Induction of mitochondrial aldehyde dehydrogenase by submergence facilitates oxidation of acetaldehyde during re-aeration in rice

Hiroyuki Tsuji[a], Naoki Meguro[a], Yasuhiro Suzuki[b], Nobuhiro Tsutsumi[a], Atsushi Hirai[a,1], Mikio Nakazono[a,*]

[a]*Laboratory of Plant Molecular Genetics, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*
[b]*National Institute of Crop Science, 2-1-18 Kannondai, Tsukuba, Ibaraki 305-8518, Japan*

Abstract Post-hypoxic injuries in plants are primarily caused by bursts of reactive oxygen species and acetaldehyde. In agreement with previous studies, we found accumulations of acetaldehyde in rice during re-aeration following submergence. During re-aeration, acetaldehyde-oxidizing aldehyde dehydrogenase (ALDH) activity increased, thereby causing the acetaldehyde content to decrease in rice. Interestingly, re-aerated rice plants showed an intense mitochondrial ALDH2a protein induction, even though *ALDH2a* mRNA was submergence induced and declined upon re-aeration. This suggests that rice *ALDH2a* mRNA is accumulated in order to quickly metabolize acetaldehyde that is produced upon re-aeration.
© 2003 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Plant; Aldehyde dehydrogenase; Mitochondrion; Submergence

1. Introduction

Extended anaerobiosis causes a decrease in ATP production and cytoplasmic acidosis, both of which can injure a plant. This type of injury is referred to as hypoxic injury or anoxic injury [1–5]. In addition, cells may be damaged during re-aeration following anaerobic conditions. This is known as post-hypoxic injury or post-anoxic injury [6–8]. The injury is associated with two kinds of molecules: reactive oxygen species (ROS) and acetaldehyde. The production of ROS is immediately induced upon exposure of anaerobic plant tissues to normal oxygen tension and, in consequence, cellular components can undergo severe peroxidation [9,10]. Re-aeration also induces the production of acetaldehyde, as a result of oxidation of ethanol, which is produced and accumulated by ethanolic fermentation under low-oxygen conditions (Fig. 1) [11,12]. Ethanol is assumed to be rapidly oxidized to acetaldehyde by alcohol dehydrogenase (ADH) and/or catalase

(CAT) (Fig. 1) [6,13,14]. In addition, residual activity of pyruvate decarboxylase (PDC), which catalyzes the conversion of pyruvate to acetaldehyde (Fig. 1), might contribute to the increase of acetaldehyde.

Acetaldehyde is harmful to cells because of its tendency to form adducts with protein and DNA [15,16]. Cells possess mechanisms to metabolize acetaldehyde. Aldehyde dehydrogenase [ALDH (aldehyde: NAD(P)$^+$ oxidoreductase, EC 1.2.1.3)] catalyzes the conversion of aldehydes to the corresponding acids [17]. In human, ALDH is encoded by a large family of genes, one of which encodes the mitochondrial ALDH2. ALDH2 plays an important role in the metabolism of ethanol-derived acetaldehyde [18]. In plants, mitochondrial *ALDH* genes have been identified in several species, including maize [19–22], rice [23,24], barley [25], tobacco [26] and *Arabidopsis* [22].

It is possible that ALDH plays a role in metabolism of post-hypoxically produced acetaldehyde in plants. To examine this possibility, we measured ALDH activities and the amounts of acetaldehyde in rice under submerged conditions and following re-aeration. Based on a comparison of mRNA levels and protein levels of mitochondrial *ALDH* genes, we propose a novel type of regulation of this enzyme in rice.

2. Materials and methods

2.1. Plant material, growth conditions and treatments
Rice (*Oryza sativa* L., cv. Nipponbare) seedlings were grown in the light at 28°C for 8 days. For treatments of submergence, aerobically grown seedlings were completely submerged in water in the dark at 28°C. After the period of submergence, seedlings were transferred to aerobic conditions in the dark at 28°C. The durations of the submergence and the following re-aeration period are given in the legends of figures. For RNA and protein extraction, leaves of seedlings were harvested and immediately frozen in liquid nitrogen. For crude enzyme extractions, the treated seedlings were processed immediately.

2.2. Crude enzyme extraction and ALDH assay
Leaves of submerged and re-aerated seedlings were collected and immediately ground in a mortar with ice-cooled extraction buffer (150 mM Tris–HCl, pH 8.0, 25% (v/v) glycerol, 2% (w/v) polyvinyl-pyrrolidone, 0.8% (v/v) β-mercaptoethanol, 5 mM dithiothreitol and 0.5% (v/v) Triton X-100). The homogenates were filtered by two layers of Miracloth (Calbiochem, La Jolla, CA, USA), and then centrifuged at 15 000 rpm for 15 min at 4°C. The supernatants were assayed for ALDH activity as described by Tsuji et al. [24].

2.3. Gas chromatography–mass spectrometry (GC-MS) analysis of acetaldehyde and ethanol
Eight-day-old rice seedlings were submerged in water for 24 h and

---

*Corresponding author. Fax: (81)-3-5841 5183.
E-mail address: anakazo@mail.ecc.u-tokyo.ac.jp (M. Nakazono).

[1] Present address: School of Agriculture, Meijo University, 1-501 Shiogamaguchi, Tenpaku-ku, Nagoya, Aichi 468-8502, Japan.

Abbreviations: ALDH, aldehyde dehydrogenase; ADH, alcohol dehydrogenase; CAT, catalase; ROS, reactive oxygen species; GC-MS, gas chromatography–mass spectrometry

then were re-aerated for 0.5, 1, 2 and 4 h in the dark. The leaves were collected and were quickly frozen in liquid nitrogen. A GC-MS (Hewlett-Packard, Cheadle Heath, UK) analysis of acetaldehyde and ethanol was conducted by the method of Suzuki et al. [27] with some modifications. At first, the frozen samples were ground in liquid nitrogen with a mortar and pestle. Approximately 20 mg of powdered samples and 100 µl of $10\,000\times$ diluted 1-propanol (an internal standard) were added to a 6-ml vial. The vials were immediately sealed with a septum secured by an aluminum cap and then were heated with boiling water for 1.5 min. These processes were conducted in a cold room (4°C). GC-MS analysis conditions were as follows: a capillary column (HP-Wax), 60 m long×0.25 mm i.d. fused silica, wall coated with cross-linked polyethylene glycol (0.25 µm film thickness); oven temperature, held at 40°C for 2 min, increased in two-step linear gradations from 40 to 240°C (5°C min^{-1} from 40 to 120°C, and 10°C min^{-1} from 120 to 240°C), and held at 240°C for 3 min; injection temperature, 200°C; injection, split (1/5 ratio) injection; carrier gas, helium; flow rate, 1 ml min^{-1}. Results were shown by total ion chromatography (TIC) of acetaldehyde [TIC(AA)] or ethanol [TIC(EtOH)] divided by dry weight [g(DW)] and TIC of 1-propanol [TIC(1-PrOH); internal control].

2.4. RNA extraction and Northern hybridization

Total RNA was extracted by the standard guanidine thiocyanate/CsCl method [28]. Electrophoresis of RNA and Northern hybridization were performed by the method of Saisho et al. [29]. Each blot was hybridized only with the specific probe and equal loading of RNA was confirmed by ethidium bromide staining of each electrophoresis gel. All experiments were repeated three times or more and the same results were obtained.

2.5. Protein extraction and immunoblotting

Total proteins were extracted from leaves of rice seedlings by the method of Nakazono et al. [23]. SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and immunoblotting were done as described previously [24]. All experiments were repeated three times or more and the same results were obtained.

3. Results

3.1. ALDH activities and acetaldehyde amount in rice under submergence and under re-aerated conditions

ALDH activity in rice plants that had been submerged for

24 h (Fig. 2A, bar labeled S24) was comparable to that in aerobic plants (bar labeled 0). However, ALDH activity increased gradually following re-aeration, reaching three times the activity in the aerobic plants after 4 h (bars labeled R0.5 to R4).

Ethanol and acetaldehyde levels in rice seedlings were low under aerobic conditions, but significantly increased after submergence for 24 h (Fig. 2B) most likely as a result of ethanolic fermentation. When the submerged rice plants were transferred to aerobic conditions, concentrations of ethanol began to decrease during the first 1 h and reached one-fifth the initial level at 4 h after re-aeration (Fig. 2B). The acetaldehyde concentration in rice slightly increased just after re-aeration (i.e. 0.5, 1 and 2 h) and then decreased significantly at 4 h after re-aeration. Thus, the decrease of acetaldehyde was slower than the decrease of ethanol.

3.2. Induction of ALDH2a protein occurs soon after re-aeration

To dissect the post-hypoxic activation of ALDH at the molecular level, we carried out a Northern blot analysis of rice ALDH2 genes (Fig. 3A). Rice possesses two mitochondrial ALDH genes, ALDH2a and ALDH2b [24]. When rice seedlings were submerged for 24 h, the ALDH2a mRNA level increased (Fig. 3A), as described previously [23]. When the submerged seedlings were re-aerated, the submergence-induced ALDH2a mRNA level gradually declined and became almost undetectable at 4 h after re-aeration (Fig. 3A). By contrast, the ALDH2b mRNA level, which was decreased by submergence, began to recover after 2 h of re-aeration (Fig. 3A). The steady-state mRNA level of the anoxia (hypoxia)-inducible ADH1 gene [5] increased significantly under submergence (data not shown), verifying the low oxygen status in the experiment. Aerated control plants did not show any changes of expression of ALDH2a and ALDH2b genes (data not shown).

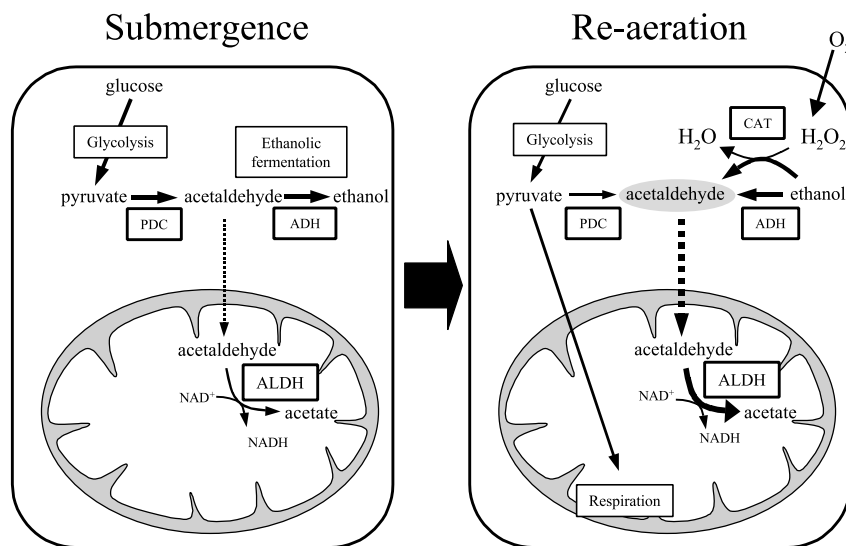In an immunoblot analysis of ALDH2 protein levels under

Fig. 1. Proposed metabolic pathways of acetaldehyde in rice under submerged conditions and following re-aeration. Under submerged conditions, pyruvate, which is produced by pathways such as glycolysis, is converted to acetaldehyde by PDC. At the same time, acetaldehyde is converted to ethanol by ADH and to acetate by ALDH. When the submerged plants are transferred to aerobic conditions (re-aeration), the anaerobically accumulated ethanol is rapidly oxidized to acetaldehyde by the reverse reaction of ADH and/or the peroxidation of ethanol by CAT during the conversion of H_2O_2 to H_2O. We suggest that ALDH efficiently metabolizes acetaldehyde by the rapid increase of mitochondrial ALDH under re-aeration.
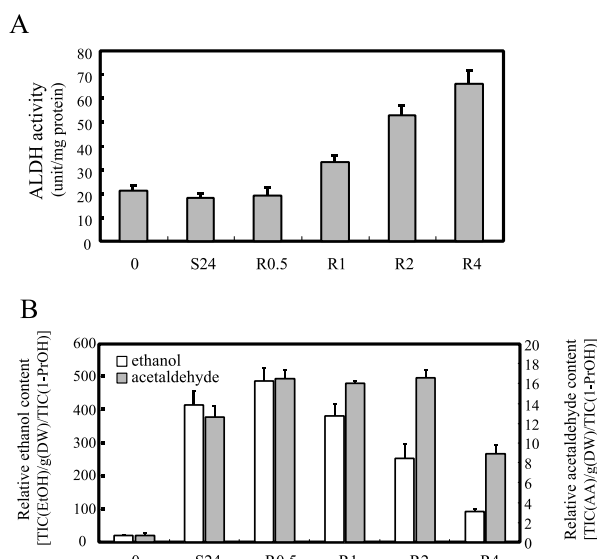
A

B

Fig. 2. A: ALDH activities of 8-day-old rice seedlings prior to submergence (0), after 24 h of submergence in the dark (S24), and after 0.5, 1, 2 and 4 h of re-aeration (R0.5, R1, R2 and R4, respectively) in the dark. Activities indicate mean values ± S.E.M. of four separate experiments. B: Ethanol and acetaldehyde contents of submerged and re-aerated rice seedlings. Amounts of ethanol and acetaldehyde in headspace vapor of leaves measured by GC-MS. Data show mean values ± S.E.M. of three separate experiments.

submergence and after re-aeration, two signals were observed (Fig. 3B). The upper and lower bands correspond to *ALDH2a* and *ALDH2b*, respectively, as reported previously [24]. The band intensities (Fig. 3C) show that ALDH2a protein levels were very low before submergence (0 h), and increased slightly

during submerged conditions, in spite of a significant increase in the mRNA of *ALDH2a*. Surprisingly, re-aerated seedlings showed an intense, but transient, *ALDH2a* induction, peaking at 4 h after re-aeration, whereas *ALDH2a* mRNA declined after re-aeration. On the other hand, ALDH2b protein levels slightly decreased under submerged conditions and slowly recovered after re-aeration. The expression patterns of *ALDH2a* and *ALDH2b* are summarized in Fig. 4.

4. Discussion

Post-hypoxic acetaldehyde accumulation has been reported in several plants [6,11,12], and may be a result of oxidation of ethanol by ADH and/or CAT or decarboxylation of pyruvate by PDC (Fig. 1). In order to fully recover from submergence, the re-aerated tissues must further metabolize acetaldehyde to non-toxic metabolites. We assume that ALDH is an important enzyme for metabolizing acetaldehyde during the re-aeration period in rice (Fig. 1). In this study, we found that the acetaldehyde-oxidizing ALDH activities of rice increased under re-aerated conditions following a period of submergence (Fig. 2A). This corresponds to the patterns of accumulation of mitochondrial ALDH proteins (Fig. 3B,C), which can oxidize acetaldehyde [23,24], indicating that the increase of ALDH2a protein in rice plants after re-aeration contributes to the increase of total ALDH activity.

The contributions of the cytosolic ALDH proteins of rice (ALDH1a and ALDH1b) to oxidation of post-hypoxically produced acetaldehyde may be less than those of mitochondrial ALDH2. This is because the transcript levels of *ALDH1a* and *ALDH1b* are very low in leaves of rice seedlings ([30]; Tsuji and Nakazono, unpublished data), and because
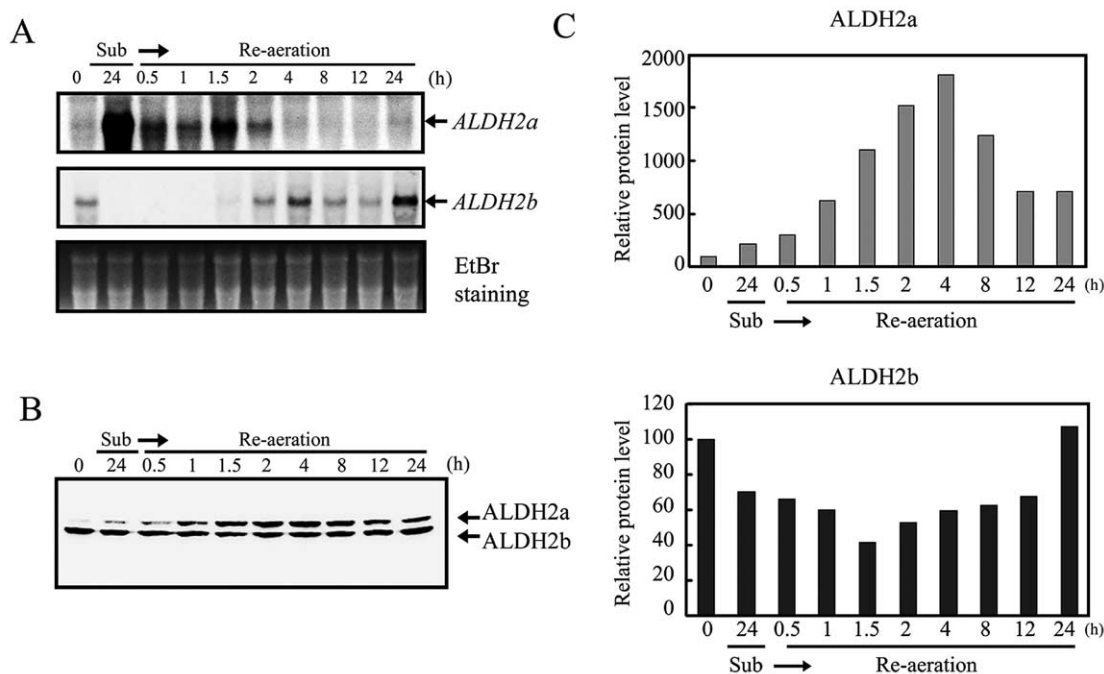

Fig. 3. Eight-day-old aerobically grown seedlings in the light (0 h) were submerged for 24 h in the dark, and then re-aerated for the indicated intervals in the dark. A: Northern blot analysis of transcripts of *ALDH2a* and *ALDH2b*. Each lane was loaded with 5 μg total RNA. Equal loadings of total RNA were checked by ethidium bromide (EtBr) staining. B: Immunoblot analysis of ALDH2a and ALDH2b. Each lane was loaded with 35 μg total protein. C: Quantification of relative protein levels of ALDH2a and ALDH2b shown in panel B. Protein levels are expressed as a percent of the level at 0 h.
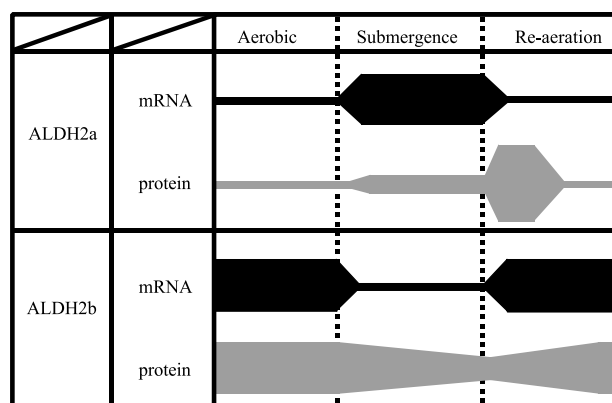
Fig. 4. Expression patterns of rice *ALDH2a* and *ALDH2b* under submergence and subsequent re-aeration in rice. During submerged conditions, the amount of ALDH2a protein increased slightly, in spite of a significant increase in the *ALDH2a* mRNA. Re-aerated seedlings showed an intense, but transient, ALDH2a induction, whereas *ALDH2a* mRNA declined after re-aeration. By contrast, the *ALDH2b* mRNA level and the ALDH2b protein level decreased during the submergence, and recovered by subsequent re-aeration.

both *ALDH1a* and *ALDH1b* transcripts decreased by treatments of submergence (Tsuji and Nakazono, unpublished data).

Although submergence caused a dramatic increase of rice *ALDH2a* mRNA, it did not greatly increase the level of ALDH2a protein (Figs. 3 and 4). One possible explanation is that *ALDH2a* mRNA is translated, but that ALDH2a protein is rapidly degraded under submergence, but not under re-aerated conditions after submergence. Another possibility is that the translation efficiency of rice *ALDH2a* mRNA is lower under submerged conditions because submerged conditions can lead to selective translation [31–35]. If translation does not occur during submergence, the induced *ALDH2a* mRNA might be pooled in preparation for the upsurge of acetaldehyde after re-aeration [12,36]. The increase in ALDH2a protein during re-aeration might be the result of the translation of this pooled mRNA. Thus, pooling of the mRNA would greatly speed up oxidation of acetaldehyde after re-aeration (Fig. 4).

In the re-aeration period, a possible role of mitochondrial ALDH is to contribute to the production of acetyl–CoA. ALDH-produced acetate could be converted to acetyl–CoA by acetyl–CoA synthetase (ACS; EC 6.2.1.1.) in plants. The acetyl–CoA may be supplied as a substrate for the TCA cycle, respiration and lipid biosynthesis, each of which has been shown to occur in tobacco pollen [37–39]. In fact, we detected significant levels of ACS proteins (ACS1 and ACS2) in rice seedlings under submergence and under subsequent re-aerated conditions (Tsuji and Nakazono, unpublished data).

Another possible role of mitochondrial ALDH is to supply NADH for respiration. The oxidation of acetaldehyde to acetate by ALDH concomitantly converts NAD^+ to NADH in mitochondria (Fig. 1). Thus, the oxidation of acetaldehyde by mitochondrial ALDH under re-aerated conditions may contribute to a rapid recovery of respiration and ATP synthesis through NADH production in rice mitochondria. Further investigations are needed to evaluate the possible physiological roles of mitochondrial ALDH proteins in re-aerated rice plants.

References

[1] Perata, P. and Alpi, A. (1990) Plant Physiol. 95, 748–752.
[2] Crawford, R.M.M. and Brändle, R. (1996) J. Exp. Bot. 47, 145–159.
[3] Sachs, M.M., Subbaiah, C.C. and Saab, I.N. (1996) J. Exp. Bot. 47, 1–15.
[4] Vartapetian, B.B. and Jackson, M.B. (1997) Ann. Bot. 79, 3–20.
[5] Das, A. and Uchimiya, H. (2002) J. Plant Res. 115, 315–320.
[6] Monk, L.S., Brändle, R. and Crawford, R.M.M. (1987) J. Exp. Bot. 38, 233–246.
[7] VanToai, T.T. and Bolles, C.S. (1991) Plant Physiol. 97, 588–592.
[8] Crawford, R.M.M., Walton, J.C. and Wollenweberratzer, B. (1994) Proc. R. Soc. Edinb. 102B, 325–332.
[9] Wollenweber-Ratzer, B. and Crawford, R.M.M. (1994) Proc. R. Soc. Edinb. 102B, 381–390.
[10] Biemelt, S., Keetman, U. and Albrecht, G. (1998) Plant Physiol. 116, 651–658.
[11] Pfister-Sieber, M. and Brändle, R. (1995) Potato Res. 38, 231–239.
[12] Zuckermann, H., Harren, F.J.M., Reuss, J. and Parker, D.H. (1997) Plant Physiol. 113, 925–932.
[13] Pavelic, D., Arpagaus, S., Rawyler, A. and Brändle, R. (2000) Plant Physiol. 124, 1285–1292.
[14] Kreuzwieser, J., Harren, F.J.M., Laarhoven, L.J.J., Boamfa, I., te Lintel-Hekkert, S., Scheerer, U., Huglin, C. and Rennenberg, H. (2001) Physiol. Plant. 113, 41–49.
[15] Perata, P., Vernieri, P., Armellini, D., Bugnoli, M., Tognoni, F. and Alpi, A. (1992) Plant Physiol. 98, 913–918.
[16] Zhang, M., Nagata, S., Miyazawa, K., Kikuchi, H. and Esashi, Y. (1997) Plant Physiol. 113, 397–402.
[17] Sophos, N.A., Pappa, A., Ziegler, T.L. and Vasiliou, V. (2001) Chem. Biol. Interact. 130-132, 323–337.
[18] Yoshida, A., Rzhetsky, A., Hsu, L.C. and Chang, C. (1998) Eur. J. Biochem. 251, 549–557.
[19] Cui, X., Wise, R.P. and Schnable, P.S. (1996) Science 272, 1334–1336.
[20] Liu, F., Cui, X., Horner, H.T., Weiner, H. and Schnable, P.S. (2001) Plant Cell 13, 1063–1078.
[21] Liu, F. and Schnable, P.S. (2002) Plant Physiol. 130, 1657–1674.
[22] Skibbe, D.S., Liu, F., Wen, T.J., Yandeau, M.D., Cui, X., Cao, J., Simmons, C.R. and Schnable, P.S. (2002) Plant Mol. Biol. 48, 751–764.
[23] Nakazono, M., Tsuji, H., Li, Y., Saisho, D., Arimura, S., Tsutsumi, N. and Hirai, A. (2000) Plant Physiol. 124, 587–598.
[24] Tsuji, H., Tsutsumi, N., Sasaki, T., Hirai, A. and Nakazono, M. (2003) Gene 305, 195–204.
[25] Meguro, N., Nakazono, M., Tsutsumi, N. and Hirai, A. (2001) Plant Biotechnol. 18, 223–228.
[26] op den Camp, R.G.L. and Kuhlemeier, C. (1997) Plant Mol. Biol. 35, 355–365.
[27] Suzuki, Y., Ise, K., Li, C., Honda, I., Iwai, Y. and Matsukura, U. (1999) J. Agric. Food Chem. 47, 1119–1124.
[28] Kingston, R.E. (1991) in: Current Protocols in Molecular Biology (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K., Eds.), pp. 4.2.1–4.2.8. Greene Publishing Associates and Wiley-Interscience, New York.
[29] Saisho, D., Nambara, E., Naito, S., Tsutsumi, N., Hirai, A. and Nakazono, M. (1997) Plant Mol. Biol. 35, 585–596.
[30] Li, Y., Nakazono, M., Tsutsumi, N. and Hirai, A. (2000) Gene 249, 67–74.

[31] Sachs, M.M., Freeling, M. and Okimoto, R. (1980) Cell 20, 761–767.

[32] Bailey-Serres, J. and Dawe, R.K. (1996) Plant Physiol. 112, 685–695.

[33] Fennoy, S.L., Nong, T. and Bailey-Serres, J. (1998) Plant J. 15, 727–735.

[34] Zeng, Y., Wu, Y., Avigne, W.T. and Koch, K.E. (1998) Plant Physiol. 116, 1573–1583.

[35] Bailey-Serres, J. (1999) Trends Plant Sci. 4, 142–148.

[36] Pfister-Sieber, M. and Brändle, R. (1994) Proc. R. Soc. Edinb. 102B, 313–324.

[37] Tadege, M. and Kuhlemeier, C. (1997) Plant Mol. Biol. 35, 343–354.

[38] Tadege, M., Dupuis, I. and Kuhlemeier, C. (1999) Trends Plant Sci. 4, 320–325.

[39] Mellema, S., Eichenberger, W., Rawyler, A., Suter, M., Tadege, M. and Kuhlemeier, C. (2002) Plant J. 30, 329–336.