of anti-
86 oxidative enzymes that protect the essential proteins and photosystems against desiccation
87 injury (Dash and Swain 2015). Proline and soluble sugars are common osmo-regulator that
88 are accumulated in response to drought (Jiang et al. 2012). (Savoure et al. (1997) reported an
89 increase in P5CS gene expression under water stress in response to 50 μM ABA but P5CR
90 gene expression was scarcely detectable in response to ABA. Proline dehydrogenase (PDH)
91 that is responsible for degradation of proline has been reported to decrease under water stress
92 condition (Sharma and Verslues 2010). Proline is also synthesized in an alternate pathway
93 using ornithine as precursors through the enzyme ornithine-δ-aminotransferases (OAT).
94 Expression of Ornithine-δ-aminotransferase (OAT) in an ABA-dependent and ABA-
95 independent manner has also been reported (You et al. 2012). Similarly, sugar accumulation
96 in response to water stress contributes in maintaining osmotic adjustment. Increase in total

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97 sugar and reducing sugars under water stress and in response to ABA has been observed with
98 increased α- and β- amylase activity (Sarfaraz-Ardakani et al. 2014). During water stress,
99 many authors have reported increased synthesis of anti-oxidative enzymes. (Jiang and Zhang
100 2001) reported increased generation of O_2 and induction of antioxidant enzyme gene
101 expression in response to exogenously applied. But reports regarding the effect of ABA on its
102 own biosynthesis enzymes like aldehyde oxidase (AO) is limited. In the present study,
103 attempts are made to characterise some identified drought tolerant and susceptible genotypes
104 on the basis of ABA modulated physiological and biochemical traits during osmotic stress.

105 **Materials and Methods**

106 *Plant material:* Four previously identified promising rice genotypes (*Oryza sativa* L.) (AC
107 43037, AC 43025, AC 43012 and Lalaijung) seems to be tolerant to drought stress at
108 vegetative stage along with one tolerant (CR 143-2-2) & susceptible check (IR 64) were
109 taken in the experimentation to further detailed study. Seeds were sterilized with 10% sodium
110 hypochlorite solution and then germinated in petri dishes on moist filter paper. Sprouting
111 seeds were placed in the holes of polystyrene plates supported by nylon mess after two days
112 of germination. The polystyrene plates were placed on the surface of plastic trays containing
113 10 lt. of full-strength nutrient medium as described by Yoshida et al. 1976. Three sets of trays
114 were arranged to maintain three replications (12 no. of trays) per treatment and the seedling
115 of all varieties were placed in each tray in separate rows. Plants were allowed to grow for 21
116 days in the nutrient solution and pH of the nutrient solution was maintained at 5.7 every day.
117 Nutrient solution was renewed in every 5 days interval. After 21 days, the seedlings were
118 exposed to four types of treatments for 6 days to study the effect of ABA on different
119 biochemical traits in the presence as well as in the absence of osmotic stress. The treatments
120 were as follows:

121 1) medium containing only nutrient solution i.e. control (C)
122 2) medium containing nutrient solution along with 2% D- mannitol (M)
123 3) medium containing nutrient solution along with 10 μM ABA (ABA)
124 4) medium containing nutrient solution, 2% D-mannitol and 10 μM ABA (M+ABA)

125 For each treatment, five replications were made. Osmotic potential of 2% D-mannitol was
126 estimated to be 0.59 MPa using water potential system, WESCOR, USA. Leaf sampling was
127 done at sixth day of treatments applied. Fully expanded second leaf was collected in a

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128 polyethylene bag at the midday (1.00-2.00 pm) for estimation of physiological and
129 biochemical traits and were stored at -80 ºC.

130 *Determination of Leaf water potential (LWP)*

131 Leaf water potential was measured following the method of H.D. Barrs and P.E.
132 Weatherley 1962 and Turner 1982 with the help of water potential system, Psypro, Wescor
133 (USA).

134 *Estimation of Proline content*

135 Total proline content was estimated following (Bates et al. 1973). Fresh leaf sample
136 of 0.5 g was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and centrifuged at
137 10,009 g for 10 min at 4 °C. Two millilitres of the supernatant was mixed with 2 ml of glacial
138 acetic acid and 2 ml of acid ninhydrin (1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml
139 6 M phosphoric acid) and kept in boiling water bath for 1 hr. The reaction was stopped by
140 placing the tubes in an ice bath. After cooling to room temperature, 4 ml toluene was added
141 to the reaction mixture and vortex shaken. The toluene layer was separated and the red colour
142 intensity was measured at 520 nm.

143 *Enzyme assay for proline metabolism*

144 Enzyme extraction from the frozen leaf samples was carried out according to Lutts et
145 al. 1999. One gram frozen leaf sample was homogenized in 100 mM potassium phosphate
146 buffer containing 1 mM EDTA, 10 mM β-mercaptoethanol, 1% polyvinylpolypyrrolidone, 5
147 mM $MgCl_2$ and 0.6 M KCl maintained at pH 7.4. The homogenate was centrifuged at 13,000
148 g for 15 min at 4 °C.

149 The activity of Δ^1-pyrroline-5-carboxylate synthase (P5CS; EC 2.7.2.11) was
150 determined according to the method of Stines et al. (1999). Reaction was started by adding
151 0.5 ml of enzyme extract to a mixture of 75 mM L-glutamate, 20 mM $MgCl_2$, 100 mM Tris–
152 HCl, 5 mM ATP and 0.4 mM NADPH. After incubation for 20 min at 37 °C, absorbance was
153 recorded at 340 nm using UV-Visible spectrophotometer (Thermo Fisher Scientific, Finland).
154 Enzyme activity was expressed as U mg protein^{-1} considering 1 U is equivalent to an increase
155 of 0.001 A_{340} per min.

156 Δ^1-pyrroline-5-carboxylate reductase (P5CR; EC 1.5.1.2) activity was measured
157 following the method of Madan et al. (1995). To a mixture of 0.06 mM NADH, 0.15 mM Δ^1-
158 pyrroline-5-carboxylic acid, 120 mM potassium phosphate buffer and 2 mM dithiothreitol,
159 0.5 ml of enzyme extract was added and the decrease in the absorbance was recorded at 340

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160 nm. P5CR activity was expressed as U mg protein^{-1} (one unit defined as a decrease in 0.01
161 A_{340} per min).
162 Ornithine-δ-aminotransferase (OAT; EC 2.6.1.68) was assayed by the method of
163 Vogel and Kopac (1960). Enzyme extract and 100 mM potassium phosphate buffer (pH 8.0)
164 was mixed in the proportion 0.2:0.8 ml. Buffer contained 50 mM L-ornithine, 20 mM α-
165 ketoglutarate and 1 mM pyridoxal-5-phosphate. The reaction was stopped by adding 0.5 ml
166 10% trichloro acetic acid and 0.5% O-aminobenzaldehyde in ethanol to the reaction mixture
167 after incubation for 30 min at 37 °C. After 1 hour, the reaction mixture was centrifuged at 12
168 0009 g for 10 min at 4 °C. Increase in absorbance was recorded at 440 nm. Enzyme activity
169 was expressed as U mg protein^{-1} (one unit defined as an increase in 0.01 $A_{440)}$.
170 For proline dehydrogenase (PDH; EC 1.5.99.8) estimation, the enzyme extract was
171 dissolved in 0.15 M dm^{-3} Na_2CO_3-HCl buffer (pH 10.3) with 13 mM dm^{-3} L-proline and 1.5
172 mM dm^{-3} NAD^+ ((Lutts et al. 1999) and absorbance was measured at 340 nm. PDH was
173 expressed as U mg protein^{-1} (one unit is defined as an increase in 0.01 A_{340} per min).

174 *Anti-oxidative enzyme Assay*

175 Leaf material (1 g) was homogenized in 1.5 ml of potassium phosphate buffer (pH
176 7.8) containing 1 mM EDTA, 1 mM ascorbate, 10% w/v sorbitol and 0.1% Triton X. The
177 homogenate was centrifuged at 15,000g for 20 mins at 4 °C and the supernatant was used for
178 catalase, peroxidase, and superoxide dismutase enzyme analysis. Protein concentration was
179 detected according to the method of (Lowry et al. 1951) using the bovine serum as a standard.
180 Catalase (EC 1.11.1.6) activity was estimated by the method followed by Pereira et al.
181 2002. To a mixture of 50 mM potassium phosphate buffer (pH 7.0) and 0.036% of H_2O_2, 0.1
182 ml of enzyme extract was added and the decrease in absorbance was recorded at 240 nm and
183 the rate of enzyme activity was expressed as U mg protein^{-1}.
184 Peroxidase (EC 1.11.1.x) activity was estimated following the method Pütter (1974).
185 To a mixture of 0.1 M potassium phosphate buffer (pH 7.0) and 0.018 M guaiacol, 0.1 ml of
186 enzyme solution was added. The increase in absorbance was recorded at 436 nm and the rate
187 of enzyme activity was expressed as U mg protein^{-1}.
188 The activity of superoxide dismutase (SOD) (EC 1.15.1.1) was estimated by the
189 method Giannopolitis and Ries (1977). To a mixture of 0.05 M phosphate buffer, 13 mM
190 methionine, 75 μM NBT and 100 nM EDTA, 0.1 ml of enzyme extract was added and placed
191 30 cm below a light bank consisting of two 30–watt-fluorescent bulbs (reference tube must be

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192 placed in the dark). The reaction was started by switching on the light and allowed to run for
193 20 min and then terminated by switching off the light followed by covering the tubes with a
194 black cloth. Each tube was wrapped with aluminum foil. Absorbance was read against the
195 blank (reference tube) at 560 nm. The volume of enzyme extract producing 50% inhibition of
196 the reaction is defined to have one unit of SOD activity.

197 *Assay of Aldehyde oxidase (AO) activity*

198 Aldehyde oxidase (AO; EC 1.2.3.1)) activity was determined following the method of
199 Zdunek and Lips (2001). Plant tissue was homogenized immediately after harvesting with
200 acid- washed sand and ice-cold extraction medium containing 250 mM TRIS-HCl (pH 8.5), 1
201 mM EDTA, 10 mM reduced glutathione (GSH), and 2 mM dithiothreitol (DTT). A ratio of 1
202 g tissue to 3 ml buffer (1:3 w/v) was used to homogenize plant material and was centrifuged
203 at 27,000g and 4 °C for 15 min. The resulting supernatant was subjected to native
204 polyacrylamide gel electrophoresis (PAGE) with 7.5% polyacrylamide gel in a Laemmli
205 buffer system (Laemmli 1970) in the absence of sodium dodecyl sulphate at 4 °C. Each lane
206 in the gel was loaded with 400 µg leaf proteins. After electrophoresis, AO activity staining
207 was developed at room temperature in a mixture containing 0.1 M TRIS-HCl, pH 7.5, 0.1
208 mM phenazine methosulphate, 1 mM MTT (3[4,5-dimethylthiazol-2-yl]-2,5-
209 diphenyltetrazolium-bromide) and 1 mM indole-3-aldehyde (substrate). The gel was
210 incubated in dark overnight for the development of bands. The activity of AO was estimated
211 based on MTT reduction, which resulted in the development of specific formazon bands
212 which were directly proportional to enzyme activity (Rothe 1974) and quantified using the
213 NIH Image J 1.6 computer software.

214

215 **Statistical Analysis**

216 Analysis of Variance was analysed using Cropstat for windows 7.2.2007.3 (IRRI,
217 Philippines). The least significant difference test was used to distinguish among individual
218 mean values where applicable with a confidence level of $p < 0.01$ and $p < 0.001$.

219

220 **Results**

221 *Effect of osmotic stress and ABA on Leaf water potential*

222 Leaf water potential substantially decreased till six days of treatment under osmotic
223 stress and osmotic stress along with ABA ($p<0.05$) compared to control (C). Exogenous
224 application of ABA did not affect LWP and there was no significant variation in LWP
225 between the control plants and the plants treated with only ABA. LWP also did not seem to

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226 be affected under osmotic stress by ABA treatment which was evident from the insignificant
227 difference in LWP between M (-2.11 MPa) and M+ABA (-2.15 MPa) treatment. However, a
228 significant difference in LWP ($p<0.05$) was observed between tolerant and susceptible
229 genotypes. Decrease in LWP in susceptible genotypes (Lalaijung and IR 64) was more than
230 two fold to that of tolerant genotypes (AC 43025, AC 43012, AC 43037 and CR 143-2-2)
231 both under M and M+ABA treatments (Table 1; Fig 1). LWP did not vary between M and
232 M+ABA treatments, despite the presence of ABA in M+ABA treatment in all the susceptible
233 genotypes.
234

235 *Effect of osmotic stress and ABA on proline content and proline biosynthesis enzymes*

236 Proline content increased significantly ($p<0.05$) under all the three treatments (M,
237 ABA and M+ABA) compared to control. Accumulation of proline was increased up to 22-
238 fold and 27-fold under M and M+ABA treatment respectively after six days of treatments
239 (Fig. 1). In ABA treatment, the accumulation of proline was only 10-fold compared to
240 control. However, proline accumulation was more in M+ABA compared to osmotic stress
241 (M) and ABA treatment. Significant variation was observed in proline content ($p<0.05$)
242 between tolerant and susceptible genotypes. Variation in proline content in susceptible
243 genotypes Lalaijung and IR 64 was insignificant in M and ABA treatments, however, in
244 M+ABA treatment, Lalaijung had significantly higher proline content compared to IR 64. To
245 elucidate the differential accumulation of proline under the three treatments, the rate of
246 activity of enzymes involved in proline biosynthesis was studied. The activity of P5CS was
247 20-fold more as compared to control under all the three treatments after six days of treatment.
248 P5CS activity was significantly lower in ABA treatments of all tolerant genotypes compared
249 to their corresponding M and M+ABA treatments. In susceptible genotypes, P5CS activity
250 was higher than M ($p<0.05$) but lower than M+ABA ($p<0.05$). Amount of proline
251 accumulated in susceptible genotypes was much lower than tolerant genotypes. (Fig. 1).

252 P5CR activity was significantly ($p<0.05$) higher in M and M+ABA compared to C
253 and ABA (7 to 9-fold increase). No significant variation in P5CR activity was observed
254 neither between C and ABA nor M and M+ABA. However, P5CR activity in tolerant
255 genotypes were significantly higher than in susceptible genotypes. (Table 1; Fig.1).

256 OAT activity varied significantly in all the treatments ($p<0.05$). OAT activity
257 increased about three-fold under all the three treatments compared to control after six days of
258 treatment. OAT activity was higher in ABA compared to M but lower than M+ABA.

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259 PDH decreased drastically in all the three treatments after six days of imposing
260 treatments. M and M+ABA caused 60-78% reduction in PDH activity whereas ABA alone
261 reduced only 20% of the enzyme activity depicted that PDH activity is less influenced by
262 ABA. (Table 1; Fig. 1).
263
264 *Effect of omotic stress and ABA on anti-oxidative enzymes*
265 Antioxidative enzyme activity was increased under all the three treatments compared
266 to control. On an average, catalase activity was increased more than 2.5 times under all the
267 three treatments compared to control. A significant difference in catalase activity between
268 tolerant and susceptible genotypes was observed (Table 1). Increase in catalase activity was
269 approximately 60% more under osmotic stress and 16- 23% more under ABA and M+ABA
270 in tolerant genotypes compared to susceptible ones. (Table 1; Fig. 2).
271 Similarly, peroxidase activity was increased under all the three treatments and reached
272 up to three-fold compared to control. Tolerant genotypes showed about 2-fold increase in
273 peroxidase activity over susceptible genotypes under M, ABA and M+ABA. (Table 2 & 4;
274 Fig 3).
275 Superoxide dismutase activity was also increased under all the three treatments and
276 amounting about 5-fold increase under M and M+ABA. Under ABA treatment, 4-fold
277 increase in SOD activity was recorded after six days of treatment. It was also observed that
278 under osmotic stress, tolerant genotypes showed 2.4-fold increase in SOD activity than
279 susceptible genotypes whereas under ABA and M+ABA treatments, SOD activity increased
280 about 1.2-fold in tolerant genotypes (Table 1; Fig. 2).
281
282 *Effect of osmotic stress and ABA on aldehyde oxidase activity*
283 A significant increase in AO activity was observed under M and M+ABA but not
284 under ABA treatment. Mean AO activity was increased up to 258.6% after 6 days of
285 treatment under M whereas, under M+ABA, it was increased up to 271.3%. Under ABA
286 treatment increase in mean AO activity was insignificant (128%) compared to M and
287 M+ABA. It clearly indicates that AO activity is stimulated in response to osmotic stress but
288 had no response in presence of ABA (Fig-3).
289
290 **Discussion**

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291 Earlier studies reported the active involvement of ABA during osmotic stress. The
292 present study represents how ABA modulates different physiological and biochemical traits
293 during osmotic stress. In many reports, ABA is known to induce stress tolerance during
294 osmotic stress at very low concentration through alteration at the physiological as well as at
295 molecular level but at high concentrations, it induces oxidative stress (Kuo and Ching 2004).
296 In our study, the decrease in LWP during osmotic stress (M) has been observed that may be
297 due to rapid transpiration and slower water absorption through roots. The effect of ABA
298 during stress may help in maintaining LWP through closure of stomata. No such variation in
299 LWP was observed between M and M+ABA that clearly indicate that ABA has no influence
300 on LWP. However, a significant difference in LWP ($p<0.05$) was observed between tolerant
301 and susceptible genotypes. Decrease in LWP in susceptible genotypes (Lalaijung and IR 64)
302 was more than double to that of tolerant genotypes (AC 43025, AC 43012, AC 43037 and CR
303 143-2-2) both under M and M+ABA treatments that indicates rapid transpiration in
304 susceptible genotypes resulting in loss of turgidity and hence very low water potential
305 compared to tolerant genotypes. Maintenance of high LWP in response to moisture stress can
306 be the result of the accumulation of compatible solutes like proline in tolerant genotypes, that
307 decreases the cellular osmotic potential to prevent water loss (Zhang et al. 1999). In our
308 study, an increase in proline content was less in ABA compared to osmotic stress (M).
309 Proline accumulation might be independent of the ABA-mediated signaling pathway.
310 According to Zhang et al. (1999), endogenous ABA accumulation is required for proline
311 accumulation but ABA alone is not sufficient to elicit the levels of proline accumulation
312 observed under low water potential. The reason for this differential response might be due to
313 the response of biosynthesis enzymes involved in proline biosynthesis towards ABA.
314 According to Savoure et al. (1997), both the expression of P5CS and P5CR were up-regulated
315 under osmotic stress but exogenous ABA application enhanced the expression of P5CS but
316 not P5CR. Therefore, the glutamate might be converted to glutamate-γ-semialdehyde (GSA)
317 but unable to form proline due to reduced expression of enzyme P5CR. This supported our
318 finding where a 24-fold increase in P5CS activity was observed in osmotic stress (M) and the
319 combined effect of osmotic stress and ABA (M+ABA). Under ABA treatment, a 21-fold
320 increase in P5CS activity was observed which was almost equal to the amount of P5CS
321 activity produced under other two treatments (M and M+ABA). The activity of P5CR was
322 insignificant from that of the control under the treatment of ABA. However, under osmotic
323 stress (M) and combined effect of osmotic stress and ABA (M+ABA), P5CR activity was
324 enhanced leading to an increase in proline content to more than 20-fold increase. The

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325 alternative pathway to proline production catalyzed by OAT also enhanced both in response
326 to osmotic stress and exogenously applied ABA. Many authors suggested that *OAT* can also
327 produce P5C and contribute to proline accumulation (Roosens et al. 2002). Funck et al,
328 (2008) discarded the involvement of OAT in proline accumulation and proline biosynthesis
329 occurred predominantly via the glutamate pathway in Arabidopsis. However, the function of
330 OAT in Arabidopsis has been questioned in part because of new information showing that it
331 is localized in the mitochondria (Funck et al. 2008). This changing role of OAT makes it of
332 even greater importance to determine its regulation under conditions leading to proline
333 accumulation. Sharma and Verslues (2010) stated that exogenous ABA did not induce OAT
334 expression which was against our findings in which 3.5-fold increase in OAT activity was
335 observed in response to ABA. Insignificant difference in OAT activity in M, ABA and M
336 treatments gave insights on the regulation of OAT activity by both ABA dependent and ABA
337 independent pathways. Moreover, it can be inferred that reduced activity of P5CR might have
338 resulted in reduced proline synthesis under ABA treatment and most of the accumulated
339 proline might have contributed by OAT activity. The difference in proline accumulation
340 between M and M+ABA treatment might be due to the contribution of OAT in response to
341 ABA under M+ABA treatment. According to You et al. (2012), OAT was strongly induced
342 by ABA in rice and both ABA-dependent and AB-independent pathway contribute to the
343 drought-induced expression of OAT. This is in accordance with our findings. Proline
344 dehydrogenase (PDH) is the enzyme, which is associated with the oxidation of proline to
345 glutamate thus responsible for the reduction in proline level in cells. In our study, the activity
346 of PDH was higher in the control condition but gradually decreased under stress. According
347 to Shevyakova et al. (2013), ABA suppressed PDH activity when treated to the roots.
348 Dallmier and Stewart (1992) also stated that exogenous ABA declines PDH activity, which is
349 very small, compared to level declined in response to drought stress suggesting that ABA is
350 not the pathway linking drought stress and PDH activity. In our experiment, no response of
351 ABA was observed on PDH activity. However, PDH activity was declined under osmotic
352 stress and combined effect of osmotic stress and ABA. There was much difference in PDH
353 activity between susceptible and tolerant genotypes that might be due to lack of intracellular
354 signal or failure in the suppression of PDH activity that contributed to low levels of proline
355 content in susceptible genotypes.

356 The first response to osmotic stress is the production of free radicals that induce
357 expression of many genes like anti-oxidative enzymes that helped in diluting the effect of

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358 osmotic or oxidative stress. ABA biosynthesis enzymes also get activated in response to free
359 radicals that induce H_2O_2 production and activated anti-oxidative enzymes like catalases,
360 peroxidases and superoxide dismutase. Interrelationship between the generation of reactive
361 oxygen species in response to PEG-induced osmotic stress and in response to ABA with
362 activation of anti-oxidative enzymes has been established (Jiang and Zhang 2002). This is in
363 accordance with our finding where peroxidase, catalase and superoxide dismutase activity
364 were enhanced by in response to M, ABA and M+ABA. Rivero et al., (2007) also reported an
365 increase in the transcript level of SOD in transgenic lines of tobacco plants under drought.
366 According to the findings of Rivero et al., (2007), efficient scavenging of ROS protects the
367 photosynthetic apparatus during drought stress that leads to improved water use efficiency
368 during and after drought stress.

369 The plant hormone abscisic acid has long been known to be involved in the response
370 of plants to various environmental stresses, particularly drought (Zhu 2011). It mediates
371 coordination between growth and development in response to the environment. Under non-
372 stressful conditions, low ABA level is maintained in the plant cell which is required for
373 normal plant growth (Finkelstein and Rock 2002). In this investigation, the effect of osmotic
374 stress, ABA and the combined effect of osmotic stress and ABA (M+ABA) on the activity of
375 aldehyde oxidase has been studied. The activity of AO was enhanced in response to osmotic
376 stress and under the combined effect of osmotic stress and ABA. According to Xiong and
377 Zhu (2003), the degradation of ABA is suppressed by osmotic stress and activated by ABA
378 and stress relief which is in accordance with our findings. No significant change in AO
379 activity was observed under ABA treatment. Qin and Zeevaart (2002) have demonstrated that
380 overproduction of ABA correlated with the over-accumulation of phaseic acid which leads to
381 the notion that ABA might restrict its own accumulation by activating its degradation even
382 under non-stressful conditions.

383 **Conclusion**

384 Abscisic acid is a phytohormone that positively regulates drought tolerance at low
385 concentration but induces senescence at high concentration. Though activation of many
386 drought responsive enzymes is ABA-dependent, some are regulated independently of ABA.
387 The present study revealed how ABA modulates different biochemical traits that are involved
388 in the drought tolerance mechanism which signifies the role of ABA in drought tolerance
389 mechanism and provided information for the development of an effective drought

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390 management strategy. Though P5CS and OAT are activated through ABA, P5CR and PDH
391 remain unresponsive which indicates proline can be synthesized independently of ABA from
392 glutamate or can be synthesized from ornithine through an ABA-dependent pathway. Anti-
393 oxidative enzymes produced through ABA-dependent pathway that helps in ameliorating the
394 effects of drought stress. ABA also regulates its own endogenous level by down-regulating its
395 own activity to maintain its optimal level inside the cell. The information provided here will
396 be useful for carrying out an extensive analysis of the role of ABA in drought tolerance.

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Fig. 1a-f Effect of osmotic stress, ABA and combined ABA and osmotic stress on leaf water potential (LWP), proline, and proline biosynthesis enzymes (P5CS- Δ^1-pyrroline-5-carboxylate synthase; P5CR- Δ^1-pyrroline-5-carboxylate reductase; OAT - Ornithine-δ-aminotransferase; PDH- proline dehydrogenase) in four drought tolerant and two drought sensitive genotypes. Error bars represent SE values (n=3). Different letters indicate significant differences ($P < 0.05$) among the treatments and within the genotypes. Control (C) corresponds to normal growth condition without any treatment. Osmotic stress (M) corresponds to 2% D-mannitol mediated osmotic stress; ABA corresponds to 10 μM of ABA applied exogenously through the nutrient medium; M+ABA corresponds to 2% D-mannitol and 10 μM ABA applied simultaneously to the nutrient medium.

Fig. 2a-c Effect of osmotic stress, ABA and combined ABA and osmotic stress on catalase (CAT), peroxidae (POX) and superoxide dismutase (SOD) in four drought tolerant and two drought sensitive genotypes. Error bars represent SE values (n=3). Different letters indicate significant differences ($P < 0.05$) among the treatments and within the genotypes. Control (C) corresponds to normal growth condition without any treatment. Osmotic stress (M) corresponds to 2% D-mannitol mediated osmotic stress; ABA corresponds to 10 μM of ABA applied exogenously through the nutrient medium; M+ABA corresponds to 2% D-mannitol and 10 μM ABA applied simultaneously to the nutrient medium.

Fig. 3 Zymograms of aldehyde oxidase (AO) activity in leaves of six rice genotypes exposed to osmotic stress, ABA and combined ABA and osmotic stress for 6 days grown in the nutrient solution. Aldehyde oxidase activity was developed using indole-3-aldehyde as substrate after native PAGE. Each lane in the gel was loaded with 400 μg of leaf proteins. Aldehyde oxidase activity is represented as the percentage after scanning and analyzing using NIH ImageJ 1.6 software assuming activity under control as 100%. Control (C) corresponds to normal growth condition without any treatment. Osmotic stress (M) corresponds to 2% D-mannitol mediated osmotic stress; ABA corresponds to 10 μM of ABA applied exogenously through the nutrient medium; M+ABA corresponds to 2% D-mannitol and 10 μM ABA applied simultaneously to the nutrient medium.

566 Table-1 Mean of all biochemical traits of six rice genotypes measured under four treatments.

Traits	Treatmets	6 DAT	Traits	Treatmets	6 DAT
LWP (-MPa)	C	-1.05 ± 0.04	PDH (U mg protein^{-1})	C	4.42 ± 0.15
	M	-2.12 ±0.36		M	1.08 ± 0.10
	ABA	-1.22 ±0.05		ABA	3.52 ± 0.11
	M+ABA	-2.14 ±0.34		M+ABA	1.08 ± 0.21
Proline (µM g DW^{-1})	C	6.6 ± 0.60	CAT (U mg protein^{-1})	C	0.28 ± 0.01
	M	148.6 ± 30.25		M	0.69 ± 0.06
	ABA	63.0 ± 7.21		ABA	0.73 ± 0.03
	M+ABA	186.0 ± 19.24		M+ABA	0.77 ± 0.03
P5CS (U mg protein^{-1})	C	0.05 ± 0.00	POX (U mg protein^{-1})	C	2.43 ± 0.11
	M	1.09 ± 0.23		M	8.08 ± 0.95
	ABA	1.04 ± 0.16		ABA	8.87 ± 0.97
	M+ABA	1.35 ± 0.17		M+ABA	9.33 ± 1.18
P5CR (U mg protein^{-1})	C	0.03 ± 0.00	SOD (U mg protein^{-1})	C	0.46 ± 0.02
	M	0.27 ± 0.04		M	2.53 ± 0.40
	ABA	0.04 ± 0.00		ABA	2.40 ± 0.37
	M+ABA	0.27 ± 0.04		M+ABA	2.77 ± 0.40
OAT (U mg protein^{-1})	C	0.31 ± 0.01			
	M	1.02 ± 0.14			
	ABA	1.29 ± 0.14			
	M+ABA	1.58 ± 0.14			

567 C-Medium containing only nutrient solution i.e. control; M-Medium containing nutrient solution along with 2% D- mannitol to study the effect of osmotic stress on
568 biochemical traits; ABA-Medium containing nutrient solution along with 60 µM ABA to study the effect of ABA on biochemical traits; M+ABA-Medium containing
569 nutrient solution, 2% D-mannitol and 60 µM ABA to study how ABA modulates biochemical traits under osmotic stress; P5CS- Δ1-pyrroline-5-carboxylate synthase; P5CR-
570 Δ1-pyrroline-5-carboxylate reductase; OAT-Ornithine-δ-aminotransferase; PDH-Proline dehydrogenase. CAT- catalase; POX- peroxidase; SOD- Superoxide dismutase.

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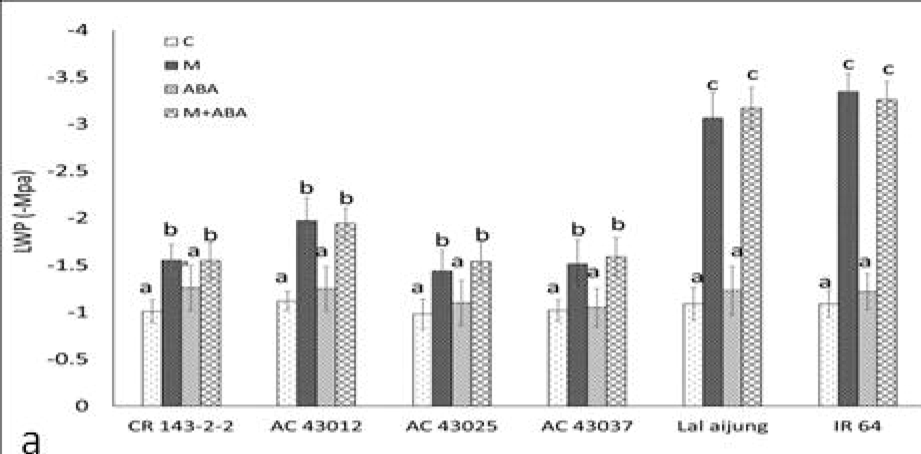
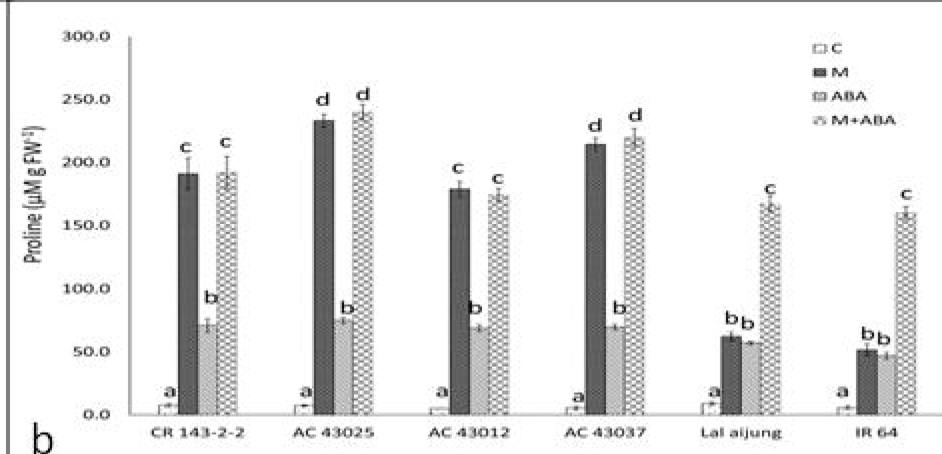
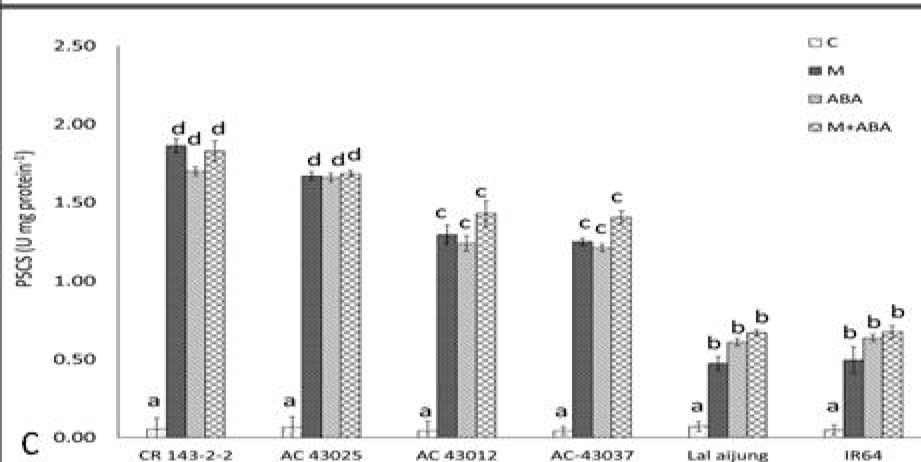


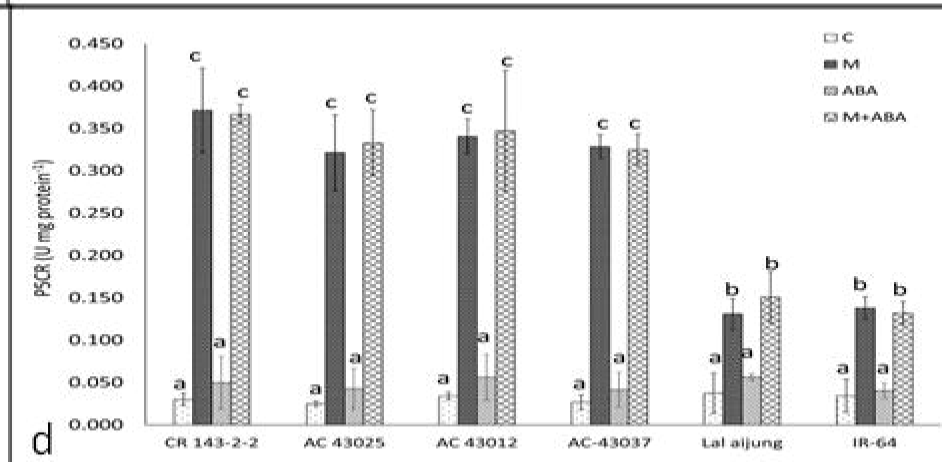
LWP (-Mpa)
C
M
ABA
M+ABA
CR 143-2-2
AC 43012
AC 43025
AC 43037
Lal aijung
IR 64
a



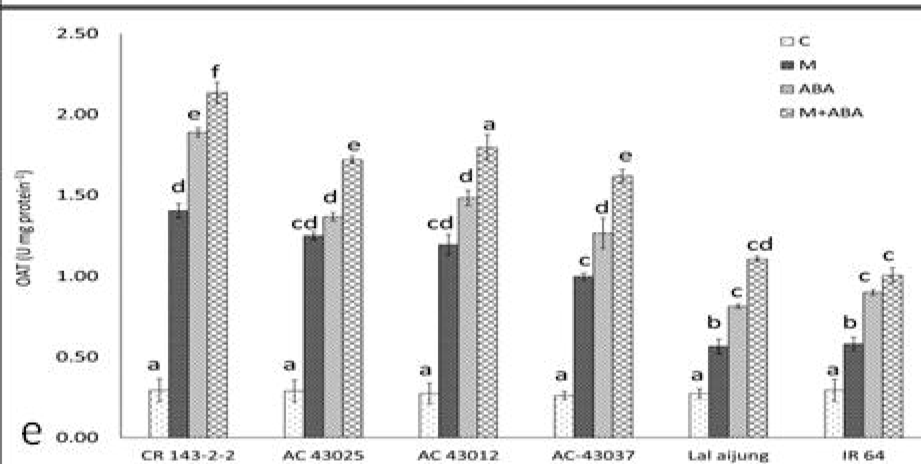
Proline (μM g FW⁻¹)
C
M
ABA
M+ABA
CR 143-2-2
AC 43025
AC 43012
AC 43037
Lal aijung
IR 64
b



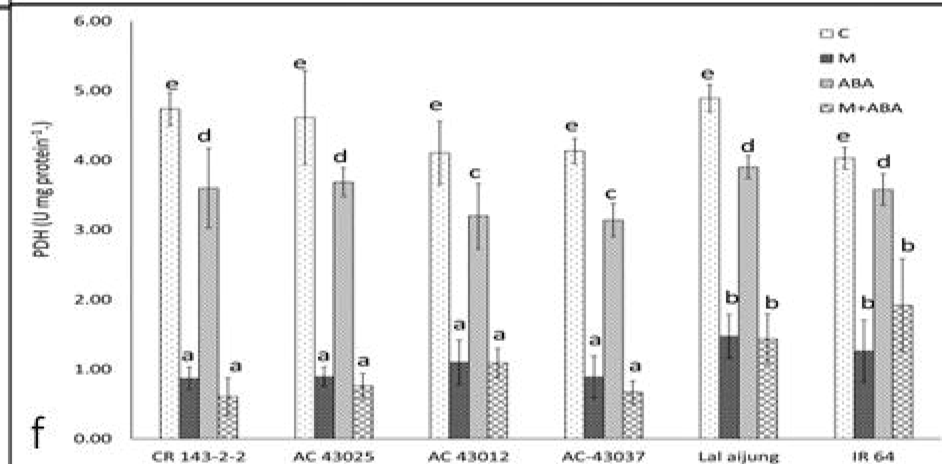
P5CS (U mg protein⁻¹)
C
M
ABA
M+ABA
CR 143-2-2
AC 43025
AC 43012
AC-43037
Lal aijung
IR64
c



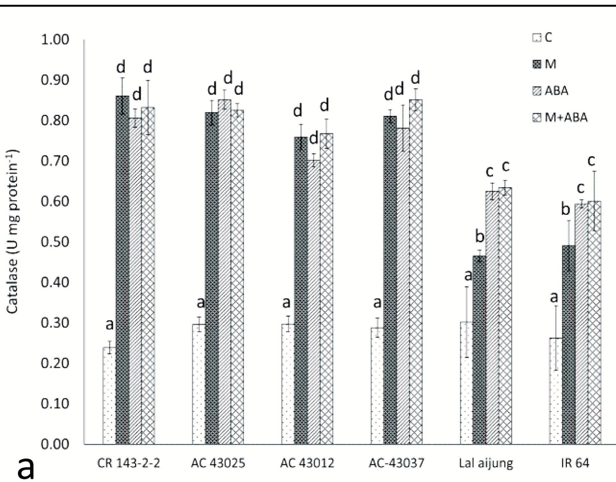
P5CR (U mg protein⁻¹)
C
M
ABA
M+ABA
CR 143-2-2
AC 43025
AC 43012
AC-43037
Lal aijung
IR-64
d

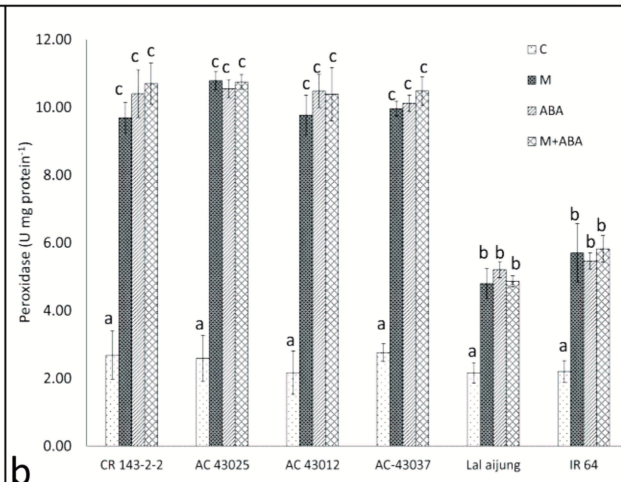


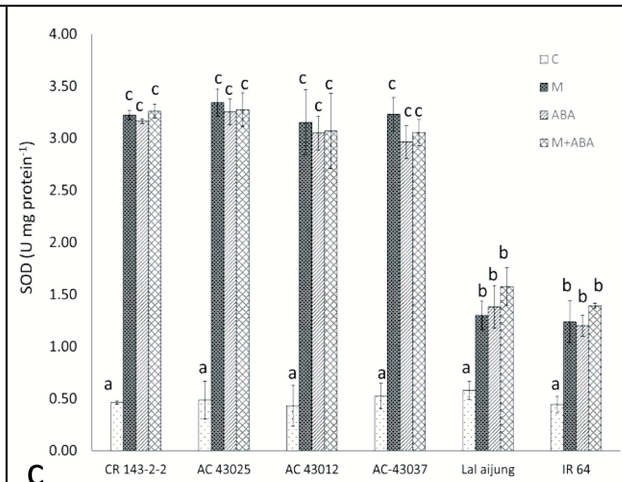
OAT (U mg protein⁻¹)
C
M
ABA
M+ABA
CR 143-2-2
AC 43025
AC 43012
AC-43037
Lal aijung
IR 64
e

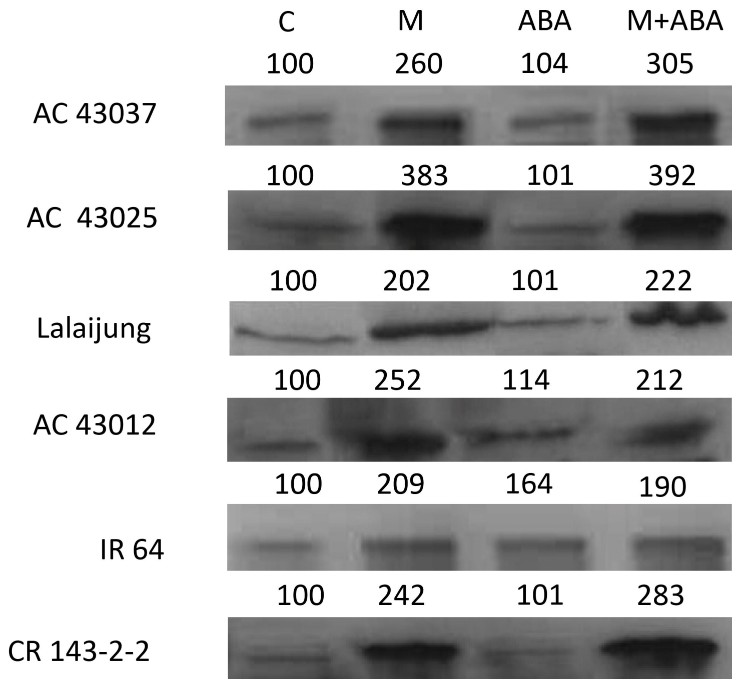


PDH (U mg protein⁻¹)
C
M
ABA
M+ABA
CR 143-2-2
AC 43025
AC 43012
AC-43037
Lal aijung
IR 64
f


Catalase (U mg protein-1)
C
M
ABA
M+ABA
CR 143-2-2
AC 43025
AC 43012
AC-43037
Lal aijung
IR 64
a


Peroxidase (U mg protein-1)
C
M
ABA
M+ABA
CR 143-2-2
AC 43025
AC 43012
AC-43037
Lal aijung
IR 64
b


SOD (U mg protein-1)
C
M
ABA
M+ABA
CR 143-2-2
AC 43025
AC 43012
AC-43037
Lal aijung
IR 64
c


C
M
ABA
M+ABA
100
260
104
305
AC 43037
100
383
101
392
AC 43025
100
202
101
222
Lalaijung
100
252
114
212
AC 43012
100
209
164
190
IR 64
100
242
101
283
CR 143-2-2