ABSTRACT

Elongation escape mediated by seedling vigor with fast shoot elongation under submergence is considered to be an important strategy for rice particularly in direct seeding cultivation systems. We compared the expression profiles of two key genes in rice for ethylene biosynthesis – 1-aminocyclopropane-1-carboxylate synthase (OsACS) and oxidase (OsACO) genes, and five Na⁺/H⁺ exchanger (OsNHX) paralogs under normal and submerged conditions. The amount of transcripts was studied by RT-PCR using seedlings of one japonica and three indica cultivars with different levels of seedling vigor under submergence. In both types, submergence induced marked accumulation of transcripts of all paralogs of OsACS and OsACO except for OsACS1, which showed complete repression under the stress in non-vigorous indica cultivars suggesting its key role in seedling vigor under submergence. OsNHX expression was also enhanced under submergence, and the levels of OsNHX1 and OsNHX5 transcripts agreed well with those of seedling vigor under submergence. The observed mode of OsACS, OsACO and OsNHX expression under submergence suggests that these genes can be potential targets for understanding the mechanism regulating seedling vigor under submergence at the post-germination stage in rice.

Keywords: OsACS, OsACO, OsNHX, rice (Oryza sativa L.), seedling vigor, submergence stress

Introduction

Submergence stress is a serious limiting factor in rainfed lowland rice cultivation fields in South and Southeast Asia, where flash flood often takes place at various stages of rice cultivation (17). Rice plants display varying degrees of adaptability to this stress, which is likely regulated by two major strategies. The “quiescent strategy” under control by the quantitative trait locus SUBMERGENCE 1 (SUB1) has been considered as a major mechanism controlling submergence tolerance in rice, whereby rice plants can maintain high levels of stored carbohydrates coupled with minimum shoot elongation and retention of high chlorophyll content under submergence (8, 9, 10, 39). This strategy is favorable to rice plants for their survival during temporal flash flood, in which the whole plants are covered by water for a period of no more than two weeks (7, 18).

On the other hand, high growth capacity with fast shoot elongation has been considered as the central core of seedling vigor under submergence and hence of rapid and optimum stand establishment in rice (28). Submergence stress in fact significantly promotes shoot elongation in young rice seedlings (15). Hence, the “elongation escape strategy”, which is associated with enhanced shoot growth and seedling vigor under submergence, has also been considered important, particularly in the direct seeding rice cultivation system in flood-prone fields (4, 44). Shoot elongation during submergence depends on genetic architecture and is affected by the submergence environment and by the state of seedling growth before submergence (18). In our previous study, using a set of 150 rice cultivars including japonica and indica types, we showed that seedling vigor evaluated based on the shoot elongation ability and the recovery rate after submergence could serve as a submergence escape or avoidance mechanism and help rice seedlings survive under submergence during the post-germination stage (38).

The role of ethylene biosynthesis and sensitivity in submergence tolerance has been well established during the vegetative stage in rice. Internode elongation in excised stem sections of floating or deepwater rice was stimulated by the phytohormone ethylene (14, 27, 31). Ethylene promoted elongation not only of internodes (24) but also of coleoptiles (20) and mesocotyls (32) in these types of rice. Most rice cultivars also possessed ability to elongate shoots during submergence, and this ability was mediated by ethylene (35). The role of the locus SUB1A in ethylene biosynthesis and sensitivity under submergence is also well established. In fact, the locus SUB1A plays an essential role in submergence tolerance by dampening the ethylene production and responsiveness, resulting in the limitation of ethylene-mediated elongation and carbohydrate consumption during submergence in rice. webinar
Much less information, however, is available on the role of ethylene biosynthesis in the regulation of seedling vigor under submergence at the post-germination stage in rice. To examine the role of ethylene during the post-germination stage as a mechanism associated with submergence tolerance, we studied the expression profiles of ethylene biosynthesis genes using rice seedlings subjected to submergence stress. A key enzyme in ethylene biosynthesis is 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) (40). A multigene family encodes ACS with five paralogous members in rice (33, 42, 43). ACC oxidase (ACO), which converts ACC to ethylene, is another key enzyme for ethylene biosynthesis (40). Seven ACO paralogs including a pseudogene ACO6 have been identified through computational analysis in the rice genome (16).

In addition, early seedling vigor is considered desirable under saline conditions because rice seedlings often encounter high salinity levels (41). The problem of high salinity in paddy fields is compounded by many factors including mineral deficiencies, drought and submergence (13). Improved varieties are needed that possess high yielding capacity together with substantial levels of submergence and salinity tolerance in some rice ecosystems. The rice genome contains five Na+/H+ exchanger genes (osNHX), which play important roles in salt tolerance in rice by regulating the uptake of sodium.

**Table 1**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence (5’-3’)</th>
<th>Product length (bp)</th>
<th>Optimal cycles</th>
<th>Orientation</th>
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<tbody>
<tr>
<td>OsACS1</td>
<td>GATTGATTCGATCGGCTGT</td>
<td>235</td>
<td>30</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td>GTCGAACGACAGCTGTT</td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>OsACS2</td>
<td>TTTTCCTTGGGGGCTGT</td>
<td>161</td>
<td>30</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td>TTCGTCGAGAGGTCGAT</td>
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<td></td>
<td>Reverse</td>
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<tr>
<td>OsACS3</td>
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</tr>
<tr>
<td></td>
<td>CCAACGACTCTCACCTTC</td>
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<td>OsACS4</td>
<td>AAGCTGAGCAGCTGATGTT</td>
<td>207</td>
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</tr>
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<td></td>
<td>GTGACAGGACATGCATACGA</td>
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</tr>
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<td>OsACS5</td>
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<td>Forward</td>
</tr>
<tr>
<td></td>
<td>CGAAGTAGCGGAGTCTCTT</td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
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<td></td>
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<td>OsACO2</td>
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<td></td>
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<td>OsACO3</td>
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<td>173</td>
<td>26</td>
<td>Forward</td>
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<tr>
<td></td>
<td>ATCTCGAAGAAGCCAGTCTT</td>
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<td></td>
<td>Reverse</td>
</tr>
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<td>30</td>
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<td></td>
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<td></td>
<td>GAAGATGTCTCCAGGTCCA</td>
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<td></td>
<td>TTTCTTCCTCCAGGTCTCGT</td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
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<td>OsNHX1</td>
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<td>28</td>
<td>Forward</td>
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<tr>
<td></td>
<td>GACTCGAGCCGACCGATT</td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>OsNHX4</td>
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<td>182</td>
<td>28</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td>GAGAGACGCTGACGGCTTA</td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
<tr>
<td>OsNHX5</td>
<td>ATTCCTTGGGACATCCATTGA</td>
<td>164</td>
<td>28</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td>CCCAGCTTCTGGGAATATTGA</td>
<td></td>
<td></td>
<td>Reverse</td>
</tr>
</tbody>
</table>
and/or potassium under saline conditions (11, 25). To examine if OsNHX expression responds to submergence stress, we studied the expression profiles of these OsNHX paralogs in rice seedlings subjected to the same submergence stress condition.

Materials and Methods

Plant materials and bioassay conditions for evaluating seedling vigor under submergence

Four cultivars of rice (Oryza sativa L.) were subjected to the test tube bioassay according to Manangkil et al. (22). FR13A and IR42 were used as well-known submergence tolerant and intolerant indica checks, respectively; Nipponbare, as a high seedling vigor japonica check; and Kasalath, as a low seedling vigor indica check (22, 37). Seeds were surface sterilized in 1% sodium hypochlorite (NaClO) solution for 10 min and rinsed with distilled water three times. They were then placed in glass Petri dishes in a dark incubator adjusted at 28 °C and relative humidity of 65% for 3 days. Seeds were washed everyday before germination. A total of 10 germinated seeds at the pigeon breast stage (about 3 days after imbibition) with normal coleoptile growth were transferred to a glass test tube with 10 cm deep distilled water. Uncovered test tubes were placed in an incubator at 28 °C in the dark (24hD) without changing the water for 5 days. Shoot (coleoptile) lengths were measured from the base to the tip of the seedlings. At the end of the submergence period, seedlings were transferred to pots with soil and placed under normal conditions (28 °C with 16hL/8hD). Seedling recovery rate (%) was recorded after 5 days and 2 weeks of further growth under the normal conditions. Seedling heights were also measured after these recovery periods. The whole experiment was repeated three times and statistical comparisons were made between Nipponbare and three indica cultivars by the LSD test.

Gene expression profiling by RT-PCR

Total RNA was extracted using guanidine thiocyanate from seedlings of the four cultivars subjected to the 5-day test tube bioassay and also from those grown in pots with soil under normal conditions for 5 days. For RT-PCR, the first strand cDNA was synthesized from 1 μg of DNaseI-treated RNA with oligo-dT primers using ReverTra Ace (TOYOBO, Osaka, Japan). Gene-specific primer sets were designed to ensure equal amplification efficiency (Table 1). The rice ubiquitin gene was used as an internal control. For each sample 4 μl cDNA template was added to 16 μl reaction mixture containing 1 μl of each forward and reverse primer, 2 μl 10x buffer, 0.8 μl MgCl₂, 1 μl dNTPs, 10 μl Q-water and 0.2 μl rTaq Polymerase to make a 20 μl PCR mixture. Negative control containing all the reaction components except for reverse transcriptase was included in the amplification to ensure no genomic DNA contamination. RT-PCR was performed using a thermal cycler, Gene Amp PCR System 9700 (Applied Biosystems). Different amplification cycles were applied to different paralogs. RT-PCR products were separated by electrophoresis in 1% agarose gel and stained with ethidium bromide.

Results and Discussion

Seedling vigor under submergence

We first measured the shoot (coleoptile) length and recovery rate as parameters for evaluating seedling vigor of four rice cultivars after incubating germinated seedlings in the dark for 5 days under submergence based on the test tube bioassay method previously developed (22). A japonica standard cultivar Nipponbare showed the highest elongation ability under this stress condition followed by indica check cultivars FR13A, Kasalath and IR42 (Fig. 1a). To examine if seedlings showing elongation under submergence stress can recover with greening after release from the stress, stressed seedlings were transferred to the normal conditions in pots with soil. Seedling recovery rates (%) were recorded after 5 days and 2 weeks of recovery period (Fig. 1b). Seedling recovery rate of submergence tolerant FR13A was nearly 100% after these recovery periods. Both FR13A and vigorous Nipponbare showed much higher recovery rates than submergence intolerant and non-vigorous Kasalath and IR42. Seedling heights were also measured after the 5-day and 2-week recovery periods (Fig. 1a). The amount of growth gained during these recovery periods was the greatest in Kasalath (Fig. 1a), which reflected its faster elongation growth than the others under the normal conditions (37). Significant positive correlations were observed between the shoot length measured under the 5-day dark submergence and the recovery rate of dark-submerged seedlings after both recovery periods (Table 2). This suggested that seedling vigor measured by shoot elongation ability reflected the recovery after release of the stress.

![Fig. 1. Seedling vigor under submergence evaluated by the test tube bioassay method. Shoot length of seedlings (a) of four cultivars subjected to 5-day dark submergence just after germination (black bars) and seedling height after the following 5-day (white bars) and 2-week (grey bars) recovery periods in pots with soil. Recovery rate (%) of the stressed seedlings (b) after the 5-day (black bars) and 2-week (white bars) recovery periods. Statistical comparisons were made between Nipponbare and the others under both conditions (P < 0.01).](image-url)
TABLE 2

Coefficients of determination ($R^2$) among the three parameters used for evaluating seedling vigor under submergence

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Recovery rate</th>
<th>Seedling height after 5-day recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot length</td>
<td>0.69</td>
<td>0.71</td>
</tr>
<tr>
<td>Recovery rate</td>
<td></td>
<td>0.15</td>
</tr>
</tbody>
</table>

$R^2$ was calculated using the data shown in Fig. 1a (shoot length in the test tube bioassay and seedling height after 5-day recovery from dark submergence) and Fig. 1b (recovery rate in 5-day-old and 2-week-old plants).

Rice ubiquitin gene was used as an internal control. Transcript of four cultivars (b): including FR13A and IR42 studied by RT-PCR. T and N respectively represent seedlings grown under submergence and normal conditions. Figures on the right of the panels indicate PCR cycle numbers.

Fig. 2. Expression profiles of OsACS genes under submergence. Transcripts of five OsACS paralogs (a) in Nipponbare and Kasalath studied by RT-PCR. Rice ubiquitin gene was used as an internal control. Transcript of four cultivars (b): including FR13A and IR42 studied by RT-PCR. T and N respectively represent seedlings grown under submergence and normal conditions. Figures on the right of the panels indicate PCR cycle numbers.

Expression profiles of OsACS and OsACO under submergence

Ethylene-mediated elongation and carbohydrate consumption during submergence is known to be under control by the SUB1A locus in rice (1, 2). We therefore next studied the expression profiles of the ethylene biosynthesis genes by RT-PCR analysis using Nipponbare and Kasalath incubated under the same test tube bioassay conditions. Marked induction occurred in all five OsACS paralogs in the stressed seedlings of Nipponbare as compared to the aerobically grown seedlings (Fig. 2a). Surprisingly, however, Kasalath showed a high amount of OsACS1 transcript under normal condition but no detectable levels of transcript under the stressed condition. We further compared transcript levels of these paralogs in all four cultivars (Fig. 2b). In the stressed seedlings, OsACS2, 3, 4 and 5 showed varying degrees of induction under the stressed condition in all cultivars (Fig. 2b). The transcript levels were the lowest in IR42. The opposite mode of expression of OsACS1 to that of the other paralogs was confirmed in both Kasalath and IR42, in which considerable amounts of transcript present in the non-stressed seedlings decreased to undetectable levels under submergence stress. With respect to OsACO, submergence stress greatly enhanced the expression of all 6 paralogs in both Nipponbare and Kasalath, although no cultivar differences were observed (Fig. 3).

Fig. 3. Expression profiles of OsACO genes under submergence. Transcripts of six OsACO paralogs studied by RT-PCR in Nipponbare and Kasalath are shown with rice ubiquitin gene as an internal control. T and N respectively represent seedlings grown under submergence and normal conditions. Figures on the right of the panels indicate PCR cycle numbers.

Expression profiles of OsNHX genes under submergence

Fig. 4. Expression profiles of OsNHX genes under submergence. Transcripts of OsNHX1, 4 and 5 paralogs (a) in Nipponbare and Kasalath studied by RT-PCR. Rice ubiquitin gene was used as an internal control. Transcript of OsNHX5 in four cultivars (b) including FR13A and IR42 studied by RT-PCR. T and N respectively represent seedlings grown under submergence and normal conditions. Figures on the right of the panels indicate PCR cycle numbers.
Expression profiles of OsNHX paralogs under submergence

We further studied the expression profiles of five paralogs of Na⁺/H⁺ exchanger gene, which were considered to be involved in salt tolerance by regulating uptake of sodium and/or potassium under saline conditions (11, 25). Under the same experimental conditions as the ones used for the ethylene biosynthesis genes, transcripts of OsNHX2 and 3 could not be detected. For three other paralogs, OsNHX1, 4 and 5, we observed marked induction under the submergence stress (Fig. 4b). The level of induction was higher in the vigorous cultivars Nipponbare and FR13A than in non-vigorous Kasalath and IR42. The amount of OsNHX1 and OsNHX5 transcripts under submergence was lowest in the most non-vigorous IR42.

Significance of seedling vigor under submergence in the ‘elongation escape strategy’ in rice seedlings

Submergence tolerance is a physiological adaptation, whereby rice plants can change metabolic pathways to adapt to the submerged environments (12). A volume of study supports the ‘quiescent strategy’ as a principal mechanism of submergence tolerance, and it has been well established that the ethylene-response-factor-like genes located at the SUB1 locus plays a key role (2, 8, 9, 10, 39). Meanwhile, the “elongation escape or avoidance strategy” has been considered as another mechanism for adaptation to submergence stress, whereby rice plants can escape from or avoid the stress to keep normal metabolic pathways (12). Cui et al. (5) reported that germination rate and early seedling growth were two major traits related to seedling vigor. Submergence stress significantly promoted shoot elongation (28). Fast shoot elongation reflected the ability of intercalary meristems that responded quickly to flood water to escape from unfavorable stresses (23). In our previous studies, we showed that the “elongation escape strategy” facilitating fast shoot elongation is advantageous for rice seedlings subjected to submergence stress just after germination (22, 37, 38). We confirmed this in the present study by showing that Nipponbare known to be submergence intolerant according to the “quiescent strategy” was most vigorous, showing both fast shoot elongation under submergence and good recovery after transferring to the soil under normal conditions (Fig. 1). These results support that seedling vigor under submergence is related to the submergence tolerance at the early seedling stages after germination. According to Perata and Voesenek (26), the ‘quiescence strategy’ has been experimentally supported based on the bioassay conditions in that 10- to 14-day-old seedlings (three-leaf stage) are completely submerged for 7 to 14 days. On the other hand, our test tube bioassay is based on the conditions that can evaluate shoot elongation of rice seedlings after 5 days of submergence just after germination. Our results suggest that there exist growth stage specific differences in the submergence tolerance mechanism and that fast and vigorous shoot elongation is advantageous and constitutes one important trait in the “elongation escape strategy” during the post-germination early seedling stage.

Expression profiles of OsACS, OsACO and OsNHX genes under submergence at the post-germination stage in rice

Rice plants show variations in their elongation capacity under submergence, which involves a transition from normoxia to hypoxia (6, 9). We previously showed that the expression of alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) genes was highly responsive to the submergence stress but was not a major determinant of the cultivar/genotype-specific level of seedling vigor under submergence (37). It is known that a very early response of rice plants to submergence is an increase in ethylene concentration due to its physical entrapment and elevated biosynthesis (24, 30). Several ethylene biosynthesis pathway genes including those encoding ACS and ACO enzymes are up-regulated by submergence stress (29, 34, 36). Ethylene production is the result of ACS activity and a rapid conversion of accumulated ACC to ethylene by ACO (44). The activity of ACO requires molecular oxygen, but the role of ethylene under anoxia is still not clear (3). Furthermore, much less information is available on the role of ACS and ACO in the regulation of post-germination seedling vigor under submergence, although the role of ethylene biosynthesis and sensitivity in submergence tolerance has been well established during the vegetative stage in rice. We therefore examined the transcript levels of OsACS and OsACO genes under both submergence and normal conditions at the post-germination seedling stage. Our results showed that the submergence stress strongly induced mRNA accumulation of all five OsACS paralogs in vigorous Nipponbare and submergence tolerant FR13A (Fig. 2). OsACS1, however, showed a contrasting mode of expression in non-vigorous and submergence intolerant Kasalath and IR42 as compared with other paralogs, i.e. its transcript was present in a high amount under non-stressed conditions but completely disappeared under submergence. According to Zarembinski and Theologis (43), the OsACS1 gene is the key gene responsible for intermodal elongation in deepwater rice, and the expression of this gene was implicated in long-term submergence-induced ethylene production. Our result showing the marked repression of OsACS1 expression in the non-vigorous and/or submergence intolerant cultivars suggest its key role in maintaining seedling vigor under submergence (Fig. 2). The OsACS5 gene was also suggested to play a fundamental role in the growth-promoting increase in ethylene biosynthesis during the first hours of submergence in deepwater rice (44). The early response of this paralog needs to be further studied. With respect to the OsACO gene expression, our results agreed with the report that it was induced 6-fold in deepwater rice upon 2-day submergence (34).

Bailey-Serres and Voesenek (1) pointed out the importance of combining submergence tolerance and salt tolerance because floodwaters are often saline. At the seedling stage, salt tolerance causes significant reduction in the germination index and seedling vigor (19, 21). Physiological studies of rice suggest that tolerance to salt within leaves can increase the ability to cope with salinity (41). Salinity caused a greater
decrease in the shoot length and shoot fresh weight in Kasalath than in Nipponbare (13). Difference in vigor among rice cultivars in fact accounted for much of the variation in their survival of salinity (41). We therefore studied the expression profiles of Na\(^+\)/H\(^+\) exchanger gene OsNHX using submerged rice seedlings (Fig. 4). Our study showed that three paralogs of OsNHX responded positively to the submergence stress and that the level of enhancement appeared to agree with the level of seedling vigor among the four cultivars studied. Although variation in gene expression itself could not be taken as a valid indicator of mechanistic evidence, this observation indicates the necessity of further study of the role of OsNHX gene expression in seedling vigor under submergence in rice.

Conclusions

Transcript levels of two key genes in the ethylene biosynthesis pathway consisting of OsACS and OsACO and a Na\(^+\)/H\(^+\) exchanger gene OsNHX were studied in rice seedlings grown under submergence. Submergence induced marked increases with varying degrees in the amount of transcripts of all these gene paralogs except for OsACS1. OsACS1 showed a unique response to the submergence stress in two non-vigorous cultivars, Kasalath and IR42, in that its expression was completely repressed under submergence, while non-stressed cultivars, Kasalath and IR42, in that its expression was response to the submergence stress in two non-vigorous

Acknowledgements

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References


